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(54) Title: P21 AND P21-ASSOCIATED MOLECULES AS BIOMARKERS

(57) Abstract: Methods for assessing sensitivity of drugs candidate to EGFR tyrosine kinase inhibitors are provided. These methods are performed with comparing test and reference levels of at least one biomarker selected from p21 and/or p21 -associated molecules. A similarity, difference or alteration of the level of p21, or a resulting difference or alteration of at least one p21 -associated molecule indicates sensitivity of drugs candidate to one or more EGFR tyrosine kinase inhibitors.



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p21 AND p21-ASSOCIATED MOLECULES AS BIOMARKERS

BACKGROUND OF THE INVENTION

Epidermal growth factor receptor (EGFR) exists on a cell surface and is activated by binding of its specific ligands, including epidermal growth factor and transforming growth factor α (TGF α). Upon activation by its growth factor ligands, EGFR is believed to undergo a transition from an inactive monomeric form to an active homodimer. EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity, which leads to the autophosphorylation of several tyrosine (Y) residues in the C-terminal domain of EGFR. This autophosphorylation elicits downstream activation and signaling by several other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. These downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, Akt and JNK pathways, leading to DNA synthesis and cell proliferation.

Importantly, mutations that lead to EGFR overexpression or overactivity have been associated with a number of cancers, including lung cancer, colon cancers, epithelial cancers, and glioblastoma multiforme. Mutations involving EGFR can lead to its constant activation, which could result in uncontrolled cell division and a predisposition for cancer.

The identification of EGFR as an oncogene has led to the development of anticancer therapeutics directed against EGFR. A number of EGFR tyrosine kinase inhibitors (EGFR-TKIs) have been developed for use in treating cancer, including monoclonal antibody-based drugs such as cetuximab and panitumumab, and small-molecule-based drugs such as gefitinib, erlotinib and lapatinib. Gefitinib and erlotinib are small molecules that reversibly target EGFR tyrosine kinase, both having proven effective in treating non-small cell lung cancer (NSCLC). Gefitinib inhibits EGFR-TK by binding to the adenosine triphosphate (ATP)-binding site of the enzyme, preventing autophosphorylation of the EGFR homodimers. This inhibits the function of the EGFR-TK in activating the signaling cascade. Like gefitinib, erlotinib specifically targets the EGFR-TK and reversibly binds to the ATP binding site of the receptor. However, prediction of a survival benefit of EGFR-TKI therapy remains difficult (Parra et al. 2004 Brit. J. of Cancer 91 : 208; Bailey et al. 2003 Proc. Am. Assoc. Cancer Res. 44: 170A).

P21 is a protein known as a strong cyclin-dependent kinase inhibitor (Ball, K.L., 1997. Progress in Cell Cycle Res., 3: 125). P21 directly inhibits the activity of cyclin-CDK2 and cyclin-CDK4 complexes and thus functions as a regulator of cell

cycle progression. In humans, the protein is encoded by the CDKN1A gene located on chromosome 6. The expression of p21 gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. Two alternatively spliced variants, which encode an identical protein, have been reported.

SUMMARY

According to one aspect of the methods described herein, a therapeutic response of a patient to treatment with an EGFR tyrosine kinase inhibitor (EGFR-TKI) can be predicted. A level of at least one biomarker in a test sample from a patient can be determined in order to determine whether or not an EGFR-TKI can be effective, where the biomarker can be selected from among p21 and one or more p21-associated molecules. The level of the at least one biomarker in the sample can be compared to a reference level, where the reference level can be obtained from diseased or non-diseased tissues depending on the circumstances as described below. The level of the at least one biomarker as compared to the reference level can be predictive of the efficacy of treatment with an EGFR-TKI. A similarity, or difference or alteration of the level of p21, or a resulting difference or alteration of at least one p21-associated molecule, indicates responsiveness of the patient to the EGFR TKI based therapy.

In one embodiment, when the reference level is derived from a non-diseased tissue and the level of the p21 biomarker is the same or decreased as compared to the reference level, the data provides an indication that predicts the patient response to treatment with an EGFR-TKI may not be successful.

In one embodiment, when the reference level is derived from a non-diseased tissue and the level of the p21 biomarker is increased compared to the reference level, the data provides an indication that predicts the patient response to treatment with an EGFR-TKI may be successful

In one embodiment, when the reference level is derived from a diseased tissue known to be responsive to EGFR-TKI treatment and the level of the p21 biomarker is the same or increased as compared to the reference level, the data provides an indication that predicts the patient response to treatment with an EGFR-TKI may be successful.

In one embodiment, when the reference level is derived from a diseased tissue known to be responsive to EGFR-TKI treatment and the level of the p21 biomarker is lower than the reference level, the data provides an indication that predicts the patient response to treatment with an EGFR-TKI may not be successful.

In one embodiment, when the reference level is derived from a diseased tissue known to be non-responsive to EGFR-TKI treatment and the level of the p21

biomarker is increased as compared to the reference level, the data provides an indication that predicts the patient response to treatment with an EGFR-TKI may be successful.

In one embodiment, when the reference level is derived from a diseased tissue known to be non-responsive to EGFR-TKI treatment and the level of the p21 biomarker is the same or lower than the reference level, the data provides an indication that predicts the patient response to treatment with an EGFR-TKI may not be successful.

According to another aspect of the methods, cancer patients can be selected for treatment with an EGFR-TKI. The selection of the patient for treatment can be based on the level of the biomarker compared to the reference level of a diseased or non-diseased tissue as described herein. Accordingly, a determination can be made from the test level in the test sample as compared to a reference level, and when the compared levels are identified to be predictive of a patient's successful response to the EGFR-TKI, the patient is selected. The type of reference tissue being non-diseased or diseased and either responsive or non-responsive to a known EGFR-TKI can be used for comparison to the test tissue levels with the comparative levels being lower, about the same, or higher as described herein.

According to another aspect of the methods, the efficacy of treatment with an EGFR-TKI can be evaluated. A level of at least one biomarker selected from among p21 and one or more p21-associated molecules in a sample is determined. The test level is compared with a reference level of the same biomarker as described herein. An evaluation of the efficacy of the treatment can be made based on the comparison. The test level of the biomarker is compared with the reference level. A patient can be selected for treatment with EGFR-TKI based on the comparison when the data indicates the patient can be successfully be treated. The test level of the biomarker compared to the reference level of the biomarker can be predictive of whether or not a cancer patient will respond to treatment with the EGFR-TKI as described herein.

According to another aspect, drug candidates can be screened for efficacy in cancer treatment. Cancer cells are exposed to at least one drug candidate. At least one of a p21 level or a level of one or more p21-associated molecules in the cancer cells in response to exposure to the at least one drug candidate is determined. The p21 level or the level of one or more p21-associated molecules can be predictive of the efficacy of the drug candidate. A similarity or difference as well as relative increase or decrease of the level of p21, or p21-associated molecule, can indicate whether or not the drug candidate can be useful in cancer therapy. The type of reference tissue being non-diseased or diseased and either responsive or non-responsive to a known EGFR-TKI can be used for comparison to the test tissue

levels with the comparative levels being lower, about the same, or higher as described herein. The foregoing summary is illustrative only and is not intended to be in any way limiting. In addition to the illustrative aspects, embodiments, and features described above, further aspects, embodiments, and features will become apparent by reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and following information as well as other features of this disclosure will become more fully apparent from the following description and appended claims, taken in conjunction with the accompanying drawings. Understanding that these drawings depict only several embodiments in accordance with the disclosure and are, therefore, not to be considered limiting of its scope, the disclosure will be described with additional specificity and detail through use of the accompanying drawings, in which:

Figures 1A-1C show Gefitinib reduced p-Akt levels, concomitant with elevation of p21 levels and suppression of cdk2/4 and cyclinE/D1 activities only in cells in which it inhibits growth. Figure 1A shows that cell death rate was in pc-9 cells (solid triangle) and H1299 cells (solid square) after 24h of Gefitinib treatment. Figure 1B shows a Western blotting analysis performed to assess p-Akt, p21, CDK2, CDK4, cyclinD1 and cyclinE protein levels in pc-9 or H1299 cells with or without Gefitinib treatment for 72 h. Figure 1C shows cell cycle pattern.

Figure 2 shows Gefitinib caused elevation of p21 through post-transcriptional pathway. Pc-9 cells were treated with or without Gefitinib. Figure 2A shows p21 and p53 protein levels assessed by Western blotting analysis. Figure 2B shows p21 mRNA expression determined by real-time PCR, normalized to beta-actin expression. Figure 2C shows p21 protein levels assessed followed by CHX treatment for 2 h.

Figures 3A-3E show the requirement of p21 for cells sensitivity to Gefitinib. Figure 3A shows cell death rate in H1299 cells transiently over-expressed with or without p21 for 24h followed by Gefitinib treatment. Figure 3B shows Western blotting analysis performed in pc-9 cells after transfected with p21 shRNA1, p21 shRNA2 or nonsense control to assess p21 protein. Figure 3C shows p21, CDK2, CDK4, cyclinD1 and cyclinE proteins 24h of Gefitinib treatment with or without p21 knockdown. Figures 3D-3E shows cell death rate was in pc-9 cells after 24h of Gefitinib treatment with or without p21 knockdown.

Figures 4A-4E show that beta-elemene enhanced sensitivity to Gefitinib in pc-9-ZD cells with acquired resistance to Gefitinib. Figure 4A shows the cell death rate in pc-9-ZD cells (*) and its parental pc-9 cells (•) after 24h of Gefitinib treatment.

Figure 4B shows cell death rate in the cells after 24 h treatment with Beta-elemene followed by 24h of Gefitinib treatment. Figures 4C-4D show Western blotting, analysis performed to assess p21 protein levels in the cells with or without treatment of Beta-elemene. Figure 4E shows cell death rate in the cells transiently over-expressed with or without p21 for 24h followed by Gefitinib treatment.

DETAILED DESCRIPTION

It is to be understood that the illustrative embodiments described herein is for illustrative purpose only, and the terminology used herein is only for the purpose of describing illustrative embodiments and is not intended to be limiting.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this embodiments disclosed herein belong.

Certain terms employed in the specification, examples and appended claims are described as follows.

The singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise.

The term “administering” includes any method of delivery of an agent or a pharmaceutical composition into the system or any region of interest of a subject such as, but not limited to, oral, mucosal, nasal, topical, parenteral administration, and the like. The phrases “systemic administration,” “administered systemically”, “peripheral administration”, and “administered peripherally” as used herein mean the administration of a compound, drug or other material such that the substance enters the subject’s system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration. Administration directly into the intended site of action is considered to be local administration and is not systemic administration. “Parenteral administration” and “administered parenterally” means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intra-articular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion. “Oral administration” means that the therapeutic composition is administered orally and is swallowed by the recipient. “Mucosal administration” means that the therapeutic composition is applied to a mucosal membrane with or without a permeation enhancer and the therapeutic agent passes through the mucosal membrane into systemic circulation. “Nasal administration” means that the therapeutic composition is administered into the nose by squirting

and/or inhalation such that the therapeutic agent may be retained in the nasopharynx or passes down to the lungs where the agent is absorbed into systemic circulation. “Topical administration” or “transdermal administration” means that the therapeutic composition is administered to skin with or without a permeation enhancer such that the therapeutic agent passes through the skin into systemic circulation.

“Epidermal growth factor receptor” or “EGFR” is a transmembrane receptor normally involved in cell proliferation among other functions. This protein is composed of an extracellular ligand binding domain, a transmembrane domain, and an intracellular domain having tyrosine kinase activity. The intracellular tyrosine kinase domain undergoes autophosphorylation in response to the binding and stimulation of EGFR by a number of ligands, including but not limited to TGF α (transforming growth factor- α). Phosphorylation of EGFR activates a number of downstream signaling molecules involved in signal transduction cascades, including but not limited to MAPK, Akt, and JNK pathways, resulting in DNA synthesis and cell proliferation. This in turn leads to activation of a variety of intracellular pathways, and these downstream events result in tumor cell proliferation in vitro.

As used herein the term “apoptosis” includes programmed cell death which can be characterized using techniques which are known in the art. Apoptotic cell death can be characterized, e.g., by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation. Cells undergoing apoptosis also display a characteristic pattern of internucleosomal DNA cleavage.

“Epidermal growth factor receptor tyrosine kinase inhibitor” or “EGFR tyrosine kinase inhibitor” or “EGFR-TKI” refers to a compound, substance, antibody, or fragment thereof, or combination that inhibits the function of the intracellular tyrosine kinase domain of EGFR. Such inhibition of the EGFR tyrosine kinase domain may inhibit phosphorylation, and thereby may inhibit downstream signaling. The inhibitor may interact with the tyrosine kinase domain by binding or associating with one or more amino acids in the tyrosine kinase domain.

The term “antibody” includes naturally occurring antibodies or immunoglobulin molecules or genetically engineered antibody molecules that bind antigen in a manner similar to natural antibody molecules. Antibodies and immunoglobulins comprise light and heavy chains, with or without an interchain covalent linkage between them. Modified forms of antibodies made from a whole precursor or parent antibody using techniques known in the art are also suitable for use in the methods provided herein. Although the general structure of essentially all antibodies is similar, a small region of the antibody structure is highly variable, which allows for different antibodies that are capable of recognizing an almost infinite variety of different molecules, known as an antigen. In the context of the immune system, recognition of an antigen by an

antibody tags it for attack by other parts of the immune system. Antibodies can also neutralize targets directly by, for example, binding to a part of a pathogen. The various types of antibodies are well known.

The term “monoclonal antibody” as used herein refers to monospecific antibodies that are made by one type of immune cell that are clones of a unique parent cell. Such monoclonal antibodies typically include an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. The unique clone is then grown and used to produce an essentially homogeneous population of antibodies. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of the embodiments disclosed herein. Monoclonal antibodies are well known.

Techniques are discussed in more detail below. In illustrative embodiments both the variable and constant regions of polypeptides are human. In some embodiments, fully human antibodies can be made using techniques that are known in the art. For example, fully human antibodies against a specific antigen can be prepared by administering the antigen to a transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Techniques that can be used to make antibodies are described, for example, in US patents: 6,150,584; 6,458,592; 6,420,140, which are incorporated by reference in their entirety.

In embodiments where the binding molecule is an antibody or modified antibody, the antigen binding site and the heavy chain portions need not be derived from the same immunoglobulin molecule. In this regard, the variable region may be derived from any type of animal that can be induced to mount a humoral response and generate immunoglobulins against the desired antigen. As such, the variable region of the polypeptides may be, for example, of mammalian origin e.g., may be human, murine, non-human primate (such as cynomolgus monkeys, macaques, etc.), lupine, camelid (e.g., from camels, llamas and related species). In another embodiment, the variable region may be chondrichthoid in origin (e.g., from sharks).

In one embodiment, the binding molecules are modified antibodies. As used

herein, the term “modified antibody” includes synthetic forms of antibodies which are altered such that they are not naturally occurring, e.g., antibodies that do not comprise complete heavy chains (such as, domain deleted antibodies or minibodies); multispecific forms of antibodies (e.g., bispecific, trispecific, etc.) altered to bind to two or more different antigens or to different epitopes on a single antigen); heavy chain molecules joined to scFv molecules and the like. ScFv molecules are known in the art and are described, e.g., in US patent 5,892,019. In addition, the term “modified antibody” includes multivalent forms of antibodies (e.g., trivalent, tetravalent, etc., antibodies that bind to three or more copies of the same antigen).

In some embodiments, an antibody useful in the present disclosure will not elicit a deleterious immune response in a human. Modifications to the constant region compatible with the embodiments described herein comprise additions, deletions or substitutions of one or more amino acids in one or more domains. That is, the antibodies may comprise alterations or modifications to one or more of the three heavy chain constant domains (CH1, CH2 or CH3) and/or to the light chain constant region domain (CL).

The term “cancer”, as used herein in its broadest sense, refers generally to any malignant neoplasm or abnormal cells having spontaneous growth or proliferation. It is contemplated that the methods provided herein may be useful for cancers in which EGFR is expressed. Examples of such cancers include but are not limited to non small cell lung cancer (NSCLC), testicular cancer, lung cancer, ovarian cancer, uterine cancer, cervical cancer, pancreatic cancer, colorectal cancer (CRC), breast cancer, as well as prostate, gastric, skin, stomach, esophageal, urothelial, and gallbladder cancer, and head and neck squamous cancer. In some embodiments, the cancer is known to be treatable by EGFR-TKI. The cancer may be in any stage or phase, primary or metastatic. In one embodiment, the patient has advanced stage cancer (e.g., Stage III/IV lung cancer). In other embodiments, the patient has early stage cancer.

The term “carcinoma” refers to any of various types of malignant neoplasias derived from epithelial cells, e.g., glandular cells (“adenoma” or “adenocarcinoma”) or squamous cells (“squamous cell carcinoma”). Carcinomas often infiltrate into adjacent tissue and spread (“metastasize”) to distant organs, e.g., bone, liver, lung or brain.

The term “chemotherapeutic agent” refers to a molecule or composition used to treat malignancy (cancer). Such agents may be used in combination with an EGFR-TKI and/or an agent that increases the level of p21 or affects the level of one or more p21-associated molecules.

The term “effective amount”, when used with reference to an EGFR-TKI, refers

to that amount of an EGFR-TKI which is sufficient to effect a desired result on a cancerous cell or tumor, including, but not limited to, for example, reduced tumor size, reduced tumor volume, prevention of metastasis, and/or decreased vascularization of a solid tumor. In certain embodiments, an effective amount of an EGFR-TKI is the amount that results in a % tumor inhibition of more than about 58%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%. The term also includes that amount of an EGFR-TKI which is sufficient to achieve a desired clinical result, including but not limited to, for example, ameliorating disease, stabilizing a patient, preventing or delaying the development of, or progression of cancer in a patient. When used with reference to an agent that increases the level of p21 or affects the level of one or more p21-associated molecules, "effective amount" refers to an amount of the agent that is sufficient to result in an increase of the p21 level or alteration in the level of one or more p21-associated molecules, as measured by any of the assays described herein or known in the art. Effective amount of the EGFR-TKI can be determined based on one administration or repeated administration. Methods of detection and measurement of indicators as mentioned above are known to those of skill in the art. Such methods include, but are not limited to measuring reduction in tumor burden, reduction of tumor size, reduction of tumor volume, reduction in proliferation of secondary tumors, decreased solid tumor vascularization, expression of genes in tumor tissue, presence of biomarkers, lymph node involvement, histologic grade, and nuclear grade.

Cancer is "inhibited" if at least one symptom of the cancer is alleviated, terminated, slowed, or prevented. As used herein, cancer is also "inhibited" if recurrence or metastasis of the cancer is reduced, slowed, delayed, or prevented.

"Treating cancer" or "treating a subject having cancer" includes inhibition of the replication of cancer cells, inhibition of the spread of cancer, reduction in tumor size, lessening or reducing the number of cancerous cells in the body, and/or amelioration or alleviation of the symptoms of cancer. A treatment is considered therapeutic if there is a decrease in mortality and/or morbidity, and may be performed prophylactically, or therapeutically.

The terms "patient" and "subject" are used interchangeably herein to refer to either a human or non-human animal. In certain embodiments, the patient or subject is a human. In certain embodiments, the patient or subject is a non-human mammal, including but not limited to a dog, cat, cow, sheep, goat, horse, pig, rabbit, mouse, rat, hamster, guinea pig, chimpanzee, macaque, or camel.

"p21", also known as CIP1, SDI1, WAF1, CAP20, CDKN1, MDA-6, p21CIP1 or CDKN1A, among others is a cyclin-dependent kinase inhibitor which binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a

regulator of cell cycle progression at G1. The expression of the p21 gene is thought to be tightly controlled by the tumor suppressor protein p53, through which p21 protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. However, p21 is known to be expressed independently of p53 in some situations. p21 protein can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair. p21 protein has been reported to be specifically cleaved by CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be instrumental in the execution of apoptosis following caspase activation. The typical amino acid sequence of human and murine version of p21 can be found in Genbank under accession number of NP_000380 and NP_031695, respectively, where the sequences of the Genbank accession numbers are incorporated herein by specific reference. However, the term is contemplated to encompass various natural or artificial variants of the p21 protein, such as the naturally occurring splicing variants of p21 and the various mutant p21 found in cancer cells.

“p21-associated molecules” are well known in the art and include, but are not limited to: proteins upstream or downstream of p21 and/or proteins interacting with p21 in p21-mediated intracellular signaling pathways. “Upstream” p21-associated molecules include proteins that have interactions with biological agents that cause a cascade signaling event to be propagated downstream to the p21 molecule, and thereby p21 receives a biological signal from upstream p21-associated molecule. “Downstream” p21-associated molecules include proteins that receive a biological signal that has been propagated from an interaction of an upstream p21 and a biological agent that causes a cascade signaling event, where the p21 interaction occurs before the p21-associated molecules receive the signal. In some embodiments, the p21-associated molecules may be molecules downstream of p21 in p21 signaling pathways. In some embodiments, the p21-associated molecules may be CDKs or cyclins. Examples of p21-associated molecules include, but are not limited to, CDK2, CDK4, cyclin D, cyclin E, etc.

The term “prognosis”, as used herein, means a prediction of the probable course and outcome of cancer therapy or the likelihood of recovery from the cancer.

The term “biopsy”, as used herein refers to the process of removing a tissue sample for diagnostic or prognostic evaluation, and to the tissue specimen itself. Any biopsy technique known in the art can be applied to the method provided herein. The biopsy technique applied will depend on the tissue type to be evaluated (e.g., lung, prostate, lymph node, liver, bone marrow, blood cell), the size and type of the tumor (e.g., solid or suspended (e.g., blood or ascites)), inter alia. Representative biopsy

techniques include, but are not limited to, excisional biopsy, incisional biopsy, needle biopsy, surgical biopsy, and bone marrow biopsy.

The term “biomarker”, as used herein, refers generally to a molecule, e.g., a gene (or nucleic acid encoding the gene) or protein, the expression of which in a biological sample derived from a patient can be detected by standard methods in the art and/or those described herein, and is indicative of, or denotes, a condition of the subject from which it was obtained.

The terms “response”, “responsive” and “respond”, or “sensitive”, as used herein, refer to the occurrence, or likelihood of occurrence, of therapeutically desirable effect, including but not limited to regression of tumor, delay in the progression or metastasis of tumor, suppressed growth or enhanced apoptosis of cancer cells, and/or alleviation of one or more clinical symptoms of cancer, in a patient or population of cancer cells subsequent to administration of an EGFR-TKI.

Gefitinib is known to its inhibition of EGFR tyrosine kinase and worldwide used for anti-tumor in non-small cell lung cancer (NSCLC). It has now been shown that Gefitinib reduces p-Akt levels, concomitant with elevation of p21 levels and suppression of cdk2/4 and cyclinE/D1 activities which result in impaired cell cycle progression through G1 arrest only in NSCLC cells in which it inhibits growth. It has been found that Gefitinib-induced p21 protein stability, rather than increased RNA accumulation, was responsible for the elevated p21 levels. More, treatment of beta-elemene, a natural plant drug extracted from *Curcuma wenyujin*, restored sensitivity to Gefitinib via the mechanism modulated the elevation of p21 levels in the cells which are acquired resistance to Gefitinib. These data suggest that administration of Gefitinib in combination with beta-elemene, may offer great opportunities for NSCLC which are acquired resistance to Gefitinib. The p21 effect on the cells to response to Gefitinib was further confirmed by p21 over-expression and knockdown studies pointing to a requirement of p21 for the cells sensitive to Gefitinib. Thus, we propose that p21 is required for Gefitinib-sensitive NSCLC cells.

Beta-elemene, a natural plant drug extracted from *Curcuma wenyujin*, has been used as an antitumor drug for different tumors, including NSCLC via mechanism that inhibits Ras/Mapk signaling and cell cycle progression. Beta-elemene acts synergistically with cytotoxic drugs against a variety of tumor cells, and the observation that beta-elemene is able to do so in drug-resistant patients and thereby overcome drug resistance is especially provocative.

Alterations in cell cycle control are a universal feature of lung cancers. P21, the product of the WAF1/CIP1/SDI1 gene, is an inhibitor of cyclin-dependent kinases and is activated through p53-dependent or p53-independent pathways. Studies have clearly indicated that p21 plays an important role in regulation of the cell cycle,

especially in G1 arrest. Recent studies have showed that p21 plays important role in antiproliferative effort of Gefitinib.

Now, correlations between p21 expression and the incidence of Gefitinib-induced cytostasis, proliferative activity, and p53 status in the cell lines have been identified. It has now been found that p21 plays an important role in NSCLC response to Gefitinib.

Use of p21 and p21-associated Molecules as Biomarkers

The present disclosure is based at least in part on the finding that p21 (waf1/cip1) is associated with gefitinib-sensitive non-small cell lung cancer (NSCLC). Although not intending to be limited by theory, it is postulated that p21 expression in NSCLC is at least partially responsible for the observation of sensitivity of NSCLC to gefitinib and other EGFR-TKI. Thus, the present disclosure relates generally to the use of p21 and p21 associated molecules as biomarkers useful in predicting whether or not a given cell type, a cancerous tumor, and/or a patient will respond positively to EGFR TKI-based therapies.

The present disclosure illustrates the use of p21 and p21 associated molecules as predictive and prognostic indicators for predicting or assessing a patient's responsiveness to EGFR TKI-based therapies, based on a comparison of the level of p21 and/or p21-associated molecules determined in a test sample derived from the patient with a reference level. For example, levels of p21 and/or p21 associated molecules can be compared to cancerous and non-cancerous tissues before and/or after exposure to EGFR-TKIs. In one embodiment, the presence of p21 expression in a tissue may be predictive of sensitivity to EGFR-TKIs in that tissue. In another embodiment, a similarity or difference of the level of p21, or a p21-associated molecule between tissue types may indicate responsiveness of a tissue to EGFR-TKI based therapy. In yet another embodiment, a similarity or difference of the level of p21, or a resulting similarity or difference of at least one p21-associated molecule before and after exposure to an EGFR-TKI may indicate responsiveness of a tissue to EGFR-TKI based therapy.

One embodiment relates to a method for predicting therapeutic response of a patient to treatment with an EGFR-TKI. In one aspect, the method for predicting therapeutic response of a patient to treatment with an EGFR tyrosine kinase inhibitor can include determining a level of at least one biomarker in a test sample. The at least one biomarker can include, but is not limited to p21 and one or more p21-associated molecules. The method further includes comparing the level of the at least one biomarker in the test sample to a reference level, wherein the test level of the at least one biomarker as compared to the reference level is predictive of a patient's

response to the EGFR-TKI.

In one aspect, the sample (e.g., a test sample and/or a reference sample) can include cells taken from one or more tissues that may have a detectable level of p21 and/or a detectable level of one or more p21-associated molecules. Alternatively, the sample may be substantially devoid of detectable p21 and/or p21-associated molecules. As such, the sample can be tested to determine whether or not the cells include the p21 and/or p21-associated molecule biomarkers.

In one aspect, a suitable example of a tissue that may have a detectable level of p21 and/or a detectable level of one or more p21-associated molecules includes cells from a cancerous tissue. Suitable examples of cancerous tissue include, but are not limited to, lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer. Another suitable example of a cancerous tissue includes, but is not limited to, non-small cell lung cancer.

In another aspect, a suitable example of a tissue that may have a detectable level of p21 and/or a detectable level of one or more p21-associated molecules includes non-cancerous cells of the same histology type as the cells from a cancerous tissue. Non-cancerous cells can be referred to as non-diseased cells for the methods described herein.

In yet another aspect, suitable examples of tissues include, but are not limited to, a surgically removed tumor mass, biopsied tissue, bronchoalveolar lavage fluid (BALF), pleural fluid, lymph nodes, or blood, a biological sample containing cancer cells, and combinations thereof.

In a further aspect, the sample of the tissue that may have a detectable level of p21 and/or a detectable level of one or more p21-associated molecules includes can be taken from the one or more tissues before, during, or after the treatment with EGFR-TKI therapy.

In one aspect, the p21-associated molecules can be molecules whose expression, cellular level, or stability is affected by p21, such as, but not limited to, downstream molecules whose expression cellular level, or stability is affected by p21. Suitable examples of p21 downstream molecules include, but are not limited to, CDK2, CDK4, cyclin D, cyclin E, and combinations thereof.

In one aspect, the level of the at least one biomarker is determined by an immunoassay. Suitable examples of immunoassays that can be used to determine the level of the at least one biomarker include, but are not limited to, western blotting, immunohistochemistry, ELISA, and combinations thereof.

One skilled in the art will appreciate that, for this and other processes and methods disclosed herein, the functions performed in the processes and methods may

be implemented in differing order. Furthermore, the outlined steps and operations are only provided as examples, and some of the steps and operations may be optional, combined into fewer steps and operations, or expanded into additional steps and operations without detracting from the essence of the disclosed embodiments.

In another embodiment, a method for evaluating the efficacy of treatment with an EGFR-TKI is disclosed. In one aspect, the method for evaluating the efficacy of treatment with an EGFR-TKI can include determining a level of at least one biomarker such as, but not limited to, p21 and/or one or more p21-associated molecules in a test sample, comparing the test level with a reference level of the same one or more biomarkers, and making an evaluation of the efficacy of the treatment based on the comparison. The test level of the at least one biomarker as compared to the reference level is predictive of the efficacy of treatment with an EGFR-TKI. The type of tissue that is used to obtain the reference level can be determinative as to whether or not the relative level provides an indication of an effective treatment.

In one aspect, the sample (e.g., a test sample and/or a reference sample) can be taken from one or more tissues that may have a detectable level of p21 and/or a detectable level of one or more p21-associated molecules. Suitable examples of a tissue having a detectable level of p21 and/or a detectable level of one or more p21-associated molecules can include, but is not limited to, cells from a cancerous tissue.

According to one aspect, the reference level can be derived from a non-diseased tissue. That is, a non-diseased tissue can be collected, assayed for the level of p21 and/or one or more p21-associated molecules, and compared to the level of p21 and/or one or more p21-associated molecules in a test sample (e.g., a tissue sample from a cell type, cancerous tumor, or a patient being evaluated for treatment with an EGFR-TKI).

In one aspect, where the reference level is derived from a non-diseased tissue, an increase of p21 level, or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR-TKI treatment. As such, the amount of increase of p21 level and/or resulting alteration of p21-associated molecule level in response to an increase in p21 level can be an indication of efficiency, where higher increases of relative levels indicate more efficiency of treatment with EGFR-TKI may be achieved. In contrast, a similarity of or a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 levels, as compared with the reference level, indicates a non-positive response to EGFR tyrosine-TKI treatment. As such, the amount of decrease of p21 and/or resulting alteration of p21-associated molecule level in

response to a decrease in p21 level can be an indication of a non-positive response, where the larger values of decreased level can be more indicative that the treatment may not be successful.

In one aspect, the reference level can be derived from a diseased tissue known to be responsive to EGFR-TKI treatment. That is, a diseased tissue known to be responsive to EGFR-TKI treatment can be collected, assayed for the level of p21 and/or one or more p21-associated molecules, and compared to the level of p21 and/or one or more p21-associated molecules in a test sample (e.g., a tissue sample from a cell type, cancerous tumor, or a patient being evaluated for treatment with an EGFR-TKI). Where the reference level is derived from a diseased tissue known to be responsive to EGFR-TKI treatment, a similarity of the p21 level or at least one p21-associated molecules from the test tissue, or an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule, as compared to the reference level, indicates a positive response to EGFR-TKI treatment. As such, the amount of increase of p21 level and/or resulting alteration of p21-associated molecule level in response to an increase in p21 level can be an indication of efficiency, where higher increases of relative levels indicate more efficiency of treatment with EGFR-TKI may be achieved. In contrast, a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates a non-positive response to EGFR-TKI treatment. This non-positive response may be no response to EGFR-TKI treatment. As such, the amount of decrease of p21 and/or p21-associated molecule level can be an indication of a non-positive response or no response to EGFR-TKI treatment, where the larger values of decreased level can be more indicative that the treatment may not be successful.

The reference level can be derived from a diseased tissue known to be non-responsive to EGFR-TKI treatment. That is, a diseased tissue known to be non-responsive to EGFR-TKI treatment can be collected, assayed for the level of p21 and/or one or more p21-associated molecules, and compared to the level of p21 and/or one or more p21-associated molecules in a test sample (e.g., a tissue sample from a cell type, cancerous tumor, or a patient being evaluated for treatment with an EGFR-TKI). Where the reference level is derived from a diseased tissue known to be non-responsive to EGFR-TKI treatment, an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR-TKI treatment. As such, the amount of increase of p21 level and/or resulting alteration of p21-associated molecule level in response to an increase in p21 level can be an indication of efficiency, where higher increases of relative levels

indicate more efficiency of treatment with EGFR-TKI may be achieved. In contrast, a similarity of the p21 level or at least one p21-associated molecule, or a decrease in the p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates a non-positive response to EGFR-TKI treatment. The non-positive response may be no response to EGFR-TKI treatment. As such, the amount of decrease of p21 and/or p21-associated molecule level can be an indication of a non-positive response or no response to EGFR-TKI treatment, where the larger values of decreased level can be more indicative that the treatment may not be successful.

In one aspect, the sample can be taken from a patient within about 1 hour before to about 15 hours after the administration of the EGFR-TKI, or within about 1 hour after to about 15 hours after, or within about 2 hour after to about 12 hours after, or within about 3 hour after to about 10 hours after, or within about 3 hour after to about 7 hours after, or any time therebetween.

The level of the at least one biomarker can be determined by an immunoassay such as, but not limited to, western blotting, immunohistochemistry, ELISA, and combinations thereof.

In yet another embodiment, a method for selecting cancer patients for treatment with an EGFR-TKI is disclosed. In one aspect, a method for selecting cancer patients for treatment with an EGFR-TKI can include, but is not limited to, determining at least one test level in the test sample (and optionally at least one reference level in the reference sample) of at least one biomarker such as but not limited to of p21 and/or one or more p21-associated molecules. This can include comparing the test level with a reference level, and selecting a patient for treatment with EGFR-TKI based on the comparison, wherein the test level of the at least one biomarker as compared to the reference level of the at least one biomarker is predictive of whether or not a cancer will respond to treatment with the EGFR-TKI.

In one aspect, a patient can be selected for treatment with an EGFR-TKI when a difference between the level in the test sample and the reference level is shown. In another aspect, a patient can be selected for treatment with an EGFR-TKI when a similarity between the level in the sample and the reference level is shown.

In one aspect, the at least one test sample and the at least one reference sample can be taken from one or more tissues that may have a detectable level of p21 and/or one or more p21-associated molecules. Suitable example of the one or more tissues that can have a detectable level of p21 and/or one or more p21-associated molecules include, but are not limited to, cells from a cancerous tissue such as, but not limited to, lung cancer, non-small cell lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer.

The p21-associated molecules can be p21 downstream molecules such as, but not limited to, CDK2, CDK4, cyclin D, cyclin E, and combinations thereof.

The level of the at least one biomarker in the test sample and/or the reference sample can be determined by an immunoassay such as, but not limited to, western blotting, immunohistochemistry, ELISA, and combinations thereof.

The EGFR-TKI such as, but not limited to, erlotinib, lapatinib, gefinitib, and combinations thereof.

In a further embodiment, a method for screening a drug candidate for efficacy in cancer treatment is disclosed. In one aspect, a method for screening a drug candidate for efficacy in cancer treatment can include but is not limited to exposing cancerous cells to at least one drug candidate, and determining at least one of a p21 level or a level of one or more p21-associated molecule in the cancer cells in response to exposure to the at least one drug candidate, wherein the p21 level or the level of one or more p21-associated molecules is predictive of the efficacy of the drug candidate.

In one aspect, the drug candidate can be a substance that induces cell death in the cancerous cells. In another aspect, the drug candidate can be an EGFR tyrosine kinase inhibitor.

In one aspect, the method for screening a drug candidate for efficacy in cancer treatment can further include providing a reference sample, and comparing a p21 level or a level of one or more p21-associated molecule in the reference sample to the p21 level or the level of one or more p21-associated molecules in the cancerous cells. In another aspect, the cancerous cells and/or the reference sample may have a detectable level of p21 and/or one or more p21-associated molecules.

In one aspect of the present disclosure, the reference level can be derived from a non-diseased tissue. That is, a non-diseased tissue can be collected, assayed for the level of p21 and/or one or more p21-associated molecules, and compared to the level of p21 and/or one or more p21-associated molecules in a test sample (i.e., a tissue sample from a cell type, cancerous tumor, or a patient being evaluated for treatment with an EGFR-TKI).

In another aspect of the present disclosure, the reference level can be derived from a diseased tissue known to be responsive to EGFR-TKI treatment. That is, a diseased tissue known to be responsive to EGFR-TKI treatment can be collected, assayed for the level of p21 and/or one or more p21-associated molecules, and compared to the level of p21 and/or one or more p21-associated molecules in a test sample (e.g., a tissue sample from a cell type, cancerous tumor, or a patient being evaluated for treatment with an EGFR-TKI).

In yet another aspect of the present disclosure, the reference level can be derived from a diseased tissue known to be non-responsive to EGFR-TKI treatment. That is,

a diseased tissue known to be non-responsive to EGFR-TKI treatment can be collected, assayed for the level of p21 and/or one or more p21-associated molecules, and compared to the level of p21 and/or one or more p21-associated molecules in a test sample (i.e., a tissue sample from a cell type, cancerous tumor, or a patient being evaluated for treatment with an EGFR-TKI).

In one aspect, the cancerous cells and the reference sample include cells having a histology type that is the same. In one embodiment, cells having the same histology type may include cells taken from the same tissue type (e.g., healthy lung cells are compared to cancerous lung cells). In another embodiment, cells having the same histology type can be described as cells as cells having the same microscopic anatomy (e.g., the cells being compared are both appear microscopically to be epithelial cells taken from healthy lung tissue and cancerous lung tissue).

In one aspect, the cancerous cells can be cells such as, but not limited to, lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer. In a further aspect, the cancerous cells are non-small cell lung cancer cells.

In one aspect, the p21-associated molecules can be p21 downstream molecules. In one aspect, the p21 downstream molecules include one or more of CDK2, CDK4, cyclin D, or cyclin E.

In one aspect, the p21 level or the level of one or more p21-associated molecules is determined by an immunoassay. Suitable examples of immunoassays include, but are not limited to, western blotting, immunohistochemistry, ELISA, and combinations thereof.

In certain embodiments, the patient is suspected of, diagnosed with, or suffering from cancer. The cancer may include, but is not limited to, lung cancer, in particular non-small cell lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer. In certain embodiments, EGFR is overexpressed or over-active in the cancer.

In the context of the methods provided herein, the level or amount of p21 and/or p21-associated molecules refers to the amount of the protein or mRNA of p21 and/or p21-associated molecules present in a tumor, a cell, or a sample. The level may be either an absolute level as measured in, e.g., molecules, moles or weight per unit volume or cells, or be a relative level, e.g., measured by densitometric analysis. The level of a biomarker, e.g., p21 and/or p21-associated molecule(s), in a tumor, a cell, or a test sample derived from a subject is "increased" or "decreased" relative to a reference level of a biomarker, if the level of the biomarker, e.g., p21 and/or p21-associated molecule(s), is greater or less, respectively, than the reference level by an amount ratio that is greater than the standard error of the assay employed to assess

the level. For the purpose of the present disclosure, the level of the biomarker is also considered “increased” where the level is determined as positive in a test sample and negative in a reference or control sample, and “decreased” where the level is determined as negative in the test sample and positive in the reference or control sample, in the particular assay as used. The level of the biomarker is “positive”, or alternatively, “negative” or “background”, when the level is higher or lower, respectively, than the cut-off or threshold of the assay used to measure the level. The level of p21 and/or p21-associated molecule(s) in a tumor, a cell, or a test sample derived from a subject is “affected”, “altered” or “modulated” when the level is increased and/or decreased as defined above. For example, the level p21 and/or p21-associated molecule(s) in a tumor, a cell, or a test sample derived from a subject is increased or decreased when the level is 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2 times, 3 times, 4 times, 5 times, 10 times or more higher/lower, respectively, than the reference level.

In some embodiments, the difference or alteration (e.g., increase or decrease) in the level of p21 or p21-associated molecules may be statistically significant, which means that the difference or alteration is greater than what might be expected to happen by chance alone. Statistical significance can be determined by any method known in the art. For example, statistical significance can be determined by p-value. The p-value is a measure of probability that a difference between groups during an experiment happened by chance. For example, a p-value of 0.01 means that there is a 1 in 100 chance the result occurred by chance. The lower the p-value, the more likely it is that the difference between groups was caused by treatment. A difference or alteration is considered to be statistically significant if the p-value is, for example, at least 0.05. The p-value may be 0.04, 0.03, 0.02, 0.01, 0.005, 0.001 or less to indicate statistical significance.

The mRNA level of p21 or p21-associated molecules can be determined using techniques known in the art for measuring mRNAs. The measuring can comprise directly measuring the mRNA or transcript obtained from a cell, or measuring the cDNA obtained from an mRNA preparation thereof. Such methods of extracting the mRNA or transcript from a cell, or preparing the cDNA thereof are well known to one skilled in the art. Techniques known in the art for analyzing or measuring mRNA include, e.g., reverse transcription and amplification of mRNA, isolation of total RNA or poly A⁺ RNA, northern blotting, dot blotting, in situ hybridization, RNase protection, probing DNA microchip arrays, and the like. These techniques can be performed with knowledge of the polynucleotide sequence of the target gene or mRNA. Detailed description of these techniques may be found, e.g., in Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press (1989).

The protein level of p21 or p21-associated molecules can be determined using suitable assays for proteins known to those skilled in the art. For example, the assay may be carried out by immunohistochemistry. The antibody used for this purpose may be polyclonal or monoclonal. It can be a direct or indirect IHC. Detailed protocols of immunohistochemistry procedures may be found in, e.g., "Diagnostic Immunohistochemistry" 2nd Edition by David Dabbs (2006) Published by Churchill Livingstone.

The protein assay may also be carried out by Western blotting, which includes steps of tissue preparation, gel electrophoresis, transfer, blocking, detection and analysis. Detailed description for general procedures of Western blotting can be found, e.g., in Sambrook, *supra*.

Immunoassays are also useful in the methods provided herein for determining the level of p21 or p21-associated molecules. Immunoassay techniques and protocols are generally described in Price and Newman, "Principles and Practice of Immunoassay," 2nd Edition, Grove's Dictionaries, 1997; and Gosling, "Immunoassays: A Practical Approach," Oxford University Press, 2000. A variety of immunoassay techniques, including competitive and non-competitive immunoassays, can be used (see, e.g., Self et al., *Curr. Opin. Biotechnol.*, 7:60-65 (1996)). The term immunoassay encompasses techniques including, but not limited to, enzyme immunoassays (EIA) such as enzyme multiplied immunoassay technique (EMIT), enzyme-linked immunosorbent assay (ELISA), IgM antibody capture ELISA (MAC ELISA), and microparticle enzyme immunoassay (MEIA); capillary electrophoresis immunoassays (CEIA); radioimmunoassay (RIA); immunoradiometric assays (IRMA); fluorescence polarization immunoassays (FPIA); and chemiluminescence assays (CL). Such immunoassays can be automated. Immunoassays can also be used in conjunction with laser induced fluorescence (see, e.g., Schmalz ing et al., *Electrophoresis*, 18:2184-93 (1 997); Bao, J. *Chromatogr. B. Biomed. Sci.*, 699:463-80 (1997)). Liposome immunoassays, such as flow-injection liposome immunoassays and liposome immunosensors, are also suitable for use in the embodiments described herein (see, e.g., Rongen et al., *J. Immunol. Methods*, 204: 105-133 (1 997)). In addition, nephelometry assays, in which the formation of protein-antibody complexes results in increased light scatter that is converted to a peak rate signal as a function of the marker concentration, are suitable for use in the methods provided herein. Nephelometry assays are commercially available from Beckman Coulter (Brea, CA; Kit #449430) and can be performed using a Behring Nephelometer Analyzer (Fink et al., *J. Clin. Chem. Clin. Biochem.*, 27:261-276 (1 989)).

In certain embodiments, a cut-off value for positive/negative expression was set

to be 10% of cancer cells displaying staining.

In certain embodiments, antibodies against p21 or p21-associated molecules are useful to detect or measure the level of p21 or p21-associated molecules. Such antibodies may be polyclonal antibodies, monoclonal antibodies, or antibody fragments. Methods for preparing such antibodies or fragments thereof are well known in the art.

For example, polyclonal antibodies may be raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}_1\text{N}=\text{C}=\text{NR}$, where R and R_1 are different alkyl groups.

Animals are immunized against the p21 or p21-associated molecules, or immunogenic fragments, conjugates or derivatives thereof, by combining, for example, 100 mg or 5 mg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. Approximately one month later, the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. In some embodiments, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., 1975, *Nature*, 256:495, or may be made by recombinant DNA methods (See, for example, U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described with polyclonal antibodies to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, 1986, *Monoclonal Antibodies: Principles and Practice*,

pp.59-103 (Academic Press)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that may contain one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

In some embodiments, myeloma cells are used which fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, some myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, 1984, *J. Immunol.*, 133:3001; Brodeur et al., 1987, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. The binding specificity of monoclonal antibodies produced by hybridoma cells may be determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *supra*). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then

transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

Antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., 1990, *Nature*, 348:552-554. Clackson et al., 1991, *Nature*, 352:624-628, and Marks et al., 1991, *J. Mol. Biol.*, 222:581-597 describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., 1992, *Bio/Technology*, 10:779-783), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., 1993, *Nuc. Acids. Res.*, 21:2265-2266). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, et al., 1984, *Proc. Natl Acad. Sci. USA*, 81:6851), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for non-immunoglobulin material (e.g., protein domains). Typically such non-immunoglobulin material is substituted for the constant domains of an antibody, or is substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

A test sample suitable for the methods provided herein may be any biological material derived from the patient in which the level of p21 or p21-associated molecules can be detected. In certain embodiments, the test sample contains populations of the cell type from which the cancer to be treated is derived. In one embodiment, the test sample comprises a lung cell (a cell obtained from the lung). Examples of the source of the test sample include, but not limited to, surgically removed tumor mass, biopsy, bronchoalveolar lavage fluid (BALF), pleural fluid, lymph nodes, or blood. Furthermore, the test sample may be cells purified from a tissue.

In certain embodiments, the test sample is a sample that contains cancer cell obtained from the subject. The cancer cell-containing sample could be any biological sample containing cancer cells, e.g., derived from surgically removed tumor mass, biopsy, bronchoalveolar lavage fluid (BALF), pleural fluid, lymph nodes,

or blood.

The test sample can be taken from the subject before, during, or after the treatment of EGFR tyrosine kinase inhibitor. More than one test samples can be taken at different times during the course of the treatment of EGFR tyrosine kinase inhibitor. For example, the test sample may be obtained from the subject before the first dose of the EGFR-TKI for providing a prognosis of the EGFR-TKI therapy, or after a dose or course of treatment of the EGFR-TKI to evaluate the efficacy of the EGFR-TKI therapy.

In certain embodiments, the reference level may be a normal control level. A normal control level may be the level of p21, or at least one of p21-associated molecules, typically found in a subject not suffering from and not likely to have the disease or disorder (e.g., cancer) in question, e.g., relative to samples collected from 10 longitudinal studies of young subjects who were monitored until advanced age and were found not to develop the disease or disorder (e.g., cancer) in question. The normal control level can be a range or an index. Alternatively, the normal control level can be a database of patterns from previously tested subjects. The normal control level of p21 and/or p21-associated molecules can also be determined in a normal sample, which means a sample that does not contain cancer cells of the cancer in question or other cancer types that involve abnormal expression of EGFR. The normal sample may be non-cancerous tissues or non-malignant cells obtained from the subject, or established non-cancerous cell lines. In certain embodiments, the normal control is of the same histology type as the cancer in question. In certain embodiments, a normal control level of p21 or p21-associated molecule is an average level determined for a population to which the subject belongs. In certain embodiments, a normal control level of p21 or p21-associated molecule is a baseline level, i.e., one that has been previously determined in the subject before the cancer in question or any other cancer type which involves abnormal expression of EGFR developed in the subject.

When the reference is a normal control level, an increase of the level of p21 or the resulting alteration in the level of at least one p21-associated molecules in the test sample as compared with the reference level indicates that the patient is responsive to the EFGR-TKI, whereas a decrease in the level of p21 or the resulting alteration in the level of at least one p21-associated molecules, or similarity in the level of p21 or the level of at least one p21-associated molecules, as compared with the reference level, indicates that the patient is not responsive to the EFGR-TKI.

The reference level may also be a responsive disease control level. A responsive disease control level may be the level of p21 or at least one of p21-associated molecules, typically found in a subject with a cancer responsive to the

EFGR-TKI. In some embodiments, the EFGR-TKI-responsive cancer is of the same type of cancer in question. In certain embodiments, the responsive disease control level is determined in a responsive disease sample, i.e., a sample containing cancer cells that are known to be responsive to the EFGR-TKI. The responsive disease sample may be a sample obtained from a subject known to be responsive to the EFGR-TKI, or a population of cancer cells known to be responsive to the EFGR-TKI. Typically, the cancer cells contained in the responsive disease sample are of the same histology type as the cancer in question. In specific embodiments, the responsive disease control level is determined as the level of p21 or at least p21-associated molecule in an established cancer cell line which is known to be responsive or sensitive to the EFGR-TKI, such as, for example, A459 cell line of NSCLC. Alternatively, the responsive disease control level can be a database of patterns from previously tested subjects who proved responsive to EFGR TKI.

When the reference is a responsive disease control level, an increase of the level of p21 or the resulting alteration in the level of at least one p21-associated molecules in the test sample as compared with the reference level, or similarity in the level of p21 or the level of at least one p21-associated molecules, as compared with the reference level, indicates that the patient is responsive to the EFGR-TKI, whereas a decrease in the level of p21 or the resulting alteration in the level of at least one p21-associated molecules indicates that the patient is not responsive to the EFGR-TKI.

The reference level may also be a non-responsive disease control level. A non-responsive disease control level may be the level of p21 or at least one of p21-associated molecules, typically found in a subject with a cancer non-responsive or resistance to the EFGR-TKI. In some embodiments, the EFGR-TKI-resistant cancer is of the same type of cancer in question. In certain embodiments, the non-responsive disease control level is determined in a non-responsive disease sample, i.e., a sample containing cancer cells that are known to be non-responsive or resistance to the EFGR-TKI. The non-responsive disease sample may be a sample obtained from a subject known to be non-responsive to the EFGR-TKI, or a population of cancer cells known to be non-responsive to the EFGR-TKI. Typically, the cancer cells contained in the non-responsive disease sample are of the same histology type as the disease or disorder in question. In specific embodiments, the non-responsive disease control level is determined as the level of p21 or at least p21-associated molecule in an established cancer cell line which is resistant to the EFGR-TKI. Alternatively, the non-responsive disease control level can be a database of patterns from previously tested subjects who showed non-responsive to EFGR-TKI.

When the reference is a non-responsive disease control level, an increase of the level of p21 or the resulting alteration in the level of at least one p21-associated molecules in the test sample as compared with the reference level indicates that the patient is responsive to the EGFR-TKI, whereas a decrease in the level of p21 or the resulting alteration in the level of at least one p21-associated molecules, or similarity in the level of p21 or the level of at least one p21-associated molecules, as compared with the reference level, indicates that the patient is not responsive to the EGFR-TKI.

As used herein, the “resulting alteration” of the level of a p21-associated molecule is the change in the level of the molecule in the cancer cell as a result of the alteration of the p21 level as recited. The change in the p21-associated molecule may be a change in the protein level, mRNA level, or activity of the molecule. The specific nature of the alteration can be readily determined by one skilled in the art based on the particular positive/negative regulatory relationship between p21 and the p21-associated molecule.

In certain embodiments of this respect, an increase in the level of p21 as compared with the reference level indicates that the patient is likely to have a positive response to EGFR-TKI therapy, and a similarity or decrease as compared with the reference level indicates that the patient is likely to have a negative response to EGFR-TKI therapy.

In certain embodiments of this respect, a decrease in the level of at least one p21-associated molecule as compared with the reference level indicates that the patient is likely to respond to EGFR-TKI therapy, and a similarity or increase as compared with the reference level indicates that the patient is unlikely to respond to EGFR-TKI therapy.

In some embodiments, the at least one p21-associated molecules such as, but not limited to, CDK2, CDK4, cyclin D, and cyclin E.

In various embodiments of this aspect, the test sample is a cancer cell-containing sample obtained from the patient. In some embodiments, the test sample is a cancer cell-containing biological sample obtained from surgically removed tumor mass, biopsy, bronchoalveolar lavage fluid (BALF), pleural fluid, lymph nodes, or blood of the patient. A sample from ascites surrounding the tumor may also be used. In some embodiments, the test sample contains lung cancer cells. In still some embodiments, the test sample contains NSCLC cells.

The level of p21 or p21-associated molecules as compared with the reference level may serve as a predictor or indicator that aids in various aspects of the treatment of cancer.

Method for predicting therapeutic response to EGFR tyrosine kinase inhibitors

For example, in one aspect, the level of p21 or p21-associated molecules as

compared with the reference level can be used as a predictor of the therapeutic response of a patient with cancer to EGFR-TKI therapy. Thus, for example, it is provided herein a method for predicting the therapeutic response of a patient with cancer to EGFR-TKI treatment, which includes providing a test sample, determining a level of at least one biomarker such as, but not limited to, p21 and one or more p21-associated molecules in the test sample from the patient, and comparing the level of the at least one biomarker in the sample to a reference level of expression.

In certain embodiments of the method, the reference level is a normal control level. In such embodiments, an increase of p21 level, or the resulting alteration in the level of at least one p21-associated molecules, as compared to the reference level, indicates that the patient will respond to EGFR-TKI treatment, whereas a similarity of or decrease in the p21 level, or the resulting alteration in the level of at least one p21-associated molecules, as compared with the reference level, indicates that the patient will not respond to EGFR-TKI treatment.

In certain embodiments of the method, the reference level is a responsive disease control level. In such embodiments, a similarity of the p21 level or at least one p21-associated molecules, or an increase in p21 level or the resulting alteration in the level of at least one p21-associated molecule, as compared to the reference level, indicates that the patient will respond to EGFR-TKI treatment, whereas a decrease in the p21 level, or the resulting alteration in the level of at least one p21-associated molecule, as compared with the reference level, indicates that the patient will not respond to EGFR-TKI treatment.

In certain embodiments of the method, the reference level is a non-responsive disease control level. In such embodiments, an increase in p21 level or the resulting alteration in the level of at least one p21-associated molecule, as compared to the reference level, indicates that the patient will respond to EGFR-TKI treatment, whereas a similarity of the p21 level or at least one p21-associated molecule, or a decrease in the p21 level or the resulting alteration in the level of at least one p21-associated molecule, as compared with the reference level, indicates that the patient will not respond to EGFR-TKI treatment.

In this and various aspects of the present disclosure, the p21-associated molecule may be a p21-downstream molecule. In some embodiments, the p21-associated molecule such as, but not limited to, CDK2, CDK4, cyclin D, and cyclin E.

In this and various aspects of the present disclosure, the EGFR-TKI such as, but not limited to, erlotinib, lapatinib, and gefinitib, particularly but not limited to gefinitib. In illustrative embodiments, the test sample is a biopsy. In illustrative embodiments, the test sample contains cancer cells. In illustrative embodiments, the cancer is NSCLC. In illustrative embodiments, the level of p21 or at least one

p21-associated molecule is a protein level. In illustrative embodiments, the level of p21 or at least one p21-associated is determined by immunohistochemistry.

Method for evaluating efficacy of EGFR tyrosine kinase inhibitors

For example, in another aspect, the level of p21 or p21-associated molecules as compared with the reference level can be utilized as an indicator on the efficacy of EGFR-TKI treatment of cancer in a patient. Thus, for example, it is provided herein a method for evaluating the efficacy of EGFR-TKI treatment of cancer in a patient, which includes providing a test sample, determining a level of at least one biomarker such as, but not limited to, p21 and one or more p21-associated molecules in the test sample from the patient, and comparing the level of the at least one biomarker in the sample to a reference level of expression.

In certain embodiments of the method, the reference level is determined in a sample obtained from the patient prior to the initiation of the EGFR-TKI treatment. In some embodiments, an increase of p21 level or the resulting alteration in the level of at least one p21-associated molecule as compared to the reference level indicates that EGFR-TKI treatment has been efficacious, whereas a similarity the level of p21 or at least one p21-associated molecule, or a decrease in the p21 level or the resulting alteration in the level of at least one p21-associated molecule, as compared with the reference level, indicates that the EGFR-TKI treatment has not been efficacious.

In certain embodiments of this respect where the EGFR-TKI includes more than one dose, the treatment regimen can be monitored and/or adjusted according to the result of the evaluation as described above. For example, after at least one dose of the EGFR-TKI regimen is administered, the efficacy of the EGFR-TKI may be evaluated according to the method provided in this aspect. The subsequent doses may be adjusted accordingly to the result of the evaluation, e.g., maintained if the administered doses show efficacious, or be abandoned if the previous doses show inefficacious, in such evaluation.

In one embodiment, a method for determining a suitable drug candidate for use as an EGFR-TKI can be performed using the p21 and p21-molecules as biomarkers as described herein. Such a method of testing drug candidates can include: exposing cancer cells to at least one drug candidate; and determining at least one of a p21 level or a level of one or more p21-associated molecule in the cancer cells in response to exposure to the at least one drug candidate, wherein the p21 level or the level of one or more p21-associated molecules is predictive of the efficacy of the drug candidate.

The method for determining a suitable drug candidate can also include comparing a p21 level or a level of one or more p21-associated molecule in a reference sample to the p21 level or the level of one or more p21-associated

molecules in the cancerous cells.

When the reference level is derived from a non-diseased tissue, and an increase of p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to an increase in p21 level, as compared to the reference level, indicates that the drug candidate is efficient in cancer treatment.

When the reference level is derived from a non-diseased tissue, and a similarity of or a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to a decrease in p21 level, as compared with the reference level, indicates that the drug candidate is not efficient in cancer treatment.

When the reference level is derived from a diseased tissue known to be responsive to EGFR tyrosine kinase inhibitor treatment, and a similarity of the p21 level or at least one p21-associated molecules, or an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates that the drug candidate is efficient in cancer treatment.

When the reference level is derived from a diseased tissue known to be responsive to EGFR tyrosine kinase inhibitor treatment, and a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates that the drug candidate is not efficient in cancer treatment.

When the reference level is derived from a diseased tissue known to be non-responsive to EGFR tyrosine kinase inhibitor treatment, and an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates that the drug candidate is efficient in cancer treatment.

When the reference level is derived from a diseased tissue known to be non-responsive to EGFR tyrosine kinase inhibitor treatment, and a similarity of the p21 level or at least one p21-associated molecule, or a decrease in the p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates that the drug candidate is not efficient in cancer treatment.

Method for selecting cancer patients for EGFR tyrosine kinase inhibitor treatment

For example, in another aspect, the level of p21 or p21-associated molecules as compared with the reference level can be utilized as an indicator on the suitability of a patient with cancer for EGFR-TKI. Thus, for example, it is provided herein a method for selecting cancer patients for treatment with an EGFR-TKI, including providing a test sample, determining the level of at least one biomarker such as, but

not limited to, p21 and one or more p21-associated molecules in the test sample obtained from the patient; comparing the level with a reference level, and selecting a patient for treatment with EGFR-TKI based on the comparison.

In certain embodiments of the method, the reference level is a normal control level. In such embodiments, an increase of p21 level, or the resulting alteration in the level of at least one p21-associated molecules, as compared to the reference level, indicates that the patient is suitable to be selected for EGFR-TKI treatment, whereas a similarity of or decrease in the p21 level, or the resulting alteration in the level of at least one p21-associated molecules, as compared with the reference level, indicates that the patient is not suitable to be selected for EGFR-TKI treatment.

In certain embodiments of the method, the reference level is a responsive disease control level. In such embodiments, a similarity of the p21 level or at least one p21-associated molecules, or increase in p21 level or the resulting alteration in the level of at least one p21-associated molecule, as compared to the reference level, indicates that the patient is suitable to be selected for EGFR-TKI treatment, whereas a decrease in the p21 level, or the resulting alteration in the level of at least one p21-associated molecule, as compared with the reference level, indicates that the patient is not suitable to be selected for EGFR-TKI treatment.

In certain embodiments of the method, the reference level is a non-responsive disease control level. In such embodiments, an increase in p21 level or the resulting alteration in the level of at least one p21-associated molecule, as compared to the reference level, indicates that the patient is suitable to be selected for EGFR-TKI treatment, whereas a similarity of the p21 level or at least one p21-associated molecule, or a decrease in the p21 level or the resulting alteration in the level of at least one p21-associated molecule, as compared with the reference level, indicates that the patient is not suitable to be selected for EGFR-TKI treatment.

In vitro or ex vivo uses

It is also contemplated that the correlation between the level p21 or p21-associated molecules and responsiveness of cancer cells to EGFR-TKI may be utilized in in vitro or ex vivo procedures, for example, as part of a diagnosis of cancer or as part of a screening for drug candidates.

For example, the level of p21 or p21-associated molecules, such as CDK2, CDK4, cyclin D, and cyclin E, may be measured in an in vitro sample suspected of containing EGFR-TKI-sensitive cancer cells. An increase in p21 level or decrease in the level of at least one p21-associated molecule, as compared with the level detected in a normal control, suggests that the sample contains EGFR-TKI-sensitive cancer cells.

It is also possible to alter the level of p21 or p21-associated molecules in a cancer

cell to modulate, i.e., decrease or increase, the cell's sensitivity or responsiveness to EGFR-TKI. Methods for decreasing or increasing the level of a protein or mRNA in a cell are well known in the art. For example, the p21 level in a cancer cell may be increased by introducing an expression vector capable of expressing p21 into the targeted cancer cell, by modifying regulatory sequences of the p21 gene so that the expression of p21 gene is under a more potent promoter, by inhibiting the degradation of p21 protein such as by using proteasome inhibitors, etc. The level of a p21-associated molecule may be decreased, for example, by using siRNA to knockdown the expression of mRNA of a p21-associated molecule. Methods for designing and using siRNA are well known in the art.

An increase in the level of p21 or a decrease in the level of p21-associated molecules may lead to increased sensitivity of the cancer cell to EGFR-TKI and thus enhanced efficacy of EGFR-TKI on these cells, as shown by an increase in various effects in the cells typical of EGFR-TKI response, including but not limited to an increase of cell apoptosis.

The present disclosure also provides for methods of drug screening using the level of p21 or p21-associated molecules in cancer cells as an index. In one embodiment, there is provided a method of screening for an agent that induces cell death in cancer cells, comprising exposing cancer cell to a candidate agent, and testing the p21 protein level or the level of one or more p21-associated molecule in the cancer cells. In some embodiments, an increase in the p21 protein level or a resulting alteration in the level of one or more p21-associated molecules as compared to the level detected before the exposure to the candidate agent indicates that the candidate agent is an agent that induces cell death in cancer cells. In still some embodiments, one or more p21-associated molecules such as, but not limited to, CDK2, CDK4, cyclin D, and cyclin E, and an resulting alteration in the level of one or more p21-associated molecules is a decrease in the protein level of one or more of CDK2, CDK4, cyclin D, and cyclin E.

In various embodiments of the method, the cancer cell may be contained in a sample obtained from a patient with cancer, and the test may be performed on the sample in various assay formats for protein or mRNA. The cancer of the patient may be, but is not limited to, lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer. In some embodiments, the lung cancer is non-small cell lung cancer. The patient may be in any stage of the cancer, including but not limited to an early stage or an advanced stage. The patient may or may not have received EGFR-TKI therapy before the sample is taken.

It is contemplated that the methods according to this aspect can be used in an ex

vivo procedure. Cells that have been treated with the methods can be delivered back to the same patient they are derived from or a different patient for, e.g., therapeutic purposes. The ex vivo procedure may be used adjunct to an EGFR-TKI therapy.

Therapeutic uses

It is contemplated that p21 or p21-associated molecules and responsiveness of cancer cells may be utilized in cancer treatment based on the correlation between the level p21 or p21-associated molecules and responsiveness of cancer to EGFR-TKI. Thus, in one respect, the present disclosure provides for the use of agents that increase or modulates the cellular level of p21 or p21-associated molecules in treatment of cancer. Such agents may be administered to a cancer patient who has previously shown resistance to EGFR-TKI treatment to render the patient responsive, or more responsive, to EGFR-TKI and thus enhance the efficacy of EGFR-TKI treatment. In certain embodiments, the patient has previously shown resistance to EGFR-TKI treatment. In certain embodiments, the agent is administered to the patient prior to the administration of at least one dose of EGFR-TKI. In certain embodiments, the agent is administered to the patient in combination of at least one dose of EGFR-TKI. The therapy efficacy of EGFR-TKI may be enhanced by this method.

Thus, it is also provided herein a pharmaceutical combination comprising at least one EGFR-TKI and at least one agent capable of enhancing the level of p21 and/or causing the resulting alteration in the level of at least one p21-associated molecules in the cancer cell to be treated. In certain embodiments, the pharmaceutical combination is a pharmaceutical composition, comprising as active ingredient an effective amount of an agent capable of enhancing the level of p21 and/or causing the resulting alteration in the level of at least one p21-associated molecule in a cancer cell, and an effective amount of an EGFR-TKI, as well as pharmaceutically acceptable carriers.

The agent, optionally with an EGFR-TKI, can be prepared into pharmaceutical formulations by mixing the agent having desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin,

gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURONICSTM or polyethylene glycol (PEG).

Lipofections or liposomes can also be used to deliver the agent into cells. Where antibody fragments are used, the smallest inhibitory fragment which specifically binds to the binding domain of the target protein may be used. For example, based upon the variable region sequences of an antibody, peptide molecules can be designed which retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology (see, e.g., Marasco et al., Proc. Natl. Acad. Sci. USA 90, 7889-7893 (1993)).

The agent may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

When desired, sterilization of the formulations to be used for in vivo administration may suitably be accomplished by filtration through sterile filtration membranes.

The agent capable of enhancing the level of p21 and/or causing the resulting alteration in the level of at least one p21-associated molecule in cancer cell may be any kind of agent which can increase the amount of p21 mRNA or protein of p21 and/or cause the resulting alteration in the amount of the mRNA or protein of at least one p21-associated molecule in the cancer cell. In certain embodiments, the agent may be a gene therapy vector carrying a p21 gene in an expressible state which targets specifically to the cancer cell of interest. In certain embodiments, the agent is an adenovirus vector carrying an expression cassette of p21 gene. In illustrative embodiments, the agent is siRNAs designed against one or more of CDK2, CDK4, cyclin D, and cyclin E.

Methods for designing and synthesizing siRNA capable of inhibiting gene expression in a target cell are well known in the art (See for example, US Patent No. 6,506,559, herein incorporated by reference in its entirety). For example, a computer

program for designing siRNAs is available from the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html).

siRNA therapy is carried out by administering to a patient afflicted with cancer a siRNA by standard vectors encoding the siRNAs as described above and/or gene delivery systems such as by delivering the synthetic siRNA molecules. Typically, synthetic siRNA molecules are chemically stabilized to prevent nuclease degradation *in vivo*. Methods for preparing chemically stabilized RNA molecules are well known in the art. Typically, such molecules comprise modified backbones and nucleotides to prevent the action of ribonucleases. Other modifications are also possible, for example, cholesterol-conjugated siRNAs have shown improved pharmacological properties. (Song et al. *Nature Med.* 9:347-351 (2003)): Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, or viral vectors such as herpes viruses, retroviruses, adenoviruses and adeno-associated viruses, among others. A therapeutic nucleic acid composition may be formulated in biologically compatible vehicles suitable for administration to an animal, e.g., physiological saline. The therapeutic composition may also include a gene delivery system as described above. The therapeutic nucleic acid composition may be formulated as described hereinabove.

The present disclosure also provides for the use of agent capable of enhancing the level of p21 and/or causing the resulting alteration in the level of at least one p21-associated molecule in cancer cell in the manufacture of a medicament for treating cancer. In certain embodiments, the medicament is formulated with at least one EGFR-TKI.

In various embodiments of this aspect, the EGFR-TKI may be selected from, but not limited to, the group consisting of gefitinib, erlotinib and lapatinib, cetuximab and panitumumab.

In various embodiments of this aspect, the cancer to be treated may be, but is not limited to, a cancer with overexpression or other otherwise abnormal expression or activity of EGFR. Cancers suitable to be treated according to the present aspect include, but are not limited to, lung cancer, in particular non-small cell lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer.

Kits and Articles of Manufacture

It is also provided a kit for carrying out any prognostic, diagnostic or therapeutic methods provided herein, which comprises an agent for determining level of at least one biomarker such as, but not limited to, p21 and at least one p21-associated molecule in a sample from a patient.

Accordingly, it is provided herein a kit comprising an agent for determining the

level of at least one biomarker such as, but not limited to, p21 and one or more p21-associated molecules in a sample from a patient, and instructions for interpreting the results. In certain embodiments, the kit may further include a reference sample from a control population of subjects without cancer. The agent may an antibody against p21 protein or one or more p21-associated molecules. The antibody may be labeled with a detectable label, such as, for example, a florescent label or chemoluminescent label such as horse radish peroxidase (HRP), to facilitate detection and quantification of the target protein, e.g., p21 or p21-associated molecules. In some embodiments, the antibody is monoclonal. The kit may include reagents, buffers or materials necessary for detection and quantification of the target protein.

It is also provided an article of manufacture which a container containing an effective amount of an agent capable of increasing level of p21 and/or decreasing one or more p21-associated molecules in a cancer cell. In an embodiment, the article further comprises a second container containing an effective amount of an EGFR-TKI, and a label having instructions on the use of the EGFR-TKI in combination with said agent in treating cancer. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent may be a gene therapy vector carrying an expression cassette of p21 and/or decreasing one or more p21-associated molecules. An instruction or label on or associated with the container indicates that the agent is used for diagnosing or treating the cancer of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments described herein, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

Examples are provided below for illustrative purposes.

Tumor specimens were surgically resected from 11 patients with NSCLC. All of these patients were admitted into Zhejiang Tumor Hospital, HangZhou, China. The tumor types and stages were histologically determined according to the WHO classification. Pathologic material was processed for conventional histologic

procedures. Patients with histologically or cytologically confirmed advanced NSCLC received 250 mg/day gefitinib orally. Treatment was primarily continued until disease progression (PD). No other systemic chemotherapy was performed during gefitinib treatment.

Cells were washed twice with PBS, trypsinized and resuspended in PBS containing 0.1% Triton X-100 and RNase (1 mg/ml) (Sigma). The cell suspension was incubated at 37 °C for 30 min. Propidium iodide (Molecular Probes) was added at a final concentration of 50 flg/ml and the cell suspension was kept at 4 °C for 1 h. The cells were filtered and the cell cycle was analyzed by flow cytometry with the FACScan system (Becton Dickinson).

Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 um. For p21 detection, sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0), and then incubated with a monoclonal antihuman p21 antibody (Cell Signaling Technology, Inc., USA) for 90 min at 25°C. Negative controls were obtained by leaving out the primary antibody. The intensities of signals were evaluated independently by three observers. Negative immunostaining was defined to be with 0 % to 10 %, and cases with >10 % were decided to be positive for immunostaining.

Cell Cycle Analysis by Fluorescence-Activated Cell Sorting

Flow cytometry was performed to determine the cell cycle pattern of NSCLC cell lines response to gefitinib treatment. Cells were washed twice with PBS, trypsinized and resuspended in PBS containing 0.1% Triton X-100 and RNase (1 mg/ml) (Sigma, St Louis, MO). The cell suspension was incubated at 37 °C for 30 min. Propidium iodide (Molecular Probes, Inc. Eugene, OR) was added at a final concentration of 50 µg/ml and the cell suspension was kept at 4 °C for 1 h. The cells were filtered and the cell cycle was analyzed by flow cytometry with the FACScan system (Becton Dickinson, Franklin Lakes, NJ).

Cells and transfection

Human lung cancer cell lines pc-9 (Gefitinib-sensitive, as shown in experiment) and H1299 cells (Gefitinib-resistant, as shown in experiment) and the Gefitinib-resistant cell line pc-9-ZD cells were cultured in Dulbecco's modified Eagle's minimum essential medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). MYC tagged p21 expression vector was generated. ShRNA sequence against p21 (e.g., P21 small interfering RNA as hairpins) were constructed according to the procedure of pSUPER RNAi system (OligoEngine) to have a sequence of CTTCGACTTTGTCACCGAG (SEQ ID NO: 6), and GACCATGTGGACCTGTCAC (SEQ ID NO: 7). Construct pSuper p21 was

generated by inserting the following sequence into HindIII/BglII pSR-EGFP1 sites, 5'-GATCCCCTGGCCAACGAAGCACTGATTTC AAGAGAATC AGTGCTTCGTTGGCCATTTTTGGAAA-3' (SEQ ID NO: 1), which were synthesized by Sangon Ltd. (Shanghai, China). Cells were transiently transfected with expression constructs using GeneJammer transfection reagents (Stratagene, La Jolla, CA). For half-life experiments, cells were treated with cycloheximide (CHX, Sigma) at a final concentration of 10 µg/ml and MG132 (Beyotime, Hangzhou, China) at 20 µM.

Measurement of cell death

Cells were seeded into 96-well microtiter plate and treated with Gefitinib (10, 100, 1000nM) or beta-elemene (40 µg/ml). A 10 µl of the CCK-8 solution was added to each well of the plate and then incubated 2 h in the incubator (37 °C and 5% CO₂) according to the procedure of Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan). The absorbance was measured at 450 nm using a microplate reader (BioTeK).

Western Blotting and Immunoprecipitation

The cells were prepared in lysis buffer of MC-CellLytics Kit (Shenergy Biocolor, Shanghai, China). The protein content was determined using the Bradford calorimetric assay method (Shenergy Biocolor, Shanghai, China). For immunoprecipitation, a 500µg aliquot of cell or tissues lysate was incubated for 12 h at 4 °C with the anti-p21 antibody (Cell Signaling Technology, Inc., USA). Immune complexes were captured by incubation with 80 µl protein A/G-plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 3h, then washed several times with NP-40 buffer and lysed with loading buffer. For immunoblotting, the lysate was resolved by 10% polyacrylamide-sodium lauryl sulfate gel electrophoresis and transferred to a Hybond-C Super membrane (Amersham, Buckinghamshire, UK). Antibodies used for detection as follows, p21 (Cell Signaling), p53(Cell Signaling), p-Akt (05-669, Upstate), CDK2 (CalBiochem), CDK4 (Santa Cruz), cyclinD1 (Santa Cruz), cyclinE (Santa Cruz), active-caspase 3 (Cell Signaling), active-caspase 8 (Cell Signaling), active-caspase 9 (Cell Signaling). Then the blot was incubated with a secondary antibody, IRDye 800 conjugated affinity purified anti-mouse or anti-rabbit IgG (Rockland Immunochemicals, Inc., Gilbertsville, PA) and detected with Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).

Real-Time RT PCR Analysis

Total RNA of tissues and cells was extracted by homogenization in 1mL TRIzol reagent (Invitrogen, California, USA), followed by chloroform reextraction and isopropanol precipitation. 1µg of total RNA were reverse transcribed using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) and random hexamer

primer (Fermentas). Real-time PCR was done in a final volume of 20 μ L containing 1.6 μ L of each cDNA template, 1 μ L of each primer (10Mm), and 10 μ L of a SYBR Green master mix (Takara, Japan). Primers used were 5'-GGCAGACCAGCATGACA GATT-3' (sense) (SEQ ID NO: 2) and 5'-GCGGATTAGGGCTTCCTCTT-3' (antisense) (SEQ ID NO: 3) for p21(waf/cip1); 5'-CACGATGGAGGGGCCGGAATCATC-3' (sense) (SEQ ID NO: 4) and 5'-TAAAGACCTCTATGCCAACACAGT-3' (antisense) (SEQ ID NO: 5) for human β -actin. The average of p21 gene was normalized to β -actin as endogenous housekeeping gene.

P21 was elevated by gefitinib in non-small lung cancer cells that are sensitive to gefitinib.

Recent studies have led to understand some mechanism of gefitinib induced cytostasis. Akt activity is commonly reduced by gefitinib in these tumors that are sensitive to gefitinib. To confirm this point, the inventors assessed gefitinib induced cytostasis and Akt activities in two NSCLC cell lines: pc-9 and H1299 cells. Western blotting analysis was performed using anti-phosphor-Akt antibody. As shown in Figure 1A, it was observed that pc-9 cells have an $IC_{50} < 1 \mu M$, indicated as gefitinib-sensitive cells, whereas H1299 cells have an $IC_{50} > 1 \mu M$, indicated as gefitinib-resistant cells. Western blotting analysis was performed using anti-phosphor-Akt antibody, where Figure 1B shows that gefitinib led to a reduction in phosphor-Akt only in the gefitinib-sensitive pc-9 cells but not in gefitinib-resistant H1299 cells. The data suggested that reducing Akt activation levels can play a role in mediating Gefitinib induced cytostasis.

Studies have shown that anti-tumor agent suppresses cell growth likely related to up-regulation of cell cycle inhibitors such as p27 and p21 in NSCLC. P21 and p27 are important cell cycle checkpoint proteins. Thus, we asked if p21 plays a role in response to gefitinib selectively in NSCLC cell lines whose growth is inhibited by gefitinib. We examined p21 levels in pc-9 and H1299 cells by Western blotting analysis.

As can be seen in Figure 1B, it was observed that the p21 protein levels were high in gefitinib-sensitive pc-9 cells. Moreover, exposure to gefitinib actually increased p21 expression, effects that were most pronounced in cells that are sensitive to gefitinib. In contrast, no detectable p21 could be seen in gefitinib-resistant H1299 cells with or without treatment of gefitinib.

It was further sought to test other key cell cycle regulators in relation to p21. A significant decrease in the levels of cdk2, cdk4, cyclinE and cyclinD1 in response to gefitinib was observed in gefitinib-sensitive pc-9 cells but not in gefitinib-resistant H1299 cells. These data are consistent with results of cell cycle analysis showing

that gefitinib induced retention of cells in the G1phase, a sharp decrease in the S phase population but no significant change in the G2/M fraction in gefitinib-sensitive pc-9 cells. In contrast, there was no apparent change of cell cycle pattern by treatment of gefitinib in gefitinib-resistant H1299 cells (see Figure 1C). These data indicate that gefitinib elevated p21 levels and suppressed cdk2/4 and cyclinE/D1 activities resulted in impaired cell cycle progression through G1 arrest. Taken together, gefitinib treatment induced cytostasis through multiple mechanisms such as reducing phosphorylation of Akt activity and suppressing cell cycle progression by induction of p21 protein in gefitinib-sensitive pc-9 cells.

It is suggested that gefitinib-promoted p21 protein elevation was at least partly responsible for the negative regulation of cell progression by gefitinib in gefitinib-sensitive pc-9 cells. As p21 expression is believed to be controlled by p53 (i.e., the p21 gene is believed to be a transcription target of p53), the amount of p53 protein was assessed in order to examine the possibility that the induction of p21 was caused by p53 transcriptional activity in pc-9 cells treated with or without gefitinib. As shown in Figure 2A, no apparent correlation between p53 levels and p21 levels was seen, which suggests that p53 does not play a role in sensitivity to gefitinib in gefitinib-sensitive cells and that p53 expression/transcriptional activation does not play a significant role in the observed elevation of p21 expression in gefitinib-sensitive cells.

We then evaluated p21 mRNA by real-time RT-PCR analysis to determine whether mRNA accumulation contributed to elevated p21 levels by Gefitinib in pc-9 cells. In contrast to the large differences in protein levels, p21 mRNA levels were no apparent difference between with and without treatment of Gefitinib in pc-9 cells (Fig 2B). Therefore p21 levels were elevated mainly due to the protein stabilization.

The elevation of p21 by gefitinib raises a possibility that gefitinib promotes p21 protein stability in post-transcriptional pathway in gefitinib-sensitive pc-9 cells. Thus, p21 protein in pc-9 cells was examined by inhibiting protein synthesis with cycloheximide (CHX). Gefitinib treated cells were treated with 10 μ g/ml CHX and p21 levels were assessed by western blotting. Gefitinib treated cells expressed elevated p21 levels that did not decline significantly over the period of CHX treatment; 78% of the p21 remained 2 h after protein synthesis was blocked (Fig. 2C). Moreover, Gefitinib could maintain the same amount of p21 levels as in the presence of proteasome inhibitor MG-132 in pc-9 cells. Taken together, these data indicate that gefitinib caused pronounced elevation of p21, possibly through post-transcriptional pathway of inhibition of proteasome-mediated protein degradation in gefitinib-sensitive pc-9 cells.

Results presented thus far suggest that gefitinib helped to stabilize p21 protein

through proteasome-dependent pathway. It was further determined whether or not p21 stabilization caused by gefitinib can be required its binding to CDKs/cyclins when their activities were low. Proteins were immunoprecipitated by an anti-p21 antibody in gefitinib-sensitive pc-9 cells treated with or without gefitinib. Then CDK2, CDK4, cyclinD1, cyclinE, and p21 proteins were assessed by Western blotting analysis in the complexes. The results demonstrate that gefitinib treatment significantly promoted p21 association with CDK2, CDK4 and cyclinD1 in gefitinib-sensitive pc-9 cells. There was no apparent difference in association between p21 and cyclin E. These results suggest that a decreased cyclinD1 level mediated by gefitinib was concomitant with reduced CDK2 and CDK4 protein levels. It is likely that gefitinib decreased CyclinD1, CDK2 and CDK4 activity contributed to increasing p21 accumulation through mediating p21 binding to CDKs/cyclinD1, as opposed to prevention of p21 degradation in a proteasome-dependent manner in gefitinib-sensitive pc-9 cells.

Requirement of p21 for NSCLC sensitivity to gefitinib

To determine whether the restoration of p21 influences gefitinib-induced cytostasis, gefitinib-resistant H1299 cells were used, which lack p21 expression due to homozygous deletion of the p53 gene. For the expression of p21, we introduced p21 into the H1299 and HCT116 (human colon tumor) cells by transient transfection. It was demonstrated that cells transfected with vector expression of p21 showed a dramatic induction of p21 expression as compared to cells transfected with empty vector. Then, the direct effect of p21 over-expression on the treatment of gefitinib-resistant H1299 cells with gefitinib was examined. Upon over-expression of p21, cell death rate was remarkably enhanced in comparison to cells transfected with a control vector (Fig. 3A). Studies were next performed to define the high level of p21 effects on the activation of caspase family in the cells treated by gefitinib.

To further examine the role of p21 in the gefitinib-induced cytostasis, a siRNA targeting p21 gene was used to silence p21 expression in gefitinib-sensitive pc-9 cells. As seen in Figure 3B, it was demonstrated that cells transfected with p21 siRNA showed a dramatic reduction of endogenous p21 expression as compared to cells transfected with control vector (Fig. 3C). Furthermore, silencing of endogenous p21 expression in the cells increased levels of CDK2, CDK4, cyclinD1 and CyclinE by gefitinib in comparison to the cells transfected with control vector (Fig. 3C). By the silencing of endogenous p21 expression, cell death rate was remarkably decreased in comparison to cells transfected with a nonsense control vector (Fig. 3D). These data are in agreement with the observation in the cells with over-expression of p21 which showed above, suggesting that p21 promoted gefitinib-induced cytostasis. Thus, it is proposed that p21 is required for gefitinib-sensitive NSCLC cells.

It was thus attempted to determine whether p21 is expressed in NSCLC patients by immunohistochemistry to verify the relationship between p21 expression and response to gefitinib in clinical study. Immunohistochemistry was used to detect the positive or negative expression of p21 in the tumor tissues of 6 randomly selected gefitinib sensitive NSCLC patients and in 5 randomly selected gefitinib resistant NSCLC patients. The p21-positive staining tumors were >10%, and <10% for p21-negative patients. The data clearly show that p21 was predominately expressed in NSCLC sensitive to gefitinib, although some of paired adjacent normal tissues had low-level p21 expression. In contract, NSCLC resistant to gefitinib showed negative p21 expression. Of 11 randomly selected NSCLC patients, p21 expression was positive in all 6 NSCLC patients that were sensitive to gefitinib, and was negative in all 5 NSCLC patients. Patients with positive expression of p21 in NSCLC were all females and nonsmokers. These data are consistent with the findings in the cellular model shown above, suggesting that p21 plays an important role in response to gefitinib in NSCLC.

To further assess the direct effects of restoration of p21 influences gefitinib-induced cytostasis in acquired resistance to gefitinib NSCLC cells, the pc-9-ZD cells were transiently transfected with vector expression of p21. As expected, cells transfected with vector expression of p21 showed a dramatic induction of p21 expression as compared to cells transfected with a control vector. The direct effect of p21 over-expression on the treatment of acquired resistance to gefitinib pc-9ZD cells with gefitinib was then examined. Upon over-expression of p21, cell death rate was remarkably increased by gefitinib in comparison to the death rate in cells transfected with a control vector. Thus, restoration of p21 seems to reverse sensitivity to gefitinib treatment in NSCLC cells with acquired resistance to gefitinib. Taken together, it is suggested that p21 promotes NSCLC sensitivity to gefitinib via a mechanism that involves p21-dependent inhibition of cdk2 activity and cell cycle arrest in G1 phase.

Studies were conducted to determine whether p21 reversed sensitivity to Gefitinib in NSCLC with acquired resistance to Gefitinib. Gefitinib has been tested as monotherapy for patients with relapsed NSCLC. In cases of NSCLC sensitive to Gefitinib, resistance might be acquired through continuous drug administration. Additional treatment for cases of NSCLC relapsing during treatment with Gefitinib is urgently needed. Beta-elemene, a natural plant drug extracted from *Curcuma wenyujin*, has been used as an antitumor drug for different tumors, including NSCLC via mechanism that inhibits Ras/Mapk signaling and cell cycle progression. Our clinical trials showed that administration of Gefitinib in combination with beta-elemene for NSCLC which are acquired resistance to Gefitinib, remarkably

enhanced the anti-tumor effect as compared to Gefitinib treatment alone (personal communication).

First, we established acquired Gefitinib-resistant pc-9 cells through continuous exposure of Gefitinib. Resistance against Gefitinib developed about six months and the relative resistant values reached 4 fold after exposure to Gefitinib. We picked the clones of Gefitinib resistant cell lines named pc-9-ZD. As it showed in Fig 4A, pc-9-ZD cells was able to survival by >50% at the concentration of >1 uM Gefitinib whereas 84.8% of its parental pc-9 cells was inhibited by that concentration of Gefitinib. To test the effect of beta-elemene on Gefitinib-induced cytostasis in the cells with acquired resistance to Gefitinib, we measured the cell death rate in pc-9-ZD cells treated with or without beta-elemene in the presence of Gefitinib. As seen in Fig 4B, beta-elemene reversed sensitivity to Gefitinib treatment in pc-9-ZD cells. These data suggested that this innovative clinical application of beta-elemene in combination with Gefitinib may offer great opportunities for NSCLC patients who are acquired resistance to Gefitinib.

Studies have reported that anti-proliferation induced by beta-elemene was likely dependent on preventing cell cycle progression by arresting in G0/G1 phase. Therefore, we speculated that p21 might involve in a mechanism that beta-elemene inhibits cell progression. To test this hypothesis, we assessed the p21 proteins expression in pc-9-ZD cells which incubated with or without optimal concentration of beta-elemene. The results shown in Fig 4C demonstrated that acquired resistance to Gefitinib cells, pc-9-ZD significantly decreased the amount of p21 protein as compared to its parental pc-9 cells. However, pc-9-ZD cells exposed to beta-elemene, induced significantly expression of p21 protein. These data indicated that beta-elemene induced p21 protein in NSCLC cells.

Our previously study suggested that pc-9-ZD cells with heightened levels of p-Akt and reduced levels of p21 resisted further Gefitinib-induced cytostasis. To assess the direct effects of restoration of p21 influences, we transiently transfected the pc-9-ZD cells with vector expression of p21. Upon over-expression of p21, Gefitinib-induced cytostasis was remarkably increased by Gefitinib in comparison to that in cells transfected with a control vector (Fig. 4D). Thus, restoration of p21 seems to reverse sensitivity to Gefitinib treatment in NSCLC cells with acquired resistance to Gefitinib. Taken together, we suggested that p21 promote NSCLC to Gefitinib via a mechanism that involves p21-dependent inhibition of cdc2 activity and cell cycle arrest in G1 phase.

The present disclosure is not to be limited in terms of the particular embodiments described in this application, which are intended as illustrations of various aspects. Many modifications and variations can be made without departing from its spirit and

scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatuses within the scope of the disclosure, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present disclosure is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled. It is to be understood that this disclosure is not limited to particular methods, reagents, compounds compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (e.g., bodies of the appended claims) are generally intended as “open” terms (e.g., the term “including” should be interpreted as “including but not limited to,” the term “having” should be interpreted as “having at least,” the term “includes” should be interpreted as “includes but is not limited to,” etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases “at least one” and “one or more” to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles “a” or “an” limits any particular claim containing such introduced claim recitation to embodiments containing only one such recitation, even when the same claim includes the introductory phrases “one or more” or “at least one” and indefinite articles such as “a” or “an” (e.g., “a” and/or “an” should be interpreted to mean “at least one” or “one or more”); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation is explicitly recited, those skilled in the art will recognize that such recitation should be interpreted to mean at least the recited number (e.g., the bare recitation of “two recitations,” without other modifiers, means at least two recitations, or two or more recitations). Furthermore, in those instances where a convention analogous to “at least one of A, B, and C, etc.” is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., “a system having at least one of A, B, and C” would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). In those instances where a convention analogous to “at least one of A, B, or C, etc.” is used, in general such a construction is intended

in the sense one having skill in the art would understand the convention (e.g., “a system having at least one of A, B, or C” would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase “A or B” will be understood to include the possibilities of “A” or “B” or “A and B.”

In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

As will be understood by one skilled in the art, for any and all purposes, such as in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as “up to,” “at least,” and the like include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 cells refers to groups having 1, 2, or 3 cells. Similarly, a group having 1-5 cells refers to groups having 1, 2, 3, 4, or 5 cells, and so forth.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the embodiments disclosed herein. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed, subject to any specifically excluded limit in the stated range.

From the foregoing, it will be appreciated that various embodiments of the present disclosure have been described herein for purposes of illustration, and that various modifications may be made without departing from the scope and spirit of the

present disclosure. Accordingly, the various embodiments disclosed herein are not intended to be limiting, with the true scope and spirit being indicated by the following claims.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes. In case of conflict, the present specification, including definitions, will control. Reference to such publications, patents, and patent applications should in no way be construed as admission that the present application is not entitled to antedate such publications, patents, and patent applications by virtue of prior invention or priority.

CLAIMS

1. A method for predicting therapeutic response of a patient to treatment with an EGFR tyrosine kinase inhibitor comprising:

determining a level of at least one biomarker in a test sample from the patient, the at least one biomarker being selected from the group consisting of p21 and one or more p21-associated molecules; and

comparing the level of the at least one biomarker in the sample to a reference level, wherein the level of the at least one biomarker as compared to the reference level is predictive of the patient's response to the EGFR tyrosine kinase inhibitor.

2. The method of claim 1, wherein the sample is taken from one or more tissues having a detectable level of p21 and/or one or more p21-associated molecules.

3. The method of claim 2, wherein the one or more tissues includes cells from a cancerous tissue.

4. The method of claim 3, wherein said cancerous tissue is selected from the group consisting of lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer.

5. The method of claim 4, wherein said cancer is non-small cell lung cancer.

6. The method of claim 3, wherein the one or more tissues include non-cancerous cells of the same histology type as the cells from the cancerous tissue.

7. The method of claim 2, wherein the one or more tissues are selected from a group consisting of a surgically removed tumor mass, biopsied tissue, bronchoalveolar lavage fluid (BALF), pleural fluid, lymph nodes, or blood, a biological sample containing cancer cells, and combinations thereof.

8. The method of claim 7, wherein the sample is taken from the one or more tissues is taken before, during, or after the treatment of EGFR tyrosine kinase inhibitor.

9. The method of claim 1, wherein said p21-associated molecules are p-21 downstream molecules.

10. The method of claim 9, wherein the p-21 downstream molecules include one or more of CDK2, CDK4, cyclin D, or cyclin E.

11. The method of claim 1, wherein the level of the at least one biomarker is determined by an immunoassay.

12. The method of claim 11, wherein the immunoassay is selected from the group consisting of western blotting, immunohistochemistry, ELISA, and combinations thereof.

13. The method of claim 1, wherein the reference level is derived from a non-diseased tissue.

14. The method of claim 13, wherein an increase of p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR tyrosine kinase inhibitor treatment.

15. The method of claim 13, wherein a similarity of or a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to a decrease in p21 level, as compared with the reference level, indicates a non-positive response to EGFR tyrosine kinase inhibitor treatment.

16. The method of claim 1, wherein the reference level is derived from a diseased tissue known to be responsive to EGFR tyrosine kinase inhibitor treatment.

17. The method of claim 16, wherein a similarity of the p21 level or at least one p21-associated molecules, or an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR tyrosine kinase inhibitor treatment.

18. The method of claim 16, wherein a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates a non-positive response to EGFR tyrosine kinase inhibitor treatment.

19. The method of claim 1, wherein the reference level is derived from a diseased tissue known to be non-responsive to EGFR tyrosine kinase inhibitor treatment.

20. The method of claim 19, wherein an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR tyrosine kinase inhibitor treatment.

21. The method of claim 19, wherein a similarity of the p21 level or at least one p21-associated molecule, or a decrease in the p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level,

as compared with the reference level, indicates a non-positive response to EGFR tyrosine kinase inhibitor treatment.

22. A method for evaluating the efficacy of treatment with an EGFR tyrosine kinase inhibitor, comprising:

determining a level of at least one biomarker selected from the group consisting of p21 and one or more p21-associated molecules in a sample;

comparing said level with a reference level of the same one or more biomarkers; and

making an evaluation of the efficacy of the treatment based on the comparison, wherein the level of the at least one biomarker as compared to the reference level is predictive of the efficacy of treatment with an EGFR tyrosine kinase inhibitor.

23. The method of claim 22, wherein the sample is taken from one or more tissues having a detectable level of p21 and/or one or more p21-associated molecules.

24. The method of claim 23, wherein the one or more tissues include cells from a cancerous tissue.

25. The method of claim 22, wherein the reference level is derived from a non-diseased tissue.

26. The method of claim 25, wherein an increase of p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR tyrosine kinase inhibitor treatment.

27. The method of claim 26, wherein a similarity of or a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to a decrease in p21 level, as compared with the reference level, indicates a non-positive response to EGFR tyrosine kinase inhibitor treatment.

28. The method of claim 22, wherein the reference level is derived from a diseased tissue known to be responsive to EGFR tyrosine kinase inhibitor treatment.

29. The method of claim 28, wherein a similarity of the p21 level or at least one p21-associated molecules, or an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR tyrosine kinase inhibitor treatment.

30. The method of claim 28, wherein a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates a non-positive response to EGFR tyrosine kinase inhibitor treatment.

31. The method of claim 22, wherein the reference level is derived from a diseased tissue known to be non-responsive to EGFR tyrosine kinase inhibitor treatment.

32. The method of claim 31, wherein an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates a positive response to EGFR tyrosine kinase inhibitor treatment.

33. The method of claim 31, wherein a similarity of the p21 level or at least one p21-associated molecule, or a decrease in the p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates a non-positive response to EGFR tyrosine kinase inhibitor treatment.

34. The method of claim 22, wherein the sample is taken from a patient within about -1 hour to about 15 hours from the administration of the EGFR tyrosine kinase inhibitor.

35. The method of claim 22, wherein the sample is taken from a patient within about 3 hours to about 10 hours from the administration of the EGFR tyrosine kinase inhibitor.

36. The method of claim 22, wherein the sample is taken from a patient within about 3 hours to about 7 hours from the administration of the EGFR tyrosine kinase inhibitor.

37. The method of claim 22, wherein the level of the at least one biomarker is determined by an immunoassay selected from the group consisting of western blotting, immunohistochemistry, ELISA, and combinations thereof.

38. A method for selecting cancer patients for treatment with an EGFR tyrosine kinase inhibitor, comprising:

determining at least one test level in a test sample of at least one biomarker selected from the group consisting of p21 and/or one or more p21-associated molecules in the test sample;

comparing said test level with a reference level; and

selecting a patient for treatment with EGFR tyrosine kinase inhibitor based on the comparison, wherein the test level of the at least one biomarker as compared to the reference level is predictive of whether or not a cancer patient will respond to treatment with the EGFR tyrosine kinase inhibitor.

39. The method of claim 38, wherein a patient is selected for treatment with EGFR tyrosine kinase inhibitor when a difference between the level in the test sample and the reference level is shown.

40. The method of claim 38, wherein a patient is selected for treatment with EGFR tyrosine kinase inhibitor when a similarity between the level in the sample and the reference level is shown.

41. The method of claim 38, wherein the at least one test sample and the at least one reference sample are taken from one or more tissues having a detectable level of p21 and/or one or more p21-associated molecules.

42. The method of claim 41, wherein the one or more tissues include cells from a cancerous tissue selected from the group consisting of lung cancer, non-small cell lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer.

43. The method of claim 38, wherein said p21-associated molecules are p-21 downstream molecules selected from a group consisting of CDK2, CDK4, cyclin D, cyclin E, and combinations thereof.

44. The method of claim 38, wherein the level of the at least one biomarker is determined by an immunoassay selected from the group consisting of western blotting, immunohistochemistry, ELISA, and combinations thereof.

45. The method of claim 38, wherein said EGFR tyrosine kinase inhibitor is selected from the group consisting of erlotinib, lapatinib, gefinitib, and combinations thereof.

46. A method for screening a drug candidate for efficacy in cancer treatment, comprising:

exposing cancer cells to at least one drug candidate; and

determining at least one of a p21 level or a level of one or more p21-associated molecule in the cancer cells in response to exposure to the at least one drug candidate, wherein the p21 level or the level of one or more p21-associated molecules is

predictive of the efficacy of the drug candidate.

47. The method of claim 46, wherein the drug candidate induces cell death in the cancerous cells.

48. The method of claim 46, wherein the drug candidate is an EGFR tyrosine kinase inhibitor.

49. The method of claim 46, further comprising:

comparing a p21 level or a level of one or more p21-associated molecule in a reference sample to the p21 level or the level of one or more p21-associated molecules in the cancerous cells.

50. The method of claim 49, wherein the cancerous cells and the reference sample have a detectable level of p21 and/or one or more p21-associated molecules.

51. The method of claim 49, wherein the reference level is derived from a non-diseased tissue.

52. The method of claim 51, wherein an increase of p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to an increase in p21 level, as compared to the reference level, indicates that the drug candidate is efficient in cancer treatment.

53. The method of claim 51, wherein a similarity of or a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecules in response to a decrease in p21 level, as compared with the reference level indicates that the drug candidate is not efficient in cancer treatment.

54. The method of claim 49, wherein the reference level is derived from a diseased tissue known to be responsive to EGFR tyrosine kinase inhibitor treatment.

55. The method of claim 54, wherein a similarity of the p21 level or at least one p21-associated molecules, or an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level indicates that the drug candidate is efficient in cancer treatment.

56. The method of claim 54, wherein a decrease in the p21 level, or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates that the drug candidate is not efficient in cancer treatment.

57. The method of claim 49, wherein the reference level is derived from a diseased tissue known to be non-responsive to EGFR tyrosine kinase inhibitor treatment.

58. The method of claim 57, wherein an increase in p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to an increase in p21 level, as compared to the reference level, indicates that the drug candidate is efficient in cancer treatment.

59. The method of claim 57, wherein a similarity of the p21 level or at least one p21-associated molecule, or a decrease in the p21 level or a resulting alteration in the level of at least one p21-associated molecule in response to a decrease in p21 level, as compared with the reference level, indicates that the drug candidate is not efficient in cancer treatment.

60. The method of claim 46, wherein the cancerous cells and the reference sample include cells having a histology type that is the same.

61. The method of claim 60, wherein said cancerous cells are selected from the group consisting of lung cancer, colorectal cancer, breast cancer, pancreatic cancer, head and neck squamous cancer, urothelial cancer, and gallbladder cancer.

62. The method of claim 46, wherein said cancerous cells are non-small cell lung cancer cells.

63. The method of claim 46, wherein said p21-associated molecules are p21 downstream molecules.

64. The method of claim 63, wherein the p21 downstream molecules include one or more of CDK2, CDK4, cyclin D, or cyclin E.

65. The method of claim 46, wherein the p21 level or the level of one or more p21-associated molecules is determined by an immunoassay.

66. The method of claim 65, wherein the immunoassay is selected from the group consisting of western blotting, immunohistochemistry, ELISA, and combinations thereof.

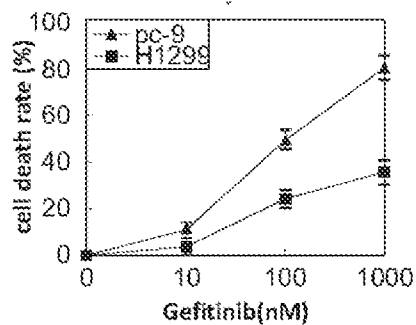


FIG. 1A

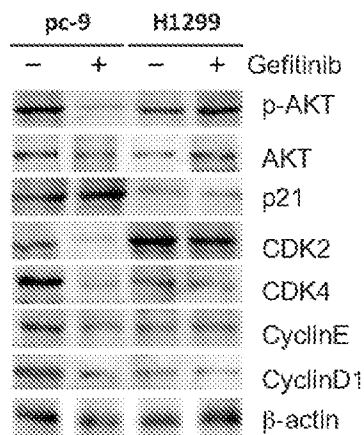


FIG. 1B

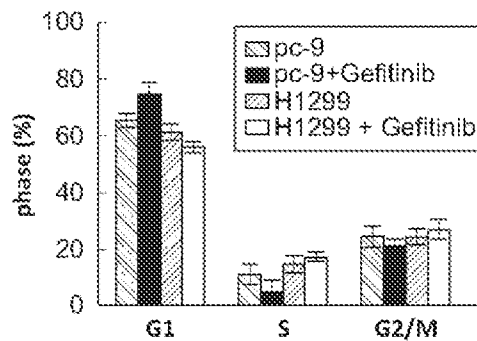


FIG. 1C

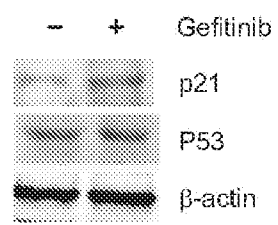


FIG. 2A

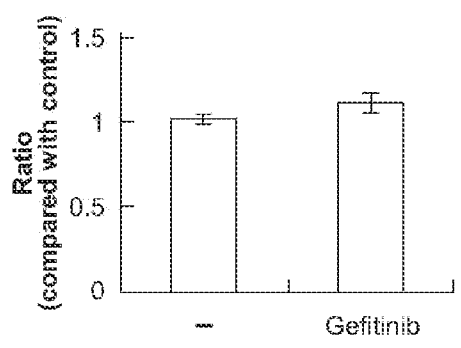


FIG. 2B

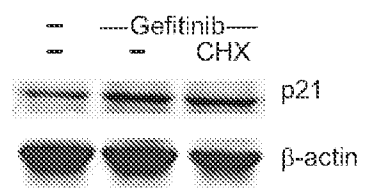


FIG. 2C

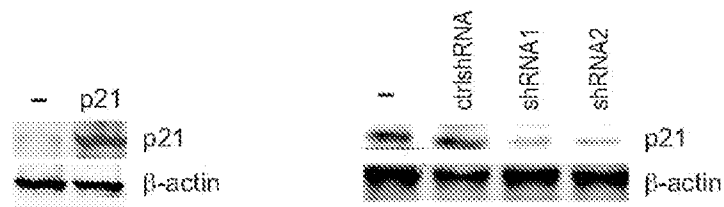


FIG. 3A

FIG. 3B

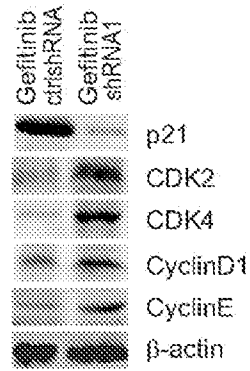


FIG. 3C

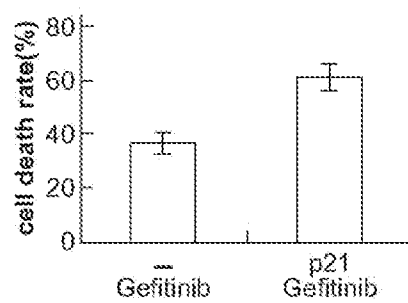


FIG. 3D

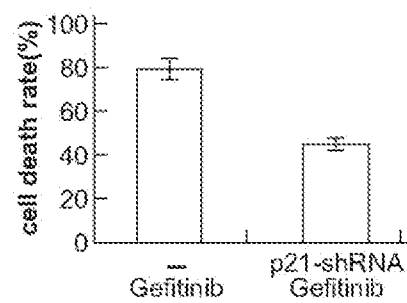


FIG. 3E

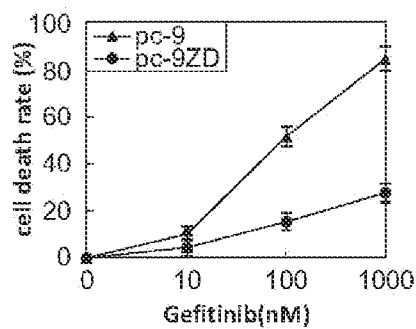


FIG. 4A

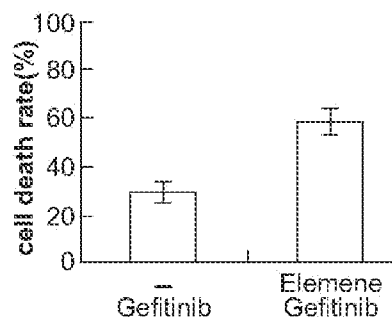


FIG. 4B

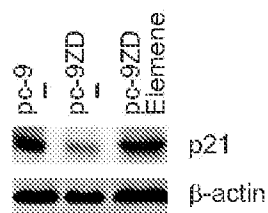


FIG. 4C

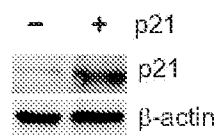


FIG. 4D

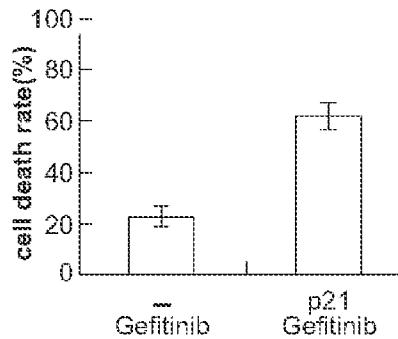


FIG. 4E

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2011/073442

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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:C07K14/-;A61K38/-;A61P35/-

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)

CNKI,CNPAT,WPLEPODOC,ISI web: p21, gefitinib, screen+, EGFR, NSCLC, non-small cell lung cancer, author: lu

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
x	JL, Yuan et al. Combined treatment with TNF- α /gefitinib alleviates the resistance to gefitinib in PC-9 cells, Anti-Cancer Drugs, 2009, Vol. 20, No. 9, pages 832-837, see page 834, right column, paragraphs 1-2, Fig.1	46-66
x	DI GENNARO, ELENA et al. Critical Role of Both p27 ^{KIP1} and p21 ^{CIP1/WAF1} in the Antiproliferative Effect of ZD1839("Iressa"), an Epidermal Growth Factor Tyrosine Kinase Inhibitor, in Head and Neck Squamous Carcinoma Cells, JOURNAL OF CELLULAR PHYSIOLOGY, 2003, Vol.195, pages 139-150, see abstract	46-66
x	CN101816649A (UNIV. PEKING) 01 Sep.2010 (01.09.2010) see claim 1	46-66
x	WO2005065723A1 (UNIV. TEXAS SYSTEM) 21 July 2005 (21.07.2005) see description	46-66

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search 12 Jan.2012 (12.01.2012)	Date of mailing of the international search report 09 Feb. 2012 (09.02.2012)
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer CHEN, Hongxia Telephone No. (86-10)82245448

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2011/073442

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-45
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-45 are directed to a method for the treatment of the human or animal body. Accordingly, claims 1-45 relate to the subject matter considered by the authority to be covered by the provisions of Rule 39.1 (iv) PCT.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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:

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 an additional fees, this Authority did not invite payment of additional fee.
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.:

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 application No.
PCT/CN2011/073442

Form PCT/ISA /210 (patent family annex) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2011/073442

CLASSIFICATION OF SUBJECT MATTER:

C07K14/81 (2006.01)i;

C07K14/435(2006.01)i;

C07K14/705(2006.01)i;

A61K38/55(2006.01)i;

A61P35/00(2006.01)i