



(51) International Patent Classification:

A61K 39/395 (2006.01) C12Q 1/686 (2018.01)

A61P 35/00 (2006.01) G01N 33/53 (2006.01)

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(21) International Application Number:

PCT/IL2018/050367

(22) International Filing Date:

29 March 2018 (29.03.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/478,647 30 March 2017 (30.03.2017) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: METHODS FOR TREATING MELANOMA

(57) Abstract: The present invention discloses methods for improved detection of melanoma, leading to optimization of melanoma treatment regimen, and methods for treating melanoma.



WO 2018/178989 A1

METHODS FOR TREATING MELANOMA

FIELD OF THE INVENTION

The present invention discloses methods for improved detection of melanoma,
5 optimization of melanoma treatment regimens and methods for treating melanoma.

BACKGROUND OF THE INVENTION

The incidence of cutaneous melanoma continues to rise throughout the globe. Despite
major advancements in its treatment in recent years, the disease continues to pose significant
10 clinical challenges and, in loco-regional and advanced stages, often leads to demise. The
prognosis for stage III melanoma (namely, involvement of regional lymph nodes without
distant metastasis) remains poor, with 5-year-survival of approximately 50%, and the
prognosis for distant metastatic disease is even worse (5-year-survival of less than 20%).
Ulceration of the primary tumor has been long known to be a negative prognostic factor, and
15 ulceration status also serves as a predictive factor for response to adjuvant therapy with
interferon-alpha in stage III patients.

Immune checkpoint inhibitors – namely, monoclonal antibodies that increase cancer
immunogenicity by decreasing inhibitory signals between cancerous cells and lymphocytes –
were shown to have significant activity in metastatic melanoma (Jessurun et al., Front.
20 Oncol., 7 (article 233): 1-12, 2017). One such agent – the anti-CTLA4 monoclonal antibody
ipilimumab – was recently shown to prolong survival in stage III melanoma (Eggermont et
al., N Engl J Med 2016; 375:1845-1855). More recently, the anti-PD1 monoclonal antibody
nivolumab was shown to be superior to ipilimumab in prolonging progression-free survival
(Weber et al., N. Engl. J. Med., 377:1824-1835, 2017). Notwithstanding this major
25 advancement, the clinical use of monoclonal antibodies is hampered by their significant
toxicity and enormous costs.

Micro-RNAs (miRNAs) are short non-coding RNAs known to have fundamental roles
in the regulation of gene expression. miRNAs have been widely implicated in physiological
processes, such as embryonal development and in pathological processes, such as cancerous
30 transformation and progression. For example, Mir-424, one of the miRNAs from the mir-
15a/16-1 (comprising of 8 miRNAs with an identical seed sequence) was implicated in the

regulation of the checkpoint mRNAs B7.1 and PD-1L in ovarian cancer. The transcription factor NF-kB was shown to upregulate the expression of mir-16 in a gastric cancer model.

There is an unmet need for personalized treatment regimens suitable for effective treatment of melanoma. In addition, there is an unmet need for assessment of responsiveness to immunotherapeutic treatments in melanoma.

SUMMARY OF THE INVENTION

The present disclosure is based on the unexpected finding that Tumor Necrosis Factor Superfamily Member 4 (TNFSF4) outperform ulceration as a prognostic marker in melanoma. Moreover, TNFSF4, alone or in combination with ulceration status and/or with mir-16 level, provides an improved assessment of responsiveness to immunotherapy in advanced (stage III or IV) patients. Thus, TNFSF4 provides a new platform for selection of a subpopulation of melanoma patients that can benefit from immunotherapy treatment, thus improving the benefit-risk ratio. Furthermore, use of TNFSF4 as disclosed herein point to patients in need of an intensified-treatment with combined immunotherapy (anti-PD1 and anti-CTLA4 antibodies).

According to some embodiments, there is provided a method for treating melanoma in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising mono-immunotherapy to a subject having a level of TNFSF4 mRNA or protein above a reference value.

According to some embodiments, the level of TNFSF4 mRNA or protein above a reference value is obtained by a method comprising: providing a sample comprising biological material from the subject; determining, in the biological material, the level of TNFSF4 mRNA or protein; and comparing the level of TNFSF4 mRNA or protein to a reference value.

According to some embodiments, the sample is a biopsy derived from a skin lesion.

According to some embodiments, the biological material is RNA and the level of TNFSF4 is the level of TNFSF4 mRNA or a level corresponding to the level of TNFSF4 mRNA.

According to some embodiments, the biological material comprises proteins and the level of TNFSF4 is the level of TNFSF4 protein or a level corresponding to the level of TNFSF4 protein.

According to some embodiments, the melanoma is stage III or IV melanoma.

5 According to some embodiments, the melanoma is selected from superficial spreading melanoma, lentigo maligna, acral lentiginous melanoma and nodular melanoma.

According to some embodiments, the mono-immunotherapy comprises at least one anti-PD1 antibody.

10 According to some embodiments, the mono-immunotherapy comprises at least one anti-CTLA4 antibody.

According to some embodiments, the method further comprising administering to said subject a pharmaceutical composition comprising an agent capable of down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family in a plurality of cell within the skin lesion.

15 According to some embodiments, said agent is an siRNA.

According to some embodiments, said siRNA is selected from SEQ ID NO: 1 and SEQ ID NO: 2.

According to some embodiments, said at least one micro RNA from the mir-15a/16-1 family is mir-16.

20 According to some embodiments, there is provided a method for treating melanoma in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising combined immunotherapy to a subject having expression level of TNFSF4 mRNA or level of TNFSF4 protein below a reference value.

According to some embodiments, the melanoma is stage III or IV melanoma.

25 According to some embodiments, the combined immunotherapy comprises at least one anti-CTLA4 antibody and at least one anti-PD1 antibody.

According to some embodiments, the combined immunotherapy comprises ipilimumab and nivolumab or ipilimumab and pembrolizumab.

30 According to some embodiments, there is provided a method for treating a subject having melanoma comprising administering to the subject a pharmaceutical composition comprising a compound that down-regulates the expression of the at least one micro RNA from the mir-15a/16-1 family.

According to some embodiments, the compound is an inhibitor of MAP3K7.

According to some embodiments, the compound comprises at least one of de-methylating compounds and HDAc inhibitors.

According to some embodiments, the compound comprises at least one de-methylating compound.

5 According to some embodiments, the at least one de-methylating compound is 5-azacytidine.

According to some embodiments, the compound comprises at least one HDAc inhibitor.

According to some embodiments, the at least one HDAc inhibitor is phenyl-butyric acid or valproic acid.

10 According to some embodiments, the compound comprises one or more of de-methylating compounds and HDAc inhibitor.

According to some embodiments, said subject is having a skin lesion and wherein administering comprises transfecting a plurality of melanoma cells within the lesion with a vector comprising at least one siRNA that inhibits at least one micro RNA from the mir-

15 15a/16-1 family.

According to some embodiments, the at least one siRNA is selected from SEQ ID NO: 1 and SEQ ID NO: 2.

20 Further embodiments, features, advantages and the full scope of applicability of the present invention will become apparent from the detailed description and drawings given hereinafter. However, it should be understood that the detailed description, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

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BRIEF DESCRIPTION OF THE DRAWINGS

30 Exemplary embodiments are illustrated in referenced figures. Dimensions of components and features shown in the figures are generally chosen for convenience and clarity of presentation and are not necessarily shown to scale. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than restrictive. The figures are listed below.

FIGs. 1A-1D present survival rates in subjects with melanoma at all stages (Fig. 1A; $p=0.0006$), Stage I/II subjects (Fig. 1B; $p=0.00384$), Stage III subjects (Fig. 1C; $p=0.00512$) and Stage IIIc-IV (Fig. 1D; $p=7 \cdot 10^{-5}$), having TNFSF4 higher than median - H, and TNFSF4 lower than median - L.

5 **FIGs. 1E-1F** present TNFSF4 mRNA expression and TNFSF4 protein levels in melanoma cell lines.

FIG. 2 presents TNFSF4 expression in malignant melanoma, lymphocytes and other cell types.

10 **FIG. 3** presents TNFSF4 expression in non-ulcerated and ulcerated primary lesions and metastasis.

FIGs. 4A-4C present survival rates with ulceration (U) or without ulceration (N) in subjects having melanoma at all stages (Fig. 4A), at Stage II (Fig. 4B) and at Stage III (Fig. 4C).

15 **FIGs. 5A-5C** present survival rates of subjects with melanoma, having TNFSF4 (low/high) and ulceration at all stages (Fig. 5A), Stage II subjects (Fig. 5B) and Stage III subjects (Fig. 5C): No ulceration + TNFSF4 low = NL; No ulceration + TNFSF4 high = NH; Ulceration + TNFSF4 low = UL; Ulceration + TNFSF4 high = UH.

20 **FIGs. 5D-5E** present overall survival (OS) and progression free survival (PFS) of patients with metastatic (stage IV) melanoma treated with anti-PD1 antibody, having TNFSF4 mRNA expression levels higher than median – H (broken lines), intermediate – I (dotted lines) and lower than median – L (solid lines).

FIGs. 6A-6I present Spearman rho correlations in the expression of pairs of 18 checkpoint mRNAs in melanoma cell lines.

25 **FIGs. 7A-7C** present Spearman correlation in the expression of checkpoint mRNAs and mir-16-1 in 17 melanoma cell lines.

FIG. 8 presents survival rate in stage III melanoma patients having high mir-16 (H) and low mir-16 (L).

30 **FIGs. 9A-9C** present the combination of mir-16-1 expression and three mRNAs as a prognostic marker in melanoma: TNFSF4 (Fig. 9A), CD274 (Fig. 9B) and CD80 (Fig. 9C); mir low, mRNA high (LH); both mir and mRNA high (HH); both mir and mRNA low (LL); mir high, mRNA low (HL).

FIGs. 10A-10C present the combination of mir-16-1 expression and three mRNAs as a prognostic marker in melanoma: CD86 (Fig. 10A), PDCD1LG2 (Fig. 10B) and C10orf54 (Fig.

10FC); mir low, mRNA high (LH); both mir and mRNA high (HH); both mir and mRNA low (LL); mir high, mRNA low (HL).

FIG. 11 presents the percentage change (relative to control) in the expression of checkpoint mRNAs following transient inhibition of mir-16 in 6 melanoma cell lines.

5 **FIGs. 12-13** present the effect of MA3K7 inhibition with increasing levels of 5Z-7-oxozeaenol on the expression levels of B7.1 in two melanoma cell lines: SB2 and SK-mel, respectively.

FIG. 14 presents the relative expression of mir-16 in four melanoma cell lines following addition of 5-azacytidine and phenyl-butyric acid (PBA).

10 **FIGs. 15A-15B** present the effect of Mir-16 on the 3'UTR of the mRNAs of two co-stimulatory checkpoint genes – CD40 and CD80, respectively, using a luciferase reporter assay.

FIGs. 15C-15E present the effect of an siRNA mimic or inhibitor on the expression of mir-16, CD40 and CD80, respectively.

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DETAILED DESCRIPTION OF THE INVENTION

Use of predictive and prognostic biomarkers may hold the key to the development of targeted cancer therapies and to improvement of drug efficacy, and may provide guidance to clinical decision making. While there are advances in melanoma treatment, current immunotherapeutic approaches remain inefficient and ineffective for a substantial percentage of patients. One reason for this is that the selected treatment is often not closely matched to the individual patient's disease. A personalized medicine approach that couples precise diagnostics with specific therapy might alleviate this problem.

25 Disclosed herein are methods for improved treatment of melanoma that includes selection of treatment regimen for melanoma, methods for selecting a subpopulation of melanoma patients which can benefit from current treatments, and methods involving new platforms for the treatment of melanoma. The methods disclosed herein are based on the understanding of the crosstalk between the immune system and cancer and the realization that cancer cells and lymphocytes communicate through cell-cell interactions, designated 'the immunological synapse', within the tumor microenvironment. The immunological synapse comprises of both co-stimulatory and co-inhibitory transmembrane proteins, that all serve to modulate the signal transmitted from the cancer cell to the lymphocyte towards either

30

activation energy or exhaustion. Several checkpoint mRNAs are under transcriptional regulation of the transcription factor NF- κ B, which has been implicated in the determination of cancer immunogenicity.

5 TNFSF4 (also known as OX40L) is a co-stimulatory checkpoint gene that can interact with its cognate receptor, TNFRSF4 (OX40) on lymphocytes.

Immune checkpoint inhibitors, namely, monoclonal antibodies that increase cancer immunogenicity by decreasing inhibitory signals between cancerous cells and lymphocytes, have revolutionized treatment in melanoma, and led to unprecedented improvement in the therapy outcome of metastatic disease (stage IV). The response rate to single-agent anti-CTLA4 antibodies (e.g. ipilimumab) is around 10%, to single-agent anti-PD1 antibodies (nivolumab or pembrolizumab) is around 30-40%, and the response rate to dual checkpoint inhibition with the combination of ipilimumab and nivolumab is as high as 60-70%, albeit at a cost of significant toxicity. Nonetheless, a clinically significant fraction of melanoma patients (30-70%, depending on the therapeutic approach) do not respond to the currently available immunotherapeutic drugs, and a significant fraction of responding patients develop resistance to immune checkpoint inhibition at later stages. There is thus an unmet clinical need to improve the activity of the current arsenal of immunotherapeutic drugs, and to introduce new immune-modulatory approaches in this yet incurable disease.

20 The anti-CTLA4 monoclonal antibody ipilimumab was recently shown to prolong survival in stage III melanoma (melanoma that has spread to regional lymph nodes but not to distant organs). Treatment of stage III melanoma patients with adjuvant ipilimumab improved the 5-year survival from ~55% to ~65%, which is both statistically and clinically significant. Notwithstanding this major improvement, adjuvant ipilimumab is currently not routinely used in stage III melanoma patients for two main reasons – first, this drug has significant toxicity, with a fatality rate of a little more than 1%. Second, its cost is enormous, and current drug reimbursement schemes across the world have not indorsed this drug. More recently, the anti-PD1 antibody nivolumab was shown to be superior to ipilimumab in prolonging progression-free survival in stage III melanoma. However, patients with stage III melanoma that poorly respond to nivolumab may need dual immunotherapeutic treatment, for example, combined immunotherapy with nivolumab and ipilimumab. Thus, the routine use of ipilimumab for all populations of stage III melanoma patients is almost not feasible. Accordingly, in most countries, these patients are monitored without active treatment, or receive interferon-alpha,

which has only a marginal survival benefit while associated with significant toxicity, or receive nivolumab, which has still not been shown to improve overall survival. Identifying in advance the subpopulation of patients that will not benefit from mono immunotherapy, and may benefit from combined immunotherapy would improve survival and would reduce the overall cost.

The methods disclosed herein permit the personalization of therapy amongst melanoma patients, wherein a subject's biomarker profile is predictive of, or indicative of, treatment efficacy and/or survival. The methods disclosed herein can be used in combination with assessment of conventional clinical factors, such as tumor size, tumor grade, lymph node status, family history, and analysis of expression level of additional biomarkers. In this manner, the methods of the present disclosure permit a more accurate evaluation of prognosis and cancer therapy effectiveness.

According to some embodiments, the methods disclosed herein include determining the level of TNFSF4 protein or the expression levels of the mRNA transcripts for TNFSF4 in a sample from a patient with melanoma.

Determining the level of TNFSF4 protein or mRNA does not require identification of the entire amino sequence of the protein or the entire ribonucleic acid sequence of the RNA (or the corresponding nucleic acid). Rather, determining the level of TNFSF4 protein or mRNA includes determining a fragment of the protein, mRNA or the corresponding cDNA, which is sufficient for identifying the presence of TNFSF4 protein or mRNA. The fragment may be a distinct epitope identified by an immunoassay or a segment for hybridization which is sufficient for determining the presence or level of TNFSF4. Accordingly, the term "TNFSF4 protein" includes a fragment of the TNFSF4 protein. Similarly, the term TNFSF4 mRNA includes a fragment of TNFSF4 mRNA, or a fragment of DNA corresponding to TNFSF4 mRNA.

The term "TNFSF4" as used herein refers to TNFSF4 mRNA or TNFSF4 protein.

According to some embodiments, the methods disclosed herein include determining the level of TNFSF4 protein in a sample from a patient with melanoma.

According to some embodiments, the methods disclosed herein include determining the expression levels of the mRNA transcripts for TNFSF4 in a sample from a patient with melanoma.

The methods described herein includes steps comprising determining the expression level of TNFSF4 in a test sample obtained from a subject; wherein an increase in the TNFSF4 expression level relative to a reference level indicates the subject can benefit from mono immunotherapy for the treatment of melanoma. According to some embodiments, the expression level of TNFSF4 can be determined by measuring the level of TNFSF4 RNA transcript. According to some embodiments, the RNA transcript level can be measured using reverse transcription polymerase chain reaction (RT-PCR). According to some embodiments, the expression level of TNFSF4 can be determined by measuring the level of TNFSF4 protein. According to some embodiments, the protein level can be measured using immunochemistry. According to some embodiments, the protein level can be measured using a small molecule which specifically binds to TNFSF4 and which is detectably labeled. According to some embodiments, the sample can comprise a material selected from the group consisting of: a biofluid sample, serum, plasma, urine, saliva and a tumor sample.

The methods of the present disclosure can be used to assist in selecting appropriate courses of treatment and to identify patients that would benefit from a particular course of therapy. Thus, determining the level of TNFSF4 protein or mRNA as described herein provides insight into which cancer treatment regimen will be most effective for the patient. This information can be used to generate treatment plans for the patient to prolong survival and minimize side effects or cancer therapy related toxicity.

According to some embodiments, there is provided a method of predicting responsiveness of melanoma in a subject to immunotherapy, comprising: obtaining a sample from the subject; measuring TNFSF4 protein level or the mRNA expression level of TNFSF4 in the sample; generating a comparison of the level of TNFSF4 in the sample to a reference level of TNFSF4; and using said comparison to predict the responsiveness of the melanoma to treatment with mono-immunotherapy, wherein high levels of TNFSF4 are predictive of responsiveness of the melanoma to treatment with mono-immunotherapy and wherein low levels of TNFSF4 are predictive of lack of responsiveness of the melanoma to treatment with mono-immunotherapy.

According to some embodiments, low levels of TNFSF4 are predictive of responsiveness of the melanoma to treatment with combined-immunotherapy

The term "mono immunotherapy" as used herein refers to treatment regimen with one monoclonal antibody. Mono-immunotherapy includes any one of anti-PD1 and anti-CTLA4

monoclonal antibodies. According to some embodiments, anti-PD1 comprises any one or more of nivolumab and pembrolizumab. According to some embodiments, anti-CTLA4 comprises ipilimumab.

5 The term "combined immunotherapy" as used herein refers to treatment regimen with a combination of monoclonal antibodies, such as, a combination of anti-PD1 and anti-CTLA4 monoclonal antibodies.

According to some embodiments, low levels of TNFSF4 indicate unfavorability of including mono-immunotherapy in the melanoma therapy.

10 According to some embodiments, identifying a subject in need of mono-immunotherapy for melanoma comprises: determining the expression level of TNFSF4 in a test sample obtained from a subject, wherein the subject is identified as being in need of mono-immunotherapy for melanoma if the expression level of TNFSF4 is increased relative to a reference level.

15 According to some embodiments, identifying a subject in need of combined-immunotherapy for melanoma comprises: determining the expression level of TNFSF4 in a test sample obtained from a subject, wherein the subject is identified as being in need of combined-immunotherapy for melanoma if the expression level of TNFSF4 is decreased relative to a reference level.

20 As used herein the term "predicting responsiveness" refers to providing a probability based analysis of how a particular subject diagnosed with melanoma and expected to receive treatment (immunotherapy) will respond to a cancer therapy, specifically, to mono-immunotherapy. The prediction of responsiveness is not a guarantee or absolute, only a statistically probable indication of the responsiveness of the subject. The prediction of responsiveness to a cancer therapy may indicate that the subject is likely to be responsive to
25 mono-immunotherapy or alternatively may indicate that the subject is not likely to be responsive to mono-immunotherapy. Alternatively, the prediction may indicate that inclusion of mono-immunotherapy in a cancer therapy regimen may be counter-productive and lead to a worse result for the subject than if no therapy was used or a placebo was used. Responsiveness includes but is not limited to, any measure of a likelihood of clinical benefit.
30 For example, clinical benefits include an increase in overall survival, an increase in progression free survival, an increase in time to progression, increased tumor response, decreased symptoms, or other quality of life benefits.

According to some embodiments there is provided a method for treating melanoma in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising mono-immunotherapy to a subject having expression level of TNFSF4 mRNA or level of TNFSF4 protein above a reference value.

5 According to some embodiments, lack of responsiveness to mono-immunotherapy indicates a significant increase in clinical benefit to treatment with combined therapy. Thus, the method disclosed herein provide a clinical guidance to the selection of treatment regimen.

As used herein, the terms "subject" and "patient" are used interchangeably and refer to both human and non-human animals. Preferably, the subject is a human patient. More
10 preferably, the subject is a human patient diagnosed with melanoma, undergoing, or about to undergo, a cancer treatment regimen.

According to some embodiments, said subject is human.

According to some embodiments, said subject is a human patient diagnosed with melanoma, undergoing, or about to undergo, cancer treatment.

15 According to some embodiments, the pharmaceutical composition comprises at least one anti-PD1 antibody. According to some embodiments, the pharmaceutical composition comprises nivolumab. According to some embodiments, the pharmaceutical composition comprises nivolumab as the only active ingredient. According to some embodiments, the pharmaceutical composition comprises pembrolizumab. According to some embodiments, the
20 pharmaceutical composition comprises pembrolizumab as the only active ingredient.

According to some embodiments, the pharmaceutical composition comprises at least one anti-CTLA4 antibody. According to some embodiments, the pharmaceutical composition comprises ipilimumab. According to some embodiments, the pharmaceutical composition comprises ipilimumab as the only active ingredient.

25 Method of developing a prognosis, in particular overall survival or progression free survival prognosis, for a subject with melanoma is also provided herein. According to some embodiments, the prognosis is independent of cancer therapeutic or cancer treatment regimen employed. According to some embodiments, the subject may have localized, advanced or metastatic melanoma. The method includes determining or detecting the expression level of
30 TNFSF4 mRNA, or the level of TNFSF4 protein, in a sample from the subject. The level of

the biomarker present in the sample is then compared to reference levels as described below. Finally, by comparison to the reference levels a prognosis for the subject can be determined .

According to some embodiments, the level of TNFSF4 is measured, and high level is indicative of a better prognosis and low level is indicative of poor prognosis.

5 Methods of developing a treatment plan for a subject with melanoma are also provided herein. Treatment plans may be developed using the predictions of the responsiveness of the cancer to treatment with a cancer therapy including mono-immunotherapy obtained using the methods described herein to determine whether treatment of the subject with mono-immunotherapy may be beneficial. The treatment plan will include mono-immunotherapy if
10 such therapy is expected to be beneficial and the treatment plan will include combined-immunotherapy if it is predicted to be clinically beneficial to the subject as described above.

According to some embodiments, there is provided a method for detecting melanoma in a subject, the method comprising:

- (a) providing a sample comprising genetic material from the subject;
- 15 (b) determining, in the genetic material, the expression level of TNFSF4 mRNA or a fragment thereof;
- (c) comparing the expression level of TNFSF4 mRNA to a reference value, wherein an expression level of TNFSF4 mRNA in the sample different than the reference value, is indicative of the presence of
20 melanoma and wherein the melanoma is metastatic melanoma, or non-metastatic melanoma with the presence of ulceration.

According to some embodiments, said providing a sample is obtaining a biopsy from a skin lesion.

According to some embodiments, said sample is blood, plasma, serum or urine.

25 According to some embodiments, the genetic material is RNA.

According to some embodiments, in the methods described herein the level of TNFSF4 protein in a sample from the subject is determined. The presence or absence of TNFSF4 protein may be determined by various methods, including, but not limited to, methods that are compatible with the detectable label used, e.g., microscopy, radiography, scintillation
30 counting, immunohistochemistry, etc. Generally, a level of TNFSF4 protein is compared to a

level of one or more reference samples, and the results evaluated to facilitate the determination of treatment regimen as disclosed herein. Control samples can be run in parallel to provide comparison levels, or the levels of specific control level may be provided as standard values for purposes of comparison.

5 As used herein, the terms “determining”, “assessing”, “assaying”, “measuring” and “detecting” refer to both quantitative and qualitative determinations and as such, the term “determining” is used interchangeably herein with “assaying,” “measuring,” and the like. Where a quantitative determination is intended, the phrase “determining an amount” of an analyte and the like is used. Where either a qualitative or a quantitative determination is
10 intended, the phrase “determining a level” of an analyte or “detecting” an analyte is used.

 The level of TNFSF4 protein in a sample from the subject may be assessed using an immunoassay. Exemplary formats include, but are not limited to, competitive binding assays in the presence of different amounts of a competitor protein which competes for binding to the TNFSF4 protein, and lateral flow immunoassay test (LFIA), also known as the
15 immunochromatographic assay. The competitor molecule can be labeled.

 Other immunoassays (e.g., Western blots) may be performed on protein gels or protein spots on filters may be adapted for use in the methods disclosed herein.

 The detection assays can be carried out in solution. Exemplary assays and reagents compatible for use in protein level assessment assays are known in the art and may be
20 adapted for use in the methods disclosed herein.

 The level of TNFSF4 protein in the sample may be determined by Mass spectrometry-based methods. Mass spectrometry can be combined with immunoassays, e.g., by first forming specific biomarker (TNFSF4 protein)-antibody immunocomplexes, and detecting the presence or absence of the specific immunocomplexes by mass spectroscopy. According to
25 some embodiments, an anti-biomarker antibody may be used to capture the TNFSF4 protein. The anti-biomarker antibody may be bound to a support, such as a bead, a plate, a membrane or a chip. After unbound materials are washed away, the captured biomarkers may be detected by mass spectrometry. Examples of mass spectrometers include time-of-flight, magnetic sector, quadmpole filter, ion trap, ion cyclotron resonance, electrostatic sector
30 analyzer and hybrids of these.

 The mass spectrometer can be a laser desorption/ionization (LDI) mass spectrometer.

The level of TNFSF4 may be determined by immunohistochemistry. Immunohistochemistry involves the process of selectively imaging TNFSF4 in the sample by exploiting labeled (directly or indirectly) anti-TNFSF4 antibodies that specifically bind to TNFSF4 in the sample. Visualizing an antibody-antigen interaction may be accomplished in a number of ways. For instance, an antibody is conjugated to an enzyme, such as peroxidase that can catalyze a color-producing reaction. Alternatively, the antibody may also be tagged to a fluorophore, such as fluorescein or rhodamine.

According to some embodiments, in the methods described herein the expression level of TNFSF4 mRNA in a sample from the subject is determined. Then the level in the sample from the subject is compared to a reference level of the biomarker (TNFSF4 mRNA).

The expression of TNFSF4 mRNA can be detected on a nucleic acid level. By "detecting or determining expression" is intended determining the quantity or presence of an RNA transcript for TNFSF4. Thus, "detecting expression" encompasses instances where a biomarker is determined not to be expressed, not to be detectably expressed, expressed at a low level, expressed at a normal level, or overexpressed.

Commonly used methods known in the art for the quantification of mRNA expression in a sample include northern blotting, in situ hybridization and quantitative reverse-transcription-PCR (RT-PCR). Alternatively, methods for deep sequencing of all mRNAs in samples (designated RNA-seq) using next-generation sequencing approaches may be employed.

The reference level may be determined empirically such as it was in the Examples, by comparison to the levels found in a set of samples from cancer patients treated with cancer therapies, specifically, with mono immunotherapy with known clinical outcomes for the patients. The results may be analyzed by dividing the data to quartiles, each corresponding to the level of the marker, such that, the upper quartile corresponds to high expression levels, the lowest quartile to low expression levels and the remaining quartile (2nd and 3rd) to intermediate expression levels. Alternatively, the reference level may be a level of the biomarker found in samples, such as plasma samples, which becomes a standard and can be used as a predictor for new samples. For example, the cut-off levels reported in the Examples may now serve as reference levels for comparison. As noted in Example 1, cutoff of 2 transcripts per million (TPM) in expression of TNFSF4 mRNA was found to be associated with a significantly improved survival following treatment with mono-immunotherapy.

According to some embodiments, the reference level is determined by analysis of a set of samples from melanoma patients treated with cancer therapies including or excluding immunotherapy with known outcomes.

5 According to some embodiments, the reference level can comprise the level of TNFSF4 (e.g. RNA transcript or protein) in a sample of the same type taken from a subject, or a plurality of subjects, not exhibiting any signs or symptoms of melanoma.

TNFSF4 mRNA expression or TNFSF4 protein level in some instances may be normalized against the expression levels of all RNA transcripts in the sample, or against a reference set of RNA transcripts in the sample or against the level of proteins in the sample.
10 The level of TNFSF4 is indicative of the prognosis for the subject or predictive of the effectiveness of a particular treatment, as disclosed herein.

According to some embodiments, the reference value corresponds to the median of the results obtained during the study described in Example 1.

15 According to some embodiments, the methods described herein further comprise a step of obtaining a sample from a subject.

According to some embodiments, prognostic and predictive performance of TNFSF4 is assessed and presented as Kaplan-Meier curves or plots. Methods for assessing statistical significance are well known in the art and include, for example, using a log-rank test, Student T-test, a Cox proportional hazards model and Kaplan-Meier curves. In some aspects of the
20 invention, a p-value of less than 0.05 constitutes statistical significance.

According to some embodiments, said melanoma is selected from superficial spreading melanoma, lentigo maligna, acral lentiginous melanoma and nodular melanoma.

According to some embodiments, the melanoma is stage III or stage IV melanoma.

25 Melanoma staging methods vary but are all approaches rely mostly on the thickness of the tumor, also known as Breslow's thickness or Breslow's depth and the appearance of microscopic ulceration (meaning that the epidermis on top of a major portion of the melanoma is not intact). Early melanomas (clinical stages I and II) are commonly determined based on T categories.

30 According to some embodiments, the method further comprises determining ulceration of the primary tumor, wherein ulceration together with an expression level of TNFSF4 mRNA in

the sample higher from the reference value, is indicative of subject's responsiveness to 'mono immunotherapy' treatment.

The terms "tumor", "lesion" and "skin lesion" as used herein are interchangeable. Preferably, these terms include melanoma (or melanoma tumor).

5 Ulceration typically refers to a breakdown of the skin over the melanoma. Melanomas that are ulcerated tend to have a worse outlook. Ulceration is commonly one of the aspects that define staging of melanoma in the T categories, which include:

- TX: Primary (main) tumor cannot be assessed.
- T0: No evidence of primary tumor.
- 10 – Tis: Melanoma in situ. (The tumor is only in the epidermis, the outermost layer of skin.)
- T1a: The melanoma is less than or equal to 1.0 mm thick (1.0 mm = 1/25 of an inch), without ulceration and with a mitotic rate of less than 1/mm².
- T1b: The melanoma is less than or equal to 1.0 mm thick. It is ulcerated and/or
15 the mitotic rate is equal to or greater than 1/mm².
- T2a: The melanoma is between 1.01 and 2.0 mm thick without ulceration.
- T2b: The melanoma is between 1.01 and 2.0 mm thick with ulceration.
- T3a: The melanoma is between 2.01 and 4.0 mm thick without ulceration.
- T3b: The melanoma is between 2.01 and 4.0 mm thick with ulceration.
- 20 – T4a: The melanoma is thicker than 4.0 mm without ulceration.
- T4b: The melanoma is thicker than 4.0 mm with ulceration.

In later stages (III and IV) important changes occur. The Breslow's thickness is less relevant but the presence of microscopic ulceration continues to be used, as it has an important effect on the progression of the disease. At this stage, the tumor spreads either to
25 the lymph nodes or to the skin between the primary tumor and the nearby lymph nodes. Usually, a tumor is assigned to Stage III if it has metastasized or spread beyond the original (primary) tumor site. This can be determined by examining a biopsy of the node nearest the tumor, known as the sentinel node. Such a biopsy may be done when a tumor is more than 1 mm in thickness, or when a thinner melanoma shows evidence of ulceration.

In-transit or satellite metastases are also included in Stage III. In this case, the spread is to skin or underlying (subcutaneous) tissue for a distance of more than 2 cm from the primary tumor, but not to the regional lymph nodes.

5 Metastases may be so tiny that they can be seen only through the microscope (micrometastases). The advancement of a tumor into Stage III depends on factors such as whether the metastases are in-transit or have reached the nodes, the number of metastatic nodes, the number of cancer cells found in them, and whether or not they are micrometastases or can be seen with the naked eye.

10 Stage IV melanoma has metastasized to lymph nodes distant from the primary tumor or to internal organs, most often the lung, followed in descending order of frequency by the liver, brain, bone, and gastrointestinal tract. The two main factors in determining how advanced the melanoma is into Stage IV are the site of the distant metastases (e.g. nonvisceral, lung) and elevated serum lactate dehydrogenase (LDH) level.

15 According to some embodiments, said sample is a sample from at least one melanoma tumor. According to some embodiments, the sample is a plurality of samples obtained from at least one tumor. According to some embodiments, the sample is a plurality of samples, each obtained from a different tumor in the same subject. According to some embodiments, the sample is a plurality of samples, each obtained from a different tumor, where at least one of said plurality of tumors is a primary tumor.

20 According to some embodiments, there is provided a method for treating melanoma, comprising down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family.

25 According to some embodiments, there is provided a method for treating melanoma, comprising administering to a subject in need thereof a pharmaceutical composition comprising an agent capable of down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family.

30 Micro-RNAs (miRNAs) are short non-coding RNAs known to have fundamental roles in the regulation of gene expression, and have been widely implicated in physiological processes such as embryonal development and in pathological processes such as cancerous transformation and progression. miRNAs can bind to the 3'UTR of mRNAs and lead to their post-transcriptional degradation or to translational repression. Mir-424, one of the miRNAs

from the mir-15a/16-1 (comprising of 8 miRNAs with an identical seed sequence) was implicated in the regulation of the checkpoint mRNAs B7.1 and PD-1L in ovarian cancer. The transcription factor NF-kB was shown to upregulate the expression of mir-16 in a gastric cancer model.

5 According to some embodiments, there is provided use of a compound that down regulates at least one micro RNA from the mir-15a/16-1 family for the treatment of melanoma.

 According to some embodiments, the technology described herein relates to a pharmaceutical composition comprising a compound that down regulates at least one micro
10 RNA from the mir-15a/16-1 family for the treatment of melanoma, and optionally a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art.

 According to some embodiments, the pharmaceutical composition comprising of a
15 compound that down regulates at least one micro RNA from the mir-15a/16-1 family as described herein can be a parenteral dose form. Since administration of parenteral dosage forms typically bypasses the patient's natural defenses against contaminants, parenteral dosage forms are preferably sterile or capable of being sterilized prior to administration to a patient. Examples of parenteral dosage forms include, but are not limited to, solutions ready for
20 injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, and emulsions. In addition, controlled-release parenteral dosage forms can be prepared for administration to the patient.

 According to some embodiments, a compound that down regulates at least one micro RNA from the mir-15a/16-1 family as described herein can be administered in a liposome
25 formulation. As used herein, "lipid vesicle" or "liposome" refers to vesicles surrounded by a bilayer formed of lipid components usually including lipids optionally in combination with non-lipidic components. The interior of a vesicle is generally aqueous.

 According to some embodiments, the methods described herein comprise administering an effective amount of compositions described herein, e.g. a composition comprising a
30 compound that down regulates at least one micro RNA from the mir-15a/16-1 family to a subject in order to treat melanoma.

As used herein, the terms "treat," "treatment," "treating," or "amelioration" are interchangeable and refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with melanoma. The term "treating" includes reducing or alleviating at least one adverse
5 effect or symptom of a condition, disease or disorder associated with melanoma. Treatment is generally "effective" if one or more symptoms and/or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be
10 expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or decreased mortality, whether detectable or undetectable. The term "treatment" of a disease
15 also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

As used herein, the term "pharmaceutical composition" refers to the one or more active agents in combination with a pharmaceutically acceptable carrier e.g. a carrier commonly used in the pharmaceutical industry. The phrase "pharmaceutically acceptable" is employed herein
20 to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

As used herein, the term "administering," refers to the placement of a compound as
25 disclosed herein into a subject by a method or route which results in at least partial delivery of the agent at a desired site. Pharmaceutical compositions comprising the compounds disclosed herein can be administered by any appropriate route which results in an effective treatment in the subject, e.g. parenteral, intravenous, intralesional, or intratumoral.

The term "statistically significant" or "significantly" refers to statistical significance and
30 generally means a two standard deviation (2SD) difference.

The term "effective amount" as used herein refers to the amount of a therapy needed for treatment of melanoma, and relates to a sufficient amount of pharmacological composition to

provide the desired effect. The term "therapeutically effective amount" therefore refers to an amount of a therapy that is sufficient to cause a particular effect when administered to a typical subject. An effective amount as used herein, in various contexts, would also include an amount sufficient to delay the development of melanoma, alter the course of melanoma (for
5 example, but not limited to, slowing the progression of the disease), or reverse the course of the disease. Thus, it is not generally practicable to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation.

According to some embodiments, an effective amount can be an amount which causes a
10 skin lesion growth to decrease or, at least, to increase at a lower rate than it would be expected to increase in a subject not receiving a composition as described herein. In some embodiments, an effective amount can be an amount which causes a lessening of pain as compared to the level of pain experienced by a subject prior to administration of a composition as described herein. In some embodiments, an effective amount can be an
15 amount that decreases the amount of mir-16 by a statistically significant amount.

Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dosage can vary depending upon the dosage form
20 employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that
25 includes the IC50 (i.e., the concentration of an therapeutic agent which achieves a half-maximal inhibition of symptoms) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. The dosage can be determined by a physician and adjusted, as necessary, to suit observed
30 effects of the treatment.

According to some embodiments, an effective dose of a composition comprising a compound that down regulates at least one micro RNA from the mir-15a/16-1 family as

described herein can be administered to a patient once. In certain embodiments, an effective dose of a composition comprising a compound that down regulates at least one micro RNA from the mir-15a/16-1 family can be administered to a patient repeatedly.

Membership in the mir-15a/16-1 family is defined on the basis of sequence I near the mature miRNAs' 5' end: all include the sequence AGCAGC (sequence 3).

According to some embodiments, down-regulating comprises administering to the subject a pharmaceutical composition comprising a compound that down-regulates the expression of the at least one micro RNA from the mir-15a/16-1 family. According to some embodiments, the compound is an inhibitor of MAP3K7. According to some embodiments, the compound comprises one or more of de-methylating compounds and histone deacetylase inhibitors (HDAC).

Demethylating compounds can inhibit methylation, resulting in the expression of the previously hypermethylated silenced genes. Cytidine analogs such as 5-azacytidine (azacitidine) and 5-azadeoxycytidine (decitabine) are the most commonly used demethylating compounds. These compounds work by binding to the enzymes that catalyse the methylation reaction, DNA methyltransferases.

According to some embodiments, the de-methylating compound is selected from 5-azacytidine and 5-azadeoxycytidine. According to some embodiments, the de-methylating compound is 5-azacytidine.

Histone deacetylase (HDAC) inhibitors (HDACi) is a group of 'targeted' anticancer agents. There are about 18 HDACs, which are generally divided into four classes, based on sequence homology to yeast counterparts. Classical HDACi such as the hydroxamic acid-based vorinostat (also known as SAHA and Zolinza) inhibits classes I, II and IV, but not the NAD⁺-dependent class III enzymes. In addition to histones, HDACs have many other protein substrates involved in regulation of gene expression, cell proliferation and cell death. Inhibition of HDACs causes accumulation of acetylated forms of these proteins, altering their function. Thus, HDACs are also referred to as 'lysine deacetylases.' HDACi induces different phenotypes in various transformed cells, including growth arrest, activation of the extrinsic and/or intrinsic apoptotic pathways, autophagic cell death, reactive oxygen species (ROS)-induced cell death, mitotic cell death and senescence. In comparison, normal cells are relatively more resistant to HDACi-induced cell death.

According to some embodiments, the HDAC inhibitor is phenyl-butyric acid or valproic acid.

According to some embodiments, down-regulating comprises transfecting the melanoma cells with an siRNA that inhibits at least one micro RNA from the mir-15a/16-1 family.

5 According to some embodiments, the siRNA comprises a ribonucleotide sequence comprising CGCCUUTUTTTUCGTGCTGCTU (SEQ ID NO:1) or GCGGUUAUAAAUGCACGACGAU (SEQ ID NO: 2).

According to some embodiments, the siRNA comprises SEQ ID NO:1.

According to some embodiments, the siRNA comprises SEQ ID NO:2.

10 According to some embodiments, the method further comprises co-administering to the subject said compound that down-regulates the expression of the at least one micro RNA from the mir-15a/16-1 family and at least one anti-melanoma therapeutic agent. According to some embodiments, the at least one anti-melanoma therapeutic agent is selected from the group consisting of at least one anti-PD1 monoclonal antibody, at least one anti-CTLA4 monoclonal
15 antibody monoclonal antibody and a combination thereof.

According to some embodiments, the at least one micro RNA from the mir-15a/16-1 family is mir-16.

According to some embodiments, there is provided a method for treating melanoma in a subject in need thereof, comprising: determining the expression level of TNFSF4 mRNA or
20 level of TNFSF4 protein in a sample obtained from a subject; and administering a pharmaceutical composition comprising mono-immunotherapy and a pharmaceutical composition comprising an agent capable of down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family, to a subject having expression level of TNFSF4 mRNA or level of TNFSF4 protein above a reference value.

25 According to some embodiment, said administering comprises administering a pharmaceutical composition comprising mono-immunotherapy and an agent capable of down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family.

According to some embodiments, there is provided mono-immunotherapy for use in the treatment of melanoma having expression level of TNFSF4 mRNA or level of TNFSF4
30 protein above a reference value.

According to some embodiments, there is provided combined-immunotherapy for use in the treatment of melanoma having expression level of TNFSF4 mRNA or level of TNFSF4 protein below a reference value.

5 According to some embodiments, there is provided a pharmaceutical composition comprising an agent capable of down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family for use in the treatment of melanoma.

According to some embodiments, there is provided a first pharmaceutical composition comprising an agent capable of down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family and a second pharmaceutical composition comprising mono-
10 immunotherapy for use in the treatment of melanoma having expression level of TNFSF4 mRNA or level of TNFSF4 protein above a reference value.

According to some embodiments, there is provided a pharmaceutical composition comprising an agent capable of down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family and mono-immunotherapy for use in the treatment of melanoma
15 having expression level of TNFSF4 mRNA or level of TNFSF4 protein above a reference value.

According to some embodiments, there is provided a kit for detecting melanoma in a subject, the kit comprises a first container comprising means for evaluating the amount of TNFSF4 mRNA, TNFSF4 protein or fragments thereof in biological materials; indication of
20 a threshold level for TNFSF4 and instructions manual, wherein level of TNFSF4 in the sample higher than the reference value, is indicative of the presence of melanoma.

According to some embodiments, the kit further comprises a sample comprising biological material from a subject having at least one skin lesion.

According to some embodiments, the sample is a biopsy of the at least one skin lesion.

25 According to some embodiments, the means for evaluating the amount of TNFSF4 mRNA or a fragment thereof the sample comprise materials for RNA purification and primers for amplification of TNFSF4 mRNA by qRT-PCR.

According to some embodiments, the means for evaluating the amount of TNFSF4 protein or a fragment thereof in the sample comprise materials for protein purification and agents for
30 immunohistochemistry.

According to some embodiments, there is provided a kit for treating a subject having at least one melanoma lesion, the kit comprising a compound that down-regulates at least one micro RNA from the mir-15a/16-1 family.

According to some embodiments, the compound is an inhibitor of MAP3K7. According to some embodiments, the compound comprises at least one of de-methylating compounds and HDAC inhibitors. According to some embodiments, the compound comprises at least one de-methylating compound. According to some embodiments, the at least one de-methylating compound is 5-azacytidine. According to some embodiments, the compound comprises at least one HDAC inhibitor. According to some embodiments, the at least one HDAC inhibitor is phenyl-butyric acid or valproic acid. According to some embodiments, the compound comprises one or more of de-methylating compounds and HDAC inhibitor.

According to some embodiments, the kit further comprises at least one anti-melanoma immuno-therapeutic agent. According to some embodiments, the at least one anti-melanoma immuno-therapeutic agent is an anti-PD-1 or an anti-CTLA-4. According to some embodiments, the at least one anti-melanoma immuno-therapeutic agent is selected from the group consisting of: ipilimumab, nivolumab and pembrolizumab.

According to some embodiments, the kit comprises a vector comprising at least one siRNA that inhibits at least one micro RNA from the mir-15a/16-1 family. According to some embodiments, the at least one siRNA is selected from SEQ ID NO:1 and SEQ ID NO:2. According to some embodiments, the vector is a plasmid.

According to some embodiments, there is provided use of a compound that down regulates at least one micro RNA from the mir-15a/16-1 family in a melanoma lesion, for the treatment of melanoma.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to". The terms "comprises" and "comprising" are limited in some embodiments to "consists" and "consisting", respectively. The term "consisting of" means "including and limited to". The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure. In the description

and claims of the application, each of the words “comprise” “include” and “have”, and forms thereof, are not necessarily limited to members in a list with which the words may be associated.

As used herein, the singular form "a", "an" and "the" include plural references unless
5 the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof .

As used herein the term "about" refers to plus/minus 10% of the value stated .

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means,
10 techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single
15 embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

20 Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

25 **Example 1: Bioinformatics analysis of mRNAs and miRNAs expression in melanoma**

Bioinformatics analyses of the expression of mRNAs and miRNAs from 451 samples was performed using the melanoma TCGA database. Correlation coefficients between the expression of mRNAs or mRNAs/miRNAs were calculated using the Spearman rho method. Survival analysis was performed using the Kaplan-Meier method resulting with survival
30 curves of survival time (months) vs. relative survival rate (a fraction of alive (or disease-free)

patients per month relative to the initial no. of patients that were analyzed). Each survival curve reflects the trend from the collective data of individual patients, where each small perpendicular line along the survival curve corresponds to a single patient. Potential 3'UTR binding sites of miRNAs were found using the web-based tool www.targetscan.org.

5 The expression of TNFSF4 above the median value was found to be associated with a significantly improved survival for all melanoma patients using a cutoff of 2 transcripts per million (Fig. 1A; TPM). This holds true for the subgroups of stage I/II patients (Fig. 1B; using a cutoff of 1.8 TPM), but is even more pronounced and significant for the subgroup of stage III patients (Fig. 1C; using a cutoff of 2.1 TPM) or the subgroup of stage IIIc/IV
10 patients (Fig. 1D; distant metastasis, cutoff 2.3 TPM). The results suggest that prognosis of melanoma is determined, at least in part, by the extent of expression of the co-stimulatory checkpoint gene TNFSF4, and that increasing its expression would improve outcome in this disease, potentially by increasing the immunogenicity of the tumor.

 The expression of TNFSF4 was found to be significantly higher in malignant
15 melanocytes than in other types of immune cells and endothelial cells, specifically, T-cells, B-cells, NK cells, cancer associated fibroblasts (CAFs), endothelial cells and macrophages (Fig. 2, in reads per kilobase per million (RPKM)). The results suggests that expression of TNFSF4 plays a role in melanoma evolution or progression. A significant variance in the expression of TNFSF4 between samples was observed. Non significant differences in
20 expression of TNFSF4 were detected between ulcerated and non-ulcerated melanoma in either the primary tumor samples or in metastatic deposits originating from ulcerated or non-ulcerated tumors. The expression of TNFSF4 was generally higher in metastatic samples than in primary tumors (Fig. 3, in transcripts per million (TPM)).

 The presence of TNFSF4 mRNA and TNFSF4 protein was determined in 16 melanoma
25 cell lines: mel-14PA (14), mel-526 (526), eng-003-mel (003), mel-624 (624), 451Lu (451), mel-33-A1 (A1), mel-793 (793), mel-15A (15), A375 (375), SK-mel (SK), WM852 (852), MEWO, mel-1617 (1617) and HS294T (294HS). TNFSF4 mRNA and TNFSF4 protein were detected in most of the cell lines (Figs. 1E and 1F, respectively).

 Ulceration, a known prognostic factor in melanoma, was evaluated for its association
30 with survival in the TCGA cohort. Whereas there was a statistically significant association between ulceration and worse prognosis in the entire cohort (Fig. 4A) and in a sub-group of stage II patients (Fig. 4B), the difference in overall survival in stage III patients between

those with ulcerated (U) primaries vs. those with non-ulcerate (N) primaries was not statistically significant (Fig. 4C).

Overall survival was determined for four (4) groups of patients: patients with non-ulcerated primaries with either high or low TNFSF4 (NH and NL, respectively), and patients with ulcerated primaries with either high or low TNFSF4 (UH and UL, respectively; Figs. 5A-5C). The results were analyzed for all subjects, at all stages (Fig. 5A), Stage II subjects (Fig. 5B) and stage III subjects (Fig. 5C). Strikingly, the differences in overall survival between the four groups were statistically significant for the entire cohort (p value $< 3 \times 10^{-9}$) and in the sub-group of stage III patients (p value < 0.0003). In the entire cohort, patients with non-ulcerated and TNFSF4-high tumor (above a cutoff of 2.0 TPM) had the best prognosis, the patients with ulcerated TNFSF4-low tumors had the worst prognosis, and the two other sub-groups had an intermediate prognosis. In contrast, in the subgroup of stage III patients, patients with ulcerated TNFSF4-low tumors (below a cutoff of 2.1 TPM) had a significantly worse prognosis compared to each of the other three (3) subgroups. The latter 3 subgroups did not significantly differ between each other, with a median overall survival of ~25 months in ulcerated-TNFSF4-low vs. 67 month in non-ulcerated-TNFSF4-low vs ~100 for any TNFSF4-high tumor, irrespective of the ulceration status.

Example 2: TNFSF4 expression for selecting treatment regimen

The expression of TNFSF4 was assessed in tumor samples of 40 patients with metastatic melanoma receiving anti-PD1 treatment. The progression free survival (PFS; defined as the time from treatment start to disease progression) and the overall survival (OS; defined as the time from treatment start to death) was calculated according to quartiles of TNFSF4 expression (lowest quartile - L, two middle/intermediate quartiles - I, high quartile - H of expression). The results indicate that metastatic melanoma patients with low TNFSF4 expression (lowest quartile, L; solid lines in Figs. 5D and 5E) had worse outcome in both PFS and OS following treatment with anti-PD1 immunotherapy. In contrast, patients with intermediate (two middle quartiles, I; dotted lines in Figs. 5D and 5E) or high (highest quartile, H; broken lines in Figs. 5D and 5E) expression of TNFSF4 had better outcome on mono immunotherapy (Figs. 5D and 5E).

Thus, TNFSF4 expression was found to be a marker for responsiveness to immunotherapy, especially to monotherapy.

Example 3: Expression of checkpoint mRNAs in melanoma

The immunological synapse – namely, the interface between immune cells and cancer cells or antigen presenting cells (APCs) – is multi-faceted and complex. The following example relates to immune checkpoints on the melanoma/APC side of the immunological synapse.

The expression of 18 checkpoint mRNAs suggested from the literature to be expressed on the melanoma/APC side of the immunological synapse) was evaluated by calculating the Spearman rho correlation co-efficient of any two of the 18 mRNAs (153 comparisons in total). Of these, the expression of 12 mRNAs (VISTA, PD-L1, PD-L2, LGALS9, B7.1 (CD80), B7.2, CD40, CD40L, ICOSLG, TNFSF18, CD70 and TNFSF4) was highly correlated to each other, with a Spearman rho value ≥ 0.3 , of which 9 were correlated with a Spearman rho value ≥ 0.5 , and 7 (VISTA, PD-L1, PD-L2, LGALS9, B7.1, B7.2 and CD40) were correlated with a Spearman rho value ≥ 0.7 (Table 1, values above 0.69 are underlined; values between 0.69 and 0.50 are in bold; values between 0.49 and 0.30 are in italic format; and values below 0.30 are not highlighted). These results were then corroborated in melanoma cell lines in vitro: SB2, WM852, SK-Mel, mel-793, A375, mel-1617, 451Lu, mel-526, mel-624, mel-1361A, eng-003-mel, HS294T, MEWO, mel-14PA, mel-15A, mel-33-A1, mel-33-B1. There was a significant correlation between several pairs of mRNAs across 17 different cell lines, with coefficient values from about 0.5 to about 0.9 (Figs. 6A-6I).

Table 1: Spearman rho correlation coefficient between two mRNAs on the side of melanoma or APC in the immunological synapse

	PD-L2	B7.1	VISTA	LGALS9	CD40	PD-L1	CD40LG	ICOSLG	CD70	TNFSF4	TNFSF18	PVR L2
B7.2	<u>0.90</u>	<u>0.89</u>	<u>0.76</u>	<u>0.79</u>	<u>0.71</u>	<u>0.72</u>	0.73	0.62	<i>0.49</i>	0.50	0.50	0.22
PD-L2		<u>0.87</u>	<u>0.70</u>	<u>0.72</u>	0.63	<u>0.81</u>	0.70	0.54	<i>0.42</i>	<i>0.48</i>	0.51	0.21
B7.1			<u>0.70</u>	<u>0.72</u>	0.68	<u>0.75</u>	0.69	0.56	<i>0.42</i>	<i>0.49</i>	<i>0.47</i>	0.18
VISTA				0.69	0.70	0.53	0.67	0.66	0.50	<i>0.37</i>	<i>0.39</i>	<i>0.32</i>
LGALS9					0.59	0.54	0.65	0.53	<i>0.46</i>	<i>0.34</i>	<i>0.34</i>	0.22
CD40						0.51	0.61	0.53	<i>0.49</i>	<i>0.43</i>	<i>0.36</i>	0.22
PD-L1							0.57	<i>0.37</i>	<i>0.36</i>	0.50	<i>0.42</i>	0.07
CD40LG								0.50	<i>0.34</i>	<i>0.32</i>	<i>0.35</i>	0.12

ICOSLG									<i>0.48</i>	<i>0.33</i>	0.26	0.23
CD70										<i>0.41</i>	0.22	0.22
TNFSF4											<i>0.39</i>	0.06
TNFSF18												0.12

Putative binding sites for the miRNAs from the mir-15a/16-1 family in the 3'UTR of all 18 checkpoint mRNAs were identified in 11 mRNAs (Table 2).

The number of putative binding sites ranged from one to five in each of the mRNAs.

- 5 The data is provided in Table 3. There were several significant, albeit weaker, positive correlations between the expression of miRNAs from the mir-15a/16-1 family (mir-15a, mir-15b, mir-16-1, mir-16-2, mir-195, mir-424, mir-497 and mir-503) and some of the checkpoint mRNAs, with Spearman rho values ≥ 0.2 (corresponding to a p value < 0.0003 ; bold in Table 3).

Table 2: Putative binding sites for mir-15a/16-1 family miRNAs

No. of putative miRNA binding sites	mRNA	Additional name	Additional name
5	C10orf54	VISTA	B7-H5
5	CD40		
4	CD80	B7.1	
2	TNFSF9	CD137L	4-1BBL
2	VTCN1	B7-H4	
1	CD112	NECTIN-2	PVRL2
1	CD155	PVR	
1	CD274	PD-L1	
1	CD276	B7-H3	
1	ICOSLG	CD275	B7-H2
1	TNFRSF14	HVEM	

Table 3: Correlation between expression of mir-15a/16-1 miRNAs and checkpoint mRNAs

mRNA	mir-497	mir-195	mir-503	mir-16-1	mir-424	mir-16-2	mir-15a	mir-15-b
TNFSF4	0.21	0.15	0.28	0.13	0.26	0.16	0.08	0.01
B7.1	0.21	0.18	0.20	0.22	0.15	0.17	0.04	0.00
CD70	0.20	0.19	0.19	0.13	0.19	0.10	0.13	0.02-
B7.2	0.23	0.20	0.19	0.18	0.15	0.11	0.02	0.06-
PD-L2	0.22	0.15	0.18	0.18	0.12	0.13	0.05	0.10-
CD40	0.22	0.24	0.12	0.11	0.11	0.12	0.07-	0.03
VISTA	0.25	0.29	0.08	0.02	0.07	0.02	0.07-	0.14-
PD-L1	0.11	0.05	0.15	0.23	0.08	0.18	0.12	0.03-
LGALS9	0.17	0.22	0.13	0.14	0.11	0.10	0.03	0.03-
CD40LG	0.24	0.29	0.03	0.14	0.01-	0.09	0.02	0.07-
TNFRSF14	0.15-	0.07-	0.03	0.14	0.04-	0.07	0.20	0.05
ICOSLG	0.28	0.27	0.01-	0.01	0.01-	0.00	0.10-	0.08-
TNFSF9	0.02-	0.04-	0.20	0.06-	0.23	0.04-	0.08	0.05-
TNFSF18	0.17	0.09	0.18	0.03	0.12	0.04-	0.05	0.02-
PVR	0.06-	0.15-	0.09	0.06-	0.09	0.02-	0.04-	0.02-
PVRL2	0.07	0.09	0.09	0.05-	0.14	0.04-	0.03-	0.00
CD276	0.01	0.02-	0.03-	0.15-	0.01	0.10-	0.13-	0.04
VTCN1	0.00	0.01-	0.04	0.07-	0.02	0.07-	0.06-	0.05-

5 The positive correlations between mir-16 was corroborated in vitro for checkpoint mRNAs B7.1, PD-L1 and PD-L2 (Figs. 7A-7C).

The association between miRNA expression and overall survival was determined using the TCGA database. Of all eight (8) miRNAs from the mir-15a/16-1 family, only the

expression of mir-16 was borderline associated with overall survival ($p=0.09$), with a higher-than-median level of mir-16 associated with worse overall survival than a lower-than-median level (Fig. 8). The overall survival with all combinations of checkpoint mRNAs (either below or above median) and mir-16 expression (either below or above median) was assessed. A statistically significant difference in survival between the four subgroups: low mir16 and low mRNA (LL); low mir16 and high mRNA (LH) high mir16 and low mRNA (HL); high mir16 and high mRNA (HH) was found for 6 mRNAs (B7.1, B7.2, PD-L1, PD-L2, VISTA, TNFSF4; Figs. 9A-9C and 10A-10C, respectively). The combination of high-mir-16-low-mRNA (HL) was associated with significantly worse outcome than the three (3) other groups (LL, LH and HH) in 5 out of those 6 mRNAs (Figs. 9A-9C and 10A-10B, where the combination of low-mir-16-high-TNFSF4 (LH; Fig. 10C) was associated with significantly better outcome than the 3 other groups. The statistical significance of the results presented in Figs. 9A-9C and 10A-10C are summarized in Table 4.

Table 4: Statistics corresponding to Figs. 9A-9C and 10A-10C

mRNA	additional name	p value
TNFSF4	OX40L	0.005
CD274	PD-L1	0.015
CD80	B7.1	0.017
C10orf54	VISTA	0.02
PDCD1LG2	PD-L2	0.025
CD86	B7.2	0.043

Example 4: Down-regulation of mir-16 by siRNA

The following melanoma cell lines were used for the tissue culture experiments – SB2, WM852, SK-Mel, mel-793, A375, mel-1617, 451Lu, mel-526, mel-624, mel-1361A, eng-003-mel, HS294T, MEWO, mel-14PA, mel-15A, mel-33-A1, mel-33-B1.

Six (6) melanoma cell lines (SB2, SK-mel, MEWO, mel-14PA, mel-1617 and mel-793) were transiently transfected with a plasmid expressing a small-interfering RNA to mir-16 (mir-16-siRNA): CGCCUUTUTTTUCGTGCTGCTU (SEQ ID NO:1) and GCGGUUAUAAAUGCACGACGAU (SEQ ID NO: 2) or to an unrelated sequence using Lipofectin™ (control cells). 48 hours after transfection, RNA was extracted from the cells using the Total RNA purification kit (Norgen Biotek), and the expression of checkpoint mRNAs assessed by qRT-PCR.

The levels of mir-16 were significantly lower in the transfected cells compared to control cells (in which an irrelevant siRNA was transfected; results not shown). In 5 out of 6 mir-16-deprived cell lines (i.e. in mel-14PA, mel-1617, mel-793, MEWO and SB2), the expression levels of seven (7) checkpoint mRNAs increased compared to control cell lines (Fig. 11).

Without wishing to be bound by any theory or mechanism of action, the positive co-regulation of many checkpoint mRNAs and mir-16, together with the existence of putative binding sites of mir-16 in the 3'UTR of several of these mRNAs, may suggest that there is a common transcription factor that regulates the mRNAs and mir-16. Furthermore, mir-16 acts to negatively regulate the expression of mRNAs, by targeting their 3'UTR directly or indirectly. This negative feedforward loop is predicted to maintain the levels of checkpoint mRNAs constant even in the face of cellular perturbations in the expression of the transcription factor that regulates the loop. NF- κ B was previously shown to regulate the transcription of mir-16, B7.1 and PD-1L.

Example 5: Down-regulation of mir-16 by pharmacologic and epigenetic modifiers

Melanoma cells were treated with the pharmacological inhibitor of MAP3K7 5Z-7-oxozeaenol (Sigma®) or the epigenetic modifiers 5-azacytidine and phenyl-butyric acid (PBA) and the level of checkpoint mRNAs were assessed by qRT-PCR.

Without being bound to any theory or mechanism of action, it is believed that MAP3K7 regulates NF- κ B. NF- κ B activation depends on MAP3K7, which phosphorylates and activates the IKK complex, leading to the phosphorylation, ubiquitination and degradation of NFKB1A. This frees NF- κ B, which is then activated, trans-located to the nucleus and activates transcription of multiple genes.

The effect of 5Z-7-oxozeaenol on the expression of B7.1 (CD 80) was determined. It was found that the expression of B7.1 decreased following inhibition of MAP3K7 achieved by 5Z-7-oxozeaenol (Figs. 12-13).

The effect of epigenetic modifiers, de-methylating compound (5-azacytidine) in combination with an HDAC inhibitor (PBA) on the expression of mir-16, was evaluated in 4 cells lines: mel-624, mel-526, mel-14PA and mel-33-b1. In 3 out of 4 cell lines, the combined treatment led to a decrease in the expression of mir-16 (FIG. 14). The results indicate that in most cell lines (75%), epigenetic modifiers lead to a decrease in the levels of mir-16.

Mir-16 was shown to target directly the 3'UTR of the mRNAs of two co-stimulatory checkpoint genes – CD40 and CD80 (FIGs. 15A-15B, respectively). This was done by cloning the 3'UTR of CD40 and CD80 to the cDNA of the reporter gene luciferase. Luciferase activity in the transfected cells was assessed in the absence (0µg) or presence of two different concentrations of a vector comprising mir-16 cDNA. The decrease in luciferase activity in the presence of mir-16 served to indicate direct targeting of both 3'UTRs by mir-16. Transient transfection of melanoma cells with either a plasmid coding for a mir-16-mimic (i.e. an RNA with the same sequence) or a plasmid coding for a mir-16-siRNA (small interfering RNA, i.e. an RNA with a complementary sequence to mir-16) led to an increase and decrease in the levels of mir-16, respectively, as expected (FIG.15C). The levels of CD40 and CD80 mRNAs were not altered following transfection with the mir-16-mimic but, surprisingly, were increased following transfection with a mir-16-siRNA (FIGs.15D-16E), indicating that inhibition of mir-16 by means of small interfering RNAs increases the expression of CD40 and CD80 mRNAs.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. A method for treating melanoma in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising mono-immunotherapy to a subject having a level of TNFSF4 mRNA or protein above a reference value.
2. The method of claim 1, wherein the level of TNFSF4 mRNA or protein above a reference value is obtained by a method comprising: providing a sample comprising a biological material from the subject; determining, in the biological material, the level of TNFSF4 mRNA or protein; and comparing the level of TNFSF4 mRNA or protein to a reference value.
3. The method of claim 2, wherein the sample is a biopsy derived from a skin lesion.
4. The method of claim 2, wherein the biological material is RNA and the level of TNFSF4 is corresponding to the level of TNFSF4 mRNA.
5. The method of claim 2, wherein the biological material comprises proteins and the level of TNFSF4 is corresponding to the level of TNFSF4 protein.
6. The method of claim 1, wherein the melanoma is stage III or IV melanoma.
7. The method of claim 6, wherein the melanoma is selected from superficial spreading melanoma, lentigo maligna, acral lentiginous melanoma and nodular melanoma.
8. The method of claim 1, wherein the mono-immunotherapy comprises at least one anti-PD1 antibody.
9. The method of claim 1, wherein the mono-immunotherapy comprises at least one anti-CTLA4 antibody.
10. The method of claim 1, further comprising administering to said subject a pharmaceutical composition comprising an agent capable of

down-regulating the expression of at least one micro RNA from the mir-15a/16-1 family in a plurality of cell within the skin lesion.

11. The method of claim 10, wherein said agent is an siRNA.
12. The method of claim 11, wherein said siRNA is one of SEQ ID NO:1 and SEQ ID NO:2.
13. The method of claim 10, wherein said at least one micro RNA is mir-16.
14. A method for treating melanoma in a subject in need thereof, the method comprising administering a pharmaceutical composition comprising combined immunotherapy to a subject having expression level of TNFSF4 mRNA or level of TNFSF4 protein below a reference value.
15. The method of claim 14, wherein the melanoma is stage III or IV melanoma.
16. The method of claim 14, wherein the combined immunotherapy comprises at least one anti-CTLA4 antibody and at least one anti-PD1 antibody.
17. The method of claim 16, wherein the combined immunotherapy comprises ipilimumab and nivolumab or ipilimumab and pembrolizumab.
18. A method for treating a subject having melanoma comprising administering to the subject a pharmaceutical composition comprising a compound that down-regulates the expression of the at least one micro RNA from the mir-15a/16-1 family.
19. The method of claim 18, wherein the compound is an inhibitor of MAP3K7.
20. The method of claim 18, wherein the compound comprises at least one of de-methylating compounds and HDAC inhibitors.
21. The method of claim 20, wherein the at least one de-methylating compound is 5-azacytidine.

22. The method of claim 20, wherein the at least one HDAC inhibitor is phenyl-butyric acid or valproic acid.
23. The method of claim 20, wherein the compound comprises one or more of de-methylating compounds and HDAC inhibitor.
- 5 24. The method of claim 18, wherein said subject is having a skin lesion and wherein administering comprises transfecting a plurality of melanoma cells within the lesion with a vector comprising at least one siRNA that inhibits at least one micro RNA from the mir-15a/16-1 family.
- 10 25. The method of claim 24, wherein the at least one siRNA is selected from SEQ ID NO:1 and SEQ ID NO:2.

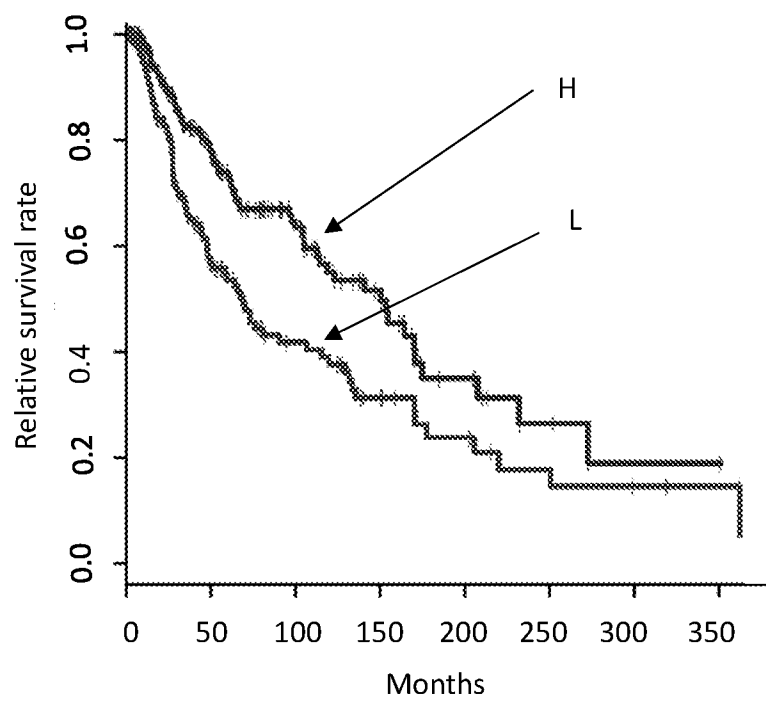


Figure 1A

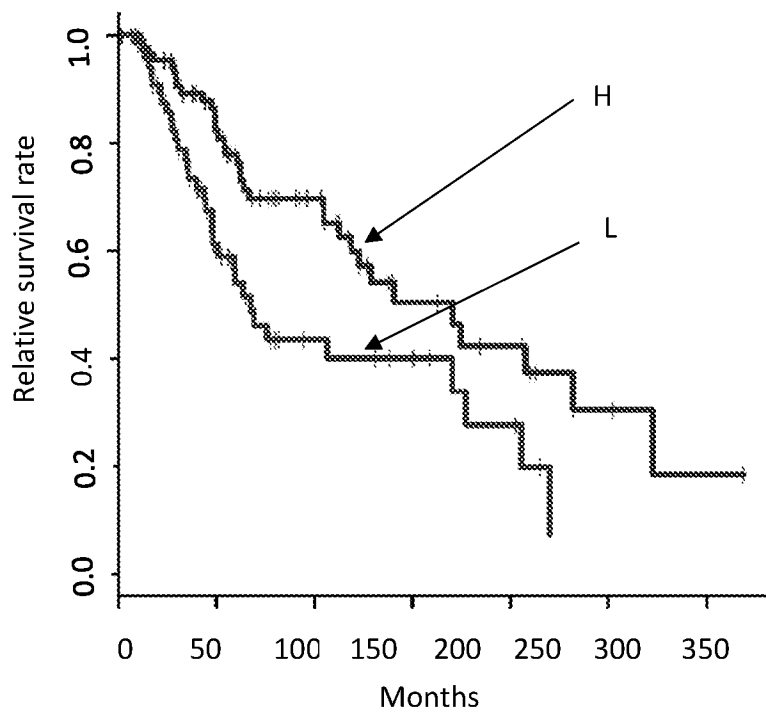


Figure 1B

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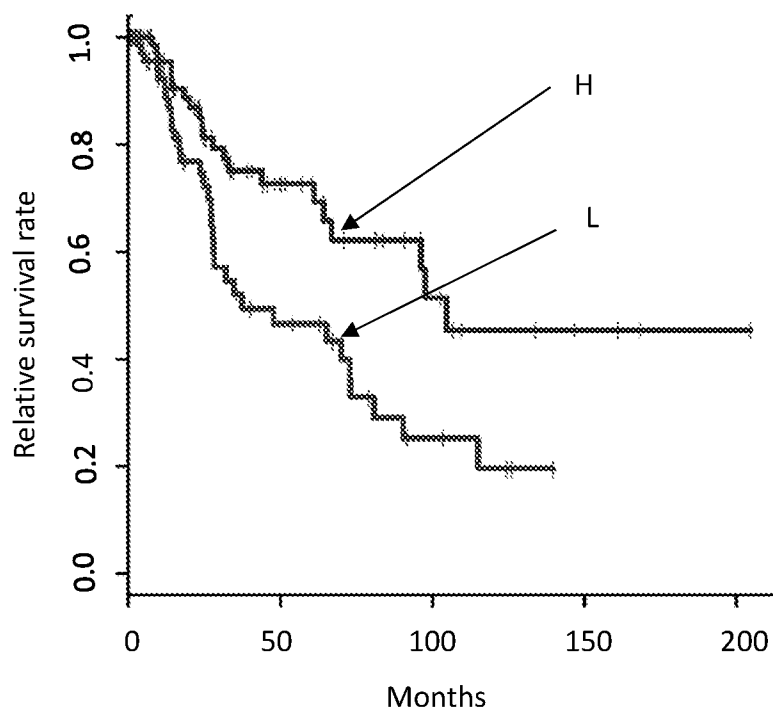


Figure 1C

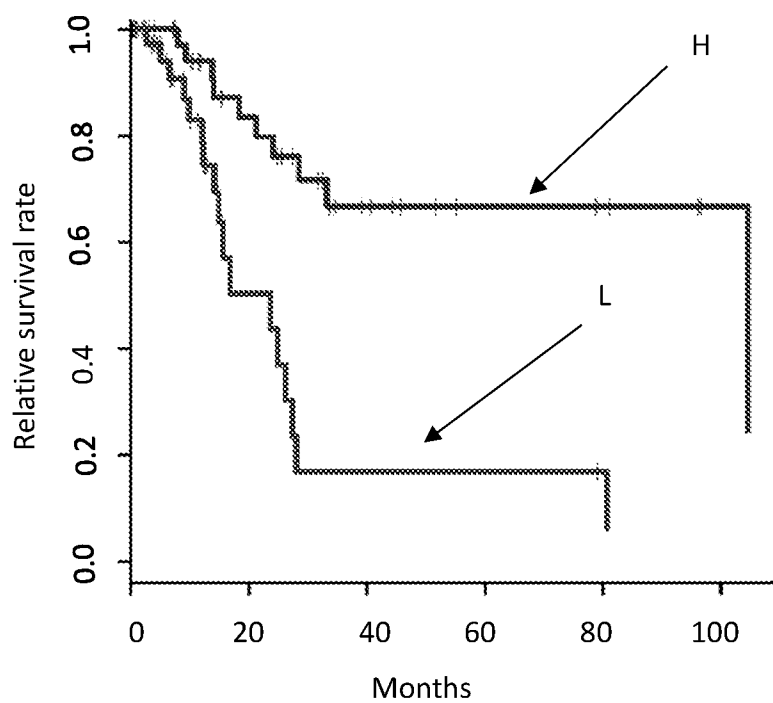


Figure 1D

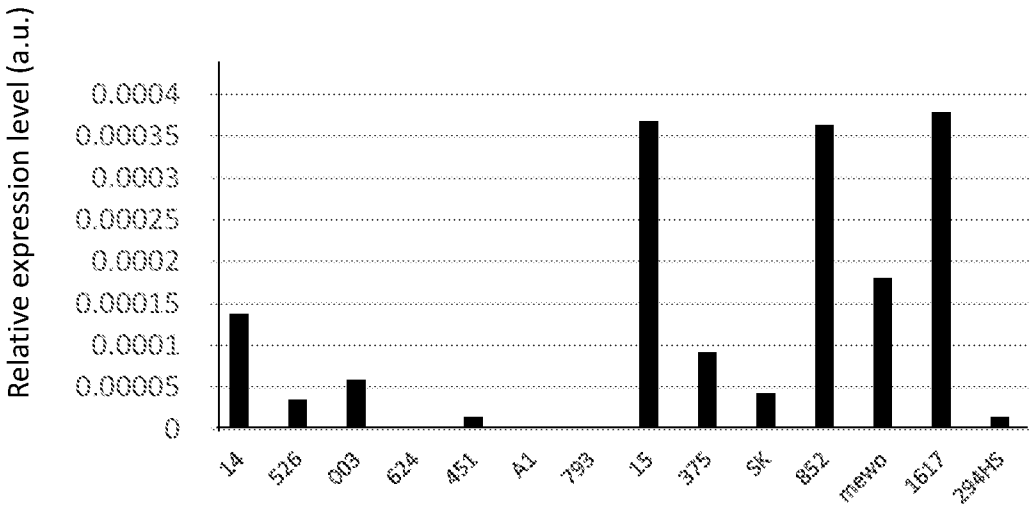


Figure 1E

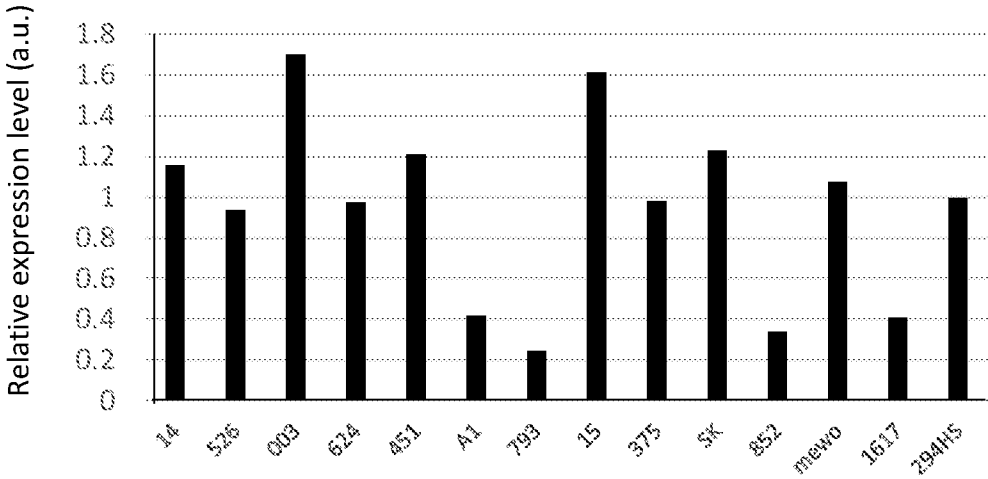


Figure 1F

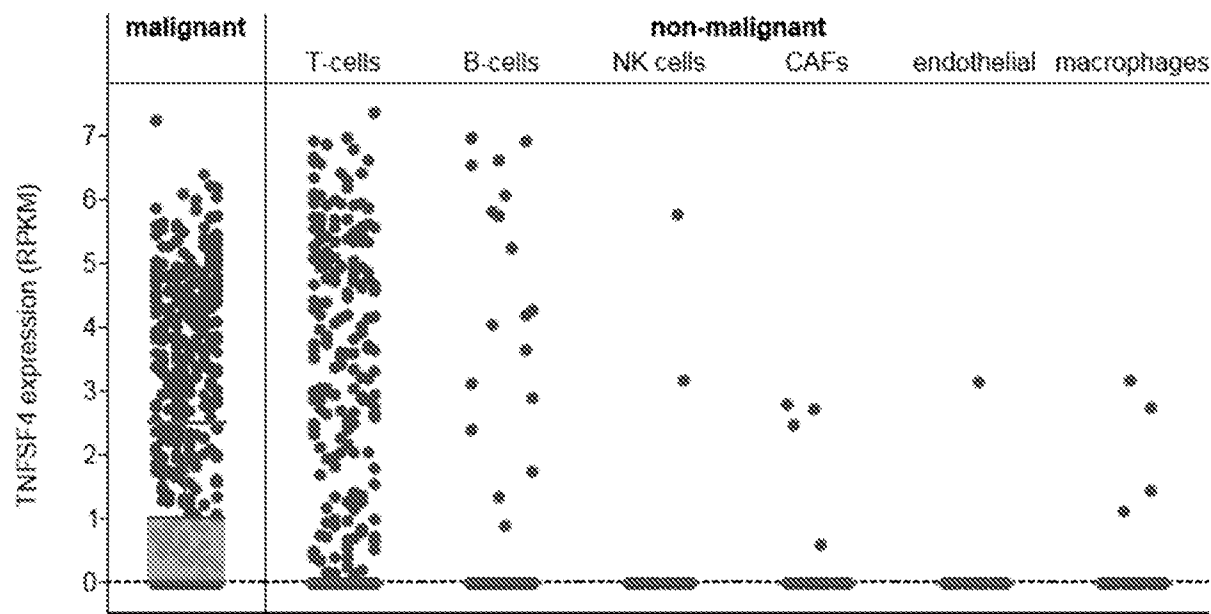


Figure 2

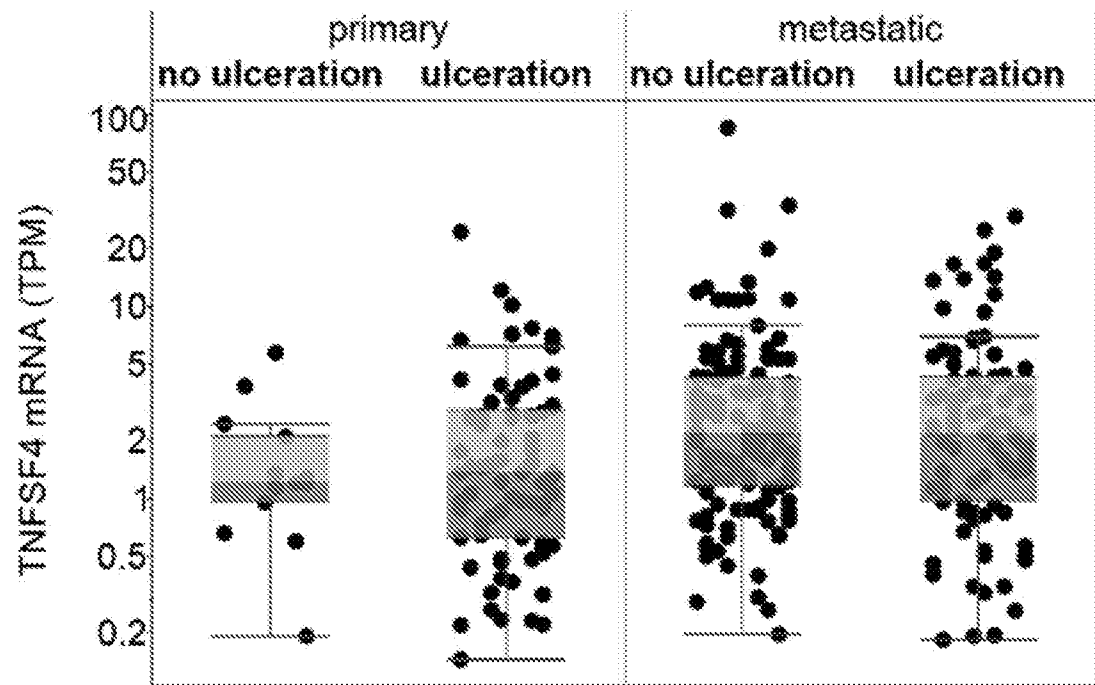


Figure 3

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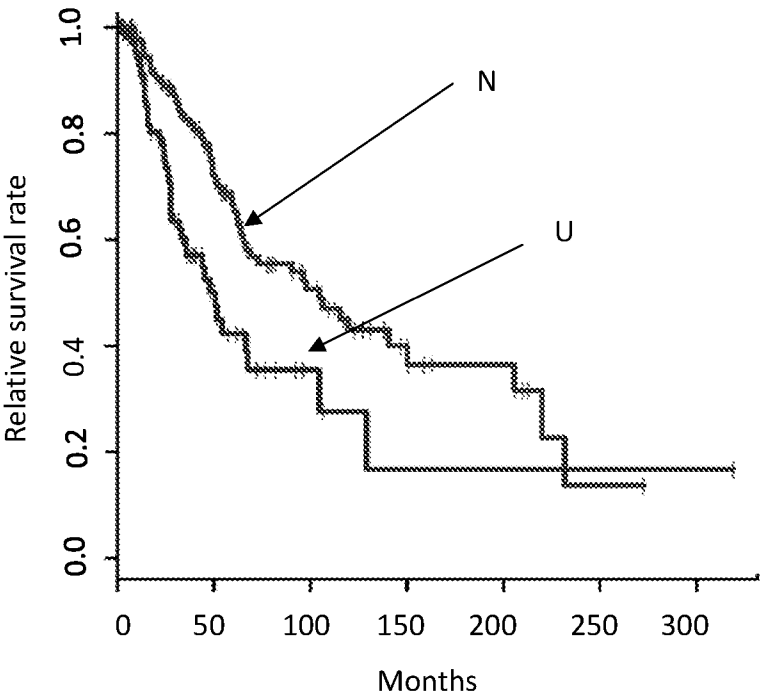


Figure 4A

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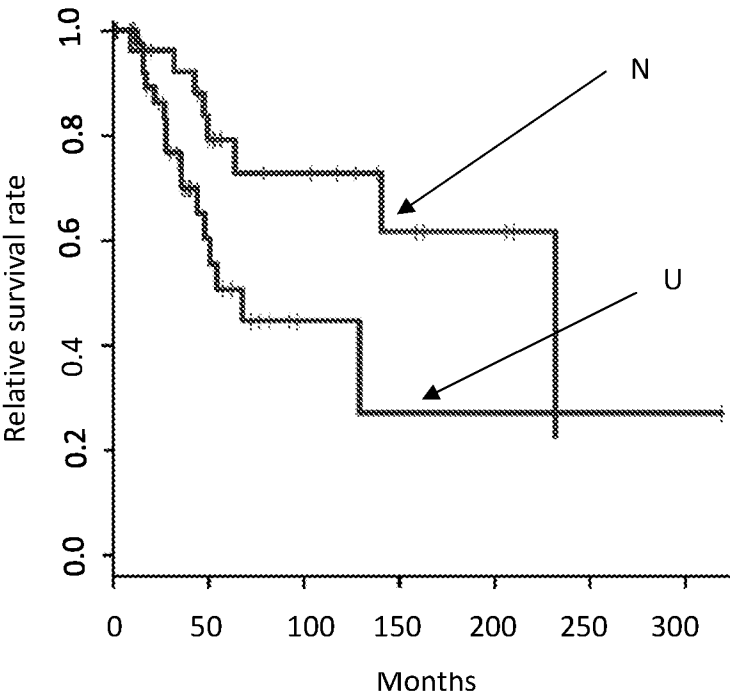


Figure 4B

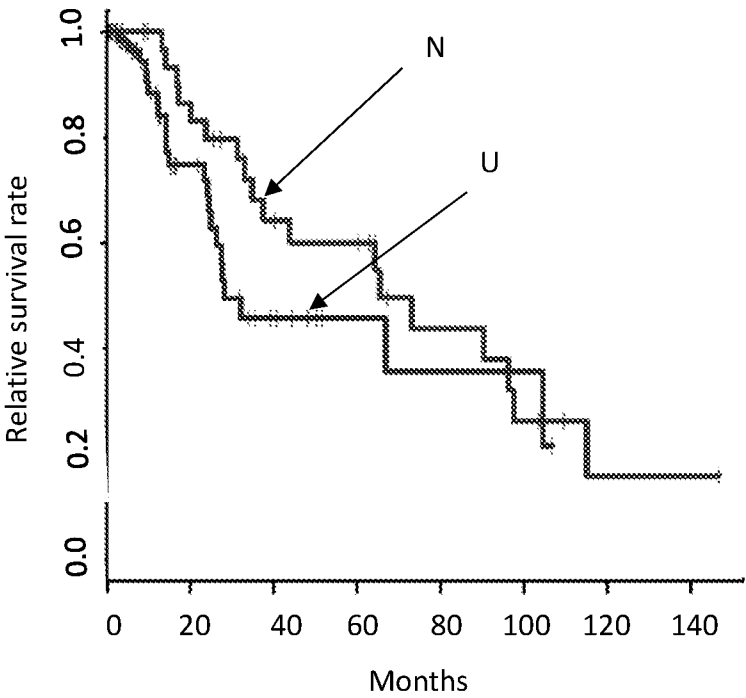
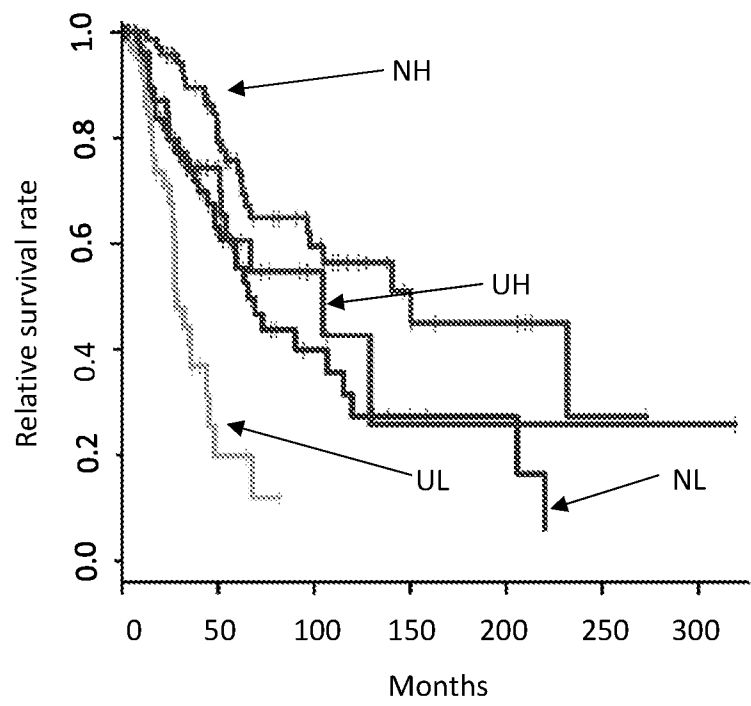
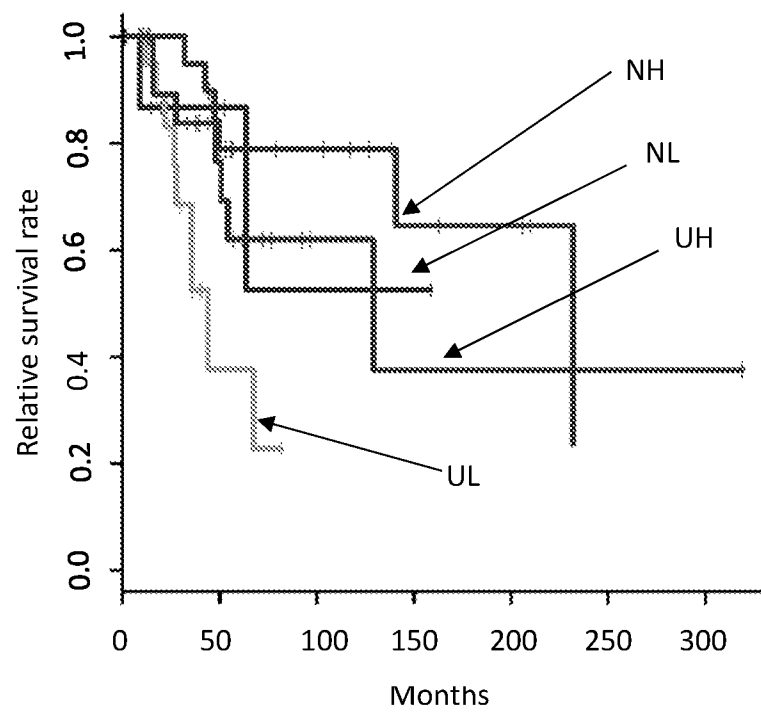
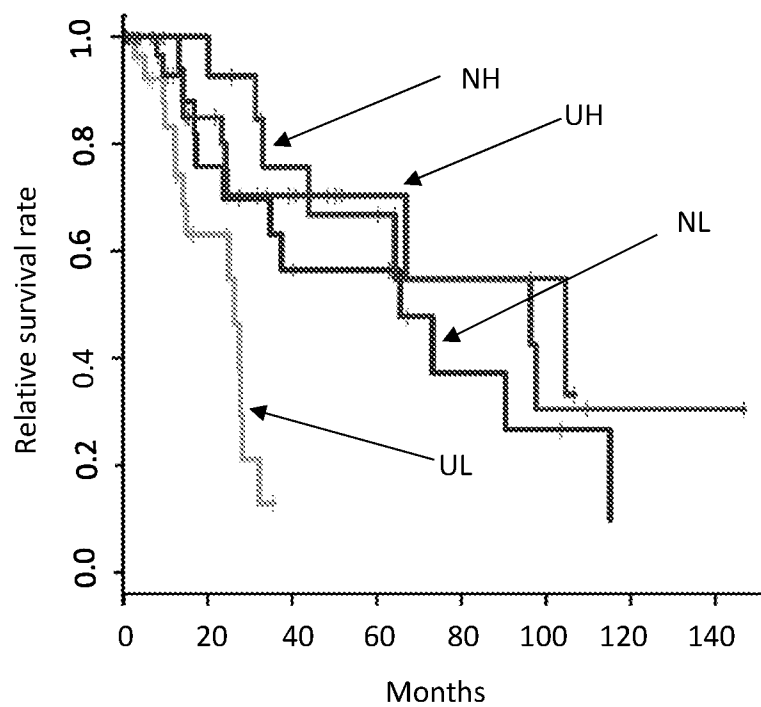


Figure 4C

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**Figure 5A**

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**Figure 5B****Figure 5C**

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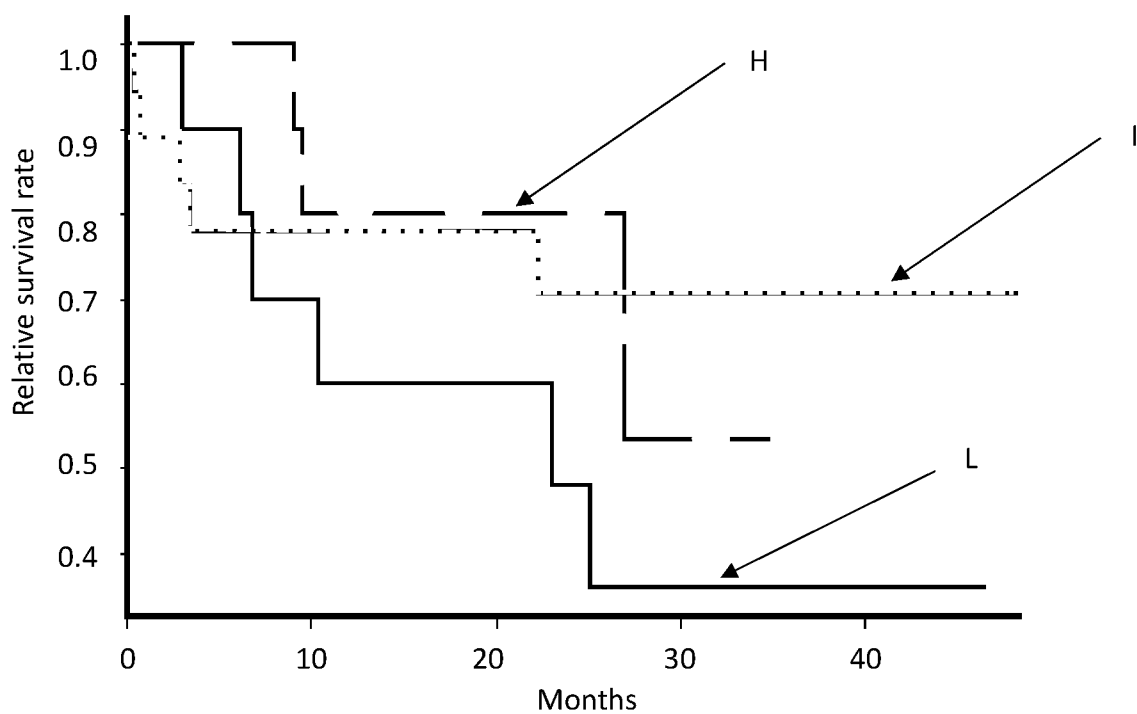


Figure 5D

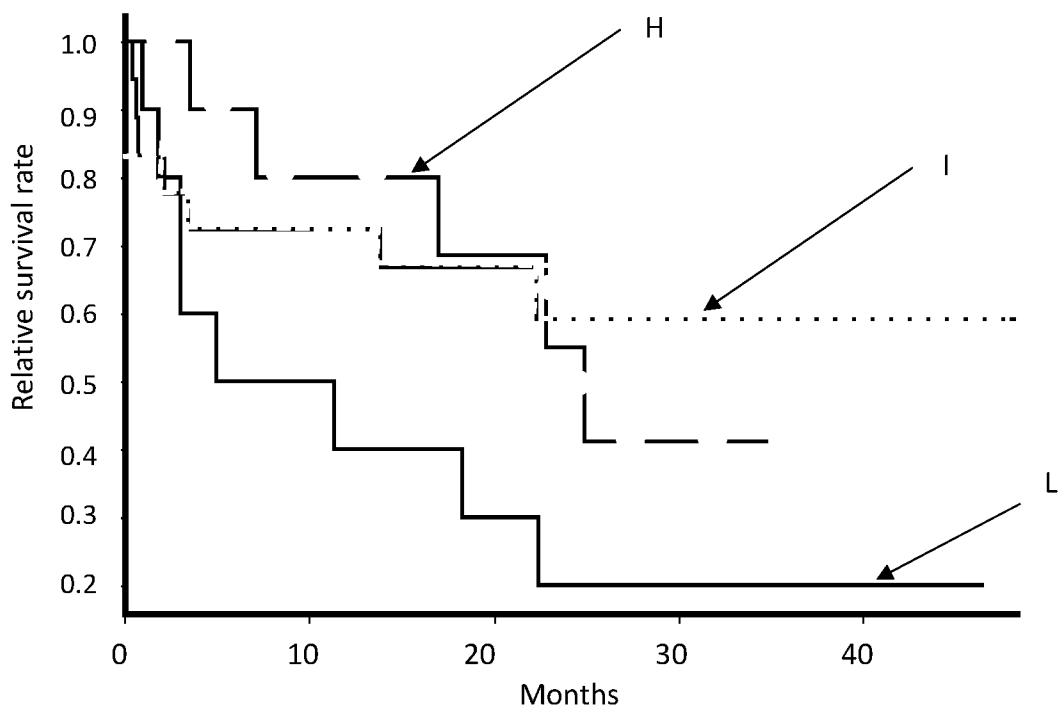


Figure 5E

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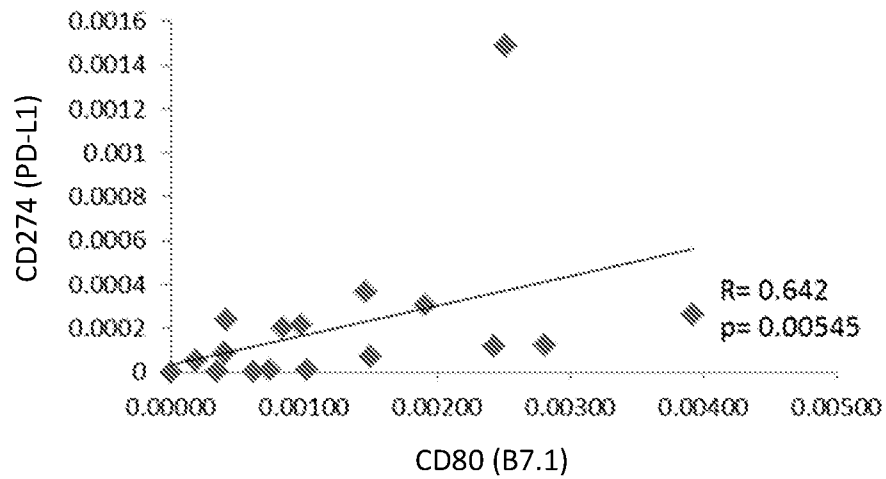


Figure 6A

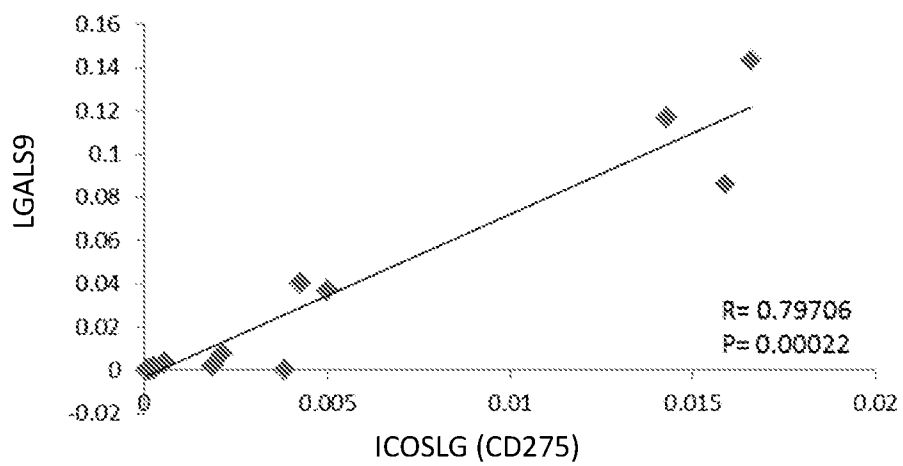


Figure 6B

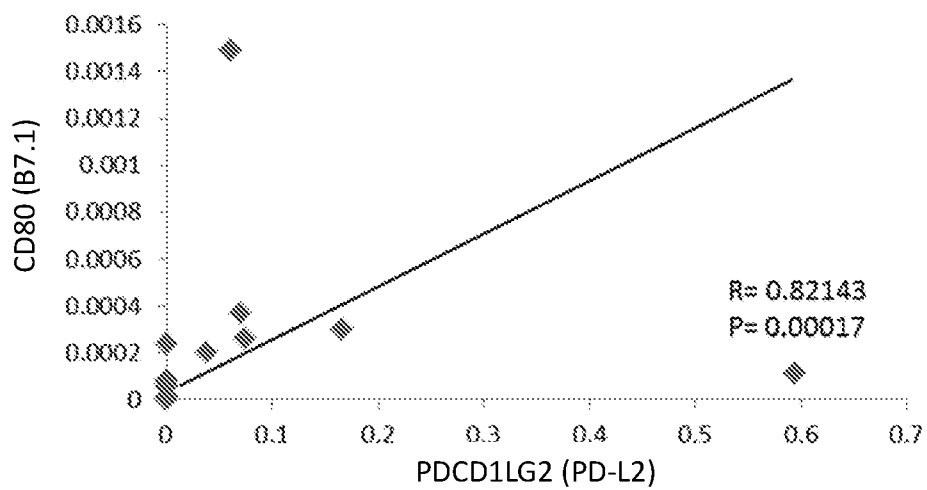


Figure 6C

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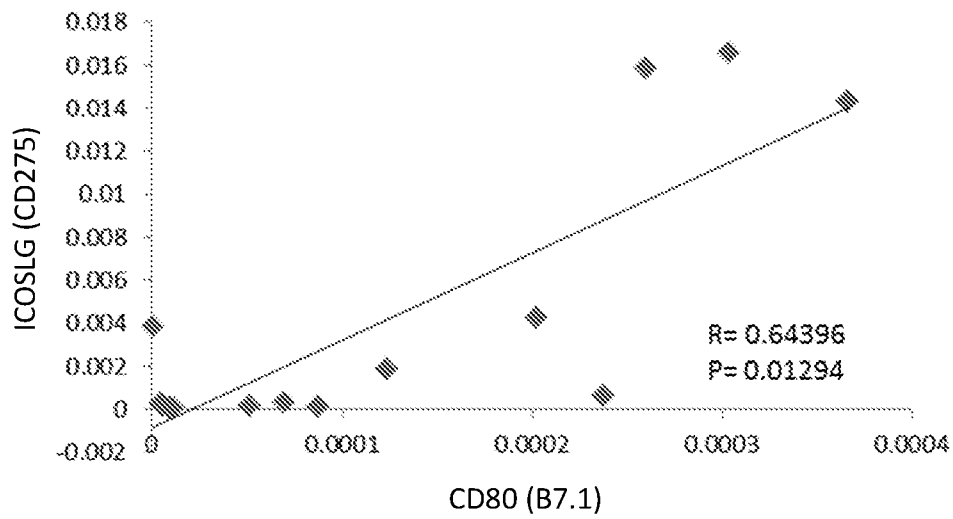


Figure 6D

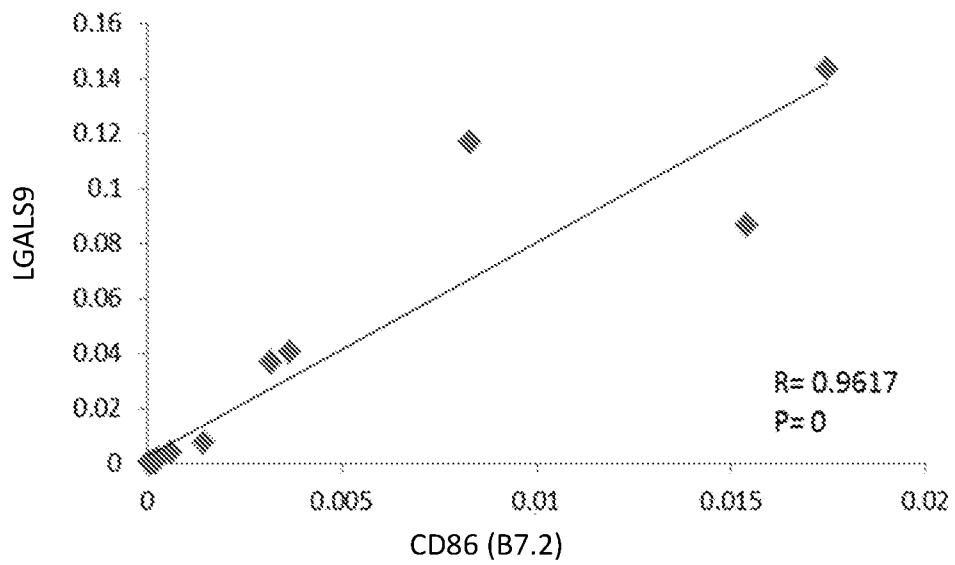


Figure 6E

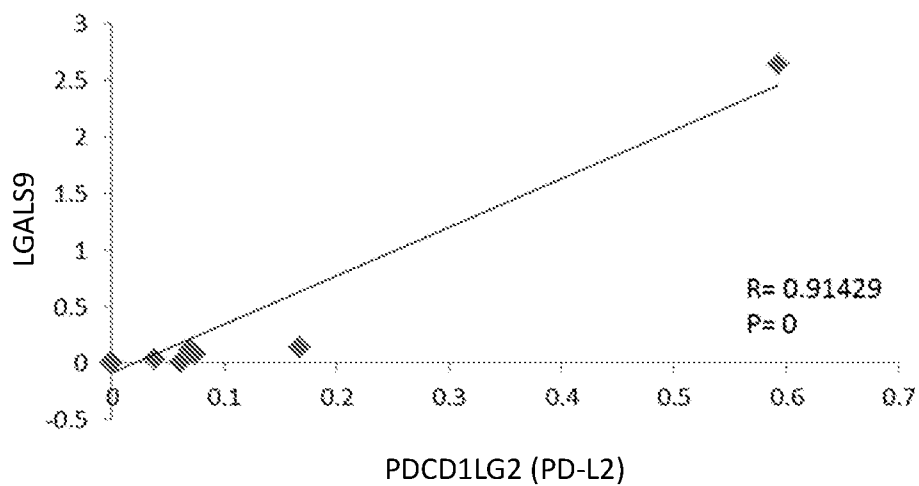


Figure 6F

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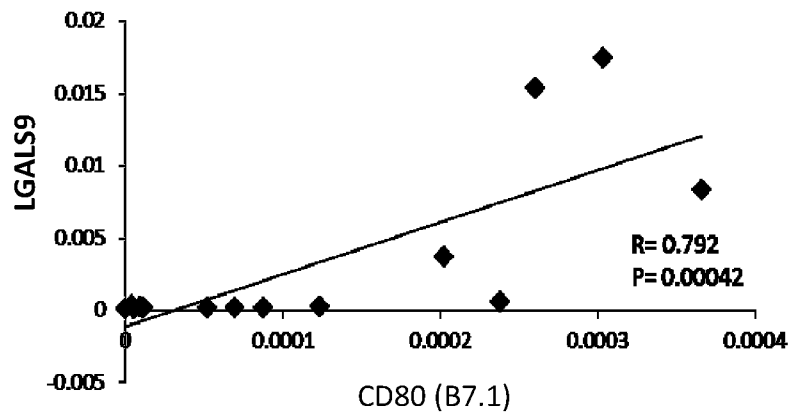


Figure 6G

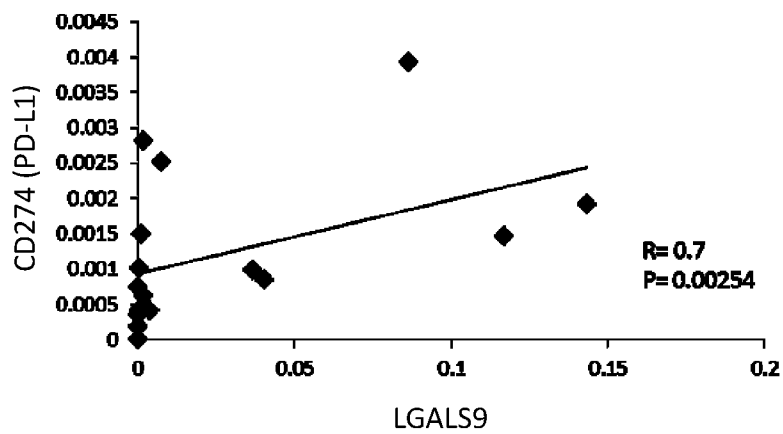


Figure 6H

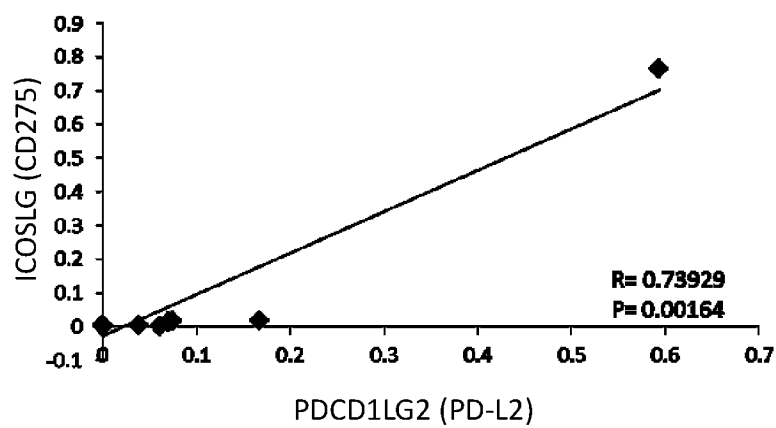


Figure 6l

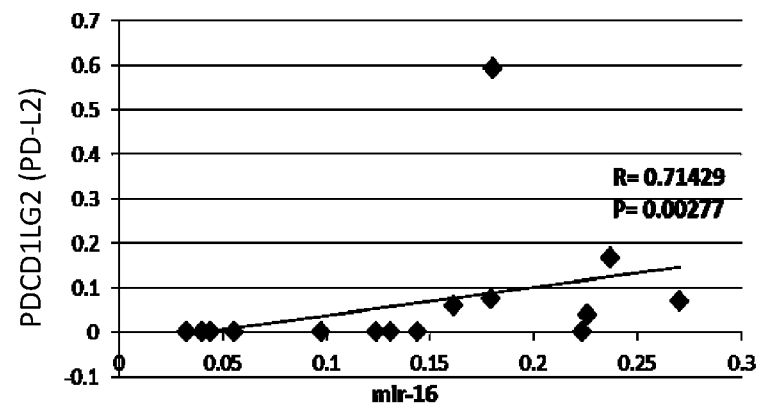


Figure 7A

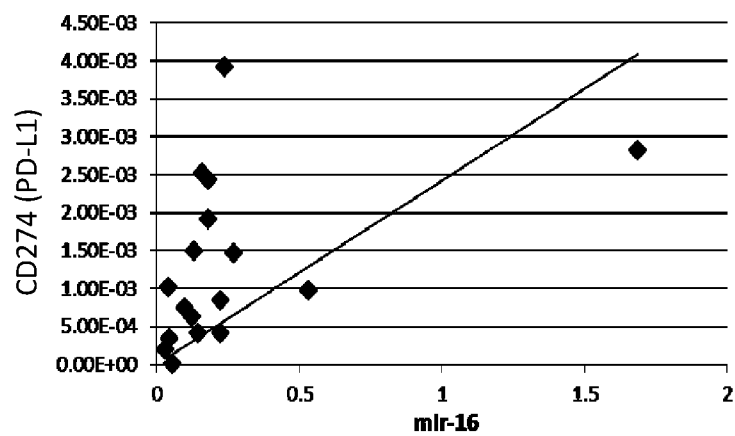


Figure 7B

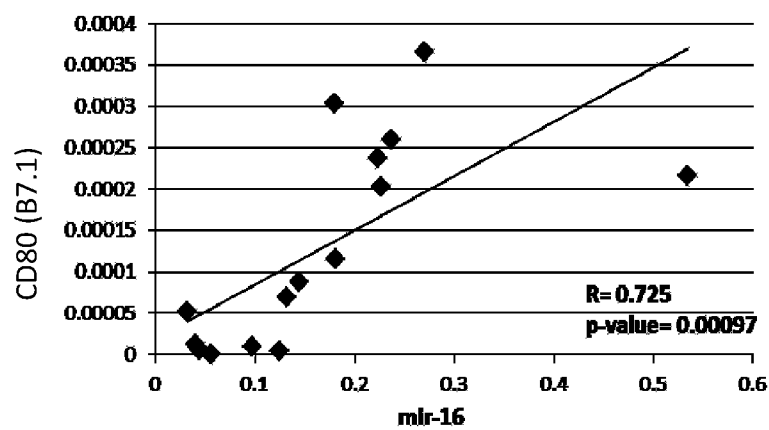


Figure 7C

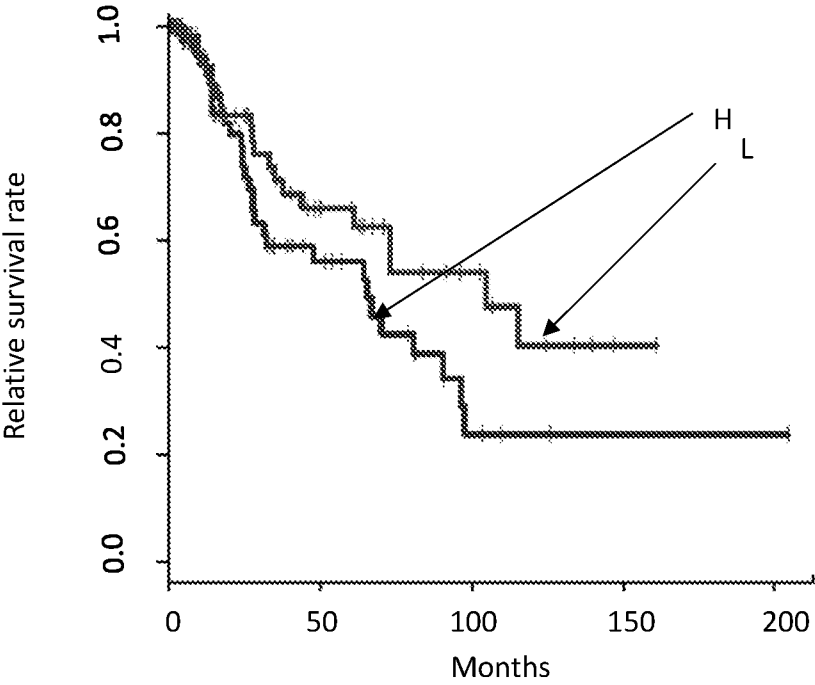


Figure 8

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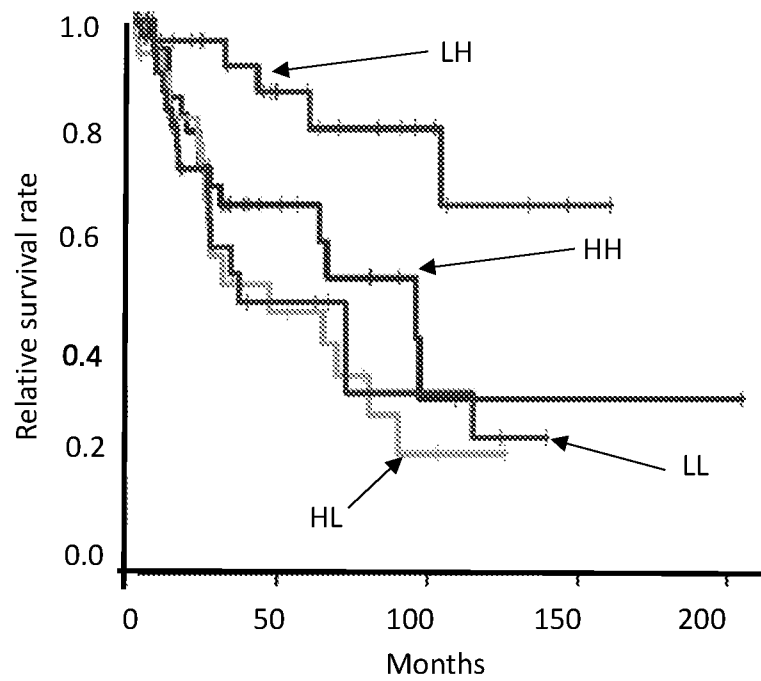


Figure 9A

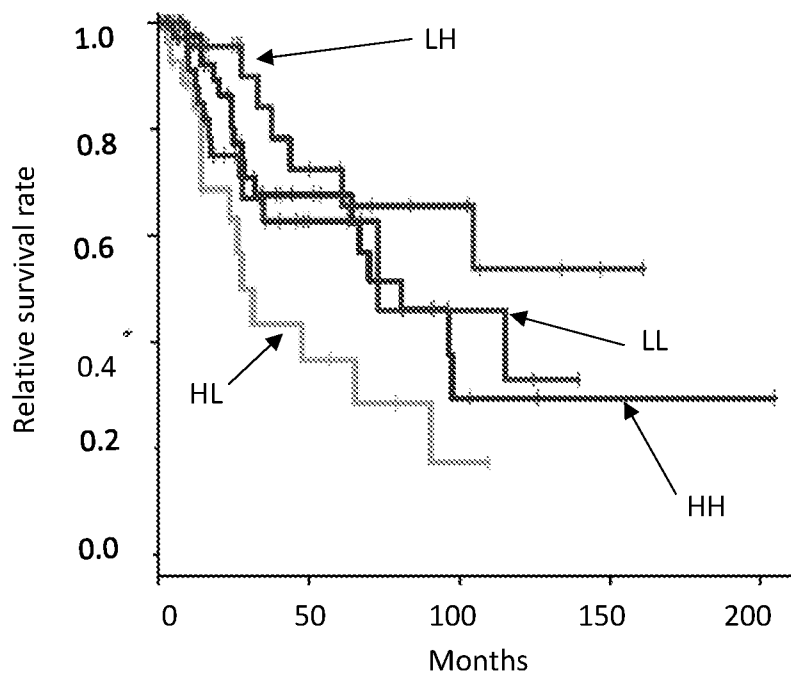


Figure 9B

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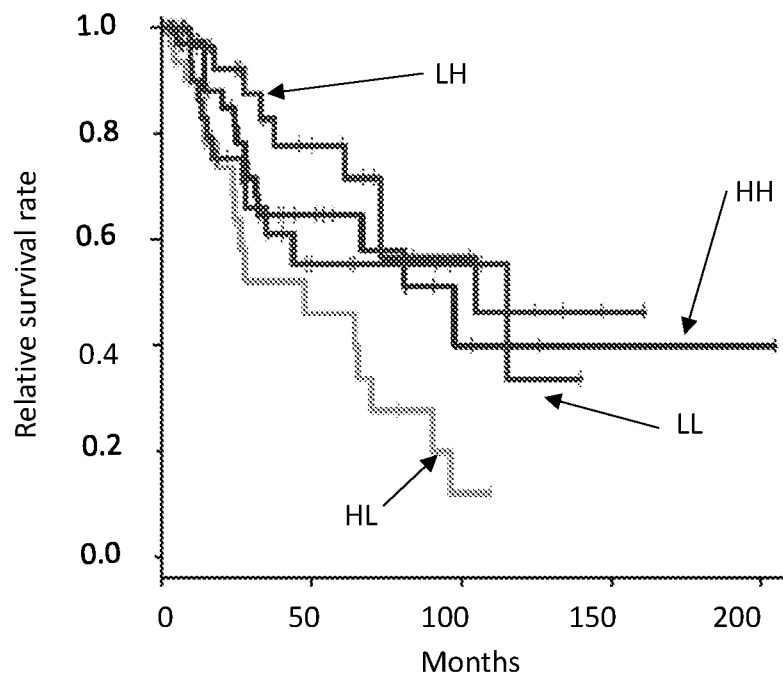


Figure 9C

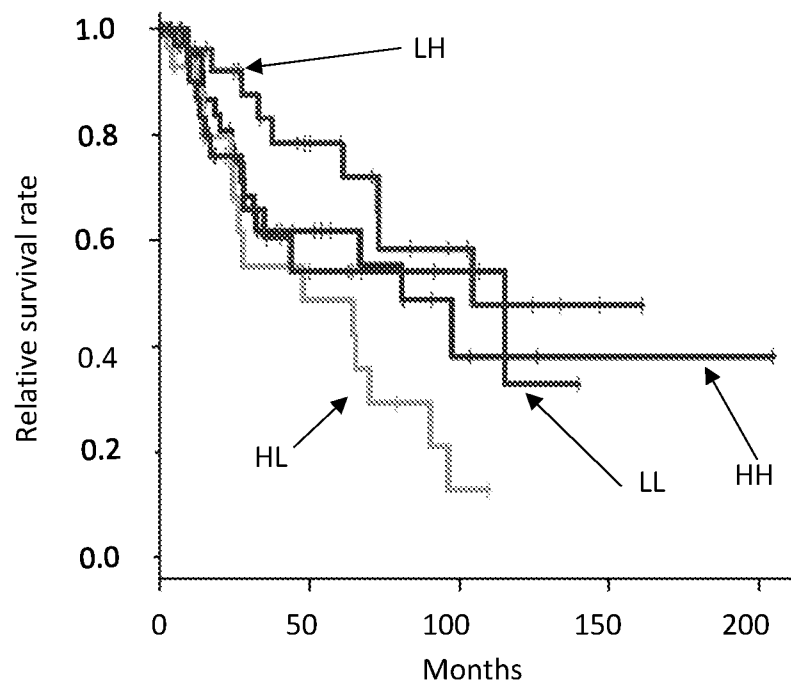


Figure 10A

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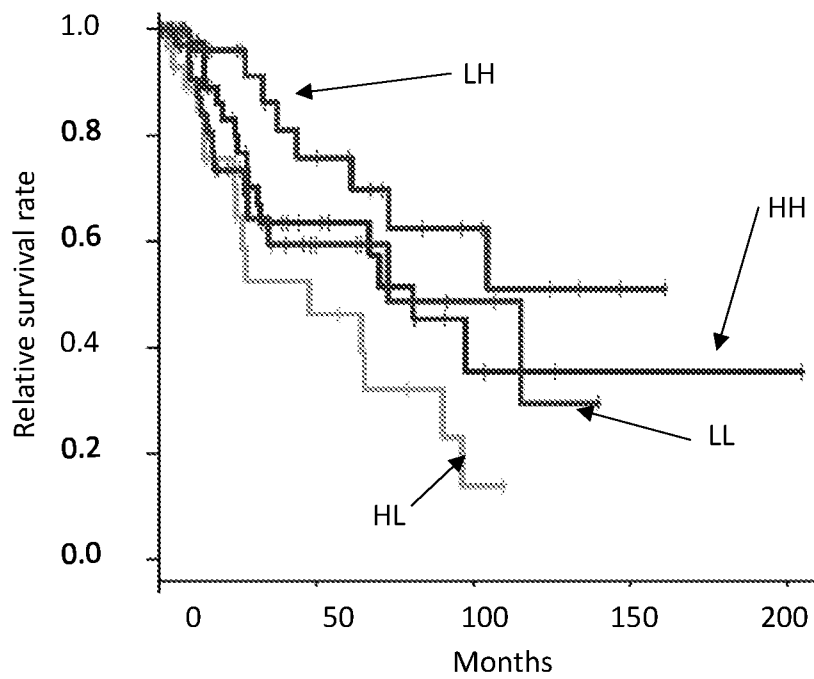


Figure 10B

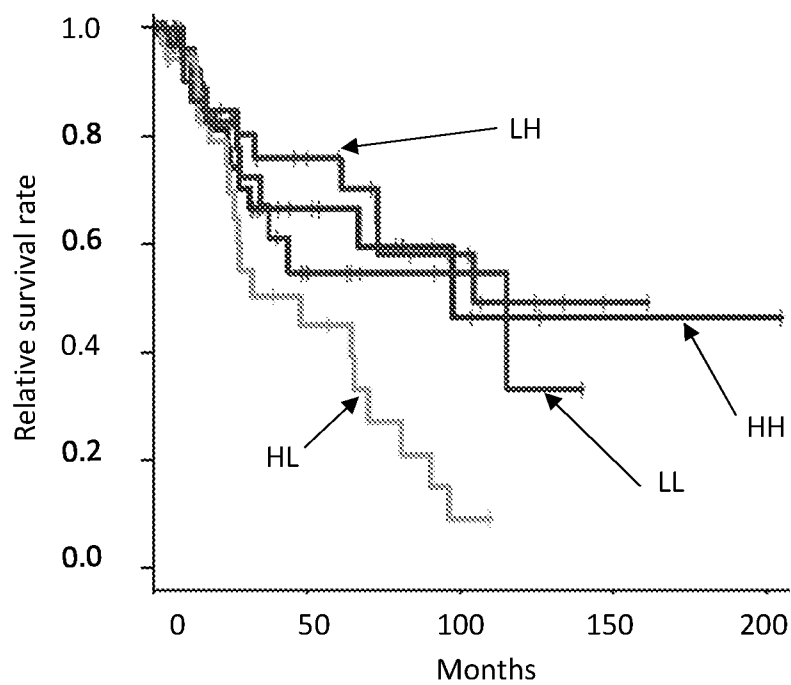


Figure 10C

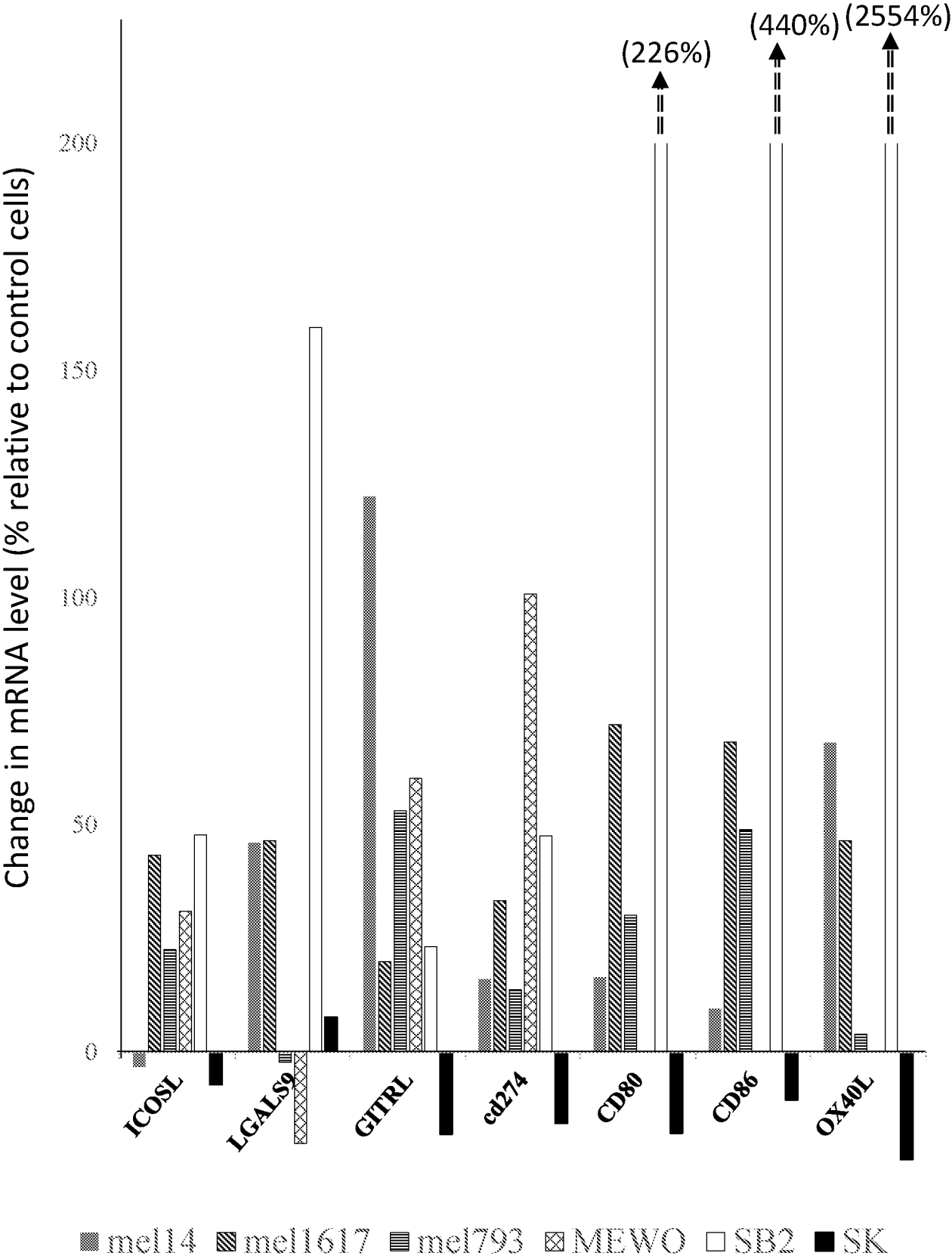
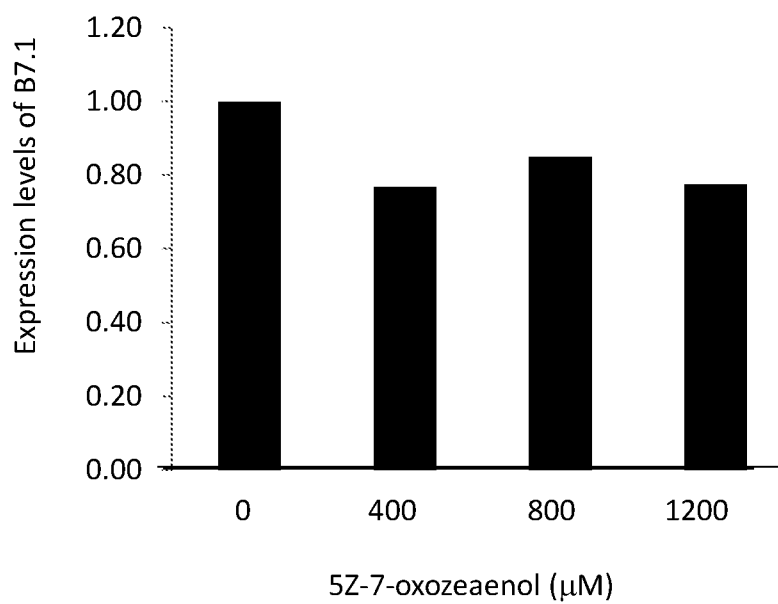
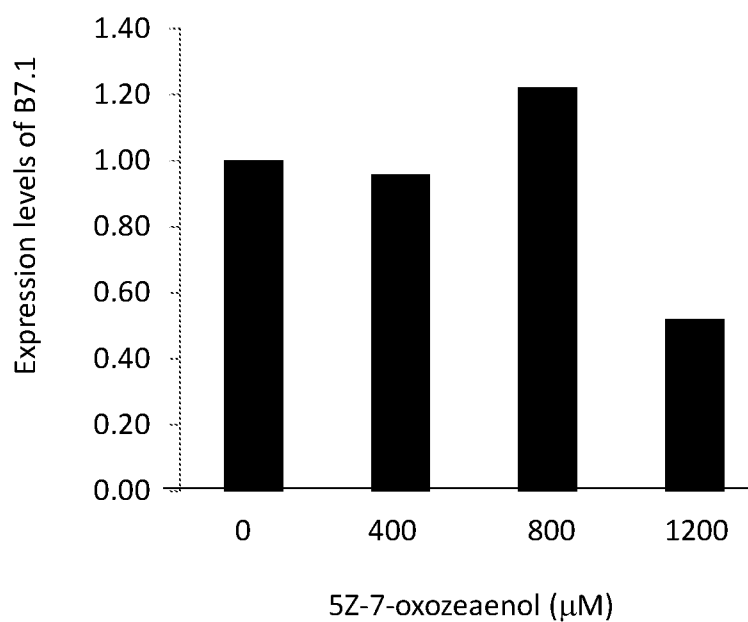


Figure 11

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**Figure 12****Figure 13**

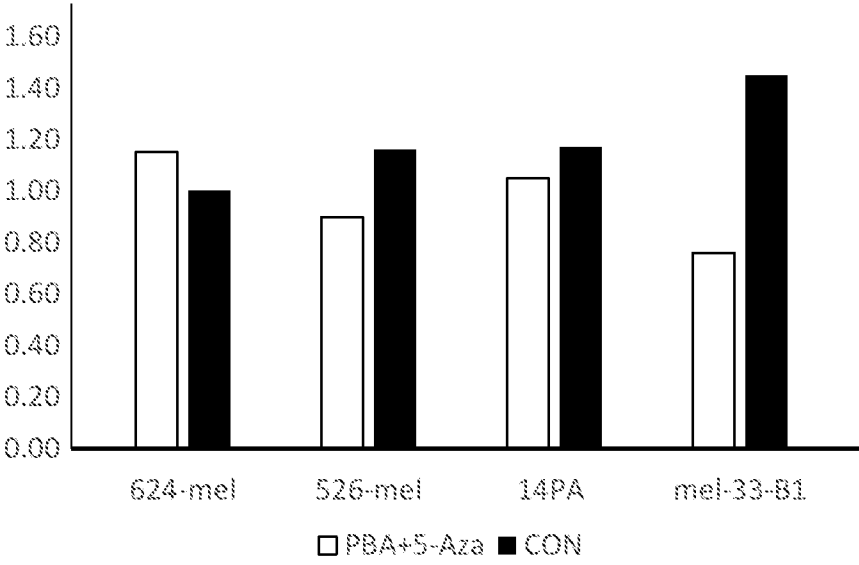


Figure 14

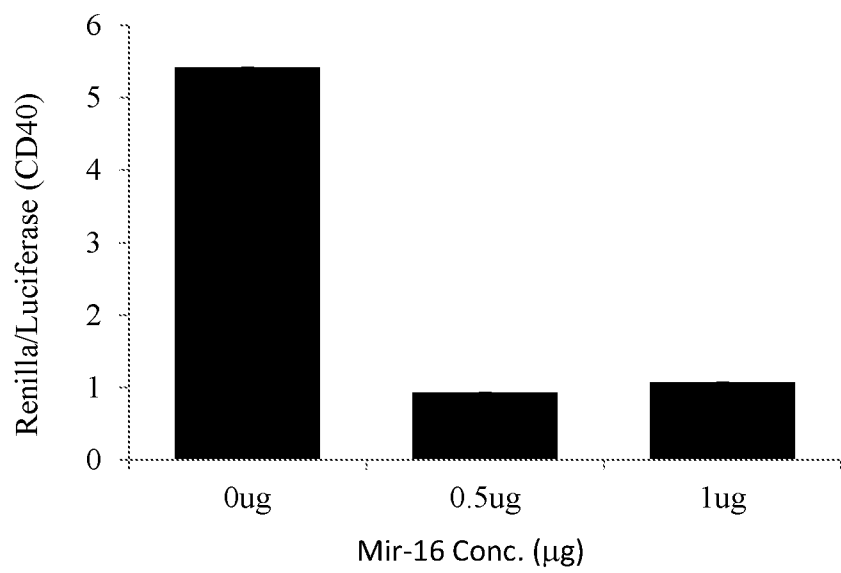


Figure 15A

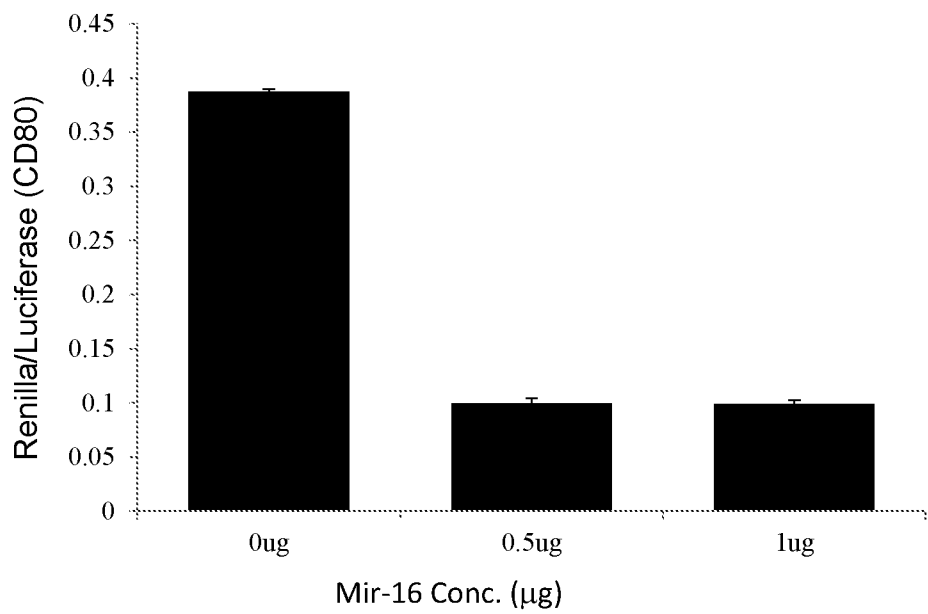


Figure 15B

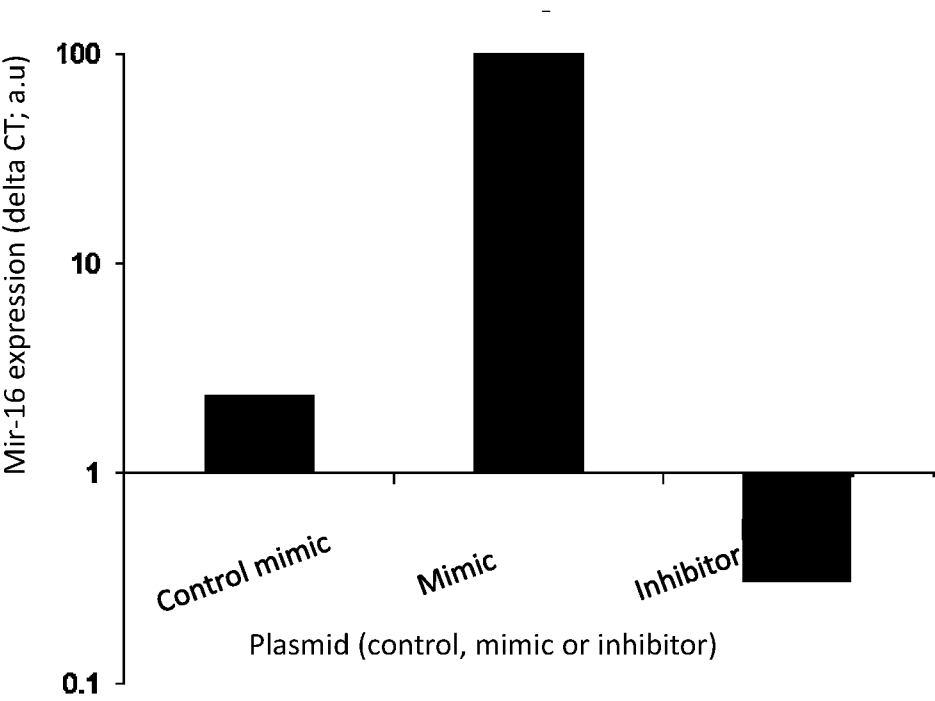


Figure 15C

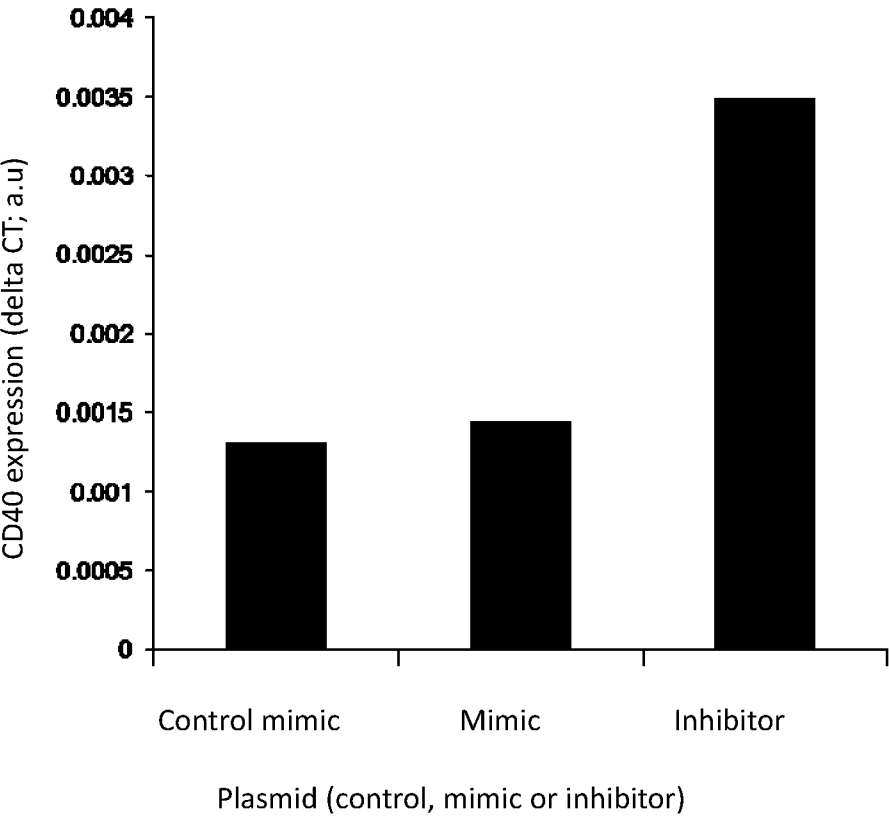


Figure 15D

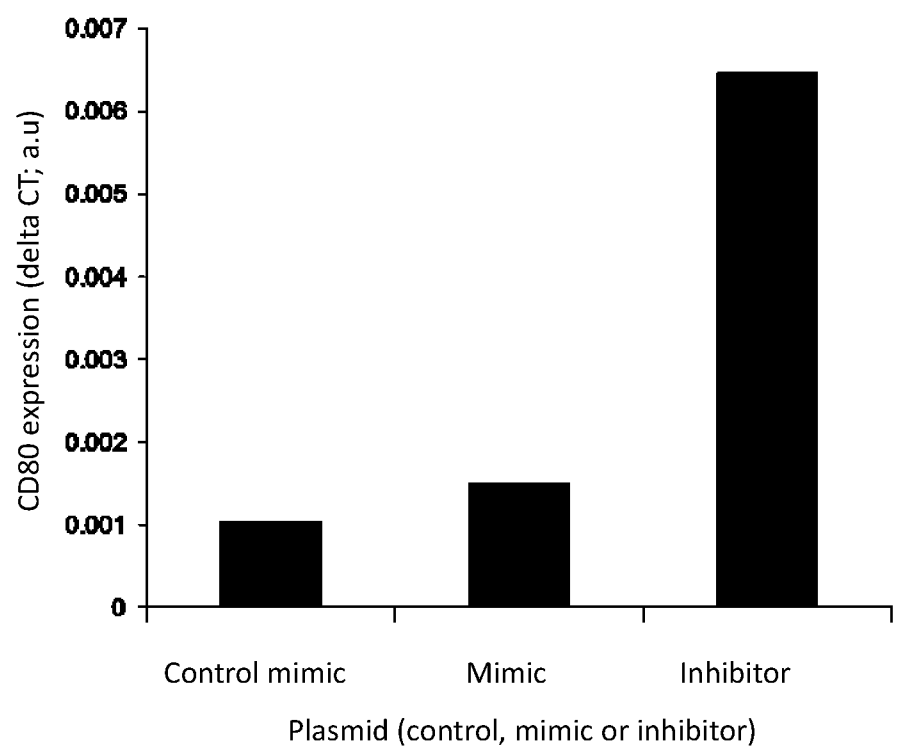


Figure 15E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2018/050367

A. CLASSIFICATION OF SUBJECT MATTER IPC (2018.01) A61K 39/395, A61P 35/00, C12Q 1/686, G01N 33/53 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) IPC (2018.01) A61K, A61P, C12Q, G01N 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) Databases consulted: Esp@cenet, Google Patents, CAPLUS, BIOSIS, EMBASE, MEDLINE, Google Scholar, DWPI, Derwent Innovation Search terms used: melanoma, immunotherapy, tnfsf4, ox40l, pd1, ctla-4, antibody, glycoprotein 34, treatment		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SNYDER, Alexandra, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. New England Journal of Medicine, 2014, 371.23: 2189-2199. https://www.nejm.org/doi/full/10.1056/NEJMoa1406498 04 Dec 2014 (2014/12/04) Abstract	1-7,9
A	DULOS, John, et al. PD-1 blockade augments Th1 and Th17 and suppresses Th2 responses in peripheral blood from patients with prostate and advanced melanoma cancer. Journal of immunotherapy, 2012, 35.2: 169-178. https://journals.lww.com/immunotherapy-journal/Abstract/2012/02000/PD_1_Blockade_Augments_Th1_and_Th17_and_Suppresses.8.aspx 01 Jan 2012 (2012/01/01) p. 4317	1-8,14,15,17
A	SIMEONE, Ester; ASCIERTO, Paolo A. Immunomodulating antibodies in the treatment of metastatic melanoma: the experience with anti-CTLA-4, anti-CD137, and anti-PD1. Journal of immunotoxicology, 2012, 9.3: 241-247. https://www.tandfonline.com/doi/full/10.3109/1547691X.2012.678021 17 Mar 2012 (2012/03/17) abstract	14-17
☒ Further documents are listed in the continuation of Box C. ☒ 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 16 Jul 2018		Date of mailing of the international search report 16 Jul 2018
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616		Authorized officer BENVENESTE - COHEN Sigalit SigalitB@justice.gov.il Telephone No. 972-2-5651724

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2018/050367

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-17

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2018/050367

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MELERO, Ignacio, et al. Evolving synergistic combinations of targeted immunotherapies to combat cancer. Nature Reviews Cancer, 2015, 15.8: 457. https://www.semanticscholar.org/paper/Evolving-synergistic-combinations-of-targeted-to-Melero-Berman/abf17d9398345400785bc035f46f11b650034235 01 Aug 2015 (2015/08/01) p. 457 right column 2nd paragraph, p.466 left column 2nd paragraph,	14-17
A	OTT, Patrick A.; HODI, F. Stephen; ROBERT, Caroline. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. 2013. http://clincancerres.aacrjournals.org/content/19/19/5300 30 Oct 2013 (2013/10/30) abstract, p.5307 left column last paragraph to right column first paragraph	14-17
A	MORRIS, Nicholas P., et al. Development and characterization of recombinant human Fc: OX40L fusion protein linked via a coiled-coil trimerization domain. Molecular immunology, 2007, 44, 12: 3112-3121. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1950941/ 31 May 2007 (2007/05/31) discussion p. 9 end	1-7
A	US 2015191525 A1 : EPSTEIN ALAN L?[US]; HU PEISHENG?[US] 09 Jul 2015 (2015/07/09) claims 1, 20, 34-35	1,6,7
A	AQEILAN, R. I.; CALIN, G. A.; CROCE, C. M. miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. Cell death and differentiation, 2010, 17.2: 215 05 Jun 2009 (2009/06/05) abstract	10,11,13
A	GUO, Sen, et al. Serum miR-16: a potential biomarker for predicting melanoma prognosis. Journal of Investigative Dermatology, 2016, 136.5: 985-993. https://www.sciencedirect.com/science/article/pii/S0022202X16003651 29 Jan 2016 (2016/01/29) abstract, results p. 986 left column, p. 988 suppressive role of mir-16,	10,11,13

International application No.
PCT/IL2018/050367

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
US 2015191525 A1	09 Jul 2015	US 2015191525 A1	09 Jul 2015

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2018/050367

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet):

* This International Searching Authority found multiple inventions in this international application, as follows:

Invention/s 1	Treatment of melanoma by comparing the level of TNFSF4 mRNA or protein, in a sample wherein, if it is above or below a reference value, mono-immunotherapy or combined immunotherapy is used, respectively.	Claim/s 1-17
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Invention/s 2	Treating melanoma by administering a compound that downregulates the expression of at least one micro RNA from the mir-15a/16-1 family.	Claim/s 18-25
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