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[Continued on next page]

(54) Title: THE KRAS VARIANT AND TUMOR BIOLOGY

(57) Abstract: The disclosure provides methods for identifying a subject at risk of developing cancer, predicting the onset of cancer, and predicting a subject's response to chemotherapy/treatment by determining the presence or absence of a SNP in the KRAS oncogene, known as the KRAS variant.

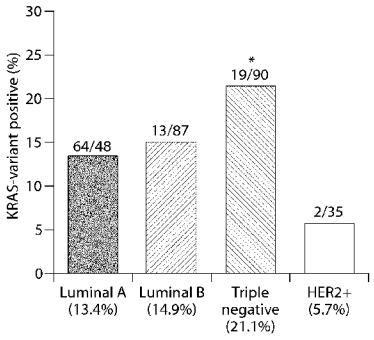

Fig. 1A

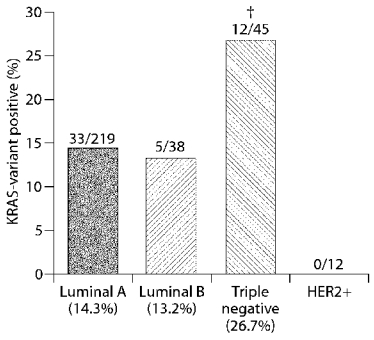

Fig. 1B

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THE KRAS VARIANT AND TUMOR BIOLOGY

RELATED APPLICATIONS

[01] This application claims the benefit of provisional application USSN 61/454,765 filed March 21, 2011; USSN 61/454,767, filed March 21, 2011; and USSN 61/454,769, filed March 21, 2011; the contents of which are each herein incorporated by reference in their entirety.

INCORPORATION BY REFERENCE

[02] The contents of the text file named "34592-515001WOST25.txt", which was created on March 16, 2012 and is 32.2 KB in size, are hereby incorporated by reference in their entirety.

GOVERNMENT SUPPORT

[03] This invention was made, in part, with U.S. Government support under Clinical and Translational Science Awards (CTSA), grant UL1 RR024139, provided by the National Center for Research Resources, a component of the National Institutes of Health.

[04] This invention was made, in part, with U.S. Government support under grant RO1 CA131301-01A1, provided by The National Cancer Institute, grant CA124484 (K08) provided by The National Institutes of Health, grant RO1 CA122728, provided by the National Institutes of Health, grant RO1 CA74415, provided by the National Institutes of Health, and grant RC4CA153828 provided by the National Cancer Institute and the Office of the Director of the National Institutes of Health.

[05] The Government has certain rights in the invention.

FIELD OF THE DISCLOSURE

[06] This disclosure relates generally to the fields of cancer, reproductive health and molecular biology. The disclosure provides methods for diagnosing and prognosing a subject having cancer by determining the presence or absence of a genetic marker. Moreover, the disclosure provides methods for determining a subject's response to treatment by determining the presence or absence of a genetic marker.

BACKGROUND

[07] The heterogeneity of cancer is reflected by the variable risk factors, treatment response and outcome in patients. While prognostic gene expression markers are highly divergent, several modules such as DNA repair deficiency, signatures of immune response or epithelial-to-mesenchymal transition are commonly found to be relevant for a subset of tumors. Thus, there is a need in the art for the identification of the drivers of these transcriptional modules as a promising approach for the discovery of specific and personalized therapies.

SUMMARY

[08] The studies presented in this disclosure relate to a central thesis regarding the role of miRNAs in cancer: disruption of miRNAs' regulation of oncogenes or tumor suppressor genes impact cancer risk, tumor development, and response to treatment. MiRNAs may regulate oncogenes or tumor suppressor genes directly or indirectly. For example, the *KRAS* variant, a SNP located in the *let-7* complementary site 6 (LCS6) of the 3' UTR of the *KRAS* gene, disrupts regulation of *KRAS* by the *let-7* family of miRNAs. In this case, *let-7*-mediated regulation of *KRAS* is disrupted; however, there are secondary effects of the *KRAS* variant. Disruption of the *let-7*/*KRAS* interaction upstream perpetuates aberrant signaling to downstream factors. Furthermore, components of signaling pathways other than the canonical RAS pathway are affected. The presence of the KRAS variant increases angiogenesis, survival (even under hypoxic conditions), metastasis, and confers resistance to frequently used chemotherapy agents. Moreover, epigenetic changes in the cancer cell, such as changes to promoter methylation of tumor suppressor and cell cycle genes, influence the development, survival, and response to treatment of a cancer cell positive for the *KRAS* variant. Finally, the cellular consequences of the *KRAS* variant are independent of other mutations in *KRAS*, including, for example, acquired mutations in a coding region of *KRAS*. For many cancer cells, the occurrence of the *KRAS* variant is mutually exclusive with the occurrence of other *KRAS* mutations. Unlike acquired mutations in *KRAS*, the *KRAS* variant is a germline mutation. Thus, the *KRAS* variant is a heritable biomarker of tumor cell biology.

[09] The occurrence of the *KRAS* variant mutation leads to increased expression and/or abundance of KRAS and decreased expression of the *let-7* family of miRNAs. The *KRAS* variant also affects the expression levels of transcription factors and miRNAs other than *let-7* family

miRNAs. For example, the *KRAS* variant is statistically significantly associated with increased expression levels of miR-23 and miR-27, which target anti-angiogenic genes such as Sprouty 2 and Sema6A. Thus, the poor outcome and resistance to traditional chemotherapy agents may result from an ability of the *KRAS* variant to drive activation of cell proliferation through RAS pathways, but also angiogenesis pathways that irrigate tumors with blood and nutrients to promote survival of cancer cells within a tumor. In the face of two aberrant pathways that have a common activator, the activity of certain chemotherapeutic agents may be insufficient to combat the progression of the cancer. The perturbation of RAS and other pathways in tumors that have the *KRAS* variant is conserved across cancer cell and tumor types (such as breast and ovarian cancers).

[10] The *KRAS* variant is associated with poor clinical outcomes in various cancers, including, but not limited to, colon, ovarian, head and neck cancer, and lung cancer. The evidence suggests that the *KRAS* variant determines a patient's response to treatment. If a carrier of the *KRAS* variant is resistant to the standard chemotherapeutic agent, then the patient's outcome is worse. The data presented herein demonstrate that the KRAS variant can confer resistance to traditional chemotherapeutic agents, while sometimes conferring increased sensitivity to monoclonal antibody therapy. For example, the *KRAS* variant increases a subject's sensitivity to Cetuximab when delivered as the only treatment, which targets an upstream regulator of the *KRAS* pathway (EGFR). Accordingly, the occurrence of the *KRAS* variant may suggest that agents specific for targets upstream of KRAS will be successful, however, conventional chemotherapeutic agents that target cell cycle checkpoints, which are downstream of *KRAS* may be ineffective. Similarly, the *KRAS* variant confers resistance to platinum-based chemotherapy. Platinum-based agents crosslink DNA molecules to prevent DNA replication, ultimately triggering apoptosis. However, DNA replication is a process that occurs downstream of KRAS activation, and, therefore, may be ineffective, particularly in light of data showing the recruitment of signaling pathways other than RAS.

[11] These discoveries about KRAS tumor biology provided herein have significant clinical value because chemotherapy as a treatment method is very hard on the patient. Chemotherapeutic agents present side effects that not only add to the patient's discomfort, but also introduce complications with otherwise functioning bodily systems. For instance, a chemotherapeutic agent that kills cancer cells may also damage or weaken the patient's heart.

Thus, the *KRAS* variant is a biomarker for determining resistance or sensitivity to known chemotherapy agents. If a patient is positive for the *KRAS* variant, then the doctor may be able to choose an optimal treatment, or at least avoid an ineffective treatment.

[12] In this disclosure the terms subject and patient are used interchangeably.

[13] The disclosure provides a method of predicting the an increased risk of vascularization of a tumor, including (a) detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a first patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and (b) determining the expression level of a miRNA selected from the group consisting of miR-23 and miR-27 in a second patient sample, wherein the presence of the mutation in (a) and an increase in the expression level of a miRNA in (b) compared to a control indicates increased transcriptional silencing of an anti-angiogenic gene, thereby predicting the an increased risk of vascularization of the tumor. The first and second patient samples are extracted from the same patient. Moreover, the first and second patient samples may include the same fluid, tissue, or biopsy. Preferably, the second patient sample is extracted or derived from the tumor or an area of non-tumor tissue in physical contact with the tumor (*i.e.,* surrounding the tumor). For example, the anti-angiogenic gene can be Sprouty2 or Sema 6A. The tumor may include a cancer cell derived from a(n) AIDS-related cancer, breast cancer, cancer of the digestive/gastrointestinal tract, anal cancer, appendix cancer, bile duct cancer, colon cancer, colorectal cancer, esophageal cancer, gallbladder cancer, islet cell tumors, pancreatic neuroendocrine tumors, liver cancer, pancreatic cancer, rectal cancer, small intestine cancer, stomach (gastric) cancer, endocrine system cancer, adrenocortical carcinoma, parathyroid cancer, pheochromocytoma, pituitary tumor, thyroid cancer, eye cancer, intraocular melanoma, retinoblastoma, bladder cancer, kidney (renal cell) cancer, penile cancer, prostate cancer, transitional cell renal pelvis and ureter cancer, testicular cancer, urethral cancer, Wilms' tumor, other childhood kidney tumors, germ cell cancer, central nervous system cancer, extracranial germ cell tumor, extragonadal germ cell tumor, ovarian germ cell tumor, gynecologic cancer, cervical cancer, endometrial cancer, gestational trophoblastic tumor, ovarian epithelial cancer, uterine sarcoma, vaginal cancer, vulvar cancer, head and neck cancer, hypopharyngeal cancer, laryngeal cancer, lip and oral cavity cancer, metastatic squamous neck cancer with occult primary, mouth cancer, nasopharyngeal cancer, oropharyngeal cancer, paranasal sinus and nasal cavity cancer, pharyngeal cancer, salivary gland cancer, throat cancer,

musculoskeletal cancer, bone cancer, Ewing's sarcoma, gastrointestinal stromal tumors (GIST), osteosarcoma, malignant fibrous histiocytoma of bone, rhabdomyosarcoma, soft tissue sarcoma, uterine sarcoma, neurologic cancer, brain tumor, astrocytoma, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, central nervous system germ cell tumor, craniopharyngioma, ependymoma, medulloblastoma, spinal cord tumor, supratentorial primitive neuroectodermal tumors and pineoblastoma, neuroblastoma, respiratory cancer, thoracic cancer, non-small cell lung cancer, small cell lung cancer, malignant mesothelioma, thymoma, thymic carcinoma, skin cancer, Kaposi's sarcoma, melanoma, or Merkel cell carcinoma. Alternatively, or in addition, the tumor or cancer is metastic.

[14] The disclosure provides a method of predicting an increased survival or proliferation of a cancer cell under hypoxic conditions, comprising (a) detecting a mutation in let-7 com*plem*entary site LCS6 of human *KRAS* in a first patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and (b) determining the expression level of a miR-210 miRNA in a second patient sample, wherein the presence of the mutation in (a) and an increase in the expression level of the miRNA in (b) compared to a control predicts an increased survival or proliferation of the cancer cell under hypoxic conditions. The first and second patient samples are extracted from the same patient. Moreover, the first and second patient samples may include the same fluid, tissue, or biopsy. The cancer cell may be derived from a(n) AIDS-related cancer, breast cancer, cancer of the digestive/gastrointestinal tract, anal cancer, appendix cancer, bile duct cancer, colon cancer, colorectal cancer, esophageal cancer, gallbladder cancer, islet cell tumors, pancreatic neuroendocrine tumors, liver cancer, pancreatic cancer, rectal cancer, small intestine cancer, stomach (gastric) cancer, endocrine system cancer, adrenocortical carcinoma, parathyroid cancer, pheochromocytoma, pituitary tumor, thyroid cancer, eye cancer, intraocular melanoma, retinoblastoma, bladder cancer, kidney (renal cell) cancer, penile cancer, prostate cancer, transitional cell renal pelvis and ureter cancer, testicular cancer, urethral cancer, Wilms' tumor, other childhood kidney tumors, germ cell cancer, central nervous system cancer, extracranial germ cell tumor, extragonadal germ cell tumor, ovarian germ cell tumor, gynecologic cancer, cervical cancer, endometrial cancer, gestational trophoblastic tumor, ovarian epithelial cancer, uterine sarcoma, vaginal cancer, vulvar cancer, head and neck cancer, hypopharyngeal cancer, laryngeal cancer, lip and oral cavity cancer, metastatic squamous neck cancer with occult

primary, mouth cancer, nasopharyngeal cancer, oropharyngeal cancer, paranasal sinus and nasal cavity cancer, pharyngeal cancer, salivary gland cancer, throat cancer, musculoskeletal cancer, bone cancer, Ewing's sarcoma, gastrointestinal stromal tumors (GIST), osteosarcoma, malignant fibrous histiocytoma of bone, rhabdomyosarcoma, soft tissue sarcoma, uterine sarcoma, neurologic cancer, brain tumor, astrocytoma, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, central nervous system germ cell tumor, craniopharyngioma, ependymoma, medulloblastoma, spinal cord tumor, supratentorial primitive neuroectodermal tumors and pineoblastoma, neuroblastoma, respiratory cancer, thoracic cancer, non-small cell lung cancer, small cell lung cancer, malignant mesothelioma, thymoma, thymic carcinoma, skin cancer, Kaposi's sarcoma, melanoma, or Merkel cell carcinoma.

[15] The disclosure provides a method of predicting an increased survival or proliferation of a cancer cell, including (a) detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a first patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and (b) determining the methylation status of a promoter of a tumor suppressor gene in a second patient sample, wherein the presence of the mutation in (a) and an increase in the methylation of a promoter (b) compared to a control predicts an increased survival or proliferation of the cancer cell. The first and second patient samples are extracted from the same patient. Moreover, the first and second patient samples may include the same fluid, tissue, or biopsy. Optionally, the tumor suppressor gene is *Notch1*. Survival may include maintaining tumorigenic potential. The cancer cell may be derived from a(n) AIDS-related cancer, breast cancer, cancer of the digestive/gastrointestinal tract, anal cancer, appendix cancer, bile duct cancer, colon cancer, colorectal cancer, esophageal cancer, gallbladder cancer, islet cell tumors, pancreatic neuroendocrine tumors, liver cancer, pancreatic cancer, rectal cancer, small intestine cancer, stomach (gastric) cancer, endocrine system cancer, adrenocortical carcinoma, parathyroid cancer, pheochromocytoma, pituitary tumor, thyroid cancer, eye cancer, intraocular melanoma, retinoblastoma, bladder cancer, kidney (renal cell) cancer, penile cancer, prostate cancer, transitional cell renal pelvis and ureter cancer, testicular cancer, urethral cancer, Wilms' tumor, other childhood kidney tumors, germ cell cancer, central nervous system cancer, extracranial germ cell tumor, extragonadal germ cell tumor, ovarian germ cell tumor, gynecologic cancer, cervical cancer, endometrial cancer, gestational

trophoblastic tumor, ovarian epithelial cancer, uterine sarcoma, vaginal cancer, vulvar cancer, head and neck cancer, hypopharyngeal cancer, laryngeal cancer, lip and oral cavity cancer, metastatic squamous neck cancer with occult primary, mouth cancer, nasopharyngeal cancer, oropharyngeal cancer, paranasal sinus and nasal cavity cancer, pharyngeal cancer, salivary gland cancer, throat cancer, musculoskeletal cancer, bone cancer, Ewing's sarcoma, gastrointestinal stromal tumors (GIST), osteosarcoma, malignant fibrous histiocytoma of bone, rhabdomyosarcoma, soft tissue sarcoma, uterine sarcoma, neurologic cancer, brain tumor, astrocytoma, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, central nervous system germ cell tumor, craniopharyngioma, ependymoma, medulloblastoma, spinal cord tumor, supratentorial primitive neuroectodermal tumors and pineoblastoma, neuroblastoma, respiratory cancer, thoracic cancer, non-small cell lung cancer, small cell lung cancer, malignant mesothelioma, thymoma, thymic carcinoma, skin cancer, Kaposi's sarcoma, melanoma, or Merkel cell carcinoma. Optionally, the cancer cell is a cancer stem cell.

Breast Cancer

[16] The disclosure provides methods for identifying a subject at risk for developing aggressive and high-risk forms of breast cancer as well as methods for predicting the onset of these forms. The data provided herein constitute the first disclosure of a mechanism delineating a detectable genomic mutation that drives the development of breast cancer tumors characterized by either a lack of expression of the estrogen receptor or the progesterone receptor. In preferred embodiments, the aggressive and high-risk form of breast cancer is triple negative breast cancer, which is further characterized by a lack of expression of the Human Epidermal growth factor Receptor 2 (HER2) gene transcript or protein.

[17] The disclosure provides a method of identifying a subject at risk for developing an estrogen receptor (ER) and progesterone receptor (PR) negative (ER/PR negative) breast cancer, including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of a mutation indicates greater risk of developing the ER/PR negative breast cancer.

[18] The disclosure provides a method of predicting the onset of developing an estrogen receptor (ER) and progesterone receptor (PR) negative (ER/PR negative) breast cancer in a

subject at risk for developing breast cancer, including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of a mutation indicates an earlier onset of developing the ER/PR negative breast cancer.

[19] In a preferred embodiment of the methods described herein, the ER/PR negative breast cancer is also negative for HER2, and therefore, is a triple negative breast cancer (TNBC). The triple negative breast cancer (TNBC) can be a basal or luminal cancer or tumor. In certain aspects of these methods, the triple negative breast cancer (TNBC) is a basal tumor that expresses a transcript or protein encoded by the epidermal growth factor receptor (*EGFR*) or the cytokeratin 5/6 (*CK5/6*) gene. In other aspects, ER/PR negative or ER/PR/HER2 negative breast cancer is further characterized by low or negative expression of the breast cancer 1 (*BRCA1*) gene.

[20] The subject (patient) is preferably a pre-menopausal female; however, the subject may be of any age. Alternatively, or in addition, the subject is less than 51 years of age, however, the subject may optionally, be less than 100, 90, 80, 70, 60, 50, 40, 30, 20, or any number of years of age in between.

Colorectal Cancer

[21] The disclosure provides a method of prognosing a subject with colorectal cancer (CRC), including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, wherein the presence of the *KRAS*-variant indicates a increased survival rate when compared to a control. In one aspect of this method, the detecting step further includes microsatellite-instability (MSI) analysis. The *KRAS*-variant is an independent marker of survival in colorectal cancer cells and patients; however, microsatellite instability (MSI) analysis may be used as a secondary analysis. Although MSI is a molecular marker for good prognosis in CRC patients (i.e. those with MSI tumors are considered to have a good prognosis), determination of the *KRAS*-variant status revealed that individuals who have developed a MSI tumor, but who are negative for the *KRAS*-variant (or, in other words, wild type) still have a poor prognosis in CRC. Thus, the disclosure provides a superior method for predicting the clinical outcome, or prognosis of CRC, particularly when the CRC patients are stratified by cancer stage.

[22] In particular embodiments of this method, the colorectal cancer (CRC) is early stage CRC. Preferably, the colorectal cancer (CRC) is stage 1 or 2.

[23] The test subject may have a second mutation in the *KRAS* gene, the KRAS-variant being the first mutation.

[24] The test or control subject may carry one or more mutations in the *BRAF* gene. Alternatively, or in addition, the test or control subject may have a hypermethylated *RASSF1A* promoter.

[25] The control subject does not carry the *KRAS*-variant (i.e. the control subject is wild type for the *KRAS*-variant mutation). However, the control subject may have CRC, or may be a cancer-free individual. Furthermore, the control subject may have a second mutation in the *KRAS* gene, which is not the *KRAS*-variant.

[26] In certain aspects of this method, the survival rate is an overall survival rate (for instance, some examples, include, but are not limited to, survival rates calculated from the time of cancer development or diagnosis until the subject succumbs to the cancer (death), enters remission, or a doctor declares the subject cured or clean of all cancer cells), five-year survival rate or one-year survival. Shorter survival periods are calculated, for instance, from either the development or diagnosis of the cancer until a determined time, such as one or five years.

Response to Treatment for Ovarian Cancer

[27] The disclosure provides methods of prognosing subjects with epithelial ovarian cancer (EOC) and, furthermore, methods of optimizing treatment by predicting the subject's response to platinum-based chemotherapy. The methods and data described herein identify a specific genomic mutation in a let-7 miRNA binding site within the 3' untranslated region (UTR) of the *KRAS* gene (known as the *KRAS* variant).

[28] The disclosure provides a method of prognosing a subject with epithelial ovarian cancer (EOC), including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, wherein the presence of the KRAS-variant indicates a decreased survival rate when compared to a control.

[29] Although the method can be applied to subjects and women of all ages, in certain embodiments of this method, the test subject is post-menopausal or 52 years of age or older.

Control subjects include healthy individuals and those women who have EOC, but who do not carry the KRAS-variant. Moreover, the control subject can be a national average based upon the expected survival of women born in the same year as the test subject, or who belong to the same generation as the test subject. In a preferred embodiment, this control value does not include those individuals who carry the KRAS-variant. In certain aspects of this method, the survival rate is an overall survival rate (for instance, some examples, include, but are not limited to, survival rates calculated from the time of cancer development or diagnosis until the subject succumbs to the cancer (death), enters remission, or a doctor declares the subject cured or clean of all cancer cells), five-year survival rate or one-year survival. Shorter survival periods are calculated, for instance, from either the development or diagnosis of the cancer until a determined time, such as one or five years.

[30] The disclosure also provides a method of predicting the response of an epithelial ovarian cancer (EOC) cell to a platinum-based chemotherapy, including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates a resistance to platinum-based chemotherapy. The EOC cell may be evaluated *in vitro* or *ex vivo*. When the EOC cell is evaluated *ex vivo*, the cell is obtained from a subject. The subject may be of any age, however, in a preferred embodiment, the subject is either postmenopausal or at least 52 years old. Alternatively, in the same embodiment, the subject is at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 years of age, or any age in between. In other aspects of this method, the subject is not post-menopausal, but presents a similar hormonal profile due to a second medical condition or medical treatment. An exemplary, but non-limiting menopausal hormonal profile includes decreased levels of estrogen and progesterone hormone, as determined by, for instance, assessment of a sample of the subject's blood or urine. Exemplary, but non-limiting, secondary medical conditions that induce a menopausal hormonal profile are surgical removal of at least one ovary (ovariectomy, also known as surgical menopause), cervical, uterine or ovarian cancer that necessitates a hysterectomy (especially if removal of the uterus is combined with removal of the Fallopian tubes and one or both ovaries). Exemplary, but non-limiting, secondary medical conditions that induce a menopausal hormonal profile are chemotherapy and anti-estrogen treatments.

[31] When the EOC cell is evaluated *in vitro*, the cell is isolated, reproduced, or derived from the BG1, CAOV3, or IGR-OV1 cell lines. These cell lines are non-limiting examples of ovarian cancer cell lines. An EOC cell may be isolated, reproduced, or derived from any ovarian cancer cell line, including, but not limited to, those cell lines that carry the *KRAS*-variant, a deleterious *BRCA1* mutation, a deleterious *BRCA2* mutation, or any combination thereof. A deleterious *BRCA1* or *BRCA2* mutation is a mutation that increases the risk or likelihood that it's carrier will develop cancer, and, in preferred embodiments, breast or ovarian cancer. A deleterious *BRCA1* or *BRCA2* mutation is a mutation that also increases the risk or likelihood that it's carrier will develop cancer at a younger age (i.e. experience an earlier onset of cancer), and, in preferred embodiments, the cancer is breast or ovarian cancer.

[32] For the methods described herein, the preferred platinum-based chemotherapy is carboplatin or paclitaxel, however, the platinum-based chemotherapy encompasses all chemotherapy agent that incorporate platinum or a platinum salt to treat or prevent cancer. In certain aspects of these methods, the platinum-based chemotherapy is an adjuvant therapy. Therefore, the methods described herein predict a patient's response to the use of a platinum-based chemotherapy as either a monotherapy or a combination therapy with other known anti-cancer agents or techniques (e.g. radiation and surgery, for example).

Response to Treatment for Colorectal Cancer

[33] The disclosure provides methods of prognosing subjects with colorectal cancer (CRC) or metastatic CRC (mCRC) and, furthermore, methods of optimizing treatment by predicting the subject's response to monoclonal antibody therapy, alone, or in combination with cytotoxic chemotherapy. The methods and data described herein identify a specific genomic mutation in a let-7 miRNA binding site within the 3' untranslated region (UTR) of the *KRAS* gene, referred to as the *KRAS* variant.

[34] The disclosure provides a method of prognosing a test subject with early stage colorectal cancer (CRC), including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of mutation indicates an increased survival rate when compared to a control subject or a subject with advanced CRC (including, for example stage III, stage IV, and metastatic CRC).

[35] The disclosure provides a method of prognosing a patient with advanced colorectal cancer (CRC), including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the *KRAS*-variant indicates a decreased survival rate when compared to a control subject or a subject with early stage CRC. Advanced CRC includes, for example, stage III, stage IV, and metastatic CRC.

[36] The disclosure provides a method of predicting the response of a cancer cell to a monoclonal antibody monotherapy, including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, wherein the presence of the mutation indicates a sensitivity to monoclonal antibody monotherapy. In certain embodiments of this method, the cancer cell is a colorectal cancer (CRC) cell. The cancer cell may be evaluated *in vitro* or *ex vivo*. A non-limiting example of the monoclonal antibody monotherapy is Cetuximab.

[37] The disclosure provides a method of predicting the response of a cancer cell to the combination of a chemotherapy and monoclonal antibody therapy, including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates a resistance to the combination. In certain embodiments of this method, the cancer cell is a colorectal cancer (CRC) cell. The cancer cell may be evaluated *in vitro* or *ex vivo*. A non-limiting example of the monoclonal antibody monotherapy is Cetuximab. The chemotherapy may be a cytotoxic agent. A non-limiting example of the cytotoxic agent is irinotecan. In certain embodiments, treatment of a subject carrying the *KRAS*-variant with a chemotherapeutic agent (*e.g.* irinotecan) results in increased expression of the *KRAS*-variant. When reporter expression is compared following irinitecan exposure in KRAS-variant versus non-variant cancer cells, no change was found in expression of the wild-type 3'UTR reporter. However, a statistically-significant increase in expression in the *KRAS*-variant 3'UTR reporter was discovered (Figures 24A and 24B). The data indicates that irinotecan exposure changes the cellular context in a manner that activates the *KRAS*-variant allele.

[38] Although the method can be applied to subjects of all ages, in certain embodiments of this method, the test subject a newborn, child, adult, or senior (aged 65 or above). The subject may be pre- or post-menopausal (aged 52 years or older).

[39] Controls or control subjects include healthy individuals and those individuals who have CRC, but who do not carry the *KRAS*-variant. Moreover, the control subject can be a national average based upon the expected survival of individuals born in the same year as the test subject, or who belong to the same generation as the test subject. In a preferred embodiment, this control value does not include those individuals who carry the *KRAS*-variant. In certain aspects of this method, the survival rate is an overall survival rate (for instance, some examples, include, but are not limited to, survival rates calculated from the time of cancer development or diagnosis until the subject succumbs to the cancer (death), enters remission, or a doctor declares the subject cured or clean of all cancer cells), five-year survival rate or one-year survival. Shorter survival periods are calculated, for instance, from either the development or diagnosis of the cancer until a determined time, such as one or five years.

[40] The disclosure also provides a method of predicting the response of a colorectal cancer (CRC) cell to a monoclonal antibody based therapy, including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates an increased sensitivity to monoclonal antibody based therapy. The CRC cell may be evaluated *in vitro* or *ex vivo*. The monoclonal antibody based therapy may be Cetuximab.

[41] The disclosure also provides a method of predicting the response of a colorectal cancer (CRC) cell to a cytotoxic chemotherapy, including detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, wherein the presence of the mutation indicates a resistance to cytotoxic chemotherapy. In certain embodiments of this method, the CRC cell is evaluated *in vitro* or *ex vivo*. The cytotoxic chemotherapy may be irinotecan. In an embodiment of this method, the cytotoxic chemotherapy is a combinatorial therapy that includes a monoclonal antibody based therapy. The monoclonal antibody based therapy may be Cetuximab.

[42] When the CRC cell is evaluated *ex vivo*, the cell is obtained from a subject. The subject may be of any age. In certain embodiments of this method the subject is at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 years of age, or any age in between.

[43] When the CRC cell is evaluated *in vitro*, the cell may be isolated, reproduced, or derived from an established cell lines, including a colon or colorectal cancer cell line included in the NCI-60 panel. A CRC cell may be isolated, reproduced, or derived from any colon or colorectal cancer cell line, including, but not limited to, those cell lines that carry the *KRAS*-variant, either alone, or in combination with a second or additional mutation in *KRAS* or another gene.

[44] For this method, the preferred monoclonal antibody monotherapy is Cetuximab, however, the monoclonal antibody monotherapy encompasses any monoclonal antibody used to treat or prevent cancer. Preferably, the monoclonal antibody is in part or entirely human or humanized. For this method, the preferred chemotherapy is a cytotoxic chemotherapy such as irinotecan, however, the chemotherapy encompasses any chemotherapy agent that is used to treat or prevent cancer. In certain aspects of this method, the chemotherapy or cytotoxic chemotherapy is an adjuvant therapy. Therefore, this method predicts a patient's response to the use of a monoclonal antibody as either a monotherapy or a combination therapy with a chemotherapy agent or other known techniques for treating or preventing cancer (*e.g.* radiation and surgery).

BRIEF DESCRIPTION OF THE DRAWINGS

[45] Figure 1A-B is a pair of graphs depicting the distribution of the *KRAS* variant in breast-cancer subtypes in all women (A) and premenopausal (≤ 51 years) women (B) from study group 2. Data are numbers of cases diagnosed with breast-cancer subtype/numbers of patients tested for the *KRAS* variant.*p=0.044 versus all other subtypes. †p=0.033 versus all other subtypes.

[46] Figure 2A-B is a pair of box plot depicting *BRCA1* gene expression among the *KRAS*-variant positive and *KRAS*-variant negative cases of triple-negative breast cancer. Y-axes are in arbitrary units. (A) *BRCA1* probe 1, p=0·06. (B) *BRCA1* probe 2, p=0·01.

[47] Figure 3 is a series of box plots depicting the expression of *let-7* family of microRNAs in the *KRAS*-variant positive versus *KRAS*-variant negative cases of triple-negative breast cancer. Y-axes are in arbitrary units.

[48] Figure 4 is a heat map showing the *KRAS*-variant differentially expressed genes in triple negative breast cancer patients analyzed by LIMMA model. The 50 most significant genes were

used for the clustering; $p<0.0001$ for clustering. *KRAS*-variant samples are dark gray; wild-type samples are light gray. White have unknown *KRAS*-variant status.

[49] Figure 5 is a graph depicting the *KRAS*-variant in ER/PR+ versus ER/PR- premenopausal breast cancer patients.

[50] Figure 6 is a series of box graphs depicting Gene expression signatures associated with the *KRAS*-variant in triple negative breast cancer patient tumors.

[51] Figure 7 is a graph showing that the *KRAS* variant predicts significantly worse overall survival for postmenopausal ovarian cancer patients over 52 years of age. Overall survivals for ovarian cancer patients with (n= 59) and without (n= 220) the *KRAS* variant are compared using the Kaplan -Meier analysis. Outcome is significantly worse for *KRAS* variant positive EOC patients over 52 years of age by log-rank test ($P = 0.0399$).

[52] Figure 8 is a graph showing that the *KRAS* variant is associated with suboptimal debulking after neoadjuvant chemotherapy. Surgical debulking after neoadjuvant chemotherapy is compared in ovarian cancer patients (n = 116) with the *KRAS* variant (n = 26) or without (n = 90). By χ^2 analysis, *KRAS*-variant patients are significantly more likely to be suboptimally debulked with greater residual disease (RD) than are non-variant patients ($P = 0.044$).

[53] Figure 9A is a signature of 50 differentially expression gene candidates in *KRAS* variant (KV) triple-negative breast tumors (TNBC KRAS Signature) that shows higher scores in KV EOC samples than in non-variant samples.

[54] Figure 9B is a signature of genes associated with KRAS-addicted tumors (KRAS Addiction Signature), which are upregulated in KV EOC tumors.

[55] Figure 9C a signature of differential expression of the top 20 genes in KV EOC tumors, reflecting a re-analysis of differential gene expression in carboplatin-sensitive and carboplatin-resistant EOC cells.

[56] Figure 9D is a heat map of the top differentially expressed genes between KV (dark gray) and non-variant (light gray) tumor samples. The color key depicts a spectrum from blue (values 0 to 5) to white (approximately 5), and from white to red (5 to 10). For a color version of this heat map, see Ratner ES, et al. Oncogene, (5 December 2011), 1-8; the contents of which are incorporated herein by reference).

[57] Figure 10 is a graph showing that the *KRAS* variant is associated with resistance to carboplatin and carboplatin/taxol chemotherapy in cell lines. Cell lines with the *KRAS* variant

(BG1) and without the *KRAS* variant (CAOV3) were treated with chemotherapy and half-maximal inhibitory concentration (IC50) is shown on the Y axis, and chemotherapeutic agent on the X axis. Higher IC50 represents resistance to the tested chemotherapeutic agent. BG1 = *KRAS* variant/*BRCA* wild-type cell line; CAOV3 = non-variant/*BRCA* wild-type cell line; IGR-OV1 = *KRAS*-variant/*BRCA1* mutant cell line. Error bars are RSE.

[58] Figure 11A is a graph showing decreased cell survival in the *KRAS*-variant line, BG1 (*$P < 0.001$), with no effect on the non-variant line, CAOV3. Cell lines, with (BG1) and without (CAOV3) the KRAS variant, were treated with siRNA/miRNA combinations that bind selectively to the variant allele.

[59] Figure 11B is a graph showing decreased KRAS protein expression in BG1 (right) concordant with the decrease in cell survival, with no effect on CAOV3 (left). Cell lines, with (BG1) and without (CAOV3) the *KRAS* variant, were treated with siRNA/miRNA combinations that bind selectively to the variant allele. Different siRNAs are denoted by numbers.

[60] Figure 12 is a graph depicting Cell lines with the *KRAS* variant (BG-1 and IGROV1) have significantly lower levels of *let-7b* compared to a non-variant cell line (CaOV3). Statistical analysis was done with a one way Anovea and Tukey's Multiple comparison test.

[61] Figure 13A-B is a schematic depicting an alignment of the *KRAS*-variant sequence with non-variant sequences. Panel A depicts a non-variant sequence of *KRAS*. Panel B depicts exemplary variant siRNA oligos targeted to the *KRAS*-variant sequence. In both panels, the underlined sequence depicts the *let-7* binding site. In both panels, the boxed nucleotide represents either the wild type (non-variant) nucleotide (A) or the *KRAS* variant single nucleotide polymorphism (B). siRNAs are shown starting with their 3' end.

[62] Figure 14 is a Kaplan-Meier curve for the *KRAS* variant and cause-specific survival in all cancer stages.

[63] Figure 15A is a Kaplan–Meier curve for the *KRAS* variant and cause-specific survival in early-stage (stage I and II) CRC.

[64] Figure 15B is a Kaplan–Meier curve for the *KRAS* variant and cause-specific survival in stage III CRC.

[65] Figure 15C is a Kaplan–Meier curve for the *KRAS* variant and cause-specific survival in stage IV CRC.

[66] Figure 16A is a Kaplan–Meier curve for the *KRAS* variant, *KRAS* mutations and cause-specific survival in early-stage (stage I and II) CRC, $P = 0.875$.

[67] Figure 16B is a Kaplan–Meier curve for the *KRAS* variant, *KRAS* mutations and cause-specific survival in stage III CRC.

[68] Figure 16C is a Kaplan–Meier curve for the *KRAS* variant, *KRAS* mutations and cause-specific survival in stage IV CRC.

[69] Figure 17 is a Kaplan–Meier curve for the *KRAS* variant, MSI status and cause-specific survival in early-stage (stage I and II) CRC.

[70] Figure 18A is a graph depicting the median progression free survival according to the *KRAS* LCS6 genotype status in patients treated with anti-EGFR moAbs monotherapy or in combination with chemotherapy as salvage treatment.

[71] Figure 18B is a graph depicting the median overall survival according to the *KRAS* LCS6 genotype status in patients treated with anti-EGFR moAbs monotherapy or in combination with chemotherapy as salvage treatment.

[72] Figure 19A is a graph depicting the median progression-free survival according to the *KRAS* LCS6 genotype status in all patients treated with anti-EGFR moAbs monotherapy as salvage treatment.

[73] Figure 19B is a graph depicting the median progression-free survival according to the *KRAS* LCS6 genotype status in all patients treated with anti-EGFR moAbs based combination chemotherapy as salvage treatment.

[74] Figure 19C is a graph depicting the median progression-free survival according to type of therapy in all *KRAS* variant carriers.

[75] Figure 19D is a graph depicting the median progression-free survival according to type of therapy in all non-*KRAS* variant carriers.

[76] Figure 20A is a graph depicting the median progression-free survival according to the *KRAS* LCS6 genotype status in the double (*KRAS* and *BRAF*) wt patients' population treated with anti-EGFR moAbs monotherapy as salvage treatment.

[77] Figure 20B is a graph depicting the median progression-free survival according to the *KRAS* LCS6 genotype status in the double (*KRAS* and *BRAF*) wt patients' population treated with anti-EGFR moAbs based combination chemotherapy as salvage treatment.

[78] Figure 20C is a graph depicting the Median progression-free survival according to type of therapy in the double (*KRAS* and *BRAF*) wt *KRAS* variant carriers

[79] Figure 20D is a graph depicting the Median progression-free survival according to type of therapy in the double (*KRAS* and *BRAF*) wt non-*KRAS* variant carriers.

[80] Figure 21A is a graph depicting the median overall survival according to the *KRAS* LCS6 genotype status in all patients treated with anti-EGFR moAbs monotherapy as salvage treatment.

[81] Figure 21B is a graph depicting the median overall survival according to the *KRAS* LCS6 genotype status in all patients treated with anti-EGFR moAbs based combination chemotherapy as salvage treatment.

[82] Figure 21C is a graph depicting the median overall survival according to type of therapy in all *KRAS* variant carriers.

[83] Figure 21D is a graph depicting the median overall survival according to type of therapy in all non-*KRAS* variant carriers.

[84] Figure 22A is a graph depicting the median overall survival according to the *KRAS* LCS6 genotype status in the double (*KRAS* and *BRAF*) wt patients' population treated with anti-EGFR moAbs monotherapy as salvage treatment.

[85] Figure 22B is a graph depicting the median overall survival according to the *KRAS* LCS6 genotype status in the double (*KRAS* and *BRAF*) wt patients' population treated with anti-EGFR moAbs based combination chemotherapy as salvage treatment.

[86] Figure 22C is a graph depicting the median overall survival according to type of therapy in the double (*KRAS* and *BRAF*) wt *KRAS* variant carriers.

[87] Figure 22D is a graph depicting the median overall survival according to type of therapy in the double (*KRAS* and *BRAF*) wt non- *KRAS* variant carriers.

[88] Figure 23A is a graph depicting the median progression-free survival according to type of therapy in the *KRAS* and *BRAF* mutated *KRAS* variant carriers.

[89] Figure 23B is a graph depicting the median progression-free survival according to type of therapy in the *KRAS* and *BRAF* mutated non- *KRAS* variant carriers.

[90] Figure 23C is a graph depicting the median overall survival according to type of therapy in the *KRAS* and *BRAF* mutated *KRAS* variant carriers.

[91] Figure 23D is a graph depicting the median overall survival according to type of therapy in the *KRAS* and *BRAF* mutated non- *KRAS* variant carriers.

[92] Figure 24A is a graph depicting the normalized luciferase expression in wild type *KRAS* and *KRAS*-variant cancer cells following treatment with the chemotherapeutic agent irinotecan.

[93] Figure 24A is a graph depicting the fold repression (expressed as KRAS variant/KRAS wild type) as a function of irinotecan concentration, when cancer cells are treated with irinotecan.

DETAILED DESCRIPTION

[94] A functional variant in a *let-7* microRNA complementary site in the 3'UTR of the *KRAS* oncogene (*rs61764370*) associated with cancer was previously identified (International Patent Application No. PCT/US2008/065302, the contents of which are incorporated herein by reference in their entirety). An investigation of the association of this variant with cancer tumor biology is described herein.

Breast Cancer

[95] Breast tumors are classified into ER (estrogen) and/or PR (progesterone) receptor positive, HER2 (Her2/neu/ERBB2) amplified, and triple-negative tumors (*i.e.*, ER/PR negative and HER2 negative) (Sørlie T, et al. Proc Natl Acad Sci USA 2001; 98: 10869–74). Gene expression and receptor profiling further classifies breast cancer into four biological subgroups: The luminal A (ER- and/or PR-receptor positive, HER2-negative) tumors, luminal B (ER- and/or PR-receptor positive, HER2 positive), HER2-positive (HER2-positive, ER/PR negative) and basal like (ER/PR/HER2-negative, also referred to as triple negative breast cancer (TNBC)) tumors (Sørlie T, et al. Proc Natl Acad Sci USA 2001; 98: 10869–74).

[96] Triple negative breast cancer (TNBC) is the most aggressive subclass with worse cause-specific survival at 5 years compared to the other subtypes (Haffty BG et al. J Clin Oncol 2006; 24: 5652–57). Recent transcriptional profiling studies suggest there is further heterogeneity within TNBC and these tumors can be categorized into two broad subgroups; the ER/PR/HER2 (triple) negative tumors that express EGFR or cytokeratin (CK) 5/6, and, therefore, termed 'basal-like', and the ER/PR/HER2 (triple) negative tumors that do not express EGFR or CK5/6. The basal-like triple negative (TN) tumors are also characterized by an earlier age (or younger age) of onset than non-basal-like forms and low expression of *BRCA1* (BReast CAncer 1); the basal-like phenotype is common among carriers of the *BRCA1* mutation (Rakha EA and Ellis IO. Pathology 2009; 41: 40–47). An aberrant luminal progenitor cell population (that may be ER

positive) is a target for transformation in *BRCA-1*-associated basal tumors (Lim E, et al. Nat Med 2009; 15: 907–13). Although prognostic gene-expression markers are highly divergent, several modules such as DNA repair deficiency, signatures of immune response, or transition from epithelium to mesenchyme are commonly noted in a subset of these tumors (Bild AH, et al. Breast Cancer Res 2009; 11: R55). Identification of the drivers of these transcriptional modules is one approach for discovery of specific and personalized therapies.

[97] Association of the triple-negative breast cancer phenotype with young age of onset and an absence of association with known risks or reproductive factors (Yang XR, et al. Cancer Epidemiol Biomarkers Prev 2007; 16: 439–43) suggests there are genetic risks for development of this cancer (Bauer KR et al. Cancer 2007; 109: 1721–28). Prior to this disclosure, few genetic markers of such increased risk existed. Although *BRCA1* mutations are often associated with triple-negative tumors, these mutations are rare and account for only 10–15% of patients with triple-negative breast cancer, dependent on ethnic background and family history (Young SR, et al. BMC Cancer 2009; 9: 86; Nanda R, et al. JAMA 2005; 294: 1925–33).

[98] The studies provided herein determined the frequency distributions of the *KRAS* variant in 415 patients with histologically confirmed breast cancer and 457 controls from Connecticut, USA (study group 1) as well as an association of this variant with breast cancer subtypes in 690 Irish women with known estrogen receptor (ER), progesterone receptor (PR), and HER2 statuses, and 360 controls (study group 2). Data for study groups 1 and 2 was pooled with a cohort of 140 women with triple-negative breast cancer and 113 controls to assess the association of the *KRAS* variant with triple-negative breast cancer risk as well as genome-wide mRNA and specific miRNA expression in patients with triple-negative breast cancer.

[99] Although frequency distributions of the KRAS variant in study group 1 did not differ between all genotyped individuals, eight (33%) of 24 premenopausal women with ER/PR-negative cancer had the *KRAS* variant, compared with 27 (13%) of 201 premenopausal controls (p=0·015). In study group 2, the *KRAS* variant was significantly enriched in women with triple-negative breast cancer (19 [21%] of 90 cases) compared with 64 (13%) of 478 for luminal A, 13 (15%) of 87 for luminal B, and two (6%) of 35 for HER2-positive subgroups (p=0.044). Multivariate analysis in the pooled study groups showed that the *KRAS* variant was associated with triple-negative breast cancer in premenopausal women (odds ratio 2·307, 95% CI 1·261–4·219, p=0.0067). Gene-expression analysis of triple negative breast-cancer tumors suggested

that *KRAS*-variant positive tumors have significantly altered gene expression, and are enriched for the luminal progenitor and *BRCA1* deficiency signatures. MiRNA analysis suggested reduced levels of *let-7* miRNA species in *KRAS*-variant tumors.

[100] The KRAS variant is a genetic marker for development of triple-negative breast cancer in premenopausal women. Altered gene and miRNA expression signatures enable molecular and biological stratification of patients with triple negative breast cancer.

Colorectal Cancer

[101] The *KRAS* variant is a prognostic biomarker in early-stage colorectal cancer (CRC). Moreover, the *KRAS* variant induces higher levels of the KRAS oncogenic protein and lower levels of the tumor suppressor *lethal-7* (*let-7*) miRNAs. The influence of *KRAS*-variant was studied in 409 early-stage (stage I and II), 182 stage III and 69 stage IV cases from the large, prospective Netherlands Cohort Study (NLCS). Early-stage patients with the *KRAS* variant had a better prognosis, especially those that also had additional *KRAS* mutations. This discovery is independent of microsatellite-instability or other prognostic factors. In addition, the influence of the *KRAS*- variant on CRC risk was also studied by using data from 1,886 subcohort members from the NLCS. The G-allele (*i.e.,* the *KRAS* variant allele) was not associated with a likelihood of developing CRC overall, but was enriched in advanced stage CRC, suggesting it may predict presenting with more advanced disease. Because this study population is the only untreated population analyzed to date, these results provide a novel insight into the natural biology of colorectal cancer with the *KRAS* variant.

[102] As the data presented herein demonstrate, the *KRAS* variant is a new biomarker in colorectal cancer (CRC) to guide treatment decisions in early-stage patients. Early-stage CRC cases with the *KRAS* variant have a better outcome, however, in advanced disease, this better outcome no longer exists. For early-stage patients, the combination of the *KRAS* variant genotype and at least one *KRAS* mutation is also a prognostic biomarker of better outcome to be considered in therapy-decision-making.

[103] Despite diagnostic and therapeutic innovations, colorectal cancer (CRC) remains the second leading cause of cancer death in the western world. The tumor-node-metastasis-system (TNM) is currently the standard tool to provide prognostic information. The TNM system is highly predictive for prognosis at the extremes (early and late stage CRC), but less predictive for intermediate stages. According to current guidelines, adjuvant chemotherapy is not given to

early-stage patients (*i.e.*, T1-3-N0-M0, according to the International Union Against Cancer TNM). Five-year survival rates in this group of early-stage patients (*i.e.*, T1-3-N0-M0) are greater than 70%. Nevertheless, 20-30% of early-stage patients (stage I and II) will die of CRC within 5 years, evoking the question whether these deaths could have been avoided if these patients were identified in advance and therapy was adapted accordingly. Previously, numerous studies have been published claiming a prognostic influence of molecular markers. In contrast to the assertions of these previous reports, the results of these studies are inconsistent. Thus, prior to the development of the methods described herein, the question of which molecular alterations influence prognosis remained unresolved (Smits KM, et al. Pharmacogenomics. 2008; 9(12): 1903-16).

[104] MicroRNAs (miRNA), have been identified as important factors in cancer development and progression. Evidence suggests that a single miRNA can regulate many mRNAs simultaneously (Paranjape T, et al. Gut. 2009; 58(11): 1546-54). Moreover, miRNAs can act as both tumor suppressors and oncogenes (Johnson SM, et al. Cell. 2005; 120(5): 635-47). The *lethal-7* (*let-7*) family of miRNAs is one of the first miRNA families to be discovered. The expression of *let-7* family miRNAs is altered in many cancers. For example, in lung cancer, *let-7* is poorly expressed (Calin GA, et al. Proc Natl Acad Sci U S A. 2004; 101(9): 2999-3004; Takamizawa J, et al. Cancer Res. 2004; 64(11): 3753-6), overexpression of *let-7* inhibits cell growth *in vitro* (Takamizawa J, et al. Cancer Res. 2004; 64(11): 3753-6) and *in vivo* (Kumar MS, et al. Proc Natl Acad Sci U S A. 2008; 105(10): 3903-8; Esquela-Kerscher A, et al. Cell Cycle. 2008; 7(6): 759-64) suggesting that *let-7* miRNAs may act as tumor suppressors (Johnson SM, et al. Cell. 2005; 120(5): 635-47).

[105] In colon cancer cells, *let-7* expression is significantly decreased in tumor tissue as compared to adjacent non-cancerous tissue (Akao Y, et al. Biol Pharm Bull. 2006; 29(5): 903-6). Moreover, *let-7* expression was increased and RAS expression was decreased in cell lines after transfection of a *let-7a-1* miRNA precursor, suggesting that *let-7* is involved in regulating colon cancer cell growth (Akao Y, et al. Biol Pharm Bull. 2006; 29(5): 903-6).

[106] MiRNAs can control gene expression by binding to complementary elements in the 3'untranslated region (UTR) of target mRNAs. *Let-7* induces RAS downregulation after binding to specific sites in the 3'-UTR of *KRAS* mRNA. The *KRAS* variant affects *let-7* mediated regulation of KRAS expression. The occurrence of the variant G-allele (*i.e.*, the *KRAS* variant)

leads to higher KRAS levels and lower *let-7* levels as compared to the wild type. G-allele carriers have an increased lung cancer risk in moderate smokers, an increased ovarian cancer risk (particularly for post-menopausal women), an increased risk of developing breast cancer (and, in particular, the triple negative breast cancer subtype), and a reduced survival in oral cancers but not in lung cancer. In *KRAS/BRAF* mutated CRC, G-allele carriers (*KRAS* variant carriers) showed a reduced survival in late-stage CRC and an altered response to cetuximab, demonstrating a role of the *KRAS* variant in colon cancer. Because the role of the *KRAS* variant genotype in early-stage CRC was unresolved, the experiments and data presented herein assessed the influence on prognosis in 409 early-stage (TNM stage I and II; T1-4, N0, M0), 182 stage III (T1-4, N1, M0) and 69 stage IV (T1-4, N0-1, M1) CRC cases from a large prospective cohort study. The influence of *KRAS* variant genotype on CRC risk was also assessed by using data from 1,886 subcohort members from the NLCS.

[107] The results of this study demonstrate that a T>G variant in the LCS6 in the 3' UTR region of *KRAS* affects prognosis in early-stage (stage I and II) CRC. The *KRAS* variant was present in 16.4% of the cases, whereas it is found in only 6% of world populations (Chin LJ, et al. Cancer Res 2008;68:8535–40), and 12% to 15% in persons from European descent (Ratner E, et al. Cancer Res 2010;70:6509–15). An increased frequency of the *KRAS* variant (G-allele) was discovered in advanced cases (early stage 14%, 19.2%, and 21.4% in stage III and IV patients, respectively), which is comparable with previously reported frequencies in stage III (Graziano F, et al. Pharmacogenomics J 2010;10:458–64). The G-allele (*KRAS* variant) was found in 18% of the subcohort members. A statistically significant association was discovered between the *KRAS*-variant and an increased presentation with advanced colon cancer, providing valuable insight into the natural biology of colon cancer in *KRAS* variant carriers. Furthermore, a statistically significant increase in survival for early-stage CRC cases with the *KRAS* variant was discovered; among *KRAS*-mutated patients none of the early-stage patients carrying the G-allele (*KRAS* variant) died from CRC. This statistically significant increase in survival for early-stage CRC cases with the *KRAS* variant was independent of other prognostic factors such as tumor differentiation or sublocation. Because T4 tumors were rare in the study group of early-stage cases, a higher frequency of stage IIb cases among *KRAS* wild types is ruled out as the cause of the observed worse outcome. A statistically significant effect was not found in stage III or IV, although the results indicate a worse prognosis for stage III cases with the *KRAS* variant (G

allele) and *KRAS* mutations. In addition, the effect of the *KRAS* variant (G allele) on CRC risk was studied. A decreased risk of early-stage CRC was found, but no effect on the risk of advanced stage CRC, indicating that the G-allele (*KRAS* variant) is not associated with a higher likelihood of developing CRC overall.

[108] In previous studies, mutations in *KRAS* have been associated with a poorer prognosis. However, results on this topic are inconsistent and, furthermore, the clinical relevance of these results are unclear (Smits KM, et al. Pharmacogenomics 2008;9:1903–16). Acquired *KRAS* mutations are not the same as the *KRAS* variant, which is a congenital mutation, and, therefore, has a different effect on tumor development, biology, and thus prognosis.

[109] The discovery that the *KRAS* variant is associated with an increased survival in early-stage CRC is intriguing. Previous research has suggested that cellular senescence can be triggered by overexpression of oncogenic Ras and might contribute to growth cessation in premalignant or benign neoplasms (Mooi WJ and Peeper DS. N Engl J Med 2006;355:1037–46). Tumor cell senescence has been reported in human cancers. Premalignant colon adenomas display features of senescence as well (Collado M and Serrano M. Nat Rev Cancer 2010;10:51–7). Oncogene-induced senescence may play a role in premalignant lesions only. Nevertheless, physiologic levels of *KRAS* can induce senescence in the absence of the transcription factor Wilms tumor 1 (WT1) (Vicent S, et al. J Clin Invest 2010;120:3940–52). Lung cancer patients with high *KRAS* gene expression had a good prognosis if they had decreased expression of WT1 related genes (Vicent S, et al. J Clin Invest 2010;120:3940–52). Together, these results imply that other molecular factors can be involved in the determination of cell fate, and that oncogene-induced senescence can occur after an altered expression of other genetic or epigenetic targets. Oncogene-induced senescence could also play a role in CRC: the KRAS-LCS6 genotype could either lead to an advanced stage tumor, or an early-stage tumor with a better prognosis based on the other (epi)genetic markers that are affected.

[110] A better outcome was found for early-stage (stage I and II) cases with the *KRAS* variant and *BRAF* mutations or *RASSF1A* hypermethylation, both of which are involved in the Ras signaling pathway. *BRAF*-associated senescence has previously been reported to occur in melanoma (Michaloglou C, et al. Nature 2005;436:720–4) but a possible role of *RASSF1A* in oncogene-induced senescence has not been demonstrated. As in the study population described herein, the coincidence of the *KRAS* variant with either a *BRAF* mutation and/or *RASSF1A*

hypermethylation is less common, and, therefore, statistical significance was not reached. When combining these (epi)genetic events, the better outcome of patients with a combination of the *KRAS* variant (G-allele) and an alternation of *KRAS*, *BRAF*, or *RASSF1A* was even more enhanced. Thus, Ras overexpression due to the KRAS variant (G-allele), in combination with (epi)genetic alterations in genes from the Ras pathway, could induce senescence in early-stage CRC, thereby influencing survival. For advanced-staged cases, an increasing number of molecular pathways are affected that influence prognosis.

[111] The *let-7* family of miRNA demonstrate a tumor growth suppression effect with decreased *let-7* expression and increased KRAS levels in the presence of the *KRAS* variant compared to wild type (13). Accordingly, patients with the KRAS-variant are expected to have a worse prognosis, as shown for, for instance, in oral cancer (Christensen BC, et al. Carcinogenesis 2009;30:1003–7). For CRC, there are two reports studying the effect of KRAS genotype on outcome in treated patients (Graziano F, et al. Pharmacogenomics J 2010;10:458–64; Zhang W et al. Ann Oncol 2011;22:104–9). The first reports poor survival among a small population of irinotecan-refractory metastatic patients with the *KRAS*-variant treated with Irinotecan and Cetuximab, as well as an association with *KRAS* mutations and the absence of *BRAF* mutations (Graziano F, et al. Pharmacogenomics J 2010;10:458–64), however, these findings could not be replicated in this study as patients were primarily untreated. The second reports a better response to cetuximab alone in metastatic CRC and a longer survival in patients with the *KRAS* variant without *KRAS* mutations, but the response was not statistically significant (Zhang W et al. Ann Oncol 2011;22:104–9). The data presented herein demonstrate a better prognosis in stage IV *KRAS* variant carriers, although the comparison is not statistically significant, which may be explained by the small size of the group of stage IV patients. Other studies used germline tissue to assess the *KRAS* genotype, however, the studies described herein used tumor DNA to assess *KRAS* genotype. It is well documented that genotype of normal and tumor tissue is the same for the *KRAS* variant.

[112] The seemingly discordant results in early and advanced stage CRC raises questions on the origin and progression of tumors in different cancer stages, and whether early-stage CRC might develop through a molecular distinct pathway as compared with advanced stage. The *KRAS*- variant is more common among cases with advanced stage disease, however, patients who are diagnosed early with the *KRAS* variant seem to have a more advantageous outcome.

Thus, the data imply a different biology in early-stage as compared with advanced stage cases. The discovery that early-stage *KRAS* wild-type patients have a poor prognosis, even if they have a MSI tumor, might indicate that these patients would benefit from additional adjuvant treatment. Further research, including randomized clinical trials, is needed to assess whether these early-stage patients with a poor prognosis would benefit from additional adjuvant treatment. Prior to the discovery of the biomarkers and methods described herein, MSI has been considered to be a marker for good prognosis (Boland CR and Goel A. Gastroenterology 2010;138:2073–87.e3) however, the data from this study demonstrate a better outcome for *KRAS* variant allele carriers independent of MSI status.

[113] The analysis presented herein of the influence of the *KRAS* variant in early-stage CRC cases demonstrates a better outcome for early-stage G-allele (*KRAS* variant) carriers with *KRAS* mutations. The population used in this study is the only group studied to date that is generally untreated, and for the first time, the data gathered from this study provides a valuable insight into the natural biology of early stage CRC with the *KRAS* variant. Consequently, the evidence presented herein is the first indication that the *KRAS* variant genotype is a possible prognostic biomarker for early-stage CRC that can be used to identify CRC patients with a good prognosis.

Response to Treatment

Ovarian cancer

[114] Epithelial ovarian cancer (EOC) is the second most common female pelvic reproductive organ cancer in the United States, and carries the highest mortality in this category in the Western world. It is the fifth overall leading cause of cancer death in females in the United States, with 13,850 women dying from this disease yearly. Despite multiple new approaches to treatment, the high rates of death from EOC have remained largely unchanged for many years, with a 5-year overall survival of only 30-39% (Parmar MK, et al. (2003). Lancet 361: 2099-2106).

[115] The standard chemotherapy regimen to treat EOC currently used is carboplatin and paclitaxel (Pfisterer J, et al. (2006). J Clin Oncol 24: 4699-4707), based on prospective randomized trials (Herzog T and Pothuri B. (2006). Nat Clin Pract Oncol 3: 604-611; Esquela-Kerscher A and Slack F. (2006). Nat Rev Cancer 6: 259-269; Iorio M, et al. (2007). Cancer Res 67: 8699-8707). Although some patients are initially resistant to platinum-based chemotherapy (referred to as 'platinum resistant'), developing recurrence within 6 months of treatment, it is the

first line treatment given to all EOC patients. An improved understanding of the fundamental biological differences in EOC tumors that could explain platinum resistance among EOC patients would allow a more rational selection of treatments (Parmar MK, et al. (2003). Lancet 361: 2099-2106; Pfisterer J, et al. (2006). J Clin Oncol 24: 4699-4707; Herzog T and Pothuri B. (2006). Nat Clin Pract Oncol 3: 604-611).

[116] MicroRNAs (miRNAs) are a class of 22-nucleotide noncoding RNAs that are aberrantly expressed in virtually all cancer types, where they can function as a novel class of oncogenes or tumor suppressors. In EOC, in addition to distinguishing normal ovarian tissue from malignant ovarian tissue (Iorio M, et al. (2007). Cancer Res 67: 8699-8707; Zhang L, et al. (2008). Proc Natl Acad Sci USA 105: 7004-7009), miRNA expression patterns have been shown to be important in EOC pathogenesis (Mezzanzanica D, et al. (2010). Int J Biochem Cell Biol 42: 1262-1272; van Jaarsveld M, et al. (2010). Int J Biochem Cell Biol 42: 1282-1290) and are associated with altered EOC patient outcome (Eitan R, et al. (2009). Gynecol Oncol 114: 253-259) and response to treatment (Lu L, et al. (2011). Gynecol Oncol 122: 366-371). MiRNA expression differences have also been associated with chemotherapy and platinum resistance in EOC (Eitan R, et al. (2009). Gynecol Oncol 114: 253-259; Lu L, et al. (2011). Gynecol Oncol 122: 366-371; Chen K, et al. (2008). Carcinogenesis 29:1306-1311).

[117] Additional insight into the importance of miRNAs in cancer has come from the discovery of inherited single-nucleotide polymorphisms that disrupt miRNA coding sequences (Chin LJ, et al. (2008). Cancer Res 68: 8535-8540) and miRNA-binding sites in the 3' untranslated regions (3 ' UTRs) of oncogenes (Chen K, et al. (2008). Carcinogenesis 29:1306-1311; Chin LJ, et al. (2008). Cancer Res 68: 8535-8540). An example of such a functional variant is rs61764370, referred to as the *KRAS* variant, which is located in the KRAS 3' UTR in a *let-7* miRNA complementary site. An association between rs61764370 and epithelial ovarian cancer (EOC) risk was previously reported (see, International Patent Application No. PCT/US2008/065302 and International Patent Application No. PCT/US2010/023412; the contents of which are each herein incorporated in their entireties). Furthermore, the methods and examples provided demonstrate that this variant is a biomarker of clinical outcome and chemotherapy resistance in epithelial ovarian cancer (EOC). The evidence supports a continued functional role of the *KRAS* variant in tumors, an association with aggressive tumor biology and poor cancer-specific outcome.

[118] The potential of the *KRAS* variant to act as a biomarker of outcome in EOC in both the presence and the absence of deleterious BRCA mutations is evaluated herein. Moreover, the potential cause of altered outcome in *KRAS*-variant EOC patients is determined by studying the response to neoadjuvant platinum-based chemotherapy, assessing platinum resistance and evaluating EOC tumor gene expression. The data demonstrate that directly targeting of this gain-of-function *KRAS* variant could reduce cell growth and survival in EOC cell lines with this lesion.

[119] The KRAS variant is a biomarker of poor outcome for postmenopausal women (over 52 years of age) with EOC. The poor outcome in *KRAS* variant-associated ovarian cancer is due, at least in part, to the association of the *KRAS* variant with resistance to platinum-based chemotherapy, based on a worse response to neoadjuvant platinum-based chemotherapy, and statistically significantly increased platinum resistance in adjuvantly-treated EOC patients with the *KRAS* variant.

[120] The biological differences between *KRAS*-variant EOC and nonvariant EOC tumors are supported by gene expression data , which indicates that KRAS addiction and AKT-mediated platinum resistance in *KRAS*-variant-associated EOC. Platinum resistance was further confirmed *in vitro* in an ovarian cancer cell line with the *KRAS* variant as compared with a non-variant line. Evidence for the continued dependence of *KRAS* variant-associated EOC on the *KRAS* variant germline lesion was shown through direct targeting of this mutation, which led to significant inhibition of both tumor growth and cell survival in a *KRAS*-variant EOC cell line versus non-variant EOC lines.

[121] The association of the *KRAS* variant with poor survival for postmenopausal women could be due to underlying biology associated with this variant. In support of the hypothesis that the discovered association reflects underlying biology, the *KRAS* variant is associated with postmenopausal ovarian cancer (Ratner E, et al. (2010). Cancer Res 15: 6509-6515),with a median age of diagnosis near 59 years of age. Relative survival varies by age, with older women twice as likely to die within 5 years of diagnosis of EOC, further supporting the hypothesis that postmenopausal women may have biologically different tumors than younger women (ACS (2010). Cancer facts & figures 2010. Cancer Facts & Figures. ACS: Atlanta, GA, pp 1-56). Furthermore, the *KRAS* variant has been shown to be a biomarker of TNBC risk in premenopausal women, aged < 52 years. Thus, the role of the *KRAS* variant in cancer risk and

biology in different tissues may depend on miRNA expression alterations in response to physiologic conditions, such as menopause. Women with the *KRAS* variant may be first at risk for breast cancer and then, subsequently, be at risk for developing postmenopausal ovarian cancer.

[122] The discovery that the *KRAS* variant does not predict for poor outcome in a cohort of EOC patients with known deleterious *BRCA* mutations may be partially explained by the fact that *BRCA* mutations are associated with platinum sensitivity. Consequences of *BRCA* mutations associated with platinum sensitivity may occur downstream of any resistance caused or exacerbated by the *KRAS* variant to platinum agents. It is possible that the younger patients in the study presented herein could have had undocumented deleterious *BRCA* mutations. Alternatively, or in addition, the younger patients in the study presented herein may also have had other subtypes of ovarian cancer seen more frequently in younger women, such as borderline tumors, resulting in a misdiagnosis of these patients. Although the data provided herein were extensively clinically annotated, *BRCA* status was not obtained on all of our EOC patients, and although pathology reports were available, tumor tissue was not available for re-review. A recent study that failed to find the association of the *KRAS* variant with poor outcome and resistance to therapy in EOC used ovarian collections used for genome-wide association studies that had very limited clinical information, *i.e.,* factors such as *BRCA* status and ovarian cancer specific survival were not available nor included in their analyses (Pharoah P, et al. (2011). Clin Cancer Res 17: 3742-3750).

[123] Similar gene mis-expression patterns were found in two different types of *KRAS* variant-associated tumors, indicating that these tumors, regardless of tissue of origin, use similar pathways in oncogenesis. Direct targeting of the *KRAS*-variant lesion in *KRAS* variant-associated EOC cell lines leads to significantly enhanced cell death and a reduction in KRAS levels. These discoveries suggest a continued critical dependence of *KRAS*-variant tumors on this single, non-coding germline lesion. Although there has been a significant effort to tailor cancer treatment by measuring tumor gene expression and determining tumor-acquired mutations, there are few, if any, germline variants that have previously been shown to be critical targets for therapy in cancer.

[124] Based upon the data provided herein, it is determined that the *KRAS* variant is a functional cancer mutation that is important in ovarian cancer and that the *KRAS* variant allows

meaningful subclassification of the ovarian tumors with which it is associated. These discoveries are useful for improving ovarian cancer patient outcome.

Colorectal cancer

[125] The incorporation in metastatic colorectal cancer (mCRC) clinical practice of two monoclonal antibodies targeting epidermal growth factor receptor (anti-EGFR moAbs), cetuximab and panitumumab, either used as monotherapy or in combination with chemotherapy, provides a modest clinical benefit in pretreated patients (Cunningham D, et al. N Engl J Med 2004; 351(4):337-345; Saltz LB, et al. J Clin Oncol 2004; 22(7):1201-1208; Saltz LB, et al. N Engl J Med 2000; 343(13):905-914; Van CE, et al. J Clin Oncol 2007; 25(13):1658-1664). Nevertheless, it soon became evident that their efficacy was restricted to a subset of patients. Non-randomized retrospective studies (Amado RG, et al. J Clin Oncol 2008; 26(10):1626-1634; De RW, et al. Ann Oncol 2008; 19(3):508-515; Lievre A, et al. Cancer Res 2006; 66(8):3992-3995; Lievre A, et al. J Clin Oncol 2008; 26(3):374-379; Moroni M, et al. Lancet Oncol 2005; 6(5):279-286; Sartore-Bianchi A, al. J Clin Oncol 2007; 25(22):3238-3245), retrospective analysis of prospective randomized trials (Bokemeyer C, et al. J Clin Oncol 2009; 27(5):663-671; Douillard J et al. AnnOncol supp . 2009; Karapetis CS, et al. N Engl J Med 2008; 359(17):1757-1765; Tol J, et al. N Engl J Med 2009; 360(6):563-572; Van Cutsem E, et al. N Engl J Med 2009; 360(14):1408-1417), and a grand European consortium study (De RW, et al. Lancet Oncol 2010; 11(8):753-762) demonstrated that the presence of tumor acquired *KRAS* mutations were predictive of resistance to anti-EGFR moAbs therapy and were associated with a worse prognosis and a shorter survival. While for some years now the *KRAS* mutational status is mandatory for the initiation of anti-EGFR moAb treatment, the issue is unresolved, since, approximately 50-65% of the mCRC patients with *KRAS* wt tumors derive no benefit from these treatments, implying that additional genetic determinants of resistance or perhaps sensitivity exist (De RW, et al. Ann Oncol 2008; 19(3):508-515; Allegra CJ, et al. J Clin Oncol 2009; 27(12):2091-2096; De RW, al. Lancet Oncol 2010; 11(8):753-762; Roock WD,et al. Lancet Oncol 2010). Mounting evidence indicates that the *BRAF* V600E mutation confers resistant to anti-EGFR MoAbs (De RW, et al. Lancet Oncol 2010; 11(8):753-762; Di NF, et al. J Clin Oncol 2008; 26(35):5705-5712; Laurent-Puig P, et al. J Clin Oncol 2009; 27(35):5924-5930; Saridaki Z, et al. IPLoS One 2011; 6(1):e15980; Souglakos J, et al. Br J Cancer 2009; 101(3):465-472), whereas, although not entirely clear yet, *PIK3CA*-mutant tumors seem to derive no or little

benefit from such a treatment (De RW, et al. Lancet Oncol 2010; 11(8):753-762; Prenen H, et al. Clin Cancer Res 2009; 15(9):3184-3188; Sartore-Bianchi A, et al. Cancer Res 2009; 69(5):1851-1857; Jhawer M, et al. Cancer Res 2008; 68(6):1953-1961; Ogino S, et al. J Clin Oncol 2009; 27(9):1477-1484).

[126] In addition to the tumoral genetic characteristics, there is mounting evidence that the germline genome of patients might also play a role in granting resistance or sensitivity to anti-EGFR moAbs therapy. In support of this notion, polymorphisms in the genes encoding for FcγRIIa and FcγRIIIa, EGFR, EGF, cyclinD1 and COX-2 have been associated with outcome in mCRC patients treated with cetuximab administered both as monotherapy and in combination with chemotherapy.

[127] MicroRNAs (miRNAs) are an abundant class of highly conserved, endogenous, non-coding, small RNA molecules, 18-25 nucleotides in length, which negatively regulate gene expression by binding to partially complementary sites in the 3'-untranslated region (UTR) of their target mRNAs. Upon processing by Dicer and Drosha RNase III endonucleases, mature miRNAs can suppress mRNA translation by directing an RNA-induced silencing complex to the target mRNA. MiRNAs regulate of a number of genes involved in basic biological processes such as proliferation, cellular differentiation and apoptosis, and act as important players in cancer development and progression by behaving either as oncogenes or as tumor suppressors. Although more than 700 miRNA sequences have been recognized in the human genome to date, this number is expected to double. Furthermore, each miRNA can control hundreds of genes by regulating many mRNAs simultaneously.

[128] MiRNA binding to mRNAs is critical for the regulation process of mRNA levels and subsequent protein expression, and this regulation can be affected by single-nucleotide polymorphisms (SNPs) occurring in the miRNA target sites. These SNPs can either create erroneous binding sites or abolish (eliminate) the correct ones, leading to resistance to miRNA regulation and reflecting another kind of genetic variability capable of playing a role in human diseases like cancer (or conferring an increased risk for certain diseases like cancer). Emerging research focuses on the systematic genomic evaluation of these sites and the functional and biological relevance of the detected SNPs, which are significant molecular markers in the rapidly growing area of personalized medicine. Such SNPs appear to affect not only gene expression, but also tumor biology and drug response and drug resistance.

[129] The Lethal-7 (*let-7*) family of miRNAs was among the first discovered and its differential expression has been detected in a number of cancers. The *KRAS* oncogene is a direct target of the *let-7* miRNA family, and more precisely, *let-7* was shown to induce *KRAS* downregulation upon binding to certain sites in the 3' untranslated region (3'-UTR) of the *KRAS* mRNA.

[130] The *KRAS* variant is a functional single nucleotide polymorphism (SNP) that occurs in a *let-7* complementary site (LCS) in the *KRAS* 3'-UTR mRNA. This SNP (rs61764370) results from a T to G base substitution, which was found to alter the binding capability of mature *let-7* to the *KRAS* mRNA and results in both an increased expression of the KRAS oncogenic protein *in vitro* and lower *let-7* miRNA levels *in vivo*, possibly due to a negative feedback loop. Consistent with the oncogenic nature of the *KRAS* gene, the *KRAS* variant (also referred to as the G-allele) has been shown to confer an increased non-small cell lung cancer (NSCLC) risk in moderate smokers, an increased risk for the development of triple negative breast cancer and, in a subset of women, ovarian cancer. In addition, an increased frequency of the *KRAS* variant allele was detected among *BRCA1* carriers in a small cohort. Furthermore, *KRAS* variant (G-allele) carriers with head and neck cancer, but not NSCLC, exhibited reduced overall survival. Statistically significantly worse survival and platinum resistance was found in ovarian cancer patients with the *KRAS* variant (G-allele). Together, the evidence demonstrates a functional and clinical significance of the *KRAS* variant (also known as the *KRAS* 3'-UTR LCS6 SNP).

[131] In the mCRC targeted anti-EGFR moAb therapy setting to date, the *KRAS* variant has been evaluated in two studies with small selected populations and with contradicting and conflicting results (Graziano F, et al. Pharmacogenomics J 2010; 10(5):458-464; Zhang W, et al. Ann Oncol 2011; 22(1):104-109). In the first study (Graziano F, et al. Pharmacogenomics J 2010; 10(5):458-464) within a patient population with *KRAS* and *BRAF* wt alleles, and treated with salvage irinotecan-cetuximab combination therapy, *KRAS* variant (G-allele) carriers were shown to have a statistically significant worse progression free survival (PFS) and overall survival (OS). In contrast, in the second study (Zhang W, et al. Ann Oncol 2011; 22(1):104-109)), where patients were exposed to salvage cetuximab monotherapy, *KRAS* variant (G-allele) carriers exhibited a longer PFS and OS and had a better objective response rate (ORR). While these studies seem to have opposite results, these patients were not treated identically, and in fact, the addition of irinotecan chemotherapy to cetuximab was also found to predict a poor response in *KRAS* variant (G-allele) carriers (Winder T, et al. J.Clin.Oncol. [27 (15S Suppl)].

2009. Abstract). The evidence indicates that unlike tumor acquired KRAS protein mutations, the combination of therapy given to *KRAS* variant (G-allele) carriers differentially impacts response to cetuximab (Winder T, et al. J.Clin.Oncol. [27 (15S Suppl)]. 2009. Abstract). This is in agreement with data that such miRNA binding site variants are dynamically regulated in disease.

[132] In this study, the *KRAS* variant, along with other molecular markers like the *KRAS* and *BRAF* mutational status, is evaluated in a series of 559 mCRC patients who underwent salvage anti-EGFR MoAbs monotherapy or MoAbs in combination with chemotherapy. The data presented herein clarify the role of the *KRAS* variant in predicting response to MoAbs therapy. In this patient cohort, as well as in cell lines, that the *KRAS* variant (G allele) predicts a positive response to MoAbs monotherapy, without any additional benefit of cytotoxic chemotherapy.

[133] The studies presented herein demonstrate a statistically significant improvement in median PFS for all *KRAS* variant carriers with metastatic colon cancer (and a trend towards improved OS in the double wt patients) who received anti-EGFR moAbs monotherapy. Moreover, a statistically significant was discovered for a favorable prognosis of these patients compared to non-*KRAS* variant carriers across all cohorts studied in the response to anti-EGFR moAbs, including *KRAS* or *RAF* mutant patients. This improved prognosis was not dependent on the addition of chemotherapy, and in fact, *KRAS* variant (G allele) carriers appeared to have no benefit to chemotherapy in addition to anti-EGFR moAbs therapy. This was in contrast to non-*KRAS* variant patients, who derived a significant benefit from the addition of chemotherapy to anti-EGFR moAbs across all cohorts, and the addition of chemotherapy brought their prognosis to the same level of *KRAS* variant allele carriers who received anti-EGFR moAbs monotherapy. Cell lines studies showed the same effect with lack of benefit of combination therapy in *KRAS* variant cell lines compared to non-variant cell lines. These findings suggest for the first time the *KRAS* variant allele patients with metastatic colon cancer could and perhaps should avoid the toxic and sometimes deadly affect of chemotherapy treatment, and could be meaningfully treated with anti-EGFR moAbs monotherapy alone.

[134] A population of patients mainly of European origin showed an elevated frequency of the *KRAS* variant of 19.5%, compared to reported baseline prevalences. While the *KRAS* variant is found in 6% of the world population, its frequency has been estimated to rise above 10% in healthy Caucasians. Furthermore, the prevalence of the *KRAS* variant is substantially increased to almost 20% in patients suffering from NSCLC, highlighting an association of increased risk. In

the Caucasian mCRC patient population with European descent studied by Graziano et al (Pharmacogenomics J 2010; 10(5):458-464) the *KRAS* variant (G allele) (incorporating both TG and GG genotypes) was found in 25%, of patients, whereas, in a more heterogeneous population in the study by Zhang et al (Ann Oncol 2011; 22(1):104-109) the frequency of the *KRAS* variant was 15.3%. Data provided herein did not find that the *KRAS* variant allele was a risk for developing colon cancer, although the *KRAS* variant was enriched in patients with Stage IV disease. Together, the evidence indicates that although the *KRAS* variant (G allele) is not a risk for all types of colon cancer, it is associated with the likelihood of developing advanced and metastatic colon cancer. The KRAS variant predicts a good prognosis in both early stage colon cancer as well as metastatic colon cancer patients when treated with Cetuximab monotherapy. However, the *KRAS* variant (G allele) may be associated with the development of metastatic disease in colon cancer, which is universally fatal.

[135] A different distribution of the *KRAS* variant genotypes according to the *KRAS* and *BRAF* mutational status was observed in this study with respect to the mCRC patient population compared to prior reports. In this study, the *KRAS* genotypes were equally distributed among the *KRAS* wt and mutated groups, but, in the *BRAF* mutated group, the frequency of the *KRAS* variant was statistically significantly increased, *i.e.*, twice as high compared to wild type. In the later stages of CRC carcinogenesis, the *KRAS* variant allele may mediate the selection of less differentiated and more aggressive clones that carry *BRAF* mutations. Additionally, a selective pressure may favor the development of *KRAS* or *BRAF* mutations in the presence of the *KRAS* variant, depending on exposure to specific therapies. Patients with the *KRAS* variant (G allele) have a different prognosis when treated with Cetuximab regardless of patients also having a *KRAS* or a *BRAF* mutation, suggesting that these groups need re-evaluation for the potential of Cetuximab treatment.

[136] When the survival outcomes were analyzed according to treatment, in the whole and the double wt patient populations treated with anti-EGFR moAbs monotherapy, the *KRAS* variant genotype carriers had a statistically significantly longer PFS ($p = 0.019$ and $p = 0.039$, respectively). Although, in the whole monotherapy patient population the *KRAS* variant genotype carriers had a longer OS of 45 weeks compared to 28.85 weeks of the wt carriers, nevertheless this difference did not reach statistical significance. In the double (*KRAS* and *BRAF*) wt patient

population a trend towards statistical significance ($p = 0.087$) was observed with a longer OS in favor of the *KRAS* variant carriers (55.43 vs. 35.71 weeks).

[137] Cetuximab/irinotecan treated *KRAS* mutated patients with the *KRAS* variant (G-allele) genotype showed a significantly worse PFS of 6.4 weeks compared to 12 weeks in those patients with the LCS6 wt genotype ($p = 0.037$, log-rank test). In our analysis, in the anti-EGFR moAbs-based combination chemotherapy group, where people were treated with a variety of agents, no statistically significant differences were found in PFS or OS in any population between the *KRAS* variant and wt genotype carriers. There was a trend for worse survival (23 versus 28 weeks) in *KRAS* variant carriers with *KRAS* or *RAF* mutations when they received chemotherapy versus monotherapy, respectively. These findings collectively may indicate that certain chemotherapy in combination with anti-EGFR moAbs-based therapy in *KRAS* variant carriers is detrimental.

[138] An important step in the development of CRC, among other cancers, is the deregulation of miRNAs. Over the past few years miRNAs have been brought to the central stage of molecular oncology and have substantially changed the way we view and understand gene regulation. The *KRAS* variant was the first SNP in a miRNA binding site to be implicated in cancer risk. The data presented herein indicate that patients carrying the *KRAS* variant allele genotype are biologically different then non-variant, or LCS6 wt, patients. Patients carrying the *KRAS* variant allele genotype have a higher probability of benefit from anti-EGFR moAbs monotherapy as well as a better overall prognosis, without a benefit from the addition of chemotherapy. Because tumors with the KRAS variant induce overexpression of the KRAS pathway, upstream inhibition of this pathway could specifically sensitize these tumors. This mechanism appears to contradict the lack of efficacy of moAbs therapy in tumor acquired *KRAS* mutant tumors, however, it is possible that the *KRAS* variant does not induce as high of a level of independent KRAS pathway signaling as tumor acquired *KRAS* mutations.

[139] *KRAS* variant tumors derive no benefit from the addition of cytotoxic therapy to moAbs monotherapy. Because the *KRAS* variant is regulated by the *let-7* family of miRNA, and because chemotherapy lowers *let-7* levels and allows higher *KRAS* expression (especially in the presence of the *KRAS* variant), treatment with chemotherapy may increase activation of this allele, thereby removing the ability of upstream moAbs therapy to overcome KRAS pathway activation. The potential of the 3' UTR functional variants, including the KRAS variant, to predict altered tumor biology and response to treatment and allow better risk stratification of patients.

MicroRNA

[140] MicroRNAs (miRNAs) are a novel class of small non-coding RNAs that regulate gene expression by base pairing with sequences within the 3'-untranslated regions (UTR) of target mRNAs, as well as 5'- untranslated regions (UTR) and coding sequence regions, causing mRNA cleavage and/or translational repression (He L, et al. Nature 2005; 435: 828–33; Esquela-Kerscher A. and Slack FJ. Nat Rev Cancer 2006; 6: 259–69). MiRNAs are misregulated in every cancer studied thus far, including, but not limited to, breast and colorectal cancers, where certain miRNA alterations (and specifically reduced *let-7)* are found in tumor-initiating cells, suggesting that low *let-7* allows self-renewal and proliferation of these cells (Yu F, et al. Cell 2007; 131: 1109–23) and increases cancer risk.

[141] Because miRNAs act as global gene regulators, inherited variations in miRNAs are associated with increased cancer risk. Evidence is quickly growing that polymorphisms disrupting miRNA coding sequences (Hoffman A, et al. Cancer Res 2009; 69: 5970–77) or 3'UTR miRNA binding sites are strong predictors of cancer risk, including, but not limited to, breast and colorectal cancers (Pongsavee M, et al. Genet Test Mol Biomarkers 2009; 13: 307–17; Tchatchou S, et al. Carcinogenesis 2009; 30: 59–64). However, none of the previously identified miRNA-altering polymorphisms has been associated with triple negative breast cancer (TNBC), or with altered gene and/or miRNA expression in tumors.

[142] A novel germline polymorphism (*rs61764370*) in a *let-7* miRNA complementary site within the 3'UTR of the *KRAS* oncogene was recently identified (International Patent Application No. PCT/US2008/065302, the contents of which are incorporated herein by reference in their entirety), referred to as the "LCS6-SNP' or the '*KRAS*-variant'.

[143] The *KRAS* variant is associated with low concentrations of *let-7* in tumors and altered KRAS regulation in lung cancer (Chin L, et al. Cancer Res 2008; 68: 8535–40). Moreover, the *KRAS* variant predicts poor cancer specific outcome in head and neck cancer (Christensen BC, et al. Carcinogenesis 2009; 30: 1003–07) and altered drug response in colon cancer (Graziano F, et al. Pharmacogenomics J 2010; 10: 458–64; Zhang W, et al. Ann Oncol 2011; 22: 104–09). The *KRAS* variant is also enriched in ovarian cancer and is most frequently associated with patients from families with Hereditary Breast and Ovarian Cancer (HBOC) (Ratner E, et al. Cancer Res 2010; 70: 6509–15). The studies provided herein further assess the role of the KRAS variant in cancer risk and tumor biology.

[144] The data provided herein demonstrate, for example, that a germline polymorphism in the *KRAS* 3'UTR, known as the '*KRAS* variant', is a genetic marker of an increased risk of developing triple negative breast cancer for premenopausal women. Because study group 1 was small and only assessed in patients with known ER and PR statuses, this association was validated in larger case controls with full receptor status. Most importantly, the data demonstrate that the tumors of patients with triple negative breast cancer (TNBC) who have the *KRAS*-variant have distinct gene expression patterns compared to other patients without the *KRAS*-variant, demonstrating that the *KRAS*-variant drives specific pathways that are known to influence tumor biology and modify tumor development. Thus, the *KRAS*-variant can classify tumors into meaningful biological subgroups to both predict prognosis as well as direct treatment decisions in the future.

[145] The finding of reduced *let-7* expression in TNBC tumors associated with the *KRAS*-variant is clinically important. *KRAS* overexpression, through NFKB, can lead to induction of *lin-28*, a negative regulator of *let-7*, and, consequently, lowering of *let-7* expression (Iliopoulos D, et al. Cell 2009; 139: 1–14; Meylan E, et al. Nature 2009; 462: 104–08; Barbie D, et al. Nature 2009; 462: 108–12). This suggests a potential mechanism whereby *let-7* is lowered in pre-malignant tissue,, and ultimately, tumors associated with the *KRAS*-variant. Furthermore, *let-7* regulates proliferation of breast like stem cells (Yu F, Yao H, Zhu P, et al. Cell 2007; 131: 1109–23), and low *let-7* expression or concentrations allow expansion of this group of cells, thereby increasing breast cancer risk in women with the *KRAS*-variant. The association of the *KRAS* variant with TNBC risk only in premenopausal women indicates a meaningful interaction between the *KRAS*-variant and hormonal exposure.

[146] Although more than half of breast tumors that carry the *BRCA1* mutation develop into the triple negative subtype (TNBC) (Atchley DP, et al. J Clin Oncol 2008; 26: 4282–88), *BRCA1* mutations are rare, and, thus, account for only about 10-15% of all TNBC cases (Young SR, et al. BMC Cancer 2009; 9: 86; Nanda R, et al. JAMA 2005; 294: 1925–33). The *KRAS*-variant is found in up to 23% of premenopausal TNBC patients, without an apparent significant enrichment in *BRCA* mutation carriers from these cohorts or in young ER/PR negative *BRCA1* mutation carriers (miRNA profiling, publicly available at www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/mirna-profiling.html (accessed Jan 1, 2008)). The *KRAS*-variant is associated with a *BRCA1* mutant-

like gene expression signature, indicating that there may be increased oncogenic risk in the presence of the *KRAS* variant, high *KRAS* expression and low *BRCA1* expression, either through mutation or other mechanisms.

[147] The *KRAS*-variant affects the regulation of KRAS expression *in vitro* and promotion of higher KRAS concentrations (Chin L, et al. Cancer Res 2008; 68: 8535–40). The *KRAS* oncogene is an important upstream mediator of the MAPK pathway, and its overexpression can result in increased activation of the Raf/MEK/MAPK pathway, thereby promoting tumorigenesis. The studies provided herein demonstrate that patients with the *KRAS*-variant and TNBC show activation of the MAPK pathway (Table X). Hyperactivation of MAPK in breast cancer cells decreases ER expression leading to an ER -negative phenotype (Atchley DP, et al. J Clin Oncol 2008; 26: 4282–88), which agrees with our finding that the *KRAS* variant is associated with even lower estrogen signaling in these histologically ER negative tumors. MAPK activation has been implicated in estrogen-independent tumor growth and insensitivity to anti-estrogen treatment (Oh AS, et al. Mol Endocrinol 2001; 15: 1344–59), and might be a mechanism by which the *KRAS*-variant drives the development of TNBC more than other breast cancer subtypes.

[148] The *KRAS*-variant is a biomarker of poor outcome in several cancers, including head and neck cancer (Christensen BC, et al. Carcinogenesis 2009; 30: 1003–07). The *KRAS*-variant is also a biomarker of poor response to targeted therapies in combination with chemotherapy in colon cancer (Graziano F, et al. Pharmacogenomics J 2010; 10: 458–64). The discovery that *KRAS*-variant positive TNBC patients have a luminal progenitor signature and differential expression of angiogenic and metastatic markers within the signature demonstrates that tumors harboring the *KRAS* variant are an aggressive sub-group of TNBC.

[149] The study provided herein demonstrates that the *KRAS*-variant is associated with tumors that maintain unique gene expression patterns. Although work is ongoing, data from these studies provide valuable insight into critical steps and pathways required for transformation and tumor development in these women. These are meaningful steps towards understanding the mechanisms of gain of function miRNA disrupting polymorphisms in cancer biology, which are unique in function from previously discovered genetic markers of cancer risk.

KRAS Variant

[150] The disclosure is based, in part, upon the unexpected discovery that the presence of a SNP in the 3' untranslated region (UTR) of *KRAS*, referred to herein as the "LCS6 SNP" or the "KRAS variant," which is predictive of an individual's risk of developing cancer and an individual's response to treatment for cancer. The KRAS variant is located in LCS6, the wild type and variant sequence of which is provided below.

[151] The KRAS variant may be represented by one or more of the following sequences. For example, the KRAS variant may be defined by the GenBank accession number *rs61764370* and the sequence GTCTCGAACTCCTGACCTCAAGTGAT**G**CACCCACCTTGGCCTCATAAACCTG (SEQ ID NO: 22, in which the SNP is bolded and underlined).

[152] There are three human RAS genes comprising HRAS, KRAS, and NRAS. Each gene comprises multiple miRNA complementary sites in the 3'UTR of their mRNA transcripts. Specifically, each human RAS gene comprises multiple *let-7* complementary sites (LCSs). The let-7 family-of-microRNAs (miRNAs) includes global genetic regulators important in controlling lung cancer oncogene expression by binding to the 3'UTRs (untranslated regions) of their target messenger RNAs (mRNAs).

[153] Specifically, the term "*let-7* complementary site" is meant to describe any region of a gene or gene transcript that binds a member of the *let-7* family of miRNAs. Moreover, this term encompasses those sequences within a gene or gene transcript that are complementary to the sequence of a *let-7* family miRNA. The term "complementary" describes a threshold of binding between two sequences wherein a majority of nucleotides in each sequence are capable of binding to a majority of nucleotides within the other sequence *in trans*.

[154] The Human KRAS 3' UTR comprises 8 LCSs named LCS1-LCS8, respectively. For the following sequences, thymine (T) may be substituted for uracil (U). LCS1 comprises the sequence GACAGUGGAAGUUUUUUUUCCUCG (SEQ ID NO: 1). LCS2 comprises the sequence AUUAGUGUCAUCUUGCCUC (SEQ ID NO: 2). LCS3 comprises the sequence AAUGCCCUACAUCUUAUUUUCCUCA (SEQ ID NO: 3). LCS4 comprises the sequence GGUUCAAGCGAUUCUCGUGCCUCG (SEQ ID NO: 4). LCS5 comprises the sequence GGCUGGUCCGAACUCCUGACCUCA (SEQ ID NO: 5). LCS6 comprises the sequence GAUUCACCCACCUUGGCCUCA (SEQ ID NO: 6). LCS7 comprises the sequence

GGGUGUUAAGACUUGACACAGUACCUCG (SEQ ID NO: 7). LCS8 comprises the sequence AGUGCUUAUGAGGGGAUAUUUAGGCCUC (SEQ ID NO: 8).

[155] Human KRAS has two wild type forms, encoded by transcripts a and b, which are provided below as SEQ ID NOs: 9 and 10, respectively. The sequences of each human KRAS transcript, containing the LCS6 SNP, are provided below as SEQ ID NOs: 11 and 12.

[156] Human KRAS, transcript variant a, is encoded by the following mRNA sequence (NCBI Accession No. NM_033360 and SEQ ID NO: 9) (untranslated regions are bolded, LCS6 is underlined):

```
   1 ggccgcggcg gcggaggcag cagcggcggc ggcagtggcg gcggcgaagg tggcggcggc
  61 tcggccagta ctcccggccc ccgccatttc ggactgggag cgagcgcggc gcaggcactg
 121 aaggcggcgg cggggccaga ggctcagcgg ctcccaggtg cgggagagag gcctgctgaa
 181 aatgactgaa tataaacttg tggtagttgg agctggtggc gtaggcaaga gtgccttgac
 241 gatacagcta attcagaatc attttgtgga cgaatatgat ccaacaatag aggattccta
 301 caggaagcaa gtagtaattg atggagaaac ctgtctcttg gatattctcg acacagcagg
 361 tcaagaggag tacagtgcaa tgagggacca gtacatgagg actggggagg gctttctttg
 421 tgtatttgcc ataaataata ctaaatcatt tgaagatatt caccattata gagaacaaat
 481 taaaagagtt aaggactctg aagatgtacc tatggtccta gtaggaaata aatgtgattt
 541 gccttctaga acagtagaca caaaacaggc tcaggactta gcaagaagtt atggaattcc
 601 ttttattgaa acatcagcaa agacaagaca gagagtggag gatgcttttt atacattggt
 661 gagggagatc cgacaataca gattgaaaaa aatcagcaaa gaagaaaaga ctcctggctg
 721 tgtgaaaatt aaaaaatgca ttataatgta atctgggtgt tgatgatgcc ttctatacat
 781 tagttcgaga aattcgaaaa cataaagaaa agatgagcaa agatggtaaa aagaagaaaa
 841 agaagtcaaa gacaaagtgt gtaattatgt aaatacaatt tgtacttttt tcttaaggca
 901 tactagtaca agtggtaatt tttgtacatt acactaaatt attagcattt gttttagcat
 961 tacctaattt ttttcctgct ccatgcagac tgttagcttt taccttaaat gcttatttta
1021 aaatgacagt ggaagttttt ttttcctcta agtgccagta ttcccagagt tttggttttt
1081 gaactagcaa tgcctgtgaa aaagaaactg aatacctaag atttctgtct tggggttttt
1141 ggtgcatgca gttgattact tcttattttt cttaccaatt gtgaatgttg gtgtgaaaca
1201 aattaatgaa gcttttgaat catccctatt ctgtgtttta tctagtcaca taaatggatt
1261 aattactaat ttcagttgag accttctaat tggtttttac tgaaacattg agggaacaca
1321 aatttatggg cttcctgatg atgattcttc taggcatcat gtcctatagt ttgtcatccc
1381 tgatgaatgt aaagttacac tgttcacaaa ggttttgtct cctttccact gctattagtc
1441 atggtcactc tccccaaaat attatatttt ttctataaaa agaaaaaaat ggaaaaaaat
1501 tacaaggcaa tggaaactat tataaggcca tttccttttc acattagata aattactata
1561 aagactccta atagcttttc ctgttaaggc agacccagta tgaaatgggg attattatag
1621 caaccatttt ggggctatat ttacatgcta ctaaattttt ataataattg aaaagatttt
1681 aacaagtata aaaaattctc ataggaatta aatgtagtct ccctgtgtca gactgctctt
1741 tcatagtata actttaaatc ttttcttcaa cttgagtctt tgaagatagt tttaattctg
1801 cttgtgacat taaaagatta tttgggccag ttatagctta ttaggtgttg aagagaccaa
1861 ggttgcaagg ccaggccctg tgtgaacctt tgagctttca tagagagttt cacagcatgg
1921 actgtgtccc cacggtcatc cagtgttgtc atgcattggt tagtcaaaat ggggagggac
1981 tagggcagtt tggatagctc aacaagatac aatctcactc tgtggtggtc ctgctgacaa
2041 atcaagagca ttgcttttgt ttcttaagaa aacaaactct tttttaaaaa ttacttttaa
2101 atattaactc aaaagttgag attttggggt ggtggtgtgc caagacatta attttttttt
2161 taaacaatga agtgaaaaag ttttacaatc tctaggtttg gctagttctc ttaacactgg
2221 ttaaattaac attgcataaa cacttttcaa gtctgatcca tatttaataa tgctttaaaa
2281 taaaaataaa aacaatcctt ttgataaatt taaaatgtta cttattttaa aataaatgaa
2341 gtgagatggc atggtgaggt gaaagtatca ctggactagg aagaaggtga cttaggttct
2401 agataggtgt cttttaggac tctgattttg aggacatcac ttactatcca tttcttcatg
2461 ttaaaagaag tcatctcaaa ctcttagttt ttttttttta caactatgta atttatattc
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2521 catttacata aggatacact tatttgtcaa gctcagcaca atctgtaaat ttttaaccta
2581 tgttacacca tcttcagtgc cagtcttggg caaaattgtg caagaggtga agtttatatt
2641 tgaatatcca ttctcgtttt aggactcttc ttccatatta gtgtcatctt gcctccctac
2701 cttccacatg ccccatgact tgatgcagtt ttaatacttg taattcccct aaccataaga
2761 tttactgctg ctgtggatat ctccatgaag ttttcccact gagtcacatc agaaatgccc
2821 tacatcttat ttcctcaggg ctcaagagaa tctgacagat accataaagg gatttgacct
2881 aatcactaat tttcaggtgg tggctgatgc tttgaacatc tctttgctgc ccaatccatt
2941 agcgacagta ggatttttca aacctggtat gaatagacag aaccctatcc agtggaagga
3001 gaatttaata aagatagtgc tgaaagaatt ccttaggtaa tctataacta ggactactcc
3061 tggtaacagt aatacattcc attgttttag taaccagaaa tcttcatgca atgaaaaata
3121 ctttaattca tgaagcttac ttttttttt tggtgtcaga gtctcgctct tgtcacccag
3181 gctggaatgc agtggcgcca tctcagctca ctgcaacctc catctcccag gttcaagcga
3241 ttctcgtgcc tcggcctcct gagtagctgg gattacaggc gtgtgccact acactcaact
3301 aatttttgta ttttaggag agacggggtt tcaccctgtt ggccaggctg gtctcgaact
3361 cctgacctca agtgattcac ccaccttggc ctcataaacc tgttttgcag aactcattta
3421 ttcagcaaat atttattgag tgcctaccag atgccagtca ccgcacaagg cactgggtat
3481 atggtatccc caaacaagag acataatccc ggtccttagg tagtgctagt gtggtctgta
3541 atatcttact aaggcctttg gtatacgacc cagagataac acgatgcgta ttttagtttt
3601 gcaaagaagg ggtttggtct ctgtgccagc tctataattg ttttgctacg attccactga
3661 aactcttcga tcaagctact ttatgtaaat cacttcattg ttttaaagga ataaacttga
3721 ttatattgtt tttttatttg gcataactgt gattctttta ggacaattac tgtacacatt
3781 aaggtgtatg tcagatattc atattgaccc aaatgtgtaa tattccagtt ttctctgcat
3841 aagtaattaa aatatactta aaaattaata gttttatctg ggtacaaata aacaggtgcc
3901 tgaactagtt cacagacaag gaaacttcta tgtaaaaatc actatgattt ctgaattgct
3961 atgtgaaact acagatcttt ggaacactgt ttaggtaggg tgttaagact tacacagtac
4021 ctcgtttcta cacagagaaa gaaatggcca tacttcagga actgcagtgc ttatgagggg
4081 atatttaggc ctcttgaatt tttgatgtag atgggcattt ttttaaggta gtggttaatt
4141 acctttatgt gaactttgaa tggtttaaca aaagatttgt ttttgtagag attttaaagg
4201 gggagaattc tagaaataaa tgttacctaa ttattacagc cttaaagaca aaaatccttg
4261 ttgaagtttt tttaaaaaaa gctaaattac atagacttag gcattaacat gtttgtggaa
4321 gaatatagca gacgtatatt gtatcatttg agtgaatgtt cccaagtagg cattctaggc
4381 tctatttaac tgagtcacac tgcataggaa tttagaacct aacttttata ggttatcaaa
4441 actgttgtca ccattgcaca attttgtcct aatatataca tagaaacttt gtggggcatg
4501 ttaagttaca gtttgcacaa gttcatctca tttgtattcc attgattttt tttttcttct
4561 aaacattttt tcttcaaaca gtatataact ttttttaggg gatttttttt tagacagcaa
4621 aaactatctg aagatttcca tttgtcaaaa agtaatgatt tcttgataat tgtgtagtaa
4681 tgtttttttag aacccagcag ttaccttaaa gctgaattta tatttagtaa cttctgtgtt
4741 aatactggat agcatgaatt ctgcattgag aaactgaata gctgtcataa aatgaaactt
4801 tctttctaaa gaaagatact cacatgagtt cttgaagaat agtcataact agattaagat
4861 ctgtgtttta gtttaatagt ttgaagtgcc tgtttgggat aatgataggt aatttagatg
4921 aatttagggg aaaaaaaagt tatctgcaga tatgttgagg gcccatctct ccccccacac
4981 ccccacagag ctaactgggt tacagtgttt tatccgaaag tttccaattc cactgtcttg
5041 tgttttcatg ttgaaaatac ttttgcattt ttcctttgag tgccaatttc ttactagtac
5101 tatttcttaa tgtaacatgt ttacctggaa tgtattttaa ctatttttgt atagtgtaaa
5161 ctgaaacatg cacattttgt acattgtgct ttcttttgtg ggacatatgc agtgtgatcc
5221 agttgttttc catcatttgg ttgcgctgac ctaggaatgt tggtcatatc aaacattaaa
5281 aatgaccact cttttaattg aaattaactt ttaaatgttt ataggagtat gtgctgtgaa
5341 gtgatctaaa atttgtaata tttttgtcat gaactgtact actcctaatt attgtaatgt
5401 aataaaaata gttacagtga caaaaaaaaa aaaaaa

[157] Human KRAS, transcript variant b, is encoded by the following mRNA sequence (NCBI Accession No. NM_004985 and SEQ ID NO: 10)(untranslated regions are bolded, LCS6 is underlined):

1 ggccgcggcg gcggaggcag cagcggcggc ggcagtggcg gcggcgaagg tggcggcggc

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  61 tcggccagta ctcccggccc ccgccatttc ggactgggag cgagcgcggc gcaggcactg
 121 aaggcggcgg cggggccaga ggctcagcgg ctcccaggtg cgggagagag gcctgctgaa
 181 aatgactgaa tataaacttg tggtagttgg agctggtggc gtaggcaaga gtgccttgac
 241 gatacagcta attcagaatc attttgtgga cgaatatgat ccaacaatag aggattccta
 301 caggaagcaa gtagtaattg atggagaaac ctgtctcttg gatattctcg acacagcagg
 361 tcaagaggag tacagtgcaa tgagggacca gtacatgagg actggggagg gctttctttg
 421 tgtatttgcc ataaataata ctaaatcatt tgaagatatt caccattata gagaacaaat
 481 taaaagagtt aaggactctg aagatgtacc tatggtccta gtaggaaata aatgtgattt
 541 gccttctaga acagtagaca caaaacaggc tcaggactta gcaagaagtt atggaattcc
 601 ttttattgaa acatcagcaa agacaagaca gggtgttgat gatgccttct atacattagt
 661 tcgagaaatt cgaaaacata aagaaaagat gagcaaagat ggtaaaaaga agaaaaagaa
 721 gtcaaagaca aagtgtgtaa ttatgtaaat acaatttgta ctttttctt aaggcatact
 781 agtacaagtg gtaatttttg tacattacac taaattatta gcatttgttt tagcattacc
 841 taatttttt cctgctccat gcagactgtt agcttttacc ttaaatgctt attttaaaat
 901 gacagtggaa gttttttttt cctctaagtg ccagtattcc cagagttttg gtttttgaac
 961 tagcaatgcc tgtgaaaaag aaactgaata cctaagattt ctgtcttggg gtttttggtg
1021 catgcagttg attacttctt atttttctta ccaattgtga atgttggtgt gaaacaaatt
1081 aatgaagctt ttgaatcatc cctattctgt gttttatcta gtcacataaa tggattaatt
1141 actaatttca gttgagacct tctaattggt ttttactgaa acattgaggg aacacaaatt
1201 tatgggcttc ctgatgatga ttcttctagg catcatgtcc tatagtttgt catccctgat
1261 gaatgtaaag ttacactgtt cacaaaggtt ttgtctcctt tccactgcta ttagtcatgg
1321 tcactctccc caaaatatta tatttttct ataaaaagaa aaaaatggaa aaaaattaca
1381 aggcaatgga aactattata aggccatttc cttttcacat tagataaatt actataaaga
1441 ctcctaatag cttttcctgt taaggcagac ccagtatgaa atggggatta ttatagcaac
1501 cattttgggg ctatatttac atgctactaa atttttataa taattgaaaa gattttaaca
1561 agtataaaaa attctcatag gaattaaatg tagtctccct gtgtcagact gctctttcat
1621 agtataactt taaatctttt cttcaacttg agtctttgaa gatagtttta attctgcttg
1681 tgacattaaa agattatttg ggccagttat agcttattag gtgttgaaga gaccaaggtt
1741 gcaaggccag gccctgtgtg aacctttgag ctttcataga gagtttcaca gcatggactg
1801 tgtccccacg gtcatccagt gttgtcatgc attggttagt caaaatgggg agggactagg
1861 gcagtttgga tagctcaaca agatacaatc tcactctgtg gtggtcctgc tgacaaatca
1921 agagcattgc ttttgtttct taagaaaaca aactcttttt taaaaattac ttttaaatat
1981 taactcaaaa gttgagattt tggggtggtg gtgtgccaag acattaattt ttttttaaa
2041 caatgaagtg aaaaagtttt acaatctcta ggtttggcta gttctcttaa cactggttaa
2101 attaacattg cataaacact tttcaagtct gatccatatt taataatgct ttaaaataaa
2161 aataaaaaca atccttttga taaatttaaa atgttactta ttttaaaata aatgaagtga
2221 gatggcatgg tgaggtgaaa gtatcactgg actaggaaga aggtgactta ggttctagat
2281 aggtgtcttt taggactctg attttgagga catcacttac tatccatttc ttcatgttaa
2341 aagaagtcat ctcaaactct tagtttttt ttttacaac tatgtaattt atattccatt
2401 tacataagga tacacttatt tgtcaagctc agcacaatct gtaaattttt aacctatgtt
2461 acaccatctt cagtgccagt cttgggcaaa attgtgcaag aggtgaagtt tatatttgaa
2521 tatccattct cgttttagga ctcttcttcc atattagtgt catcttgcct ccctaccttc
2581 cacatgcccc atgacttgat gcagttttaa tacttgtaat tcccctaacc ataagattta
2641 ctgctgctgt ggatatctcc atgaagtttt cccactgagt cacatcagaa atgccctaca
2701 tcttatttcc tcagggctca agagaatctg acagatacca taaagggatt tgacctaatc
2761 actaattttc aggtggtggc tgatgctttg aacatctctt tgctgcccaa tccattagcg
2821 acagtaggat ttttcaaacc tggtatgaat agacagaacc ctatccagtg gaaggagaat
2881 ttaataaaga tagtgctgaa agaattcctt aggtaatcta taactaggac tactcctggt
2941 aacagtaata cattccattg ttttagtaac cagaaatctt catgcaatga aaaatacttt
3001 aattcatgaa gcttactttt ttttttggt gtcagagtct cgctcttgtc acccaggctg
3061 gaatgcagtg gcgccatctc agctcactgc aacctccatc tcccaggttc aagcgattct
3121 cgtgcctcgg cctcctgagt agctgggatt acaggcgtgt gccactacac tcaactaatt
3181 tttgtatttt taggagagac ggggtttcac cctgttggcc aggctggtct cgaactcctg
3241 acctcaagtg attcacccac cttggcctca taaacctgtt ttgcagaact catttattca
3301 gcaaatattt attgagtgcc taccagatgc cagtcaccgc acaaggcact gggtatatgg
3361 tatccccaaa caagagacat aatcccggtc cttaggtagt gctagtgtgg tctgtaatat
3421 cttactaagg cctttggtat acgacccaga gataacacga tgcgtatttt agttttgcaa
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3481 agaaggggtt tggtctctgt gccagctcta taattgtttt gctacgattc cactgaaact
3541 cttcgatcaa gctactttat gtaaatcact tcattgtttt aaaggaataa acttgattat
3601 attgtttttt tatttggcat aactgtgatt cttttaggac aattactgta cacattaagg
3661 tgtatgtcag atattcatat tgacccaaat gtgtaatatt ccagttttct ctgcataagt
3721 aattaaaata tacttaaaaa ttaatagttt tatctgggta caaataaaca ggtgcctgaa
3781 ctagttcaca gacaaggaaa cttctatgta aaaatcacta tgatttctga attgctatgt
3841 gaaactacag atctttggaa cactgtttag gtagggtgtt aagacttaca cagtacctcg
3901 tttctacaca gagaaagaaa tggccatact tcaggaactg cagtgcttat gaggggatat
3961 ttaggcctct tgaatttttg atgtagatgg gcatttttt aaggtagtgg ttaattacct
4021 ttatgtgaac tttgaatggt ttaacaaaag atttgttttt gtagagattt taaaggggga
4081 gaattctaga aataaatgtt acctaattat tacagcctta aagacaaaaa tccttgttga
4141 agtttttta aaaaaagcta aattacatag acttaggcat taacatgttt gtggaagaat
4201 atagcagacg tatattgtat catttgagtg aatgttccca agtaggcatt ctaggctcta
4261 tttaactgag tcacactgca taggaattta gaacctaact tttataggtt atcaaaactg
4321 ttgtcaccat tgcacaattt tgtcctaata tatacataga aactttgtgg ggcatgttaa
4381 gttacagttt gcacaagttc atctcatttg tattccattg attttttttt tcttctaaac
4441 atttttttctt caaacagtat ataacttttt ttaggggatt ttttttaga cagcaaaaac
4501 tatctgaaga tttccatttg tcaaaaagta atgatttctt gataattgtg tagtaatgtt
4561 ttttagaacc cagcagttac cttaaagctg aatttatatt tagtaacttc tgtgttaata
4621 ctggatagca tgaattctgc attgagaaac tgaatagctg tcataaaatg aaactttctt
4681 tctaaagaaa gatactcaca tgagttcttg aagaatagtc ataactagat taagatctgt
4741 gttttagttt aatagtttga agtgcctgtt tgggataatg ataggtaatt tagatgaatt
4801 taggggaaaa aaaagttatc tgcagatatg ttgagggccc atctctcccc ccacaccccc
4861 acagagctaa ctgggttaca gtgttttatc cgaaagtttc caattccact gtcttgtgtt
4921 ttcatgttga aaatactttt gcatttttcc tttgagtgcc aatttcttac tagtactatt
4981 tcttaatgta acatgtttac ctggaatgta ttttaactat ttttgtatag tgtaaactga
5041 aacatgcaca ttttgtacat tgtgctttct tttgtgggac atatgcagtg tgatccagtt
5101 gttttccatc atttggttgc gctgacctag gaatgttggt catatcaaac attaaaaatg
5161 accactcttt taattgaaat taacttttaa atgtttatag gagtatgtgc tgtgaagtga
5221 tctaaaattt gtaatatttt tgtcatgaac tgtactactc ctaattattg taatgtaata
5281 aaaatagtta cagtgacaaa aaaaaaaaaa aa
```

[158] Human KRAS, transcript variant a, comprising the LCS6 SNP, is encoded by the following mRNA sequence (SEQ ID NO: 11) (untranslated regions are bolded, LCS6 is underlined, SNP is capitalized):

```
   1 ggccgcggcg gcggaggcag cagcggcggc ggcagtggcg gcggcgaagg tggcggcggc
  61 tcggccagta ctcccggccc ccgccatttc ggactgggag cgagcgcggc gcaggcactg
 121 aaggcggcgg cggggccaga ggctcagcgg ctcccaggtg cgggagagag gcctgctgaa
 181 aatgactgaa tataaacttg tggtagttgg agctggtggc gtaggcaaga gtgccttgac
 241 gatacagcta attcagaatc attttgtgga cgaatatgat ccaacaatag aggattccta
 301 caggaagcaa gtagtaattg atggagaaac ctgtctcttg gatattctcg acacagcagg
 361 tcaagaggag tacagtgcaa tgagggacca gtacatgagg actggggagg gctttctttg
 421 tgtatttgcc ataaataata ctaaatcatt tgaagatatt caccattata gagaacaaat
 481 taaaagagtt aaggactctg aagatgtacc tatggtccta gtaggaaata aatgtgattt
 541 gccttctaga acagtagaca caaaacaggc tcaggactta gcaagaagtt atggaattcc
 601 ttttattgaa acatcagcaa agacaagaca gagagtggag gatgcttttt atacattggt
 661 gagggagatc cgacaataca gattgaaaaa aatcagcaaa gaagaaaaga ctcctggctg
 721 tgtgaaaatt aaaaaatgca ttataatgta atctgggtgt tgatgatgcc ttctatacat
 781 tagttcgaga aattcgaaaa cataaagaaa agatgagcaa agatggtaaa aagaagaaaa
 841 agaagtcaaa gacaaagtgt gtaattatgt aaatacaatt tgtacttttt tcttaaggca
 901 tactagtaca agtggtaatt tttgtacatt acactaaatt attagcattt gttttagcat
 961 tacctaattt ttttcctgct ccatgcagac tgttagcttt taccttaaat gcttatttta
1021 aaatgacagt ggaagttttt ttttcctcta agtgccagta ttcccagagt tttggttttt
1081 gaactagcaa tgcctgtgaa aaagaaactg aatacctaag atttctgtct tggggttttt
```

1141 ggtgcatgca gttgattact tcttattttt cttaccaatt gtgaatgttg gtgtgaaaca
1201 aattaatgaa gcttttgaat catccctatt ctgtgtttta tctagtcaca taaatggatt
1261 aattactaat ttcagttgag accttctaat tggtttttac tgaaacattg agggaacaca
1321 aatttatggg cttcctgatg atgattcttc taggcatcat gtcctatagt ttgtcatccc
1381 tgatgaatgt aaagttacac tgttcacaaa ggttttgtct cctttccact gctattagtc
1441 atggtcactc tccccaaaat attatatttt ttctataaaa agaaaaaaat ggaaaaaaat
1501 tacaaggcaa tggaaactat tataaggcca tttccttttc acattagata aattactata
1561 aagactccta atagcttttc ctgttaaggc agacccagta tgaaatgggg attattatag
1621 caaccatttt ggggctatat ttacatgcta ctaaattttt ataataattg aaaagatttt
1681 aacaagtata aaaaattctc ataggaatta aatgtagtct ccctgtgtca gactgctctt
1741 tcatagtata actttaaatc ttttcttcaa cttgagtctt tgaagatagt tttaattctg
1801 cttgtgacat taaaagatta tttgggccag ttatagctta ttaggtgttg aagagaccaa
1861 ggttgcaagg ccaggccctg tgtgaacctt tgagctttca tagagagttt cacagcatgg
1921 actgtgtccc cacggtcatc cagtgttgtc atgcattggt tagtcaaaat ggggagggac
1981 tagggcagtt tggatagctc aacaagatac aatctcactc tgtggtggtc ctgctgacaa
2041 atcaagagca ttgcttttgt ttcttaagaa aacaaactct tttttaaaaa ttacttttaa
2101 atattaactc aaaagttgag attttggggt ggtggtgtgc caagacatta attttttttt
2161 taaacaatga agtgaaaaag ttttacaatc tctaggtttg gctagttctc ttaacactgg
2221 ttaaattaac attgcataaa cacttttcaa gtctgatcca tatttaataa tgctttaaaa
2281 taaaaataaa aacaatcctt ttgataaatt taaaatgtta cttattttaa aataaatgaa
2341 gtgagatggc atggtgaggt gaaagtatca ctggactagg aagaaggtga cttaggttct
2401 agataggtgt cttttaggac tctgattttg aggacatcac ttactatcca tttcttcatg
2461 ttaaaagaag tcatctcaaa ctcttagttt tttttttta caactatgta atttatattc
2521 catttacata aggatacact tatttgtcaa gctcagcaca atctgtaaat ttttaaccta
2581 tgttacacca tcttcagtgc cagtcttggg caaaattgtg caagaggtga agtttatatt
2641 tgaatatcca ttctcgtttt aggactcttc ttccatatta gtgtcatctt gcctccctac
2701 cttccacatg ccccatgact tgatgcagtt ttaatacttg taattccccc aaccataaga
2761 tttactgctg ctgtggatat ctccatgaag ttttcccact gagtcacatc agaaatgccc
2821 tacatcttat ttcctcaggg ctcaagagaa tctgacagat accataaagg gatttgacct
2881 aatcactaat tttcaggtgg tggctgatgc tttgaacatc tctttgctgc ccaatccatt
2941 agcgacagta ggatttttca aacctggtat gaatagacag aaccctatcc agtggaagga
3001 gaatttaata aagatagtgc tgaaagaatt ccttaggtaa tctataacta ggactactcc
3061 tggtaacagt aatacattcc attgttttag taaccagaaa tcttcatgca atgaaaaata
3121 ctttaattca tgaagcttac tttttttttt tggtgtcaga gtctcgctct tgtcacccag
3181 gctggaatgc agtggcgcca tctcagctca ctgcaacctc catctcccag gttcaagcga
3241 ttctcgtgcc tcggcctcct gagtagctgg gattacaggc gtgtgccact acactcaact
3301 aatttttgta tttttaggag agacggggtt tcaccctgtt ggccaggctg gtctcgaact
3361 cctgacctca agtgatGcac ccaccttggc ctcataaacc tgttttgcag aactcattta
3421 ttcagcaaat atttattgag tgcctaccag atgccagtca ccgcacaagg cactgggtat
3481 atggtatccc caaacaagag acataatccc ggtccttagg tagtgctagt gtggtctgta
3541 atatcttact aaggcctttg gtatacgacc cagagataac acgatgcgta ttttagtttt
3601 gcaaagaagg ggtttggtct ctgtgccagc tctataattg ttttgctacg attccactga
3661 aactcttcga tcaagctact ttatgtaaat cacttcattg ttttaaagga ataaacttga
3721 ttatattgtt tttttatttg gcataactgt gattctttta ggacaattac tgtacacatt
3781 aaggtgtatg tcagatattc atattgaccc aaatgtgtaa tattccagtt ttctctgcat
3841 aagtaattaa aatatactta aaaattaata gttttatctg ggtacaaata aacaggtgcc
3901 tgaactagtt cacagacaag gaaacttcta tgtaaaaatc actatgattt ctgaattgct
3961 atgtgaaact acagatcttt ggaacactgt ttaggtaggg tgttaagact tacacagtac
4021 ctcgtttcta cacagagaaa gaaatggcca tacttcagga actgcagtgc ttatgagggg
4081 atatttaggc ctcttgaatt tttgatgtag atgggcattt ttttaaggta gtggttaatt
4141 acctttatgt gaactttgaa tggtttaaca aaagatttgt ttttgtagag attttaaagg
4201 gggagaattc tagaaataaa tgttacctaa ttattacagc cttaaagaca aaaatccttg
4261 ttgaagtttt tttaaaaaaa gctaaattac atagacttag gcattaacat gtttgtggaa
4321 gaatatagca gacgtatatt gtatcatttg agtgaatgtt cccaagtagg cattctaggc
4381 tctatttaac tgagtcacac tgcataggaa tttagaacct aacttttata ggttatcaaa
4441 actgttgtca ccattgcaca attttgtcct aatatataca tagaaacttt gtggggcatg
4501 ttaagttaca gtttgcacaa gttcatctca tttgtattcc attgattttt tttttcttct

4561 **aaacattttt tcttcaaaca gtatataact ttttttaggg gatttttttt tagacagcaa**
4621 **aaactatctg aagatttcca tttgtcaaaa agtaatgatt tcttgataat tgtgtagtaa**
4681 **tgttttttag aacccagcag ttaccttaaa gctgaattta tatttagtaa cttctgtgtt**
4741 **aatactggat agcatgaatt ctgcattgag aaactgaata gctgtcataa aatgaaactt**
4801 **tctttctaaa gaaagatact cacatgagtt cttgaagaat agtcataact agattaagat**
4861 **ctgtgtttta gtttaatagt ttgaagtgcc tgtttgggat aatgataggt aatttagatg**
4921 **aatttagggg aaaaaaaagt tatctgcaga tatgttgagg gcccatctct ccccccacac**
4981 **ccccacagag ctaactgggt tacagtgttt tatccgaaag tttccaattc cactgtcttg**
5041 **tgttttcatg ttgaaaatac ttttgcattt ttcctttgag tgccaatttc ttactagtac**
5101 **tatttcttaa tgtaacatgt ttacctggaa tgtattttaa ctatttttgt atagtgtaaa**
5161 **ctgaaacatg cacattttgt acattgtgct ttcttttgtg ggacatatgc agtgtgatcc**
5221 **agttgttttc catcatttgg ttgcgctgac ctaggaatgt tggtcatatc aaacattaaa**
5281 **aatgaccact cttttaattg aaattaactt ttaaatgttt ataggagtat gtgctgtgaa**
5341 **gtgatctaaa atttgtaata tttttgtcat gaactgtact actcctaatt attgtaatgt**
5401 **aataaaaata gttacagtga caaaaaaaaa aaaaaa**

[159] Human KRAS, transcript variant b, comprising the LCS6 SNP, is encoded by the following mRNA sequence (SEQ ID NO: 12)(untranslated regions are bolded, LCS6 is underlined, SNP is capitalized):

1 **ggccgcggcg gcggaggcag cagcggcggc ggcagtggcg gcggcgaagg tggcggcggc**
61 **tcggccagta ctcccggccc ccgccatttc ggactgggag cgagcgcggc gcaggcactg**
121 **aaggcggcgg cggggccaga ggctcagcgg ctcccaggtg cgggagagag gcctgctgaa**
181 **a**atgactgaa tataaacttg tggtagttgg agctggtggc gtaggcaaga gtgccttgac
241 gatacagcta attcagaatc attttgtgga cgaatatgat ccaacaatag aggattccta
301 caggaagcaa gtagtaattg atggagaaac ctgtctcttg gatattctcg acacagcagg
361 tcaagaggag tacagtgcaa tgagggacca gtacatgagg actggggagg gctttctttg
421 tgtatttgcc ataaataata ctaaatcatt tgaagatatt caccattata gagaacaaat
481 taaaagagtt aaggactctg aagatgtacc tatggtccta gtaggaaata aatgtgattt
541 gccttctaga acagtagaca caaaacaggc tcaggactta gcaagaagtt atggaattcc
601 ttttattgaa acatcagcaa agacaagaca gggtgttgat gatgccttct atacattagt
661 tcgagaaatt cgaaaacata aagaaaagat gagcaaagat ggtaaaaaga agaaaaagaa
721 gtcaaagaca aagtgtgt**aa ttatgtaaat acaatttgta ctttttctt aaggcatact**
781 **agtacaagtg gtaatttttg tacattacac taaattatta gcatttgttt tagcattacc**
841 **taatttttt cctgctccat gcagactgtt agctttacc ttaaatgctt attttaaaat**
901 **gacagtggaa gttttttttt cctctaagtg ccagtattcc cagagttttg gtttttgaac**
961 **tagcaatgcc tgtgaaaaag aaactgaata cctaagattt ctgtcttggg gtttttggtg**
1021 **catgcagttg attacttctt atttttctta ccaattgtga atgttggtgt gaaacaaatt**
1081 **aatgaagctt ttgaatcatc cctattctgt gttttatcta gtcacataaa tggattaatt**
1141 **actaatttca gttgagacct tctaattggt ttttactgaa acattgaggg aacacaaatt**
1201 **tatgggcttc ctgatgatga ttcttctagg catcatgtcc tatagtttgt catccctgat**
1261 **gaatgtaaag ttacactgtt cacaaaggtt ttgtctcctt tccactgcta ttagtcatgg**
1321 **tcactctccc caaaatatta tatttttttct ataaaaagaa aaaaatggaa aaaaattaca**
1381 **aggcaatgga aactattata aggccatttc cttttcacat tagataaatt actataaaga**
1441 **ctcctaatag cttttcctgt taaggcagac ccagtatgaa atggggatta ttatagcaac**
1501 **cattttgggg ctatatttac atgctactaa atttttataa taattgaaaa gattttaaca**
1561 **agtataaaaa attctcatag gaattaaatg tagtctccct gtgtcagact gctctttcat**
1621 **agtataactt taaatctttt cttcaacttg agtctttgaa gatagtttta attctgcttg**
1681 **tgacattaaa agattatttg ggccagttat agcttattag gtgttgaaga gaccaaggtt**
1741 **gcaaggccag gccctgtgtg aacctttgag ctttcataga gagtttcaca gcatggactg**
1801 **tgtccccacg gtcatccagt gttgtcatgc attggttagt caaaatgggg agggactagg**
1861 **gcagtttgga tagctcaaca agatacaatc tcactctgtg gtggtcctgc tgacaaatca**
1921 **agagcattgc ttttgtttct taagaaaaca aactcttttt taaaaattac ttttaaatat**
1981 **taactcaaaa gttgagattt tggggtggtg gtgtgccaag acattaattt ttttttaaa**
2041 **caatgaagtg aaaaagtttt acaatctcta ggtttggcta gttctcttaa cactggttaa**

2101 attaacattg cataaacact tttcaagtct gatccatatt taataatgct ttaaaataaa
2161 aataaaaaca atccttttga taaatttaaa atgttactta ttttaaaata aatgaagtga
2221 gatggcatgg tgaggtgaaa gtatcactgg actaggaaga aggtgactta ggttctagat
2281 aggtgtcttt taggactctg attttgagga catcacttac tatccatttc ttcatgttaa
2341 aagaagtcat ctcaaactct tagtttttt tttttacaac tatgtaattt atattccatt
2401 tacataagga tacacttatt tgtcaagctc agcacaatct gtaaattttt aacctatgtt
2461 acaccatctt cagtgccagt cttgggcaaa attgtgcaag aggtgaagtt tatatttgaa
2521 tatccattct cgttttagga ctcttcttcc atattagtgt catcttgcct ccctaccttc
2581 cacatgcccc atgacttgat gcagttttaa tacttgtaat tcccctaacc ataagattta
2641 ctgctgctgt ggatatctcc atgaagtttt cccactgagt cacatcagaa atgccctaca
2701 tcttatttcc tcagggctca agagaatctg acagatacca taaagggatt tgacctaatc
2761 actaattttc aggtggtggc tgatgctttg aacatctctt tgctgcccaa tccattagcg
2821 acagtaggat ttttcaaacc tggtatgaat agacagaacc ctatccagtg gaaggagaat
2881 ttaataaaga tagtgctgaa agaattcctt aggtaatcta taactaggac tactcctggt
2941 aacagtaata cattccattg ttttagtaac cagaaatctt catgcaatga aaaatacttt
3001 aattcatgaa gcttactttt ttttttggt gtcagagtct cgctcttgtc acccaggctg
3061 gaatgcagtg gcgccatctc agctcactgc aacctccatc tcccaggttc aagcgattct
3121 cgtgcctcgg cctcctgagt agctgggatt acaggcgtgt gccactacac tcaactaatt
3181 tttgtatttt taggagagac ggggtttcac cctgttggcc aggctggtct cgaactcctg
3241 acctcaagtg atGcacccac cttggcctca taaacctgtt ttgcagaact catttattca
3301 gcaaatattt attgagtgcc taccagatgc cagtcaccgc acaaggcact gggtatatgg
3361 tatccccaaa caagagacat aatcccggtc cttaggtagt gctagtgtgg tctgtaatat
3421 cttactaagg cctttggtat acgacccaga gataacacga tgcgtatttt agttttgcaa
3481 agaaggggtt tggtctctgt gccagctcta taattgtttt gctacgattc cactgaaact
3541 cttcgatcaa gctactttat gtaaatcact tcattgtttt aaaggaataa acttgattat
3601 attgtttttt tatttggcat aactgtgatt cttttaggac aattactgta cacattaagg
3661 tgtatgtcag atattcatat tgacccaaat gtgtaatatt ccagttttct ctgcataagt
3721 aattaaaata tacttaaaaa ttaatagttt tatctgggta caaataaaca ggtgcctgaa
3781 ctagttcaca gacaaggaaa cttctatgta aaaatcacta tgatttctga attgctatgt
3841 gaaactacag atctttggaa cactgtttag gtagggtgtt aagacttaca cagtacctcg
3901 tttctacaca gagaaagaaa tggccatact tcaggaactg cagtgcttat gaggggatat
3961 ttaggcctct tgaatttttg atgtagatgg gcatttttt aaggtagtgg ttaattacct
4021 ttatgtgaac tttgaatggt ttaacaaaag atttgttttt gtagagattt taaaggggga
4081 gaattctaga aataaatgtt acctaattat tacagcctta aagacaaaaa tccttgttga
4141 agtttttta aaaaaagcta aattacatag acttaggcat taacatgttt gtggaagaat
4201 atagcagacg tatattgtat catttgagtg aatgttccca agtaggcatt ctaggctcta
4261 tttaactgag tcacactgca taggaattta gaacctaact tttataggtt atcaaaactg
4321 ttgtcaccat tgcacaattt tgtcctaata tatacataga aactttgtgg ggcatgttaa
4381 gttacagttt gcacaagttc atctcatttg tattccattg attttttttt tcttctaaac
4441 attttttctt caaacagtat ataacttttt ttaggggatt ttttttaga cagcaaaaac
4501 tatctgaaga tttccatttg tcaaaaagta atgatttctt gataattgtg tagtaatgtt
4561 ttttagaacc cagcagttac cttaaagctg aatttatatt tagtaacttc tgtgttaata
4621 ctggatagca tgaattctgc attgagaaac tgaatagctg tcataaaatg aaactttctt
4681 tctaaagaaa gatactcaca tgagttcttg aagaatagtc ataactagat taagatctgt
4741 gttttagttt aatagtttga agtgcctgtt tgggataatg ataggtaatt tagatgaatt
4801 taggggaaaa aaaagttatc tgcagatatg ttgagggccc atctctcccc ccacaccccc
4861 acagagctaa ctgggttaca gtgttttatc cgaaagtttc caattccact gtcttgtgtt
4921 ttcatgttga aaatactttt gcatttttcc tttgagtgcc aatttcttac tagtactatt
4981 tcttaatgta acatgtttac ctggaatgta ttttaactat ttttgtatag tgtaaactga
5041 aacatgcaca ttttgtacat tgtgctttct tttgtgggac atatgcagtg tgatccagtt
5101 gttttccatc atttggttgc gctgacctag gaatgttggt catatcaaac attaaaaatg
5161 accactcttt taattgaaat taacttttaa atgtttatag gagtatgtgc tgtgaagtga
5221 tctaaaattt gtaatatttt tgtcatgaac tgtactactc ctaattattg taatgtaata
5281 aaaatagtta cagtgacaaa aaaaaaaaaa aa

[160] The KRAS variant is the result of a substitution of a G for a U at position 4 of SEQ ID NO: 6 of LCS6. This KRAS variant comprises the sequence GAUGCACCCACCUUGGCCUCA (SNP bolded for emphasis) (SEQ ID NO: 13).

[161] The KRAS variant leads to altered *KRAS* expression by disrupting the miRNA regulation of a *KRAS*. The identification and characterization of the KRAS variant is further described in International Application No. PCT/US08/65302 (WO 2008/151004), the contents of which are incorporated by reference in their entirety.

Let-7 family miRNAs

[162] Expression of *let-7* family miRNAs is increased in cells that carry the KRAS variant. Interestingly, the let-7 family of miRNAs bind to the let-7 complementary site in which the KRAS variant in located. The presence of the KRAS variant interferes with let-7 binding to KRAS. By interfering, the KRAS variant either induces let-7 to bind more or less tightly to LCS6 of KRAS. It was discovered that cells containing the KRAS variant have lower levels of KRAS mRNA compared to wild type cells, and increased levels of the KRAS protein. Thus, while not wishing to be bound by theory, the presence of the KRAS variant within cells may interfere with the ability of let-7 to bind to KRAS and inhibit protein translation, allowing higher KRAS protein levels.

[163] The presence of the KRAS-Variant in triple negative breast cancer is also associated with significantly lower levels of let-7 miRNAs. For instance, let-7 miRNA expression is decreased by 2-fold (2X), 3-fold (3X), 4-fold (4X), 5-fold (5X), 6-fold (6X), 7-fold (7X), 8-fold (8X), 9-fold (9X), 10-fold (10X), 20-fold (20X), 50-fold (50X), 100-fold (100X), 200-fold (200X), 500-fold (500X), 1000-fold (1000X), or any multiplier in between. Alternatively, or in addition, the statistically significant difference between the reduction of let-7 miRNA expression in a cell obtained from a subject who has triple negative breast cancer compared to the level of let-7 miRNA expression in a cell obtained from a subject who does not have triple negative breast cancer (i.e. a normal or control cell) is exemplified by a p-value of less than 0.05, preferably, a p-value of less than 0.01, or most preferably, a p-value of less than 0.001. The level of let-7 miRNA expression present in a cell obtained from a subject who has triple negative breast cancer may also be compared to a known standard level in the art. Moreover, the level of let-7 expression may be compared between an affected cell and an unaffected cell within a subject

who has breast cancer or, specifically triple negative breast cancer, wherein the unaffected cell serves as an internal control.

[164] Exemplary *let-7* miRNAs include, but are not limited to, *let-7a (let-7a-1, let-7a-2, let-7a-3), let-7b, let-7c, let-7d, let-7e, let-7f (let-7f-1 and let-7f-2), let-7g,* and *let-7i.* For the following sequences, thymine (T) may be substituted for uracil (U). *let-7a* comprises the sequence UUGAUAUGUUGGAUGAUGGAGU (SEQ ID NO: 14). *let-7b* comprises the sequence UUGGUGUGUUGGAUGAUGGAGU (SEQ ID NO: 15). *let-7c* comprises the sequence UUGGUAUGUUGGAUGAUGGAGU (SEQ ID NO: 16). *let-7d* comprises the sequence UGAUACGUUGGAUGAUGGAGA (SEQ ID NO: 17). *let-7e* comprises the sequence UAUAUGUUGGAGGAUGGAGU (SEQ ID NO: 18). *let-7f* comprises the sequence UUGAUAUGUUAGAUGAUGGAGU (SEQ ID NO: 19). *let-7g* comprises the sequence GACAUGUUUGAUGAUGGAGU (SEQ ID NO: 20). *let-7i* comprises the sequence UGUCGUGUUUGUUGAUGGAGU (SEQ ID NO: 21).

[165] Sequences of additional let-7 family members are publicly available from miRBase at (www.mirbase.org).

Therapeutic Methods

[166] Identification of the KRAS variant mutation indicates an increased risk of developing triple negative breast cancer. "Risk" in the context of the present disclosure, relates to the probability that an event will occur over a specific time period, and can mean a subject's "absolute" risk or "relative" risk. Absolute risk can be measured with reference to either actual observation post-measurement for the relevant time cohort, or with reference to index values developed from statistically valid historical cohorts that have been followed for the relevant time period. Relative risk refers to the ratio of absolute risks of a subject compared either to the absolute risks of low risk cohorts or an average population risk, which can vary by how clinical risk factors are assessed. Odds ratios, the proportion of positive events to negative events for a given test result, are also commonly used (odds are according to the formula p/(1-p) where p is the probability of event and (1- p) is the probability of no event) to no-conversion.

[167] "Risk evaluation," or "evaluation of risk" in the context of the present disclosure encompasses making a prediction of the probability, odds, or likelihood that an event or disease state may occur, the rate of occurrence of the event or conversion from one disease state to another, i.e., from a primary tumor to a metastatic tumor or to one at risk of developing a

metastatic, or from at risk of a primary metastatic event to a secondary metastatic event or from at risk of a developing a primary tumor of one type to developing a one or more primary tumors of a different type. Risk evaluation can also comprise prediction of future clinical parameters, traditional laboratory risk factor values, or other indices of cancer, either in absolute or relative terms in reference to a previously measured population.

[168] An "increased risk" is meant to describe an increased probably that an individual who carries the *KRAS* variant will develop or has developed cancer, when compared to an individual who does not carry the *KRAS* variant. In certain embodiments, a *KRAS* variant carrier is 1.5X, 2X, 2.5X, 3X, 3.5X, 4X, 4.5X, 5X, 5.5X, 6X, 6.5X, 7X, 7.5X, 8X, 8.5X, 9X, 9.5X, 10X, 20X, 30X, 40X, 50X, 60X, 70X, 80X, 90X, or 100X more likely to develop or have cancer than an individual who does not carry the *KRAS* variant.

[169] By poor prognosis is meant that the probability of the individual surviving the development of a particularly aggressive, high-risk, severe, or inherited form of cancer (*e.g.*, triple negative breast cancer), or that the probability of surviving the development or progression of an aggressive, high-risk, severe, or inherited form is less than the probability of surviving the development or progression of a more benign form.

[170] Poor prognosis is also meant to describe a less satisfactory recovery, longer recovery period, more invasive or high-risk therapeutic regime, or an increased probability of reoccurrence of cancer or a metastasis thereof. For example, triple negative breast cancer or a metastasis thereof is correlated with the worst prognosis of breast cancer subtypes, resulting in a poor prognosis for the subject.

[171] The terms subject, patient, and individual are used interchangeably throughout the description. A subject is preferably a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but are not limited to these examples. A subject is male or female. A subject may not have been previously diagnosed as having cancer, a particular type of cancer (*e.g.*, breast cancer), or a subtype of cancer (*e.g.*, triple negative breast cancer as a subtype of breast cancer). The subject may exhibit one or more risk factors for cancer, a particular type of cancer (*e.g.*, breast cancer), or a subtype of cancer (*e.g.*, triple negative breast cancer as a subtype of breast cancer). Alternatively, the subject does not exhibit a risk factor for cancer, a particular type of cancer (*e.g.*, breast cancer), or a subtype of cancer (*e.g.*, triple negative breast cancer as a subtype of breast cancer).

[172] Breast cancer, including triple negative breast cancer, risk factors include, but are not limited to, the presence of the *KRAS* variant; being female, aging, obesity, lack of childbearing or breastfeeding, higher hormone levels, smoking, exposure to radiation, personal history of breast cancer, family history of breast cancer, and particular breast changes (e.g. those changes associated with fibrocystic conditions, including, but not limited to, Atypical hyperplasia and lobular carcinoma *in situ*). Exemplary protective measures against the development of triple negative breast cancer, include, but not limited to, regular exercise, avoiding environmental triggers (e.g. smoking, drinking, high fat diet leading to obesity, radiation exposure through occupation), choosing to breastfeed children, and, for those at the most severe risk, prophylactic bilateral mastectomy. Subjects of the disclosure may present one or more risk factors that may further be mitigated or modified by a protective measure.

[173] The methods described herein provide for obtaining a sample from a subject. The sample can be any tissue or fluid that contains nucleic acids. Various embodiments include, but are not limited to, paraffin imbedded tissue, frozen tissue, surgical fine needle aspirations, and cells of the breast (including cells harvested from a duct, a lobule, or connective tissue), a lymph node (including a sentinel or axillary node), a thoracic or abdominal muscle or connective tissue, an organ (including any potential deposit site for a potential metastatic cell, such as the brain, liver, kidney, stomach, intestines, bone marrow, pancreas, colon, or lung). Other embodiments include fluid samples such as blood, plasma, serum, lymph fluid, ascites, serous fluid, and urine.

SNP Genotyping Methods

[174] The *KRAS* variant is a single nucleotide polymorphism that occurs within the 3' UTR of the human *KRAS* gene. Linkage disequilibrium (LD) refers to the co-inheritance of alleles (e.g., alternative nucleotides) at two or more different SNP sites at frequencies greater than would be expected from the separate frequencies of occurrence of each allele in a given population. The expected frequency of co-occurrence of two alleles that are inherited independently is the frequency of the first allele multiplied by the frequency of the second allele. Alleles that co-occur at expected frequencies are said to be in "linkage equilibrium". In contrast, LD refers to any non-random genetic association between allele(s) at two or more different SNP sites, which is generally due to the physical proximity of the two loci along a chromosome. LD can occur when two or more SNPs sites are in close physical proximity to each other on a given chromosome and therefore alleles at these SNP sites will tend to remain unseparated for multiple generations with

the consequence that a particular nucleotide (allele) at one SNP site will show a non-random association with a particular nucleotide (allele) at a different SNP site located nearby. Hence, genotyping one of the SNP sites will give almost the same information as genotyping the other SNP site that is in LD.

[175] For screening individuals for genetic disorders (e.g. prognostic or risk) purposes, if a particular SNP site is found to be useful for screening a disorder, then the skilled artisan would recognize that other SNP sites which are in LD with this SNP site would also be useful for screening the condition. Various degrees of LD can be encountered between two or more SNPs with the result being that some SNPs are more closely associated (*i.e.,* in stronger LD) than others. Furthermore, the physical distance over which LD extends along a chromosome differs between different regions of the genome, and therefore the degree of physical separation between two or more SNP sites necessary for LD to occur can differ between different regions of the genome.

[176] For screening applications, polymorphisms (e.g., SNPs and/or haplotypes) that are not the actual disease-causing (causative) polymorphisms, but are in LD with such causative polymorphisms, are also useful. In such instances, the genotype of the polymorphism(s) that is/are in LD with the causative polymorphism is predictive of the genotype of the causative polymorphism and, consequently, predictive of the phenotype (e.g., disease) that is influenced by the causative SNP(s). Thus, polymorphic markers that are in LD with causative polymorphisms are useful as markers, and are particularly useful when the actual causative polymorphism(s) is/are unknown.

[177] Linkage disequilibrium in the human genome is reviewed in: Wall et al., "Haplotype blocks and linkage disequilibrium in the human genome", Nat Rev Genet. 2003 August; 4(8):587-97; Gamer et al., "On selecting markers for association studies: patterns of linkage disequilibrium between two and three diallelic loci", Genet Epidemiol. 2003 January; 24(1):57-67; Ardlie et al., "Patterns of linkage disequilibrium in the human genome", Nat Rev Genet. 2002 April; 3(4):299-309 (erratum in Nat Rev Genet 2002 July; 3(7):566); and Remm et al., "High-density genotyping and linkage disequilibrium in the human genome using chromosome 22 as a model"; Curr Opin Chem Biol. 2002 February; 6(1):24-30.

[178] The screening techniques of the present disclosure may employ a variety of methodologies to determine whether a test subject has a SNP or a SNP pattern associated with an

increased or decreased risk of developing a detectable trait or whether the individual suffers from a detectable trait as a result of a particular polymorphism/mutation, including, for example, methods which enable the analysis of individual chromosomes for haplotyping, family studies, single sperm DNA analysis, or somatic hybrids. The trait analyzed using the diagnostics of the disclosure may be any detectable trait that is commonly observed in pathologies and disorders.

[179] The process of determining which specific nucleotide (*i.e.,* allele) is present at each of one or more SNP positions, such as a SNP position in a nucleic acid molecule disclosed in SEQ ID NO: 11, 12, 13 or 22, is referred to as SNP genotyping. The present disclosure provides methods of SNP genotyping, such as for use in screening for a variety of disorders, or determining predisposition thereto, or determining responsiveness to a form of treatment, or prognosis, or in genome mapping or SNP association analysis, etc.

[180] Nucleic acid samples can be genotyped to determine which allele(s) is/are present at any given genetic region (e.g., SNP position) of interest by methods well known in the art. The neighboring sequence can be used to design SNP detection reagents such as oligonucleotide probes, which may optionally be implemented in a kit format. Exemplary SNP genotyping methods are described in Chen et al., "Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput", Pharmacogenomics J. 2003; 3(2):77-96; Kwok et al., "Detection of single nucleotide polymorphisms", Curr Issues Mol. Biol. 2003 April; 5(2):43-60; Shi, "Technologies for individual genotyping: detection of genetic polymorphisms in drug targets and disease genes", Am J Pharmacogenomics. 2002; 2(3):197-205; and Kwok, "Methods for genotyping single nucleotide polymorphisms", Annu Rev Genomics Hum Genet 2001; 2: 235-58. Exemplary techniques for high-throughput SNP genotyping are described in Marnellos, "High-throughput SNP analysis for genetic association studies", Curr Opin Drug Discov Devel. 2003 May; 6(3):317-21. Common SNP genotyping methods include, but are not limited to, TaqMan assays, molecular beacon assays, nucleic acid arrays, allele-specific primer extension, allele-specific PCR, arrayed primer extension, homogeneous primer extension assays, primer extension with detection by mass spectrometry, pyrosequencing, multiplex primer extension sorted on genetic arrays, ligation with rolling circle amplification, homogeneous ligation, OLA (U.S. Pat. No. 4,988,167), multiplex ligation reaction sorted on genetic arrays, restriction-fragment length polymorphism, single base extension-tag assays, and the Invader assay. Such methods may be used in combination with detection mechanisms such as, for example,

luminescence or chemiluminescence detection, fluorescence detection, time-resolved fluorescence detection, fluorescence resonance energy transfer, fluorescence polarization, mass spectrometry, and electrical detection.

[181] Various methods for detecting polymorphisms include, but are not limited to, methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al., Science 230:1242 (1985); Cotton et al., PNAS 85:4397 (1988); and Saleeba et al., Meth. Enzymol. 217:286-295 (1992)), comparison of the electrophoretic mobility of variant and wild type nucleic acid molecules (Orita et al., PNAS 86:2766 (1989); Cotton et al., Mutat. Res. 285:125-144 (1993); and Hayashi et al., Genet. Anal. Tech. Appl. 9:73-79 (1992)), and assaying the movement of polymorphic or wild-type fragments in polyacrylamide gels containing a gradient of denaturant using denaturing gradient gel electrophoresis (DGGE) (Myers et al., Nature 313:495 (1985)). Sequence variations at specific locations can also be assessed by nuclease protection assays such as RNase and SI protection or chemical cleavage methods.

[182] In a preferred embodiment, SNP genotyping is performed using the TaqMan assay, which is also known as the 5' nuclease assay (U.S. Pat. Nos. 5,210,015 and 5,538,848). The TaqMan assay detects the accumulation of a specific amplified product during PCR. The TaqMan assay utilizes an oligonucleotide probe labeled with a fluorescent reporter dye and a quencher dye. The reporter dye is excited by irradiation at an appropriate wavelength, it transfers energy to the quencher dye in the same probe via a process called fluorescence resonance energy transfer (FRET). When attached to the probe, the excited reporter dye does not emit a signal. The proximity of the quencher dye to the reporter dye in the intact probe maintains a reduced fluorescence for the reporter. The reporter dye and quencher dye may be at the 5' most and the 3' most ends, respectively, or vice versa. Alternatively, the reporter dye may be at the 5' or 3' most end while the quencher dye is attached to an internal nucleotide, or vice versa. In yet another embodiment, both the reporter and the quencher may be attached to internal nucleotides at a distance from each other such that fluorescence of the reporter is reduced.

[183] During PCR, the 5' nuclease activity of DNA polymerase cleaves the probe, thereby separating the reporter dye and the quencher dye and resulting in increased fluorescence of the reporter. Accumulation of PCR product is detected directly by monitoring the increase in fluorescence of the reporter dye. The DNA polymerase cleaves the probe between the reporter

dye and the quencher dye only if the probe hybridizes to the target SNP-containing template which is amplified during PCR, and the probe is designed to hybridize to the target SNP site only if a particular SNP allele is present.

[184] Preferred TaqMan primer and probe sequences can readily be determined using the SNP and associated nucleic acid sequence information provided herein. A number of computer programs, such as Primer Express (Applied Biosystems, Foster City, Calif.), can be used to rapidly obtain optimal primer/probe sets. It will be apparent to one of skill in the art that such primers and probes for detecting the SNPs of the present disclosure are useful in prognostic assays for a variety of disorders including cancer, and can be readily incorporated into a kit format. The present disclosure also includes modifications of the Taqman assay well known in the art such as the use of Molecular Beacon probes (U.S. Pat. Nos. 5,118,801 and 5,312,728) and other variant formats (U.S. Pat. Nos. 5,866,336 and 6,117,635).

[185] The identity of polymorphisms may also be determined using a mismatch detection technique, including but not limited to the RNase protection method using riboprobes (Winter et al., Proc. Natl. Acad Sci. USA 82:7575, 1985; Meyers et al., Science 230:1242, 1985) and proteins which recognize nucleotide mismatches, such as the E. coli mutS protein (Modrich, P. Ann. Rev. Genet. 25:229-253, 1991). Alternatively, variant alleles can be identified by single strand conformation polymorphism (SSCP) analysis (Orita et al., Genomics 5:874-879, 1989; Humphries et al., in Molecular Diagnosis of Genetic Diseases, R. Elles, ed., pp. 321-340, 1996) or denaturing gradient gel electrophoresis (DGGE) (Wartell et al., Nuci. Acids Res. 18:2699-2706, 1990; Sheffield et al., Proc. Natl. Acad. Sci. USA 86:232-236, 1989).

[186] A polymerase-mediated primer extension method may also be used to identify the polymorphism(s). Several such methods have been described in the patent and scientific literature and include the "Genetic Bit Analysis" method (WO92/15712) and the ligase/polymerase mediated genetic bit analysis (U.S. Pat. No. 5,679,524). Related methods are disclosed in WO91/02087, WO90/09455, WO95/17676, U.S. Pat. Nos. 5,302,509, and 5,945,283. Extended primers containing a polymorphism may be detected by mass spectrometry as described in U.S. Pat. No. 5,605,798. Another primer extension method is allele-specific PCR (Ruano et al., Nucl. Acids Res. 17:8392, 1989; Ruano et al., Nucl. Acids Res. 19, 6877-6882, 1991; WO 93/22456; Turki et al., J Clin. Invest. 95:1635-1641, 1995). In addition, multiple

polymorphic sites may be investigated by simultaneously amplifying multiple regions of the nucleic acid using sets of allele-specific primers as described in Wallace et al. (WO89/10414).

[187] Another preferred method for genotyping the KRAS variant is the use of two oligonucleotide probes in an OLA (see, e.g., U.S. Pat. No. 4,988,617). In this method, one probe hybridizes to a segment of a target nucleic acid with its 3' most end aligned with the SNP site. A second probe hybridizes to an adjacent segment of the target nucleic acid molecule directly 3' to the first probe. The two juxtaposed probes hybridize to the target nucleic acid molecule, and are ligated in the presence of a linking agent such as a ligase if there is perfect complementarity between the 3' most nucleotide of the first probe with the SNP site. If there is a mismatch, ligation would not occur. After the reaction, the ligated probes are separated from the target nucleic acid molecule, and detected as indicators of the presence of a SNP.

[188] The following patents, patent applications, and published international patent applications, which are all hereby incorporated by reference, provide additional information pertaining to techniques for carrying out various types of OLA: U.S. Pat. Nos. 6,027,889, 6,268,148, 5494810, 5830711, and 6054564 describe OLA strategies for performing SNP detection; WO 97/31256 and WO 00/56927 describe OLA strategies for performing SNP detection using universal arrays, wherein a zipcode sequence can be introduced into one of the hybridization probes, and the resulting product, or amplified product, hybridized to a universal zip code array; U.S. application US01/17329 (and Ser. No. 09/584,905) describes OLA (or LDR) followed by PCR, wherein zipcodes are incorporated into OLA probes, and amplified PCR products are determined by electrophoretic or universal zipcode array readout; U.S. application 60/427,818, 60/445,636, and 60/445,494 describe SNPlex methods and software for multiplexed SNP detection using OLA followed by PCR, wherein zipcodes are incorporated into OLA probes, and amplified PCR products are hybridized with a zipchute reagent, and the identity of the SNP determined from electrophoretic readout of the zipchute. In some embodiments, OLA is carried out prior to PCR (or another method of nucleic acid amplification). In other embodiments, PCR (or another method of nucleic acid amplification) is carried out prior to OLA.

[189] Another method for SNP genotyping is based on mass spectrometry. Mass spectrometry takes advantage of the unique mass of each of the four nucleotides of DNA. SNPs can be unambiguously genotyped by mass spectrometry by measuring the differences in the mass of nucleic acids having alternative SNP alleles. MALDI-TOF (Matrix Assisted Laser Desorption

Ionization--Time of Flight) mass spectrometry technology is preferred for extremely precise determinations of molecular mass, such as SNPs. Numerous approaches to SNP analysis have been developed based on mass spectrometry. Preferred mass spectrometry-based methods of SNP genotyping include primer extension assays, which can also be utilized in combination with other approaches, such as traditional gel-based formats and microarrays.

[190] Typically, the primer extension assay involves designing and annealing a primer to a template PCR amplicon upstream (5') from a target SNP position. A mix of dideoxynucleotide triphosphates (ddNTPs) and/or deoxynucleotide triphosphates (dNTPs) are added to a reaction mixture containing template (e.g., a SNP-containing nucleic acid molecule which has typically been amplified, such as by PCR), primer, and DNA polymerase. Extension of the primer terminates at the first position in the template where a nucleotide complementary to one of the ddNTPs in the mix occurs. The primer can be either immediately adjacent (i.e., the nucleotide at the 3' end of the primer hybridizes to the nucleotide next to the target SNP site) or two or more nucleotides removed from the SNP position. If the primer is several nucleotides removed from the target SNP position, the only limitation is that the template sequence between the 3' end of the primer and the SNP position cannot contain a nucleotide of the same type as the one to be detected, or this will cause premature termination of the extension primer. Alternatively, if all four ddNTPs alone, with no dNTPs, are added to the reaction mixture, the primer will always be extended by only one nucleotide, corresponding to the target SNP position. In this instance, primers are designed to bind one nucleotide upstream from the SNP position (i.e., the nucleotide at the 3' end of the primer hybridizes to the nucleotide that is immediately adjacent to the target SNP site on the 5' side of the target SNP site). Extension by only one nucleotide is preferable, as it minimizes the overall mass of the extended primer, thereby increasing the resolution of mass differences between alternative SNP nucleotides. Furthermore, mass-tagged ddNTPs can be employed in the primer extension reactions in place of unmodified ddNTPs. This increases the mass difference between primers extended with these ddNTPs, thereby providing increased sensitivity and accuracy, and is particularly useful for typing heterozygous base positions. Mass-tagging also alleviates the need for intensive sample-preparation procedures and decreases the necessary resolving power of the mass spectrometer.

[191] The extended primers can then be purified and analyzed by MALDI-TOF mass spectrometry to determine the identity of the nucleotide present at the target SNP position. In one

method of analysis, the products from the primer extension reaction are combined with light absorbing crystals that form a matrix. The matrix is then hit with an energy source such as a laser to ionize and desorb the nucleic acid molecules into the gas-phase. The ionized molecules are then ejected into a flight tube and accelerated down the tube towards a detector. The time between the ionization event, such as a laser pulse, and collision of the molecule with the detector is the time of flight of that molecule. The time of flight is precisely correlated with the mass-to-charge ratio (m/z) of the ionized molecule. Ions with smaller m/z travel down the tube faster than ions with larger m/z and therefore the lighter ions reach the detector before the heavier ions. The time-of-flight is then converted into a corresponding, and highly precise, m/z. In this manner, SNPs can be identified based on the slight differences in mass, and the corresponding time of flight differences, inherent in nucleic acid molecules having different nucleotides at a single base position. For further information regarding the use of primer extension assays in conjunction with MALDI-TOF mass spectrometry for SNP genotyping, see, e.g., Wise et al., "A standard protocol for single nucleotide primer extension in the human genome using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry", Rapid Commun Mass Spectrom. 2003; 17(11):1195-202.

[192] The following references provide further information describing mass spectrometry-based methods for SNP genotyping: Bocker, "SNP and mutation discovery using base-specific cleavage and MALDI-TOF mass spectrometry", Bioinformatics. 2003 July; 19 Suppl 1:144-153; Storm et al., "MALDI-TOF mass spectrometry-based SNP genotyping", Methods Mol. Biol. 2003;212:241-62; Jurinke et al., "The use of MassARRAY technology for high throughput genotyping", Adv Biochem Eng Biotechnol. 2002; 77:57-74; and Jurinke et al., "Automated genotyping using the DNA MassArray technology", Methods Mol. Biol. 2002; 187:179-92.

[193] SNPs can also be scored by direct DNA sequencing. A variety of automated sequencing procedures can be utilized ((1995) Biotechniques 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO94/16101; Cohen et al., Adv. Chromatogr. 36:127-162 (1996); and Griffin et al., Appl. Biochem. Biotechnol. 38:147-159 (1993)). The nucleic acid sequences of the present disclosure enable one of ordinary skill in the art to readily design sequencing primers for such automated sequencing procedures. Commercial instrumentation, such as the Applied Biosystems 377, 3100, 3700, 3730, and 3730.times.1 DNA Analyzers (Foster City, Calif.), is commonly used in the art for automated sequencing.

[194] Other methods that can be used to genotype the *KRAS* variant include single-strand conformational polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE) (Myers et al., Nature 313:495 (1985)). SSCP identifies base differences by alteration in electrophoretic migration of single stranded PCR products, as described in Orita et al., Proc. Nat. Acad. Single-stranded PCR products can be generated by heating or otherwise denaturing double stranded PCR products. Single-stranded nucleic acids may refold or form secondary structures that are partially dependent on the base sequence. The different electrophoretic mobilities of single-stranded amplification products are related to base-sequence differences at SNP positions. DGGE differentiates SNP alleles based on the different sequence-dependent stabilities and melting properties inherent in polymorphic DNA and the corresponding differences in electrophoretic migration patterns in a denaturing gradient gel (Erlich, ed., PCR Technology, Principles and Applications for DNA Amplification, W. H. Freeman and Co, New York, 1992, Chapter 7).

[195] Sequence-specific ribozymes (U.S. Pat. No. 5,498,531) can also be used to score SNPs based on the development or loss of a ribozyme cleavage site. Perfectly matched sequences can be distinguished from mismatched sequences by nuclease cleavage digestion assays or by differences in melting temperature. If the SNP affects a restriction enzyme cleavage site, the SNP can be identified by alterations in restriction enzyme digestion patterns, and the corresponding changes in nucleic acid fragment lengths determined by gel electrophoresis

[196] SNP genotyping can include the steps of, for example, collecting a biological sample from a human subject (e.g., sample of tissues, cells, fluids, secretions, etc.), isolating nucleic acids (e.g., genomic DNA, mRNA or both) from the cells of the sample, contacting the nucleic acids with one or more primers which specifically hybridize to a region of the isolated nucleic acid containing a target SNP under conditions such that hybridization and amplification of the target nucleic acid region occurs, and determining the nucleotide present at the SNP position of interest, or, in some assays, detecting the presence or absence of an amplification product (assays can be designed so that hybridization and/or amplification will only occur if a particular SNP allele is present or absent). In some assays, the size of the amplification product is detected and compared to the length of a control sample; for example, deletions and insertions can be detected by a change in size of the amplified product compared to a normal genotype.

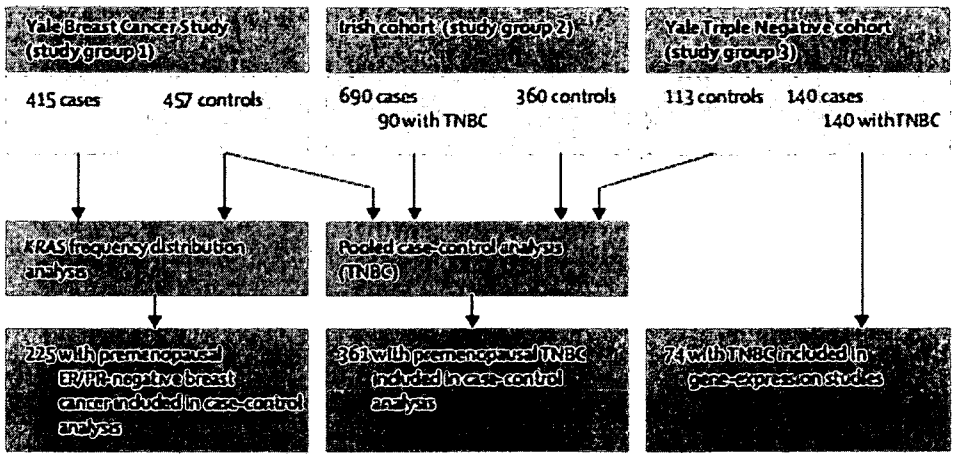
EXAMPLES

Example 1: The *KRAS* variant in Triple-Negative Breast Cancer (TNBC)

Study Populations

[197] In this case-control study and genetic analysis, data were assessed from four cohorts (Table 1). To assess frequency distributions of the *KRAS*-variant genotype, individuals from the Yale Breast Cancer Study (study group 1) were assessed. Individuals from the Yale Breast Cancer Study (study group 1) were enrolled in a breast cancer case-control study in Connecticut, USA; which was approved by the Yale institutional review board (Hoffman A, et al. Cancer Res 2009; 69: 5970–77). Briefly, patients were aged 30–80 years and had incident, histologically confirmed breast cancer and no history of cancer (other than non-melanoma skin cancer). ER and PR statuses were established for all cases but HER2 statuses were not known and not obtainable. Controls were recruited either from Yale-New Haven Hospital (New Haven, CT, USA) or Tolland County, CT, USA. Controls from the Yale–New Haven Hospital underwent breast-related surgery for histologically confirmed benign breast diseases. Controls from Tolland County were identified either through random-digit dialing (for individuals aged <65 years) or through the Health Care Finance Administration files (=65 years). Informed consent and data for family histories of cancer, reproductive history, demographic factors, and blood sample were obtained from all participants. 415 cases and 457 controls had DNA samples available for this study, which were obtained between 1990 and 1999.

[198] **Table 1.** Study Groups



TNBC = triple negative breast cancer, ER = estrogen receptor, and PR = progesterone receptor.

[199] To define the association of the *KRAS* variant with receptor status and breast cancer subtype, a cohort of 690 Irish women diagnosed with breast cancer with complete receptor status and subtype classification was assessed. Patients from this cohort (study group 2) had histologically confirmed breast cancer and were recruited from the west of Ireland after appropriate ethical approval from the Galway University Hospital (Galway, Ireland) ethics committee. Informed consent and a detailed family history of breast cancer or ovarian cancer, and a blood sample were obtained from all cases. 710 cases of breast cancer of all stages and histological types, apart from preinvasive carcinomas. ER, PR, and HER2 statuses were established for all samples by use of standard histopathological analysis and immunohistochemistry, and confirmed by fluorescence *in-situ* hybridization for HER2 positivity. These samples were classified as luminal A, luminal B, HER2, or triple-negative breast cancer by receptor status (Table 2). 690 of the 710 patients had complete information and were assessed in this study. The 360 controls in this cohort were healthy women from the same geographical area, and were mainly older than 60 years, with no self-reported personal history of any cancer and no family history of breast cancer or ovarian cancer. Cases and controls were mainly recruited from July, 2006, to July, 2010.

[200] **Table 2.** Receptor Status of Subtypes.

Breast Cancer Subtypes	ER	PR	Her-2
Luminal A	+/-	+/-	-
Luminal B	+/-	+/-	+
Her-2 +	-	-	+
Triple Negative	-	-	-

[201] To establish whether the *KRAS* variant predicted an increased risk of development of triple-negative breast cancer, a pooled analysis was performed of a cohort of patients with triple-negative breast cancer and controls from Yale (study group 3), patients with triple-negative breast cancer and controls from study group 2, and controls from study group 1. Patients in study group 3 were receiving treatment either at Yale-New Haven Hospital or at the Bridgeport Hospital (Bridgeport, CT, USA). After approval by the Yale Human Investigation Committee, tissue or saliva specimens were obtained from 156 patients. Complete data were available for

140 patients who were diagnosed in 1990–2007 and were included in this study. 130 cases of triple-negative breast cancer had samples of tumor available before any treatment for gene and miRNA-expression analysis, 78 of whom were also genotyped for the *KRAS* variant. 113 controls in this cohort were healthy women who presented to the Yale–New Haven Hospital and who had no personal history of cancer apart from nonmelanoma skin cancer and were recruited between 2000 and 2007. Clinical information, age, ethnic origin, and family history were obtained for all cases and controls. Table 3 summarizes basic information for these three cohorts.

[202] **Table 3.** Description of the three separate breast cancer case-control cohorts utilized in the study.

Cohort Name	Ascertainment criteria	Available receptor status	Age (years)
Yale Case-Control			
Cases	Histologically confirmed BC cases, no prior history of cancer (except non-melanoma skin cancer) from CT, USA	ER and PR	30-80
Controls	Cancer free healthy subjects or subjects who underwent surgery for histologically confirmed benign breast disease.		35-85
Irish Cohort			
Cases	Histologically confirmed BC cases from west of Ireland	ER, PR, and HER2	30-80
Controls	Healthy females, no self-reported personal history of any cancer, no family history of breast or ovarian cancer		> 60
Yale TN cohort			
TNBC cases	Patients being treated eithe at YNHH in New Haven or at Bridgeport Hospita in Bridgeport, CT.	ER, PR, and HER2	30-85
Controls	Subjects with no prior history of cancer (except non-melanoma skin cancer)		30-80

[203] To assess association of the *KRAS* variant with *BRCA* mutations in ER-negative tumors, *BRCA1*-mutation carriers with breast cancer and known *KRAS*-variant status from our previous study of the Rotterdam population were analyzed. The Rotterdam population has been described (Hollestelle A, et al. Breast Cancer Res Treat 2010; published online July 30. DOI:10.1007/s10549-010-1080-z) but, briefly, this population included Dutch patients with breast cancer and documented *BRCA1* mutations who were identified by investigators at the Erasmus University through the Rotterdam Family Clinic (Rotterdam, Netherlands).

Procedures

[204] *KRAS-variant genotyping assays*: DNA from all samples was genotyped for the *KRAS* variant using a custom Taqman SNP genotyping assay. Samples heterozygous or homozygous

for the variant G allele were considered positive for the *KRAS*-variant (Chin L, et al. Cancer Res 2008; 68: 8535–40).

[205] *Gene expression analysis*: Genome-wide mRNA expression was measured in 78 patients from the Yale triple-negative cohort who were also tested for the *KRAS* variant. Total RNA was isolated from tissue specimen with the RecoverAll total nucleic acid isolation kit (Applied Biosystems) and hybridized to the whole genome DASL assay (HumanRef-8 version 3.0, Illumina, San Diego, CA, USA). Data preprocessing and statistical analysis were done with the lumi package in Bioconductor/R software. Gene-expression data from three whole-genome DASL runs were combined and processed together. Samples with less than 30% detectable probes and probes that were detectable in less than 10% of the samples were discarded before quantile-normalization. 74 samples and 18345 probes remained after filtering.

[206] *MicroRNA analysis:* MicroRNA arrays were performed using the Multiplex RT and TaqMan low density array human miRNA panel-real-time PCR system (Applied Biosystems) as per the manufacturer's protocol (miRNA profiling, publicly available at www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/mirna-profiling.html (accessed Jan 1, 2008). Expression levels of miRNAs of interest were examined.

Statistical Analysis

[207] Genotype distributions of all cases and controls were tested for Hardy-Weinberg equilibrium and were found to be in equilibrium. Unconditional logistic regression was performed to estimate the relative risk associated with every genotype. Controls were adjusted for age (continuous) and ethnic origin (white, black, Hispanic, or other). The population was stratified by menopausal status (estimated by age ≤ 51 years or >51 years), and separate risk estimates were obtained by ER and PR statuses with multinomial logistic regression with a three-level outcome variable coded as 0 for controls, 1 for cases with ER-positive and/or PR-positive tumors, and 2 for ER/PR-negative tumors. Wald χ^2 tests for interaction were performed, comparing the parameter estimates obtained for every genotype in cases of ER-positive and/or PR-positive disease compared with ER/PR-negative disease.

[208] Patients in study group 2 were stratified according to the subtype of breast cancer and a $\chi 2$ test was performed using the GraphPad Prism4 software to calculate the p values, odds ratios (Ors), and 95% confidence interval (CI). The dominant model was used for all genetic association analysis due to the low frequency of *KRAS* variant.

[209] Categorical variables (*e.g.*, ethnic origin, stage, and study site) were compared between study groups with a χ^2 test or two-sided Fisher's exact test, and continuous variables (*e.g.*, age) with a t test. ORs and a 95% CI were calculated for the *KRAS* variant in controls and cases of triple-negative breast cancer with an unconditional logistic regression model with a binary outcome variable. Multivariate logistic regression analyses with a binary outcome variable coded as controls and cases included variables such as *KRAS*-variant status, age, ethnic origin, and study site. The population was also stratified by age group, and separate logistic regression analyses were done for patients aged 51 years or younger (premenopausal group) or older than 51 years (postmenopausal group). Statistical analyses were done with SAS version 9.1.3.

[210] Pathway activation was measured as correspondence with previously published expression signatures and axes derived from principal component analysis of the expression set. Principal component analysis was used to separate biological from technical sources of information in the gene-expression dataset. Every component was characterized by correspondence to RNA quality, the structure of a batch effect, and biological annotations of the contributing probes (*i.e.*, probes with expression profiles that have high absolute projection values for the specified component). Signatures of gene expression are provided as lists of genes and their changes in expression in a specific condition. Such signatures are especially valuable for noisy data because they require coordinated differential expression of multiple probes, typically in the order of 100. Because mRNA was extracted from formalin-fixed, paraffin-embedded (FFPE) blocks that were up to 20 years old, analysis of the data set with a signature approach was justified (Kibriya M, et al. BMC Genomics 2010; 11: 622). S signature scores were calculated as Pearson correlation between the respective signature vector of gene contributions and a sample's expression profile for these genes. Association of the *KRAS* variant with the outcomes described by the respective signature was analyzed by a paired Kolmogorov-Smirnov test between signatures scores of KRAS variant and wild type samples. Differential gene expression was assessed with a linear model, taking into account technical batch artifacts as an offset. Model fitting and empirical Bayesian error moderation of the fold changes were performed with the LIMMA package for R (Smyth GK. Limma: linear models for microarray data. In: Gentleman R, et al, eds. Bioinformatics and computational biology solutions using R and bioconductor. New York, USA: Springer, 2005: 397–420).

[211] MiRNA expression was analyzed in 8 batches of 46 miRNAs and 2 endogenous controls each. MicroRNA expression was normalized using the geometric mean over all expressed samples: A miRNA was judged have been expressed if threshold fluorescence was detected after less than 35 cycles (ct < 35) and the geometric mean cycle number of all expressed miRNAs was subtracted. MiRNAs that were not expressed in more than two thirds of all samples were removed, followed by scale-normalization over all remaining threshold cycle (C_t) values.

[212] Frequency distributions of the *KRAS*-variant genotype did not differ between cases and controls who were genotyped from study group 1 (Table 1 and Table 4). However, the *KRAS* variant was significantly associated with breast cancer in premenopausal patients with ER/PR negative tumors (Table 4). This association was not observed for postmenopausal women. Eight (33%) of 24 premenopausal women with ER/PR-negative cancer had the *KRAS* variant, compared with 27 (13%) of 201 controls and four (9%) of 44 premenopausal women with cancer that was positive for ER and/or PR (Figure 5). Thus, the *KRAS* variant might be a genetic marker of increased risk of development of receptor-negative breast cancer for premenopausal women.

[213] **Table 4.** Association of the *KRAS*-variant with ER/PR positive versus ER/PR negative breast cancer.

	Controls	All		ER and/or PR positive		ER/PR negative		P[illegible]
		Cases	Odds ratio (95% CI)*	Cases	Odds ratio (95% CI)*	Cases	Odds ratio (95% CI)*	
All ages								
Non-variant (T/T)	391	347	Reference	145	Reference	62	Reference	-
Variant (T/G or G/G)	79	68	0·95 (0·67–1·36)	28	0·93 (0·58–1·49)	18	1·59 (0·88–2·86)	0·118
Premenopausal								
Non-variant (T/T)	174	84	Reference	40	Reference	16	Reference	-
Variant (T/G or G/G)	37	16	1·64 (0·79–3·43)	4	0·87 (0·28–2·75)	8	4·78 (1·71–13·38)	0·015
Postmenopausal								
Non-variant (T/T)	217	263	Reference	105	Reference	46	Reference	-
Variant (T/G or G/G)	52	52	0·77 (0·51–1·16)	24	0·90 (0·53–1·53)	10	0·90 (0·43–1·90)	0·991

Data are number or odds ratio (95% CI), unless otherwise stated. ER=oestrogen receptor. PR=progesterone receptor. *Age, ethnic origin, and menopausal status were adjusted in monomial unconditional logistic regression. G/G phenotype occurs in less than 5% of cases and controls and was combined with the G/T phenotype. Minor allele frequency (controls) 0·087, p for Hardy-Weinberg equilibrium 0·783.

[214] In study group 2, 478 women had luminal A breast cancer, 87 had luminal B breast cancer, 90 had triple-negative breast cancer, and 35 had HER2-positive breast cancer. 98 (14%) of 690 breast-cancer cases from this cohort had the *KRAS* variant, but prevalence varied between the breast cancer subtypes: The *KRAS* variant was statistically significantly enriched in women with triple-negative breast cancer (19 [21%] of 90 cases) compared with 64 (13%) of 478 for

luminal A, 13 (15%) of 87 for luminal B, and two (6%) of 35 for HER2-positive subgroups (p=0.044; Figure 1). This association with triple-negative breast cancer was also noted in women younger than 51 years (p=0.033, Figure 1).

[215] By comparison of cases of triple-negative breast cancer from groups 2 and 3 and controls across all three cohorts (n=1160), a statistically significant difference was found between cases or between controls for the prevalence of the *KRAS* variant (Table 5). There were more non-white women in the controls from study groups 1 and 3 than there were in the study group 2, which allowed assessment of the association of the *KRAS* variant in non-white women with triple-negative breast cancer in the multivariate analysis. After controlling for age, ethnic origin, and study site, the *KRAS* variant did not predict an increased risk of development of triple-negative breast cancer for all women in multivariate analysis (Table 6 and Table 7). However, the *KRAS* variant was associated with a statistically significant increased risk of development of triple-negative breast cancer in the'361 premenopausal women in this pooled group in multivariate analysis (Table 6, Table 8, and Table 9).

[216] **Table 5.** Demographic variables for TNBC cases (A) and controls (B) from the Irish cohort versus Yale cohort using Chi-square test for categorical variable such as ethnicity and t-test for the continuous variable (*i.e.* age.)

A. TNBC cases

Variable	Ireland (n = 90)	Yale (n = 140)	P value
Age	52.09 (10.66)	53.2 (13.03)	0.4995
Ethnicity			<0.0001
Caucasian (n = 166)	90 (100.00)	76 (54.29)	
African American (n = 50)	0 (0.00)	50 (35.71)	
Hispanic (n = 11)	0 (0.00)	11 (7.86)	
Asian American (n = 3)	0 (0.00)	3 (2.14)	
KRAS status			0.3863
Wild type (n = 188)	71 (78.89)	117 (83.57)	
Variant (n = 42)	19 (21.11)	23 (16.43)	

B. Controls

Variable	Ireland (n = 360)	Yale (n = 570)	P value
Age	70.78 (6.78)	55.14 (11.02)	<0.0001
Ethnicity			<0.0001
Caucasian (n = 881)	360 (100.00)	521 (91.40)	
African American (n = 44)	0 (0.00)	44 (7.72)	
Hispanic (n = 5)	0 (0.00)	5 (0.88)	
KRAS status			0.9271
Wild type (n = 780)	303 (84.17)	477 (83.68)	
Variant (n = 150)	57 (15.83)	93 (16.32)	

[217] **Table 6.** Association of the KRAS-variant in 230 patients with triple negative breast cancer compared with 930 controls from pooled analysis of study groups 1-3.

	Odds ratio (95% CI)	p value
All ages		
Univariate analysis		
KRAS variant	1·162 (0·797–1·694)	0·4363
Multivariate analysis		
KRAS variant	1·352 (0·901–2·028)	0·1455
Age	0·913 (0·942–0·967)	<0·0001
Ethnic origin	2·536 (2·784–5·999)	<0·0001
Premenopausal women		
Univariate analysis		
KRAS variant	1·879 (1·067–3·310)	0·029
Multivariate analysis		
KRAS variant	2·307 (1·261–4·219)	0·0067
Age	0·913 (0·871–0·956)	0·0001
Ethnic origin	2·536 (1·582–4·067)	0·0001

Age, ethnic origin, menopausal status, and study site were adjusted in a logistic regression model. G/G phenotype occurs in less than 5% of cases and controls and was combined with the G/T phenotype.

[218] **Table 7.** Demographic variables for TNBC cases and controls of all ages using Chi-square test for a categorical variable such as ethnicity and t-test for a continuous variable (*e.g.*, age).

Demographics

Variable	Controls (n = 930)	Cases (n = 230)	P value
Age	61.20 (12.26)	52.77 (12.14)	<0.0001
KRAS			0.4293
Wild type (n = 968)	780 (83.87)	188 (81.74)	
Variant (n = 192)	150 (16.13)	42 (18.26)	
Ethnicity			<0.0001
Caucasian (n = 1047)	881 (94.73)	166 (72.17)	
African American (n = 94)	44 (4.73)	50 (21.74)	
Hispanic (n = 16)	5 (0.54)	11 (4.78)	
Asian (n = 3)	0 (0.00)	3 (1.30)	

[219] **Table 8.** Demographic variables for premenopausal TNBC cases and controls of using Chi-square test for a categorical variable such as ethnicity and t-test for a continuous variable (*e.g.*, age).

Demographics

Variable	Controls (n = 250)	Cases (n = 111)	P value
Age	45.37 (4.65)	42.70 (5.80)	<0.0001
KRAS			0.0331
Wild type (n = 300)	215 (86.00)	85 (76.58)	
Variant (n = 61)	35 (14.00)	26 (23.42)	
Ethnicity			<0.0001
Caucasian (n = 297)	219 (87.60)	78 (70.27)	
African American (n = 52)	28 (11.20)	24 (21.62)	
Hispanic (n = 9)	3 (1.20)	6 (5.41)	
Asian (n = 3)	0 (0.00)	3 (2.70)	

[220] **Table 9.** Association of the KRAS variant with triple negative breast cancer cases under 51 years of age versus controls in the Irish and Yale cohorts.

Irish cohort*

Variable	OR	95% CI	P value
KRAS -variant	1.933	0.942 - 3.966	0.0723

* Univariate analysis against all controls

Yale cohort

Variable	OR	95% CI	P value
KRAS -variant	2.457	1.121 - 5.384	0.0248

Multivariate Analysis, controlled for race and age

[221] Because *BRCA1* coding sequence mutations are associated with risk of triple-negative breast cancer, and because the *KRAS* variant is enriched in *BRCA1* mutation-carriers with breast cancer (Hollestelle A, et al. Breast Cancer Res Treat 2010; published online July 30. DOI:10.1007/s10549-010-1080-z), it was determined whether the association of the *KRAS* variant with premenopausal triple-negative breast cancer was due only to its association with carriers of *BRCA1* mutation. Of 36 women with triple negative breast cancer from cohort 2 and 3 who were *BRCA* tested, 25 (69%) were *BRCA* negative and 11 (31%) were *BRCA* positive. Of

these patients, eight (32%) *BRCA*-negative women had the *KRAS* variant compared with three (27%) women who were *BRCA* positive. These findings suggest that the *KRAS* variant is associated with an independent group of patients with triple-negative breast cancer without *BRCA* mutations.

[222] An association was discovered between *KRAS*-variant status and ER or PR negative statuses in the Rotterdam population cohort (Hollestelle A, et al. Breast Cancer Res Treat 2010; published online July 30. DOI:10.1007/s10549-010-1080-z; Kibriya M, et al. BMC Genomics 2010; 11: 622), however, menopausal status was not considered in these studies. With respect to the results of the study described herein, an enrichment of the *KRAS* variant was not observed in 126 premenopausal *BRCA1*-mutation carriers who had ER/PR-negative breast cancer compared with 268 *BRCA1*-mutation-carriers from the Rotterdam cohort (21·8% vs 23·5%, p=0.95). Thus, association of the *KRAS* variant with premenopausal triple-negative breast cancer is independent of its association with *BRCA1* mutations.

[223] To further assess potential biological interaction between the *KRAS* variant and altered *BRCA1* expression in triple-negative breast cancer, *BRCA1* expression levels were determined in 74 triple-negative tumors from study group 3 (Table 1). Those patients with the *KRAS* variant demonstrated a statistically significant reduction of *BRCA1* expression compared with *KRAS* variant-negative triple-negative tumors (p=0.06 for probe 1 [ILMN_2311089] and p=0.01 for probe 2 [ILMN_1738027], Figure 2). Furthermore, the *KRAS* variant demonstrated a statistically significant association with a gene expression signature of decreased *BRCA1* activity (p=0.04) (van't Veer LJ, et al. Nature 2002; 415: 530–36). The data provided herein indicate that, although the *KRAS* variant is not restricted to patients with triple negative breast cancer with known *BRCA1* mutations, a biological interaction between the *KRAS* variant, altered *BRCA1* expression or functionality, and development of triple-negative breast cancer may exist.

[224] Signaling pathways in triple-negative breast-cancer tumors that were KRAS-variant positive were compared with those that were *KRAS*-variant negative from patients in study group 3. Although analysis of *KRAS* mRNA did not vary by *KRAS*-variant status, the data are consistent with the other publications with respect to the effect of miRNA binding to the 3′-UTR of *KRAS* (Chin L, et al. Cancer Res 2008; 68: 8535–40; Johnson SM, et al. Cell 2005; 120: 635–47). An increase was found in both an *NRAS* mutation (Croonquist PA, et al. Blood 2003; 102: 2581–92) and a MAP-kinase activation signature (Creighton CJ, et al. Cancer Res 2006; 66:

3903–11) (Table 10) in tumors with the *KRAS* variant. The data indicate that the *KRAS* variant alters gene expression of canonical RAS pathways. Moreover, the data provide the first *in-vivo* evidence that the *KRAS* variant leads to continued altered downstream gene expression in tumors with which it is associated.

[225] Table 10. Association of the *KRAS*-variant with pathway signatures in tumors of patients with triple negative breast cancer and positive *KRAS* variant status.

	Signature expression	Kolmogorov-Smirnov p value
NRAS	Upregulated	0·02
BRCA mutant-like	Upregulated	0·04
Luminal progenitor	Upregulated	0·04
MAPK (Creighton)	Upregulated	0·06
PCA oestrogen	Downregulated	0·04

Signature scores were computed as Pearson correlation between the signature vector of gene contributions and each sample's expression profile for these genes. The Kolmogorov-Smirnov test was used to analyse the association of the KRAS-variant with signature activation.

[226] Because concentrations of *let-7* miRNA are altered in lung tumors with the *KRAS* variant, let-7 concentrations were examined in triple-negative breast cancer tumors with the *KRAS* variant. The data demonstrated lower concentrations of all *let-7* miRNA family members in *KRAS*-variant-associated tumors (Figure 3).

[227] To establish how the *KRAS* variant integrates with known gene-expression signatures of triple-negative breast cancer, known signatures that are differentially expressed in such tumors were assessed. *KRAS*-variant tumors have several features of triple negative and basal-like tumor biology, including decreased estrogen signaling in a main component derived from the expression set (p=0.04). Furthermore, *KRAS*-variant tumors have a luminal progenitor signature (p=0.04), which is a candidate progenitor for basal-like breast cancer (Lim E, et al. Nat Med 2009; 15: 907–13) (Table 10 and Figure 6). Within the luminal progenitor and the *BRCA* mutation-like signatures, markers of cell adhesion, tissue invasion, proliferation, and angiogenesis (such as α5 integrin, DUSP6, and aurora kinase B) were differentially regulated (Table 11). This discovery agrees with the slight enrichment by functional annotations that were observed in three of 41 genes for wound healing (p=0.02), three of 151 genes for glycan expression (p=0.05), and four of 148 genes for MEK activation (p=0.009) on the basis of the differentially expressed genes in a linear model comparing *KRAS* variant versus non-variant for the dataset (Figure 4, Table 12 and Table 13).

[228] **Table 11.** KRAS variant differentially expressed genes within a luminal progenitor and BRCA mutant signature by LIMMA analysis in triple negative breast cancer patients.

Within luminal progenitor signature nuID	gene	logFC	p	p.adj
W5dWOuc9PtPXl'IOHmo	SORBS2	0.77024109	0.001	0.165
rK6quXGu26dLdfeAJ4	PPP1R1B	0.75990879	0.003	0.212
0VIU5B1erOXhhF0r8	EPAS1	-1.5277527	0.003	0.212
W047wnl3994KR9_d.o	WFDC2	0.76664801	0.003	0.212
3t4p_pol6lla12q.l0	FABP4	0.6895776	0.009	0.338
NRBlFzZ2ltCuhzC9Vk	AURKB	-1.5353021	0.011	0.338
Brh6EzR4xBDW178mE	AKR1C3	-0.8935685	0.012	0.338
raaulol?SnkqpBIJBE	MATK	0.49838612	0.012	0.338
0SUcSsfN4XwSS3fTro	TLR5	-1.0574883	0.014	0.338
6BV+9xJ77TR4UIRek	FABP4	-0.8803527	0.014	0.338
TJc6LpVwNL4e7J35ek	NCALD	-1.0265287	0.015	0.338
ropelTUh1SaPWDlif.0	UBE2C	-0.6930782	0.029	0.498
lO0dd56nvT7TS7vSoU	NCALD	-1.0912361	0.031	0.498
cRUpDE96.fUIlL_lf0	FHL2	-0.9577935	0.034	0.498
KzhYQpF167cHVUCEOI	CAMK2N1	-0.898999	0.034	0.498
f7r9CcMS9S_H1LcV64	PNMA2	0.60230064	0.035	0.498
3IN_kumvr9Ut3OnSqY	C4orf7	2.14470518	0.035	0.498
xJ1J7qZL0S0hjUj1U	COL4A5	-1.3394053	0.038	0.498
No174RVAVBCigl6quU	SLPI	1.32821488	0.038	0.498
itHUgo.Di1NkG1FXIE	CSN3	1.69487059	0.046	0.561
Nrlw71O3gv5VLnEb6I	MAOA	-1.2134788	0.053	0.597
NKr_sVQH8gDQOQXS6Q	MAF	0.42034031	0.057	0.597
0luaqfpUfrolEn_qP0	PIK3R1	0.37430905	0.057	0.597
uQV9XRfpFIRUofPwE	ITGA5	-0.31653	0.058	0.597
uVwtXd.AgfeatId4JY	C1QTNF1	-1.0556671	0.061	0.598
it1lSdTSgUJAzSMl4Q	HSPB8	-1.1720388	0.064	0.607
upHrr.S3YSzUQyeGno	NQO1	-0.8463655	0.067	0.607
TgCvBlxJ2l_4kdxSCE	CPE	-0.7809367	0.075	0.651
EMXdPdfU745Uq0d_c	C1QTNF1	-0.9906795	0.077	0.651
9iGSGSK2JkpYCe9Fv8	BMP4	0.49379544	0.083	0.651
N_SIAbV_UdVHdNKlLb	KRT15	0.97595087	0.085	0.651
fargRN4IRzv16dwp20	NKD2	-0.4423883	0.086	0.651
Z1Jj2hGjuC1Fznp1CQ	C19orf33	-0.9290315	0.087	0.651
rdxCJVSV3vopEvBqwo	PIGR	-0.7374176	0.091	0.662
iSupIMfzEnQ1wS4010	SRPK2	0.36689011	0.097	0.680
r_iBJ6cKOHcLse.k.U	TMEM45A	0.72481799	0.100	0.685

Within the BRCA mutant like signature nuID	gene	logFC	p	p.adj
reP_FIlUMuQSHaOstY	DUSP6	-1.5635473	0.000	0.06
Zr6CDmnTPxQt.eBl94	TFPI	0.88726644	0.001	0.11
33oJ15BdCIfVK20peg	GPRC5C	-1.3098662	0.002	0.18
lcmjQs6EeHSHlS4R4	OGG1	-0.8517652	0.004	0.24
HSpcnaOBxYjCQuJZ90	GLRX2	0.47679698	0.005	0.24
NVteQjfOacrl6qsEkI	ZNF644	1.15014517	0.005	0.24
fCkeqM.Snv6eIexVLU	UNC119	0.73173575	0.006	0.24
fKVBOKu10UBIWSIeCE	VPREB3	0.92362289	0.025	0.47
rCOmieZudsntCkzNhw	ETFB	-1.0538852	0.025	0.47
IAbVmX1kWR1lAInU4	SORD	-0.8414537	0.026	0.47
ul6loQqDCFHTLsnpQk	AFF1	-0.7123633	0.028	0.47
cS7uUl4hr15e36q_uq	RBM38	0.57005612	0.029	0.47
WQoNqrg37cE9WK0Kc	WT1	0.35637204	0.029	0.47
xnfXiClfDUJePscu.0	PPP1CB	0.44366224	0.031	0.47
HIcJBIYc9oot4Ro3Q	ZFHX3	1.05484205	0.032	0.47
utBKruOuCkJ7qAbilo	P2RY10	0.58824966	0.033	0.47
iXO7UFFVnBOKn9T.e4	AHNAK	-0.5840241	0.034	0.47
KzhYQpF167cHVUCEOI	CAMK2N1	-0.898999	0.034	0.47
96nSuqQo6IOSne.kjw	C14orf115	0.26249749	0.035	0.47
Z6Lq_nS46eeicGenAs	KCNN4	0.41693431	0.035	0.47
KotS1gojafuv0V4oHo	NME1	-0.469841	0.038	0.48
o.7wbeUVUd0VU3CVU	C1orf38	0.37671966	0.039	0.48
WFwa905mw_yOTsfo4	NPTN	-0.2142517	0.046	0.54
WLF_H_9Ek7KsO3HPD4	CA2	0.4793348	0.049	0.54
EqPaeX9VKg13UjfBQq	BPI	0.41133271	0.051	0.54
ccuFel7ASQuqnkkko	DUSP13	0.37229168	0.056	0.54
6p1QpTggjsQpzXQsVY	STARD10	-0.640911	0.060	0.54
lUfekOoxruER_DO7jk	KLHL2	0.40422699	0.062	0.54
Npd2qV2SJe2WpXh2F0	NME2	-1.282517	0.065	0.54
ZWm_wXShfV_vgSX92A	PUS1	-0.6502441	0.065	0.54
upHrr.S3YSzUQyeGno	NQO1	-0.8463655	0.066	0.54
uwgl3oT4cTqooEnWpU	TNNT1	0.37817669	0.068	0.54
iH7SA5ONA5BdEF_nl0	MYC	-0.5960534	0.068	0.54
rMDue4oOgDOuSu3604	ZNF644	0.30516255	0.070	0.54
TgCvBlxJ2l_4kdxSCE	CPE	-0.7809367	0.074	0.54
ooT1lUS2EnhQoTjknAo	DCI	-0.8140602	0.076	0.54
HHS16RdB7hDSQJVFSk	INPPS1	-0.6589636	0.077	0.54
rXfTkN64kuuSmqJSIA	DDX58	0.45766024	0.077	0.54
ExQ1zRe9Pil7konrlE	GPM6B	-0.9870874	0.080	0.55
lK8gpErprOJasUm9el	CRYM	0.33291961	0.083	0.55
ZKcgDHZH10gV29_6Go	BMPR2	0.20205644	0.084	0.55
9dX6CEziOHV_ch14SU	TCF7	0.83647149	0.091	0.55
ofV37VqjeZdy5f_Kr0	FAM129B	-0.2404329	0.096	0.55
uSlqgLlkJeIep6Jfc	ORMDL3	0.90327229	0.096	0.55
Q1xpkX5rqcuB2PkfY4	NME2	-0.5310338	0.097	0.55
cXSOUqtS3JNLCr8kgE	SSR1	0.36145747	0.098	0.55
rozl_REnvjonvOxs	ITGB5	-0.5851932	0.099	0.55

[229] **Table 12.** Enrichment of selected literature-derived signatures with genes identified to be KRAS variant differentially expressed by LIMMA analysis in triple negative breast cancer patients.

Signature	p.adj	p.raw	maxG	diff. Exp
ctnnb_bild2006	0.61600883	0.045721354	61	2
glyc_potapenko09	0.61600883	0.052426283	151	3
intrinsic_hu06	1	0.688155238	823	4
mek_dry2010	0.43918195	0.009344297	148	4
safb12_chiptargets	0.7154859	0.091338625	429	5
safb12_mmatargets	0.64952229	0.069098116	519	6
wound.chang.down	0.51678369	0.021990795	41	2
wound.chang.up	1	0.387976745	87	1

Abbreviations : p.adj: FDR-adjusted p-value, maxG: The number of genes in the signature represented on the Illumina microarray, diff. Exp: the number of genes in that signature found to be differentially expressed.

[230] Table 13. List of 50 differentially expressed genes in triple negative cancer patients who are KRAS variant positive, as identified by LIMMA analysis.

saID	gene	logFC	p-value	p.adj
xu0rGtKPdOHt8XceDM	KAL1	1.161	5.27E-006	0.060
51179x05bW7t66WfXg	ABHD2	1.197	3.08E-005	0.211
6p2cSR6PJdAXXqDXES	CHTF8	-1.762	5.41E-005	0.211
63x3kkE6KIv_7Li7qY	DNSC	0.952	5.80E-005	0.211
tES9SLO6FVQLUtPIco	LIX1	0.862	7.67E-005	0.211
lno3WLlWlql4ompEqQ	BTBD3	0.892	9.30E-005	0.211
nqeojiwejf50tbdTk	SIDT2	0.704	9.45E-005	0.211
a5gLvder_5UU65g14U	CAMK2A	1.081	1.01E-004	0.211
95fXtx_pFVFF7ye68	BAG3	-2.012	1.21E-004	0.211
rVUch7idfp5b83e9k	NA	-2.011	1.25E-004	0.211
Z6n_xvX3TdHxtHH2U0	SEL1L	1.606	1.49E-004	0.211
Bpeyv1kQv0mIgogBSc	ARMCX6	-1.394	1.62E-004	0.211
3UCajp6zXipJBv0TCo	C9orf43	0.689	1.66E-004	0.211
TYoWYd0HWC0JWB3jxc	NUP93	-1.655	1.71E-004	0.211
HkskAssd7tq8Nr_KSc	PRDM1	1.024	1.83E-004	0.211
0F8FI88H_7XU0efV4	TUBA1B	-0.59	1.84E-004	0.211
cKeVUq.eR61Xf9X0t8	MLH3	-1.911	1.99E-004	0.214
Em7e.HJ5tl_hq6eE3s	ETS2	-1.661	2.27E-004	0.214
reF_FtFUMuQ5HaOstY	DUSP6	-1.564	2.28E-004	0.214
ie1dN1nl3Ulf_SV78	NA	-1.379	2.40E-004	0.214
3gq7osynhfRUieXOVI	TNNC1	0.94	2.65E-004	0.214
rmqB9Bd3eQp7bQjlQI	CDCP1	-1.343	2.73E-004	0.214
oWlPAI4KSFnkWWXkAA	NA	-1.87	2.84E-004	0.214
z_h054gFRKAke1Kyg	MFAP1	-1.08	3.04E-004	0.214
xU75QpS3gNep0LjXXk	NA	0.991	3.10E-004	0.214
Qepfmoeledex5zWUg	C9orf89	-1.841	3.12E-004	0.214
KyB4s96IAUOtJH.r_I	MYNN	1.152	3.26E-004	0.214
o017n3rGln2X0laUXE	GALR3	0.764	3.34E-004	0.214
TfSd3QigkFQhvRbijY	NF2	-1.571	3.39E-004	0.214
ZHdUeJCbe61zr2BSzc	ARHGAP24	0.524	4.02E-004	0.232
NLTejns0VUSrNidBmo	HIPK1	-1.277	4.16E-004	0.232
6k8150.rafeULn.dL4	TMTC2	0.728	4.19E-004	0.232
tjpnYVZIUk35E7YAI4	CMC1	-1.158	4.28E-004	0.232
W5dWOuc9PtRXFIOHmo	SORBS2	0.77	4.45E-004	0.232
3ddSK3kR1H0TlVh3RI	TBC1D14	-1.495	4.51E-004	0.232
fadTveHmgqBgITIXo	FAH	-1.265	4.55E-004	0.232
10fqIh.8QdN5J1q5o	CACNG6	0.695	5.00E-004	0.244
0Cb9ek_mecjpZu5dKS	AGRN	-1.358	5.05E-004	0.244
r5VtgoIRK45ogIPlPU	LARP1	0.864	5.44E-004	0.247
fb1S33UT57TptL0Ub0	VPS8	-1.787	5.60E-004	0.247
6faTpd.EqRfed0r6uE	VIP	0.655	5.60E-004	0.247
QovQuig56f4ldxHar4	NA	-1.636	5.77E-004	0.247
Qdj_t1ZKf5ABUPkbuo	PWWP2B	-1.276	5.79E-004	0.247
Qp9WAlX7cu6IXQ7pE4	APEX1	-0.558	6.01E-004	0.251
HAzSPkVV3VT16f_VU	CADM4	-1.225	6.30E-004	0.252
opX0uY0C9xuYiWWD1A	NDUFC1	-1.197	6.70E-004	0.252
QuRcd6i5Qd4r5ZIS4I	FAM192A	1.657	6.74E-004	0.252
rh2CVIv_u6OJKhA_el	HMHB1	0.738	6.98E-004	0.252
xyeqUp93VRReSP3558	PCBP4	-0.742	6.99E-004	0.252
6KL5K6C6EnZIKLIk7E	MYH10	0.77	7.04E-004	0.252

Example 2: Prevalence of the *KRAS* variant in various cancer cell lines

Materials and Methods

[231] *Genotyping*. DNA from the NCI-60 cell line panel was obtained from the NCI's Developmental Therapeutics Program. Taqman genotyping was performed to determine the presence of the *KRAS* variant allele as described previously(Bussey KJ, et al. Mol Cancer Ther

2006; 5:853-67). Cells were cultured under standard conditions (see, dtp.cancer.gov/branches/btb/ivclsp.html; Monks A, et al. J Natl Cancer Inst 1991; 83:757-66), for a maximum of 20 passages from frozen stock. DNA was isolated using the Qiagen QIAamp DNA blood maxi kit procedure (cat. 51192).

[232] *Statistical analyses*. The *KRAS* variant allele data were coded numerically, with 1 representing the presence of *KRAS* variant allele and 0 representing the absence of the *KRAS* variant allele. This pattern was used as a "seed" in COMPARE analyses (Paull KD, et al. J Natl Cancer Inst 1989; 81:1088-9248) to probe the existing NCI-60 data sets in the NCI-DTP databases. Correlations included, for example, miRNA measurements and DNA methylation measurements. A positive correlation indicates, for example, that cell lines with the variant allele tend to have higher expression of the miRNA/mRNA or greater percentage DNA methylation. Conversely, negative correlations indicate that cell lines with the variant allele tend to have lower expression of a given miRNA/mRNA or lower percentage DNA methylation at the indicated gene. These data sets can be queried or downloaded at dtp.cancer.gov.

[233] The presence of the *KRAS* variant is a genetic marker for prediction of risk and tumor biology as well as response to treatment in multiple cancers. The presence of the *KRAS* variant results in altered regulation by the *KRAS* 3' UTR. This study elucidates the biological significance of the *KRAS* variant in cancer cells. The data provided herein elucidate exemplary molecular pathways that are affected by the presence of the *KRAS* variant. To simultaneously analyze a broad range of cancer types, the comprehensive NCI-60 panel of cancer cell lines (Blower PE, et al. Mol Cancer Ther 2007; 6:1483-91; Liu H, et al. Mol Cancer Ther 2010; 9:1080-91) was used. Various molecular parameters were studied to determine which molecular events correlate with the presence of the *KRAS* variant in these cancer cell lines (Kundu, S.T. et al. 2012 Jan 15. Cell Cycle 11:2, 361-366).

[234] Seven of 60 cell lines in the NCI-60 panel harbor the KRAS variant allele (Table 14). When the NCI-60 panel of cell lines were categorized based on the presence of either an acquired dominant mutation in the *KRAS* coding region (*KRAS* mutation) or the presence of the *KRAS* variant, it was determined that all seven cell lines that contained the *KRAS* variant were negative for the presence of *KRAS*-activating mutations. Similarly, the cell lines that carried a *KRAS* coding sequence mutation lacked the *KRAS* variant allele. Thus, the presence or occurrence of either a *KRAS* coding mutation or the *KRAS* variant allele is mutually exclusive in

these cell lines. Furthermore, because this mutual exclusivity occurs in cell lines derived from a variety of cancer types, this mutual exclusivity is not specific to a particular tissue type. Rather, this mutual exclusivity is a common feature of these cancer cell lines regardless of origin. These results indicate that the occurrence of either of these two events alone (*i.e.*, the occurrence of the *KRAS* variant or the occurrence of a *KRAS* coding mutation), is sufficient to affect tumorigenesis in these cancer types. These results also indicate that the level of KRAS activation caused by a canonical coding sequence mutation is functionally comparable to the elevated *KRAS* expression induced by the presence of the *KRAS* variant in the 3' UTR. This mutual exclusivity of acquired *KRAS* coding mutations and the *KRAS* variant was also found in non-small cell lung cancer patients (Chin LJ, et al. Cancer Res 2008; 68:8535-40) and in ovarian cancer patients (Ratner E, Cancer Res 2010; 70:6509-15), but not in colon cancer patients (Zhang W, et al. Ann Oncol 2011; 22:484-5; Zhang W, et al. Ann Oncol 2011; 22:104-9).

[235] Table 14. Cell lines in the NCI-60 panel that harbor the *KRAS* variant allele or a functional mutation in the coding sequence of *KRAS*.

NCI-60 Cell Lines	Tissue Type	*KRAS* LC S6 Variant	*KRAS* Mutation in coding sequence
HCT-116	Colon	0	1
NCI-H460	NSCLC	0	1
A549/ATCC	NSCLC	0	1
OVCAR-5	Ovarian	0	1
CCRF-CEM	Leukemia	0	1
HCT-15	Colon	0	1
SN12C	Renal	0	1
NCI-H23	NSCLC	0	1
SW-620	Colon	0	1
MDA-MB-231/ATCC	Breast	0	1
RPMI-8226	Leukemia	0	1
HOP-62	NSCLC	0	1
MCF7	Breast	1	0
786-0	Renal	1	0
IGROV1	Ovarian	1	0
HCC-2998	Colon	1	0
DU-145	Prostate	1	0
EKVX	NSCLC	1	0
U251/SNB-19	CNS	1	0

[236] To determine whether the cell lines having the *KRAS* variant allele show a conserved alteration in the expression of miRNAs, a statistical analysis was performed on the miRNA expression profiles that were generated from seven cell lines that contain the *KRAS* variant allele compared with the miRNA expression profiles of the remaining cell lines of the NCI-60 panel (Blower PE, et al. Mol Cancer Ther 2007; 6:1483-91; Gaur A, et al. Cancer Res 2007; 67:2456-68). The presence of the *KRAS* variant allele shows a statistically significant positive correlation with increased expression of miR-23, miR-27 and miR-210 (Table 15). MiR-23 and miR-27 are expressed from the same cluster and advance progression of angiogenesis and metastasis (Zhou Q, et al. Proc Natl Acad Sci USA 2011; 108:8287-92). For example, miR-23 and miR-27 are enriched in endothelial cells and highly vascularized tissue. Moreover, miR-23 and miR-27 elevate signaling pathways that are essential for angiogenesis by reducing the expression of Sprouty2 and Sema6A, which have anti-angiogenic functions. Blocking the function of either miR-23 or miR-27 leads to a decrease in capillary tube formation and migration in response to VEGF *in vitro* and reduced vascularization of postnatal retinas *in vivo* (Zhou Q, et al. Proc Natl Acad Sci USA 2011; 108:8287-92). The statistically significant positive correlation of the *KRAS* variant with increased expression of miR-23, miR-27 suggests that tumor cells having the *KRAS* variant allele are prone to growth and metastatic progression as a result of elevated levels of miR-23 and miR-27.

[237] **Table 15**. MicroRNAs with statistically significant increased expression in cell lines having the *KRAS*-variant allele.

Kras SNP	By Variable	Correlation	Count	Significance	MicroRNAs upregulated
Kras SNP	MT3049	0.51	59	4.27E-05	microRNA hsa-miR-210N
Kras SNP	MT3048	0.49	59	8.31E-05	microRNA hsa-miR-210
Kras SNP	MT3076	0.47	59	0.000184	microRNA hsa-miR-27b
Kras SNP	MT3066	0.45	59	0.000373	microRNA hsa-miR-23b
Kras SNP	MT3077	0.44	59	0.000546	microRNA hsa-miR-27bN

[238] The expression of miR-210 is statistically significantly correlated with the presence of the *KRAS* variant allele in cells. MiR-210 is a marker of chronic hypoxia. Moreover, miR-210 is associated with proliferation and metastasis of breast and melanoma tumors as well as poor prognosis. MiR-210 is a direct transcriptional target of HIF proteins. Elevated levels of miR-210 are required for tumor cell survival under conditions of hypoxia. MiR-210 directly regulates the expression of MNT, a MYC antagonist that is required for cell cycle arrest under hypoxia. Consequently, increased levels of miR-210 contribute to an override of cell cycle arrest under

conditions of hypoxic stress in tumor cells. Because increased miR-210 expression is associated with the presence of the *KRAS* variant, tumor cells containing the *KRAS* variant survive and proliferate under hypoxic conditions.

[239] The data provided herein demonstrate that the *KRAS* variant contributes to or initiates aberrant signaling pathways that control the expression of several miRNAs (including, for example, miR-23, miR-27 and miR-210). Perturbation of signaling pathways that regulate expression of miRNAs, such as miR-23, miR-27 and miR-210, results in the initiation, development, maintenance or augmentation of tumor proliferation and metastatic transformation.

[240] Promoter methylation is one mechanism through which gene expression is silenced in many cancers because changes in the methylation status of gene promoters lead to reduction in gene expression. Specifically, DNA methylation is an epigenetic effect caused when CpG dinucleotides are methylated, often in the promoter region of genes. Because methylation blocks access to the promoter by molecules that mediate gene transcription, methylation of the promoter results in gene silencing. Different cancers show distinct methylation patterns, the result of which is alterations in gene expression signatures. Therefore, to determine whether there is an alteration in DNA methylation patterns in the tumor cell lines having the *KRAS* variant, the methylation status of these cell lines was compared with the non-*KRAS* variant lines in the NCI-60 panel (Ehrich M, et al. Proc Natl Acad Sci USA 2008; 105:4844-9). The presence of the *KRAS* variant allele shows a statistically significant positive correlation with increased methylation of the promoter of many genes, including, for example, *Notch1*, *cyclin D3* and *CNBP* (also known as *ZNF9*) (Table 16).

[241] **Table 16.** Genes with statistically significant promoter hyper-methylation in *KRAS* variant positive cell lines.

Kras SNP	By Variable	Correlation	Count	Significance	Promoter Locus	Gene names
Kras SNP	MT9686	0.56	58	5.89E-06	156_ZNF9_001_CpG [1]	ZNF9 or CNBP
Kras SNP	MT9698	0.52	58	2.47E-05	156_ZNF9_001_CpG [3-7]	ZNF9 or CNBP
Kras SNP	MT9695	0.52	58	0.00002697	156_ZNF9_001_CpG [17-20]	ZNF9 or CNBP
Kras SNP	MT9697	0.51	58	4.82E-05	156_ZNF9_001_CpG [43-45]	ZNF9 or CNBP
Kras SNP	MT9694	0.49	58	0.00011305	156_ZNF9_001_CpG [35-36]	ZNF9 or CNBP
Kras SNP	MT9689	0.43	58	0.00082522	156_ZNF9_001_CpG [17-19]	ZNF9 or CNBP
Kras SNP	MT5347	0.73	51	8.59E-10	014_CCND3_001_CpG [18-20]	Cyclin D3
Kras SNP	MT5363	0.59	52	3.63E-06	014_CCND3_001_CpG [6-8]	Cyclin D3
Kras SNP	MT5346	0.55	35	0.00055557	014_CCND3_001_CpG [13-17]	Cyclin D3
Kras SNP	MT5345	0.46	52	0.00058183	014_CCND3_001_CpG [11-12]	Cyclin D3
Kras SNP	MT7559	0.57	59	2.63E-06	091_NOTCH1_001_CpG [9]	Notch 1 Ligand
Kras SNP	MT7549	0.53	59	0.00001846	091_NOTCH1_001_CpG [1-2]	Notch 1 Ligand
Kras SNP	MT7557	0.5	59	6.15E-05	091_NOTCH1_001_CpG [22-23]	Notch 1 Ligand
Kras SNP	MT7555	0.42	59	0.00079928	091_NOTCH1_001_CpG [17-20]	Notch 1 Ligand

[242] The role of *Notch1* expression in cancers is diverse. In many tumors, *Notch1* overexpression or activation drives cancer progression and metastasis. For example, *Notch1* activation results in an increase in invasive and migratory characteristics of breast cancer cells. Alternatively, *Notch1* overexpression in a MYC background induces adenomas in the mouse lung, leading to the formation of lung adenocarcinoma. Thus, the evidence indicates that *Notch1* may function as an oncogene. In contrast, Notch1 may also function as a tumor suppressor. For example, inhibitory mutations in *Notch1* have been identified in squamous cell carcinomas of the head and neck. Depletion of *Notch1* in mouse skin keratinocytes results in enhanced tumorigenesis by chemical carcinogens or by oncogenic Ras. In cervical cancers positive for the human papillomavirus (HPV), *Notch1* expression is decreased when compared with normal adjacent tissue. Overexpression of activated *Notch1* in HPV-positive cervical cancers and neuroblastoma cells (Zage PE, et al. Pediatr Blood Cancer 2011) leads to growth inhibition. Considered together, the evidence show that *Notch1* is dysregulated in many cancers and, in some instances, may function as a putative tumor suppressor. Because methylation of the *Notch1* promoter is increased in *KRAS* variant-positive cancer cells, *Notch1* expression may be reduced in cells carrying the KRAS variant allele, and, therefore, *KRAS*-variant cell lines may induce or maintain their tumorigenic potential by inhibiting the tumor suppressing effects of *Notch1*.

[243] Cyclin D3 is the member of the cyclin family of cell cycle proteins that is required for the G_1/S transition of the cell cycle. In *KRAS* variant cell lines, promoter methylation of *cyclin D3* is increased, which indicates repression of *cyclin D3* transcription. Consequently, the evidence

suggests two exemplary mechanisms in which either *cyclin D3* is not required for the transformed phenotype of these cell lines or methylation of the *cyclin D3* promotor blocks a transcriptional repressor of *cyclin D3*.

[244] In contrast to *Notch1* and *cyclin D3*, *CNBP* (*cellular nucleic acid binding protein*), also called *ZNF9*, is not associated with the development or progression of cancer. However, *CNBP/ ZNF9* is part of a complex that binds to the MYC promoter. When expression of MYC is dysregulated, MYC contributes to the development and progression of cancer. The mechanism by which the association of the *KRAS* variant with the methylation status of *ZNF9* contributes to cancer progression in *KRAS*-variant cells is unclear.

[245] Gene expression in the seven cell lines harboring the *KRAS* variant allele was compared with the profiles of the remaining cell lines in the NCI-60 panel to determine specific alterations in gene expression in these cell lines. As shown in Table 17, a gene whose elevated expression is statistically significantly correlated with the presence of the *KRAS* variant in the cell lines is *glutathione S-transferase theta1* (*GSTT1*). The *GSTT1* gene encodes a member of the glutathione S transferase family of human phase II detoxifying enzymes, which detoxifies complex metabolic byproducts, xenobiotics and drugs by conjugating a glutathione group to these compounds, thus making them more soluble and easily excreted out of the cell. The theta1 isoform has been implicated in several cancers. For example, increased expression of *GSTT1* is statistically significantly correlated with aggressive bladder cancers. In other different tumors types, GSTT1 is nonfunctional or absent due to genetic polymorphism, thus leading to increased risk of carcinogenesis and poor prognosis as a result of an accumulation or increased accumulation of toxic metabolites.

[246] **Table 17.** Genes with statistically significantly higher mRNA expression in *KRAS* positive cell lines.

Kras SNP	By Variable,	Correlation	Count	Significance	Genes upregulated
Kras SNP	GSTT1	0.62	59	1.72E-07	Glutathione S-transferase theta1
Kras SNP	SYT12	0.54	59	1.06E-05	Synaptotagmin XII
Kras SNP	ITIH1	0.54	59	1.11E-05	Inter-alpha (globulin) inhibitor H1
Kras SNP	MAPK3	0.53	59	1.77E-05	Mitogen-activated protein kinase 3
Kras SNP	POLD1	0.52	59	2.26E-05	Polymerase (DNA directed), delta1, catalytic subunit 125 kDa
Kras SNP	TNFAIP2	0.52	59	2.76E-05	Tumor necrosis factor, alpha-induced protein 2
Kras SNP	SELE	0.51	59	3.15E-05	Selectin E (endothelial adhesion molecule 1)
Kras SNP	GPLD1	0.51	59	4.02E-05	Glycosylphosphatidylinositol specific phospholipase D1
Kras SNP	HINT2	0.5	59	5.39E-05	Histidine triad nucleotide binding protein 2
Kras SNP	EFNA4	0.5	59	6.48E-05	Ephrin-A4
Kras SNP	MFAP1	0.49	59	7.77E-05	Microfibrillar-associated protein 1
Kras SNP	P4HB	0.49	59	7.93E-05	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase-associated 1)
Kras SNP	SULT1E1	0.49	59	8.28E-05	Sulfotransferase family 1E, estrogen-preferring, member 1
Kras SNP	BARX1	0.49	59	9.16E-05	BarH-like homeobox 1
Kras SNP	RCE1	0.48	59	0.000129	RCE1 homolog, prenyl protein peptidase (*S. cerevisiae*)
Kras SNP	KNG1	0.47	59	0.000147	Kininogen 1
Kras SNP	MAP2K4	0.47	59	0.000158	Mitogen-activated protein kinase kinase 4
Kras SNP	BCR	0.47	59	0.000179	Breakpoint cluster region
Kras SNP	HSC20	0.47	59	0.000198	J-type co-chaperone HSC20
Kras SNP	NR2E1	0.46	59	0.000288	Nuclear receptor subfamily 2, group E, member 1
Kras SNP	SRP14	0.45	59	0.000328	Signal recognition particle 14 kDa (homologous Alu RNA binding protein)
Kras SNP	DDR1	0.45	59	0.000357	Discoidin domain receptor family, member 1
Kras SNP	DSG2	0.45	59	0.000384	Desmoglein 2
Kras SNP	CD151	0.45	59	0.000399	CD151 antigen
Kras SNP	ACP2	0.44	59	0.00047	Acid phosphatase 2, lysosomal
Kras SNP	GNAI1	0.44	59	0.000479	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1

[247] Mitogen-activated protein kinase 3 (MAPK3) is a member of the MAP kinase family. Moreover, increased expression of mitogen-activated protein kinase 3 (MAPK3) is associated with the *KRAS* variant in cancer cells. MAPK3 transduces signals from extracellular cues to regulate intracellular processes, such as cell proliferation and differentiation. For example, increased expression of phosphorylated MAPK3 has been associated with aggressive colorectal tumors and metastatic meduloblastoma. Increased levels of KRAS in *KRAS* variant positive cancer cells are associated with an increase in MAPK3 mRNA. At least in part, increased MAPK3 expression induces an increase cellular proliferation and neoplastic progression in these cells. Similarly, the expression of another MAPK (MAP2K4) was increased in the *KRAS*-variant positive expression profile. Furthermore, KRAS and MAPK (MAPK3 and/or MAP2K4) may contribute to a synergistic interaction between KRAS and MAPK signaling in *KRAS*-variant cancer cells that induces or enhances cell proliferation and/or neoplastic progression.

[248] Increased expression of Synaptotagmin-12 and increased expression of inter-α globulin inhibitor-H1 are positively correlated with the presence of the *KRAS* variant in cancer cell lines. Under normal conditions, synaptotagmins regulate calcium-dependent membrane trafficking during synaptic transmission. Although there is no evidence of an involvement of synaptotagmin-12 with cancer, overexpression of synaptotagmin-13, a family member of synaptotagmin-12, suppresses a transformed phenotype of cells derived from a rat liver tumor cell line. Overexpression of synaptotagmin-12 in *KRAS* variant-positive cancer cell lines indicates a deregulation of novel pathways involving syntaptotagmins in cancer cells. The inter-α (globulin) inhibitor H1 is the heavy chain of the plasma serine protease inhibitor. Functionally, the inter-α (globulin) inhibitor H1 is required for extracellular matrix stability. Though the role of the inter-α (globulin) inhibitor H1 in cancer remains unexplored, recent evidence indicates that the expression of inter-α (globulin) inhibitor H1 is either lost or repressed in various solid tumors, including, for example, tumors of the lung, colon and breast.

Example 3: The *KRAS* variant and patient response to treatment (ovarian cancer)

Materials and Methods

[249] *Overall survival analysis cohorts.* Complete clinical data and DNA from women diagnosed with EOC without known BRCA mutations were included from the following three institutions under individual International Review Board approvals. All protocols accrued patients prospectively at the time of their diagnosis to avoid selection bias. References indicate previous detailed descriptions of these patients: (1) Turin, Italy #1 (n = 197) (Lu L, et al. (2007). Cancer Res 67:10117-10122), (2) Brescia, Italy #2 (n = 59) (Ratner E, et al. (2010). Cancer Res 15: 6509-6515), and (3) the Yale New Haven Hospital (YNHH) (n = 198). Yale patients were collected prospectively on two clinical trials at the Yale Medical School of newly diagnosed EOC patients diagnosed between 2000 and 2009 (Table 18).

[250] **Table 18.** Clinicopathologic parameters for overall survival analysis.

Variable name	Non-variant (n=351)	*KRAS*-variant (n=103)	p value
Age (standard deviation)	60.44 (11.89)	58.77 (11.59)	0.2115
Stage			0.8627
I	52 (14.81)	15 (14.56)	
II	22 (6.27)	6 (5.83)	
III	193 (54.99)	52 (50.49)	
IV	80 (22.79)	29 (28.16)	
Unknown	4 (1.14)	1 (0.97)	
Grade			0.0507
Well differentiated	31 (8.83)	14 (13.59)	
Moderately differentiated	60 (17.09)	8 (7.77)	
Poorly differentiated	228 (64.96)	74 (71.84)	
Unknown	32 (9.12)	7 (6.80)	
Histology			0.1887
Serous	203 (57.83)	52 (50.49)	
Endometrioid	37 (10.54)	16 (15.53)	
Undifferentiated	7 (1.99)	0 (0.00)	
Clear Cell	21 (5.98)	10 (9.71)	
Mucinous	19 (5.41)	2 (1.94)	
Carcinosarcoma	13 (3.70)	7 (6.80)	
Mixed	19 (5.41)	6 (5.83)	
Unknown	32 (9.12)	10 (9.71)	
Center			0.2670
Yale New Haven Hospital	160 (45.48)	38 (36.89)	
Italy #1	146 (41.60)	51 (49.51)	
Italy #2	45 (12.82)	14 (13.59)	
Follow up Time (std deviation)	40.40 (33.57)	36.02 (29.40)	0.2324

[251] Documented *BRCA* mutant EOC cases with known outcome were collected from the following two institutions: (1) the YNHH (n = 17) and (2) the City of Hope Comprehensive Cancer Center (n = 62) (Table 19).

[252] **Table 19**. Clinicopathologic parameters for *BRCA* mutant EOC patients.

Variable name	Non-variant (n=69)	*KRAS*-variant (n=10)	P value
Age	52.77 (10.20)	52.60 (12.47)	0.9623
Stage			0.1771
I	5 (7.25)	2 (20.00)	
II	8 (11.59)	2 (20.00)	
III	51 (73.91)	5 (50.00)	
IV	5 (7.25)	1 (10.00)	
Grade			0.5275
Well differentiated	2 (2.90)	1 (10.00)	
Moderately differentiated	13 (18.84)	1 (10.00)	
Poorly differentiated	49 (71.01)	8 (80.00)	
Unknown	5 (7.25)	0 (0.00)	
Histology *			0.9913
Serous	8 (11.59)	1 (10.00)	
Endometrioid	2 (2.90)	0 (0.00)	
Undifferentiated	1 (1.45)	0 (0.00)	
Clear Cell	1 (1.45)	0 (0.00)	
Mucinous	1 (1.45)	0 (0.00)	
Carcinosarcoma	1 (1.45)	0 (0.00)	
Mixed	1 (1.45)	0 (0.00)	
Unknown	54 (78.26)	9 (90.00)	
BRCA status			0.7206
BRCA 1	51 (73.91)	7 (70.00)	
BRCA 2	18 (26.09)	3 (30.00)	
Center			0.6808
Yale New Haven Hospital	16 (23.19)	1 (10.00)	
City of Hope	53 (76.81)	9 (90.00)	

* Histology information was not available for City of Hope patients

[253] As not all stage 1 ovarian cancer patients receive adjuvant chemotherapy, when substage information was not available for patients with stage 1 tumors, these patients were excluded from the analysis. Otherwise, stage 1B and 1C tumors were included with stages 2-4. To minimize inadvertent inclusion of borderline tumors, tumors with an unknown grade were excluded from this analysis. For women treated with neoadjuvant chemotherapy, the date of pathological diagnosis was considered the start date of treatment. For women treated with adjuvant chemotherapy, the date of surgery was considered the start date of treatment. A total of 386 patients with wild-type *BRCA* or not tested for *BRCA* mutations and 79 patients with documented *BRCA* mutations fit the above-described parameters and were included in the two survival analyses.

[254] *Neoadjuvant chemotherapy cohort.* Women with EOC who received neoadjuvant platinum-based chemotherapy followed by cytoreductive surgery at the YNHH between 1996 and 2010 were identified on an International Review Board-approved protocol (n = 125) (Table 20). This cohort of patients received chemotherapy as a primary treatment due to tumor burden that was too extensive for optimal surgical debulking at presentation. After chemotherapy,

patients underwent cytoreductive surgery and additional adjuvant treatment. Only patients treated with four or more cycles of neoadjuvant platinum-containing combinations were included in this analysis (n = 116). Optimal cytoreduction was defined as residual disease measuring < 1 cm remaining after surgery, whereas suboptimal cytoreduction was defined as residual disease measuring ≥ 1 cm at the completion of surgery. Only women operated on at Yale by the same group of surgeons were included to avoid bias in surgical skill as a factor impacting residual disease.

[255] Table 20. Clinicopathologic parameters of patients receiving neoadjuvant chemotherapy.

Variable name	Non-variant (n=97)	*KRAS*-variant (n=28)	p value
Age (standard deviation)	64.30 (12.12)	62.57 (13.33)	0.5170
Ethnicity			0.5889
Caucasian	90 (92.78)	27 (96.43)	
Other or unknown	7 (7.21)	1 (3.57)	
Stage			0.0175
II	1 (1.03)	0 (0.00)	
III	41 (42.27)	4 (14.29)	
IV	51 (52.58)	23 (82.14)	
Unknown	4 (4.12)	1 (3.57)	
Grade			0.1308
Well differentiated	2 (2.06)	0 (0.00)	
Moderately differentiated	13 (13.40)	0 (0.00)	
Poorly differentiated	68 (70.10)	25 (89.29)	
Unknown	14 (14.43)	3 (10.71)	
Histology			0.8176
Serous	73 (75.26)	19 (67.86)	
Endometrioid	2 (2.06)	0 (0.00)	
Undifferentiated	2 (2.06)	0 (0.00)	
Clear Cell	4 (4.12)	2 (7.14)	
Mucinous	1 (1.03)	0 (0.00)	
Carcinosarcoma	1 (1.03)	1 (3.57)	
Mixed	6 (6.19)	3 (10.71)	
Unknown	8 (8.25)	3 (10.71)	
Neoadjuvant Chemotherapy			0.2765
Carboplatin/ Paclitaxel	85 (87.63)	21 (75.00)	
Carboplatin/ Taxotere	1 (1.03)	1 (3.57)	
Carboplatin/ Cyclophosphamide	7 (7.22)	5 (17.86)	
Other	4 (4.12)	1 (3.57)	
Neoadjuvant cycles completed:			0.3502
2	2 (2.06)	0 (0.00)	
3	4 (4.12)	2 (7.14)	
4	18 (18.56)	2 (7.14)	
5	4 (4.12)	4 (14.29)	
6	64 (65.98)	20 (71.43)	
7	3 (3.09)	0 (0.00)	
9	1 (1.03)	0 (0.00)	
Unknown	1 (1.03)	0 (0.00)	
Follow up time	30.53 (25.45)	36.54 (36.06)	0.3229

[256] *Patients for analysis of platinum resistance.* Platinum resistance was defined as progression-free survival of < 6 months from the completion of platinum-containing adjuvant chemotherapy to the date of recurrence. The progression-free survival interval was available from women from Italy #1, Italy #2 and the YNHH patients (n = 291). Table 21 describes the clinicopathological parameters of these patients.

[257] **Table 21**. Clinicopathologic parameters for platinum resistance analysis.

Variable name	Non-variant (n=225)	*KRAS*-variant (n=66)	p value
Age	58.66 (11.70)	56.11 (10.16)	0.1129
Stage:			0.9652
I	41 (18.22)	10 (15.15)	
II	19 (8.44)	6 (9.09)	
III	142 (63.11)	43 (65.15)	
IV	22 (9.78)	7 (10.61)	
Unknown	1 (0.44)	0 (0.00)	
Grade:			0.0728
Well differentiated	18 (8.00)	12 (18.18)	
Moderately differentiated	42 (18.67)	7 (10.61)	
Poorly differentiated	150 (66.67)	44 (66.67)	
Unknown	15 (6.67)	3 (4.55)	
Histology:			0.6319
Serous	114 (50.67)	31 (46.97)	
Endometrioid	33 (14.67)	10 (15.15)	
Undifferentiated	27 (12.00)	7 (10.61)	
Clear Cell	14 (6.22)	8 (12.12)	
Other	37 (16.44)	10 (15.15)	
Platinum response:			0.0340
Sensitive	208 (92.44)	55 (83.33)	
Resistant	17 (7.56)	11 (16.67)	
Cytoreductive surgery:			0.4808
Optimal cytoreduction (<1cm residual disease)	129 (57.33)	38 (57.58)	
Suboptimal cytoreduction (>1cm residual disease)	89 (39.56)	28 (42.42)	
Unknown	7 (3.11)	0 (0.00)	
Center			0.2808
Yale New Haven Hospital	55 (24.44)	10 (15.15)	
Italy #1	137 (60.89)	46 (69.70)	
Italy #2	33 (14.67)	10 (15.15)	
Follow up Time	39.08 (24.97)	36.47 (26.81)	0.4635

[258] *Detection of the KRAS variant.* DNA was isolated using standard methods from tumor, blood or saliva. The KRAS variant does not appear to be somatically acquired nor does it require a loss of heterozygosity (Chin LJ, et al. (2008). Cancer Res 68: 8535-8540); hence, blood and saliva, for example, are appropriate to test and the results are identical regardless of the tissue tested. The *KRAS* variant allele was detected using a primer specific to the *KRAS* variant and a TaqMan (Applied Biosystems, Foster City, CA, USA) PCR assay on all samples. Genotyping was performed at the YNHH, except for on samples from COH, for which the genotyping was

performed in their facility. Less than 3% of populations carry 2 copies of the *KRAS* variant (Chin LJ, et al. (2008). Cancer Res 68: 8535-8540). As such, patients who carried at least one copy of the *KRAS* variant allele were classified as KRAS-variant carriers.

[259] *Gene expression analysis of EOC with and without the KRAS variant.* Gene expression in fresh-frozen tumor samples obtained from 16 patients (9 non-variant and 7 *KRAS* variant) was profiled on the Affymetrix GeneChip Human Genome U133 Plus 2.0 platform (Affymetrix, Santa Clara, CA, USA). All samples were from high-grade serous epithelial ovarian tumors that were stage IIIC or IV. Images were processed with the MAS5 algorithm and probes that were judged absent in at least 75% of the samples were removed. Intensity values were log transformed and quantile normalized. Differential gene expression was assessed in samples obtained from patients over 52 years of age (n = 6 non-variant and 4 *KRAS* variant) using a linear model and empirical Bayesian error moderation as implemented in the LIMMA package for R statistical software (R Foundation for Statistical Computing, publicly available at www.r-project.org) (Smyth G. (2005). Limma. in Gentleman R, et al. (eds) Bioinformatics and Computational Biology Solutions using R and Bioconductor. Springer: New York, pp. 397-420).

[260] Association of published results with the *KRAS* variant in this data set was assessed using a signature approach to reduce cross-platform effects (Paranjape T, et al. (2011). Lancet Oncol 12: 377-386). In brief, signature scores were computed as Pearson's correlation between the respective signature vector of gene contributions and each sample's expression profile for these genes. Differences between signature scores in *KRAS*-variant and non-variant EOC samples were assessed using the paired Kolmogorov-Smirnov test. Unless otherwise indicated, gene lists from the respective publications were used as signature vectors. Data from the study by Peters et al. (Mol Cancer Ther 4: 1605-1616) were obtained from the Gene Expression Omnibus (GSE1926) and re-analyzed to generate a signature from the 50 most significantly differentially expressed genes between platinum sensitive and resistant samples.

[261] *Chemosensitivity and cell viability assays.* The activity of drugs alone or in combination was determined by a high-through-put CellTiter-Blue cell viability assay. For these assays, 1.2 x 10^3 cells were plated in each well of 384-well plates using a Precision XS liquid handling station (Bio-Tek Instruments Inc., Winooski, VT, USA) and allowed to attach overnight with incubation at 37°C, 5% CO_2 . Using the liquid handling station, all drugs were serially diluted 2:3 or 1:2 in media, and 5 µl of these dilutions were added to appropriate wells at indicated times. Four

replicate wells were used for each drug concentration and an additional four control wells received a diluent control without drug. At the end of the incubation period with drugs, 5 µl CellTiter-Blue reagent (Promega Corp., Madison, WI, USA) was added to each well. Cell viability was assessed by the ability of the remaining viable cells to bioreduce resazurin to resorufin. The fluorescence of resorufin (579 nm Ex/584 nm Em) was measured using a Synergy 4 microplate reader (Bio-Tek Instruments Inc.). The fluorescence data were transferred to Microsoft Excel (Microsoft) to calculate the percentage viability relative to the four replicate cell wells that did not receive the drug. IC50s were determined using a sigmoidal equilibrium model regression using XLfit version 5.2 (ID Business Solutions Ltd). The IC50 was defined as the concentration of drug required for a 50% reduction in growth/viability. All experiments were carried out a minimum of three times.

[262] *Targeting the KRAS variant.* Small-interfering RNA sequences were designed to target the *KRAS*-variant sequence by placing the single-nucleotide polymorphism at varying positions of the 6 nucleotides at the 5' end of the siRNA guide strand corresponding to the so-called 'seed sequence'. Blast searches were performed to minimize cross-reactivity. In some of the siRNA sequences, DNA nucleotides were introduced to optimize thermoenergetic features for preferred incorporation of the guide strand into the argonaute effector complex or to increase specificity for the variant.

[263] Small-interfering RNA guide strand sequences used in the experiments are as follows (lower case = RNA, upper case = DNA; GS = guide strand, PS = passenger strand):

2-1 GS ugcaucacuugaggucaggag (SEQ ID NO: 23)

2-1 PS ccugaccucaagugaugcacc(SEQ ID NO: 24)

2-3 GS TGCATCACuugaggucaggag(SEQ ID NO: 25) (passenger strand same as 2-1)

3-2 GS ucaucacuugaggucaggagu(SEQ ID NO: 26)

3-2 PS uccugaccucaagugaugcac(SEQ ID NO: 27)

[264] The negative control used was purchased from Qiagen (Valencia, CA, USA) (AllStars Negative-Control siRNA). Knockdown efficiency and specificity to the *KRAS* variant of these sequences were confirmed using a dual luciferase assay (see WO/2009/155100, the contents of which are incorporated herein by reference). Oligonucleotide combinations were annealed using standard conditions and then transfected into cells using standard protocols. Cell survival was assayed using MTT assays and experiments were conducted in quadruplicate, and repeated in

four independent experiments for all lines. Cell lysates were collected 72 hours after transfection and KRAS protein levels measured by western analysis using a probe specific to KRAS as described previously (Chin LJ, et al. (2008). Cancer Res 68: 8535-8540).

[265] *Statistics.* To assess the significance of demographic variables, a χ^2 test or a two-sided Fisher's exact test was used for categorical variables. A t-test was used for continuous variables, such as age. The overall survival time of *KRAS*-variant and wild-type patients was compared using the Kaplan-Meier method (Kaplan E and Meier P. (1958). J Am Stat Assoc 53: 457-481), and the statistical significance of the survival curves was determined by the log-rank test (Mantel N. (1966). Cancer Chemother Rep 50: 163-170). A Cox proportional hazards regression model (Cox D. (1972). J R Stat Soc 34: 187-220) was used to assess the impact of the *KRAS* variant and demographic and prognostic variables (such as age, stage, grade and histology) on overall survival. Multivariate logistic regression analyses (Cox D. (1970). The Analysis of Binary Data. Methuen, London) were used to determine the impact of the *KRAS* variant and other demographic and prognostic factors on the probability of suboptimal cytoreduction. Multivariate logistic regression analyses (Cox D. (1970). The Analysis of Binary Data. Methuen, London) were used to assess the association of the *KRAS* variant and other prognostic factors on the probability of platinum resistance. All statistical analyses were performed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and in R 2.12.1 (R Foundation for Statistical Computing)

Data and Results

[266] The association of the *KRAS* variant with overall survival in 454 EOC patients either tested and negative or untested for deleterious *BRCA* mutations was evaluated. When the entire cohort was considered, the *KRAS* variant did not predict worse survival by Kaplan-Meier analysis. Because the *KRAS* variant is most strongly associated with postmenopausal ovarian cancer (Chin LJ, et al. (2008). Cancer Res 68: 8535-8540), survival in women over 52 years of age (n = 279) were evaluated. Over and including 52 years of age is considered to be an appropriate surrogate for menopausal status. By Kaplan-Meier analysis, survival was significantly reduced in postmenopausal *KRAS*-variant EOC patients (n = 59) compared with non-variant EOC patients (n = 220, Figure 7, logrank $P = 0.0399$, non-*KRAS*-variant survival median 60 months, *KRAS*-variant survival median 34 months). When other variables including age, stage, grade, histology and treatment center were included with *KRAS*-variant status in a multivariate Cox proportional hazards regression model, the *KRAS* variant was a statistically

significant predictor of reduced overall survival for postmenopausal women with EOC (Table 22); the hazard ratio for the *KRAS* variant was 1.67 (95% confidence interval: 1.09--2.57, P = 0.019).

[267] **Table 22**. The KRAS variant is associated with reduced survival in postmenopausal (> 52 years of age) ovarian cancer patients (n = 279).

Variable	*HR*	*95% CI*	*P-value*
KRAS status	1.671	1.087 - 2.568	0.0192
Age	1.025	1.002 - 1.049	0.0307
Stage	1.380	1.185 - 1.607	<0.0001
Grade	1.341	0.912 - 1.972	0.1360
Histology	0.970	0.900 - 1.045	0.4168
Center (Non-Yale vs Yale)	1.868	1.438 - 2.427	<0.0001

Abbreviations: CI, confidence interval; HR, hazard ratio obtained from Cox proportional hazards multivariate analysis.
Studies included the Yale New Haven Hospital, Italy #1, Italy #2.

[268] The association of the *KRAS* variant with survival in a separate cohort of EOC patients carrying deleterious *BRCA1* or *BRCA2* mutations (n = 79) was evaluated. EOC patients carrying *BRCA* mutations were statistically significantly younger than EOC patients without *BRCA* mutations (52.7 vs 60.8 years of age, $P < 0.0001$). In addition, EOC patients with *BRCA* mutations had a significantly longer median survival by multivariate analysis controlling for age, stage, grade and histology than did EOC patients without *BRCA* mutations (120 vs 52 months, P = 0.0036). There was no significant difference in survival between EOC patients with *BRCA* mutations with or without the *KRAS* variant in a multivariate analysis using a multivariate Cox proportional hazards regression model (Table 23, KRAS-variant hazard ratio = 0.75, 95% confidence interval: 0.21-2.72, P = 0.66). In this study, there were too few patients to evaluate the impact of the *KRAS* variant on survival in postmenopausal EOC patients with deleterious *BRCA* mutations.

[269] **Table 23.** The *KRAS*-variant and overall survival in EOC patients with deleterious *BRCA* mutations (n = 79).

Variable	HR	95% CI	p value
KRAS status	0.75	0.21 – 2.72	0.66
Age	1.01	0.98 – 1.05	0.45
Stage			0.0005
Stage III vs. Stage I and II	14.79	1.87 – 117.29	0.01
Stage IV vs. Stage I and II	69.98	7.00 – 699.87	0.0003
Grade			
Grade 2 and 3 vs. Grade 1	4.32	1.29 – 14.46	0.02
Center (non Yale vs. Yale)	0.66	0.23 – 1.87	0.43

HR: hazards ratio obtained from Cox proportional Hazards multivariate analysis
CI: confidence interval
Studies Included: Yale New Haven Hospital, City of Hope

[270] To explain the reduced survival in postmenopausal *KRAS* variant-positive EOC patients, the association of *KRAS*-variant positivity with response to platinum-based chemotherapy was evaluated. Platinum-based chemotherapy is the standard first-line chemotherapy in the treatment of EOC. First, all women with EOC who were treated at the Yale-New Haven Hospital (YNHH) with neoadjuvant platinum-containing chemotherapy followed by surgical cytoreduction (n = 116) were evaluated. Residual disease after surgery (cytoreduction) was used as a surrogate marker of patient response to chemotherapy. It was determined that 15.4% of *KRAS*-variant patients (n = 26) were suboptimally cytoreduced (41 cm of residual disease after surgery), compared with only 3.33% of non-variant patients (n = 90) (Figure 8, $P = 0.044$). The *KRAS* variant was also significantly associated with suboptimal cytoreduction after neoadjuvant chemotherapy and surgery in a multivariate logistic regression model controlling for age, stage, grade and histology (Table 24, odds ratio = 9.36, 95% confidence interval: 1.34--65.22, $P = 0.024$).

[271] **Table 24**. The *KRAS*-variant predicts suboptimal debulking after neoadjuvant chemotherapy (n = 116).

KRAS-variant Genotype	Univariate			Multivariate[3]		
	OR[1]	95% CI[2]	p	OR	95% CI	p
All						
Wild-type (*n*=90)	1.00			1.00		
Variant (*n*=26)	5.27	1.10 – 25.30	0.0377	9.36	1.34 – 65.22	0.0240

1. OR: odds ratio obtained from logistic regression
2. CI: confidence interval
3. Multivariate: adjusted for age, stage, grade, histology, type of chemotherapy regimen, and numbers of cycles received prior to surgery.

[272] To determine whether the cause of poor response to neoadjuvant platinum-based chemotherapy seen in *KRAS*-variant EOC patients was due to resistance to platinum chemotherapy, platinum resistance in all EOC patients treated adjuvantly with platinum chemotherapy without documented *BRCA* mutations with available response data (n = 291) were evaluated. It was determined that platinum resistance (defined in this example as disease recurrence within 6 months of receiving platinum-based chemotherapy) was significantly more likely in *KRAS* variant-positive EOC patients than in non-*KRAS* variant EOC patients (16.67 vs 7.56%, $P = 0.034$). The *KRAS* variant was a statistically significant predictor for platinum resistance for EOC patients of all ages in a multivariate logistic regression analysis controlling for residual disease remaining after cytoreductive surgery, stage, histology, age and grade (Table 25, odds ratio = 3.18, 95% confidence interval: 1.31--7.72, $P = 0.0106$).

[273] **Table 25**. The *KRAS* variant is associated with platinum resistance.

KRAS variant genotype	*Univariate*			*Multivariate*[a]		
	OR	*95% CI*	*P-value*	*OR*	*95% CI*	*P-value*
All						
Non-variant (n = 225)	1.00			1.00		
Variant (n = 66)	2.45	1.08 - 5.53	0.0313	3.18	1.31 - 7.72	0.0106

Abbreviations: CI: confidence interval; OR: odds ratio obtained from logistic regression.
Studies: Yale, Italy #1, Italy #2. [a]Multivariate: adjusted for age, stage, grade, histology, residual disease after cytoreductive surgery and treatment center.

[274] Gene expression studies were performed on a small cohort of ovarian cancer patients who had fresh-frozen tissue available (Brescia cohort), and compared between seven serous EOC samples with the *KRAS* variant and nine without the *KRAS* variant (n = 16). Within this cohort,

in postmenopausal EOC patients over 52 years of age with EOC (n = 10), a gene signature previously found to be associated with *KRAS* variant-associated TNBC (Paranjape T, et al. (2011). Lancet Oncol 12: 377-86) was also upregulated in *KRAS* variant-associated EOC (Figure 9a). Similar to the previous analysis in TNBC, overexpression of KRAS-associated downstream pathways in EOC KRAS-variant tumors was discovered, which is consistent with 'KRAS addiction' (Singh A, et al. (2009). Cancer Cell 15: 489-500)(Figure 9b).

[275] Using previous analyses of gene expression data identifying platinum-resistant vs sensitive signatures (Peters D, et al. (2005). Mol Cancer Ther 4: 1605-1616), it was determined that *KRAS*-variant EOC samples had a lower carboplatin sensitivity signature compared with non-variant EOC samples (Figure 9c). In agreement with findings showing that the activation of the AKT pathway was frequently involved in platinum resistance, it was determined that AKT3 was one of the most significantly upregulated transcripts in *KRAS*-variant EOC tumors (Figure 9d).

[276] Although miRNA expression data were not available on tumor samples, the expression of *let-7b* miRNA in two cell lines with the *KRAS* variant (BG-1 and IGROV1) was compared with the expression of let-7b in a non-*KRAS* variant line (CAOV3). The expression of *let-7b* miRNA is altered in *KRAS* variant-positive lung tumors (Chin LJ, et al. (2008). Cancer Res 68: 8535-8540) and triple-negative breast tumors (Paranjape T, et al. (2011). Lancet Oncol 12: 377-386).

[277] It was determined that *let-7b* was statistically significantly lower in cells with the *KRAS* variant (Figure 12).

[278] To confirm altered chemosensitivity in the presence of the *KRAS* variant, EOC cell lines with and without the *KRAS* variant were used to test their sensitivity to different chemotherapeutic agents. For example, a cell line that is *KRAS* variant positive/*BRCA* wild-type (BG1), a non-variant/*BRCA* wild-type cell line (CAOV3) and a cell line *KRAS*-variant positive/*BRCA1* mutant (IGR-OV1) were tested. It was determined that the *KRAS*-variant line, BG1, was statistically significantly resistant to carboplatin ($P < 0.04$) and carboplatin/paclitaxel combination chemotherapy ($P < 0.0001$) compared with CAOV3, the cell line without the *KRAS* variant. In contrast, IGROV1, the cell line with the *KRAS* variant and a deleterious *BRCA1* mutation, was not resistant to these agents when compared with CAOV3 (Figure 10). These results agree with corresponding clinical results demonstrating that the *KRAS* variant is associated with platinum resistance, but not in the presence of deleterious *BRCA* mutations.

[279] Additionally, agents frequently used as second line therapy for patients who have failed carboplatin/paclitaxel chemotherapy were evaluated. These second line therapeutic agents included doxorubicin, topotecan and gemcitabine. The KRAS-variant line, BG1, was significantly resistant to each of these agents compared with CAOV3, the nonvariant cell line (Table 26).

[280] **Table 26.** Chemosensitivity in a KRAS-variant cell line (BG1) vs a non-variant line (CAOV3).

	Gemcitabine	*Doxorubicin*	*Topotecan*	*RSE*
BG1	30.4 10^6	307.5 10^9	161.8 10^9	21.69
CAOV3	2.2 10^9	75.9 10^9	30.8 10^9	19.67

Abbreviation: RSE, relative standard error which is the s.e. divided by the mean and expressed as a percentage.
Numbers are IC50 values from a minimum of four separate experiments.
Differences are statistically significant ($P<0.01$), indicating that the KRAS-variant line is more resistant to these agents.

[281] Because the data presented herein demonstrate a continued use of KRAS signaling in *KRAS* variant-associated tumors, the impact of directly targeting the *KRAS*-variant was evaluated. Small-interfering RNA (siRNA)/miRNA-like complexes were designed to directly bind the altered allele in *KRAS* variant transcripts, but not bind to non-*KRAS*-variant transcripts (Figure 13). It was determined that transfecting these oligonucleotide duplexes that target the KRAS variant caused a statistically significant decrease in cell survival in the *KRAS* variant carrying BG1 cell line ($P < 0.001$), but had no effect in CAOV3 (Figure 11a) or SKOV3, two non-variant EOC cell lines. This result is concordant with a moderate decrease in KRAS protein levels by western blot in BG1, but not in CAOV3 (Figure 11b) or SKOV3after treatment.

Example 4: The KRAS variant as a prognostic biomarker in early-stage colorectal cancer (CRC)

Materials and methods

[282] *Study population.* Until 1994, 925 incident CRC cases (ICD-O:153.0-154.1) were identified within the Netherlands Cohort Study on diet and cancer (NLCS) which started in 1986 with 120,852*b* healthy persons between 55 and 69 years. Incident cancer cases were identified by linkage with the Netherlands Cancer Registry (NCR) and PALGA, a nationwide registry of histopathology and cytopathology (Van den Brandt PA,et al. Int J Epidemiol. 1990; 19(3): 553-8). The NLCS has been described in detail elsewhere (Van den Brandt PA, et al. J Clin Epidemiol. 1990; 43(3): 285-95. 815 CRC cases could be linked to PALGA and paraffin-

embedded tumor tissue was collected from 54 pathology registries throughout the Netherlands. A sufficient amount of good quality DNA was extracted for 734 (90%) cases (Brink M, et al. Carcinogenesis. 2003; 24(4): 703-10). At baseline, a subcohort of 5000 healthy persons was randomly sampled from the entire cohort to estimate personyears at risk of the cohort through biennial follow-up of vital status. For 1,886 persons, DNA from buccal swabs was available for *KRAS* variant genotyping.

[283] *Data collection.* Information on tumor localization, stage, differentiation grade, incidence date and treatment in the 3 months after diagnosis, was available through the NCR. Vital status until May 2005 was retrieved from the Central Bureau of Genealogy and the municipal population registries and could be obtained for all 734 cases. Causes of death were retrieved through linkage with Statistics Netherlands. CRC-related deaths were defined as deaths as a result of a carcinoma in the colon, rectosigmoid, rectum, gastro-intestinal tract (non-specific) or liver metastases. In the case of gastro-intestinal (non-specified) or liver metastases, information from NCR and PALGAwas used to eliminate the possibility of another primary cancer as cause of death.

[284] *DNA isolation and KRAS-variant determination.* A 5 μm section of each tumor tissue block was stained with haematoxylin and eosin and revised by a pathologist. Five sections of 20 μm were deparaffinated and DNA was extracted using the Puregene® DNA isolation kit (Gentra systems) according to the manufacturers' instructions. In brief, cell lysis solution and proteinase K (20 mg/ml, Qiagen) were added to the tissue and incubated overnight at 55°C. DNA was extracted for 72 hours at 37°C, protein was removed, and DNA was precipitated using 100% 2-propanol. Finally, DNA was rehydrated in hydration buffer. Isolated DNA was amplified using TaqMan PCR assays designed specifically to identify the T or G allele (wild type and variant alleles, respectively) of the *let-7* complementary site 6 (LCS6) within the 3'UTR of *KRAS*(Applied Biosciences). Although tumor DNA was used to assess genotype, it is well documented that the genotype of normal and tumor tissue is the same in *KRAS* variant allele carriers (Chin LJ, et al. Cancer Res. 2008; 68(20): 8535-40).

[285] *KRAS* and *BRAF* mutations were assessed by nested polymerase chain reaction (PCR) and direct sequencing (*KRAS*), and restriction fragment length polymorphism (*BRAF*) as described previously (Brink M, et al. Carcinogenesis. 2003; 24(4): 703-10; de Vogel S, et al. Carcinogenesis. 2008; 29(9): 1765-73). Promoter methylation of *RASSF1A, O^6-MGMT, CHFR*

and CIMP markers as proposed by Weisenberger (Weisenberger DJ, et al.Nat Genet. 2006; 38(7): 787-93) was assessed by chemical modification of genomic DNA with sodium bisulfite and methylation-specific PCR (MSP) (de Vogel Set al. Carcinogenesis. 2008; 29(9):1765-73; 26. Herman JG,et al. Proc Natl Acad Sci U S A.1996; 93(18): 9821-6; Derks S,et al. Cell Oncol. 2004; 26(5-6): 291-9). MSI status was determined using BAT-26, BAT-25, NR-21, NR-22 and NR-24 as described previously (Suraweera N, et al. Gastroenterology. 2002; 123(6): 1804-11). All assays were performed and analyzed while blinded to the main study endpoint, *i.e.* CRC-related death.

[286] *Statistical analyses*. Cause-specific survival was defined as time from cancer diagnosis until CRC-related death or end of follow-up. Kaplan–Meier curves and log-rank tests were used to estimate the influence of the *KRAS* variant on cause-specific survival. HR and corresponding 95% CI were assessed by use of Cox proportional hazard models adjusted for potential confounders. Factors were considered possible confounders if they were known prognostic factors for CRC and influenced the crude HR by more than 10%. Confounders that were included were age at diagnosis (continuous), sex, tumor differentiation grade (well, moderate, poor, and undifferentiated), and location (proximal, distal, rectosigmoid, and rectum). The proportional hazard assumption was tested using the Schoenfeld residuals and the log (-log) hazards plots. Survival analyses were restricted to 10 years after diagnosis as CRC-related cause of death was unlikely after that point. Incidence rate ratios (RR) and 95% CI were estimated using Cox proportional hazards models. Standard errors were estimated using the robust Huber–White sandwich estimator to account for additional variance introduced by sampling from the cohort. All analyses were done with the statistical package STATA10.0.

Data and Results

[287] Patients in this study were more often male (55.6%), diagnosed with an early-stage tumor (62.0%) or a proximal or distal tumor (65.3%; Table 27). During follow-up, 41.4% of the patients died of CRC. The KRAS-LCS6 variant was detected in 14.0% of early-stage (stage I and II), in 19.2% of stage III and 21.4% of stage IV patients ($P = 0.160$; $P_{trend} = 0.060$). *KRAS* variant patients were more often diagnosed with advanced stage disease (47.5% versus. 36.9% in wild-type patients, $P = 0.046$). Other statistically significant differences were not found between wild type and *KRAS* variant carriers for sex, age at diagnosis, differentiation grade, tumor location, MSI, or mutations in *KRAS* (Table 27), BRAF ($P = 0.640$), or *RASSF1A* promoter CpG island

methylation ($P = 0.423$). As expected, patients with stage III or IV disease more often died from CRC ($P < 0.001$) and more often had a poorly differentiated tumor ($P < 0.001$). Advanced stage patients more often had a proximal ($P = 0.036$) or MSS tumor ($P = 0.047$) as compared with early-stage patients.

[288] **Table 27.** Baseline characteristics for the total population, KRAS variant and wild type carriers and early stage and advanced stage CRC cases within the NLCS on diet and cancer, between 1986 and 1994, inclusively.

		Overall	*KRAS-LCS6* wild-type TT	*KRAS-LCS6* variant G-allele (Ho + He)	P	Early-stage (stage I and II) CRC	Stage III	Stage IV	P
Total population, *n* (%)		734 (100)	567 (83.6)	111 (16.4)		409 (62.0)	182 (27.6)	69 (10.5)	
Sex (male, *n* (%))	Male	406 (55.6)	308 (54.3)	66 (59.5)	0.320	219(53.6)	102 (56.0)	33(47.8)	0.506
Age at diagnosis (mean, SD)		67.9 (4.3)	67.9 (4.3)	67.9 (4.4)	0.885	68.0 (4.4)	67.5 (4.1)	68.5 (3.8)	0.203
CRC-related death (yes, *n* (%))	Yes	302 (41.4)	230 (40.6)	46 (42.2)	0.761	95 (23.3)	107 (8.8)	65(95.6)	<0.001
Cancer stage, *n* (%)	Early stage (I and II)	409 (62.0)	326 (63.1)	53 (52.5)					
	III	182 (27.6)	137 (26.5)	33 (32.7)					
	IV	69 (10.5)	54 (10.4)	15 (14.9)	0.124				
Differentiation, *n* (%)	Well	74 (11.5)	58 (11.8)	9 (8.7)		46 (12.7)	13 (7.8)	3 (5.0)	
	Moderate	457 (71.0)	354 (71.8)	72 (69.9)		277(76.5)	109 (65.3)	36 (60.0)	
	Poor	106 (16.5)	75 (15.2)	21 (20.4)		37 (10.2)	41 (24.6)	20 (33.3)	
	Undifferentiated	7 (1.1)	6 (1.2)	1 (1.0)	0.532	2 (0.6)	4 (2.4)	1 (1.7)	<0.001
Location, *n* (%)	Proximal	239 (33.2)	196 (35.4)	34 (31.2)		128 (31.5)	63 (34.8)	33 (49.3)	
	Distal	231 (32.1)	177 (32.0)	37 (33.9)		125 (30.7)	61 (33.7)	22 (32.8)	
	Rectosigmoid	80 (11.1)	59 (10.6)	11 (10.1)		53 (13.0)	17 (9.4)	5 (7.5)	
	Rectum	169 (23.5)	122 (22.0)	27 (24.8)	0.824	101 (24.8)	40 (22.1)	7 (10.5)	0.036
Molecular characteristics, *n* (%)	MSS	578 (87.3)	463 (87.5)	88 (84.6)		314 (84.9)	149 (88.7)	63 (95.5)	
	MSI	84 (12.7)	66 (12.5)	16 (15.4)	0.420	56 (15.1)	19 (11.3)	3 (4.5)	0.047
	CIMP+	167 (27.7)	127 (24.5)	34 (35.4)					
	CIMP –	436 (72.3)	352 (73.5)	62 (64.6)	0.076				0.121
KRAS mutations, *n* (%)	Wild type	464 (63.2)	362 (63.8)	69 (62.2)		263 (64.3)	121 (66.5)	39 (56.5)	
	KRAS mutated	270 (36.8)	205 (36.2)	42 (37.8)	0.736	146 (35.7)	61 (33.5)	30 (43.5)	0.336
KRAS variant	Wild type	567 (83.6)				326 (86.0)	137 (80.6)	54 (78.3)	
	Variant He	107 (15.8)				51 (13.5)	32 (18.8)	15 (21.7)	
	Variant Ho	4 (0.6)				2 (0.5)	1 (0.6)		0.298

[289] Stage IV G-allele (*KRAS* variant) carriers were more likely to be female (66.7%; $P = 0.097$) and to present with a proximal tumor (71.4%; $P = 0.004$) as compared with G-allele (*KRAS* variant) carriers in other stages (Table 28).

[290] **Table 28.** Baseline and molecular characteristics for early stage, stage III and IV patients according to *KRAS* variant status.

		KRAS-LCS6 wild-type TT				*KRAS-LCS6* variant G-allele (He+Ho)			
		Stage I and II	Stage III	Stage IV	*P*	Stage I and II	Stage III	Stage IV	*P*
Total population, *n* (%)		326 (63.1)	137 (26.5)	54 (10.4)		53 (52.5)	33 (32.7)	15 (14.9)	
T1		43 (13.2)	1 (0.7)	–		8 (15.1)	–	–	
T2		95 (29.1)	13 (9.6)	3 (5.6)		17 (32.1)	5 (15.6)	–	
T3		174 (53.4)	113 (83.1)	41 (75.9)		27 (50.9)	24 (75.0)	12 (80.0)	
T4		14 (4.3)	9 (6.6)	10 (185)	<0.001	1 (1.9)	3 (9.4)	3 (20.0)	0.001
Sex [male, *n* (%)]	Male	173 (53.1)	75 (54.7)	28 (51.9)	0.920	29 (54.7)	22 (66.7)	5 (33.3)	0.097
Age at diagnosis (mean, SD)		68.0 (0.3)	67.5 (0.4)	68.5 (0.5)	0.283	68.0 (0.6)	67.5 (0.7)	68.5 (1.2)	0.756
CRC-related death [yes, *n* (%)]	Yes	79 (24.2)	79 (57.7)	51 (96.2)	<0.001	8 (15.7)	19 (57.6)	14 (93.3)	<0.001
Differentiation, *n* (%)	Well	38 (13.2)	9 (7.3)	2 (4.4)		5 (10.6)	2 (6.3)	1 (7.1)	
	Moderate	224 (77.8)	77 (62.1)	31 (67.4)		35 (74.5)	24 (75.0)	5 (35.7)	
	Poor	25 (8.7)	34 (27.4)	12 (26.1)		6 (12.8)	6 (18.8)	8 (57.1)	
	Undifferentiated	1 (0.4)	4 (3.2)	1 (2.2)	<0.001	1 (2.1)	–	–	0.028
Location, *n* (%)	Proximal	107 (33.0)	55 (40.2)	23 (43.4)		15 (28.3)	6 (18.8)	10 (71.4)	
	Distal	100 (30.9)	42 (30.7)	20 (37.7)		14 (26.4)	17 (53.1)	2 (14.3)	
	Rectosigmoid	40 (12.4)	12 (8.8)	4 (7.6)		8 (15.1)	2 (6.3)	1 (7.1)	
	Rectum	77 (23.8)	28 (20.4)	6 (11.3)	0.230	16 (30.2)	7 (21.9)	1 (7.1)	0.004
Molecular characteristics, *n* (%)	MSS	258 (85.2)	113 (86.9)	51 (100)	0.013	40 (81.6)	30 (93.8)	12 (80.0)	0.259
	CIMP+	72 (26.5)	34 (28.3)	16 (34.8)	0.504	14 (30.4)	8 (27.6)	8 (57.1)	0.126
KRAS mutations, *n* (%)	KRAS mutated	115 (35.3)	44 (32.1)	23 (42.6)	0.393	20 (37.7)	11 (33.3)	7 (46.7)	0.676

[291] The *KRAS* variant is associated with better survival in early-stage CRC. A statistically significant difference was not observed in Kaplan–Meier analyses for the *KRAS* variant and cause-specific survival in the total population (log-rank test, $P = 0.864$) (Figure 14).

[292] As survival depends on cancer stage, the analyses conducted were stratified for stage. Early-stage G-allele (*KRAS* variant) carriers showed a statistically significantly better survival as compared with wild-type cases (log-rank test, $P = 0.038$; Figure 15A). This difference was not observed for advanced stage cases (Fig. 1B and C; log rank, P = 0.775 and 0.875 for stage III and IV cases, respectively).

[293] KRAS/BRAF mutation status enhances the association between the KRAS variant and survival. Figure 16A shows Kaplan–Meier analyses for early-stage (stage I and II) CRC cases with the *KRAS* variant and *KRAS* mutations. None of the 20 G-allele (*KRAS* variant) carriers with *KRAS* mutations died due to CRC. *KRAS* wild-type patients had a poorer survival, especially if they had *KRAS* mutations (log-rank test, $P = 0.043$; log-rank test *KRAS*-variant allele carriers

with *KRAS* mutations compared with *KRAS*-variant allele carriers without *KRAS* mutations $P = 0.017$). This discovery was independent of T stage; among 115 *KRAS* wild-type cases with *KRAS* mutations, only 5 (4%) were diagnosed as high-risk stage IIb (T4N0M0). Among G-allele (*KRAS* variant allele) carriers, no patients were diagnosed as stage IIb. For advanced stage patients, a survival difference was not found (Figure 16B and 16C, log-rank test, $P = 0.535$ for stage III and $P = 0.989$ for stage IV)). Results for stage III patients indicate that KRAS wild type patients with *KRAS* mutations have the worst prognosis. Subgroup analysis showed that the better outcome for early-stage *KRAS* variant carriers was found predominantly in stage II cases. Analyses stratified for T stage were not possible due to limited patient numbers.

[294] BRAF mutated CRCs carrying the G-allele showed a similar better outcome, although this was not statistically significant (log-rank test, $P = 0.166$) possibly due to small number of patients carrying both *KRAS* variant and *KRAS* mutations (9 patients). Similarly, G-allele (*KRAS* variant allele) carriers with aberrant *RASSF1A* promoter hypermethylation, another gene involved in the Ras pathway, had a better prognosis, although less statistically significant, as compared with wild-type carriers without *RASSF1A* hypermethylation (log-rank test, $P = 0.062$). Analyses combining *KRAS*, *BRAF*, and *RASSF1A* status showed that early-stage G-allele (*KRAS* variant) carriers with additional alterations in *KRAS*, *BRAF*, or *RASSF1A* have a better prognosis (log-rank test, $P = 0.026$). In contrast, when adding methylation status of genes not involved in the Ras pathway such as *MGMT* or *CHFR*, survival differences were not observed (*MGMT*: log-rank test, $P = 0.220$; *CHFR*: log-rank test, $P = 0.118$).

[295] The survival impact of the *KRAS* variant combined with *KRAS* mutation status is independent of other prognostic factors. In multivariate analyses, statistically significant differences in cause-specific survival were not found for early-stage (HR 0.46; 95% CI: 0.18–1.14), stage III (HR 0.98, 95% CI: 0.55–1.74) or stage IV cases (HR 0.42; 95% CI: 0.17–1.06) with the G-allele (*KRAS* variant) as compared with wild types, although early-stage and stage IV G-allele (*KRAS* variant) carriers demonstrated an improved survival (Table 29).

[296] **Table 29.** HRs and 95% CI for cause-specific mortality and clinicopathologic parameters and the *KRAS* variant in 734 CRC cases from the Netherlands Cohort Study on diet and cancer.

		Early stage (stage I and II) CRC	Stage III CRC	Stage IV CRC
KRAS-LCS6 variant		0.46 (0.18–1.14)	0.98 (0.55–1.74)	0.42 (0.17–1.06)
KRAS-LCS6 variant without KRAS mutations		0.77 (0.30–1.97)	0.95 (0.44–2.05)	0.35 (0.11–1.13)
KRAS-LCS6 variant with KRAS mutations		No CRC-related deaths	1.52 (0.66–3.54)	0.60 (0.19–1.91)
Sex (male)		0.97 (0.60–1.57)	0.92 (0.59–1.45)	0.85 (0.44–1.64)
Age at diagnosis		0.99 (0.94–1.05)	1.01 (0.96–1.06)	1.02 (0.93–1.10)
Grade	1	1 (reference)	1 (reference)	1 (reference)
	2	1.40 (0.51–5.70)	0.91 (0.34–2.45)	2.14 (0.28–16.38)
	3	0.77 (0.09–6.72)	1.90 (0.52–6.94)	14.47 (1.25–167.07)
	4	–	4.17 (0.72–24.05)	62.36 (2.11–1837.24)
Sublocation of the tumor	Proximal	1 (reference)	1 (reference)	1 (reference)
	Distal	0.76 (0.41–1.43)	0.67 (0.37–1.19)	0.55 (0.24–1.24)
	Rectosigmoid	0.32 (0.11–0.94)	0.60 (0.24–1.48)	0.95 (0.27–3.35)
	Rectum	0.49 (0.18–1.36)	0.24 (0.08–0.69)	0.35 (0.06–1.87)

[297] Early-stage G-allele (*KRAS* variant) carriers with *KRAS* mutations have a good prognosis; because none of these patients died due to CRC. In contrast, statistically significant differences in survival were not found between KRAS nonmutated early-stage (HR 0.77; 95% CI: 0.30–1.97), stage III (HR 0.95; 95% CI: 0.44–2.05) or stage IV cases (HR 0.35; 95% CI: 0.11–1.13) with the *KRAS* variant. However, stage III G-allele (*KRAS* variant) carriers with *KRAS* mutations presented a poor prognosis (HR 1.52; 95% CI: 0.66–3.54) although the comparison was not statistically significant. Because Dutch guidelines did not advise adjuvant treatment at the time patients were diagnosed with CRC in the NLCS, the proportion of patients that received adjuvant treatment was very low. Within the early-stage cases, 9% received adjuvant chemotherapy. With respect to more advanced stage, 31% of stage III and 19% of stage IV patients received adjuvant chemotherapy. Exclusion of adjuvant chemotherapy-treated patients did not alter our conclusions. In fact, exclusion of adjuvant chemotherapy-treated patients enhanced the difference between early-stage and stage III G-allele (*KRAS* variant) carriers with *KRAS* mutations (early stage: no CRC-related deaths; stage III: HR 2.36 95% CI: 0.99–5.67), implying that stage III G-allele (*KRAS* variant) carriers have a worse natural course of the disease. However, this analysis is based on small patient numbers.

[298] The survival impact of the *KRAS* variant is independent of microsatellite instability (MSI). Prior to the development of the biomarkers and methods provided herein, MSI was the only established molecular prognostic marker in CRC. Therefore, the effect of KRAS variant

genotype was studied in patient populations stratified for MSI. Exclusion of patients that had an MSI tumor, which is associated with a good prognosis, did not alter the conclusions provided herein; both MSI and MSS cases with the *KRAS* variant had a good prognosis. In contrast, patients with the *KRAS* wild type had a poor prognosis, even if they had an MSI tumor (log-rank test, $P = 0.036$) (Figure 17). Additional analyses stratified for sex, tumor sublocation or differentiation grade within MSI patients were not possible due to limited patient numbers.

[299] The risk of advanced stage CRC is not associated with the *KRAS* variant. To study the possibility that the KRAS variant allele predisposes for advanced stage CRC, the association between *KRAS* genotype and CRC risk was studied. The KRAS variant (G-allele) was found in 18% of the subcohort members. For CRC, a decreased risk of developing early-stage (stage I or II) CRC was found when carrying the *KRAS* variant (G-allele) (RR 0.68, 95% CI: 0.49–0.94). The risk of developing advanced stage CRC (stage III or IV) was not influenced by the KRAS-genotype (RR stage III: 1.02, 95% CI: 0.68–1.53; RR stage IV: 1.15, 95% CI: 0.63–2.09).

Example 5: The KRAS variant, patient outcome in metastatic colorectal cancer, and response to treatment.

Materials and methods

[300] *Patient characteristics*. A total of 559 mCRC patients, 300 treated in the University Hospital of Leuven with anti-EGFR moAb monotherapy and MoAb in combination with chemotherapy, as well as 148 patients from the Universite Paris Descartes treated with cetuximab-based salvage combination chemotherapy (De RW, et al. Lancet Oncol 2010; 11(8):753-762), and 111 previously published (Zhang W, al. Ann Oncol 2011; 22(1):104-109) mCRC patients treated with cetuximab monotherapy after failing fluoropyrimidine, irinotecan and oxaliplatin containing regimens (Zhang W, al. Ann Oncol 2011; 22(1):104-109; Lenz HJ, et al. J Clin Oncol 2006; 24(30):4914-4921) had tissue available and amenable for analysis of the *KRAS* variant polymorphism. The mutational status of the *KRAS* and *BRAF* genes in the above mentioned patient populations is publicly available (De RW, et al. Lancet Oncol 2010; 11(8):753-762; Zhang W, al. Ann Oncol 2011; 22(1):104-109). The above mentioned molecular characteristics were correlated with ORR, PFS and OS. From the 559 mCRC patients entered in the study the *KRAS* 3'-UTR LCS6 variant was determined in 512 due to exhaustion of available DNA from other molecular testing.

[301] *Genetic analyses.* Formalin-fixed, paraffin-embedded normal tissue from the patients' specimens was macroscopically dissected using a scalpel blade and DNA was isolated as previously described (De RW, et al. Lancet Oncol 2010; 11(8):753-762; Zhang W, al. Ann Oncol 2011; 22(1):104-109). DNA was amplified using, as previously described (Hollestelle A, et al. Breast Cancer Res Treat 2010), a custom-made Taqman genotyping assay (Applied Biosystems, Foster City, CA) designed specifically to identify the T or variant G allele of the *KRAS*-variant (rs61764370) with the forward primer: 5'-GCCAGGCTGGTCTCGAA-3' (SEQ ID NO: 28), reverse primer: 5'-CTGAATAAATGAGTTCTGCAAAACAGGT T-3'(SEQ ID NO: 29), VIC reporter probe: 5'-CTCAAGTGATTCACCCA C-3' (SEQ ID NO: 30), and FAM reporter probe: 5'-CAAGTGATTCACCCAC- 3' (SEQ ID NO: 31). The *KRAS* and *BRAF* mutational status was determined as previously described (De RW, et al. Lancet Oncol 2010; 11(8):753-762; Zhang W, al. Ann Oncol 2011; 22(1):104-109).

[302] *Cell line studies.* A cell line with the *KRAS* variant (G-allele) (HCC2998) and a cell line without the allele and without a *KRAS* tumor acquired mutation (HT-29) were studied to evaluate the impact of treatment with chemotherapy alone or in combination with Cetuximab. Cell lines were treated with Cetuximab (100nM) or none and dilutions of Irinotecan (1mg/ml-100mg/ml). Cells were plated, treated with agents 24 hours after plating, media was changed after a 24 hour exposure, and then survival was scored 48 hours later using the MTT assay.

[303] *Statistical analyses.* The distribution of genotypes was tested for Hardy-Weinberg Equilibrium and the χ^2 test was $p = 0.8$. Because of the low frequency of homozygotes for the *KRAS* variant allele, patient samples that were either heterozygous (TG) or homozygous (GG) for the *KRAS* variant allele were considered positive for the LCS6 (KRAS-variant or G allele) and entered the analyses as one group of at least one *KRAS* variant (G allele) genotypes. PFS and OS were measured as previously described (De RW, et al. Lancet Oncol 2010; 11(8):753-762; Zhang W, al. Ann Oncol 2011; 22(1):104-109)

[304] The two-tailed Fisher's exact test was used to compare proportions between carriers of the wild-type (wt) TT genotype and carriers of at least one G allele genotypes (TG and GG). PFS and OS were estimated with the use of the Kaplan-Meier method and their association with genotypes was tested with the use of the log-rank test. The association of genotypes with objective response was determined by contingency table and the Fisher's exact test. To fully explore the possible influence of the *KRAS* variant, analyses were performed in the whole mCRC

population, in the patients harboring no mutations in the *KRAS* and *BRAF* genes (double wt population) and in the *KRAS* variant population. The level of significance was set at a two-sided *p* value of <0.05. All statistical tests were performed using the statistical package SPSS version 13.

Results

[305] *KRAS LCS6 in the entire patient cohort.* In these 512 mCRC patients there were 403 carriers of the wt LCS6 TT genotype (72%), 102 (18%) carriers of the heterozygous *KRAS* variant TG allele and 7 (1.3%) of the homozygous *KRAS* variant GG allele, thus 109 (19.5%) carriers of at least one G allele genotype. *KRAS* mutations in codons 12, 13 and 61 were found in 184 patients (33%) and the *BRAF* V600E was found in 29 patients (5.3%). All patients had received anti-EGFR moAbs-based salvage treatment, 169 as monotherapy and 377 in combination with chemotherapy. No statistically significant differences were found between *KRAS* wt and *KRAS* variant carriers for sex and age at diagnosis. The characteristics of the 559 patients have been previously published (De RW, et al. Lancet Oncol 2010; 11(8):753-762; Zhang W, al. Ann Oncol 2011; 22(1):104-109).

[306] As shown in Table 30 the distribution of the *KRAS* genotypes was different among patients harboring *KRAS* and *BRAF* mutations. In particular, whereas the percentage of at least one G variant allele genotype was equally distributed among the *KRAS* wt and mutant groups (20% in each), the *KRAS* variant (G allele) was twice as frequent in the *BRAF* V600E mutated group (40%) compared to the wt one (20%), resulting in a statistically significant difference (Fisher's exact test $p = 0.030$).

[307] **Table 30.** Distribution of the *KRAS* 3'-UTR LCS6 genotypes according to *KRAS* and *BRAF* mutational status in the mCRC patients' cohort.

				***KRAS* 3'-UTR LCS6 genotypes**		
Feature	**Patients' population (No of patients)**			**TT No of patients**	**TG+GG No of patients**	***p* value (fisher's exact test)**
KRAS status	n=484	Mutant	(n=174)	138	36	0.818
		WT	(n=310)	242	68	
BRAF status	n=504	Mutant	(n=28)	17	11	**0.030**
		WT	(n=476)	379	97	

Abbreviations: 3'-UTR LCS6, 3' untranslated region of the *Let-7* complementary site, WT, wild type.

[308] *Outcome and Survival analysis in the entire patient cohort.* In the cohort as a whole, for patients with PFS and OS information and LCS6 genotyping (n=510 and 503, respectively) no significant differences were detected regarding median PFS and OS between the LCS6 wt TT genotype carriers and the LCS6 G variant (*KRAS* variant) genotype carriers (Figure 18A and 18B). Similarly, no differences in PFS and OS were observed in the double (*KRAS* and *BRAF*) wt or in the *KRAS* variant patient cohort. Furthermore, no significant correlations regarding response (n=483) and skin rash (n=359) were observed between the *KRAS* variant and wt carriers in the whole and in the double wt patients' cohorts (Table 31).

[309] **Table 31.** Outcome and survival analysis according to *KRAS* genotypes and other clinical variables for the entire population.

		KRAS 3'-UTR LCS6 genotypes		
Population cohort	**Variables**	**TT**	**TG+GG**	***p* value**
All patients	**median PFS (weeks) (95%CI)**	16 (14.3-17.6)	18 (12.8-23.1)	0.144 (log-rank test)
	median OS (weeks) (95%CI)	38 (34.74-41.26)	45 (36.01-53.98)	0.339 (log-rank test)
Double (*KRAS* and *BRAF*) wt patients	**median PFS (weeks)**	23.3	25.3	0.13 (log-rank test)
	median OS (weeks)	46	54	0.256 (log-rank test)
***KRAS* mutated patients**	**median PFS (weeks)**	11	12	0.834 (log-rank test)
	median OS (weeks)	28	33	0.496 (log-rank test)
All patients	**responders (n) (CR+PR)**	79	29	0.142 (Fisher's exact test)
	non-responders (n) (SD+PD)	301	74	
Double (*KRAS* and *BRAF*) wt patients	**responders (n) (CR+PR)**	72	25	0.165 (Fisher's exact test)
	non-responders (n) (SD+PD)	141	32	
All patients	**skin rash (no/grade 1, n)**	149	48	0.2 (Fisher's exact test)
	skin rash (grade 2/3, n)	132	30	
Double (*KRAS* and *BRAF*) wt patients	**skin rash (no/grade 1, n)**	71	23	0.149 (Fisher's exact test)
	skin rash (grade 2/3, n)	81	15	

Abbreviations: 3'-UTR LCS6, 3' untranslated region of the *Let-7* complementary site; WT, wild type; PFS, progression-free survival; OS, overall survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

[310] *Progression free survival analysis correlated with treatment.* Patients who received moAbs monotherapy and moAbs combination therapy were analyzed separately. From the 501 patients evaluable for LCS6 SNP genotyping and treatment administration, 160 (32%) received anti-EGFR moAbs as monotherapy. Of the monotherapy patients, 128 (80%) were carriers of the LCS6 wt TT genotype and 32 (20%) were carriers of the LCS6 G variant genotype. There were

341 (68%) patients who received multiple chemotherapy combinations. Of the combination treatment patients, 266 (78%) were carriers of the LCS6 wt TT genotype and 75 (22%) were carriers of the LCS6 at least one G variant genotype.

[311] The median PFS of the whole monotherapy patients' population was 10.43 weeks (95% CI: 7.73-13.12 weeks) and a statistically significant difference ($p = 0.019$, log-rank test) was observed between the LCS6 wt TT genotype carriers, 7.85 weeks (95% CI: 3.897-11.817 weeks), and the LCS6 G variant (*KRAS* variant) genotype carriers, 16.86 weeks (95% CI: 10.2-23.51 weeks) (Figure 19A). The median PFS of the whole combination therapy patients' population was 18 weeks (95% CI: 15.87-20.12 weeks) and no statistically significant difference ($p = 0.760$, log-rank test) was observed between the LCS6 wt TT genotype carriers, 18.43 weeks (95% CI: 16.16-20.69 weeks), and the LCS6 G variant genotype carriers, 18 weeks (95% CI: 9.97-26.02 weeks) (Figure 19B). There was also no significant difference ($p = 0.291$, log-rank test) between PFS for *KRAS* variant patients that received moAbs therapy [16.86 weeks, (95% CI: 8.55-25.18 weeks)] versus combination therapy [18 weeks, (95% CI: 13.37-22.64 weeks)] (Figure 19C), while there was a significant benefit with the addition of chemotherapy for non-*KRAS* variant patients [$p < 0.0001$, log-rank test, PFS for moAbs monotherapy 7.86 weeks, (95% CI: 3.9-11.82 weeks) versus combination therapy 19.29 weeks, (95% CI: 17-21.58 weeks) (Figure 19D). Of note, there was no significant difference in PFS between *KRAS* variant patients treated with monotherapy therapy versus non-*KRAS* variant patients treated with combination therapy.

[312] In the double (*KRAS* and *BRAF*) wt patients' population the median PFS of the monotherapy patients was 12 weeks (95% CI: 8.38-15.61 weeks) and a statistically significant difference ($p = 0.039$, log-rank test) was again observed between the LCS6 wt TT genotype carriers, 10.43 weeks (95% CI: 6.74-14.11 weeks), and the LCS6 G variant genotype carriers, 18 weeks (95% CI: 5.16-30.83 weeks) (Figure 20A). In the double wt patients' population the median PFS of the combination therapy patients was 28.71 weeks (95% CI: 24.98-32.43 weeks) and no statistically significant difference ($p = 0.39$, log-rank test) was observed between the LCS6 wt TT genotype carriers, 28.3 weeks (95% CI: 24.15-32.45 weeks), and the LCS6 G variant genotype carriers, 28.85 weeks (95% CI: 14.82-42.87 weeks) (Figure 20B). There was no significant improvement ($p = 0.096$, log-rank test) between PFS for LCS6 variant patients that received moAbs monotherapy [23 weeks, (95% CI: 9.5-36.5 weeks)] versus combination therapy [28 weeks, (95% CI: 14.83-42.87 weeks)] (Figure 20C), while there was for non-LCS6 patients

[$p < 0.0001$, log-rank test, PFS for moAbs monotherapy 10.43 weeks, (95% CI: 6.75-14.15 weeks) versus combination therapy 28.71 weeks, (95% CI: 24.8-32.6 weeks) (Figure 20D). There was no difference in PFS between *KRAS* variant (G allele) patients receiving moAbs monotherapy and non-*KRAS* variant patients receiving combination therapy.

[313] *Overall survival analysis correlated with treatment.* The median OS of the whole monotherapy patients' population was 33.14 weeks (95% CI: 26.70-39.57 weeks) and no statistically significant difference ($p = 0.139$, log-rank test) was observed between the LCS6 wt TT genotype carriers, 28.85 weeks (95% CI: 22.53-35.18 weeks), and the LCS6 G variant genotype carriers, 45 weeks (95% CI: 35.02-54.97 weeks) (Figure 21A). The median OS of the whole combination therapy patients' population was 44 weeks (95% CI: 40.11-47.88 weeks) and no statistically significant difference ($p = 0.759$, log-rank test) was observed between the LCS6 wt TT genotype carriers, 44 weeks (95% CI: 40.06-47.93 weeks), and the LCS6 at least one G variant genotype carriers, 43 weeks (95% CI: 29.8-56.2 weeks) (Figure 21B). Again, there was no significant improvement ($p = 0.574$, log-rank test) between OS for *KRAS* variant patients that received moAbs monotherapy [45 weeks, (95% CI: 35-55 weeks)] versus combination therapy [43 weeks, (95% CI: 29.8-56.2 weeks)] (Figure 21C), while there was a benefit of chemotherapy addition for non-*KRAS* variant patients [$p < 0.0001$, log-rank test, OS for moAbs monotherapy 28.86 weeks, (95% CI: 22.53-35.18 weeks) versus combination therapy 44 weeks, (95% CI: 40-47.93 weeks) (Figure 21D). Again, there was no significant difference in OS between LCS6 G variant carriers treated with monotherapy, and non-*KRAS* variant carriers treated with combination therapy.

[314] In the double (*KRAS* and *BRAF*) wt patients' population the median OS of the monotherapy patients was 37 weeks (95% CI: 30.82-43.17 weeks) and a trend towards a statistically significant difference ($p = 0.087$, log-rank test) was observed between the LCS6 wt TT genotype carriers, 35.71 weeks (95% CI: 32.03-39.4 weeks), and the LCS6 at least one G variant genotype carriers, 55.43 weeks (95% CI: 36.98-73.87 weeks) (Figure 22A). In the double wt patients' population, the median OS of the combination therapy patients was 55 weeks (95% CI: 48.3-61.7 weeks) and no statistically significant difference ($p = 0.649$, log-rank test) was observed between the LCS6 wt TT genotype carriers, 57 weeks (95% CI: 49.4-64.6 weeks), and the LCS6 at least one G variant genotype carriers, 54 weeks (95% CI: 45.46-62.53 weeks) (Figure 22B). There was no significant improvement ($p = 0.705$, log-rank test) between OS for

KRAS variant (G allele) patients that received moAbs monotherapy [55.43 weeks, (95% CI: 37-73.87 weeks)] versus combination therapy [54 weeks, (95% CI: 45.47-62.54 weeks)] (Figure 22C), while there was for non- *KRAS* variant patients [$p < 0.0001$, log-rank test, OS for moAbs monotherapy 35.71 weeks, (95% CI: 32-39.4 weeks) versus combination therapy 57 weeks, (95% CI: 49.4-64.6 weeks) (Figure 22D). There was no significant difference between double wild-type patients *KRAS* variant carriers treated with monotherapy versus non-LCS6 carriers treated with combination therapy.

[315] *The LCS6 variant is prognostic in KRAS and BRAF mutated patients.* In the *KRAS* and *BRAF* mutated patients' population no statistical significant differences regarding PFS and OS were observed in patients treated with both anti-EGFR moAbs monotherapy and in combination with chemotherapy (data not shown). Median PFS times were identical between *KRAS* variant and non- *KRAS* variant patients, with no significant improvement ($p = 0.641$, log-rank test) between PFS for *KRAS* variant patients that received moAbs monotherapy [6 weeks, (95% CI: 0-13.25 weeks)] versus combination therapy [12 weeks, (95% CI: 6.45-17.56 weeks)] (Figure 23A). There was a significant improvement in PFS for non- *KRAS* variant patients [$p < 0.0001$, log-rank test, PFS for moAbs monotherapy 6 weeks, (95% CI: 4.46-7.53 weeks) versus combination therapy 12 weeks, (95% CI: 9.72-14.28 weeks) (Figure 23B). For OS, there was no significant difference ($p = 0.303$, log-rank test) between OS for *KRAS* variant (G allele) patients that received moAbs monotherapy [28.43 weeks, (95% CI: 9.47-47.39 weeks)] versus combination therapy [23 weeks, (95% CI: 10.8-35.19 weeks)] (Figure 23C), while there was for non- *KRAS* variant patients [$p = 0.002$, log-rank test, OS for moAbs monotherapy 21.29 weeks, (95% CI: 15-27.55 weeks) versus combination therapy 31 weeks, (95% CI: 25.65-36.34 weeks) (Figure 23D).

[316] *The KRAS variant and response.* From the whole population of 483 patients that were evaluable for both response and *KRAS* variant genotyping, 147 (30.4%) had received anti-EGFR moAbs as monotherapy and 336 (69.6%) with multiple chemotherapy combinations. In the monotherapy group 123 (83.6%) patients were non-responders (SD and PD), 104 LCS6 wt and 19 LCS6 variant (*KRAS* variant) carriers, and 24 (16.4%) were responders (PR and CR), 13 LCS6 wt and 11 LCS6 variant (*KRAS* variant) carriers. A statistically significant difference was observed between the wt and *KRAS* variant genotype carriers distribution in the responders and non-responders groups (Fisher's exact test p=0.002). In the combination with chemotherapy

group 252 (75%) patients were non-responders (SD and PD) and 84 (25%) were responders (PR and CR). No statistically significant difference was observed between the wt and *KRAS* variant genotype carriers, 197 vs. 55 non-responders and 66 vs. 18 responders, respectively (Fisher's exact test p=1).

[317] In the 270 double (*KRAS* and *BRAF*) wt population 90 (33.3%) had received anti-EGFR moAbs as monotherapy and 180 (66.6%) with multiple chemotherapy combinations. In the monotherapy group 71 (78.8%) patients were non-responders (SD and PD), 60 LCS6 wt and 11 LCS6 variant (*KRAS* variant) carriers and 19 (21.2%) were responders (PR and CR), 10 LCS6 wt and 9 LCS6 variant (*KRAS* variant) carriers. A statistically significant difference was observed between the wt and *KRAS* variant genotype carriers distribution in the responders and non-responders groups (Fisher's exact test p=0.010). In the combination with chemotherapy group 102 (56.6%) patients were non-responders (SD and PD) and 78 (43.4%) were responders (PR and CR). No statistically significant difference was observed between the wt and *KRAS* variant genotype carriers, 81 vs. 21 non-responders and 62 vs. 16 responders, respectively (Fisher's exact test p=1).

[318] *Cell line studies of the effect of moAbs monotherapy and combination therapy and the LCS6 variant.* To confirm that the *KRAS* variant (G allele) predicts response to moAbs monotherapy, without any benefit of additional cytotoxic therapy, the impact of monotherapy versus combination therapy in colon cancer cell lines with and without the LCS6 G variant was evaluated. It was discovered that in non-*KRAS* variant cell lines, the addition of Cetuximab to cytotoxic therapy, both radiation as well as irinotecan chemotherapy, increased cell death as compared to cytotoxic therapy alone. In contrast, in a cell line with the *KRAS* variant (G allele), there was no additional cell kill with the addition of Cetuximab to cytotoxic therapy, and in the case of radiation in fact higher cell survival when Cetuximab was added. These findings are consistent with our *in vivo* findings, that there is no benefit of the combination of Cetuximab with cytotoxic therapy in *KRAS* variant (G allele) carriers.

OTHER EMBODIMENTS

[319] While the disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the

disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

[320] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

[321] While this disclosure has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the disclosure encompassed by the appended claims.

What is claimed is:

CLAIMS

1. A method of identifying a subject or patient at risk for developing an estrogen receptor (ER) and progesterone receptor (PR) negative (ER/PR negative) breast cancer, comprising detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates an increased risk of developing the ER/PR negative breast cancer in the subject.

2. A method of predicting the onset of developing an estrogen receptor (ER) and progesterone receptor (PR) negative (ER/PR negative) breast cancer in a subject or patient at risk for developing breast cancer, comprising detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates an earlier onset of developing the ER/PR negative breast cancer.

3. The method of claim 2, wherein the ER/PR negative breast cancer is also negative for HER2, and therefore, is a triple negative breast cancer (TNBC).

4. The method of claim 3, wherein the triple negative breast cancer (TNBC) is a basal or luminal tumor.

5. The method of claim 4, wherein the triple negative breast cancer (TNBC) is a basal tumor that expresses a transcript or protein encoded by the epidermal growth factor receptor (*EGFR*) or the cytokeratin 5/6 (*CK5/6*) gene.

6. The method of claim 1, 2, or 3, wherein the breast cancer is further characterized by low or negative expression of the breast cancer 1 (*BRCA1*) gene.

7. The method of claim 1, 2, or 3, wherein the subject or patient is pre-menopausal.

8. The method of claim 1, 2, or 3, wherein the subject or patient is 51 years of age or younger.

9. A method of prognosing a subject or patient with epithelial ovarian cancer (EOC), comprising detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates a decreased survival rate when compared to a control.

10. The method of claim 9, wherein the subject or patient is post-menopausal, 52 years of age, or at least 52 years of age.

11. The method of claim 9, wherein the control does not carry the mutation.

12. The method of claim 1, wherein the survival rate is overall survival, five-year survival or one-year survival.

13. A method of predicting the response of an epithelial ovarian cancer (EOC) cell to a platinum-based chemotherapy, comprising detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, wherein the presence of the mutation indicates a resistance to platinum-based chemotherapy.

14. The method of claim 13, wherein the EOC cell is evaluated *in vitro* or *ex vivo*.

15. The method of claim 14, wherein the EOC cell is evaluated *ex vivo* from a subject who is post-menopausal, 52 years of age, or at least 52 years of age.

16. The method of claim 14, wherein the EOC cell is evaluated *in vitro* and wherein the EOC cell is isolated, reproduced, or derived from the BG1, CAOV3, or IGR-OV1 cell line.

17. The method of claim 13, wherein the platinum-based chemotherapy is carboplatin or paclitaxel.

18. The method of claim 13, wherein the platinum-based chemotherapy is an adjuvant therapy.

19. A method of prognosing a subject or patient with colorectal cancer (CRC), comprising detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, wherein the presence of the mutation indicates a increased survival rate when compared to a control.

20. The method of claim 19, wherein the detecting step further comprises microsatellite-instability (MSI) analysis.

21. The method of claim 19, wherein the colorectal cancer (CRC) is early stage CRC.

22. The method of claim 19, wherein the colorectal cancer (CRC) is stage 1 or 2 CRC.

23. The method of claim 19, wherein the control does not carry the *KRAS*-variant.

24. The method of claim 23, wherein the control has a second mutation in the *KRAS* gene.

25. The method of claim 19, wherein the subject or patient has a second mutation in the *KRAS* gene.

26. The method of claim 19, wherein the subject or control carries one or more mutations in the *BRAF* gene.

27. The method of claim 19, wherein the subject or control has a hypermethylated *RASSF1A* promoter.

28. The method of claim 19, wherein the survival rate is overall survival, five-year survival or one-year survival.

29. A method of predicting the response of a cancer cell to a monoclonal antibody monotherapy, comprising detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates a sensitivity to monoclonal antibody monotherapy.

30. The method of claim 29, wherein the cancer cell is a colorectal cancer (CRC) cell.

31. The method of claim 29, wherein the cancer cell is evaluated *in vitro* or *ex vivo*.

32. The method of claim 29, wherein the monoclonal antibody monotherapy is Cetuximab.

33. A method of predicting the response of a cancer cell to the combination of a chemotherapy and monoclonal antibody therapy, comprising detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and wherein the presence of the mutation indicates a resistance to the combination.

34. The method of claim 33, wherein the cancer cell is a colorectal cancer (CRC) cell.

35. The method of claim 33, wherein the cancer cell is evaluated *in vitro* or *ex vivo*.

36. The method of claim 33, wherein the monoclonal antibody monotherapy is Cetuximab.

37. The method of claim 33, wherein the chemotherapy is a cytotoxic agent.

38. The method of claim 37, wherein the cytotoxic agent is irinotecan.

39. A method of predicting the an increased risk of vascularization of a tumor, comprising

(a) detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a first patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and

(b) determining the expression level of a miRNA selected from the group consisting of miR-23 and miR-27 in a second patient sample,

wherein the presence of the mutation in (a) and an increase in the expression level of a miRNA in (b) compared to a control indicates transcriptional silencing of an anti-angiogenic gene, thereby predicting the an increased risk of vascularization of the tumor.

40. The method of claim 39, wherein the anti-angiogenic gene is Sprouty2 or Sema 6A.

41. The method of claim 39 or 40, wherein the tumor comprises a cancer cell derived from a(n) AIDS-related cancer, breast cancer, cancer of the digestive/gastrointestinal tract, anal cancer, appendix cancer, bile duct cancer, colon cancer, colorectal cancer, esophageal cancer, gallbladder cancer, islet cell tumors, pancreatic neuroendocrine tumors, liver cancer, pancreatic cancer, rectal cancer, small intestine cancer, stomach (gastric) cancer, endocrine system cancer, adrenocortical carcinoma, parathyroid cancer, pheochromocytoma, pituitary tumor, thyroid cancer, eye cancer, intraocular melanoma, retinoblastoma, bladder cancer, kidney (renal cell) cancer, penile cancer, prostate cancer, transitional cell renal pelvis and ureter cancer, testicular cancer, urethral cancer, Wilms' tumor, other childhood kidney tumors, germ cell cancer, central nervous system cancer, extracranial germ cell tumor, extragonadal germ cell tumor, ovarian germ cell tumor, gynecologic cancer, cervical cancer, endometrial cancer, gestational trophoblastic tumor, ovarian epithelial cancer, uterine sarcoma, vaginal cancer, vulvar cancer, head and neck cancer, hypopharyngeal cancer, laryngeal cancer, lip and oral cavity cancer, metastatic squamous neck cancer with occult primary, mouth cancer, nasopharyngeal cancer, oropharyngeal cancer, paranasal sinus and nasal cavity cancer, pharyngeal cancer, salivary gland cancer, throat cancer, musculoskeletal cancer, bone cancer, Ewing's sarcoma, gastrointestinal stromal tumors (GIST), osteosarcoma, malignant fibrous histiocytoma of bone, rhabdomyosarcoma, soft tissue sarcoma, uterine sarcoma, neurologic cancer, brain tumor, astrocytoma, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central

nervous system embryonal tumors, central nervous system germ cell tumor, craniopharyngioma, ependymoma, medulloblastoma, spinal cord tumor, supratentorial primitive neuroectodermal tumors and pineoblastoma, neuroblastoma, respiratory cancer, thoracic cancer, non-small cell lung cancer, small cell lung cancer, malignant mesothelioma, thymoma, thymic carcinoma, skin cancer, Kaposi's sarcoma, melanoma, or Merkel cell carcinoma.

42. The method of claim 39 or 41, wherein the tumor is metastic.

43. A method of predicting an increased survival or proliferation of a cancer cell under hypoxic conditions, comprising

(a) detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a first patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and

(b) determining the expression level of a miR-210 miRNA in a second patient sample,

wherein the presence of the mutation in (a) and an increase in the expression level of the miRNA in (b) compared to a control predicts an increased survival or proliferation of the cancer cell under hypoxic conditions.

44. The method of claim 43, wherein the cancer cell is derived from a(n) AIDS-related cancer, breast cancer, cancer of the digestive/gastrointestinal tract, anal cancer, appendix cancer, bile duct cancer, colon cancer, colorectal cancer, esophageal cancer, gallbladder cancer, islet cell tumors, pancreatic neuroendocrine tumors, liver cancer, pancreatic cancer, rectal cancer, small intestine cancer, stomach (gastric) cancer, endocrine system cancer, adrenocortical carcinoma, parathyroid cancer, pheochromocytoma, pituitary tumor, thyroid cancer, eye cancer, intraocular melanoma, retinoblastoma, bladder cancer, kidney (renal cell) cancer, penile cancer, prostate cancer, transitional cell renal pelvis and ureter cancer, testicular cancer, urethral cancer, Wilms' tumor, other childhood kidney tumors, germ cell cancer, central nervous system cancer, extracranial germ cell tumor, extragonadal germ cell tumor, ovarian germ cell tumor, gynecologic cancer, cervical cancer, endometrial cancer, gestational trophoblastic tumor, ovarian epithelial cancer, uterine sarcoma, vaginal cancer, vulvar cancer, head and neck cancer, hypopharyngeal cancer, laryngeal cancer, lip and oral cavity cancer, metastatic squamous neck

cancer with occult primary, mouth cancer, nasopharyngeal cancer, oropharyngeal cancer, paranasal sinus and nasal cavity cancer, pharyngeal cancer, salivary gland cancer, throat cancer, musculoskeletal cancer, bone cancer, Ewing's sarcoma, gastrointestinal stromal tumors (GIST), osteosarcoma, malignant fibrous histiocytoma of bone, rhabdomyosarcoma, soft tissue sarcoma, uterine sarcoma, neurologic cancer, brain tumor, astrocytoma, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, central nervous system germ cell tumor, craniopharyngioma, ependymoma, medulloblastoma, spinal cord tumor, supratentorial primitive neuroectodermal tumors and pineoblastoma, neuroblastoma, respiratory cancer, thoracic cancer, non-small cell lung cancer, small cell lung cancer, malignant mesothelioma, thymoma, thymic carcinoma, skin cancer, Kaposi's sarcoma, melanoma, or Merkel cell carcinoma.

45. A method of predicting an increased survival or proliferation of a cancer cell, comprising

(a) detecting a mutation in let-7 complementary site LCS6 of human *KRAS* in a first patient sample, wherein the mutation is a SNP comprising a uracil (U) or thymine (T) to guanine (G) transition at position 4 of LCS6, and

(b) determining the methylation status of a promoter of a tumor suppressor gene in a second patient sample,

wherein the presence of the mutation in (a) and an increase in the methylation of a promoter (b) compared to a control predicts an increased survival or proliferation of the cancer cell.

46. The method of claim 45, wherein tumor suppressor gene is *Notch1*.

47. The method of claim 45, wherein the cancer cell is derived from a(n) AIDS-related cancer, breast cancer, cancer of the digestive/gastrointestinal tract, anal cancer, appendix cancer, bile duct cancer, colon cancer, colorectal cancer, esophageal cancer, gallbladder cancer, islet cell tumors, pancreatic neuroendocrine tumors, liver cancer, pancreatic cancer, rectal cancer, small intestine cancer, stomach (gastric) cancer, endocrine system cancer, adrenocortical carcinoma, parathyroid cancer, pheochromocytoma, pituitary tumor, thyroid cancer, eye cancer, intraocular melanoma, retinoblastoma, bladder cancer, kidney (renal cell) cancer, penile cancer, prostate

cancer, transitional cell renal pelvis and ureter cancer, testicular cancer, urethral cancer, Wilms' tumor, other childhood kidney tumors, germ cell cancer, central nervous system cancer, extracranial germ cell tumor, extragonadal germ cell tumor, ovarian germ cell tumor, gynecologic cancer, cervical cancer, endometrial cancer, gestational trophoblastic tumor, ovarian epithelial cancer, uterine sarcoma, vaginal cancer, vulvar cancer, head and neck cancer, hypopharyngeal cancer, laryngeal cancer, lip and oral cavity cancer, metastatic squamous neck cancer with occult primary, mouth cancer, nasopharyngeal cancer, oropharyngeal cancer, paranasal sinus and nasal cavity cancer, pharyngeal cancer, salivary gland cancer, throat cancer, musculoskeletal cancer, bone cancer, Ewing's sarcoma, gastrointestinal stromal tumors (GIST), osteosarcoma, malignant fibrous histiocytoma of bone, rhabdomyosarcoma, soft tissue sarcoma, uterine sarcoma, neurologic cancer, brain tumor, astrocytoma, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, central nervous system germ cell tumor, craniopharyngioma, ependymoma, medulloblastoma, spinal cord tumor, supratentorial primitive neuroectodermal tumors and pineoblastoma, neuroblastoma, respiratory cancer, thoracic cancer, non-small cell lung cancer, small cell lung cancer, malignant mesothelioma, thymoma, thymic carcinoma, skin cancer, Kaposi's sarcoma, melanoma, or Merkel cell carcinoma.

48. The method of claim 45, wherein survival comprises maintaining tumorigenic potential.

49. The method of claim 45 or 48, wherein the cancer cell is a cancer stem cell.

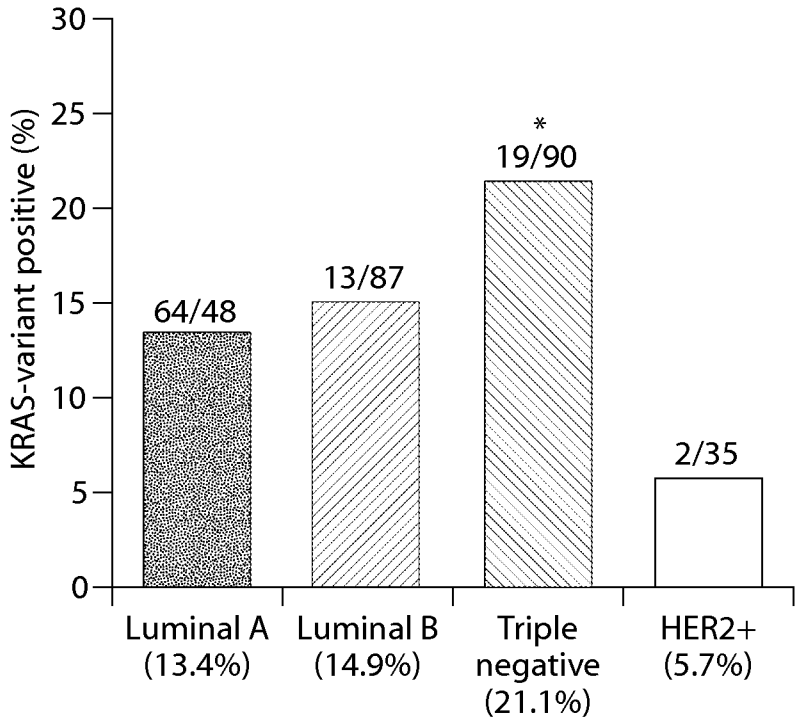

KRAS-variant positive (%)
30
25
20
15
10
5
0
64/48
13/87
*
19/90
2/35
Luminal A (13.4%)
Luminal B (14.9%)
Triple negative (21.1%)
HER2+ (5.7%)

Fig. 1A

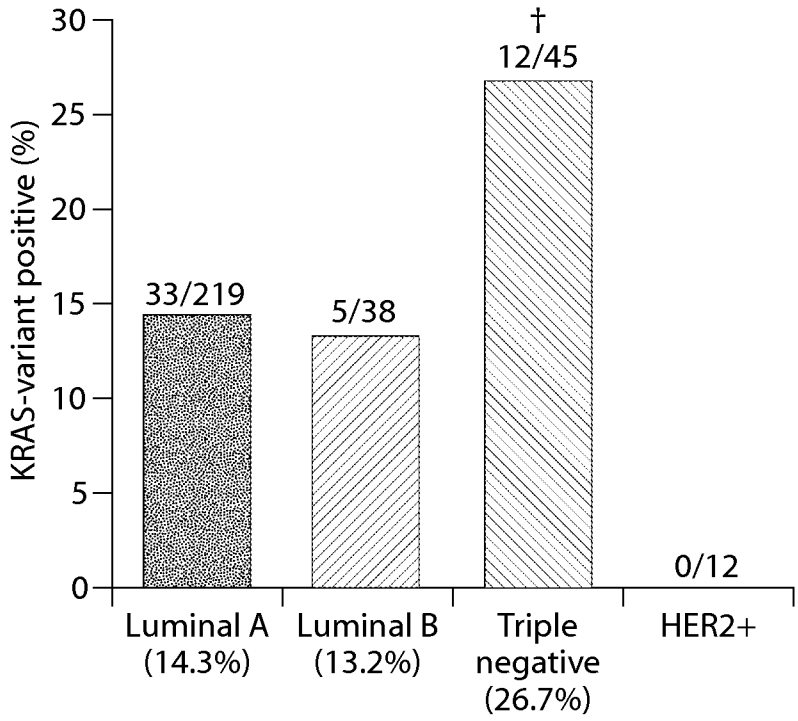

KRAS-variant positive (%)
30
25
20
15
10
5
0
33/219
5/38
†
12/45
0/12
Luminal A (14.3%)
Luminal B (13.2%)
Triple negative (26.7%)
HER2+

Fig. 1B

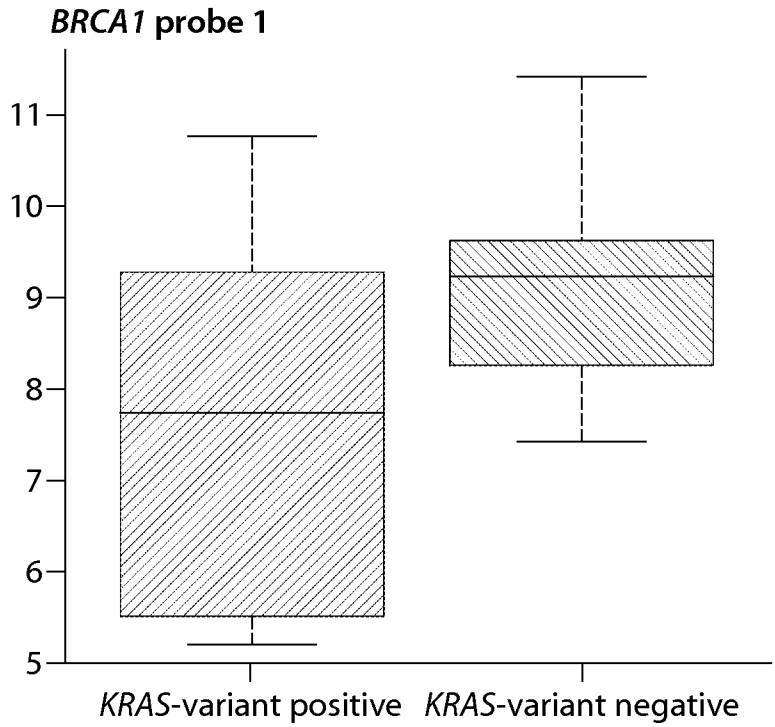

BRCA1 probe 1
11
10
9
8
7
6
5
KRAS-variant positive
KRAS-variant negative

Fig. 2A

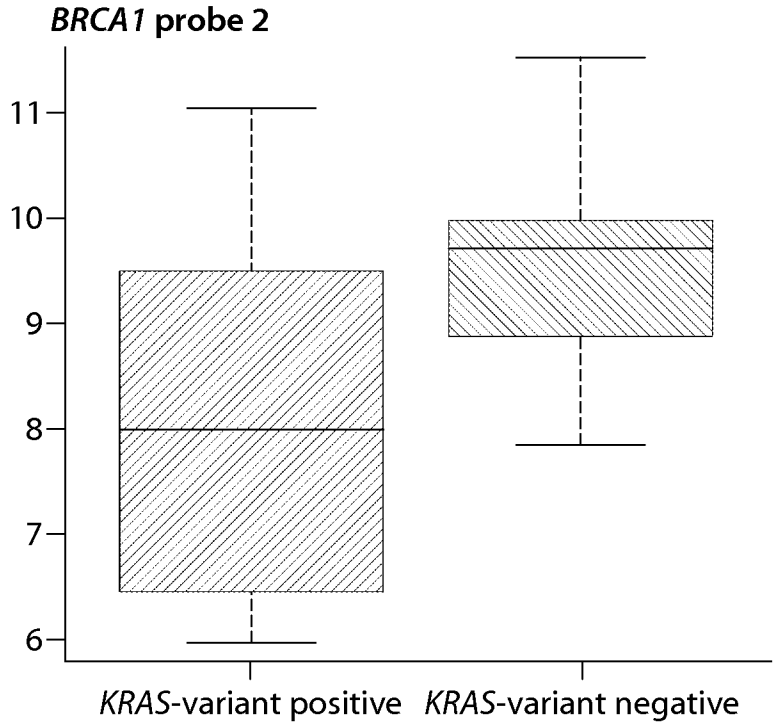

BRCA1 probe 2
11
10
9
8
7
6
KRAS-variant positive
KRAS-variant negative

Fig. 2B

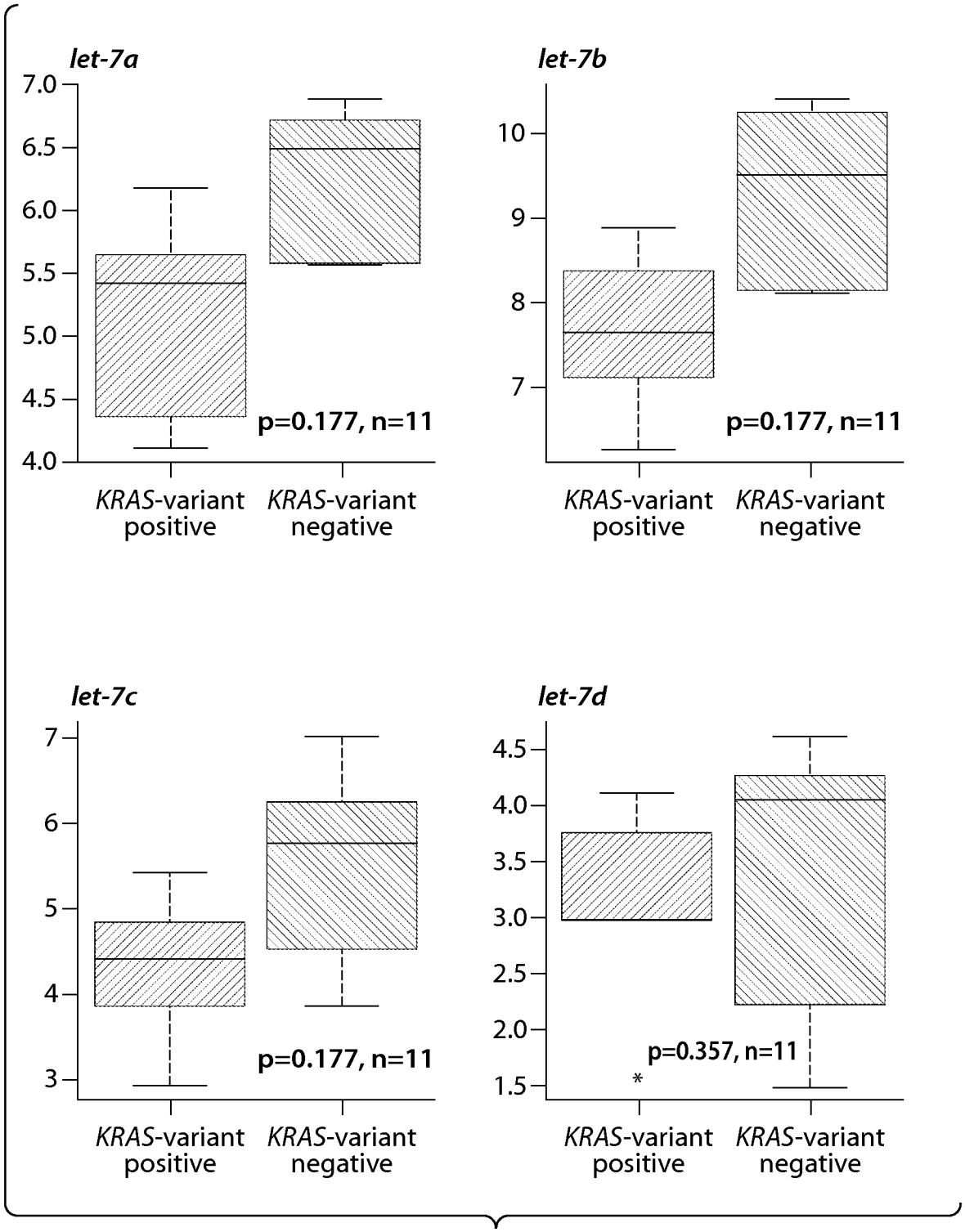

let-7a
7.0
6.5
6.0
5.5
5.0
4.5
4.0
p=0.177, n=11
KRAS-variant positive
KRAS-variant negative
let-7b
10
9
8
7
p=0.177, n=11
KRAS-variant positive
KRAS-variant negative
let-7c
7
6
5
4
3
p=0.177, n=11
KRAS-variant positive
KRAS-variant negative
let-7d
4.5
4.0
3.5
3.0
2.5
2.0
1.5
p=0.357, n=11
*
KRAS-variant positive
KRAS-variant negative

Fig. 3-1

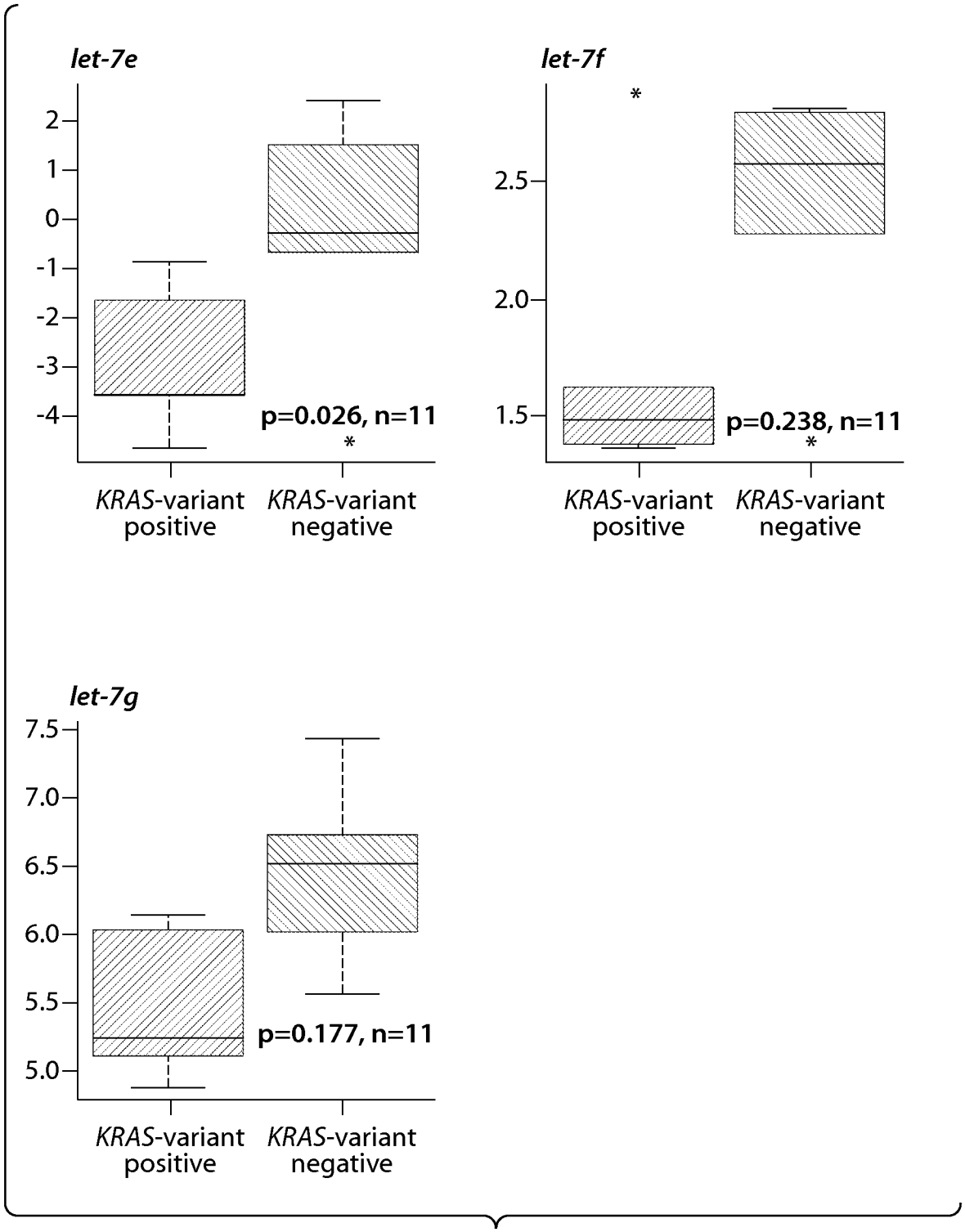

let-7e
2
1
0
-1
-2
-3
-4
p=0.026, n=11
*
KRAS-variant positive
KRAS-variant negative
let-7f
*
2.5
2.0
1.5
p=0.238, n=11
*
KRAS-variant positive
KRAS-variant negative
let-7g
7.5
7.0
6.5
6.0
5.5
5.0
p=0.177, n=11
KRAS-variant positive
KRAS-variant negative

Fig. 3-2

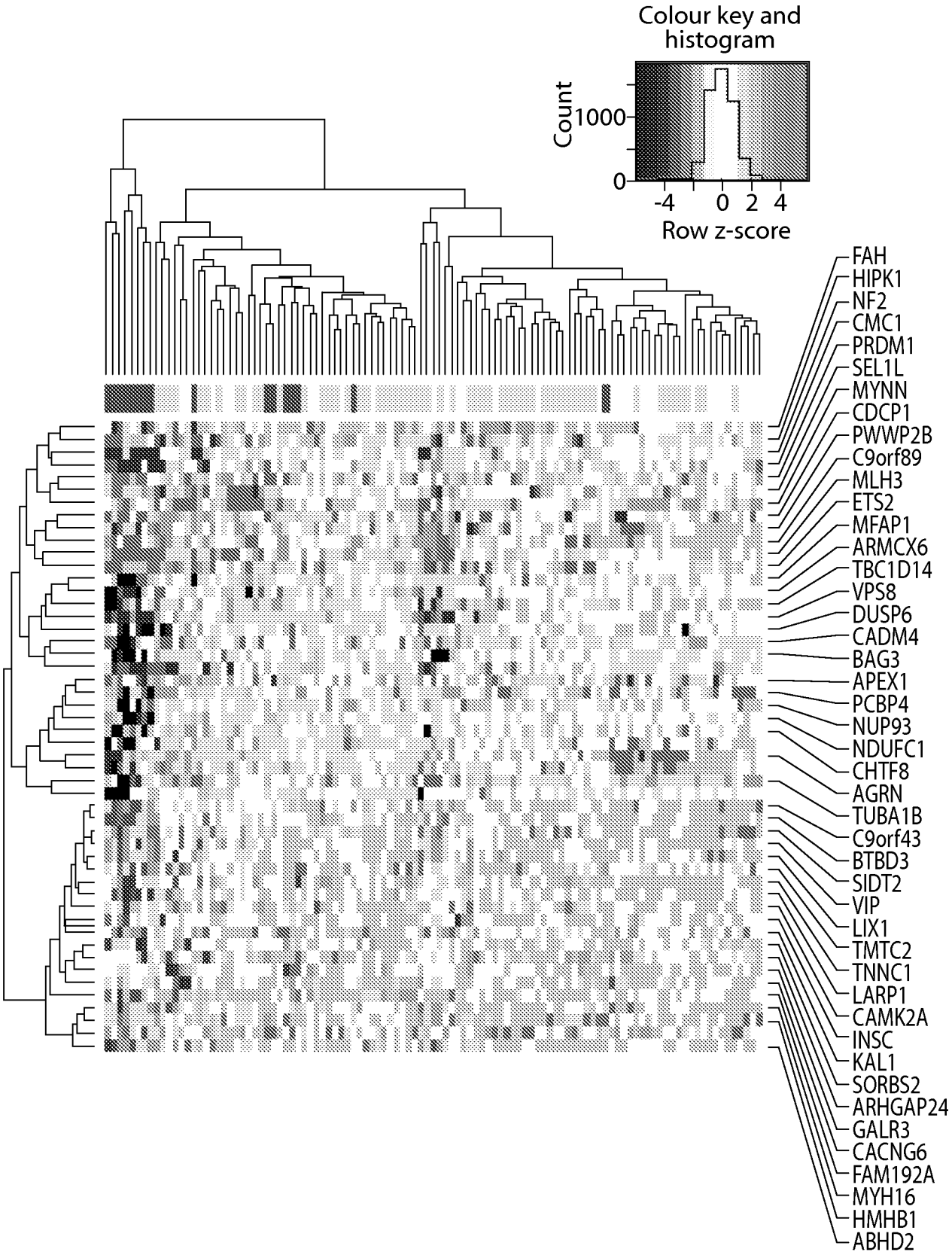

Colour key and histogram
Count
1000
0
-4
0
2
4
Row z-score
FAH
HIPK1
NF2
CMC1
PRDM1
SEL1L
MYNN
CDCP1
PWWP2B
C9orf89
MLH3
ETS2
MFAP1
ARMCX6
TBC1D14
VPS8
DUSP6
CADM4
BAG3
APEX1
PCBP4
NUP93
NDUFC1
CHTF8
AGRN
TUBA1B
C9orf43
BTBD3
SIDT2
VIP
LIX1
TMTC2
TNNC1
LARP1
CAMK2A
INSC
KAL1
SORBS2
ARHGAP24
GALR3
CACNG6
FAM192A
MYH16
HMHB1
ABHD2

Fig. 4

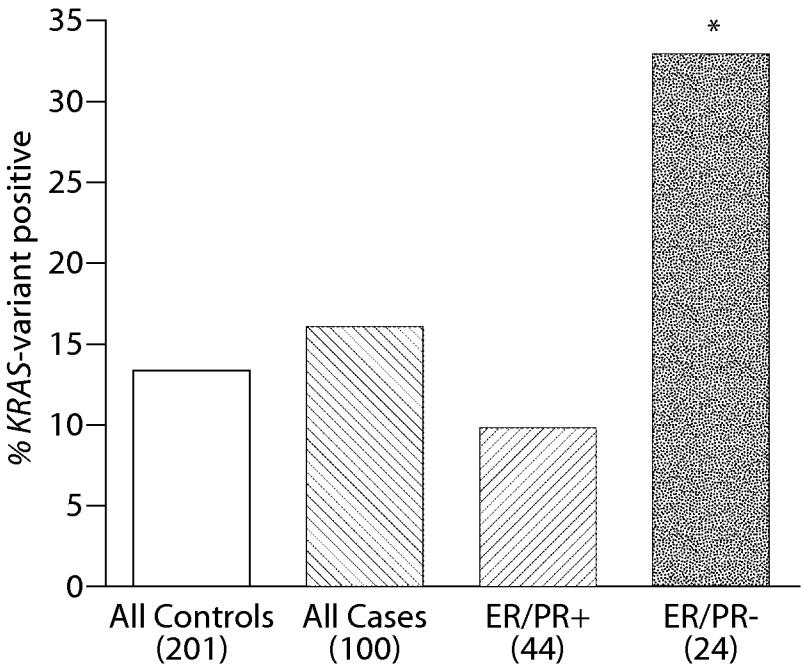

35
30
25
20
15
10
5
0
% KRAS-variant positive
*
All Controls (201)
All Cases (100)
ER/PR+ (44)
ER/PR- (24)

Fig. 5

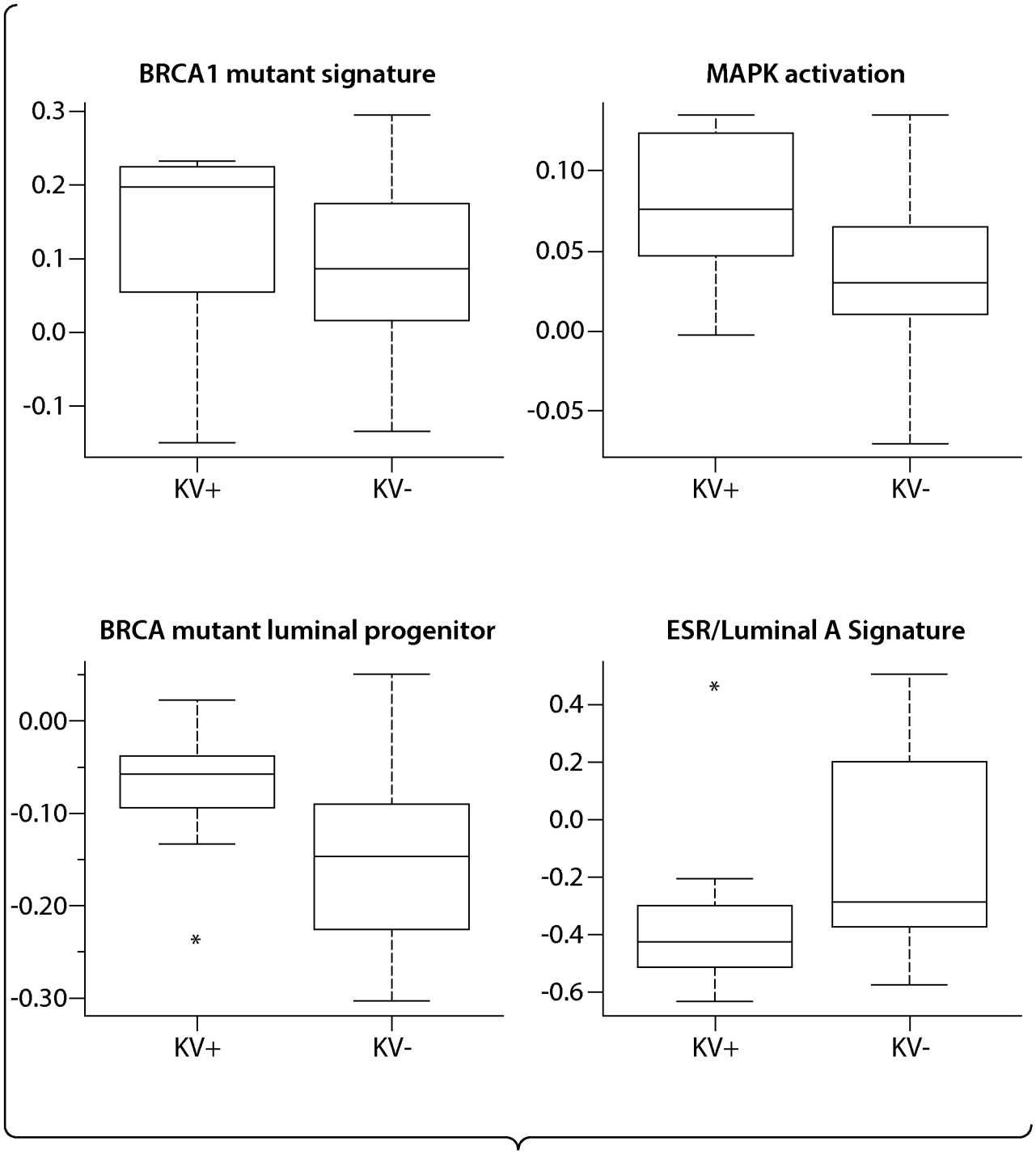

BRCA1 mutant signature
0.3
0.2
0.1
0.0
-0.1
KV+
KV-
MAPK activation
0.10
0.05
0.00
-0.05
KV+
KV-
BRCA mutant luminal progenitor
0.00
-0.10
-0.20
-0.30
*
KV+
KV-
ESR/Luminal A Signature
*
0.4
0.2
0.0
-0.2
-0.4
-0.6
KV+
KV-

Fig. 6

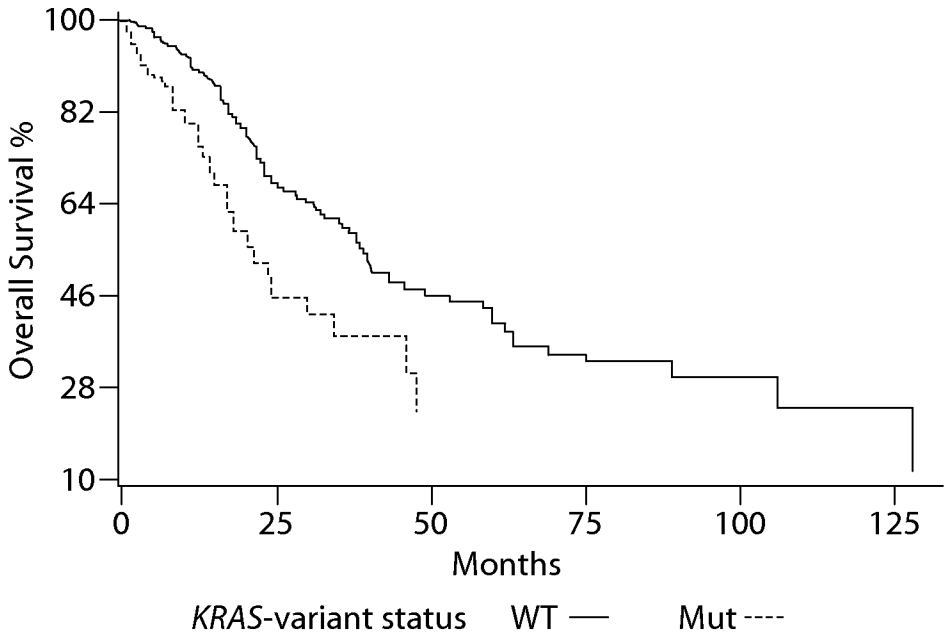

100
82
64
46
28
10
Overall Survival %
0
25
50
75
100
125
Months
KRAS-variant status WT — Mut ----

Fig. 7

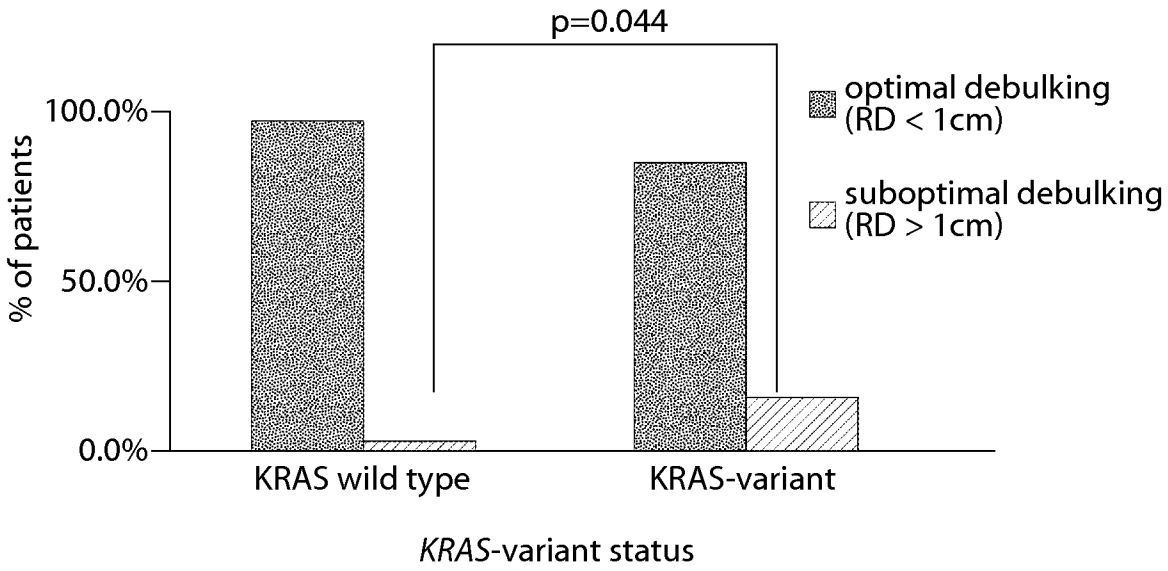

p=0.044
optimal debulking (RD < 1cm)
suboptimal debulking (RD > 1cm)
100.0%
50.0%
0.0%
% of patients
KRAS wild type
KRAS-variant
KRAS-variant status

Fig. 8

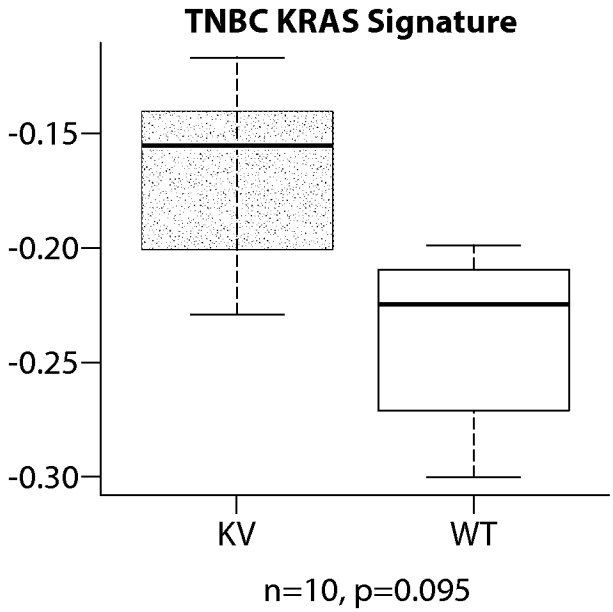


Fig. 9A

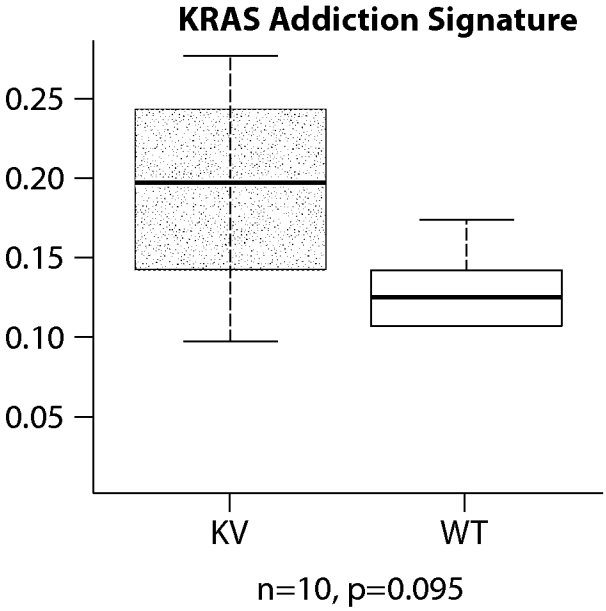


Fig. 9B

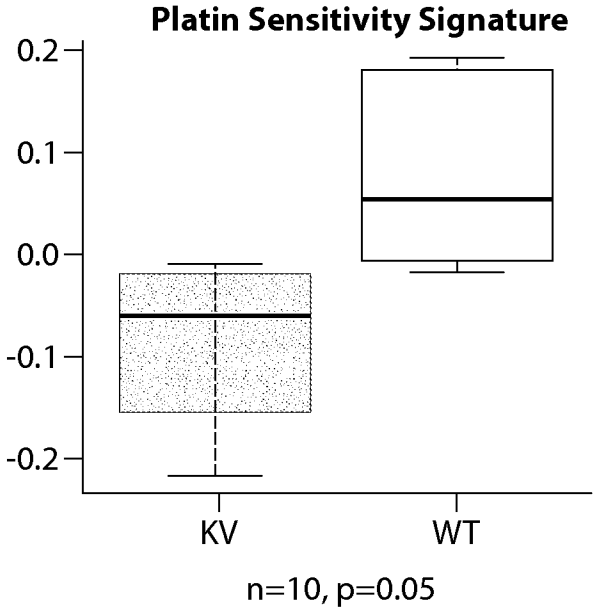


Fig. 9C

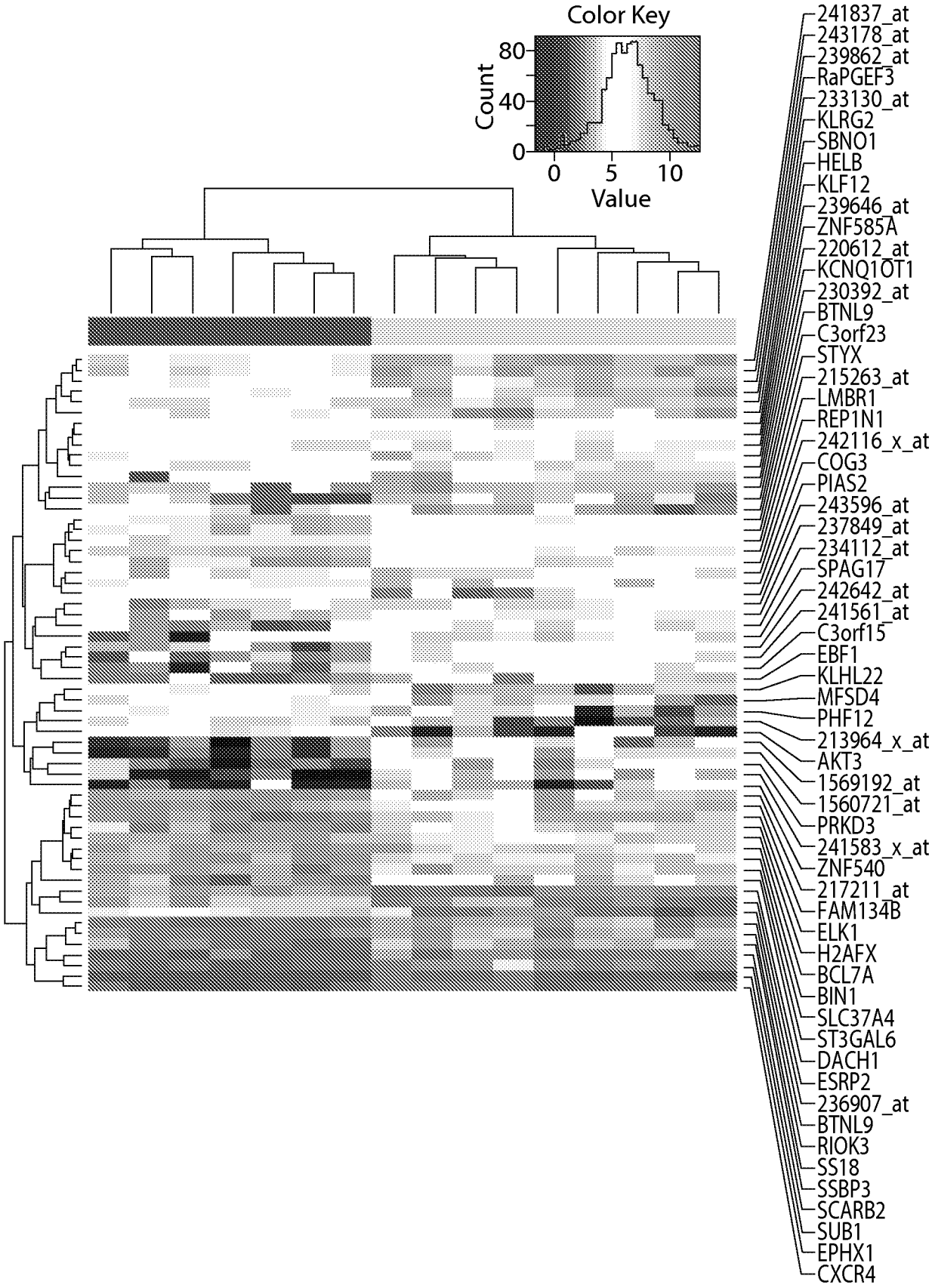

Color Key
Count
80
40
0
0
5
10
Value
241837_at
243178_at
239862_at
RaPGEF3
233130_at
KLRG2
SBNO1
HELB
KLF12
239646_at
ZNF585A
220612_at
KCNQ1OT1
230392_at
BTNL9
C3orf23
STYX
215263_at
LMBR1
REP1N1
242116_x_at
COG3
PIAS2
243596_at
237849_at
234112_at
SPAG17
242642_at
241561_at
C3orf15
EBF1
KLHL22
MFSD4
PHF12
213964_x_at
AKT3
1569192_at
1560721_at
PRKD3
241583_x_at
ZNF540
217211_at
FAM134B
ELK1
H2AFX
BCL7A
BIN1
SLC37A4
ST3GAL6
DACH1
ESRP2
236907_at
BTNL9
RIOK3
SS18
SSBP3
SCARB2
SUB1
EPHX1
CXCR4

Fig. 9D

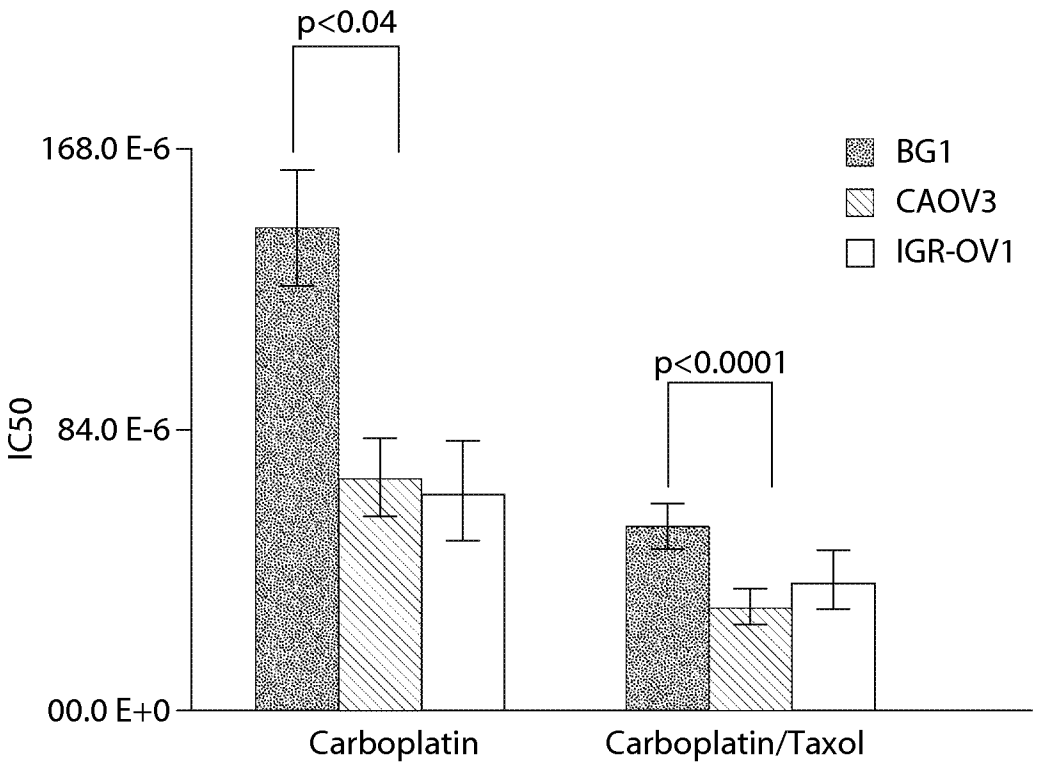

p<0.04
168.0 E-6
84.0 E-6
00.0 E+0
IC50
BG1
CAOV3
IGR-OV1
p<0.0001
Carboplatin
Carboplatin/Taxol

Fig. 10

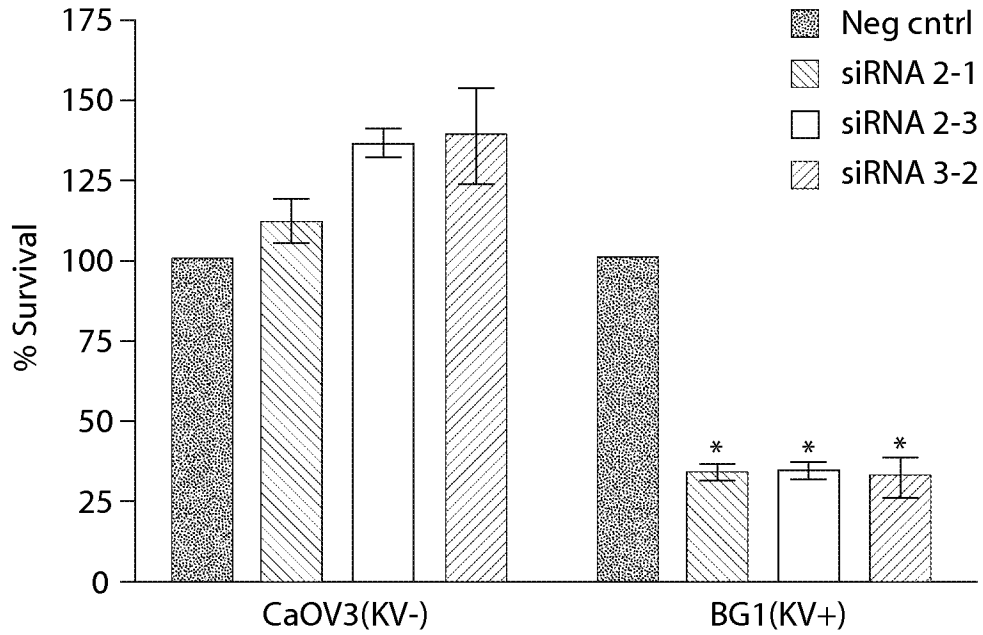


Fig. 11A

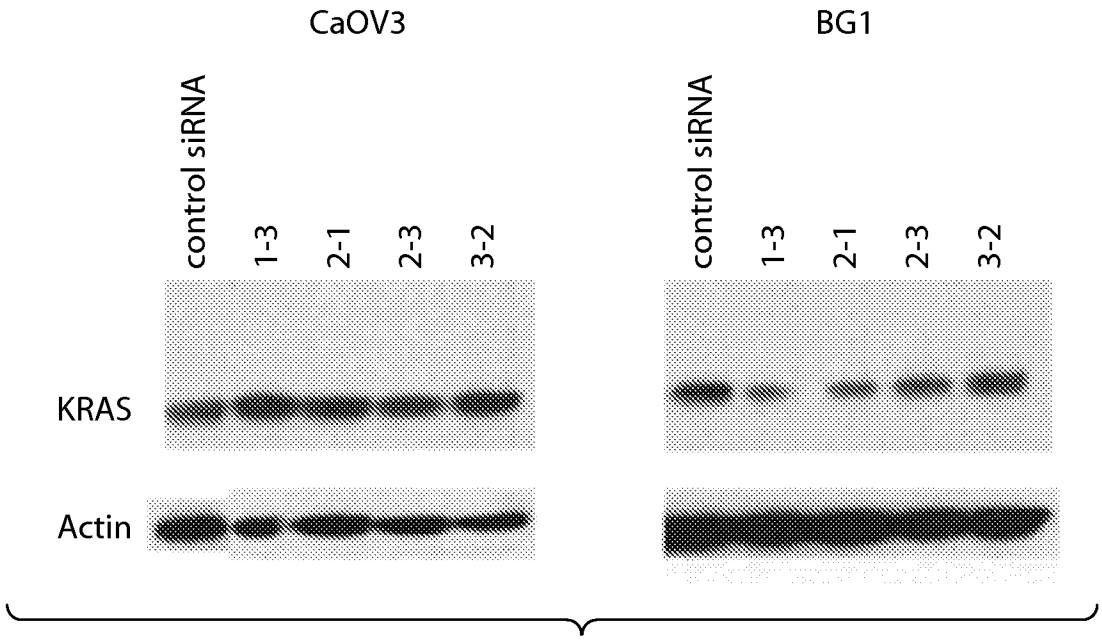
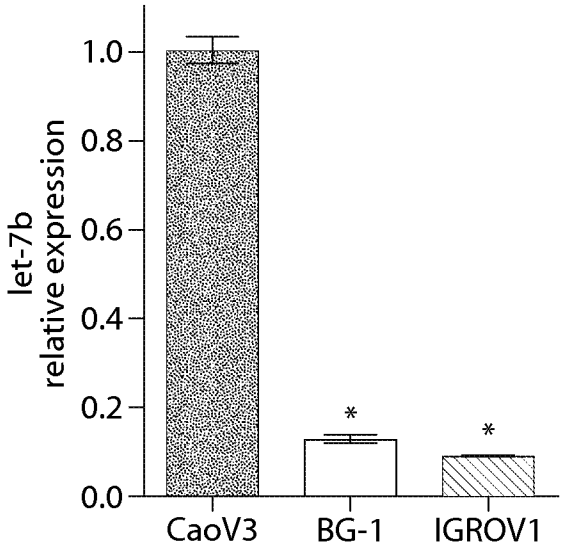


Fig. 11B



* P <0.001 vs. CaoV3

Fig. 12

KRAS non-variant sequence

5′ CCUGACCUCAAGUGAUUCACCCACCUUGGCCUCAUAAACCUG 3′

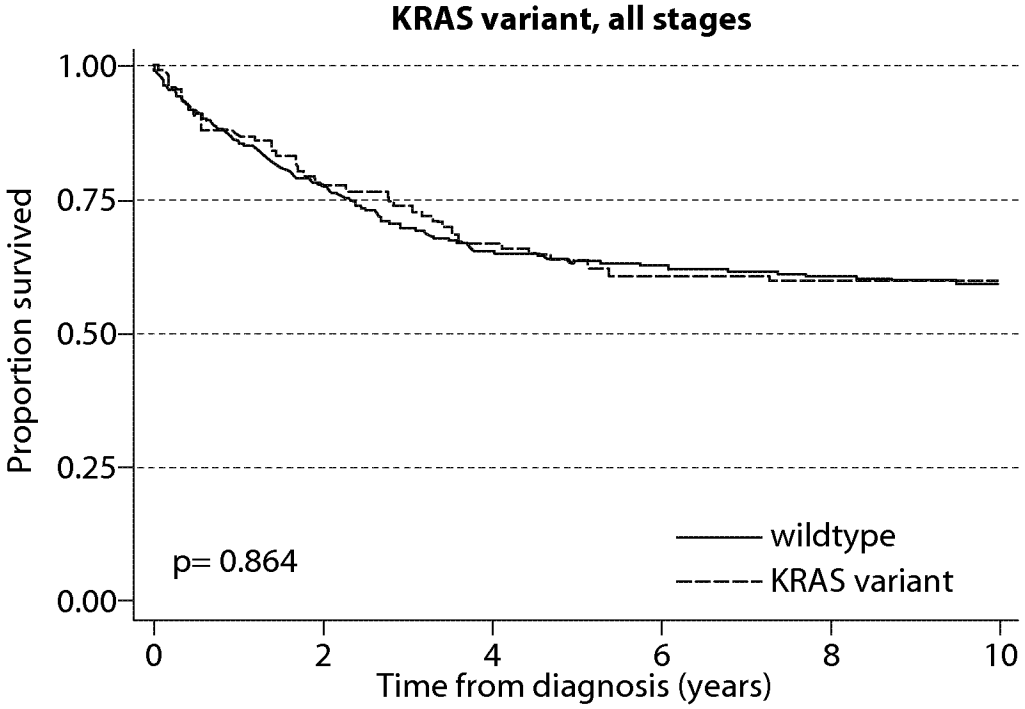
Fig. 13A

***KRAS*-variant sequence**

5′ CCUGACCUCAAGUGAUGCACCCACCUUGGCCUCAUAAACCUG 3′

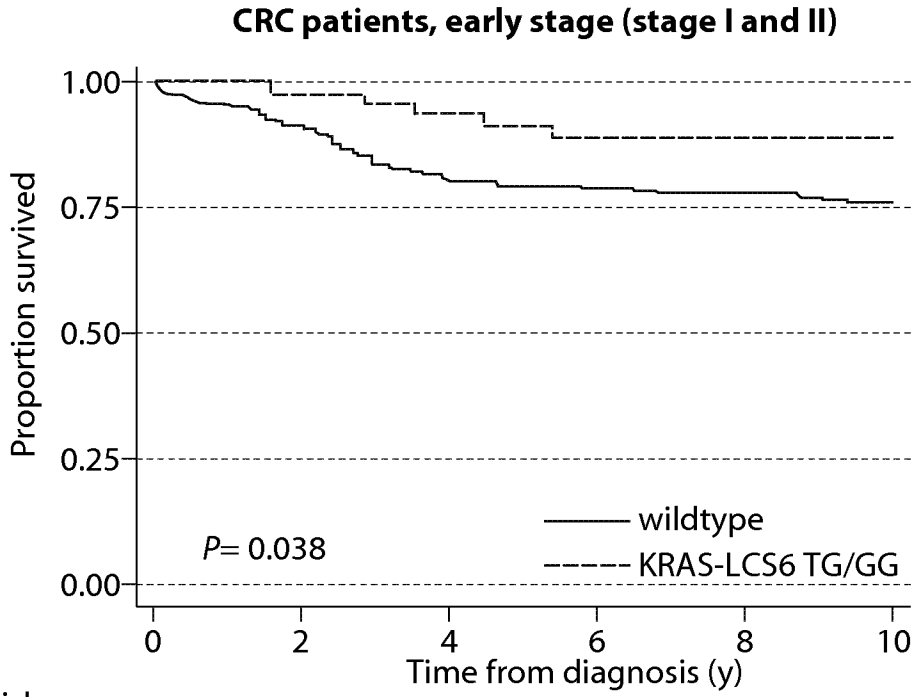
ggacuggaguucacuacgugg	1-3 variant targeting siRNA
gaggacuggaguucacuacgu	2-3 variant targeting siRNA
ggacuggaguucacuacgugu	1-2 variant targeting siRNA
ugaggacuggaguucacuacu	3-2 variant targeting siRNA

Fig. 13B



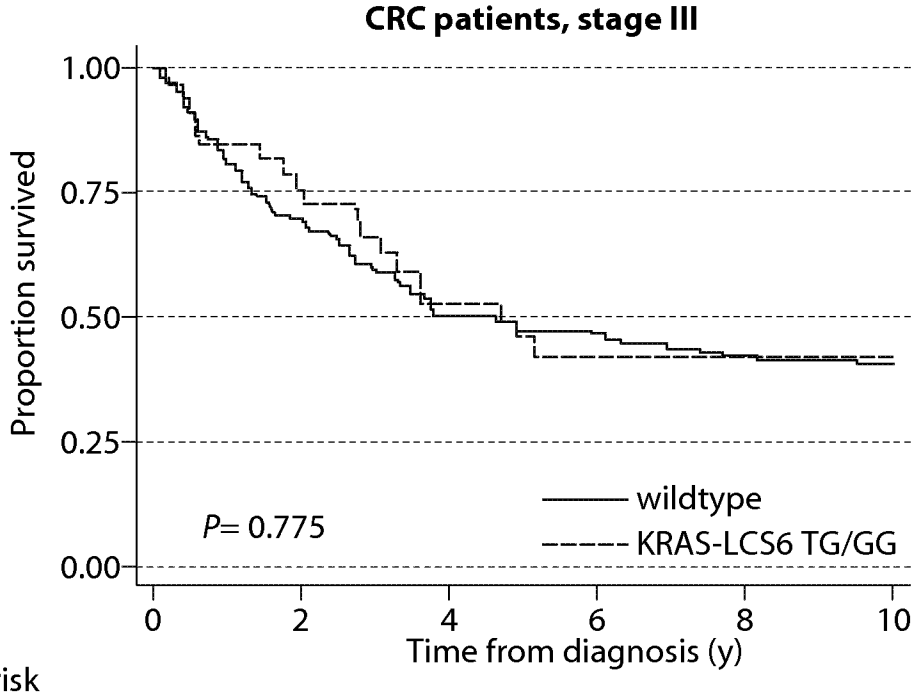
Number at risk						
KRAS wildtype	567	422	342	312	287	252
KRAS variant	111	83	67	55	52	51

Fig. 14



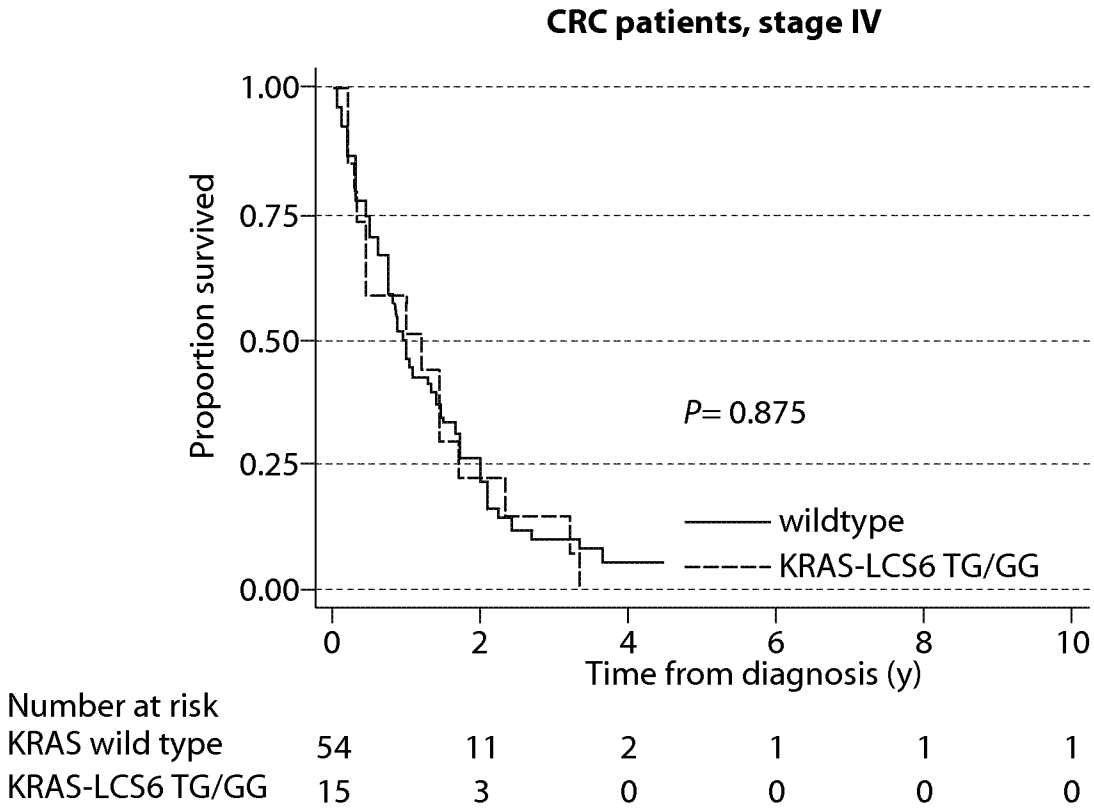
Number at risk						
KRAS wild type	326	287	246	231	217	191
KRAS-LCS6 TG/GG	53	49	46	38	37	37

Fig. 15A



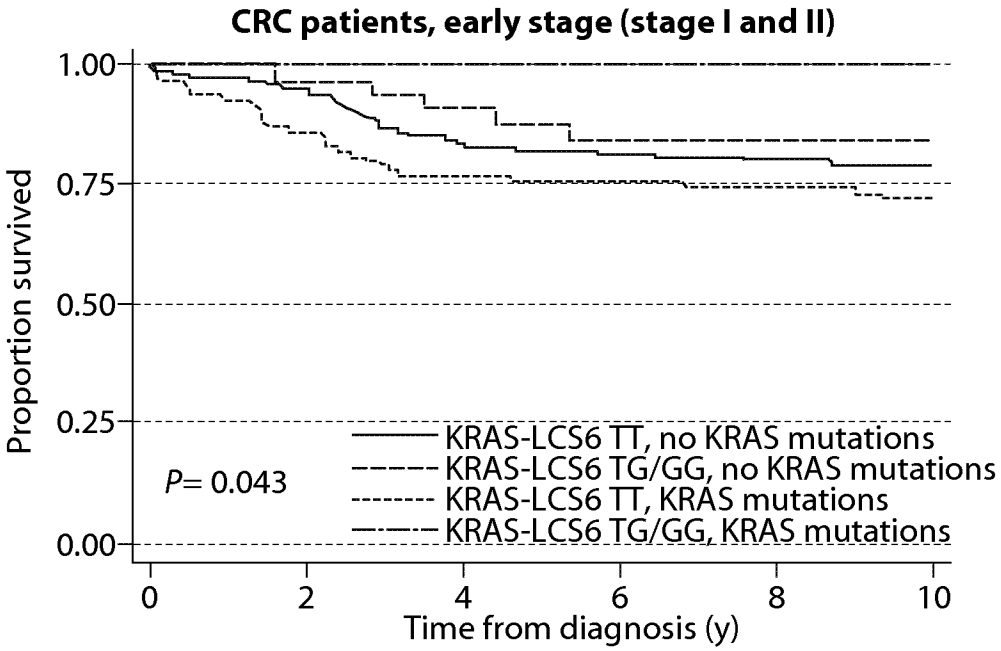
Number at risk						
KRAS wild type	137	90	65	56	49	44
KRAS-LCS6 TG/GG	33	24	15	12	11	10

Fig. 15B



Number at risk						
KRAS wild type	54	11	2	1	1	1
KRAS-LCS6 TG/GG	15	3	0	0	0	0

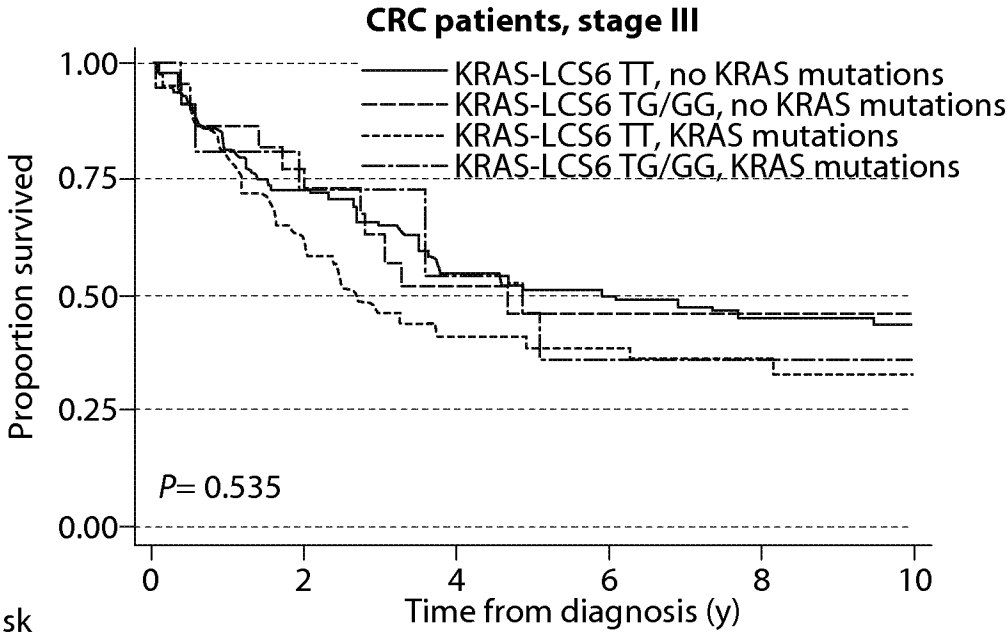
Fig. 15C



Number at risk

TT, no KRAS mutations	211	191	162	152	143	126
TG/GG, no KRAS mutations	33	31	29	24	23	23
TT, KRAS mutations	115	96	84	79	74	65
TG/GG, KRAS mutations	20	18	17	14	14	14

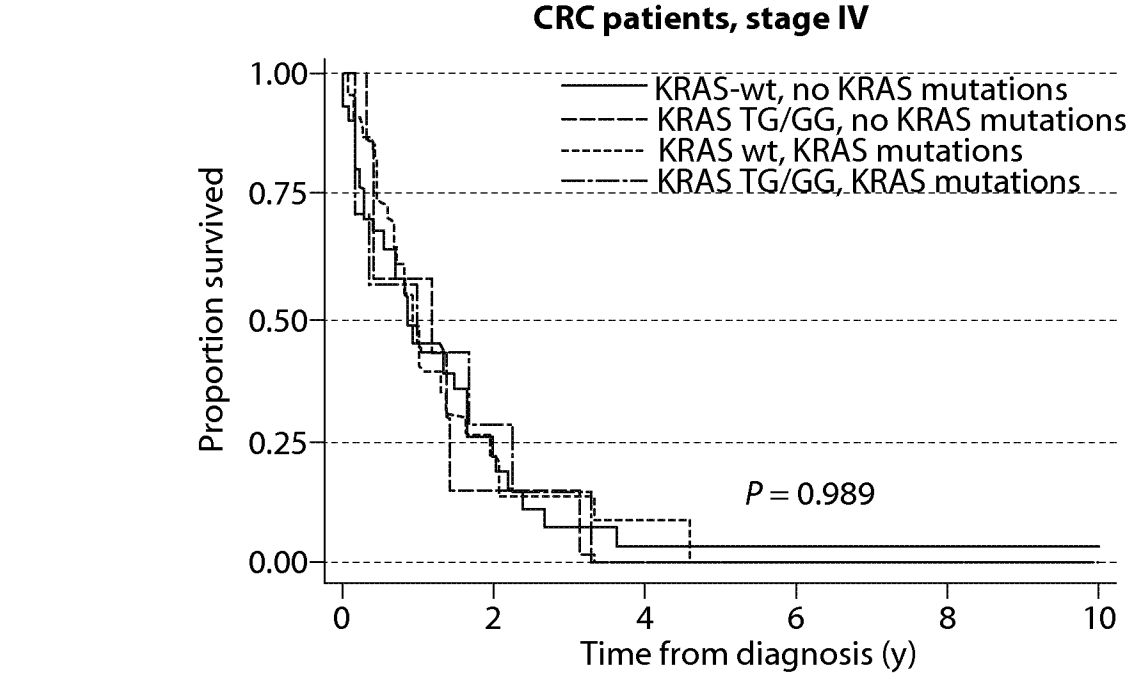
Fig. 16A



Number at risk

TT, no KRAS mutations	93	65	48	41	36	32
TG/GG, no KRAS mutations	22	16	9	8	8	7
TT, KRAS mutations	44	25	17	15	13	12
TG/GG, KRAS mutations	11	8	6	4	3	3

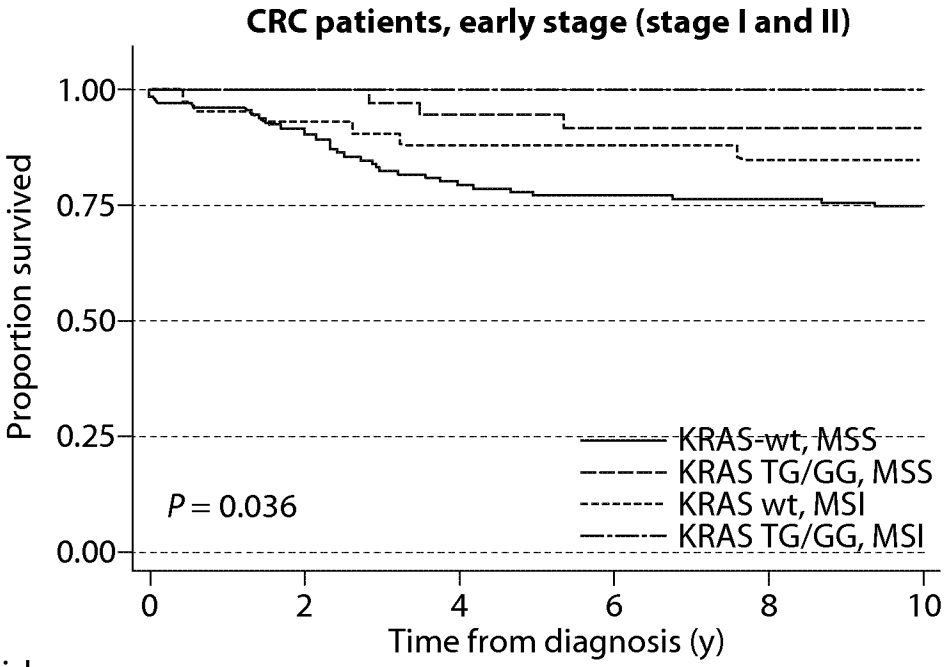
Fig. 16B



Number at risk

KRAS-wt, no KRAS mutations	31	6	1	1	1	1
KRAS TG/GG, no KRAS mutations	8	1	0	0	0	0
KRAS wt, KRAS mutations	23	5	1	0	0	0
KRAS TG/GG, KRAS mutations	2	2	0	0	0	0

Fig. 16C



Number at risk						
KRAS-wt, MSS	258	229	194	181	172	155
KRAS TG, MSS	40	38	35	30	30	30
KRAS TT/GG,	45	40	35	34	29	23
KRAS TG/GG, MSI	9	8	8	7	6	6

Fig. 17

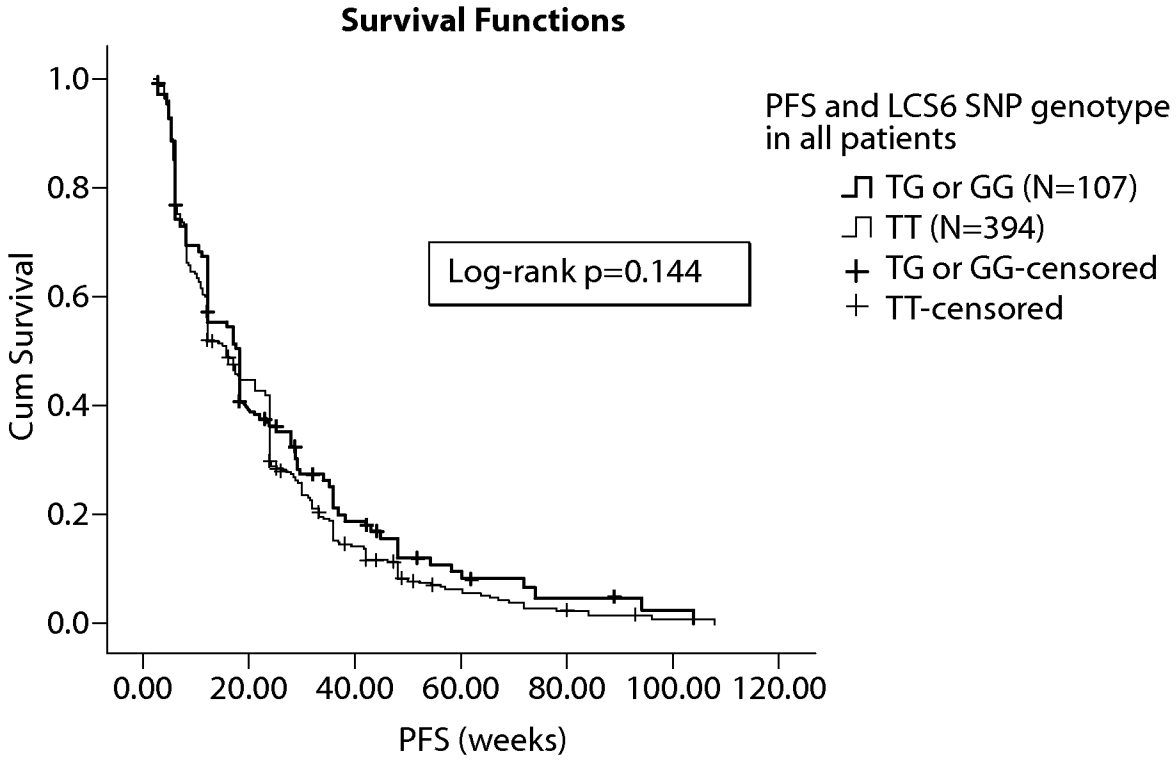


Fig. 18A

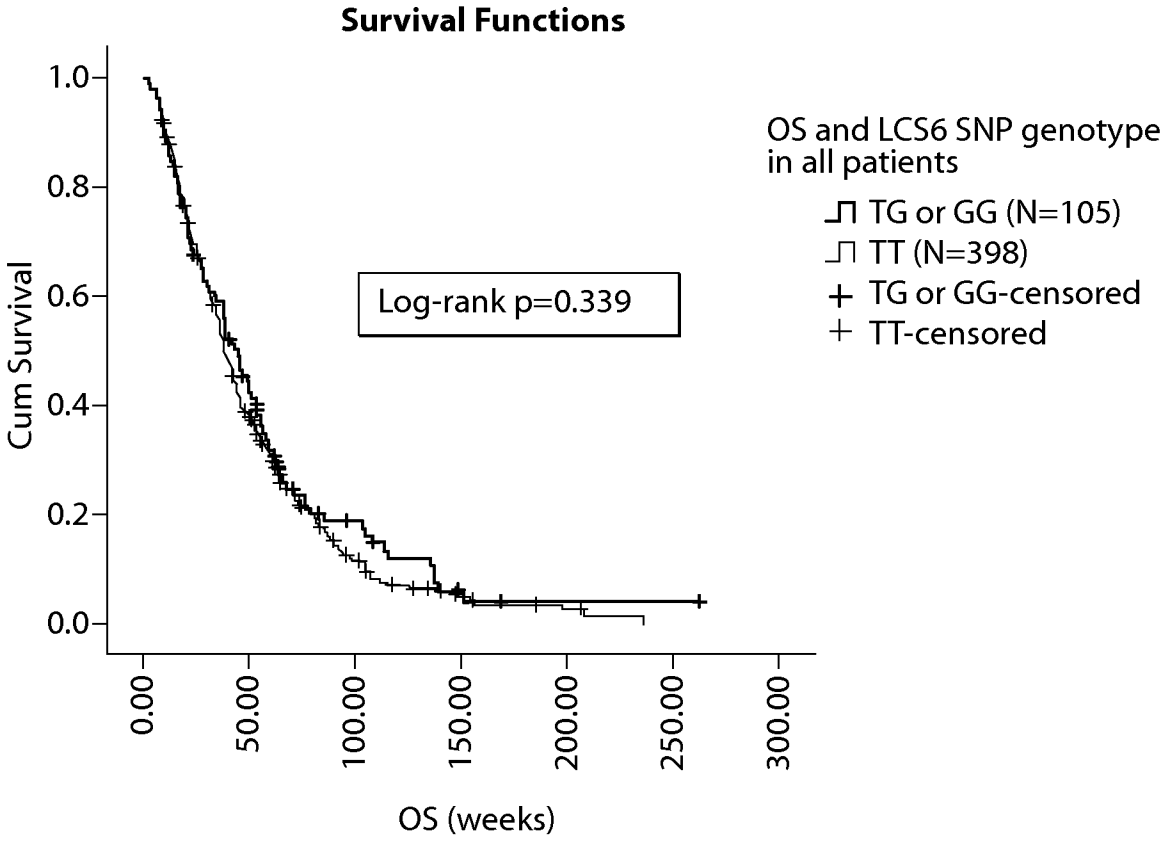


Fig. 18B

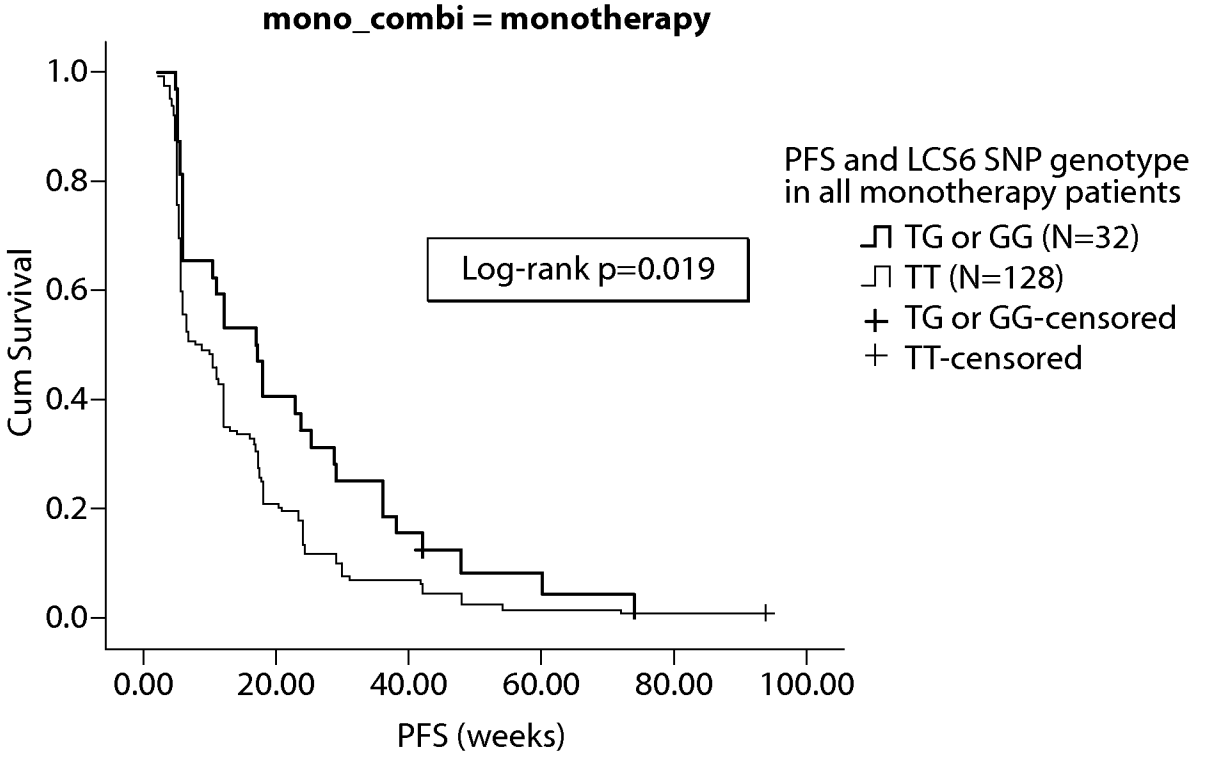


Fig. 19A

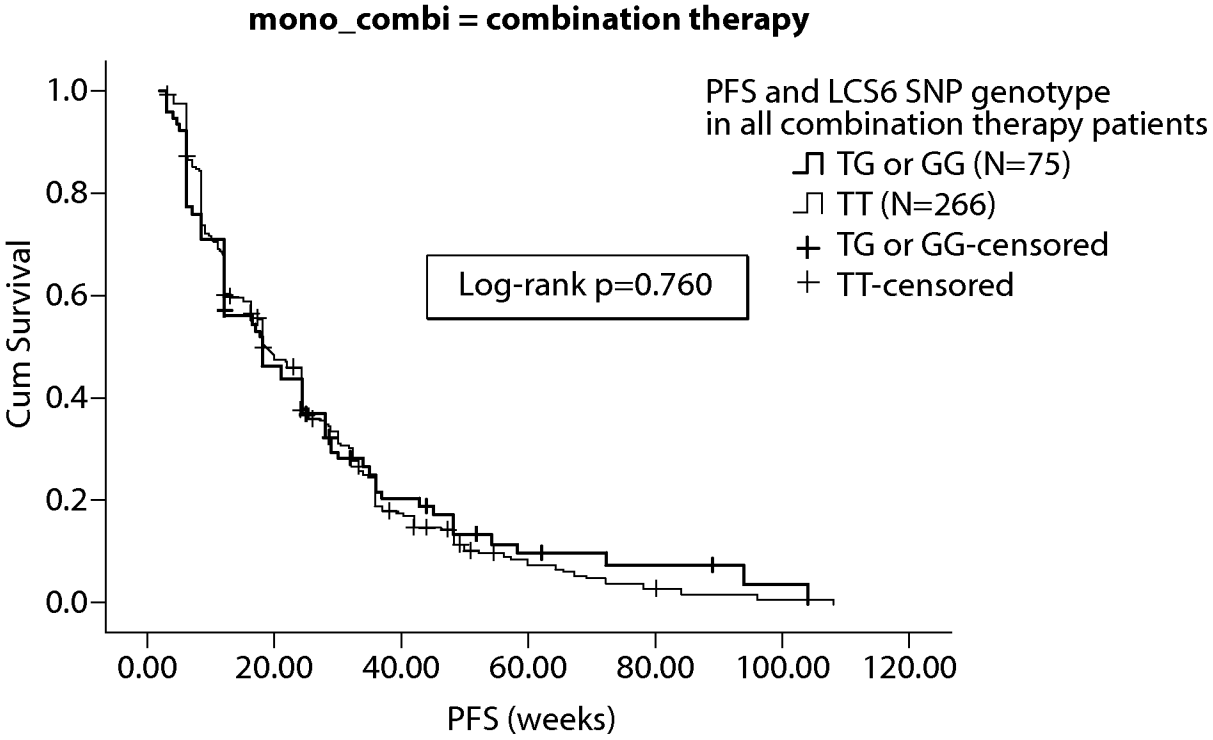


Fig. 19B

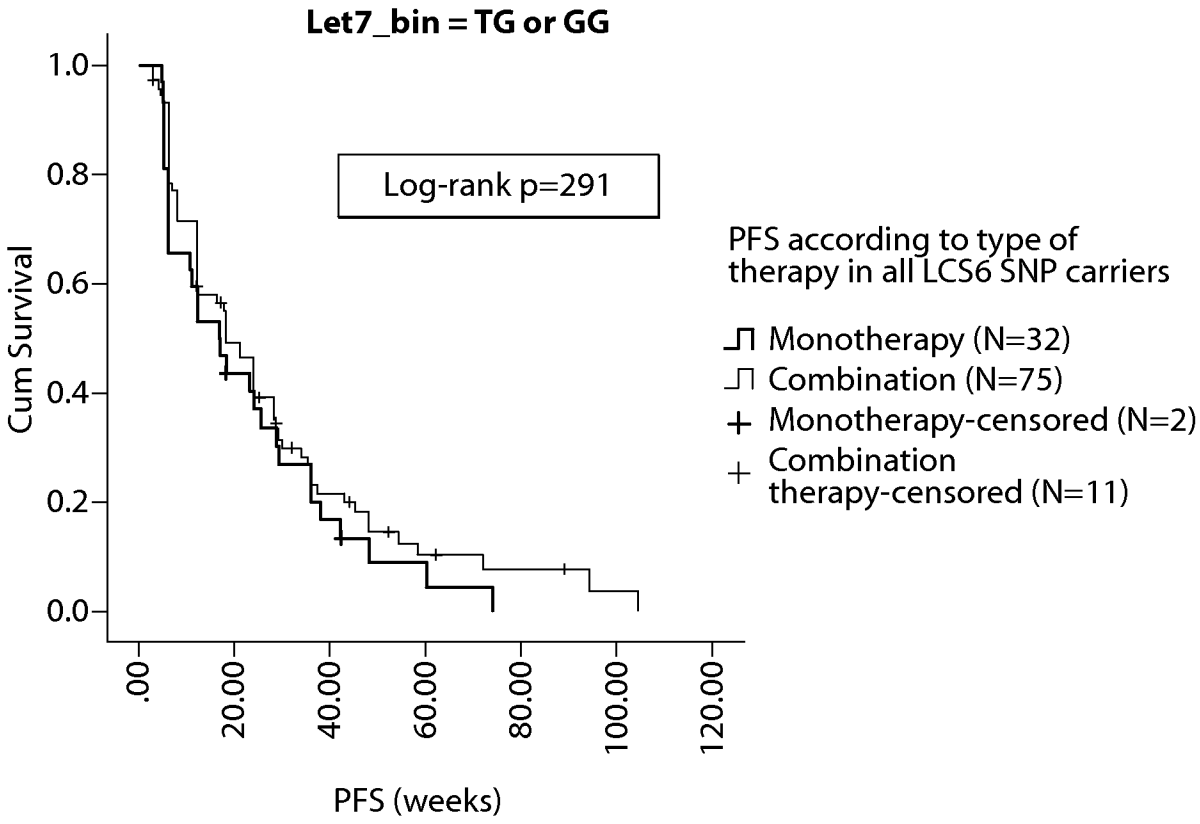


Fig. 19C

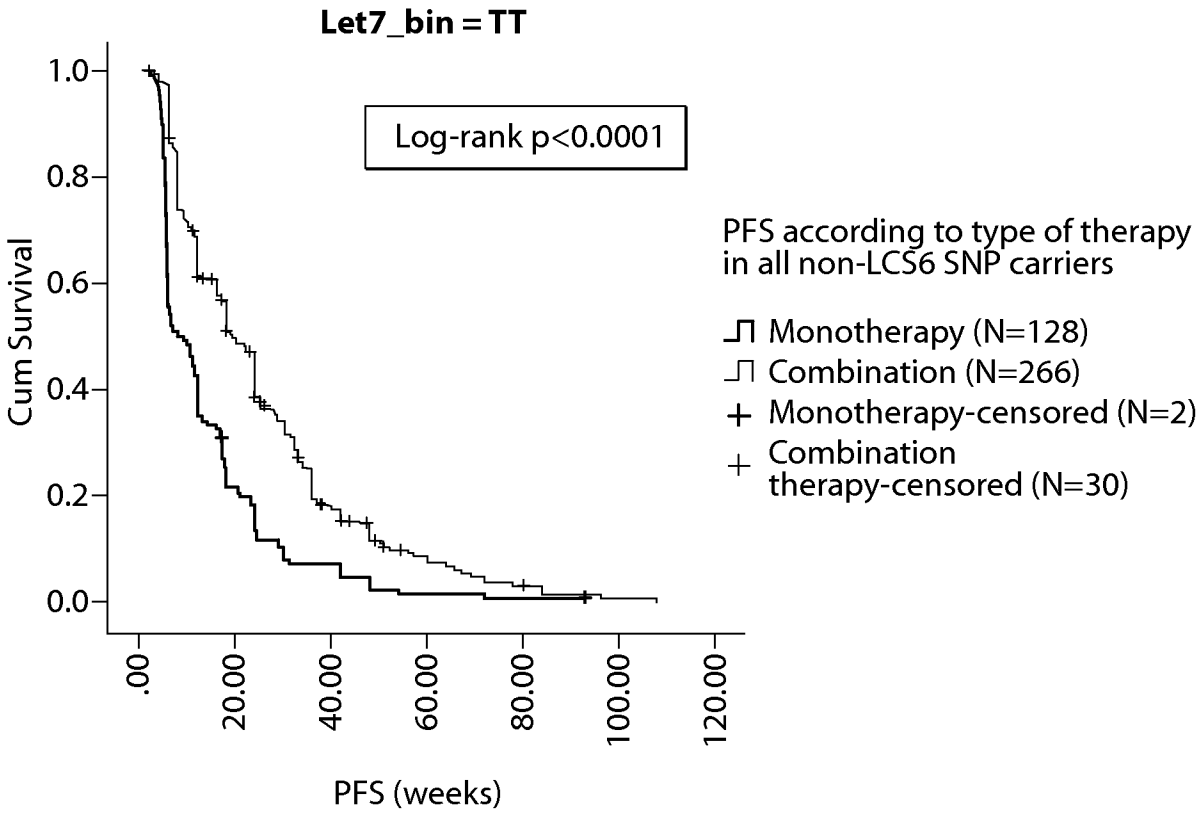


Fig. 19D

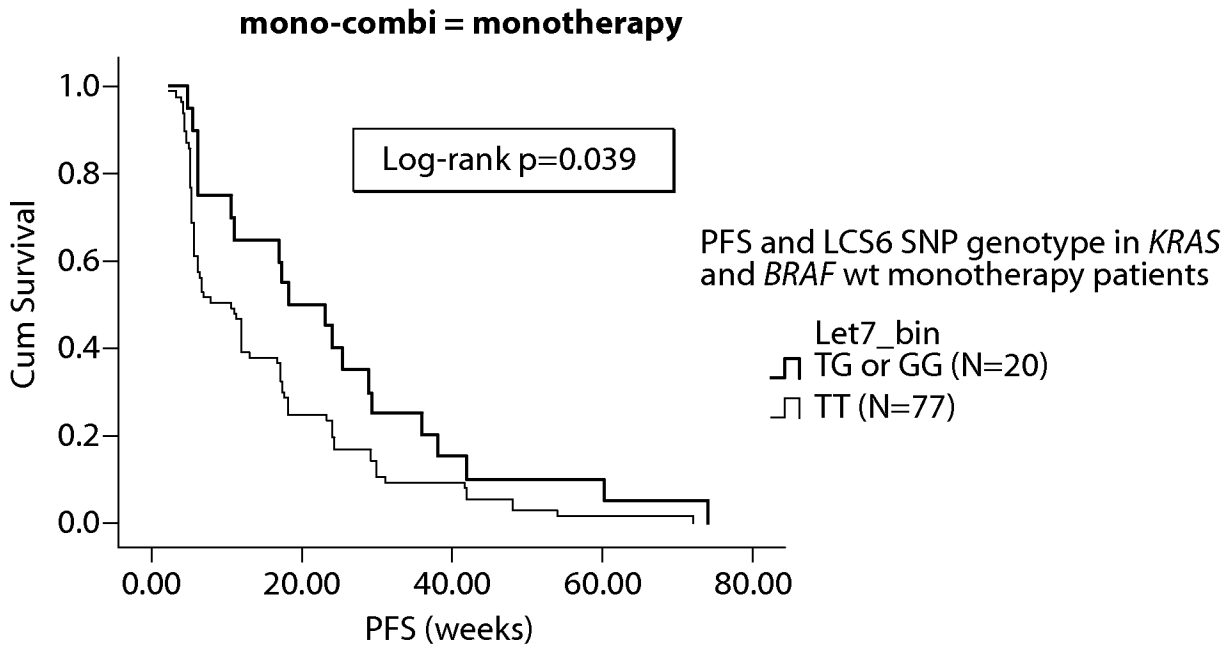


Fig. 20A

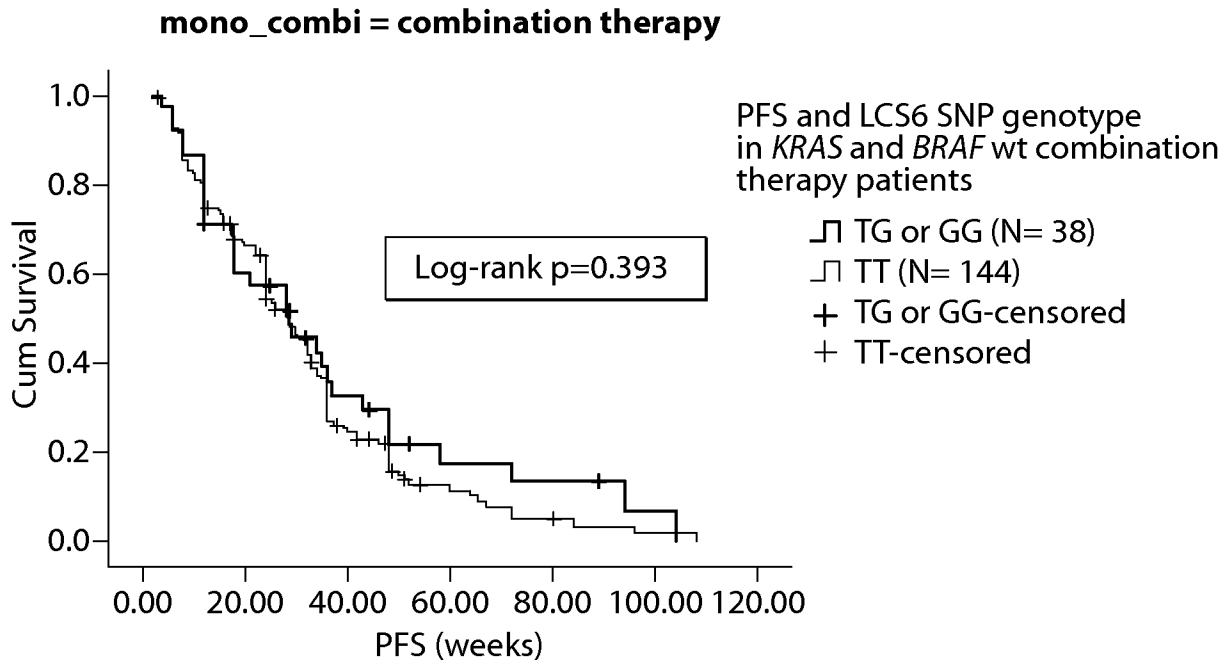


Fig. 20B

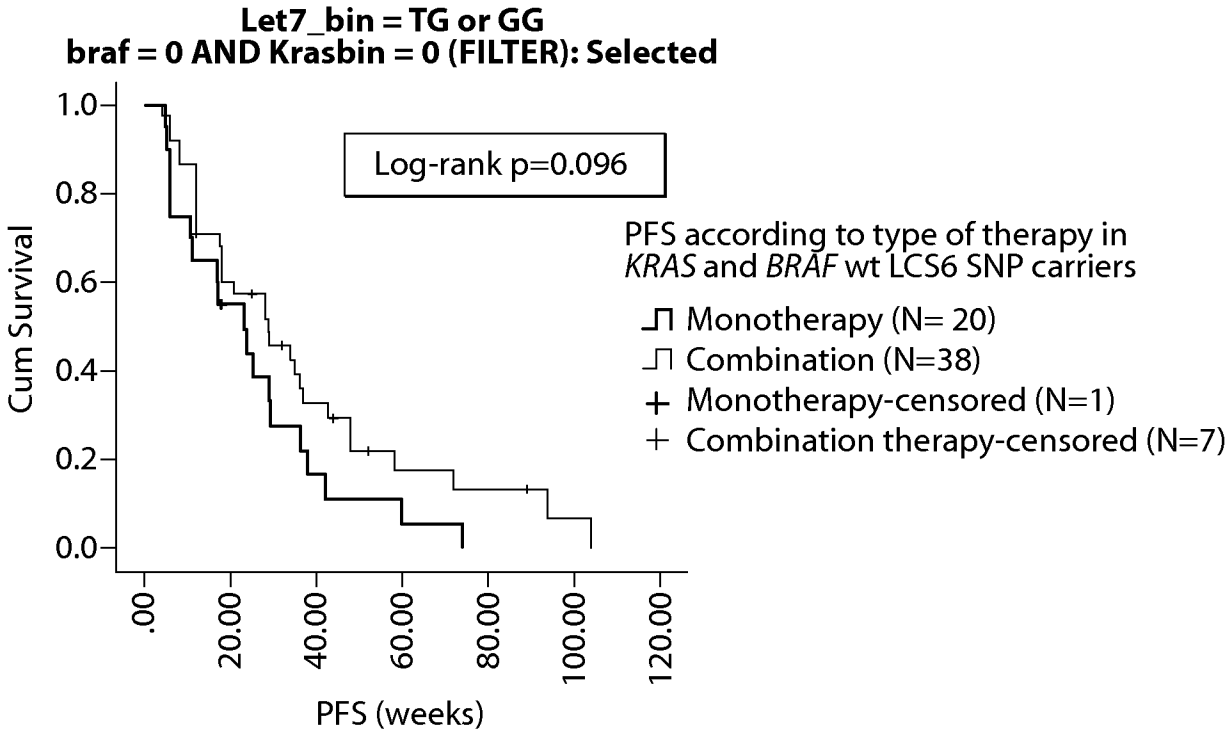


Fig. 20C

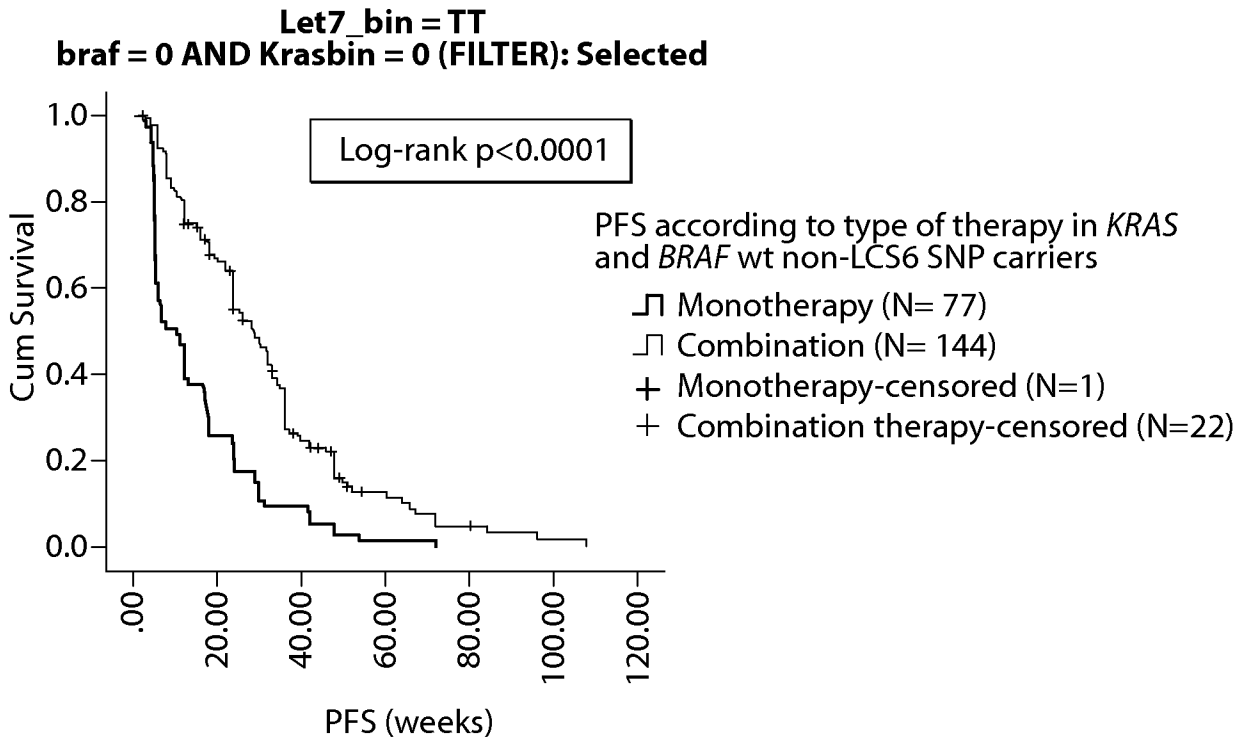


Fig. 20D

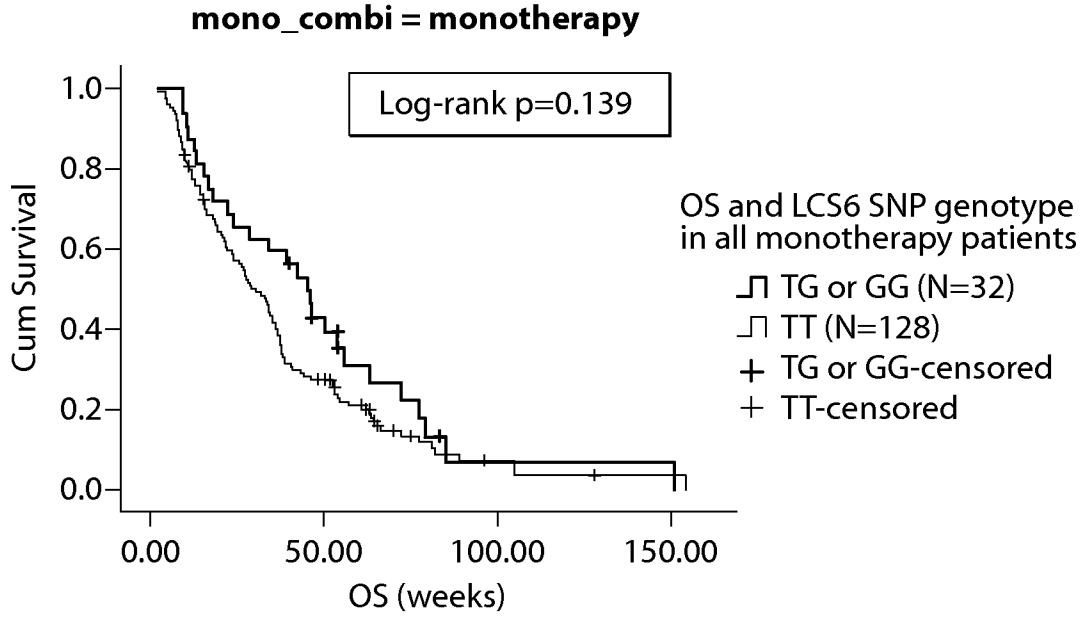


Fig. 21A

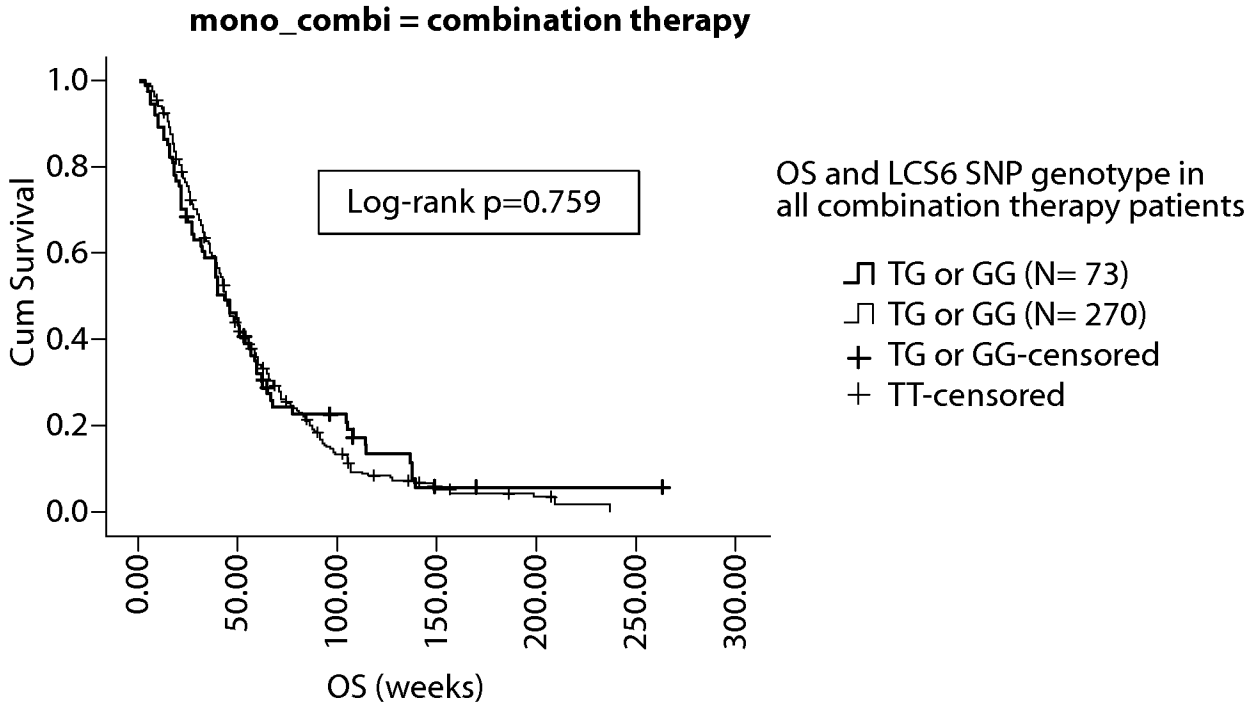


Fig. 21B

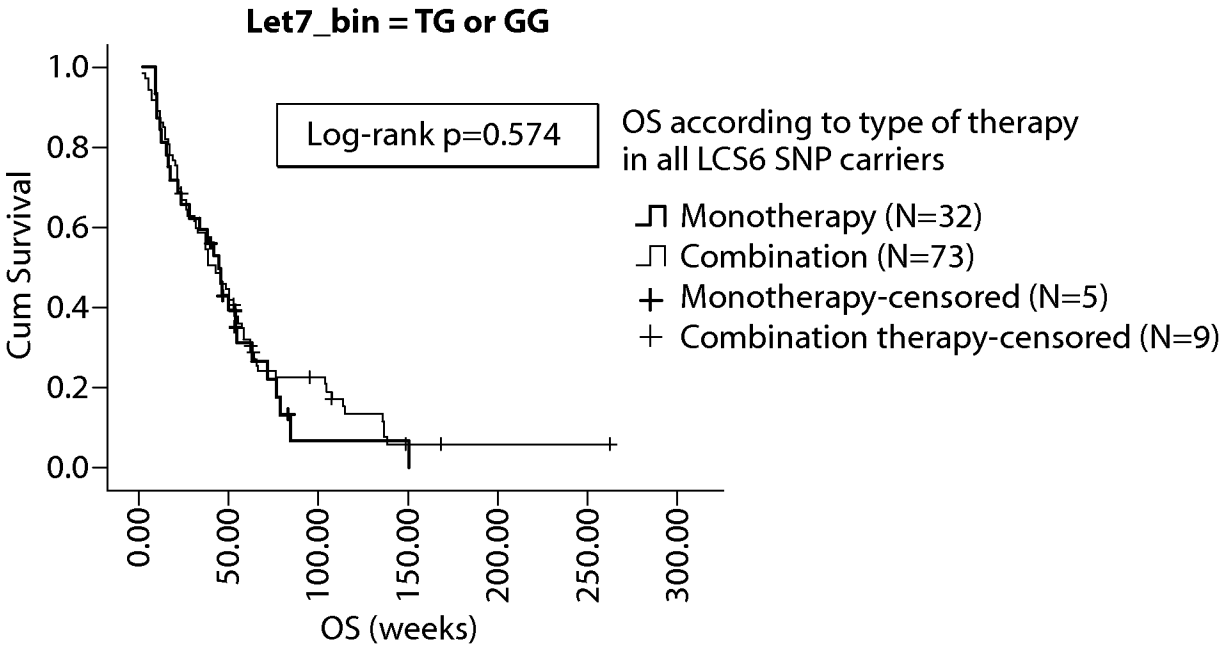


Fig. 21C

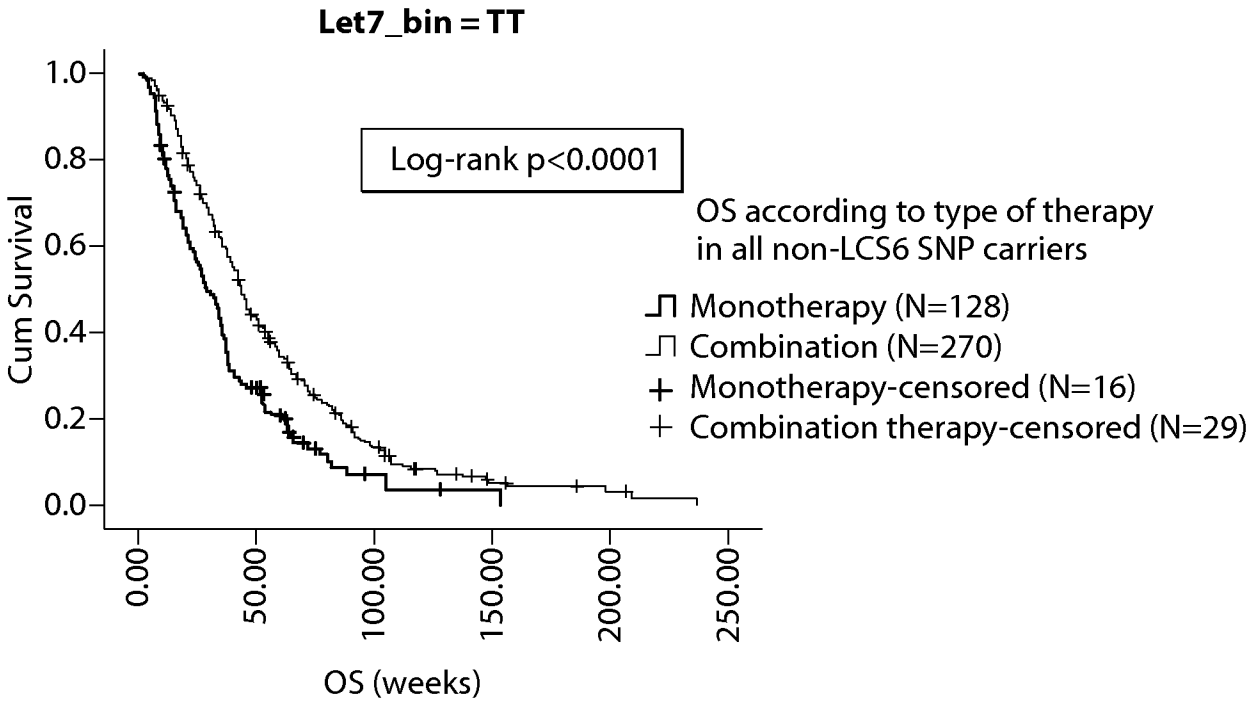


Fig. 21D

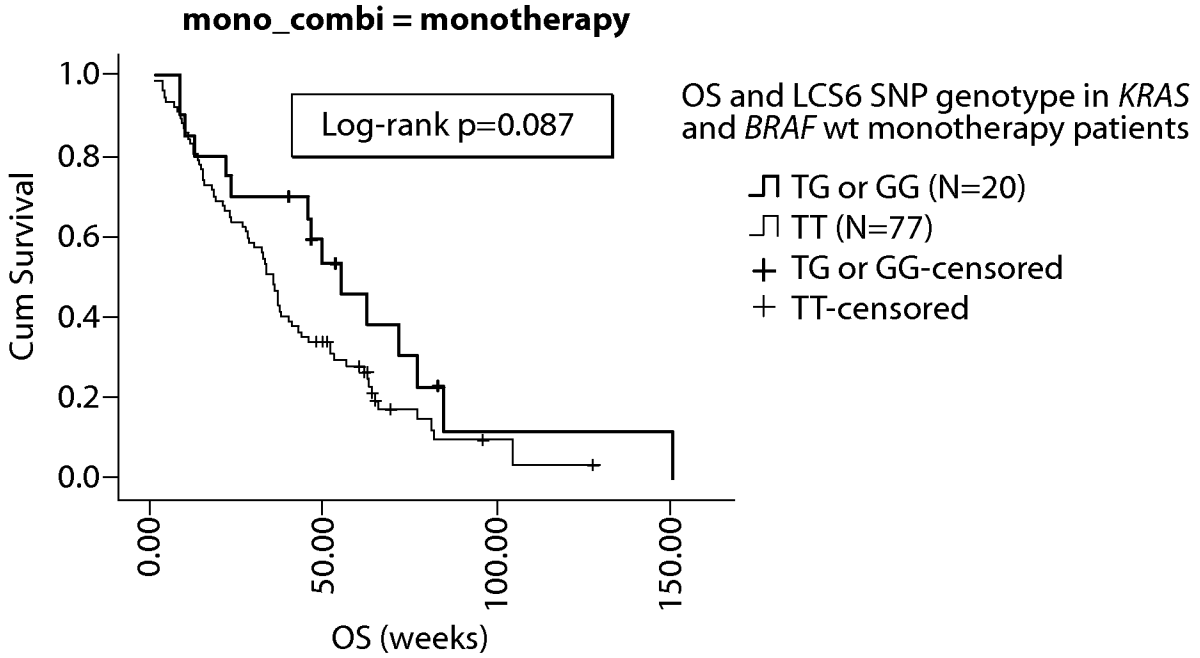


Fig. 22A

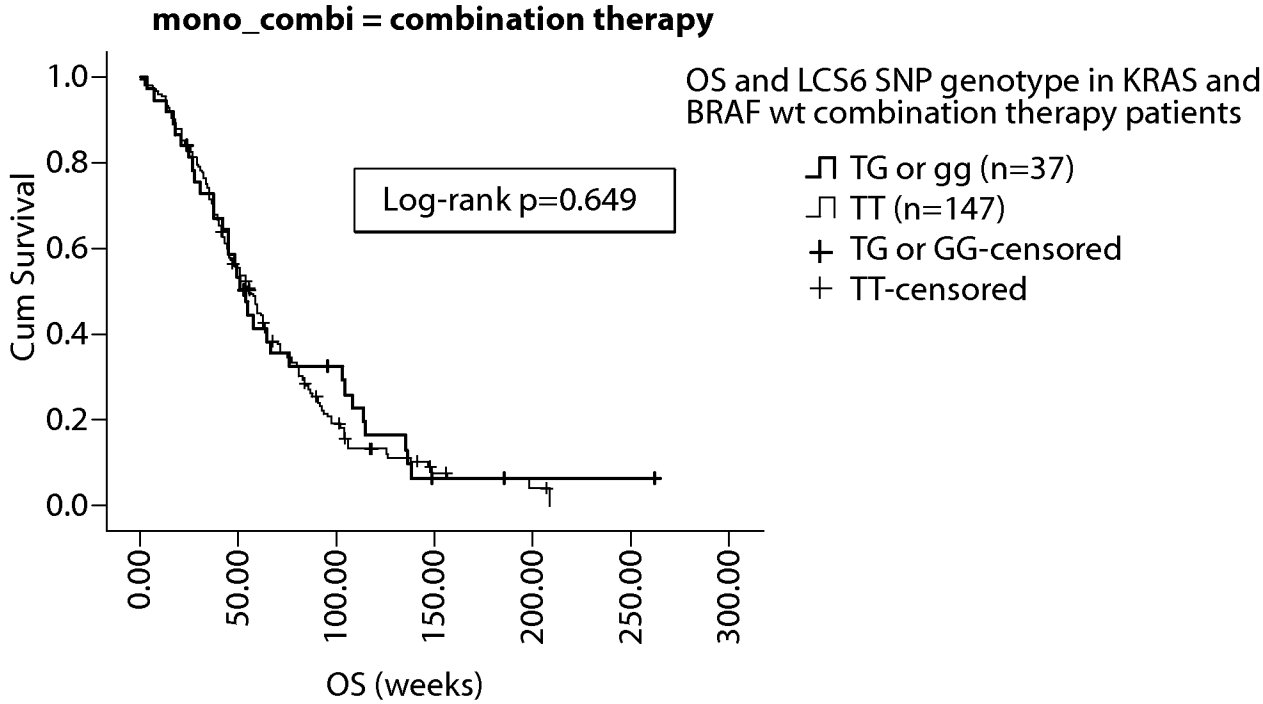


Fig. 22B

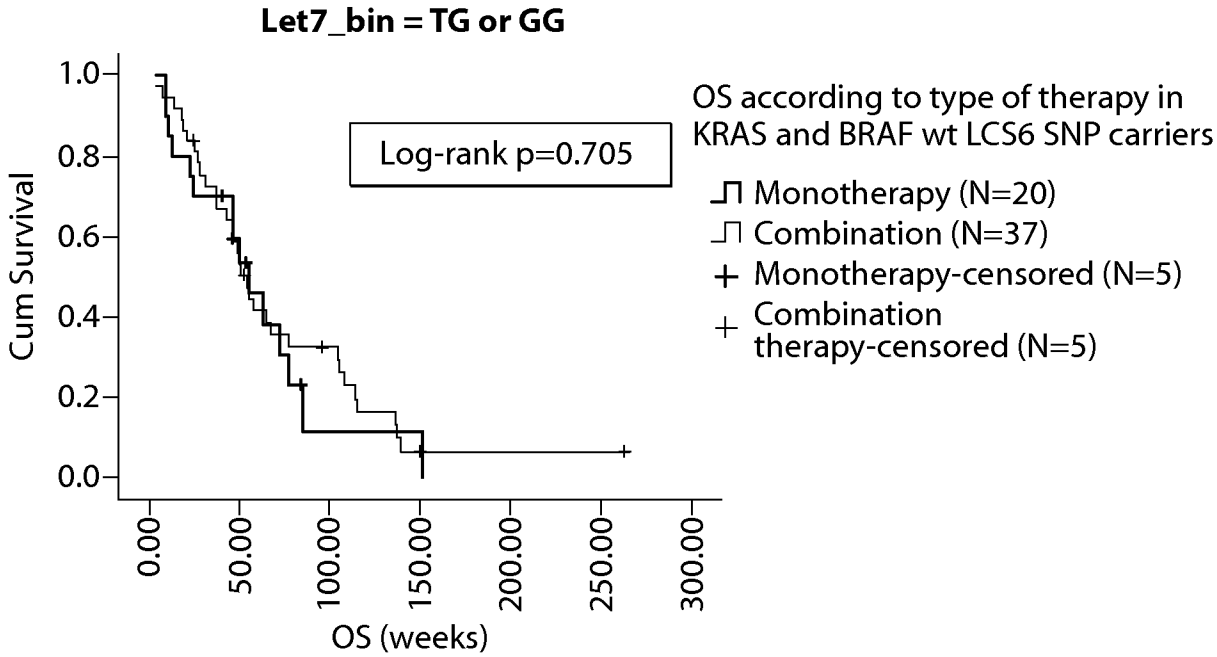


Fig. 22C

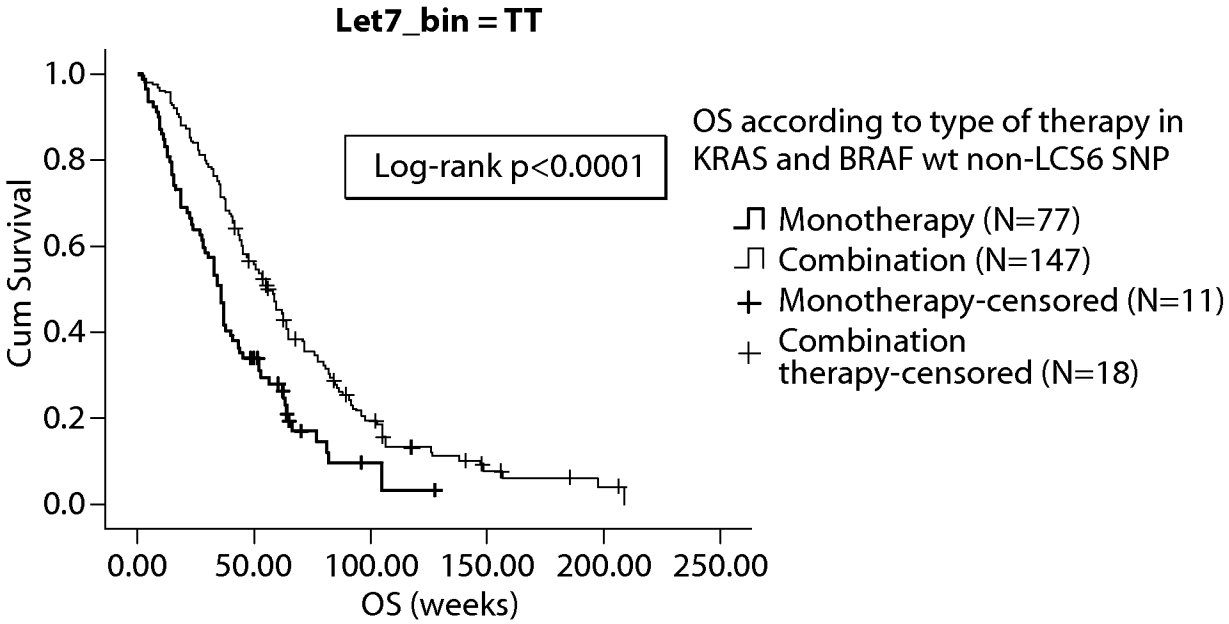


Fig. 22D

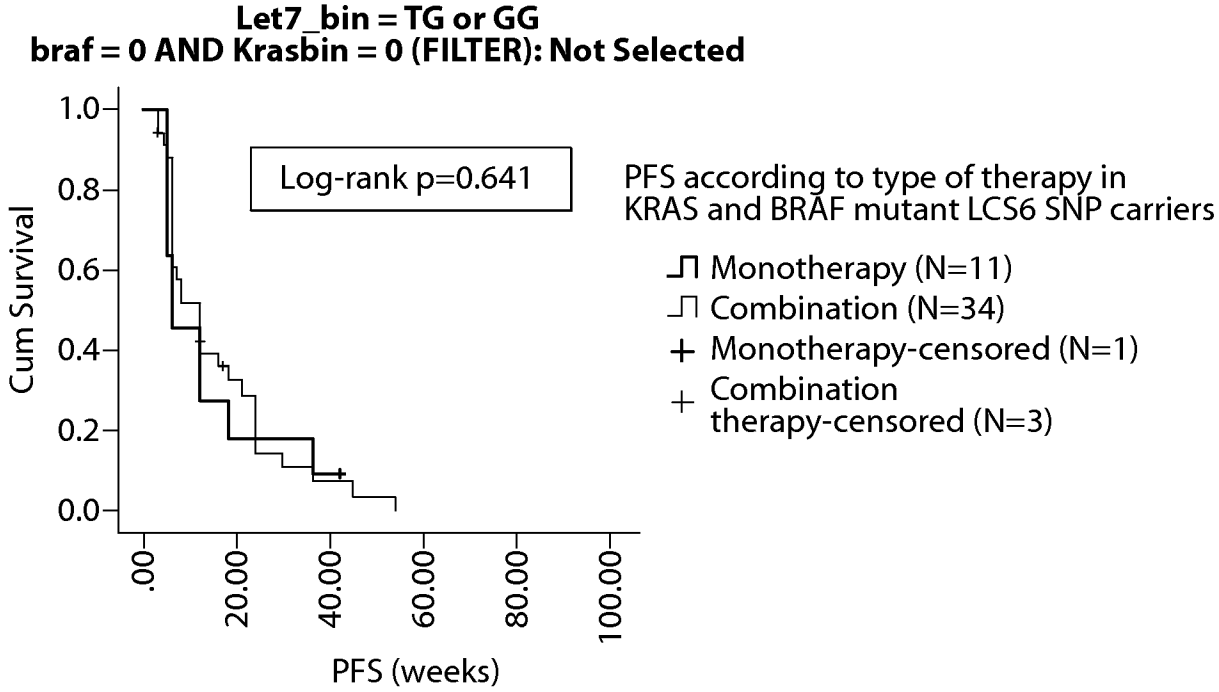


Fig. 23A

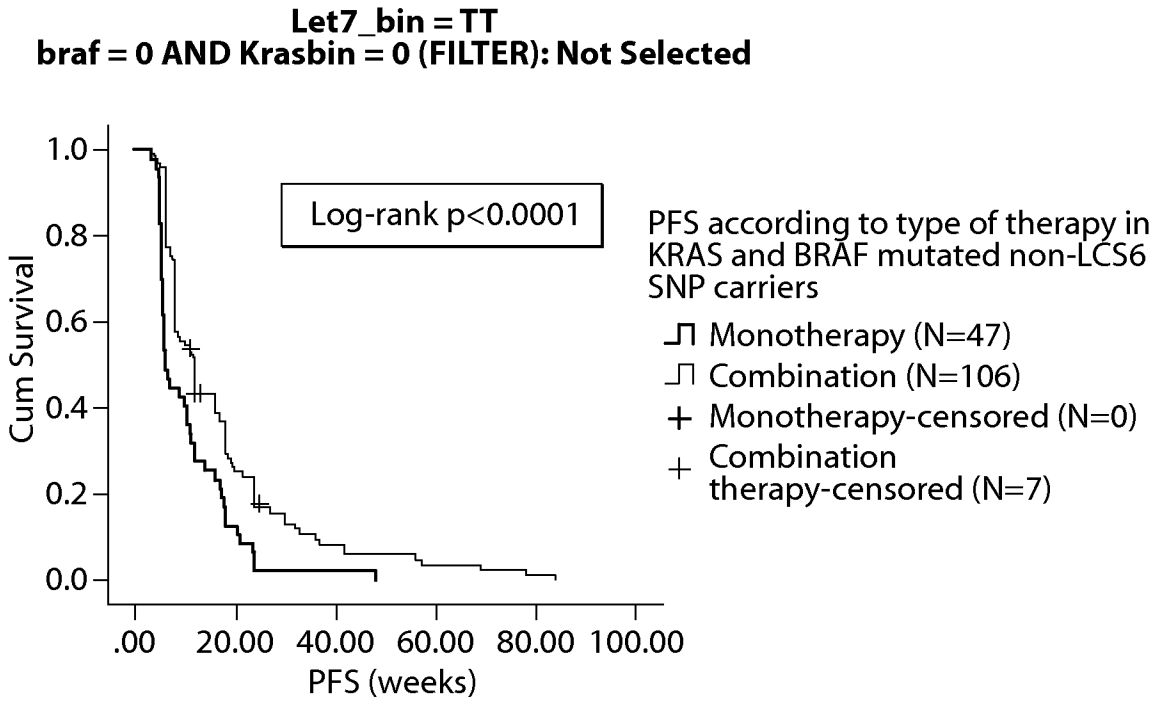


Fig. 23B

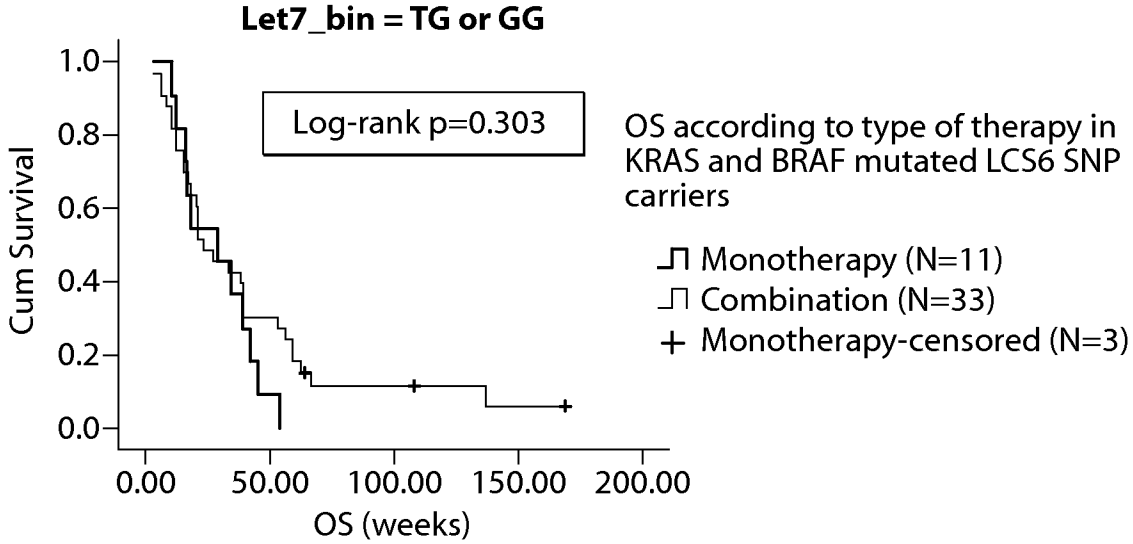


Fig. 23C

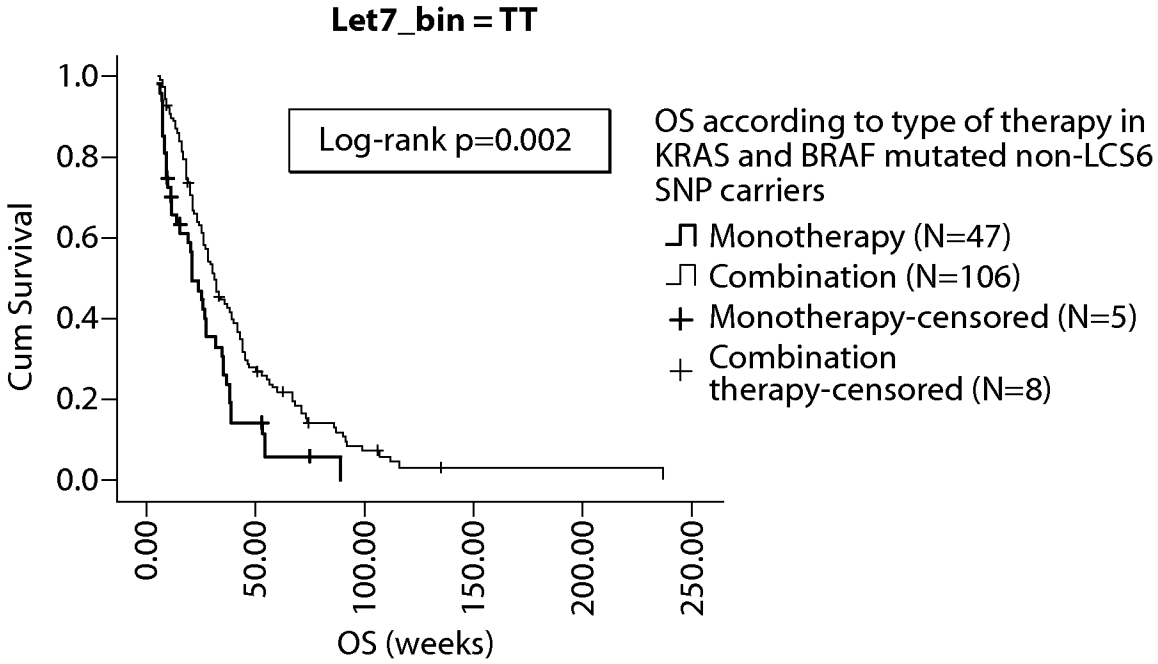


Fig. 23D

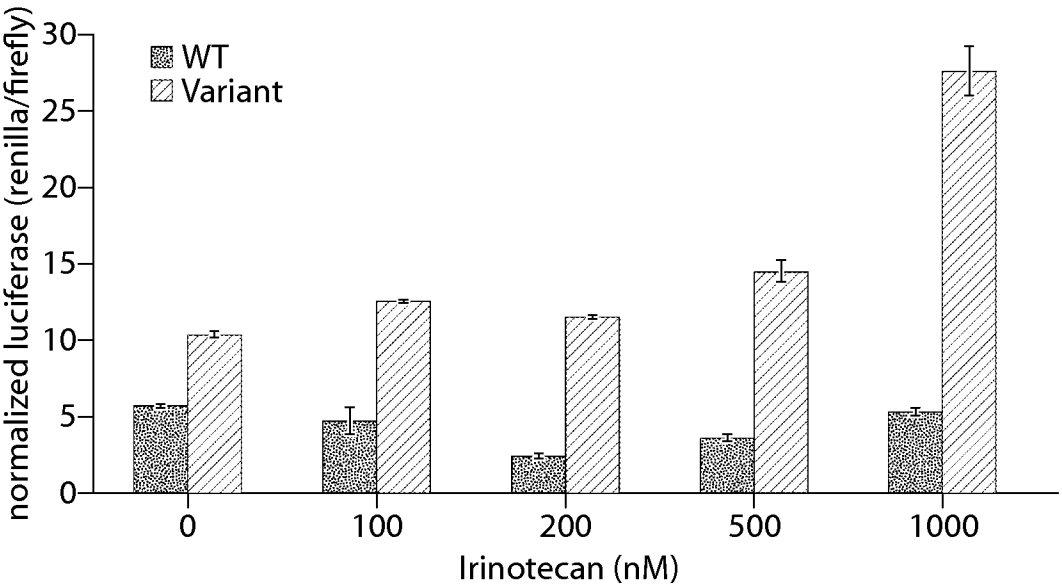


Fig. 24A

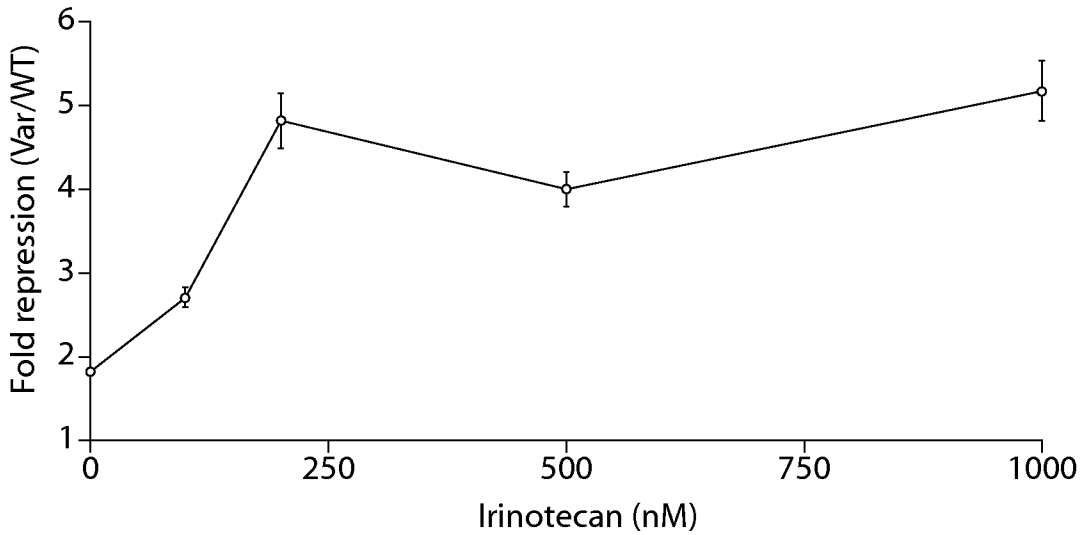


Fig. 24B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/030019

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARANJAPE TRUPTI S ET AL: "A KRAS microRNA binding site polymorphism as a novel biomarker of risk in triple negative breast cancer", PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 51, April 2010 (2010-04), pages 739-740, XP008152926, & 101ST ANNUAL MEETING OF THE AMERICAN-ASSOCIATION-FOR-CANCER-RESEARCH; WASHINGTON, DC, USA; APRIL 17 -21, 2010 ISSN: 0197-016X the whole document	1-3,7-11

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 June 2012	04/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Botz, Jürgen

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/151004 A1 (UNIV YALE [US]; SLACK FRANK J [US]; WEIDHAAS JOANNE B [US]; CHIN LENA) 11 December 2008 (2008-12-11) paragraphs [0007], [0008], [0097]; example 10	1-3,7-11
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/030019

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