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This invention provides methods for determining or predicting response to cancer therapy in an individual using differential image analysis of immunohistochemically stained tumor samples.

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(54) Title: METHOD AND QUANTIFICATION ASSAY FOR DETERMINING C-KIT/SCF/PAKT STATUS

(57) Abstract: This invention provides methods for determining or predicting response to cancer therapy in an individual using differential image analysis of immunohistochemically stained tumor samples.

METHOD AND QUANTIFICATION ASSAY FOR DETERMINING C-KIT/SCF/PAKT STATUS

This application claims priority to U.S. provisional application Serial no. 60/314,188, filed August 21, 2001.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to methods for determining or predicting response to cancer therapy in an individual. The invention also relates to methods for using image analysis of immunohistochemically-stained tissue biopsies to quantify c-kit/SCF/pAKT expression and activation, and to identify efficacious anticancer compounds.

2. Background of the Invention

A primary goal of cancer therapy is to selectively kill or inhibit uncontrolled growth of malignant cells while not adversely affecting normal cells. Traditional chemotherapeutic drugs are highly cytotoxic agents that preferably have greater affinity for malignant cells than for normal cells, or at least preferentially affect malignant cells based on their high rate of cell growth and metabolic activity. However, these agents often harm normal cells.

Generally, anticancer drugs, monoclonal antibodies, chemotherapeutic or chemopreventive agents are used to produce growth arrest, terminal differentiation and cell death of the cancerous or precancerous cells (Mendelsohn, 1990, *Semin. Cancer Biol.* 1:339-44; Hancock *et al.*, 1991, *Cancer Res.* 51:4575-80; Arteaga *et al.*, 1994, *Cancer Res.*, 54:3758-65; Pietras *et al.*, 1994, *Oncogene* 9:1829-38; Bacus *et al.*, 1997, *Anal. Quant. Cytol. Histol.* 19:316-28; Bacus *et al.*, 1999, *Breast J.*; Baselga *et al.*, 1999, Proceedings of AACR NCI EORTC International Conference, Abstract 98; Cobleigh *et al.*, 1999, *J. Clin. Oncol.*17:2639-48; DiGiovanna, 1999, *PPO Updates: Princ. Practice Oncol.* 13:1-9; Hortobagyi, 1999, *J. Clin. Oncol.* 17:25-29; Shak, 1999, *Semin. Oncol.* 26:71-77; Sliwkowski *et al.*, 1999, *Semin. Oncol.* 26:60-70; Vincent *et al.*, 2000, *Cancer Chemother. Pharmacol.* 45: 231-38). Drug-induced growth arrest or cell death is often characterized by morphological and biochemical changes associated with programmed cell death or terminal differentiation (as opposed to mitotic cell death).

Although chemotherapeutic drugs can be administered at doses high enough to bring about cell death, such doses typically produce deleterious effects on normal as well as tumor cells. Differentiating agents, and the use of lower doses of chemotherapeutic drugs and agents frequently results in growth arrest rather than cell death. Such growth arrest can be followed by apoptosis and cell death, or continued proliferation once the chemotherapeutic drugs are withdrawn. Administration of cytotoxic and chemotherapeutic drugs or ionizing radiation may also induce transient growth arrest, a state that depends largely on the function of p53 and p53-regulated cyclin-dependent kinase inhibitors (such as p16, p27, and p19) or growth inhibitors (such as TGF-β, IL-4, and IL-6). Upon removal of the chemotherapeutic drug, cells subjected to the drug treatment will eventually resume division and either continue to proliferate or die. Some drug-treated tumor cells undergo prolonged growth arrest and fail to resume cell division upon release from the drug.

Apoptosis is generally regarded as an active suicide response to various physiological or pathological stimuli. Recent studies have shown that a variety of DNA-damaging agents, including X-ray irradiation and several chemotherapeutic drugs (*e.g.*, alkylating agents and topoisomerase II inhibitors) initiate pathways leading to apoptosis. The exact mechanism by which apoptosis is induced by these agents is not yet known. However, expression of the tumor suppressor gene p53 has been implicated in this process (Kwok and Sutherland, 1989, *J. Natl. Cancer Inst.* 81:1020-24; Kwok and Sutherland, 1991, *Int. J. Cancer* 49:73-76; Lane, 1992, *Nature* 358:15-16; Kuerbitz *et al.*, 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:7491-95; Luo *et al.*, 1995, *Nature* 375:159-61; Liu *et al.*, 1996, *Cancer Res.* 56:31-35; Mellinghoff and Sawyers, 2000, *PPO Updates* 14:1-11). In addition, the up-regulation of caspases (*e.g.*, caspase 9 or caspase 3) or their chaperone molecules (*e.g.*, heat shock protein 60) has been associated with apoptosis.

Cells can be rendered resistant to apoptosis in a variety of ways, including deletion of cellular genes such as the tumor suppressor gene PTEN, overexpression of active Ras, and overexpression of active PI3K. A particular cellular protein, AKT (the protein product of the *c-akt* gene) has been identified as a key regulator of cellular survival and an inhibitor of apoptosis, which has significant implications for oncogenesis and drug resistance. For example, the loss of PTEN correlates with increased AKT activity (Li *et al.*, 1997, *Science* 275:1943-47; Liaw *et al.*, 1997, *Nat. Genet.* 16:64-67; Nelen *et al.*, 1997, *Hum. Mol. Genet.* 6:1383-87; Cantley and Neel, 1999, *Proc. Natl. Acad. Sci. U.S.A.* 96:4240-45; Datta *et al.*, 1999, *Genes Dev.* 13:2905-27). In addition,

suppression of apoptosis is not the only function that AKT may have in promoting oncogenesis. In some circumstances, AKT can also induce cell cycle progression. However, the observation that AKT can suppress apoptosis suggests that oncogenes may block adaptive cellular apoptosis by hyperactivating AKT.

Given the complexity of the apoptotic machinery, there are a number of pathways by which AKT might act to promote cell survival and inhibit cell death. AKT may block apoptosis by regulating expression or activity of members of *Bcl-2* gene family (that are known to play a role in cell survival or cell death). Alternatively, AKT may regulate expression or activity of the caspase family of proteins, or the function of death receptor pathways. The regulatory effect of AKT may be through a direct mechanism – the phosphorylation of components of the apoptotic machinery, *for example* – or an indirect mechanism – such as by altering the expression level of genes that encode components of the death machinery. Recent studies suggest that AKT regulates apoptosis at multiple sites. A number of AKT targets, all playing critical roles in mediating cell death, have been identified, including BAD, caspase-9, the Forkhead family of transcription factors, and the NFκB regulator IKK (Datta *et al.*, 1999, *supra*).

The c-*kit* proto-oncogene encodes a transmembrane tyrosine kinase growth factor receptor that is placed in the same class as the receptors for PDGF and CSF-1 by virtue of their similar immunoglobulin-like extracellular domains and the interruptions of their cytoplasmic tyrosine kinase domain by a hydrophilic insert. (Yarden *et al.*, 1987, *EMBO J.* 6:3341-3351). Its ligand, known alternatively as stem cell growth factor ("SCF"), mast cell growth factor, kit ligand, or steel factor, is an early hematopoietic growth factor that, in conjunction with other growth factors, supports the proliferation and differentiation of multiple hematopoietic lineages. *Id.*

In several small-cell lung cancer cell lines and breast cancer cell lines, it has been demonstrated that co-expression of c-*kit* and SCF occurs, suggesting that autocrine growth stimulation may play a role in non-hematopoietic tumors. Autocrine growth requires co-expression of SCF and c-*kit*. In two independent studies, immunostaining of frozen sections using polyclonal antisera directed against the carboxyl terminus of c-*kit* has demonstrated uniformly strong staining of normal breast ductal epithelial cells. These studies also demonstrated that, using the same methodology, at least 10-20% of breast carcinomas retain c-*kit* expression. Neither of these studies, however, addressed the expression of SCF in breast tumors. It is possible, therefore, that the co-expression of c-*kit* and SCF could play a role in the growth regulation of some breast tumors. (Hines, *et*

al., 1995, *Cell Growth & Differentiation*, 6:769-779; Natali *et al.*, 1992, *Int. J. Cancer*, 52:713-717; Chui *et al*, 1996, *British J. of Cancer*, 73:1233-1236). In addition, co-expression of c-*kit* and other growth factors in breast cancer results in enhanced sensitivity to the EGF family of growth factors. (Hines *et al.*, 1999, *Breast Cancer Research and Treatment* 58: 1-10).

Therefore, there exists a need in the art for improved detection of expression and activation of proteins and ligands that are directly or indirectly responsible for the expression or activation of AKT. There is also a need for improved detection of expression and activation of c-*kit* and expression of its ligand SCF.

SUMMARY OF THE INVENTION

This invention provides reagents and methods for identifying and detecting expression or activation of biological markers of tumorigenesis in cells and tissue samples from cancer patients. The methods provided herein are useful for predicting or assessing a response of an individual cancer patient to a particular treatment regimen, and methods useful for identifying efficacious anticancer compounds.

In a first aspect, the invention provides methods for assessing a response to administration of a chemotherapeutic or chemopreventive agent to an individual, comprising:

(a) obtaining a first tissue or cell sample from the individual before exposing the individual to the chemotherapeutic or biological therapeutic agent;

(b) obtaining a second tissue or cell sample from the individual after exposing the individual to the chemotherapeutic or biological therapeutic agent;

(c) detecting the amount of one or a plurality of biological markers in the first tissue or cell sample and in the second tissue or cell sample;

(d) comparing the amount of the one or a plurality of biological markers in said first tissue or cell sample with the amount in said second tissue or cell sample;

(e) determining whether expression or activation of the one or a plurality of biological markers decreases following exposure to the chemotherapeutic or biological therapeutic agent when the amount of the one or a plurality of biological markers in the second tissue sample is less than the amount of the one or a plurality of biological markers in the first tissue sample;

wherein a response to the administration of a chemotherapeutic or biological therapeutic agent to an individual is assessed.

In certain embodiments, the one or a plurality of biological markers are expressed from tumor related genes. In other embodiments, the one or a plurality biological markers are cellular components of a tumor-related signaling pathway. In yet other embodiments, the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*. In certain embodiments, the method of detecting the amount of the one or a plurality of biological markers comprises using an antibody or antibodies that are immunologically specific for the one or a plurality of biological markers. In other embodiments, the amount of one or a plurality of biological markers are determined immunohistochemically.

In yet other embodiments, the method of detecting the amount of one or a plurality of biological markers comprises,

(a) staining the first and second tissue or cell samples using one or a plurality of antibodies detectably-labeled with an optical density label, wherein at least one antibody is immunologically specific for one or a plurality of biological markers, wherein the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*; and

(b) determining an optical density of the stained first and second tissue sample in subpart (a);

wherein said optical density corresponds to the amount of said one or a plurality of biological markers.

In other embodiments, the optical density is determined using image analysis. In further embodiments, the detectable label is a chromagen or a fluorophore. In yet further embodiments, the detectable label is DAB. In other embodiments, the agent is Glivac™.

In a second aspect, the invention provides methods for predicting the response to administration of a chemotherapeutic or chemopreventive agent to an individual, comprising:

(a) obtaining a tissue or cell sample from the individual before exposing the individual to the chemotherapeutic or biological therapeutic agent;

(b) detecting the amount of one or a plurality of biological markers in the tissue or cell sample;

(c) comparing the amount of one or a plurality of biological markers determined in subpart (b) with the amount of one or a plurality of samples

expressing known amounts of the markers in one or a plurality of control samples; and

(e) determining the expression or activation level of the one or a plurality of biological markers compared with the expression or activation level markers in one or a plurality of control samples;

wherein a response to the administration of a chemotherapeutic or biological therapeutic agent to an individual is predicted based on the determination of decreased expression or activation level of the one or a plurality of biological markers.

In certain embodiments, the one or a plurality of biological markers are expressed from tumor related genes. In other embodiments, the one or a plurality biological markers are cellular components of a tumor-related signaling pathway. In yet other embodiments, the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*. In certain embodiments, the method of detecting the amount of the one or a plurality of biological markers comprises using an antibody or antibodies that are immunologically specific for the one or a plurality of biological markers. In other embodiments, the amount of one or a plurality of biological markers are determined immunohistochemically.

In yet other embodiments, the method of detecting the amount of one or a plurality of biological markers comprises,

(a) staining the first and second tissue or cell samples using one or a plurality of antibodies detectably-labeled with an optical density label, wherein at least one antibody is immunologically specific for one or a plurality of biological markers, wherein the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*; and

(b) determining an optical density of the stained first and second tissue sample in subpart (a);

wherein said optical density corresponds to the amount of said one or a plurality of biological markers.

In other embodiments, the optical density is determined using image analysis. In further embodiments, the detectable label is a chromagen or a fluorophore. In yet further embodiments, the detectable label is DAB. In other embodiments, the agent is Glivac™.

In a third aspect, the invention provides methods for identifying a compound as a chemotherapeutic or biological therapeutic agent, comprising:

(a) obtaining a first tissue or cell sample;

(b) obtaining a second tissue or cell sample;

(c) exposing the second tissue or cell sample to said compound;

(d) detecting the amount of one or a plurality of biological markers in the first tissue or cell sample and the second tissue or cell sample;

(e) comparing the amount of the one or a plurality of biological markers in said first tissue or cell sample and second tissue or cell sample;

(d) (f) determining whether expression or activation of the one or a plurality of biological markers decreases following exposure to said compound when the amount of the one or more biological markers in the second tissue or cell sample is less than the amount of the one or a plurality of biological markers in the first tissue or cell sample;

wherein the compound is identified as a chemotherapeutic or biological therapeutic agent.

In certain embodiments, the one or a plurality of biological markers are expressed from tumor related genes. In other embodiments, the one or a plurality biological markers are cellular components of a tumor-related signaling pathway. In yet other embodiments, the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*. In certain embodiments, the method of detecting the amount of the one or a plurality of biological markers comprises using an antibody or antibodies that are immunologically specific for the one or a plurality of biological markers. In other embodiments, the amount of one or a plurality of biological markers are determined immunohistochemically.

In yet other embodiments, the method of detecting the amount of one or a plurality of biological markers comprises,

(a) staining the first and second tissue or cell samples using one or a plurality of antibodies detectably-labeled with an optical density label, wherein at least one antibody is immunologically specific for one or a plurality of biological markers, wherein the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*; and

(b) determining an optical density of the stained first and second tissue sample in subpart (a);

wherein said optical density corresponds to the amount of said one or a plurality of biological markers.

In other embodiments, the optical density is determined using image analysis. In further embodiments, the detectable label is a chromagen or a fluorophore. In yet further embodiments, the detectable label is DAB.

This invention provides reagents and methods for detecting expression and activation of proteins and ligands that are directly responsible for inducing c-*akt* gene expression and/or activating AKT protein. Disclosed herein is the discovery that the c-*kit* proto-oncogene and its ligand, stem cell growth factor ("SCF" or "SCGF") are co-expressed in various amounts in breast cancers and that c-*kit* activation results in production of phosphorylated AKT (pAKT).

The invention also provides diagnostic methods for detecting and measuring c-*kit*, SCF, pc-*kit* and pAKT expression and protein levels and detecting and measuring their activation states for the treatment of tumors. The invention provides reliable assays for determining c-*kit*, SCF, pc-*kit* and pAKT protein levels and their activation state in cells or tissue samples obtained from patients that also permits a pathologist to exclude normal tissue from the analysis. Moreover, the invention provides methods for determining whether a cancer patient will benefit from a course of treatment using therapeutic agents directed at components of the c-*kit* signaling pathways. The invention further provides methods for monitoring a course of treatment using therapeutic agents directed at components of the c-*kit* signaling pathway. The invention also provides therapeutic agents identified by the inventive methods.

Specific embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DESCRIPTION OF THE DRAWINGS

Figures 1A through 1D illustrate an immunohistochemical analysis of a human breast tissue sample identified by No. 12669b9. Figure 1A shows a photomicrograph of the sample tissue immunohistochemically stained with an antibody immunohistochemically-specific for SCF, as described in Example 2, at 400x magnification using a technique described in Example 5. Figure 1B shows the same breast cancer sample stained with an antibody to c-*kit* (400x), as described in Example 1. Figure 1C shows a different field of the same cancer tissue stained for SCF (400x). Figure 1D shows another field stained for c-*kit* (400x).

Figures 2A through 2E show the results of immunohistochemical analyses of tumor samples. Figure 2A is a tumor stained for pAKT. Figure 2B show tumor sample 747 stained for c-*kit*. Figure 2C shows sample 848 stained for SCF. Figure 2D and 2E

are different fields of tumor sample 920 stained for either c-*kit* (Figure 2D) or SCF (Figure 2E).

Figure 3 is a bar graph showing the levels of c-kit, SCF, and pAKT measured by immunohistochemistry, combined with image analysis. The bar graph is expressed in units of optical density/pixel.

Figure 4 is photograph of a western blot showing c-*kit* activation in breast cancer cells treated with SCF ligand in the presence of absence of STI-571 (Glivac, obtained from Novartis Pharmaceuticals). pc-*kit* was immunoprecipitated from MDA-MB-361 cell lysates (50 μg total protein), separated by gel electrophoresis and screened with anti-phosphotyrosine antibodies to detect active c-*kit* receptor. As expected, the c-*kit* receptor was activated by SCF treatment, but inhibited with as little as 1 μM STI-571.

Figure 5 is a photograph of a western blot of c-*kit* prepared from HeLa cells. Rabbit polyclonal antibodies against c-*kit* were obtained from NeoMarkers (1:200 dilution) and DAKO (1:400 dilution), and detected using HPR-conjugated antibodies against rabbit IgG (Amersham) and chemiluminescent substrate (NEN).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides methods for quantitatively determining expression and activation levels of tumor-related genes. The invention specifically provides methods for quantitatively determining expression and activation levels for cellular proteins encoded by c-*kit*, SCF and AKT, in tumor cells, including human tumor cells, as detected in cell or tissue samples from an individual.

c-*kit* and its ligand SCF are co-expressed in various amounts in breast and bladder cancers, and the downstream signal associated with the c-*kit* activation by SCF is phosphorylation of substrate AKT to phosphorylated AKT (pAKT). According to the invention, expression and activation of c-*kit*, SCF, and AKT are analyzed and monitored to identify patients that can benefit from treatment with a chemotherapeutic or biological therapeutic agent, to monitor patients treated with such agents, or to identify chemotherapeutic or biological therapeutic agents. For example, quantitative image analysis together with immunohistochemistry are used according to the invention to

analyze expression of these three tumor markers, in order to identify patients that can benefit from treatment with a chemotherapeutic or biological therapeutic agent, including but not limited to tyrosine kinase inhibitors. In particular embodiments, such tyrosine kinase inhibitors include but are not limited to STI-571, which is known to inhibit c-*kit* kinase. (Drucker, 2001, *Am. Soc. Of Clinical Oncology*, 2001 Educational Book 37th Annual Meeting, San Francisco, CA).

In contrast to traditional anticancer methods, where chemotherapeutic drug treatment is undertaken as an adjunct to and after surgical intervention, neoadjuvant (or primary) chemotherapy consists of administering drugs as an initial treatment in cancer patients. One advantage of such an approach is that, for primary tumors of more than 3 cm, it permits the use of conservative surgical procedures (as opposed to, *e.g.*, radical mastectomy in breast cancer patients) for the majority of patients. Another advantage is that for many cancers, a partial and/or complete response is achieved in about two-thirds of all cases. Finally, because the majority of patients are responsive after two to three cycles of chemotherapeutic treatment, it is possible to monitor the *in vivo* efficacy of the chemotherapeutic regimen employed, which is important for a timely identification of those cancers which are non-responsive to chemotherapeutic treatment. Timely identification of non-responsive tumors, in turn, allows the clinician to limit the cancer patient's exposure to unnecessary side-effects of treatment and to institute alternative treatments. However, the methods present in the art, including histological examination, are insufficient for such a timely and accurate identification. The present invention provides methods by which a more informed and effective region of therapy can be administered. In addition, the present invention provides methods by which the responsiveness or non-responsiveness of a therapy regimen can be assessed, thereby limiting the patient's exposure to the chemotherapeutic drug.

A cancer diagnosis, both initial diagnosis of disease and subsequent monitoring of the disease course, before, during, or after treatment, is conventionally confirmed through histological examination of cell or tissue samples removed from a patient. Clinical pathologists need to be able to accurately determine whether such samples are benign or malignant and to classify the aggressiveness of tumor samples deemed to be malignant, because these determinations often form the basis for selecting a suitable course of patient treatment. Similarly, the pathologist needs to be able to detect the extent to which a cancer has grown or gone into remission, particularly as a result of or consequent to treatment, most particularly treatment with chemotherapeutic or biological agents.

Histological examination traditionally entails tissue-staining procedures that permit morphological features of a sample to be readily observed under a light microscope. A pathologist, after examining the stained sample, typically makes a qualitative determination of whether the tumor sample is malignant. It is difficult, however, to ascertain a tumor's aggressiveness merely through histological examination of the sample, because a tumor's aggressiveness is often a result of the biochemistry of the cells within the tumor, such as protein expression or suppression and protein activation, which may or may not be reflected by the morphology of the sample. Therefore, it is important to be able to assess the biochemistry of the cells within a tumor sample. Further, it is desirable to be able to observe and quantitate both gene expression and protein activation of tumor related genes or proteins, or more specifically cellular components of a tumor-related signally pathway.

Automated (computer-aided) image analysis systems known in the art can augment visual examination of samples. In a representative system, the cell or tissue sample is exposed to detectably labeled reagents specific for a particular biological marker, and the magnified image of the cell is then processed by a computer that receives the image from a charge-coupled device (CCD) or camera such as a television camera. Such a system can be used, for example, to detect and measure expression and activation levels of the c-*kit*, SCF, pc-*kit*, and pAKT in a sample. This methodology provides more accurate diagnosis of cancer and a better characterization of gene expression in histologically identified cancer cells, most particularly with regard to expression of tumor marker genes or genes known to be expressed in particular cancer types and subtypes (i.e., different degrees of malignancy). This information permits a more informed and effective regimen of therapy to be administered, because drugs with clinical efficacy for certain tumor types or subtypes can be administered to patients whose cells are so identified. A non-limiting example is administration of tyrosine kinase inhibitors to patients having tumor biopsy or other test samples that test positive for c-*kit*, SCF, pc-*kit*, and pAKT.

Another drawback of conventional anticancer therapies is that the efficacy of specific chemotherapeutic agents in treating a particular cancer in an individual human patient is unpredictable. In view of this unpredictability, the art is unable to determine, prior to starting therapy, whether one or more selected agents would be active as anti-tumor agents or to render an accurate prognosis of course of treatment in an individual patient. This is especially important because the same clinical cancer may present the

clinician with a choice of treatment regimens, without any current way of assessing which regimen will be most efficacious for a particular individual. It is an advantage of the methods of this invention that they are able to better assess the expected efficacy of a proposed therapeutic agent (or combination of agents) in an individual patient. Additional features of the claimed methods for assessing the efficacy of chemotherapeutic regimens are that they are both time- and cost-effective and minimally traumatic to cancer patients.

In the practice of one embodiment of the methods of this invention, a two-component immunohistochemical staining system is used. In this embodiment, cell pellets or tissues are counterstained with a dye, stain or detectable label producing a first color while proteins of interest in the cell pellet or tissue sample are stained with a dye, stain or detectable label producing a second, different color. The image of the cells in the cell pellet and tissue sample is then magnified in a light microscope and split into a pair of separated images. A pair of optical filters that are specifically matched to have a maximum absorption for each specific stain is used to enhance the separated images. One of the optical filters preferentially transmits light having a wavelength at the absorption wavelength of the counterstained tissue. The other, preferably narrow bandpass optical filter preferentially transmits light in the regions of spectral absorption for the dye, stain or detectable label used to detect the protein of interest. Using image analysis filters, different cellular proteins in various components, such as the membrane, cytoplasm and nucleus, can be quantified. For optimal results, the imaging system is calibrated prior to taking any measurements.

One of the advantages of the use of methods of this invention that utilize two-component immunohistochemical staining is that the subcellular localization of a protein of interest can be determined.

In the practice of another embodiment of the methods of the invention, the amount of target protein in a biological sample is quantitated (*i.e.*, the amount of target protein is measured) using calibration curves that relate the amount and degree of target protein-specific staining to the amount of the target protein in the sample. This is most generally accomplished by comparing experimental or patient samples with cell samples prepared using cells, most preferably cultured cell lines that produce a consistent amount of the target protein that can be determined with high degrees of accuracy and precision. In certain preferred embodiments, a plurality of cell populations are assayed that each express different amounts of the target protein, to permit a calibration curve to be

produced relating staining intensity with amount of protein in a sample. Such cell populations are used to determine the amount of target protein-specific staining associated with varying amounts of target protein in the different cell populations. In certain preferred embodiments the amount of target protein-specific staining is normalized or expressed relative to the amount of total cell protein, to provide a convenient measure for analyzing biological tissues, preferably tumor tissue and most preferably malignant tumor tissue, in which a determination of cell number would be impractical or unreliable. In the practice of the invention, the correlation between target protein-specific staining and the amount of target protein expressed in a cell is expressed as a calibration curve relating the amount of target protein to a physical parameter, most preferably optical density, associated with target protein-specific staining. The calibration curves produced according to and used with the methods of the invention are also advantageously expressed as an algorithm, most preferably in the form of a linear or logarithmic equation, and are preferably automated, *for example*, by programming a device such as a computer to convert a sample staining intensity to an amount of a target protein in the sample.

In the practice of another embodiment of the methods of the invention, the subcellular localization of a protein of interest can be determined. This is advantageous because some proteins, such as the estrogen receptor, are localized in the cytoplasm of the cell until they are activated, at which point they translocate into the nucleus where they can participate in, for example, gene expression.

A description of exemplary procedures for quantitative immunohistochemical image analysis of tissue samples can be found, generally, in co-pending U.S. Patent Application Serial Numbers 09/760,120 and 09/760,121, both filed Jan. 12, 2001, and WO 01/51924 and WO 01/51928, both filed Jan. 12, 2001, all of which are incorporated herein by reference in their entirety. As an example, Quantitative image analysis was performed on a Cell Analysis Systems Model 200, available from Becton Dickinson Company, Mountain View, CA. Quantitative image analysis, as encompassed by this invention, can be performed on systems from other vendors, such as, but not limited to, ChromaVision Medical Systems (San Juan Capistrano, CA).

In the practice of the methods of this invention, target proteins including but not limited to c-*kit*, SCF, pc-*kit*, and pAKT can be detected using a specific reagent, most preferably an antibody, that is itself detectably labeled, or using an unlabeled, target protein-specific antibody and a second antibody that is detectably labeled and recognizes

the target protein-specific antibody. Alternatively, any molecule that can be detectably labeled and that specifically binds to the target protein can be used in the practice of the methods of the invention. In a preferred embodiment of the methods of the present invention, a two-component immunohistochemical staining system is used to differentially stain the target protein and the tissue or cell sample so that the stained target protein can be more readily distinguished from the counterstained tissue or cell sample. Following immunohistochemical staining, the optical image of the tissue or cell sample preferably generated by a computer-aided image analysis system is then magnified under a light microscope and separated into a pair of images. The separated images are enhanced using a pair of optical filters, one having a maximum absorption corresponding to the stain and the other having a maximum absorption corresponding to the counterstain, thereby providing an optimum discrimination between the two stains. In other embodiments of the method of the present invention, a plurality of image analysis filters are used to detect, differentiate, and quantitate staining levels of different cellular proteins in various components (*e.g.*, membrane, cytoplasm, and nucleus). In a non-limiting example, the target can be stained using diaminobenzidine (DAB) and the tissue or cell sample can be counterstained using ethyl green or methylene blue.

It will be recognized by those with skill in the immunohistochemistry arts that dye lot variations and variability in certain environmental conditions, such as relative humidity can affect results obtained at different times using the methods of the invention. In certain embodiments of the methods of the present invention, stain and counterstain lot variability is controlled by using the same reagents to stain the sample cells and first and second control cell pellets. In other certain embodiments of the methods of the present invention, environmental differences and variability in the staining procedure are controlled for by staining the sample cells and first and second control cell pellets at the same time.

In certain embodiments, target protein-specific staining is detected, measured and quantitated using image analysis equipment, defined herein as comprising a light or fluorescence microscope, an image-transmitting camera and a view screen, most preferably also comprising a computer that can be used to direct the operation of the device and also to store and manipulate the information collected, most preferably in the form of optical density of certain regions of a stained tissue preparation. Image analysis devices useful in the practice of this invention include but are not limited to the CAS 200 system (Becton Dickenson, Mountain View, CA).

In certain embodiments, the inventive methods are practiced using such an image analysis system as follows. After immunohistochemical staining as described above, a quantified measure of the percentage of expressing cells can be taken by digitizing microscope images of stained samples, and converting light intensity values in each picture element (pixel) of the digitized image to optical density values, which correspond to the percentage of stained cell components (such as nuclei). More specifically, computerized image analysis can be used to determine a quantity of cells having a particular stain using a digital grey scale image. Grey scale images are representative of the amount of an optical enhancement factor, such as a chromagen, which binds to a specific target under study and thereby allows optical amplification and visualization of the target.

As used in the practice of the methods of this invention, a computerized image analysis apparatus includes a means such as a lens for magnifying and displaying the image of a group of cells of a specimen from a field on a microscope slide. The specimen cell population is prepared using staining and counterstaining techniques known in the immunohistochemical arts, such as enzymatic staining methods and chemical staining methods. Appropriate stains and counterstains are selected by their ability to enable cells to be distinguished, *inter alia*, those that contain antibody sandwich complexes comprising specific enzymes or markers and those that do not contain such complexes, through cameras used with the computerized image analysis system. After staining, the image field is digitized by the apparatus and stored in a memory provided by the system. From the digitized image, a nuclear or cytoplasmic image mask is formed by using one wavelength of light such as red wavelength or green optical filter for form the image. The tissue mask is stored and a second filter is used to form another filtered image of the same areas with the optical enhancement factor. Differentiation of cellular characteristics can be made by comparing the first image with the second image to obtain a quantification of material stained with the optical enhancement factor and thus, an assay of the amount of the particular target under study.

In a first step, any expressed target protein in the cells is identified by adding a detectably-labeled primary antibody specific for the target protein, or alternatively an unlabeled primary antibody and a detectably-labeled secondary antibody specific for the primary antibody. The antibodies are incubated with the sample for a time to form complexes if these antigens are present. The complexes are then visualized, either by directly detecting the label, or, if the detectable label is an enzyme, incubating the

sections with a compound, such as a chromagen under appropriate conditions. As an example, the primary antibody can be labeled peroxidase, with the subsequent use of the chromagen DAB. In a second step, the tissue is counterstained with another optical enhancement factor, such as, but not limited to ethyl green. Although a staining technique using peroxidase and ethyl green is exemplary, other stains and optical enhancement factors are also suitable. For example, other chromagens can be used with peroxidase, including, but not limited to 3-Amino-9-Ethylcarbazole (AEC), which produces a red color, and 4-Chloro-1-Naphthol. In addition, other enzymes that can be used to label the primary antibody are encompassed by this invention, including, but not limited alkaline phosphatase. Examples of chromagens that can be used with alkaline phosphatase include, but are not limited to Fast Red, Fast Green, and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT). In addition, the use of florescent dyes is encompassed by methods of the present invention. As non-limiting examples of florescent dyes, the use of Fluorescein (fluoroisothiocyanate or FITC) and Rhodamine.

Further, the use of ethyl green is exemplary of counter stains, but use of other counter stains are encompassed by the methods of the present invention. For example, a non-limiting list of additional counter stains include hematoxylin, fast red, methyl green. A counter stain is chosen for a particular stain based on the spectral separation that can be achieved such that filtering at two separate wavelengths can be achieved. For example, ethyl green offers good spectral separation from the DAB precipitate, as described in more detail below. Other examples of pairs of stains/counter stains that offer good spectral separation include, but are not limited to, Fast Red and Hematoxylin, AEC and Hematoxylin, and FITC and Texas Red. Those skilled in the art will recognize that this list of stains and counter-stain is merely exemplary and, therefore, does not serve to limit the invention.

As an example of the an embodiment of a method of the present invention, spectral studies have shown that the ethyl green stain offers good spectral separation from the DAB precipitate produced by immunoperoxidase and permits different features of the image to be readily separated by filtering the image at two different wavelengths. This allows the image to be digitized into two separate images, for example, one in which all the cell nuclei are optically enhanced (using, *for example*, ethyl green or Fast Green) and one in which only those tissue areas with receptor staining (DAB) are optically enhanced. The resulting tissue preparation stained with ethyl green will have green nuclei with varying degrees of brown DAB precipitate localized to the cytoplasm or nuclei,

proportional to the level of expression of the marker protein. Although a staining technique using peroxidase and ethyl green is described, other stains and optical enhancement factors are also suitable (such as alkaline phosphatase coupled with a specific chromagen such as Fast Red or Fast Green counterstain). Spectral studies have shown that the ethyl green stain offers good spectral separation from the DAB precipitate of the immunoperoxidase technique such that filtering at two different wavelengths can readily separate different features of the image. This allows the image to be digitized into two separate images, one in which all the cell nuclei are optically enhanced (ethyl green or Fast Green) and one in which only those tissue areas with receptor staining (DAB) are optically enhanced. In a particular embodiment, the images can be separated by a 600 nanometer (red) filter to produce an image of the counterstained area and a 500 nanometer (green) filter to produce an image of the tissue areas staining with the DAB precipitate.

To further differentiate the stained areas, an interactive threshold setting technique can be used where an operator visualizing the images can set a boundary on the areas under consideration. When such boundaries are set, images are formed by eliminating all parts of the image that are below the thresholds in optical density. As practiced according to the teachings contained herein, a threshold is advantageously set for the first image, and a second threshold is set for the second image.

Further, the image processing method performed preferably using the image analysis systems of the invention comprises first forming a mask image of the tissues under consideration with, for example, a red filter. This mask image is stored, preferably in the system's computer memory and another image for expressed protein quantification is acquired by using, for example, a green-filtered version of the same image. The effect of the filters in combination is to optically enhance (*i.e.*, make darker) those areas of the tissue mask where tissue components are stained with DAB and to make lighter those tissue components stained only with the green counterstain. An image analysis can then be performed using only those areas of the image that are stained and are within the mask.

The combination of red and green optical filters and DAB and ethyl green or other green counterstains are advantageously used in the practice of the methods of the invention. This combination provides a convenient and advantageous way to discriminate between two counterstained areas. Those with skill in the art will recognize that there are a plurality of other stains, filters, staining or optical enhancement methods and filtering

methods that can be used to optically enhance one particular area or feature over another cell feature, such as Fast green, eosin.

For example, expression and activation of proteins expressed from tumor-related genes can be detected and quantitated using methods of the present invention. Further, expression and activation of proteins that are cellular components of a tumor-related signaling pathway can be detected and quantitated using methods of the present invention. Further, proteins associated with breast cancer can be quantified by image analysis using a suitable primary antibody against Her-1, Her-2, p-Her-1, p-Her-2, p-ERK, c-*kit*, p-AKT, or SCF, or to their phosphorylated forms, and a secondary antibody (such as rabbit anti-mouse IgG when using mouse primary antibodies) and/or a tertiary avidin (or Strepavidin) biotin complex ("ABC"). Examples of reagents include a rabbit polyclonal antibody specific for pAKT and obtained from Cell Signaling Technology (Beverly, MA, Cat. No. 9277); anti-SCF antibody, obtained from Santa Cruz Biotechnology (Santa Cruz, CA, Cat. No. SC-9132); and a polyclonal anti-c-*kit* antibody obtained from Neomarkers, Inc. (Fremont, CA, Cat. No. RB-1518).

In practicing the method of the present invention, staining procedures can be carried out by a technician in the laboratory. Alternatively, the staining procedures can be carried out using automated systems.

The amount of target protein can then be quantitated by the average optical density of the stained antigens. Also, the proportion or percentage of total tissue area stained may be readily calculated, as the area stained above an antibody threshold level in the second image. Following visualization of nuclei containing c-*kit*, SCF, and pAKT, the percentage or amount of such cells in tissue derived from patients after treatment may be compared to the percentage or amount of such cells in untreated tissue.

From these comparisons, the potential effectiveness of a putative therapeutic or chemopreventive agent against a tumor can be determined. Specifically, if there is a greater amount of expression and activation of protein expressed from a tumor-related gene in the untreated portion of the sample than there is in the treated portion of the sample, then the therapeutic agent used in the treatment will be predicted to be an effective agent for treating the cancer. Further, if there is a greater amount of expression and activation of protein that is a cellular component of a tumor-related signaling pathway in the untreated portion of the sample than there is in the treated portion of the sample, then the therapeutic agent used in the treatment will be predicted to be an effective agent for treating the cancer. Further, if there is a greater amount of expression

and activation of c-*kit*, SCF, and pAKT in the untreated portion of the sample than there is in the treated portion of the sample, then the therapeutic agent used in the treatment will be predicted to be an effective agent for treating the cancer. Conversely, if there is a substantially equal percentage of cells expressing c-*kit*, SCF, and pAKT in both the treated and untreated portions of the sample, or there is less protein expression in the untreated portion of the sample, then the putative therapeutic agent will not be predicted to be effective for treatment of the cancer. For example, this procedure can be used to establish that a c-*kit* tyrosine kinase inhibitor, Novartis agent STI-571 (GLIVEC™), is predicted to be a potentially effective agent for treating a cancer sample.

Particularly useful embodiments of the present invention and the advantages thereof can be understood by referring to Examples 1-7. These Examples are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

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Example 1

Staining procedure for c-Kit

Staining for c-*kit* was performed on a Benchmark™ automated staining module (Ventana Medical Systems, Inc., Tucson, AZ) using a polyclonal antibody to c-*kit* (NeoMarkers, Fremont, CA). 10% Neutral Buffered Formalin fixed 4 micron paraffin sections of the sample were place onto coated slides. The Benchmark bar-code specific for the c-*kit* staining protocol was placed onto the slide and the slide placed onto the Benchmark. All subsequent steps, drying, deparaffinization antigen retrieval (Cell Conditioning 1 mM EDTA), and detection were performed automatically by the Benchmark. All test reagents are available from Ventana Medical Systems, including the I-View DAB Detection Kit, unless otherwise noted. The protocol implemented in using the automated system is described in Table 1. Where indicated, one drop is one reagent dispense. The sample was then manually counterstained with 4% Ethyl Green 0.1 M pH 4 acetate buffer for 10 minutes. The results of staining for c-*kit* are described below in Example 5.

Table I

1	****Select EZ Prep****

2	****Start Timed Steps
3	****Mixers Off****
4	Warmup Slide to 75°C, and Incubate for 4 minutes
5	Apply EZPrep Volume Adjust
6	Incubate for 4 minutes
7	Rinse Slide +
8	Apply EZPrep Volume Adjust
9	Incubate for 4 minutes
10	Rinse Slide +
11	Apply EZPrep Volume Adjust
12	Apply Coverslip
13	Warmup Slide to 76°C, and Incubate for 4 minutes
14	Rinse Slide +
15	Apply Depar Volume Adjust
16	Apply Coverslip
17	Warmup Slide to 42°C, and Incubate for 2 minutes
18	****Mixers On****
19	Rinse Slide +
20	Apply Medium Cell Conditioner #1
21	Apply CC Long Coverslip
22	Warmup Slide to 95°C, and Incubate for 8 minutes
23	Apply Medium Cell Conditioner #1
24	Apply Coverslip
25	Warmup Slide to 100°C, and Incubate for 4 minutes
26	Apply Coverslip
27	Apply Cell Conditioner #1
28	Incubate for 4 minutes
29	Apply Coverslip
30	Apply EZPrep CC Volume Adjust
31	Incubate for 4 minutes
32	Apply Coverslip
33	Apply Medium Cell Conditioner #1

34	Incubate for 4 minutes
35	Apply Coverslip
36	Apply Cell Conditioner #1
37	Incubate for 4 minutes
38	Disable Slide Heater
39	Incubate for 8 minutes
40	Rinse Slide +
41	Adjust Slide Volume
42	Apply Coverslip
43	****Select Reaction Buffer****
44	Warmup Slide to 42°C, and Incubate for 2 minutes
45	Rinse Slide +
46	Adjust Slide Volume
47	Apply One Drop of I-VIEW INHIBITOR, Apply Coverslip, and Incubate 4 min.
48	Rinse Slide +
49	Adjust Slide Volume
50	Apply One Drop of c-*kit* primary antibody [PREP KIT 4], Apply Coverslip, and Incubate 32 min.
51	Rinse Slide +
52	Adjust Slide Volume
53	Apply One Drop of I-VIEW BIOTIN Ig, Apply Coverslip, and Incubate 8 min.
54	Rinse Slide +
55	Adjust Slide Volume
56	Apply One Drop of I-VIEW SA-HRP, Apply Coverslip, and Incubate 8 min.
57	Rinse Slide +
58	Adjust Slide Volume
59	Apply Coverslip
60	Rinse Slide +
61	Adjust Slide Volume
62	Apply One Drop of I-VIEW DAB and One Drop I-VIEW H_2O_2, Apply Coverslip, and Incubate 8 min.
63	Rinse Slide +

64	Adjust Slide Volume
65	Apply One Drop of I-VIEW COPEER, Apply Coverslip, and Incubate 4 min.
66	Rinse Slide +

Example 2

Staining procedure for SCF

SCF staining was performed using a polyclonal antibody to SCF (Santa Cruz Biotechnology, Santa Cruz, CA). A biotinylated anti-rabbit secondary antibody (Jackson Research Labs, West Lake, PA). the "StreptABComplex-HRP" (Streptavidin/Biotin Complex with a Horseradish Peroxidase) label kit (DAKO Corporation, Carpinteria, CA), and the DAB chromagen (Dako) were used as the detection system for SCF. 10% Neutral Buffered Formalin fixed 4 micron paraffin sections of the sample were place onto coated slides. To prepare the sample for staining, the sample was first deparaffinized and hydrated to water. Antigen retrieval was preformed enzymatically with Digest-All 1 (Zymed Labs), a Ficin digestion solution. 1-2 drops of the manufacture's ready-to-use solution was added to the sections and incubated for 20 minutes at 37 °C. The sections were then washed well with deionized water and rinsed with TBS. Endogenous peroxidase was blocked by incubating the sections with a 3% hydrogen peroxide/methanol solution for 10 minutes, followed by a deionized water wash. The sections were then blocked using 10% blocking goat serum in 0.1% BSA/0.1% Triton X for 10 minutes. The goat serum was then shaken off.

The sections were incubated with a 1:20 dilution of SCF primary antibody at 37 °C for 45 minutes. The StreptABC label was prepared while the primary antibody was incubating with the sections by adding 4 µls of Streptavidin solution with 4 µls biotin solution in 1 ml of 50 mM Tris-HCL buffer, pH 7.6. After SCF primary antibody incubation, the sections were washed well with TBS and incubated with a 1:300 dilution of biotinylated Goat anti-rabbit IgG at 37 °C for 20 minutes. The sections were washed well with TBS, followed by incubation with the StreptABC label at 37 °C for 20 minutes. The sections were washed well with TBS, and DAB liquid chromagen was applied at room temperature for 5 minutes. The sections were then washed well with deionized water. The slides were counterstained with 4% ethyl green. The results of staining for SCF are described below in Example 5.

Example 3

Staining procedure for phospho-AKT

p-AKT staining was performed using a Dako Autostainer with the LSAB2 kit (described above). 10% Neutral Buffered Formalin fixed 4 micron paraffin sections of the sample were place onto coated slides. To prepare the sample for staining, the sample was first deparaffinized and hydrated to water. Antigen retrieval was preformed with 0.1 M citrate retrieval buffer, pH 6.0 in a Decloaker Chamber (Biocare Medical, Walnut Grove, CA) following the manufacturer's instructions. The sections were allowed to cool for 15 minutes, and then washed well with deionized water. Endogenous peroxidase was blocked by incubating the sections with a 3% hydrogen peroxide/Methanol solution for 10 minutes, followed by a deionized water wash. The sections were then blocked using 10% blocking goat serum in 0.1% BSA/0.1% Triton X for 10 minutes. The goat serum was then shaken off.

The sections were then incubated with a 1:75 dilution of p-AKT primary antibody (Cell Signaling Technology, Beverly, MA) at 4°C overnight. The next morning, the sections were removed from the refrigerator and allowed to come to room temperature, about 30 minutes. The slides were washed well with TBS and loaded into the Dako Autostainer. The Autostainer was programmed to apply the biotinylated link for 30 minutes, the streptavidin-HRP reagent for 30 minutes, and the DAB chromagen for 5 minutes. The slides were rinsed with TBS/Tween by the autostainer between the linking antibody, labeling, and DAB chromagen applications. After DAB chromagen, the slides were removed from the autostainer and washed well with deionized water. The slides were counterstained with 4% ethyl green. The results of staining for SCF are described below in Example 5

Example 4

Staining procedure for phospho-KIT

pc-*kit* staining was performed using a Dako Autostainer with a reagent kit obtained from DAKO Corp. (the LSAB2 kit (labeled streptavidin-biotin) containing a Horseradish Peroxidase Label). 10% Neutral Buffered Formalin fixed 4 micron paraffin sections of the sample were place onto coated slides. To prepare the sample for staining, the sample was first deparaffinized and hydrated to water. Antigen retrieval was preformed with 1 mM EDTA in a Decloaker Chamber (Biocare Medical, Walnut Grove, CA), an electric pressure cooker that can be utilized for heat-induced epitope retrieval

(HIER) procedures, following the manufacturer's instructions. The sections were allowed to cool for 15 minutes, and then washed well with deionized water. Endogenous peroxidase was blocked by incubating the sections with a 3% hydrogen peroxide/methanol solution for 10 minutes, followed by a deionized water wash. The sections were then blocked using 10% blocking goat serum in 0.1% BSA/0.1% Triton X for 10 minutes. The goat serum was then shaken off.

The sections were then incubated with a 1:50 dilution of p-*kit* (Try 719) primary antibody (Cell Signaling Technology, Beverly, MA) at 4°C overnight. The next morning, the sections were removed from the refrigerator and allowed to come to room temperature, about 30 minutes. The slides were washed well with TBS and loaded into the Dako Autostainer. The Autostainer was programmed to apply the biotinylated link for 30 minutes, the streptavidin-HRP reagent for 30 minutes, and the DAB chromagen for 5 minutes. The slides were rinsed with TBS/Tween by the autostainer between the linking antibody, labeling, and DAB chromagen applications. After DAB chromagen, the slides were taken off the autostainer and washed well with deionized water. The slides were counterstained with 4% ethyl green. The result was the ability to detect the level of expression or activation of pc-*kit* in the sample (data not shown).

Example 5

Immunohistochemical Staining of Tumor Sample for c-*kit*, SCF, and pAKT

To identify a patient to be a candidate for c-*kit* inhibitors, such as Novartis' Glivec™, tumor samples were examined for c-*kit* and SCF expression. Paraffin blocks of tumor samples were sectioned at 4-5 microns and the sections were placed onto coated slides. The slides were then stained as described above for either c-*kit*, SCF, or pAKT. The results of these experiments are represented in Figures 1A through 1D. These Figures represent microphotographs of one individual's breast cancer sample. Two microphotographs (Figure 1A and 1C) represent fields stained with antibodies to SCF and two microphotographs (Figure 1B and 1D) represent fields stained with antibodies to c-*kit*. All photographs show staining and thus that the same tumor expresses both c-*kit* and SCF. Therefore, this breast cancer sample identified the patient as a good candidate for c-*kit* inhibitors.

As a further example of tumor samples that were determined to be candidates for c-kit inhibitors, Figure 2A-2E represent either bladder cancer samples (2A, 2D, and 2E) or breast cancer samples (2B and 2C) stained with antibodies to c-*kit*, SCF, and pAKT, as

described above. Figure 2B represents breast tumor sample 747 stained for c-*kit*. Figure 2C represents breast tumor sample 848 stained for SCF. Figure 2D and 2E represent bladder tumor sample 920 stained for both c-*kit* (Figure 2D) and for SCF (Figure 2E), indicating that the patient is a good candidate for c-*kit* inhibitors. Figure 2A represents the same bladder tumor sample 920 stained for pAKT.

Example 6

Image Analysis of c-*kit*, SCF, and pAKT protein expression

Tumor samples were stained with antibodies to c-*kit*, SCF, and pAKT, and image analysis was used to determine the level of expression of these three proteins. The individual tumor samples analyzed included both samples of breast cancer (747, 848, 864, 895) and bladder cancer (920). The measurements were made using the CAS system as described in Bacus *et al.* (1997, *Anal. Quant. Cytol. Histol.* 19:316-28, incorporated herein by reference in its entirety). The levels of c-*kit*, SCF, and pAKT protein, expressed in units of optical density per pixel, are shown in Table II. Figure 3 is a bar graph showing the levels of c-*kit*, SCF, and pAKT measured by image analysis and IHC staining in these five tumors showing that all three proteins were expressed, that all three proteins could be measured, and that there was considerable variation in the expression level of the three proteins. Presumably, these variations will affect drug susceptibility of the cancer.

Table II

Image analysis quantification of c-kit, pAKT, and SCF in Tumor Sample, Expressed as Optical Density per Pixel

Sample	c-kit	PAKT	SCF
"747"	0.3	0.15	0.07
"848"	0.32	0.13	0.1
"864"	0.14	0.06	0.25
"895"	0.13	0.04	0.1
"920"	0.29	0.4	0.07

Example 7

Response to STI-571 in Breast Cancer Cell Lines

The breast cancer cell line MDA-MB-361 was used to determine the response of a breast cancer line to a c-*kit* tyrosine kinase inhibitor, specifically Novartis agent STI-571 (GLIVEC™). Figure 4 is a representation of a western blot showing c-*kit* activity in breast cancer cells treated with SCF ligand in the presence or absence of STI-571 in cell culture. The cell line was exposed to STI-571, varying from 1 μM to 40 μM, during a three-day cell culture. The cultured cell line and the control, untreated cell line were then lysed and cell lysates with 50 μg total protein were immunoprecipitated with both the Neomarkers CD117/c-Kit antibody clone Ab-6, and the DAKO CD117/c-Kit antibody clone A4502. Figure 5 is a representation of a western blot of c-*kit* expression in HeLa cells probed with the same antibodies used to immunoprecipitate c-*kit* in the present experiment, clearly showing that both of the antibodies were capable of isolating c-Kit. Figure 4, lane 1 shows that there is no activated c-*kit* expressed in the absence of its ligand, SCF. Lane 2 shows a distinct band at 145 kDa that indicates the isolation of activated c-*kit* when the cell culture is exposed to SCF. The remaining lanes (3-6) do not have a band at 145 kDa, demonstrating that there is no c-*kit* expression in the presence of as little as 1 μM STI-571. This result shows that STI-571 (GLIVEC™) is capable decreasing the amount of expression or activation of c-*kit*, in the presence of its ligand, SCF. Therefore, STI-571 (GLIVEC™) is identified as a compound that is useful to decrease expression or activation of c-*kit*, and correspondingly decrease activation of AKT.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WHAT WE CLAIM IS:

1. A method for assessing a response to administration of a chemotherapeutic or biological therapeutic agent to an individual, comprising:

(a) obtaining a first tissue or cell sample from the individual before exposing the individual to the chemotherapeutic or biological therapeutic agent;

(b) obtaining a second tissue or cell sample from the individual after exposing the individual to the chemotherapeutic or biological therapeutic agent;

(c) detecting the amount of one or a plurality of biological markers in the first and second tissue or cell sample;

(d) comparing the amount of the one or a plurality of biological markers in said first and second tissue or cell sample;

(e) determining whether expression or activation of the one or a plurality of biological markers decreases following exposure to the chemotherapeutic or biological therapeutic agent when the amount of the one or a plurality of biological markers in the second tissue sample is less than the amount of the one or a plurality of biological markers in the first tissue sample;

wherein a response to the administration of a chemotherapeutic or biological therapeutic agent to an individual is assessed.

2. The method of claim 1, wherein the one or a plurality of biological markers is expressed from a tumor-related gene.

3. The method of claim 1, wherein the one or a plurality of biological markers is a cellular component of a tumor-related signaling pathway.

4. The method of claim 1, wherein said biological markers are c-*kit*, SCF, pAKT, or pc-*kit*.

5. The method of claim 4, wherein the method of detecting the amount of said one or a plurality of biological markers comprises using an antibody or antibodies that are immunologically specific for said one or a plurality of biological markers.

6. The method of claim 5, wherein the amount one or a plurality of biological markers is determined immunohistochemically.

7. The method of claim 6, wherein the method of detecting the amount of said one or a plurality of biological markers comprises,

(a) staining the first and second tissue or cell samples using one or a plurality of antibodies detectably-labeled with an optical density label, wherein at least one antibody is immunologically specific for one or a plurality of biological markers, wherein the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*; and

(b) determining an optical density of the stained first and second tissue sample in subpart (a);

wherein said optical density corresponds to the amount of said one or a plurality of biological markers.

8. The method of claim 7, wherein optical density is determined using image analysis.

9. The method of claim 8, wherein the detectable label is a chromagen or a fluorophore.

10. The method of claim 9, wherein the detectable label is DAB.

11. The method of claim 10, wherein the chemotherapeutic or biological therapeutic agent is Glivac™.

12. A method for predicting the response to administration of a chemotherapeutic or biological therapeutic agent to an individual, comprising:

(a) obtaining a tissue or cell sample from the individual before exposing the individual to the chemotherapeutic or biological therapeutic agent;

(b) detecting the amount of one or a plurality of biological markers in the tissue or cell sample;

(c) comparing the amount of one or a plurality of biological markers determined in subpart (b) with the amount of one or a plurality of samples expressing known amounts of the markers; and

(d) determining the expression or activation level of the one or a plurality of biological markers compared with the expression or activation level of the control samples;

wherein a response to the administration of a chemotherapeutic or biological therapeutic agent to an individual is predicted based on the determination of decreased expression or activation level of the one or a plurality of biological markers.

13. The method of claim 12, wherein the one or a plurality of biological markers is expressed from a tumor-related gene.

14. The method of claim 12, wherein the one or a plurality of biological markers is a cellular component of a tumor-related signaling pathway.

15. The method of claim 12, wherein said biological markers are c-*kit*, SCF, pAKT, or pc-*kit*.

16. The method of claim 15, wherein the method of detecting the amount of said one or a plurality of biological markers comprises using an antibody or antibodies that are immunologically specific for said one or a plurality of biological markers.

17. The method of claim 16, wherein the amount one or a plurality of biological markers is determined immunohistochemically.

18. The method of claim 17, wherein the method of detecting the amount of said one or a plurality of biological markers comprises,

(a) staining the tissue or cell samples using one or a plurality of antibodies detectably-labeled with an optical density label, wherein at least one antibody is immunologically specific for one or a plurality of biological markers, wherein the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*; and

(b) determining an optical density of the stained tissue or cell sample in subpart (a);

wherein said optical density corresponds to the amount of said one or more biological markers.

19. The method of claim 18, wherein optical density is determined using image analysis.

20. The method of claim 19, wherein the detectable label is a chromagen or a fluorophore.

21. The method of claim 20, wherein the detectable label is DAB.

22. The method of claim 21, wherein the chemotherapeutic or biological therapeutic agent is Glivac^{TM}.

23. A method for identifying a compound as a chemotherapeutic or biological therapeutic agent, comprising:

(a) obtaining a first tissue or cell sample;

(b) obtaining a second tissue or cell sample;

(c) exposing the second tissue or cell sample to said compound;

(d) detecting the amount of one or a plurality of biological markers in the first tissue or cell sample and the second tissue or cell sample;

(e) comparing the amount of the one or a plurality of biological markers in said first tissue or cell sample and second tissue or cell sample;

(f) determining whether expression or activation of the one or a plurality of biological markers decreases following exposure to said compound when the amount of the one or more biological markers in the second tissue or cell sample is less than the amount of the one or a plurality of biological markers in the first tissue or cell sample;

wherein the compound is identified as a chemotherapeutic or biological therapeutic agent.

24. The method of claim 23, wherein the one or a plurality of biological markers is expressed from a tumor-related gene.

25. The method of claim 23, wherein the one or a plurality of biological markers is a cellular component of a tumor-related signaling pathway.

26. The method of claim 23, wherein said biological markers are c-*kit*, SCF, pAKT, or pc-*kit*.

27. The method of claim 26, wherein the method of detecting the amount of said one or a plurality of biological markers comprises using an antibody or antibodies that are immunologically specific for said one or a plurality of biological markers.

28. The method of claim 27, wherein the amount one or a plurality of biological markers is determined immunohistochemically.

29. The method of claim 28, wherein the method of detecting the amount of said one or a plurality of biological markers comprises,

(a) staining the first and second tissue or cell samples using one or more antibodies detectably-labeled with an optical density label, wherein at least one antibody is immunologically specific for one or a plurality of biological markers, wherein the biological markers are c-*kit*, SCF, pAKT, or pc-*kit*; and

(b) determining an optical density of the stained first and second tissue sample in subpart (a);

wherein said optical density corresponds to the amount of said one or more biological markers.

30. The method of claim 31, wherein optical density is determined using image analysis.

31. The method of claim 30, wherein the detectable label is a chromagen or a fluorophore.

32. The method of claim 31, wherein the detectable label is DAB.



Figure 1A

Immunohistochemistry for C-KIT and it's ligand, SCGF, on sample 12669b9

Immunohistochemistry for C-KIT and it's ligand, SCGF, on sample 12669b

C-KIT



Figure 1B


Immunohistochemistry for C-KIT and it's ligand, SCGF, on sample 1269b9
SCGF

Figure 1C

Immunohistochemistry for C-KIT and it's ligand, SCGF, on sample 1266969

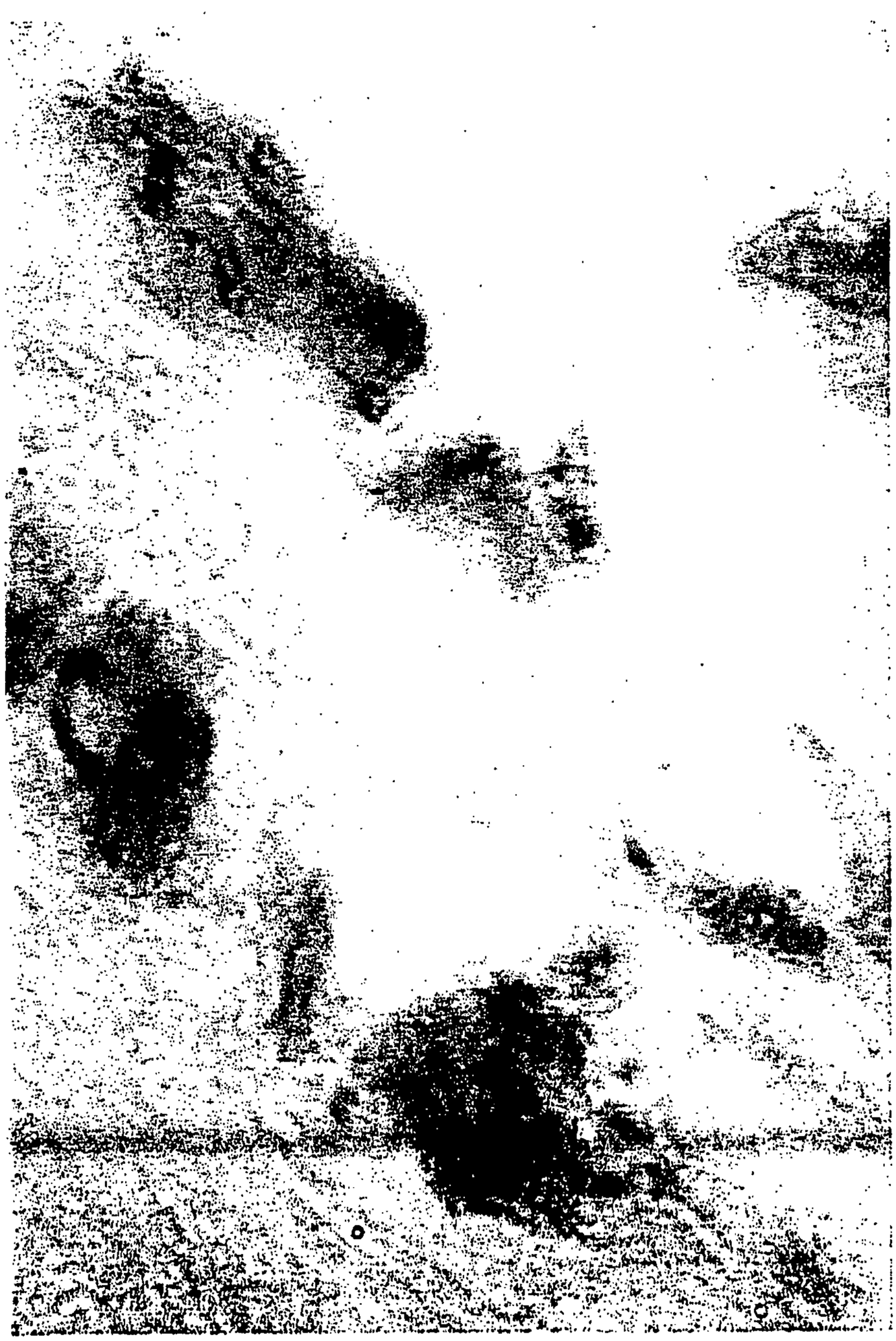
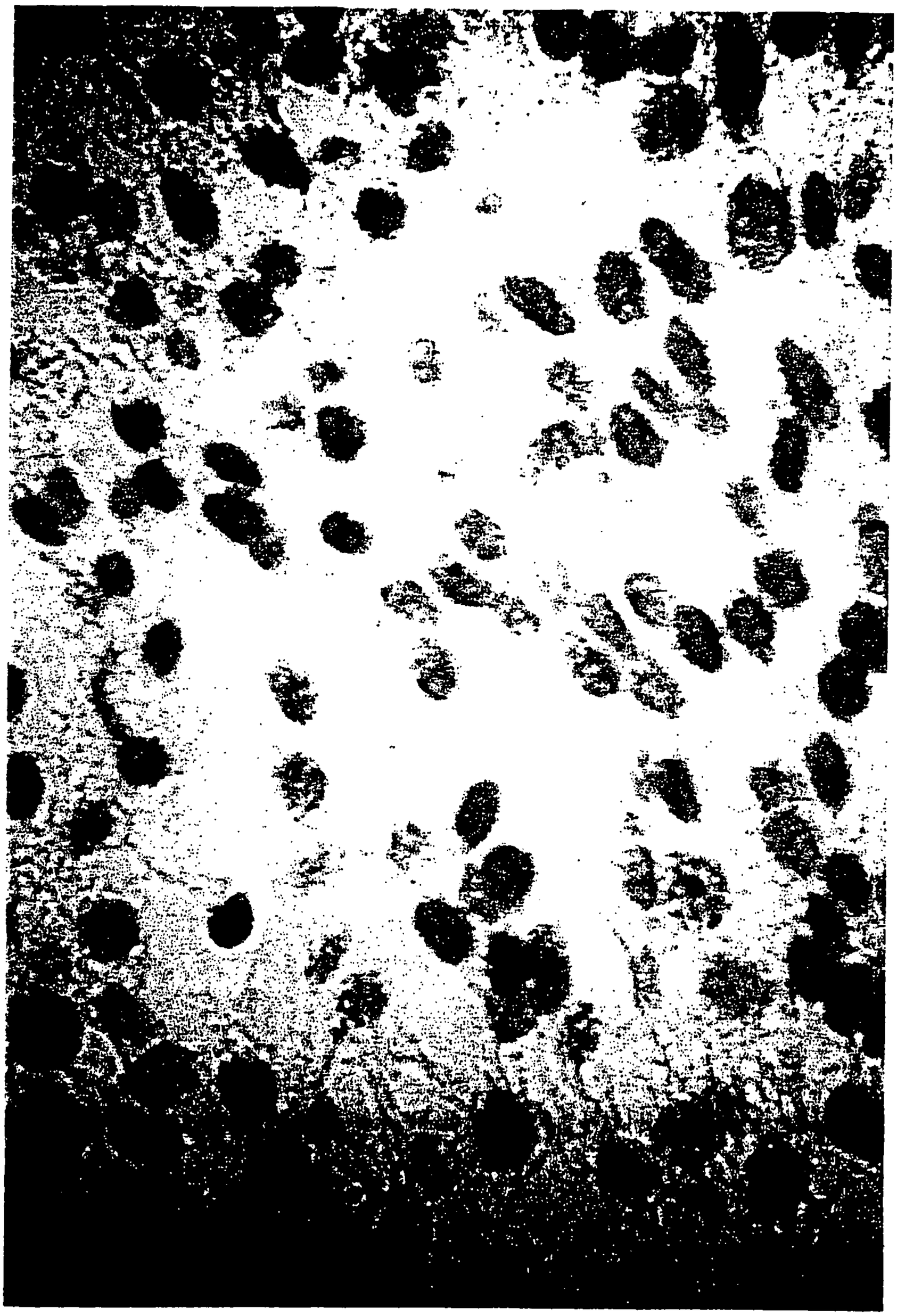


Figure 1D



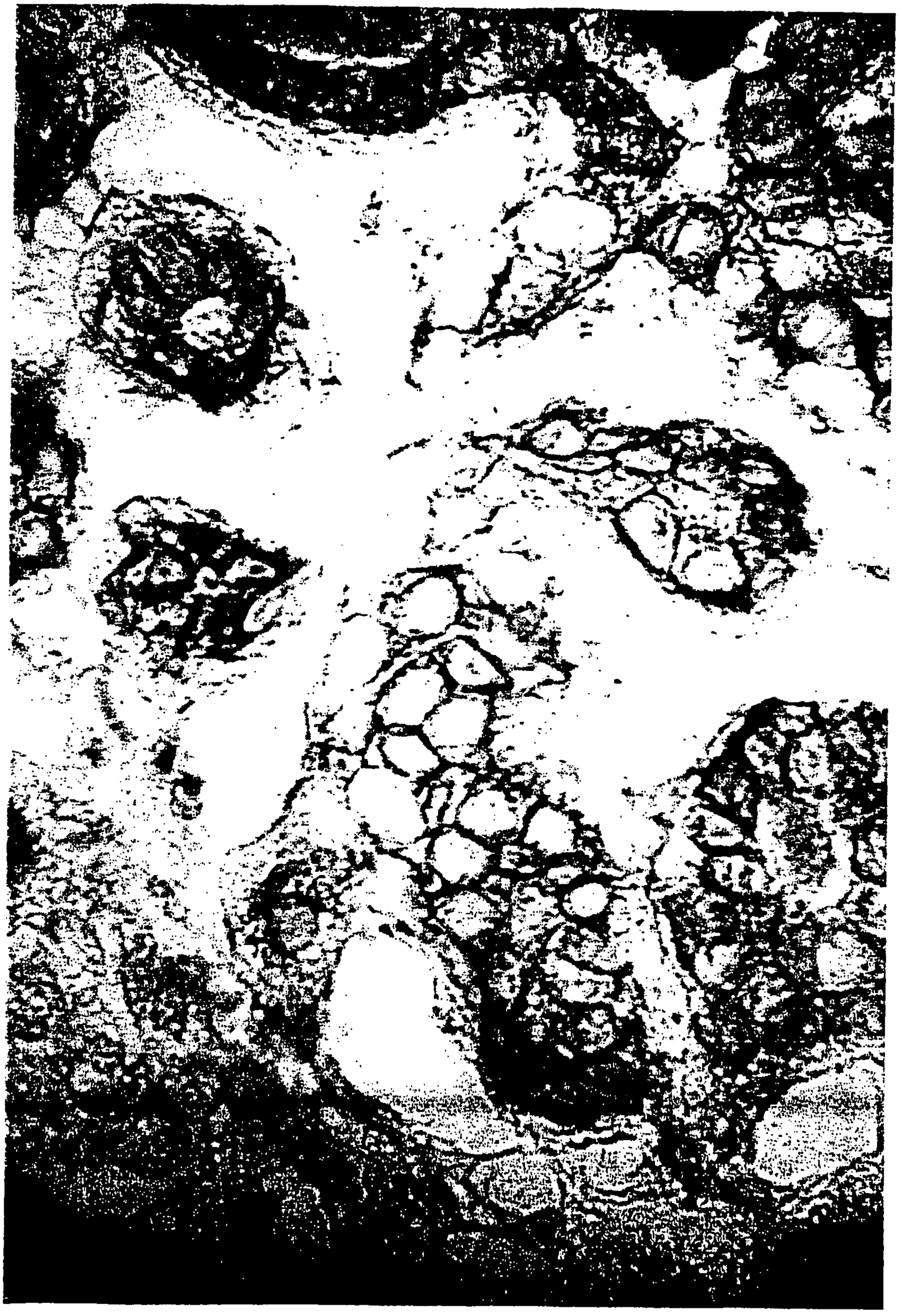
pAKT920

Figure 2A



C-kit 747 Breast Cancer

Figure 2B



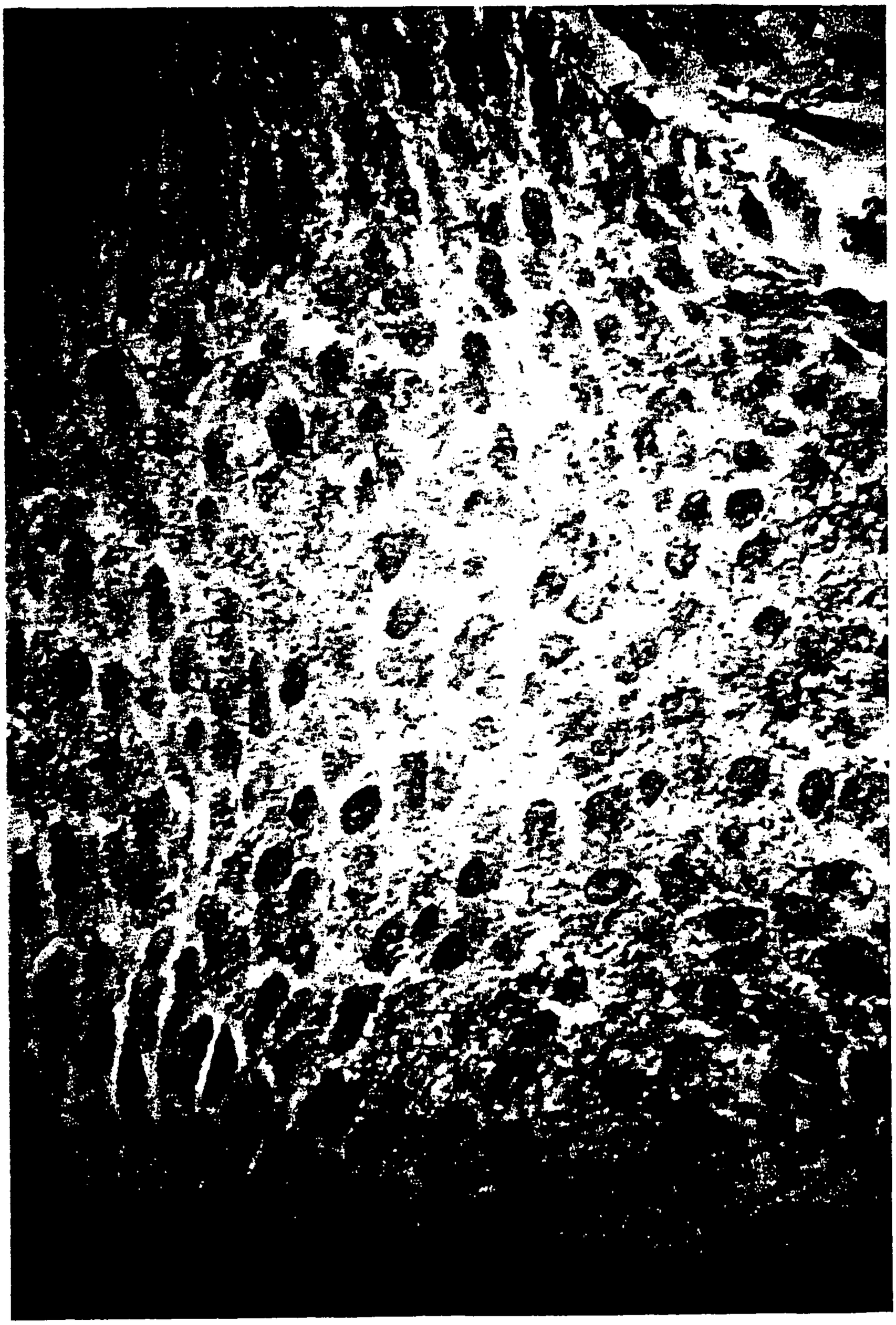
SCF 848 Breast Cancer

Figure 2C



C-kit 920 Bladder Cancer

Figure 2D



SCF 920 Bladder Cancer
Figure 2E

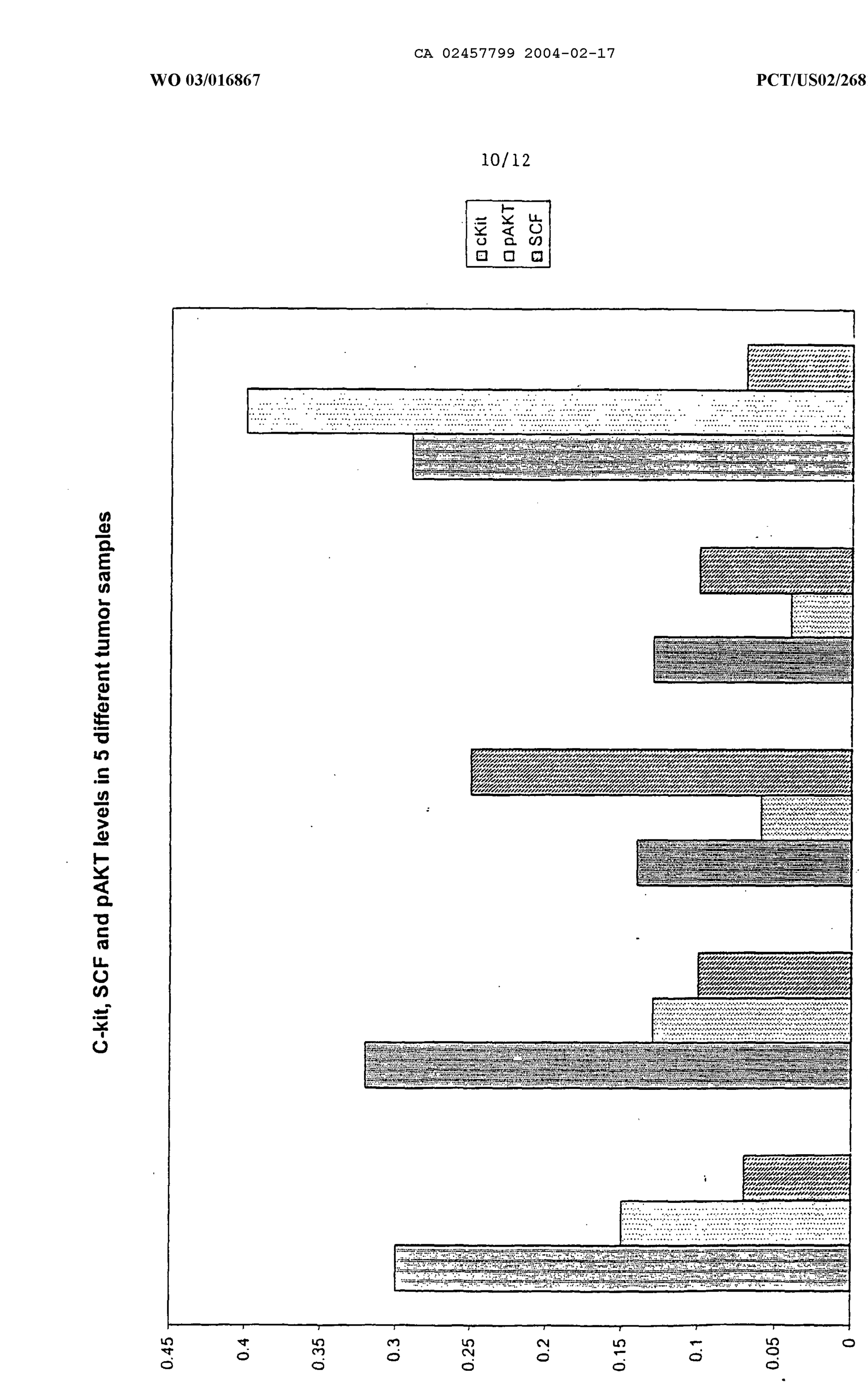
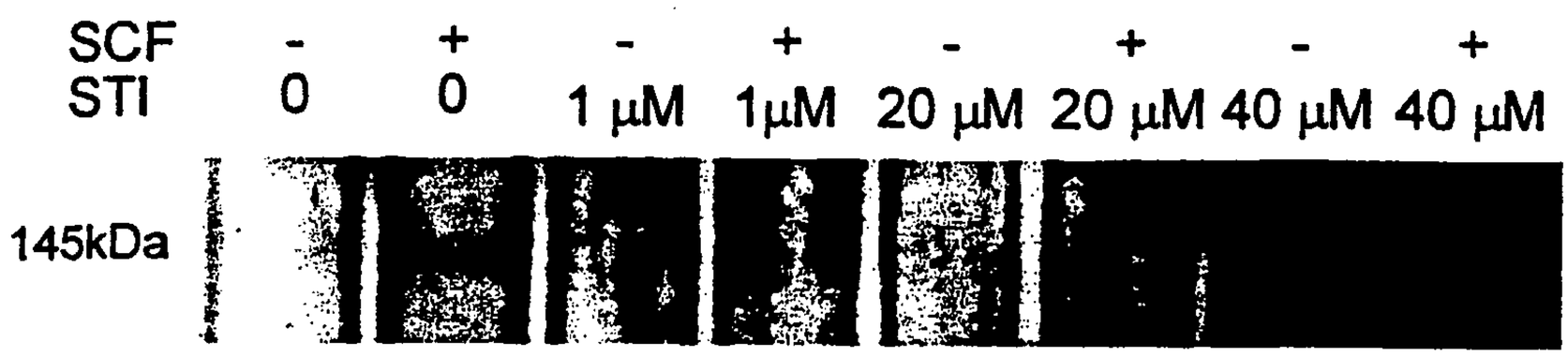
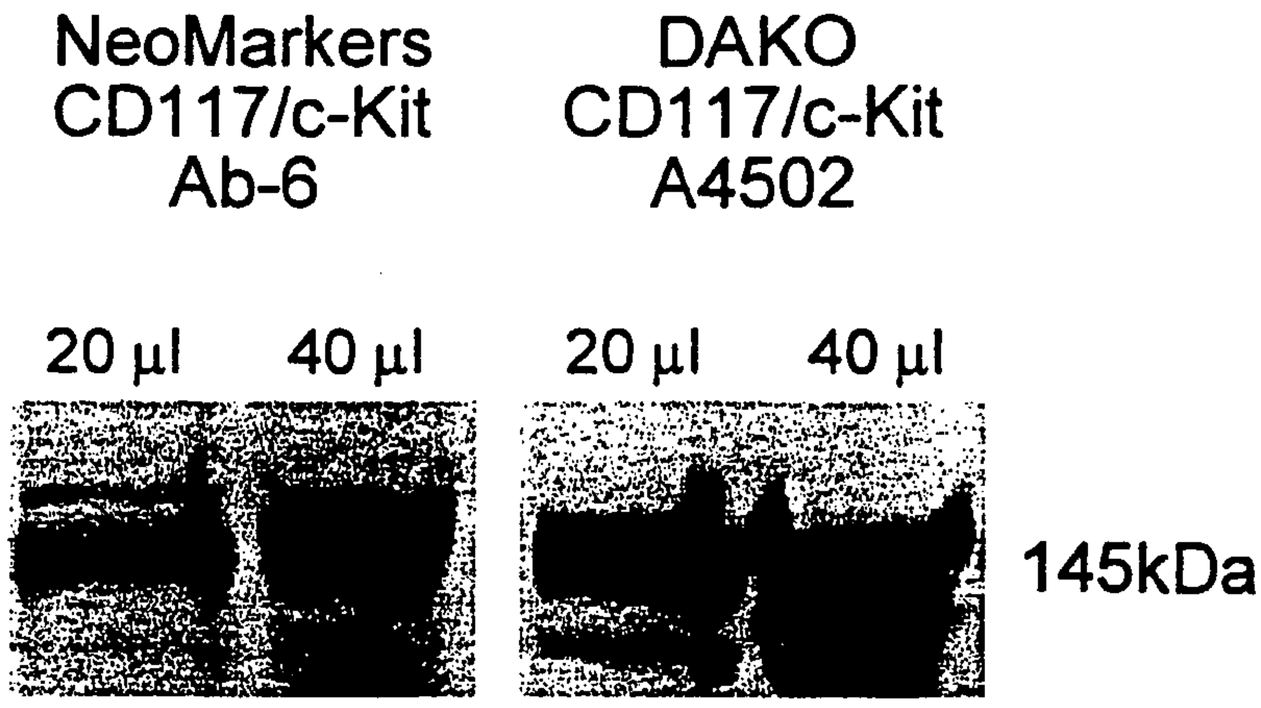

cKit
pAKT
SCF
C-kit, SCF and pAKT levels in 5 different tumor samples
920 Bladder
895 Breast
864 Breast
848 Breast
747 Breast
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0

Figure 3



c-Kit activity in breast cancer cells treated with SCF ligand and/or STI inhibitor. c-Kit was immunoprecipitated from MDA-MB-361 cell lysates (50 μg total protein), western blotted and screened with anti-phosphotryosine antibodies to detect active c-Kit receptor. As expected, the c-Kit receptor was activated by SCF treatment, but inhibited with as little as 1 μM STI.

Figure 4



Western Blot of c-Kit in HeLa cells. Rabbit polyclonal antibodies against human c-Kit from NeoMarkers (1:200 dilution) and DAKO (1:400 dilution), detected with HPR-conjugated antibodies against rabbit IgG (Amersham) and chemiluminescent substrate (NEN).

Figure 5