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(54) **DIAGNOSTIC MARKERS**

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CPC ***C12Q 1/6827*** (2013.01)

USPC .. **424/133.1**; 435/6.11; 514/266.4; 424/142.1

(57) **ABSTRACT**

The present invention provides methods for determining epithelial and mesenchymal phenotype of tumors and predicting whether tumor growth will be sensitive or resistant to treatment with an EGFR inhibitor.

Fluidigm-Based EMT Gene Expression Panel

Gene Symbol	ABI Assay ID	Gene description	Epithelial/Mesenchymal
CDH1	Hs01023894_m1	E-cadherin	E
CDH3	Hs00354998_m1	P-cadherin	E
CLDN7	Hs00600772_m1	Claudin 7	E
ERBB3	Hs00176538_m1	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3	E
GJB3	Hs02378125_s1	Gap junction protein, beta 3	E
JUP	Hs00158408_m1	Junction plakoglobin	E
KRT7	Hs00559840_m1	Keratin 7	E
LAD1	Hs00194326_m1	Ladinin 1	E
LAMB3	Hs00165078_m1	Laminin, beta 3	E
PTK6	Hs00178742_m1	Protein tyrosine kinase 6	E
FGFR1	Hs00241111_m1	Fibroblast growth factor receptor 1	M
HOXC6	Hs00171690_m1	Homeobox C6	M
MAGED4	Hs00602656_g1	Melanoma antigen family D, 4	M
STX2	Hs00181827_m1	Syntaxin 2	M
TGFB1	Hs00998133_m1	Transforming growth factor, beta 1	M
TUBB	Hs00962420_g1	Tubulin, beta	M
UCHL1	Hs00985157_m1	Ubiquitin carboxyl-terminal esterase L1	M
EMP3	Hs00171319_m1	Epithelial membrane protein 3	M
VIM	Hs00958116_m1	Vimentin	M
ZEB1	Hs00232783_m1	Zinc finger E-box binding homeobox 1	M

FIG. 1

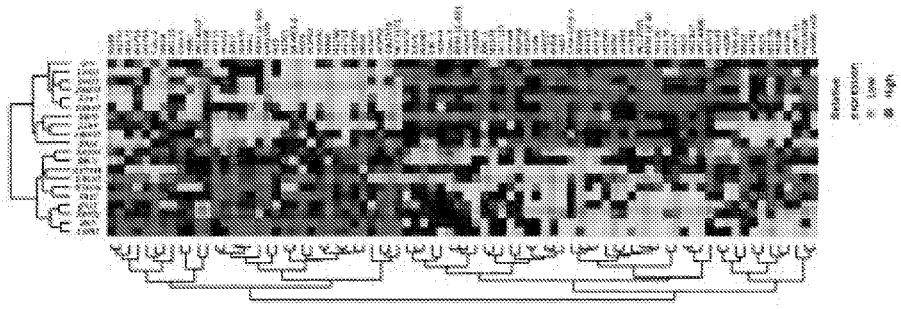


FIG. 2

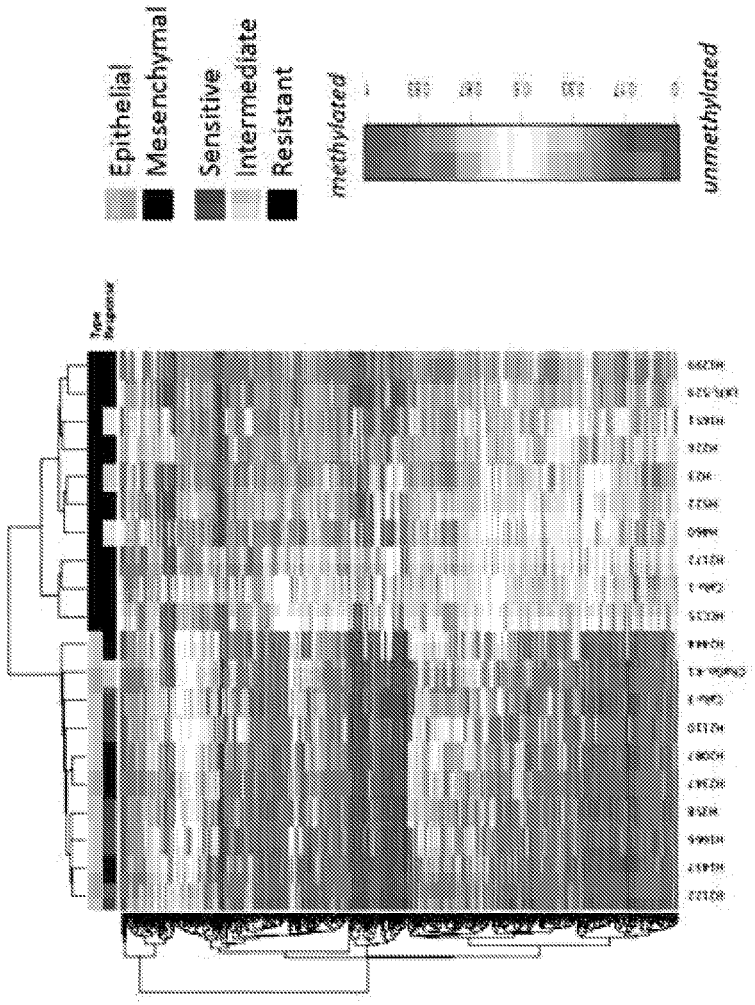
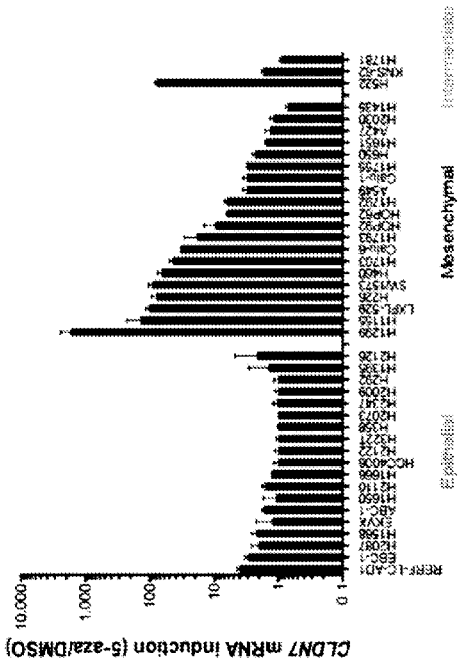

Epithelial
Mesenchymal
Sensitive
Intermediate
Resistant
methylated
unmethylated

FIG. 3

Gene symbol	Gene name	Chromosomal location	Regulatory element	5-aza-dC induction	PBMCs	Normal lung	Epithelial/mesenchymal	P (E vs. R)	P (S vs. R)
ZEB2	Zinc finger E-box binding homeobox 2	chr2: 145,069,350-144,989,169	Conserved regulatory potential	Yes	Unmethylated	No	E	0.0012	0.3432
HKR3-2	Homeobox protein Hox-2.2	chr10: 134,448,836-134,449,879	CpG island	NE	Partially methylated	No	E	0.0963	0.8353
PTK7	Pseudogene tyrosine-protein kinase 7-like	chr3: 181,236,928-181,337,788	CpG island	Yes	Unmethylated	No	F	0.0094	0.6966
CALCR1	Calcitonin receptor 1	chr19: 73,060,412-73,060,797	CpG island	NE	Unmethylated	No	F	NF	NF
PTPRM	Protein tyrosine phosphatase, receptor type, M	chr18: 7,602,674-7,933,993	Conserved regulatory potential	Yes	Methylated	Yes	E	NE	NE
MES	NADP-dependent malic enzyme 3	chr11: 86,060,344-86,061,158	CpG island	Some	Unmethylated	No	E	0.0295	0.5771
SYK	Spleen tyrosine kinase	chr9: 92,631,210-92,652,740	None defined	Yes	Methylated	Yes	E	NE	NE
PCDH8	Protocadherin 8	chr13: 52,321,012-52,321,485	CpG island	Yes	Unmethylated	No	E	0.0963	0.5407
HOXC6	Homeobox C6	chr12: 52,712,686-52,713,529	CpG island	NE	Unmethylated	No	M	0.7493	0.0008
miR200c	microRNA 200c	chr12: 6,942,890-6,943,200	None defined	NE	Methylated	No	M	NE	NE
SERPINB5	Serine (or cysteine) proteinase inhibitor, clade B, member 5, Maspin	chr18: 59,264,906-59,265,319	Conserved regulatory potential	Yes	Methylated	Yes	M	NE	NE
BCAR3	Breast cancer antiestrogen resistance 3	chr1: 93,862,869-93,863,416	Conserved regulatory potential	Some	Methylated	Yes	M	0.0951	0.2071
FAM110A	Family with sequence similarity 110, member A	chr20: 802,485-802,870	None defined	Some	Methylated	No	M	<0.0001	0.0007
CLDN7	Claudin 7	chr17: 7,103,446-7,106,446	CpG island	Yes	Partially methylated	No	M	<0.0001	0.0011
ESRP1	Epithelial splicing regulatory protein	chr8: 95,853,500-95,854,340	Yes	Yes	Unmethylated	No	M	<0.0001	0.0043
GRHL2	Grainyhead like 2	chr8: 102,575,373-102,575,783	CpG island	Yes	Unmethylated	No	M	<0.0001	0.0004
RGM	Macrophage stimulating 1 receptor	chr3: 49,915,089-49,916,545	CpG island	Yes	Unmethylated	Some	M	0.0029	0.0028
SLIK2	syntaxin 2	chr12: 129,865,904-129,869,427	CpG island	No	Partially methylated	Yes	M	0.0001	0.0023
TBCD	Tubulin-specific chaperone D	chr17: 79,446,425-79,448,951	None defined	NE	Methylated	Yes	M	NE	NE
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	chr17: 37,861,109-37,863,650	Promoter enhancer	No	Methylated	No	M	<0.0001	0.0004

FIG. 4


CLDN7 mRNA induction (5-aza/DMSO)
10,000
1,000
100
10
1
0.1
Epithelial
Mesenchymal
% Methylation of 7 CLDN7 CpG sites
120
100
80
60
40
20
0
Epithelial
Mesenchymal

B

A

FIG. 5

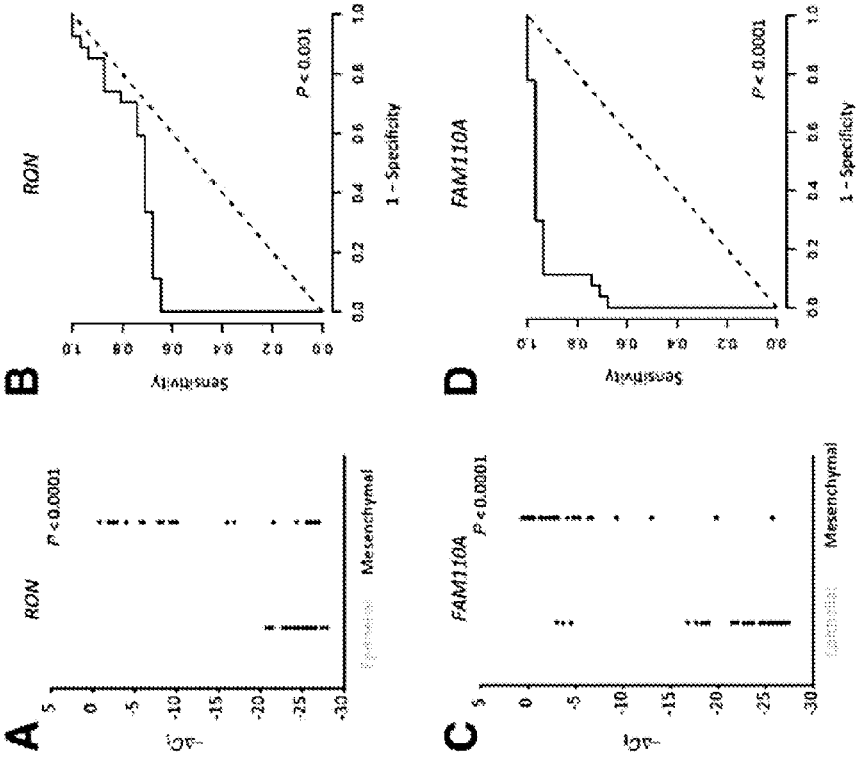

A
RON
P < 0.0001
log2
5
0
-5
-10
-15
-20
-25
-30
Epithelial
Mesenchymal
B
RON
P < 0.031
Sensitivity
1 – Specificity
0.0
0.2
0.4
0.6
0.8
1.0
C
FAM110A
P < 0.0001
log2
5
0
-5
-10
-15
-20
-25
-30
Epithelial
Mesenchymal
D
FAM110A
P < 0.0001
Sensitivity
1 – Specificity
0.0
0.2
0.4
0.6
0.8
1.0

FIG. 6

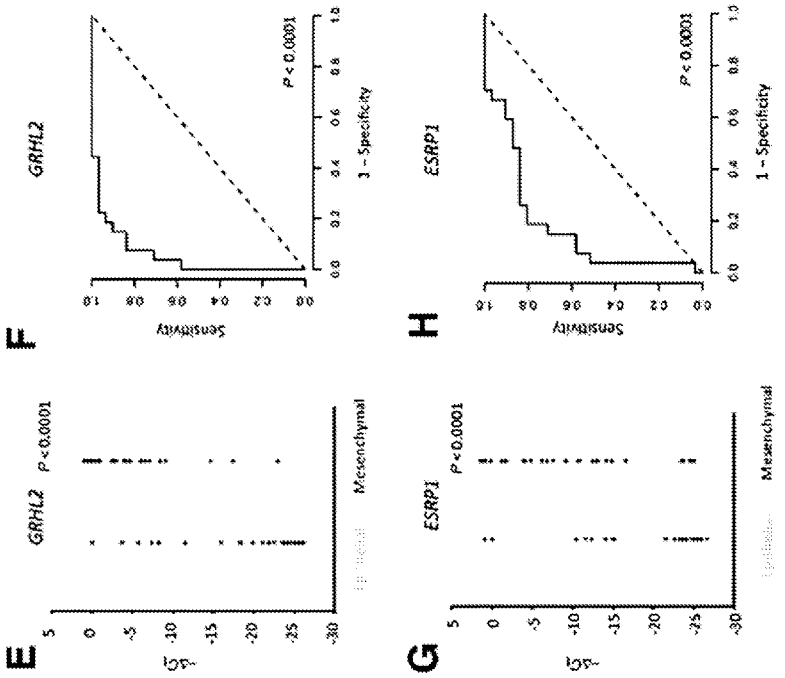

E
GRHL2
P < 0.0001
Mesenchymal
F
GRHL2
P < 0.0001
Sensitivity
1 – Specificity
G
ESRP1
P < 0.0001
Mesenchymal
H
ESRP1
P < 0.0001
Sensitivity
1 – Specificity

FIG. 6

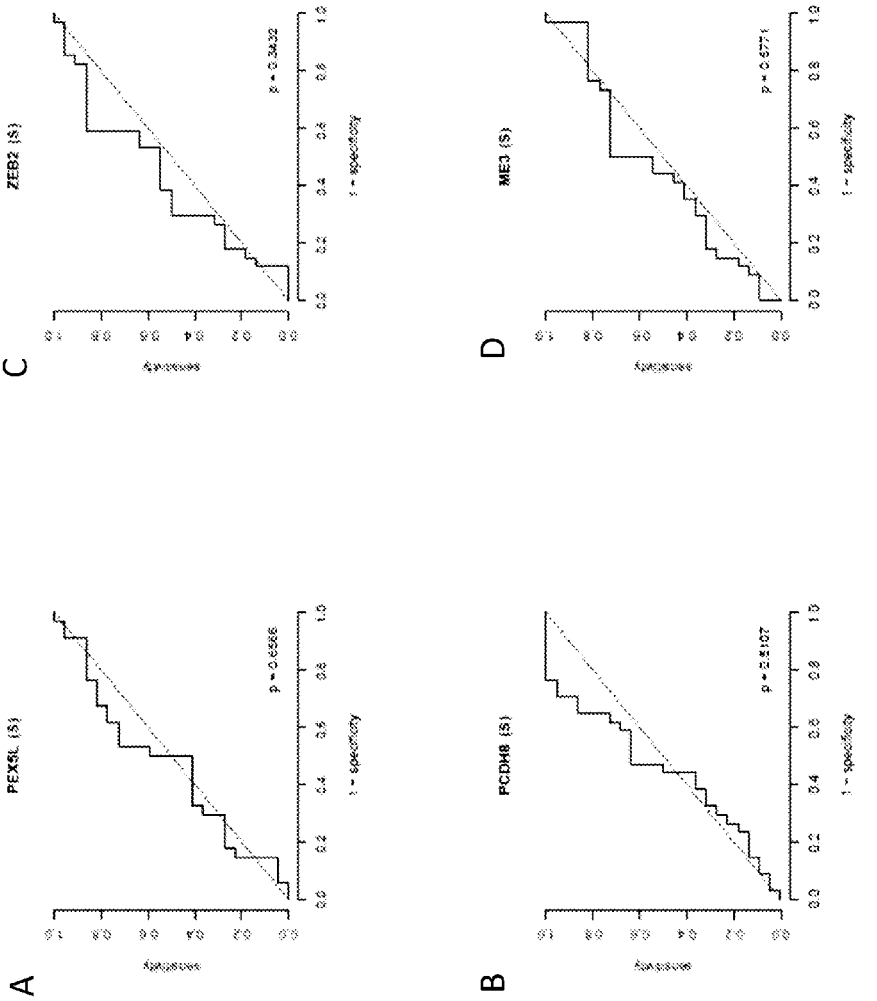


FIG. 7

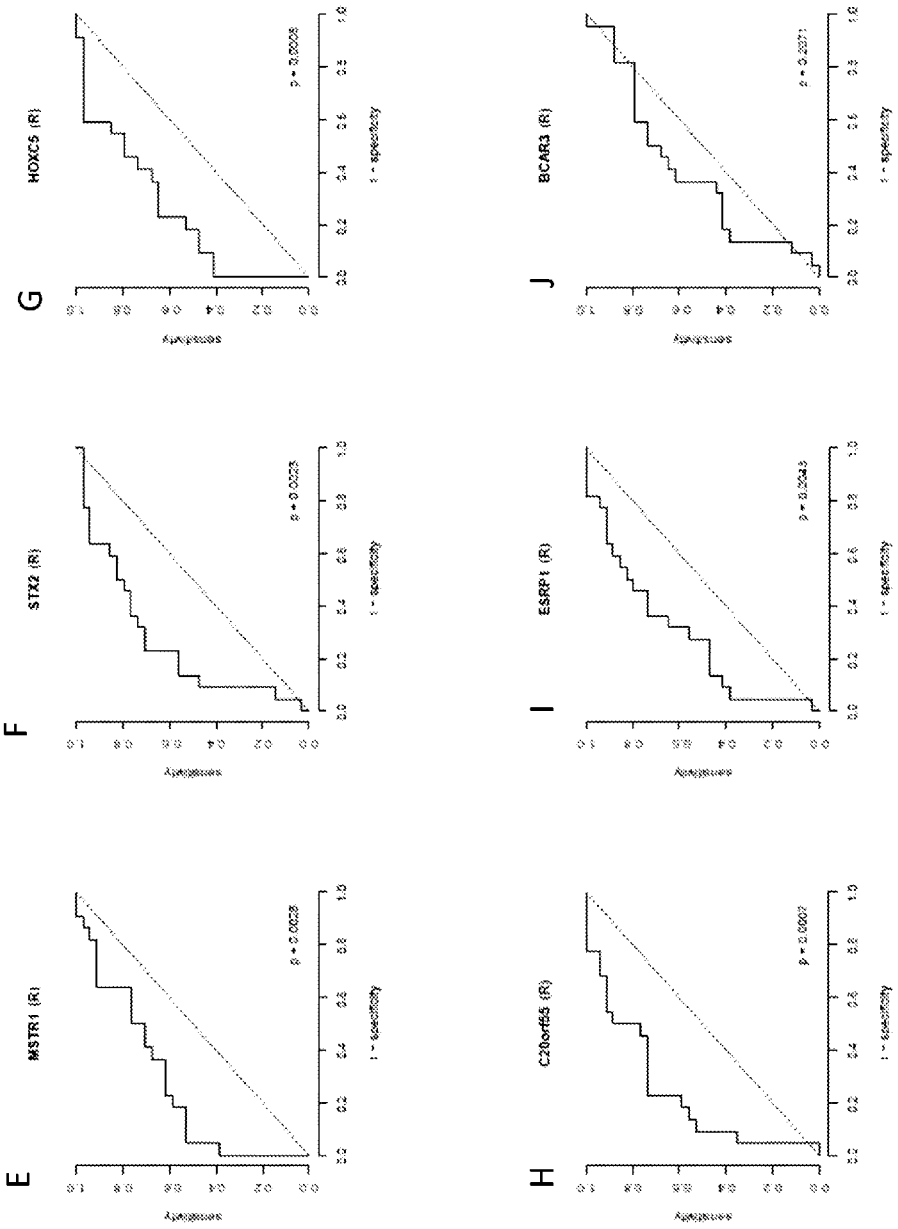


FIG. 7

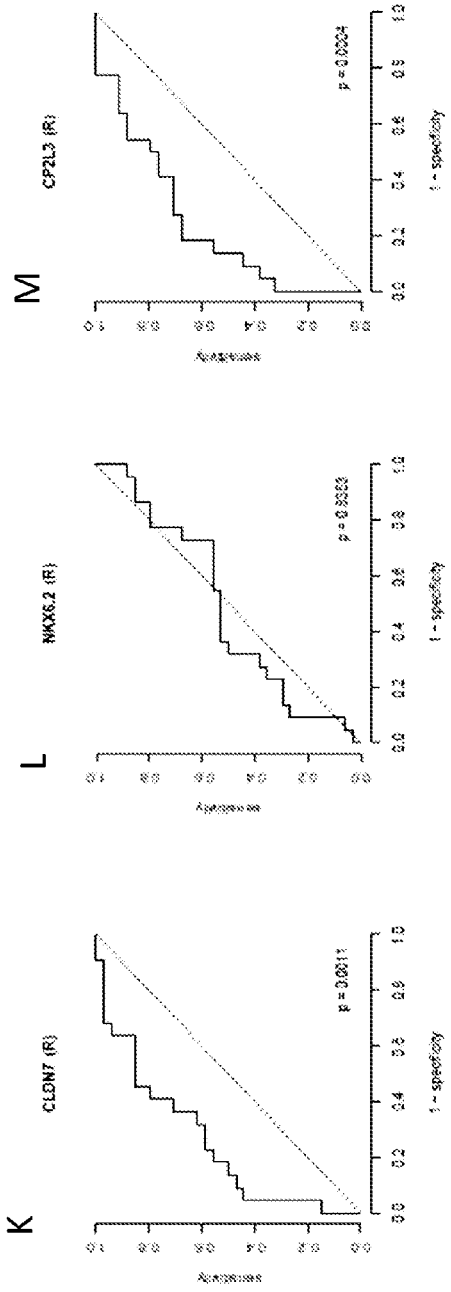


FIG. 7

Cell Line	Tissue Source	Histology	E/M	erlotinib + (TGFα) IC50 (μM)	EGFR
H2122	PE	Adeno	E	0.7	WT
H358	Lung	BAC	E	1.9	WT
H1437	PE	Adeno	E	10.0	WT
H1666	PE	Adeno	E	0.8	WT
H3119	PE	NSCLC (NOS)	E	0.9	WT
H2347	Lung	Adeno	E	10.0	WT
H2087	LN	Adeno	E	10.0	WT
Calu-3	PE	Adeno	E	1.0	WT
H2444	Lung	NSCLC (NOS)	E	10.0	WT
ChaGo-K-1	N/A	Bronchogenic carcinoma	E	4.7	WT
H1650	Lung	Adeno	E	0.5	[c.2235_2249del15, p.E746_A750del; (Sanger), 2235-2249 del (E746-A750 del) (Gazdar)
EKVX	Lung	Adeno	E	1.6	WT
RBE-1	N/A	N/A	E	10	N/A
RERF-LC-Ad1	N/A	N/A	E	1.53	N/A
H2009	Lung	Adeno	E	1.5	WT
ABC-1	N/A	N/A	E	10	N/A
H1373	Lung	Adeno	E	N/A	WT
HCC515	Lung	Adeno	E	8.3	WT
H1563	Lung	Adeno	E	1.7	WT
HOP62	N/A	N/A	E	0.5	N/A
H1975	Lung	Adenosquamous	E	10	[c.2369C>T; p.T790M]; [c.2573T>G; p.L858R] (Sanger), T790M, L858R (Gazdar)
H596	Lung	Adenosquamous	E	1.1	WT
H322T	N/A	N/A	E	0.7	N/A
H441	Lung	Adeno	E	2.8	WT
CHA-GO-K-1	N/A	Bronchogenic carcinoma	E	4.65	WT
H1395	Lung	Adeno	E	1.7	WT
H292	Lung	NSCLC (NOS)	E	0.8	WT
H1993	Lung	Adeno	E	10	WT
H1838	Lung	Adeno	E	1.6	WT
HCC2935	Lung	Adeno	E	0.11	2235-2252 del, 2254T>A, 2255C>T (E746-S752 del, I ins) (Gazdar)
RERF-LC-MJ	N/A	N/A	E	0.34	N/A
H2126	Lung	Adeno	E	0.9	WT
H2170	Lung	SCC	E	1.938	WT
H727	Lung	Carcinoid	E	10	WT
H820	Lung	Adeno	E	1	2235-2249 del (E746-E749 del), T790M (Gazdar)
HCC827	Lung	Adeno	E	0.01	2236-2250 del (E746-A750 del) (Gazdar)
HCC4006	Lung	Adeno	E	0.11	2239-2247 del, 2248G>C (L747-A750 del, P ins) (Gazdar)
H2073	Lung	Adeno	E	0.63	WT

FIG. 8A

Cell Line	Tissue Source	Histology	E/M	erlotinib C50 (μM) + (TGFα)	EGFR
A549	Lung	Adeno	M	3.9	WT
H1792	Lung	Adeno	M	5	WT
H1755	Lung	Adeno	M	10	WT
H1793	Lung	Adeno	M	N/A	WT
Calu-6	Lung	Adeno	M	10	WT
H838	Lung	Adeno	M	1.2	WT
H647	Lung	Adenosquamous	M	6.3	WT
HCC4017	Lung	Large-Cell	M	10	WT
H2228	Lung	Adeno	M	10	WT
H1155	Lung	Large Cell Neuroendocrine	M	10	WT
A427	Lung	N/A	M	4.9	WT
H1435	Lung	Adeno	M	0.9	WT
H661	Lung	LCC	M	6	WT
RERF-LC-MS	N/A	N/A	M	0.47	WT
H1915	Lung	LCC	M	8.65	N/A
SK-MES-1	N/A	N/A	M	0.3	N/A
HOP62	Lung	Adeno	M	3.6	WT
H2030	Lung	Adeno	M	2	WT
H1703	Lung	Adeno	M	8.4	WT
H1355	Lung	Adeno	M	10	WT
H650	Lung	Adeno	M	10	WT
H2405	Lung	Adeno	M	5.6	WT
HOP92	Lung	LCC	M	8.3	WT
SW1573	N/A	N/A	M	7.1	N/A
HCC3171	Lung	NSCLC (NOS)	M	10	WT
H23	Lung	Adeno	M	6.1	WT
H1299	LN	LCC	M	9.6	WT
Calu-1	PE	SCC	M	10.6	WT
H522	Lung	Adeno	M	10.6	WT
H3122	Lung	NSCLC (NOS)	M	10	WT
HCC15	Axillary FNA	SCC	M	10	WT
H226	PE	Epidermoid	M	10	WT
H1651	Lung	Adeno	M	6.2	WT
LXF529	N/A	LCC	M	10	WT
H460	PE	LCC	M	6.7	WT
KNS-62	N/A	N/A	Intermediate	10	N/A
H1781	Lung	Adeno	Intermediate	10	WT
H520	Lung	SCC	Intermediate	10	WT
H1770	Lung	Neuroendocrine	Intermediate	10	WT
H2106	Lung	Large Cell Neuroendocrine	Intermediate	10	N/A
H1385	Lung	Squamous	Intermediate	10	WT
UMC-11	Lung	Carcinoid-endocrine	Intermediate	10	WT
HCC4018	Lung	Adeno	Intermediate	10	WT
VMRC-LCD	N/A	N/A	Intermediate	10	N/A
H1693	Lung	Adeno	Intermediate	2.24	WT

FIG. 8B

DIAGNOSTIC MARKERS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/542,141 filed Sep. 30, 2011, the disclosure of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention provides methods of predicting response to a cancer therapy based on gene methylation status.

BACKGROUND OF THE INVENTION

[0003] The present invention is directed to methods for diagnosing and treating cancer patients. In particular, the present invention is directed to methods for determining which patients will most benefit from treatment with an epidermal growth factor receptor (EGFR) kinase inhibitor.

[0004] Cancer is a generic name for a wide range of cellular malignancies characterized by unregulated growth, lack of differentiation, and the ability to invade local tissues and metastasize. These neoplastic malignancies affect, with various degrees of prevalence, every tissue and organ in the body.

[0005] A multitude of therapeutic agents have been developed over the past few decades for the treatment of various types of cancer. The most commonly used types of anticancer agents include: DNA-alkylating agents (e.g., cyclophosphamide, ifosfamide), antimetabolites (e.g., methotrexate, a folate antagonist, and 5-fluorouracil, a pyrimidine antagonist), microtubule disrupters (e.g., vincristine, vinblastine, paclitaxel), DNA intercalators (e.g., doxorubicin, daunomycin, cisplatin), and hormone therapy (e.g., tamoxifen, flutamide).

[0006] The epidermal growth factor receptor (EGFR) family comprises four closely related receptors (HER1/EGFR, HER2, HER3 and HER4) involved in cellular responses such as differentiation and proliferation. Over-expression of the EGFR kinase, or its ligand TGF-alpha, is frequently associated with many cancers, including breast, lung, colorectal, ovarian, renal cell, bladder, head and neck cancers, glioblastomas, and astrocytomas, and is believed to contribute to the malignant growth of these tumors. A specific deletion-mutation in the EGFR gene (EGFRvIII) has also been found to increase cellular tumorigenicity. Activation of EGFR stimulated signaling pathways promote multiple processes that are potentially cancer-promoting, e.g. proliferation, angiogenesis, cell motility and invasion, decreased apoptosis and induction of drug resistance. Increased HER1/EGFR expression is frequently linked to advanced disease, metastases and poor prognosis. For example, in NSCLC and gastric cancer, increased HER1/EGFR expression has been shown to correlate with a high metastatic rate, poor tumor differentiation and increased tumor proliferation.

[0007] Mutations which activate the receptor's intrinsic protein tyrosine kinase activity and/or increase downstream signaling have been observed in NSCLC and glioblastoma. However the role of mutations as a principle mechanism in conferring sensitivity to EGF receptor inhibitors, for example erlotinib (TARCEVA®) or gefitinib (IRESSA™), has been controversial. Recently, a mutant form of the full length EGF receptor has been reported to predict responsiveness to the EGF receptor tyrosine kinase inhibitor gefitinib (Paez, J. G. et al. (2004) Science 304:1497-1500; Lynch, T. J. et al. (2004) N. Engl. J. Med. 350:2129-2139). Cell culture studies have shown that cell lines which express the mutant form of the EGF receptor (i.e. H3255) were more sensitive to growth inhibition by the EGF receptor tyrosine kinase inhibitor gefitinib, and that much higher concentrations of gefitinib was required to inhibit the tumor cell lines expressing wild type EGF receptor. These observations suggests that specific mutant forms of the EGF receptor may reflect a greater sensitivity to EGF receptor inhibitors but do not identify a completely non-responsive phenotype.

[0008] The development for use as anti-tumor agents of compounds that directly inhibit the kinase activity of the EGFR, as well as antibodies that reduce EGFR kinase activity by blocking EGFR activation, are areas of intense research effort (de Bono J. S. and Rowinsky, E. K. (2002) Trends in Mol. Medicine. 8:S19-S26; Dancey, J. and Sausville, E. A. (2003) Nature Rev. Drug Discovery 2:92-313). Several studies have demonstrated, disclosed, or suggested that some EGFR kinase inhibitors might improve tumor cell or neoplasia killing when used in combination with certain other anticancer or chemotherapeutic agents or treatments (e.g. Herbst, R. S. et al. (2001) Expert Opin. Biol. Ther. 1:719-732; Solomon, B. et al (2003) Int. J. Radiat. Oncol. Biol. Phys. 55:713-723; Krishnan, S. et al. (2003) Frontiers in Bioscience 8, el-13; Grunwald, V. and Hidalgo, M. (2003) J. Nat. Cancer Inst. 95:851-867; Seymour L. (2003) Current Opin. Investig. Drugs 4(6):658-666; Khalil, M. Y. et al. (2003) Expert Rev. Anticancer Ther.3:367-380; Bulgaru, A. M. et al. (2003) Expert Rev. Anticancer Ther. 3:269-279; Dancey, J. and Sausville, E. A. (2003) Nature Rev. Drug Discovery 2:92-313; Ciardiello, F. et al. (2000) Clin. Cancer Res. 6:2053-2063; and Patent Publication No: US 2003/0157104).

[0009] Erlotinib (e.g. erlotinib HCl, also known as TARCEVA® or OSI-774) is an orally available inhibitor of EGFR kinase. In vitro, erlotinib has demonstrated substantial inhibitory activity against EGFR kinase in a number of human tumor cell lines, including colorectal and breast cancer (Moyer J. D. et al. (1997) Cancer Res. 57:4838), and preclinical evaluation has demonstrated activity against a number of EGFR-expressing human tumor xenografts (Pollack, V. A. et al (1999) J. Pharmacol. Exp. Ther. 291:739). More recently, erlotinib has demonstrated promising activity in phase I and II trials in a number of indications, including head and neck cancer (Soulieres, D., et al. (2004) J. Clin. Oncol. 22:77), NSCLC (Perez-Soler R, et al. (2001) Proc. Am. Soc. Clin. Oncol. 20:310a, abstract 1235), CRC (Oza, M., et al. (2003) Proc. Am. Soc. Clin. Oncol. 22:196a, abstract 785) and MBC (Winer, E., et al. (2002) Breast Cancer Res. Treat. 76:5115a, abstract 445). In a phase III trial, erlotinib monotherapy significantly prolonged survival, delayed disease progression and delayed worsening of lung cancer-related symptoms in patients with advanced, treatment-refractory NSCLC (Shepherd, F. et al. (2004) J. Clin. Oncology, 22:14 S (July 15 Supplement), Abstract 7022). While much of the clinical trial data for erlotinib relate to its use in NSCLC, preliminary results from phase I/II studies have demonstrated promising activity for erlotinib and capecitabine/erlotinib combination therapy in patients with wide range of human solid tumor types, including CRC (Oza, M., et al. (2003) Proc. Am. Soc. Clin. Oncol. 22:196a, abstract 785) and MBC (Jones, R. J., et al. (2003) Proc. Am. Soc. Clin. Oncol. 22:45a, abstract 180). In November 2004 the U.S. Food and Drug

Administration (FDA) approved erlotinib for the treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) after failure of at least one prior chemotherapy regimen. Erlotinib is the only drug in the epidermal growth factor receptor (EGFR) class to demonstrate in a Phase III clinical trial an increase in survival in advanced NSCLC patients.

[0010] An anti-neoplastic drug would ideally kill cancer cells selectively, with a wide therapeutic index relative to its toxicity towards non-malignant cells. It would also retain its efficacy against malignant cells, even after prolonged exposure to the drug. Unfortunately, none of the current chemotherapies possess such an ideal profile. Instead, most possess very narrow therapeutic indexes. Furthermore, cancerous cells exposed to slightly sub-lethal concentrations of a chemotherapeutic agent will very often develop resistance to such an agent, and quite often cross-resistance to several other antineoplastic agents as well. Additionally, for any given cancer type one frequently cannot predict which patient is likely to respond to a particular treatment, even with newer gene-targeted therapies, such as EGFR kinase inhibitors, thus necessitating considerable trial and error, often at considerable risk and discomfort to the patient, in order to find the most effective therapy.

[0011] Thus, there is a need for more efficacious treatment for neoplasia and other proliferative disorders, and for more effective means for determining which tumors will respond to which treatment. Strategies for enhancing the therapeutic efficacy of existing drugs have involved changes in the schedule for their administration, and also their use in combination with other anticancer or biochemical modulating agents. Combination therapy is well known as a method that can result in greater efficacy and diminished side effects relative to the use of the therapeutically relevant dose of each agent alone. In some cases, the efficacy of the drug combination is additive (the efficacy of the combination is approximately equal to the sum of the effects of each drug alone), but in other cases the effect is synergistic (the efficacy of the combination is greater than the sum of the effects of each drug given alone).

[0012] Target-specific therapeutic approaches, such as erlotinib, are generally associated with reduced toxicity compared with conventional cytotoxic agents, and therefore lend themselves to use in combination regimens. Promising results have been observed in phase I/II studies of erlotinib in combination with bevacizumab (Mininberg, E. D., et al. (2003) Proc. Am. Soc. Clin. Oncol. 22:627a, abstract 2521) and gemcitabine (Dragovich, T., (2003) Proc. Am. Soc. Clin. Oncol. 22:223a, abstract 895). Recent data in NSCLC phase III trials have shown that first-line erlotinib or gefitinib in combination with standard chemotherapy did not improve survival (Gatzemeier, U., (2004) Proc. Am. Soc. Clin. Oncol. 23:617 (Abstract 7010); Herbst, R. S., (2004) Proc. Am. Soc. Clin. Oncol. 23:617 (Abstract 7011); Giaccone, G., et al. (2004) J. Clin. Oncol. 22:777; Herbst, R., et al. (2004) J. Clin. Oncol. 22:785). However, pancreatic cancer phase III trials have shown that first-line erlotinib in combination with gemcitabine did improve survival.

[0013] Several groups have investigated potential biomarkers to predict a patient's response to EGFR inhibitors (see for example, WO 2004/063709, WO 2005/017493, WO 2004/111273, WO 2004/071572; US 2005/0019785, and US 2004/0132097). One such biomarker is epithelial and mesenchymal phenotype. During most cancer metastases, an important change occurs in a tumor cell known as the epithelial-to-mesenchymal transition (EMT) (Thiery, J. P. (2002) Nat. Rev. Cancer 2:442-454; Savagner, P. (2001) Bioessays 23:912-923; Kang Y. and Massague, J. (2004) Cell 118:277-279; Julien-Grille, S., et al. Cancer Research 63:2172-2178; Bates, R. C. et al. (2003) Current Biology 13:1721-1727; Lu Z., et al. (2003) Cancer Cell. 4(6):499-515)). Epithelial cells, which are bound together tightly and exhibit polarity, give rise to mesenchymal cells, which are held together more loosely, exhibit a loss of polarity, and have the ability to travel. These mesenchymal cells can spread into tissues surrounding the original tumor, invade blood and lymph vessels, and travel to new locations where they divide and form additional tumors. EMT does not occur in healthy cells except during embryogenesis. Under normal circumstances TGF-β acts as a growth inhibitor, however, during cancer metastasis, TGF-β begins to promote EMT.

[0014] Epithelial and mesenchymal phenotypes have been associated with particular gene expression patterns. For example, epithelial phenotype was shown in WO2006101925 to be associated with high expression levels of E-cadherin, Brk, γ-catenin, α-catenin, keratin 8, keratin 18, connexin 31, plakophilin 3, stratafin 1, laminin alpha-5 and ST14 whereas mesenchymal phenotype was associated with high expression levels of vimentin, fibronectin, fibrillin-1, fibrillin-2, collagen alpha-2(IV), collagen alpha-2(V), LOXL1, nidogen, Cllorf9, tenascin, N-cadherin, embryonal EDB+fibronectin, tubulin alpha-3 and epimorphin.

[0015] Epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence—hence the name epi- (Greek: over, above, outer)-genetics. Examples of such changes include DNA methylation and histone modifications, both of which serve to modulate gene expression without altering the sequence of the associated genes. These changes can be somatically heritable through cell division for the remainder of the life of the organism and may also be passed on to subsequent generations of the organism. However, there is no change in the underlying DNA sequence of the organism; instead, non-genetic factors cause the organism's genes to behave or express differently.

[0016] DNA methylation is a crucial part of normal organismal development and cellular differentiation in higher organisms. DNA methylation stably alters the gene expression pattern in cells such that cells can "remember where they have been"; for example, cells programmed to be pancreatic islets during embryonic development remain pancreatic islets throughout the life of the organism without continuing signals telling them that they need to remain islets. In addition, DNA methylation suppresses the expression of viral genes and other deleterious elements that have been incorporated into the genome of the host over time. DNA methylation also forms the basis of chromatin structure, which enables cells to form the myriad characteristics necessary for multicellular life from a single immutable sequence of DNA. DNA methylation also plays a crucial role in the development of nearly all types of cancer. DNA methylation at the 5 position of cytosine has the specific effect of reducing gene expression and has been found in every vertebrate examined. In adult somatic tissues, DNA methylation typically occurs in a CpG dinucleotide context while non-CpG methylation is prevalent in embryonic stem cells.

[0017] "CpG" is shorthand for "—C-phosphate-G-", that is, cytosine and guanine separated by only one phosphate; phosphate links any two nucleosides together in DNA. The

"CpG" notation is used to distinguish this linear sequence from the CG base-pairing of cytosine and guanine. Cytosines in CpG dinucleotides can be methylated to form 5-methylcytosine (5-mC). In mammals, methylating the cytosine within a gene can turn the gene off. Enzymes that add a methyl group to DNA are called DNA methyltransferases. In mammals, 70% to 80% of CpG cytosines are methylated. There are regions of the genome that have a higher concentration of CpG sites, known as CpG islands. These "CpG islands" also have a higher than expected GC content (i.e. >50%). Many genes in mammalian genomes have CpG islands associated with the start of the gene. Because of this, the presence of a CpG island is used to help in the prediction and annotation of genes. CpG islands are refractory to methylation, which may help maintain an open chromatin configuration. In addition, this could result in a reduced vulnerability to transition mutations and, as a consequence, a higher equilibrium density of CpGs surviving. Methylation of CpG sites within the promoters of genes can lead to their silencing, a feature found in a number of human cancers (for example the silencing of tumor suppressor genes). In contrast, the hypomethylation of CpG sites has been associated with the over-expression of oncogenes within cancer cells.

SUMMARY OF THE INVENTION

[0018] One aspect of the invention provides for a method of determining whether a tumor cell has an epithelial phenotype comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in the tumor cell, wherein the presence of methylation at any of the CpG sites indicates that the tumor cell has an epithelial phenotype. In certain embodiments, the CpG sites are in the PCDH8, PEX5L, GALR1 or ZEB2 gene. In certain embodiments, the tumor cell is a NSCLC cell.

[0019] Another aspect of the invention provides for a method of determining whether a tumor cell has an epithelial phenotype comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 1 or Table 3, wherein the absence of methylation at any of the CpG sites indicates that the tumor cell has an epithelial phenotype. In certain embodiments, the CpG sites are in the CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, or C20orf55 gene. In certain embodiments, the tumor cell is a NSCLC cell.

[0020] Another aspect of the invention provides for a method of determining the sensitivity of tumor growth to inhibition by an EGFR kinase inhibitor, comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in a sample tumor cell, wherein the presence of DNA methylation at any one of the CpG sites indicates that the tumor growth is sensitive to inhibition with the EGFR inhibitor. In one embodiment, the EGFR inhibitor is erlotinib, cetuximab, or panitumumab. In certain embodiments, the tumor cell is a NSCLC cell.

[0021] Another aspect of the invention provides for a method of identifying a cancer patient who is likely to benefit from treatment with an EFGR inhibitor comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 1 or Table 3 in a sample from the patient's cancer, wherein the patient is identified as being likely to benefit from treatment with the EGFR inhibitor if the absence of DNA methylation at any one of the CpG sites is detected. In certain embodiments, the CpG sites are in the CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, or C20orf55 gene. In certain embodiments, the EGFR inhibitor is erlotinib, cetuximab, or panitumumab. In certain embodiments, the cancer is NSCLC.

[0022] Yet another aspect of the invention provides for a method of identifying a cancer patient who is likely to benefit from treatment with an EFGR inhibitor comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in a sample from the patient's cancer, wherein the patient is identified as being likely to benefit from treatment with the EGFR inhibitor if the presence of DNA methylation at any one of the CpG sites is detected. In certain embodiments, the patient is administered a therapeutically effective amount of an EGFR inhibitor if the patient is identified as one who will likely benefit from treatment with the EGFR inhibitor. In certain embodiments, the EGFR inhibitor is erlotinib, cetuximab, or panitumumab. In certain embodiments, the cancer is NSCLC.

[0023] Another aspect of the invention provides for a method of determining whether a tumor cell has a mesenchymal phenotype comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in the tumor cell, wherein the absence of methylation at any of the CpG sites indicates that the tumor cell has a mesenchymal phenotype. In certain embodiments, the CpG sites are in the PCDH8, PEX5L, GALR1 or ZEB2 gene. In certain embodiments, the tumor cell is a NSCLC cell.

[0024] Another aspect of the invention provides for a method of determining whether a tumor cell has a mesenchymal phenotype comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 1 or Table 3, wherein the presence of methylation at any of CpG sites indicates that the tumor cell has a mesenchymal phenotype. In certain embodiments, the CpG sites are in the CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, or C20orf55 gene. In certain embodiments, the tumor cell is a NSCLC cell.

[0025] Yet another aspect of the invention provides for a method of determining the sensitivity of tumor growth to inhibition by an EGFR kinase inhibitor, comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in a sample tumor cell, wherein the absence of DNA methylation at any one of the CpG sites indicates that the tumor growth is resistant to inhibition with the EGFR inhibitor. In certain embodiments, the EGFR inhibitor is erlotinib, cetuximab, or panitumumab. In certain embodiments, the tumor cell is a NSCLC cell.

[0026] Another aspect of the invention provides for a method of determining the sensitivity of tumor growth to inhibition by an EGFR kinase inhibitor, comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 1 or Table 3 in a sample tumor cell, wherein the presence of DNA methylation at any one of the CpG sites indicates that the tumor growth is resistant to inhibition with the EGFR inhibitor, such as for example, erlotinib, gefitinib, lapatinib, cetuximab or panitumumab. In certain embodiments, the CpG sites are in the CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, or C20orf55 gene. In certain embodiments, the EGFR inhibitor is erlotinib, cetuximab, or panitumumab. In certain embodiments, the tumor cell is a NSCLC cell.

[0027] Another aspect of the invention provides for a method of treating a cancer in a patient comprising adminis-

tering a therapeutically effective amount of an EGFR inhibitor to the patient, wherein the patient, prior to administration of the EGFR inhibitor, was diagnosed with a cancer which exhibits presence of methylation of DNA at one of the CpG sites identified in Table 2 or Table 4. In certain embodiments, the EGFR inhibitor is erlotinib, cetuximab, or panitumumab. In certain embodiments, the cancer is NSCLC.

[0028] Another aspect of the invention provides for a method of treating a cancer in a patient comprising administering a therapeutically effective amount of an EGFR inhibitor to the patient, wherein the patient, prior to administration of the EGFR inhibitor, was diagnosed with a cancer which exhibits absence of methylation of DNA at one of the CpG sites identified in Table 1 or Table 3. In certain embodiments, the EGFR inhibitor is erlotinib, cetuximab, or panitumumab. In certain embodiments, the cancer is NSCLC.

[0029] Another aspect of the invention provides for a method of selecting a therapy for a cancer patient, comprising the steps of detecting the presence or absence of DNA methylation at one of the CpG sites identified in Table 2 or Table 4 in a sample from the patient's cancer, and selecting an EGFR inhibitor for the therapy when the presence of methylation at one of the one of the CpG sites identified in Table 2 or Table 4 is detected. In one embodiment, the patient is administered a therapeutically effective amount of the EGFR inhibitor, such as erlotinib, cetuximab, or panitumumab, if the EGFR therapy is selected. In certain embodiments, the patient is suffering from NSCLC.

[0030] Another aspect of the invention provides for a method of selecting a therapy for a cancer patient, comprising the steps of detecting the presence or absence of DNA methylation at one of the CpG sites identified in Table 1 or Table 3 in a sample from the patient's cancer, and selecting an EGFR inhibitor for the therapy when the absence of methylation at one of the CpG sites identified in Table 1 or Table 3 is detected. In one embodiment, the patient is administered a therapeutically effective amount of the EGFR inhibitor, such as erlotinib, cetuximab, or panitumumab, if the EGFR therapy is selected. In certain embodiments, the patient is suffering from NSCLC.

[0031] In certain embodiments of the above aspects, the presence or absence of methylation is detected by pyrosequencing. In certain embodiments of the above aspects, the DNA is isolated from a formalin-fixed paraffin embedded (FFPE) tissue or from fresh frozen tissue. In one embodiment, the DNA isolated from the tissue sample is preamplified before pyrosequencing.

BRIEF DESCRIPTION OF THE FIGURES

[0032] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0033] FIG. **1**. NSCLC cell lines classified as epithelial and mesenchymal phenotype according to Fluidigm EMT gene expression panel.

[0034] FIG. **2**. Hierarchical clustering characterizing cell lines as epithelial-like or mesenchymal-like.

[0035] FIG. **3**. DNA methylation of patterns of epithelial and mesenchymal NSCLC cell lines classified as sensitive, intermediate, and resistant to EGFR inhibitor erlotinib

[0036] FIG. **4**. Annotation of DMRs selected for sodium bisulfite sequencing or qMSP and pyrosequencing array design.

[0037] FIG. **5**A. Pyrosequencing of the CLDN7 promoter region differentiates 42 NSCLC cell lines on the basis of epithelial-like/mesenchymal-like phenotype

[0038] FIG. **5**B. Relative expression of CLDN7 mRNA determined using a standard ΔCt method in 42 (n=20 epithelial-like, 19 mesenchymal-like, 3 intermediate) DMSO-treated and 5-aza-dC-treated NSCLC cell lines.

[0039] FIG. **6** A-H. TaqMan-based methylation detection assays specific for DMRs associated with the genes (A) MST1R/RON, (C) FAM110A, (E) CP2L3/GRHL2, and (G) ESRP1 and Receiver operating characteristic (ROC) plots for (B) RON, (D) FAM110A, (F) GRHL2, and (H) ESRP1.

[0040] FIG. **7** A-M. Receiver operating characteric (ROC) curves of quantitative methylation specific PCR assays in erlotinib sensitive versus erlotinib resistant NSCLC cell lines—PEX5L (A), PCDH8 (B), ZEB2 (C), ME3 (D), MSTR1 (E), STX2 (F), HOXC5 (G), C20orf55 (H), ESRP1 (I), BCAR3 (J), CLDN7 (K), NKX6.2 (L), CP2L3 (M).

[0041] FIG. **8**A-B. Table showing the epithelial (E) or mesenchymal (M) classification of 82 NSCLC Cell Lines and erlotinib IC50 values.

LIST OF TABLES

[0042] Table 1. Methylated cytosine nucleotides (CpG) associated with mesenchymal phenotype.

[0043] Table 2. Methylated cytosine nucleotides (CpG) associated with epithelial phenotype.

[0044] Table 3. Methylated cytosine nucleotides (CpG) associated with mesenhymal phenotype identified by chromosome number, nucleotide position and Entrez ID of the gene.

[0045] Table 4. Methylated cytosine nucleotides (CpG) associated with epithial phenotype identified by chromosome number, nucleotide position and Entrez ID of the gene.

DETAILED DESCRIPTION OF THE INVENTION

[0046] The term "cancer" in an animal refers to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, and certain characteristic morphological features. Often, cancer cells will be in the form of a tumor, but such cells may exist alone within an animal, or may circulate in the blood stream as independent cells, such as leukemic cells.

[0047] "Abnormal cell growth", as used herein, unless otherwise indicated, refers to cell growth that is independent of normal regulatory mechanisms (e.g., loss of contact inhibition). This includes the abnormal growth of: (1) tumor cells (tumors) that proliferate by expressing a mutated tyrosine kinase or overexpression of a receptor tyrosine kinase; (2) benign and malignant cells of other proliferative diseases in which aberrant tyrosine kinase activation occurs; (4) any tumors that proliferate by receptor tyrosine kinases; (5) any tumors that proliferate by aberrant serine/threonine kinase activation; and (6) benign and malignant cells of other proliferative diseases in which aberrant serine/threonine kinase activation occurs.

[0048] The term "treating" as used herein, unless otherwise indicated, means reversing, alleviating, inhibiting the progress of, or preventing, either partially or completely, the

growth of tumors, tumor metastases, or other cancer-causing or neoplastic cells in a patient. The term "treatment" as used herein, unless otherwise indicated, refers to the act of treating.

[0049] The phrase "a method of treating" or its equivalent, when applied to, for example, cancer refers to a procedure or course of action that is designed to reduce or eliminate the number of cancer cells in an animal, or to alleviate the symptoms of a cancer. "A method of treating" cancer or another proliferative disorder does not necessarily mean that the cancer cells or other disorder will, in fact, be eliminated, that the number of cells or disorder will, in fact, be reduced, or that the symptoms of a cancer or other disorder will, in fact, be alleviated.

[0050] The term "therapeutically effective agent" means a composition that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician.

[0051] The term "therapeutically effective amount" or "effective amount" means the amount of the subject compound or combination that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician.

[0052] The terms "ErbB1", "HER1", "epidermal growth factor receptor" and "EGFR" and "EGFR kinase" are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter et al. Ann. Rev. Biochem. 56:881-914 (1987), including naturally occurring mutant forms thereof (e.g. a deletion mutant EGFR as in Humphrey et al. PNAS (USA) 87:4207-4211 (1990)). erbB1 refers to the gene encoding the EGFR protein product.

[0053] As used herein, the term "EGFR kinase inhibitor" and "EGFR inhibitor" refers to any EGFR kinase inhibitor that is currently known in the art or that will be identified in the future, and includes any chemical entity that, upon administration to a patient, results in inhibition of a biological activity associated with activation of the EGF receptor in the patient, including any of the downstream biological effects otherwise resulting from the binding to EGFR of its natural ligand. Such EGFR kinase inhibitors include any agent that can block EGFR activation or any of the downstream biological effects of EGFR activation that are relevant to treating cancer in a patient. Such an inhibitor can act by binding directly to the intracellular domain of the receptor and inhibiting its kinase activity. Alternatively, such an inhibitor can act by occupying the ligand binding site or a portion thereof of the EGF receptor, thereby making the receptor inaccessible to its natural ligand so that its normal biological activity is prevented or reduced. Alternatively, such an inhibitor can act by modulating the dimerization of EGFR polypeptides, or interaction of EGFR polypeptide with other proteins, or enhance ubiquitination and endocytotic degradation of EGFR. EGFR kinase inhibitors include but are not limited to low molecular weight inhibitors, antibodies or antibody fragments, antisense constructs, small inhibitory RNAs (i.e. RNA interference by dsRNA; RNAi), and ribozymes. In a preferred embodiment, the EGFR kinase inhibitor is a small organic molecule or an antibody that binds specifically to the human EGFR.

[0054] Inhibitors of EGF receptor function have shown clinical utility and the definition of key EGF receptor signaling pathways which describe patient subsets most likely to benefit from therapy has become an important area of investigation. Mutations which activate the receptor's intrinsic protein tyrosine kinase activity and/or increase downstream signaling have been observed in NSCLC and glioblastoma. In vitro and clinical studies have shown considerable variability between wt EGF receptor cell lines and tumors in their cellular responses to EGF receptor inhibition, which in part has been shown to derive from EGF receptor independent activation of the phosphatidyl inositol 3-kinase pathway, leading to the continued phosphorylation of the anti-apoptotic serine-threonine kinase Akt. The molecular determinants to alternative routes of PI3-kinase activation and consequent EGF receptor inhibitor insensitivity are an active area of investigation. For example the insulin-like growth factor-1 receptor (IGF-1 receptor), which strongly activates the PI3-kinase pathway, has been implicated in cellular resistance to EGF inhibitors. The roles of cell-cell and cell-adhesion networks, which can also exert survival signals through the PI3-kinase pathway in mediating insensitivity to selective EGF receptor inhibition are less clear and would be postulated to impact cell sensitivity to EGF receptor blockade. The ability of tumor cells to maintain growth and survival signals in the absence of adhesion to extracellular matrix or cell-cell contacts is important not only in the context of cell migration and metastasis but also in maintaining cell proliferation and survival in wound-like tumor environments where extracellular matrix is being remodeled and cell contact inhibition is diminished.

[0055] An EMT gene expression signature that correlates with in vitro sensitivity of NSCLC cell lines to erlotinib was previously developed. (Yauch et al., 2005, Clin Cancer Res 11, 8686-8698). A fluidigm-based EMT expression signature associated with epithelial and mesenchymal phenotypes was developed based on this work (FIG. **1**).

[0056] The present invention is based, in part, on the use of an integrated genomics approach combining gene expression analysis with whole genome methylation profiling to show that methylation biomarkers are capable of classifying epithelial and mesenchymal phenotypes in cancer (such as NSCLC), demonstrating that genome-wide differences in DNA methylation patterns are associated with distinct biologic and clinically relevant subsets of that cancer. The use of methylation patterns to classify phenotypic subsets of cancers using the methods described herein is advantageous as it requires less quantity of test tissue as compared to more traditional methods of DNA- and RNA-based analyses. This feature is particularly useful when analyzing clinical samples where tissue is limited.

[0057] A major challenge in the development of predictive biomarkers is the need to establish a robust "cut-point" for prospective evaluation. This is particularly problematic for protein-based assays such as immunohistochemistry. While widely used, immunohistochemistry is subject to a number of technical challenges that limit its use in the context of predictive biomarker development. These limitations include antibody specificity and sensitivity, epitope availability and stability, and the inherent subjectivity of data interpretation by different pathologists (24, 25). Molecular assays that can leverage the dynamic range and specificity of PCR are much more desirable. However, there are also limitations with PCR-based assays: RNA is highly unstable and requires that a cutoff point be defined prospectively. Mutation detection assays, while potentially binary, are limited by the availability of high prevalence mutation hot spots and target sequences. As shown in the Examples, PCR-based methylation assays potentially address many of these issues because they have many of the properties of mutation assays, including a broad

dynamic range and an essentially binary readout with similar sensitivity to mutation assays, yet due to the locally correlated behavior of CpG methylation states, the target regions for assay design can be quite large. Most importantly, DNA methylation can be used to infer the biologic state of tumors in much the same way as gene expression has been used in the past.

[0058] The data presented in the Examples herein demonstrate that tumor cells, such as NSCLC or pancreatic cancer cells, containing wild type EGFR, grown either in cell culture or in vivo, show a range of sensitivities to inhibition by EGFR kinase inhibitors, dependent on whether they have undergone an epithelial to mesenchymal transition (EMT). Prior to EMT, tumor cells are very sensitive to inhibition by EGFR kinase inhibitors such as erlotinib HCl (Tarceva®), whereas tumor cells which have undergone an EMT are substantially less sensitive to inhibition by such compounds. The data indicates that the EMT may be a general biological switch that determines the level of sensitivity of tumors to EGFR kinase inhibitors. It is demonstrated herein that the level of sensitivity of tumors to EGFR kinase inhibitors can be assessed by determining the level of biomarkers expressed by a tumor cell that are characteristic for cells either prior to or subsequent to an EMT event. For example, high levels of tumor cell expression of epithelial biomarkers such as E-cadherin, indicative of a cell that has not yet undergone an EMT, correlate with high sensitivity to EGFR kinase inhibitors. Conversely, high levels of tumor cell expression of mesenchymal biomarkers such as vimentin or fibronectin, indicative of a cell that has undergone an EMT, correlate with low sensitivity to EGFR kinase inhibitors. Thus, these observations can form the basis of diagnostic methods for predicting the effects of EGFR kinase inhibitors on tumor growth, and give oncologists a tool to assist them in choosing the most appropriate treatment for their patients.

[0059] As described in the Examples, cancer can be differentiated into epithelial-like (EL) and mesenchymal-like (ML) tumors based on DNA methylation patterns. Mesenchymal phenotype (or a tumor cell that has undergone EMT) is associated with methylation of particular genes shown in Table 1 and Table 3. Accordingly, the present invention provides a method of determining whether a tumor cell has a mesenchymal phenotype comprising detecting the presence or absence of methylation of DNA at anyone of the CpG sites identified in Table 1 or Table 3 in the tumor cell, wherein the methylation at any of the CpG sites indicates that the tumor cell has a mesenchymal phenotype. Conversely, the absence of DNA methylation at any one of the CpG sites identified in Table 1 or Table 3 indicates the tumor has an epithelial phenotype.

[0060] In a particular embodiment, the method of determining whether a tumor cell has a mesenchymal phenotype comprises detecting the presence or absence of methylation at CpG sites in one or more of CLDN7 (claudin-7), HOXC4 (homeobox C4), CP2L3 (grainyhead like-3), STX2 (syntaxin 2), RON (macrophage stimulating 1 receptor), TBCD (tubulin-specific chaperone D), ESRP1 (epithelial splicing regulatory protein 1), GRHL2 (grainyhead-like 2), ERBB2, and C20orf55 (chromosome 20 open reading frame 55) genes, wherein the presence of methylation at any one of the CpG sites indicates the tumor has a mesenchymal phenotype. Conversely, the absence of DNA methylation at any one of the CpG sites indicates the tumor has an epithelial phenotype. In a particular embodiment, the method comprises detecting methylation at CpG sites in one or more of CLDN7, HOXC4, CP2L3, STX2, RON, TBCD, ESRP1, GRHL2. ERBB2, and C20orf55 genes, wherein the presence of methylation at any one of the CpG sites indicates the tumor has a mesenchymal phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in two of the genes in Table 1 or Table 3 indicates that the tumor has a mesenchymal phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in three of the genes in Table 1 or Table 3 indicates that the tumor has a mesenchymal phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in four of the genes in Table 1 or Table 3 indicates that the tumor has a mesenchymal phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in five of the genes in Table 1 or Table 3 indicates that the tumor has a mesenchymal phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in two, three, or four, five, six, seven, eight, or all nine of CLDN7, HOXC4, CP2L3, STX2, RON, TBCD, ESRP1, GRHL2 and C20orf55 genes indicates that the tumor has a mesenchymal phenotype. In another embodiment, detecting the presence of methylation at CpG sites in two, three, or four of CLDN7, RON, ESRP1, and GRHL2 indicates that the tumor has a mesenchymal phenotype. In another embodiment, detecting the presence of methylation at CpG sites in all four of CLDN7, RON, ESRP1, and GRHL2 indicates that the tumor has a mesenchymal phenotype.

[0061] Further, the invention provides a method of predicting the sensitivity of tumor growth to inhibition by an EGFR inhibitor, comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 1 or Table 3 in a sample cell taken from the tumor, wherein the presence of DNA methylation at any one of the CpG sites indicates the tumor growth is resistant to inhibition with an EGFR inhibitor. Conversely, the absense of methylation of DNA at any one of the CpG sites indicates the tumor growth is sensitive (i.e. responsive) to inhibition by an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in two of the genes in Table 1 or Table 3 indicates the tumor growth is resistant to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in three of the genes in Table 1 or Table 3 indicates the tumor growth is resistant to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in four of the genes in Table 1 or Table 3 indicates the tumor growth is resistant to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in five of the genes in Table 1 or Table 3 indicates the tumor growth is resistant to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in two, three, or four, five, six, seven, eight, or all nine of CLDN7, HOXC4, CP2L3, STX2, RON, TBCD, ESRP1, GRHL2, ERBB2, and C20orf55 genes indicates the tumor growth is resistant to inhibition with an EGFR inhibitor. In another embodiment, detecting the presence of methylation at CpG sites in two, three, or four of CLDN7, RON, ESRP1, and GRHL2 indicates the tumor growth is resistant to inhibition with an EGFR inhibitor. In another embodiment, detecting the presence of methylation at CpG sites in all four of CLDN7, RON, ESRP1, and GRHL2 indicates the tumor growth is resistant to inhibition with an EGFR inhibitor.

[0062] Another aspect of the invention provides a method of identifying a cancer patient who is likely to benefit from

treatment with EGFR inhibitor, comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 1 or Table 3 in a sample from the patient's cancer, wherein the patient is identified as being likely to benefit from treatment with an EGFR inhibitor if the absence of DNA methylation at any one of the CpG sites is detected. Conversely, the presence of methylation of DNA at any one of the CpG sites indicates patient is less likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the absence of methylation at CpG sites in two of the genes in Table 1 or Table 3 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the absence of methylation at CpG sites in three of the genes in Table 1 or Table 3 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the absence of methylation at CpG sites in four of the genes in Table 1 or Table 3 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the absence of methylation at CpG sites in five of the genes in Table 1 or Table 3 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the absence of methylation at CpG sites in two, three, or four, five, six, seven, eight, or all nine of CLDN7, HOXC4, CP2L3, STX2, RON, TBCD, ESRP1, GRHL2, ERBB2, and C20orf55 genes indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In another embodiment, detecting the absence of methylation at CpG sites in two, three, or four of CLDN7, RON, ESRP1, and GRHL2 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In another embodiment, detecting the absence of methylation at CpG sites in all four of CLDN7, RON, ESRP1, and GRHL2 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In certain embodiments, the patient who has been deemed likely to benefit from treatment with an EGFR inhibitor is administered a therapeutically effective amount of an EGFR inhibitor.

[0063] As described in the Examples, epithelial phenotype in a tumor cell is associated with methylation of particular genes shown in Table 2 and in Table 4. Accordingly, the present invention provides a method of determining whether a tumor cell has an epithelial phenotype comprising detecting the presence or absence of methylation of DNA at any one of the cytosine nucleotides (CpG sites) identified in Table 2 or in Table 4 in the tumor cell, wherein the presence of methylation at any of the cytosine nucleotides (CpG sites) indicates that the tumor cell has an epithelial phenotype. Conversely, the present invention further provides a method of determining whether a tumor cell has an epithelial phenotype comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in the tumor cell, wherein the absence of methylation at CpG sites indicates that the tumor has a mesenchymal phenotype.

[0064] In a particular embodiment, the method comprises detecting the presence or absence of methylation at CpG sites in one or more of PCDH8 (protocadherin 8), PEX5L (peroxisomal biogenesis factor 5-like), GALR1 (galanin receptor 1), ZEB2 (zinc finger E-box binding homeobox 2) and ME3 (malic enzyme 3) genes, wherein the presence of methylation at CpG sites indicates that the tumor has an epithelial phenotype. In a particular embodiment, the method comprises detecting the presence or absence of methylation at CpG sites in the ZEB2 gene, wherein the presence of methylation at CpG sites indicates that the tumor has an epithelial phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in two of the genes in Table 2 or Table 4 indicates that the tumor has an epithelial phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in three of the genes in Table 2 or Table 4 indicates that the tumor has an epithelial phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in four of the genes in Table 2 or Table 4 indicates that the tumor has an epithelial phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in five of the genes in Table 2 or Table 4 indicates that the tumor has an epithelial phenotype. In a particular embodiment, detecting the presence of methylation at CpG sites in each of PCDH8, PEX5L, GALR1 or ZEB2 genes indicates that the tumor has an epithelial phenotype.

[0065] Further, the invention provides a method of predicting the sensitivity of tumor growth to inhibition by an EGFR inhibitor, comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in a sample cell taken from the tumor, wherein the presence of DNA methylation at any one of the CpG sites indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor. Conversely, the absense of methylation of DNA at any one of the CpG sites indicates the tumor growth is resistant to inhibition by an EGFR inhibitor. In a particular embodiment, the method comprises detecting methylation of CpG sites of one or more of PCDH8, PEX5L, GALR1 or ZEB2 genes, wherein the presence of methylation at any one of the CpG sites indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor. In a particular embodiment, the method comprises detecting methylation of CpG sites in the ZEB2 gene, wherein the presence of methylation of CpG sites in the ZEB2 gene indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in two of the genes in Table 2 or Table 4 indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in three of the genes in or Table 4 indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in four of the genes in or Table 4 indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in five of the genes in or Table 4 indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in each of PCDH8, PEX5L, GALR1 or ZEB2 genes indicates the tumor growth is sensitive to inhibition with an EGFR inhibitor.

[0066] Another aspect of the invention provides a method of identifying a cancer patient who is likely to benefit from treatment with EGFR inhibitor, comprising detecting the presence or absence of methylation of DNA at any one of the CpG sites identified in Table 2 or Table 4 in a sample from the patient's cancer, wherein the patient is identified as being likely to benefit from treatment with an EGFR inhibitor if the presence of DNA methylation at any one of the CpG sites is detected. Conversely, the absence of methylation of DNA at any one of the CpG sites indicates patient is less likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG

sites in two of the genes in Table 2 or Table 4 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in three of the genes in Table 2 or Table 4 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in four of the genes in Table 2 or Table 4 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites in five of the genes in Table 2 or Table 4 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In a particular embodiment, detecting the presence of methylation at CpG sites two, three, or four of PCDH8, PEX5L, GALR1 or ZEB2 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In another embodiment, detecting the presence of methylation at CpG sites in ZEB2 indicates the patient is likely to benefit from treatment with an EGFR inhibitor. In certain embodiments, the patient who has been deemed likely to benefit from treatment with an EGFR inhibitor is administered a therapeutically effective amount of an EGFR inhibitor.

[0067] Another aspect of the invention provides for a method of treating a cancer patient who has previously been identified as one likely to benefit from treatment with an EGFR inhibitor using the DNA methylation profiling described herein, comprising administering to the patient a therapeutically effective amount of an EGFR inhibitor.

[0068] Another aspect of the invention provides for a method of selecting a therapy for a cancer patient based on the DNA methylation profiling methods described herein. In one embodiment, the method comprises detecting the presence or absence of DNA at one of the CpG sites identified in Table 2 or Table 4 in a sample from the patient's cancer and selecting an EGFR inhibitor for the therapy when the presence of methylation at one of the CpG sites identified in Table 2 or Table 4 is detected. In another embodiment, the method comprises detecting the presence or absence of DNA methylation at one of the CpG sites identified in Table 1 or Table 3 in a sample from the patient's cancer and selecting an EGFR inhibitor for the therapy when the absence of methylation at one of the one of the CpG sites identified in Table 1 or Table 3 is detected. In certain embodiments, the patient is administered therapeutically effective amount of the EGFR inhibitor, such as is erlotinib, cetuximab, or panitumumab, if the EGFR inhibitor therapy is selected.

[0069] One of skill in the medical arts, particularly pertaining to the application of diagnostic tests and treatment with therapeutics, will recognize that biological systems may exhibit variability and may not always be entirely predictable, and thus many good diagnostic tests or therapeutics are occasionally ineffective. Thus, it is ultimately up to the judgement of the attending physician to determine the most appropriate course of treatment for an individual patient, based upon test results, patient condition and history, and his own experience. There may even be occasions, for example, when a physician will choose to treat a patient with an EGFR inhibitor even when a tumor is not predicted to be particularly sensitive to EGFR kinase inhibitors, based on data from diagnostic tests or from other criteria, particularly if all or most of the other obvious treatment options have failed, or if some synergy is anticipated when given with another treatment. The fact that the EGFR inhibitors as a class of drugs are relatively well tolerated compared to many other anti-cancer drugs, such as more traditional chemotherapy or cytotoxic agents used in the treatment of cancer, makes this a more viable option.

[0070] Accordingly, the present invention provides a method of predicting the sensitivity of tumor cell growth to inhibition by an EGFR kinase inhibitor, comprising: assessing the DNA methylation level of one or more (or a panel of) epithelial biomarkers in a tumor cell; and predicting the sensitivity of tumor cell growth to inhibition by an EGFR inhibitor, wherein simultaneous high DNA methylation levels of all of the tumor cell epithelial biomarkers correlates with high sensitivity to inhibition by EGFR inhibitors. In one particular embodiment of this method the epithelial biomarkers comprise genes PCDH8, PEX5L, GALR1, ZEB2 and ME3, wherein simultaneous high expression level of the two tumor cell epithelial biomarkers correlates with high sensitivity to inhibition by EGFR kinase inhibitor.

[0071] The present invention also provides a method of predicting the sensitivity of tumor cell growth to inhibition by an EGFR kinase inhibitor, comprising: assessing the level of one or more (or a panel of) mesenchymal biomarkers in a tumor cell; and predicting the sensitivity of tumor cell growth to inhibition by an EGFR inhibitor, wherein simultaneous high levels of all of the tumor cell mesenchymal biomarkers correlates with resistance to inhibition by EGFR inhibitors. In one particular embodiment of this method the mesenchymal biomarkers comprise genes CLDN7, HOXC4, CP2L3, TBCD, ESRP1, GRHL2, and C20orf55, wherein simultaneous high DNA methylation levels of at least two tumor cell mesenchymal biomarkers correlates with resistance to inhibition by EGFR inhibitor.

[0072] The present invention also provides a method of predicting whether a cancer patient is afflicted with a tumor that will respond effectively to treatment with an EGFR kinase inhibitor, comprising: assessing the DNA methylation level of one or more (or a panel of) epithelial biomarkers PCDH8, PEX5L, GALR1, ZEB2 and ME3 in cells of the tumor; and predicting if the tumor will respond effectively to treatment with an EGFR inhibitor, wherein simultaneous high expression levels of all of the tumor cell epithelial biomarkers correlates with a tumor that will respond effectively to treatment with an EGFR inhibitor.

[0073] The present invention also provides a method of predicting whether a cancer patient is afflicted with a tumor that will respond effectively to treatment with an EGFR kinase inhibitor, comprising: assessing the level of one or more (or a panel of) mesenchymal biomarkers CLDN7, HOXC4, CP2L3, TBCD, ESRP1, GRHL2, and C20orf55 in cells of the tumor; and predicting if the tumor will respond effectively to treatment with an EGFR inhibitor, wherein high DNA methylation levels of all of such tumor cell mesenchymal biomarkers correlates with a tumor that is resistant to treatment with an EGFR inhibitor.

[0074] In the methods described herein the tumor cell will typically be from a patient diagnosed with cancer, a precancerous condition, or another form of abnormal cell growth, and in need of treatment. The cancer may be lung cancer (e.g. non-small cell lung cancer (NSCLC)), pancreatic cancer, head and neck cancer, gastric cancer, breast cancer, colon cancer, ovarian cancer, or any of a variety of other cancers described herein below. The cancer is one known to be potentially treatable with an EGFR inhibitor. Tumor cells may be obtained from a patients sputum, saliva, blood, urine, feces, cerebrospinal fluid or directly from the tumor, e.g. by fine needle aspirate.

[0075] Presence and/or level/amount of various biomarkers in a sample can be analyzed by a number of methodologies, many of which are known in the art and understood by the skilled artisan, including, but not limited to, immunohistochemical ("IHC"), Western blot analysis, immunoprecipitation, molecular binding assays, ELISA, ELIFA, fluorescence activated cell sorting ("FACS"), MassARRAY, proteomics, quantitative blood based assays (as for example Serum ELISA), biochemical enzymatic activity assays, in situ hybridization, Southern analysis, Northern analysis, whole genome sequencing, polymerase chain reaction ("PCR") including quantitative real time PCR ("qRT-PCR") and other amplification type detection methods, such as, for example, branched DNA, SISBA, TMA and the like), RNA-Seq, FISH, microarray analysis, gene expression profiling, and/or serial analysis of gene expression ("SAGE"), as well as any one of the wide variety of assays that can be performed by protein, gene, and/or tissue array analysis. Typical protocols for evaluating the status of genes and gene products are found, for example in Ausubel et al., eds., 1995, Current Protocols In Molecular Biology, Units 2 (Northern Blotting), 4 (Southern Blotting), 15 (Immunoblotting) and 18 (PCR Analysis). Multiplexed immunoassays such as those available from Rules Based Medicine or Meso Scale Discovery ("MSD") may also be used.

[0076] Methods for evaluation of DNA methylation are well known. For example, Laird (2010) Nature Reviews Genetics 11:191-203 provides a review of DNA methylation analysis. In some embodiments, methods for evaluating methylation include randomly shearing or randomly fragmenting the genomic DNA, cutting the DNA with a methylation-dependent or methylation-sensitive restriction enzyme and subsequently selectively identifying and/or analyzing the cut or uncut DNA. Selective identification can include, for example, separating cut and uncut DNA (e.g., by size) and quantifying a sequence of interest that was cut or, alternatively, that was not cut. See, e.g., U.S. Pat. No. 7,186,512. In some embodiments, the method can encompass amplifying intact DNA after restriction enzyme digestion, thereby only amplifying DNA that was not cleaved by the restriction enzyme in the area amplified. See, e.g., U.S. patent application Ser. Nos. 10/971,986; 11/071,013; and 10/971,339. In some embodiments, amplification can be performed using primers that are gene specific. Alternatively, adaptors can be added to the ends of the randomly fragmented DNA, the DNA can be digested with a methylation-dependent or methylation-sensitive restriction enzyme, intact DNA can be amplified using primers that hybridize to the adaptor sequences. In some embodiments, a second step can be performed to determine the presence, absence or quantity of a particular gene in an amplified pool of DNA. In some embodiments, the DNA is amplified using real-time, quantitative PCR.

[0077] In some embodiments, the methods comprise quantifying the average methylation density in a target sequence within a population of genomic DNA. In some embodiments, the method comprises contacting genomic DNA with a methylation-dependent restriction enzyme or methylation-sensitive restriction enzyme under conditions that allow for at least some copies of potential restriction enzyme cleavage sites in the locus to remain uncleaved; quantifying intact copies of the locus; and comparing the quantity of amplified product to a control value representing the quantity of methylation of control DNA, thereby quantifying the average methylation density in the locus compared to the methylation density of the control DNA.

[0078] The quantity of methylation of a locus of DNA can be determined by providing a sample of genomic DNA comprising the locus, cleaving the DNA with a restriction enzyme that is either methylation-sensitive or methylation-dependent, and then quantifying the amount of intact DNA or quantifying the amount of cut DNA at the DNA locus of interest. The amount of intact or cut DNA will depend on the initial amount of genomic DNA containing the locus, the amount of methylation in the locus, and the number (i.e., the fraction) of nucleotides in the locus that are methylated in the genomic DNA. The amount of methylation in a DNA locus can be determined by comparing the quantity of intact DNA or cut DNA to a control value representing the quantity of intact DNA or cut DNA in a similarly-treated DNA sample. The control value can represent a known or predicted number of methylated nucleotides. Alternatively, the control value can represent the quantity of intact or cut DNA from the same locus in another (e.g., normal, non-diseased) cell or a second locus.

[0079] By using methylation-sensitive or methylation-dependent restriction enzyme under conditions that allow for at least some copies of potential restriction enzyme cleavage sites in the locus to remain uncleaved and subsequently quantifying the remaining intact copies and comparing the quantity to a control, average methylation density of a locus can be determined. If the methylation-sensitive restriction enzyme is contacted to copies of a DNA locus under conditions that allow for at least some copies of potential restriction enzyme cleavage sites in the locus to remain uncleaved, then the remaining intact DNA will be directly proportional to the methylation density, and thus may be compared to a control to determine the relative methylation density of the locus in the sample. Similarly, if a methylation-dependent restriction enzyme is contacted to copies of a DNA locus under conditions that allow for at least some copies of potential restriction enzyme cleavage sites in the locus to remain uncleaved, then the remaining intact DNA will be inversely proportional to the methylation density, and thus may be compared to a control to determine the relative methylation density of the locus in the sample. Such assays are disclosed in, e.g., U.S. patent application Ser. No. 10/971,986.

[0080] In some embodiments, quantitative amplification methods (e.g., quantitative PCR or quantitative linear amplification) can be used to quantify the amount of intact DNA within a locus flanked by amplification primers following restriction digestion. Methods of quantitative amplification are disclosed in, e.g., U.S. Pat. Nos. 6,180,349; 6,033,854; and 5,972,602, as well as in, e.g., Gibson et al., Genome Research 6:995-1001 (1996); DeGraves et al., Biotechniques 34(1):106-10, 112-5 (2003); Deiman B et al., Mol. Biotechnol. 20(2):163-79 (2002).

[0081] Additional methods for detecting DNA methylation can involve genomic sequencing before and after treatment of the DNA with bisulfite. See, e.g., Frommer et al., Proc. Natl. Acad. Sci. USA 89:1827-1831 (1992). When sodium bisulfite is contacted to DNA, unmethylated cytosine is converted to uracil, while methylated cytosine is not modified.

[0082] In some embodiments, restriction enzyme digestion of PCR products amplified from bisulfite-converted DNA is used to detect DNA methylation. See, e.g., Sadri & Hornsby,

Nucl. Acids Res. 24:5058-5059 (1996); Xiong & Laird, Nucleic Acids Res. 25:2532-2534 (1997).

[0083] In some embodiments, a MethyLight assay is used alone or in combination with other methods to detect DNA methylation (see, Eads et al., Cancer Res. 59:2302-2306 (1999)). Briefly, in the MethyLight process genomic DNA is converted in a sodium bisulfite reaction (the bisulfite process converts unmethylated cytosine residues to uracil). Amplification of a DNA sequence of interest is then performed using PCR primers that hybridize to CpG dinucleotides. By using primers that hybridize only to sequences resulting from bisulfite conversion of unmethylated DNA, (or alternatively to methylated sequences that are not converted) amplification can indicate methylation status of sequences where the primers hybridize. Similarly, the amplification product can be detected with a probe that specifically binds to a sequence resulting from bisulfite treatment of an unmethylated (or methylated) DNA. If desired, both primers and probes can be used to detect methylation status. Thus, kits for use with MethyLight can include sodium bisulfite as well as primers or detectably-labeled probes (including but not limited to Taqman or molecular beacon probes) that distinguish between methylated and unmethylated DNA that have been treated with bisulfite. Other kit components can include, e.g., reagents necessary for amplification of DNA including but not limited to, PCR buffers, deoxynucleotides; and a thermostable polymerase.

[0084] In some embodiments, a Ms-SNuPE (Methylation-sensitive Single Nucleotide Primer Extension) reaction is used alone or in combination with other methods to detect DNA methylation (see Gonzalgo & Jones Nucleic Acids Res. 25:2529-2531 (1997)). The Ms-SNuPE technique is a quantitative method for assessing methylation differences at specific CpG sites based on bisulfite treatment of DNA, followed by single-nucleotide primer extension. Briefly, genomic DNA is reacted with sodium bisulfite to convert unmethylated cytosine to uracil while leaving 5-methylcytosine unchanged. Amplification of the desired target sequence is then performed using PCR primers specific for bisulfite-converted DNA, and the resulting product is isolated and used as a template for methylation analysis at the CpG site(s) of interest.

[0085] In some embodiments, a methylation-specific PCR ("MSP") reaction is used alone or in combination with other methods to detect DNA methylation. An MSP assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA. See, Herman et al., Proc. Natl. Acad. Sci. USA 93:9821-9826, (1996); U.S. Pat. No. 5,786,146. In some embodiments, DNA methylation is detected by a QIAGEN PyroMark CpG Assay predesigned Pyrosequencing DNA Methylation assays.

[0086] In some embodiments, cell methylation status is determined using high-throughput DNA methylation analysis to determine sensitivity to EGFR inhibitors. Briefly, genomic DNA is isolated from a cell or tissue sample (e.g. a tumor sample or a blood sample) and is converted in a sodium bisulfite reaction (the bisulfite process converts unmethylated cytosine residues to uracil) using standard assays in the art. The bisulfite converted DNA product is amplified, fragmented and hybridized to an array containing CpG sites from across a genome using standard assays in the art. Following hybridization, the array is imaged and processed for analysis of the DNA methylation status using standard assays in the art. In some embodiments, the tissue sample is formalin-fixed paraffin embedded (FFPE) tissue. In some embodiments, the tissue sample is fresh frozen tissue. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion by using the Invitrogen Superscript III One-Step RT-PCR System with Platinum Taq. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion using a Taqman based assay. In some embodiments, the sodium bisulfite reaction is conducted using the Zymo EZ DNA Methylation Kit. In some embodiments, the bisulfite converted DNA is amplified and hybridized to an array using the Illumina Infinium HumanMethylation450 Beadchip Kit. In some embodiments, the array is imaged on an Illumina iScan Reader. In some embodiments, the images are processed with the GenomeStudio software methylation module. In some embodiments, the methylation data is analyzed using the Bioconductor lumi software package. See Du et al., Bioinformatics, 24(13): 1547-1548 (2008).

[0087] In some embodiments, DNA methylation sites are identified using bisulfite sequencing PCR (BSP) to determine sensitivity to EGFR inhibitors. Briefly, genomic DNA is isolated from a cell or tissue sample (e.g., a tumor sample or a blood sample) and is converted in a sodium bisulfite reaction (the bisulfite process converts unmethylated cytosine residues to uracil) using standard assays in the art. The bisulfite converted DNA product is amplified using primers designed to be specific to the bisulfite converted DNA (e.g., bisulfite-specific primers) and ligated into vectors for transformation into a host cell using standard assays in the art. After selection of the host cells containing the PCR amplified bisulfite converted DNA product of interest, the DNA product is isolated and sequenced to determine the sites of methylation using standard assays in the art. In some embodiments, the tissue sample is formalin-fixed paraffin embedded (FFPE) tissue. In some embodiments, the tissue sample is an FFPE tissue that has been processed for IHC analysis; for example, for gene expression. In some embodiments, the tissue sample is an FFPE tissue that showed little or no gene expression by IHC. In some embodiments, the tissue sample is fresh frozen tissue. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion using the Invitrogen Superscript III One-Step RT-PCR System with Platinum Taq. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion using a Taqman based assay. In some embodiments, the sodium bisulfite reaction is conducted using the Zymo EZ DNA Methylation-Gold Kit. In some embodiments, the primers designed to be specific to the bisulfite converted DNA are designed using Applied Biosystems Methyl Primer Express software. In some embodiments, the bisulfite converted DNA product is PCR amplified using the Invitrogen Superscript III One-Step RT-PCR System with Platinum Taq. In further embodiments, the PCR amplified bisulfite converted DNA product is ligated into a vector using the Invitrogen TOPO TA Cloning kit. In some embodiments, the host cell is bacteria. In some embodiments, the isolated PCR amplified bisulfite converted DNA product of interest is sequenced using Applied Biosystems 3730×1 DNA Analyzer. In some embodiments,

the primers designed to be specific to the bisulfite converted DNA are designed using Qiagen PyroMark Assay Design software. In some embodiments, the bisulfite converted DNA product is PCR amplified using the Invitrogen Superscript III One-Step RT-PCR System with Platinum Taq. In further embodiments, the PCR amplified bisulfite converted DNA product is sequenced using Qiagen Pyromark Q24 and analyzed Qiagen with PyroMark software.

[0088] In some embodiments, DNA methylation sites are identified using quantitative methylation specific PCR (QMSP) to determine sensitivity to EGFR inhibitors. Briefly, genomic DNA is isolated from a cell or tissue sample and is converted in a sodium bisulfite reaction (the bisulfite process converts unmethylated cytosine residues to uracil) using standard assays in the art. In some embodiments, the tissue sample is formalin-fixed paraffin embedded (FFPE) tissue. In some embodiments, the tissue sample is an FFPE tissue that has been processed for IHC analysis. In some embodiments, the tissue sample is an FFPE tissue that showed little or no gene expression by IHC. In some embodiments, the tissue sample is fresh frozen tissue. The bisulfite converted DNA product is amplified using primers designed to be specific to the bisulfite converted DNA (e.g., quantitative methylation specific PCR primers). The bisulfite converted DNA product is amplified with quantitative methylation specific PCR primers and analyzed for methylation using standard assays in the art. In some embodiments, the tissue sample is formalin-fixed paraffin embedded (FFPE) tissue. In some embodiments, the tissue sample is fresh frozen tissue. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion using the Invitrogen Superscript III One-Step RT-PCR System with Platinum Taq. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion. In some embodiments, the DNA isolated from the tissue sample is preamplified before bisulfite conversion using a Taqman based assay. In some embodiments, the sodium bisulfite reaction is conducted using a commercially available kit. In some embodiments, the sodium bisulfite reaction is conducted using the Zymo EZ DNA Methylation-Gold Kit. In some embodiments, the primers designed to be specific to the bisulfite converted DNA are designed using Applied Biosystems Methyl Primer Express software. In some embodiments, the bisulfite converted DNA is amplified using a Taqman based assay. In some embodiments, the bisulfite converted DNA is amplified on an Applied Biosystems 7900HT and analyzed using Applied Biosystems SDS software.

[0089] In some embodiments, the invention provides methods to determine methylation by 1) IHC analysis of tumor samples, followed by 2) quantitative methylation specific PCR of DNA extracted from the tumor tissue used in the IHC ananlysis of step 1. Briefly, coverslips from IHC slides are removed by one of two methods: the slide are placed in a freezer for at least 15 minutes, then the coverslip is pried off of the microscope slide using a razor blade. Slides are then incubated in xylene at room temp to dissolve the mounting media. Alternatively, slides are soaked in xylene until the coverslip falls off. This can take up to several days. All slides are taken through a deparaffinization procedure of 5 min xylene (×3), and 5 min 100% ethanol (×2). Tissues are scraped off slides with razor blades and placed in a tissue lysis buffer containing proteinase K and incubated overnight at 56° C. In cases where tissue is still present after incubation, an extra 10 μL1 Proteinase K may be added and the tissue is incubated for another 30 min. DNA extraction was continued; for example, by using a QIAamp DNA FFPE Tissue kit. DNA extracted directly from IHC slides was subject to QMSP analysis using the QMSP3 primers and probes as described above.

[0090] In some embodiments, the bisulfite-converted DNA is sequenced by a deep sequencing. Deep sequencing is a process, such as direct pyrosequencing, where a sequence is read multiple times. Deep sequencing can be used to detect rare events such as rare mutations. Ultra-deep sequencing of a limited number of loci may been achieved by direct pyrosequencing of PCR products and by sequencing of more than 100 PCR products in a single run. A challenge in sequencing bisulphite-converted DNA arises from its low sequence complexity following bisulfite conversion of cytosine residues to thymine (uracil) residues. Reduced representation bisulphite sequencing (RRBS) may be introduced to reduce sequence redundancy by selecting only some regions of the genome for sequencing by size-fractionation of DNA fragments (Laird, P W Nature Reviews 11:195-203 (2010)). Targeting may be accomplished by array capture or padlock capture before sequencing. For example, targeted capture on fixed arrays or by solution hybrid selection can enrich for sequences targeted by a library of DNA or RNA oligonucleotides and can be performed before or after bisulphite conversion. Alternatively, padlock capture provides improved enrichment efficiency by combining the increased annealing specificity of two tethered probes, and subsequent amplification with universal primers allows for a more uniform representation than amplification with locus-specific primers.

[0091] Additional methylation detection methods include, but are not limited to, methylated CpG island amplification (see Toyota et al., Cancer Res. 59:2307-12 (1999)) and those described in, e.g., U.S. Patent Publication 2005/0069879; Rein et al., Nucleic Acids Res. 26 (10): 2255-64 (1998); Olek et al., Nat. Genet. 17(3): 275-6 (1997); Laird, P W Nature Reviews 11:195-203 (2010); and PCT Publication No. WO 00/70090).

[0092] The level of DNA methylation may be represented by a methylation index as a ratio of the methylated DNA copy number (cycle time) to the cycle time of a reference gene, which amplifies equally both methylated and unmethylated targets. A high level of DNA methylation may be the determined by comparison of the level of DNA methylation in a sample of non-neoplastic cells, particularly of the same tissue type of from peripheral blood mononuclear cells. In a particular embodiment, a high level of DNA methylation of the particular gene is detectable at a higher level compared to that in a normal cell. In another particular embodiment, a high level of DNA methylation is about 2× or greater compared to that in a normal cell. In a particular embodiment, a high level of DNA methylation is about 3× or greater compared to that in a normal cell. In a particular embodiment, a high level of DNA methylation is about 4× or greater compared to that in a normal cell. In a particular embodiment, a high level of DNA methylation is about 5× or greater compared to that in a normal cell. In a particular embodiment, a high level of DNA methylation is about 6× or greater compared to that in a normal cell. In a particular embodiment, a high level of DNA methylation is about 7× or greater compared to that in a normal cell. In a particular embodiment, a high level of DNA methylation is about 8× or greater compared to that in a normal cell. In a particular embodiment, a high level of DNA methylation is about 9× or greater compared to that in a

normal cell. In a particular embodiment, a high level of DNA methylation is about 10× or greater compared to that in a normal cell.

[0093] By "hypomethylation" is meant that a majority of the possibly methylated CpG sites are unmethylated. In certain embodiments, hypomethylation means that less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, or less than 1% of the possible methylation sites in a part of the gene is methylated. In yet another embodiment, hypomethylation means that fewer possible methylation sites are methylated compared to a gene that is expressed at a normal level, for example, in a non-tumor cell. In another embodiment, hypomethylation means that none of the CpG sites are methylated.

[0094] By "hypermethylation" is meant that a majority of the possibly methylated CpG sites are methylated. In certain embodiments, hypermethylation means that more than 50%, more than 55%, more than 60%, more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 95%, or more than 99% of the possible methylation sites in a part of the gene is methylated. In yet another embodiment, hypermethylation means that more of the possible methylation sites are methylated compared to a gene that is expressed at a normal level, for example, in a non-tumor cell. In another embodiment, hypermethylation means that all of the CpG sites are methylated.

[0095] In some embodiments, the expression of a biomarker in a cell is determined by evaluating mRNA in a cell. Methods for the evaluation of mRNAs in cells are well known and include, for example, hybridization assays using complementary DNA probes (such as in situ hybridization using labeled riboprobes specific for the one or more genes, Northern blot and related techniques) and various nucleic acid amplification assays (such as RT-PCR using complementary primers specific for one or more of the genes, and other amplification type detection methods, such as, for example, branched DNA, SISBA, TMA and the like). In some embodiments, the expression of a biomarker in a test sample is compared to a reference sample. For example, the test sample may be a tumor tissue sample and the reference sample may be from normal tissue or cells such as PBMCs.

[0096] Samples from mammals can be conveniently assayed for mRNAs using Northern, dot blot or PCR analysis. In addition, such methods can include one or more steps that allow one to determine the levels of target mRNA in a biological sample (e.g., by simultaneously examining the levels a comparative control mRNA sequence of a "housekeeping" gene such as an actin family member). Optionally, the sequence of the amplified target cDNA can be determined

[0097] Optional methods of the invention include protocols which examine or detect mRNAs, such as target mRNAs, in a tissue or cell sample by microarray technologies. Using nucleic acid microarrays, test and control mRNA samples from test and control tissue samples are reverse transcribed and labeled to generate cDNA probes. The probes are then hybridized to an array of nucleic acids immobilized on a solid support. The array is configured such that the sequence and position of each member of the array is known. For example, a selection of genes whose expression correlates with increased or reduced clinical benefit of anti-angiogenic therapy may be arrayed on a solid support. Hybridization of a labeled probe with a particular array member indicates that the sample from which the probe was derived expresses that gene.

[0098] According to some embodiments, presence and/or level/amount is measured by observing protein expression levels of an aforementioned gene. In certain embodiments, the method comprises contacting the biological sample with antibodies to a biomarker described herein under conditions permissive for binding of the biomarker, and detecting whether a complex is formed between the antibodies and biomarker. Such method may be an in vitro or in vivo method.

[0099] In certain embodiments, the presence and/or level/amount of biomarker proteins in a sample are examined using IHC and staining protocols. IHC staining of tissue sections has been shown to be a reliable method of determining or detecting presence of proteins in a sample. In one aspect, level of biomarker is determined using a method comprising: (a) performing IHC analysis of a sample (such as a subject cancer sample) with an antibody; and b) determining level of a biomarker in the sample. In some embodiments, IHC staining intensity is determined relative to a reference value.

[0100] IHC may be performed in combination with additional techniques such as morphological staining and/or fluorescence in-situ hybridization. Two general methods of IHC are available; direct and indirect assays. According to the first assay, binding of antibody to the target antigen is determined directly. This direct assay uses a labeled reagent, such as a fluorescent tag or an enzyme-labeled primary antibody, which can be visualized without further antibody interaction. In a typical indirect assay, unconjugated primary antibody binds to the antigen and then a labeled secondary antibody binds to the primary antibody. Where the secondary antibody is conjugated to an enzymatic label, a chromogenic or fluorogenic substrate is added to provide visualization of the antigen. Signal amplification occurs because several secondary antibodies may react with different epitopes on the primary antibody.

[0101] The primary and/or secondary antibody used for IHC typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories: (a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I; (b) colloidal gold particles; (c) fluorescent labels including, but are not limited to, rare earth chelates (europium chelates), Texas Red, rhodamine, fluorescein, dansyl, Lissamine, umbelliferone, phycocrytherin, phycocyanin, or commercially available fluorophores such SPECTRUM ORANGE7 and SPECTRUM GREEN7 and/or derivatives of any one or more of the above; (d) various enzyme-substrate labels are available and U.S. Pat. No. 4,275,149 provides a review of some of these. Examples of enzymatic labels include luciferases (e.g., firefly luciferase and bacterial luciferase; U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like.

[0102] Examples of enzyme-substrate combinations include, for example, horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate; alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and β-D-galactosidase (β-D-Gal) with a chromogenic

substrate (e.g., p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate (e.g., 4-methylumbelliferyl-β-D-galactosidase). For a general review of these, see U.S. Pat. Nos. 4,275,149 and 4,318,980.

[0103] Specimens thus prepared may be mounted and coverslipped. Slide evaluation is then determined, e.g., using a microscope, and staining intensity criteria, routinely used in the art, may be employed. In some embodiments, a staining pattern score of about 1+ or higher is diagnostic and/or prognostic. In certain embodiments, a staining pattern score of about 2+ or higher in an IHC assay is diagnostic and/or prognostic. In other embodiments, a staining pattern score of about 3 or higher is diagnostic and/or prognostic. In one embodiment, it is understood that when cells and/or tissue from a tumor or colon adenoma are examined using IHC, staining is generally determined or assessed in tumor cell and/or tissue (as opposed to stromal or surrounding tissue that may be present in the sample).

[0104] In alternative methods, the sample may be contacted with an antibody specific for the biomarker under conditions sufficient for an antibody-biomarker complex to form, and then detecting the complex. The presence of the biomarker may be detected in a number of ways, such as by Western blotting and ELISA procedures for assaying a wide variety of tissues and samples, including plasma or serum. A wide range of immunoassay techniques using such an assay format are available, see, e.g., U.S. Pat. Nos. 4,016,043, 4,424,279 and 4,018,653. These include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labeled antibody to a target biomarker.

[0105] Presence and/or level/amount of a selected biomarker in a tissue or cell sample may also be examined by way of functional or activity-based assays. For instance, if the biomarker is an enzyme, one may conduct assays known in the art to determine or detect the presence of the given enzymatic activity in the tissue or cell sample.

[0106] In certain embodiments, the samples are normalized for both differences in the amount of the biomarker assayed and variability in the quality of the samples used, and variability between assay runs. Such normalization may be accomplished by detecting and incorporating the level of certain normalizing biomarkers, including well known housekeeping genes, such as ACTB. Alternatively, normalization can be based on the mean or median signal of all of the assayed genes or a large subset thereof (global normalization approach). On a gene-by-gene basis, measured normalized amount of a subject tumor mRNA or protein is compared to the amount found in a reference set. Normalized expression levels for each mRNA or protein per tested tumor per subject can be expressed as a percentage of the expression level measured in the reference set. The presence and/or expression level/amount measured in a particular subject sample to be analyzed will fall at some percentile within this range, which can be determined by methods well known in the art.

[0107] In certain embodiments, relative expression level of a gene is determined as follows:

Relative expression gene1 sample1=2 exp (*Ct* housekeeping gene−*Ct* gene1) with Ct determined in a sample.

Relative expression gene1 reference RNA=2 exp (*Ct* housekeeping gene−*Ct* gene1) with Ct determined in the reference sample.

Normalized relative expression gene1 sample1=(relative expression gene1 sample1/relative expression gene1 reference RNA)×100

[0108] Ct is the threshold cycle. The Ct is the cycle number at which the fluorescence generated within a reaction crosses the threshold line.

[0109] All experiments are normalized to a reference RNA, which is a comprehensive mix of RNA from various tissue sources (e.g., reference RNA #636538 from Clontech, Mountain View, Calif.). Identical reference RNA is included in each qRT-PCR run, allowing comparison of results between different experimental runs.

[0110] In one embodiment, the sample is a clinical sample. In another embodiment, the sample is used in a diagnostic assay. In some embodiments, the sample is obtained from a primary or metastatic tumor. Tissue biopsy is often used to obtain a representative piece of tumor tissue. Alternatively, tumor cells can be obtained indirectly in the form of tissues or fluids that are known or thought to contain the tumor cells of interest. For instance, samples of lung cancer lesions may be obtained by resection, bronchoscopy, fine needle aspiration, bronchial brushings, or from sputum, pleural fluid or blood. In some embodiments, the sample includes circulating tumor cells; for example, circulating cancer cells in blood, urine or sputum. Genes or gene products can be detected from cancer or tumor tissue or from other body samples such as urine, sputum, serum or plasma. The same techniques discussed above for detection of target genes or gene products in cancerous samples can be applied to other body samples. Cancer cells may be sloughed off from cancer lesions and appear in such body samples. By screening such body samples, a simple early diagnosis can be achieved for these cancers. In addition, the progress of therapy can be monitored more easily by testing such body samples for target genes or gene products.

[0111] In certain embodiments, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is a single sample or combined multiple samples from the same subject or individual that are obtained at one or more different time points than when the test sample is obtained. For example, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is obtained at an earlier time point from the same subject or individual than when the test sample is obtained. Such reference sample, reference cell, reference tissue, control sample, control cell, or control tissue may be useful if the reference sample is obtained during initial diagnosis of cancer and the test sample is later obtained when the cancer becomes metastatic.

[0112] In certain embodiments, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is a combined multiple samples from one or more healthy individuals who are not the subject or individual. In certain embodiments, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is a combined multiple samples from one or more individuals with a disease or disorder (e.g., cancer) who are not the subject or individual. In certain embodiments, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is pooled RNA samples from normal tissues or pooled plasma or serum samples from one or more individuals who are not the subject or individual. In certain embodiments, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue

is pooled RNA samples from tumor tissues or pooled plasma or serum samples from one or more individuals with a disease or disorder (e.g., cancer) who are not the subject or individual.

[0113] In the methods of this invention, the tissue samples may be bodily fluids or excretions such as blood, urine, saliva, stool, pleural fluid, lymphatic fluid, sputum, ascites, prostatic fluid, cerebrospinal fluid (CSF), or any other bodily secretion or derivative thereof. By blood it is meant to include whole blood, plasma, serum or any derivative of blood. Assessment of tumor epithelial or mesenchymal biomarkers in such bodily fluids or excretions can sometimes be preferred in circumstances where an invasive sampling method is inappropriate or inconvenient.

[0114] In the methods of this invention, the tumor cell can be a lung cancer tumor cell (e.g. non-small cell lung cancer (NSCLC)), a pancreatic cancer tumor cell, a breast cancer tumor cell, a head and neck cancer tumor cell, a gastric cancer tumor cell, a colon cancer tumor cell, an ovarian cancer tumor cell, or a tumor cell from any of a variety of other cancers as described herein below. The tumor cell is preferably of a type known to or expected to express EGFR, as do all tumor cells from solid tumors. The EGFR kinase can be wild type or a mutant form.

[0115] In the methods of this invention, the tumor can be a lung cancer tumor (e.g. non-small cell lung cancer (NSCLC)), a pancreatic cancer tumor, a breast cancer tumor, a head and neck cancer tumor, a gastric cancer tumor, a colon cancer tumor, an ovarian cancer tumor, or a tumor from any of a variety of other cancers as described herein below. The tumor is preferably of a type whose cells are known to or expected to express EGFR, as do all solid tumors. The EGFR can be wild type or a mutant form.

Inhibitors and Pharmaceutical Compositions

[0116] Exemplary EGFR kinase inhibitors suitable for use in the invention include, for example quinazoline EGFR kinase inhibitors, pyrido-pyrimidine EGFR kinase inhibitors, pyrimido-pyrimidine EGFR kinase inhibitors, pyrrolo-pyrimidine EGFR kinase inhibitors, pyrazolo-pyrimidine EGFR kinase inhibitors, phenylamino-pyrimidine EGFR kinase inhibitors, oxindole EGFR kinase inhibitors, indolocarbazole EGFR kinase inhibitors, phthalazine EGFR kinase inhibitors, isoflavone EGFR kinase inhibitors, quinalone EGFR kinase inhibitors, and tyrphostin EGFR kinase inhibitors, such as those described in the following patent publications, and all pharmaceutically acceptable salts and solvates of the EGFR kinase inhibitors: International Patent Publication Nos. WO 96/33980, WO 96/30347, WO 97/30034, WO 97/30044, WO 97/38994, WO 97/49688, WO 98/02434, WO 97/38983, WO 95/19774, WO 95/19970, WO 97/13771, WO 98/02437, WO 98/02438, WO 97/32881, WO 98/33798, WO 97/32880, WO 97/3288, WO 97/02266, WO 97/27199, WO 98/07726, WO 97/34895, WO 96/31510, WO 98/14449, WO 98/14450, WO 98/14451, WO 95/09847, WO 97/19065, WO 98/17662, WO 99/35146, WO 99/35132, WO 99/07701, and WO 92/20642; European Patent Application Nos. EP 520722, EP 566226, EP 787772, EP 837063, and EP 682027; U.S. Pat. Nos. 5,747,498, 5,789,427, 5,650,415, and 5,656,643; and German Patent Application No. DE 19629652. Additional non-limiting examples of low molecular weight EGFR kinase inhibitors include any of the EGFR kinase inhibitors described in Traxler, P., 1998, Exp. Opin. Ther. Patents 8(12):1599-1625.

[0117] Specific preferred examples of low molecular weight EGFR kinase inhibitors that can be used according to the present invention include [6,7-bis(2-methoxyethoxy)-4-quinazolin-4-yl]-(3-ethynylphenyl) amine (also known as OSI-774, erlotinib, or TARCEVA™ (erlotinib HCl); OSI Pharmaceuticals/Genentech/Roche) (U.S. Pat. No. 5,747,498; International Patent Publication No. WO 01/34574, and Moyer, J. D. et al. (1997) Cancer Res. 57:4838-4848); CI-1033 (formerly known as PD183805; Pfizer) (Sherwood et al., 1999, Proc. Am. Assoc. Cancer Res. 40:723); PD-158780 (Pfizer); AG-1478 (University of California); CGP-59326 (Novartis); PKI-166 (Novartis); EKB-569 (Wyeth); GW-2016 (also known as GW-572016 or lapatinib ditosylate; GSK); and gefitinib (also known as ZD1839 or IRESSA™; Astrazeneca) (Woodburn et al., 1997, Proc. Am. Assoc. Cancer Res. 38:633). A particularly preferred low molecular weight EGFR kinase inhibitor that can be used according to the present invention is [6,7-bis(2-methoxyethoxy)-4-quinazolin-4-yl]-(3-ethynylphenyl) amine (i.e. erlotinib), its hydrochloride salt (i.e. erlotinib HCl, TARCEVA™), or other salt forms (e.g. erlotinib mesylate).

[0118] Antibody-based EGFR kinase inhibitors include any anti-EGFR antibody or antibody fragment that can partially or completely block EGFR activation by its natural ligand. Non-limiting examples of antibody-based EGFR kinase inhibitors include those described in Modjtahedi, H., et al., 1993, Br. J. Cancer 67:247-253; Teramoto, T., et al., 1996, Cancer 77:639-645; Goldstein et al., 1995, Clin. Cancer Res. 1:1311-1318; Huang, S. M., et al., 1999, Cancer Res. 15:59(8):1935-40; and Yang, X., et al., 1999, Cancer Res. 59:1236-1243. Thus, the EGFR kinase inhibitor can be the monoclonal antibody Mab E7.6.3 (Yang, X. D. et al. (1999) Cancer Res. 59:1236-43), or Mab C225 (ATCC Accession No. HB-8508), or an antibody or antibody fragment having the binding specificity thereof. Suitable monoclonal antibody EGFR kinase inhibitors include, but are not limited to, IMC-C225 (also known as cetuximab or ERBITUX™; Imclone Systems), ABX-EGF (Abgenix), EMD 72000 (Merck KgaA, Darmstadt), RH3 (York Medical Bioscience Inc.), and MDX-447 (Medarex/ Merck KgaA).

[0119] The methods of this invention can be extended to those compounds which inhibit EGFR and an additional target. These compounds are referred to herein as "bispecific inhibitors". In one embodiment, the bispecific inhibitor is a bispecific HER3/EGFR, EGFR/HER2, EGFR/HER4 or EGFR c-Met, inhibitor. In one embodiment, the bispecific inhibitor is a bispecific antibody. In one embodiment, the bispecific inhibitor is a bispecific antibody which comprises an antigen binding domain that specifically binds to EGFR and a second target. In one embodiment, the bispecific inhibitor is a bispecific antibody which comprises an antigen binding domain that specifically binds to HER3 and EGFR. In one embodiment, the bispecific HER3/EGFR inhibitor is a bispecific antibody which comprises two identical antigen binding domains. Such antibodies are described in U.S. Pat. No. 8,193,321, 20080069820, WO2010108127, US20100255010 and Schaefer et al, Cancer Cell, 20: 472-486 (2011). In one embodiment, the bispecific HER2/EGFR is lapatinib/GW572016.

[0120] Additional antibody-based inhibitors can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production.

[0121] Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and Milstein (Nature, 1975, 256: 495-497); the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci. USA 80: 2026-2030); and the EBV-hybridoma technique (Cole et al, 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

[0122] Alternatively, techniques described for the production of single chain antibodies (see, e.g., U.S. Pat. No. 4,946,778) can be adapted to produce single chain antibodies with desired specificity. Antibody-based inhibitors useful in practicing the present invention also include antibody fragments including but not limited to F(ab').sub.2 fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab').sub.2 fragments. Alternatively, Fab and/or scFv expression libraries can be constructed (see, e.g., Huse et al., 1989, Science 246: 1275-1281) to allow rapid identification of fragments having the desired specificity.

[0123] Techniques for the production and isolation of monoclonal antibodies and antibody fragments are well-known in the art, and are described in Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, and in J. W. Goding, 1986, Monoclonal Antibodies: Principles and Practice, Academic Press, London. Humanized anti-EGFR antibodies and antibody fragments can also be prepared according to known techniques such as those described in Vaughn, T. J. et al., 1998, Nature Biotech. 16:535-539 and references cited therein, and such antibodies or fragments thereof are also useful in practicing the present invention.

[0124] Inhibitors for use in the present invention can alternatively be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of target mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of the target protein, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding EGFR or HER2 can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

[0125] Small inhibitory RNAs (siRNAs) can also function as inhibitors for use in the present invention. Target gene expression can be reduced by contacting the tumor, subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that expression of the target gene is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschi, T., et al. (1999) Genes Dev. 13(24): 3191-3197; Elbashir, S. M. et al. (2001) Nature 411:494-498; Hannon, G. J. (2002) Nature 418:244-251; McManus, M. T. and Sharp, P. A. (2002) Nature Reviews Genetics 3:737-747; Bremmelkamp, T. R. et al. (2002) Science 296:550-553; U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

[0126] Ribozymes can also function as inhibitors for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

[0127] Both antisense oligonucleotides and ribozymes useful as inhibitors can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

[0128] In the context of the methods of treatment of this invention, inhibitors (such as an EGFR inhibitor) are used as a composition comprised of a pharmaceutically acceptable carrier and a non-toxic therapeutically effective amount of an EGFR kinase inhibitor compound (including pharmaceutically acceptable salts thereof).

[0129] The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic bases or acids. When a compound of the present invention is acidic, its corresponding salt can be conveniently prepared from pharmaceutically acceptable non-toxic bases, including inorganic bases and organic bases. Salts derived from such inorganic bases include aluminum, ammonium, calcium, copper (cupric and cuprous), ferric, ferrous, lithium, magnesium, manganese (manganic and manganous), potassium, sodium, zinc and the like salts. Particularly preferred are the ammonium, calcium, magnesium, potassium and sodium

salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, as well as cyclic amines and substituted amines such as naturally occurring and synthesized substituted amines. Other pharmaceutically acceptable organic non-toxic bases from which salts can be formed include ion exchange resins such as, for example, arginine, betaine, caffeine, choline, N',N'-dibenzylethylenediamine, diethylamine, 2-diethylaminoethanol, 2-dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethylmorpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydrabamine, isopropylamine, lysine, methylglucamine, morpholine, piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylameine, trimethylamine, tripropylamine, tromethamine and the like.

[0130] When a compound used in the present invention is basic, its corresponding salt can be conveniently prepared from pharmaceutically acceptable non-toxic acids, including inorganic and organic acids. Such acids include, for example, acetic, benzenesulfonic, benzoic, camphorsulfonic, citric, ethanesulfonic, fumaric, gluconic, glutamic, hydrobromic, hydrochloric, isethionic, lactic, maleic, malic, mandelic, methanesulfonic, mucic, nitric, pamoic, pantothenic, phosphoric, succinic, sulfuric, tartaric, p-toluenesulfonic acid and the like. Particularly preferred are citric, hydrobromic, hydrochloric, maleic, phosphoric, sulfuric and tartaric acids.

[0131] Pharmaceutical compositions used in the present invention comprising an inhibitor compound (including pharmaceutically acceptable salts thereof) as active ingredient, can include a pharmaceutically acceptable carrier and optionally other therapeutic ingredients or adjuvants. Other therapeutic agents may include those cytotoxic, chemotherapeutic or anti-cancer agents, or agents which enhance the effects of such agents, as listed above. The compositions include compositions suitable for oral, rectal, topical, and parenteral (including subcutaneous, intramuscular, and intravenous) administration, although the most suitable route in any given case will depend on the particular host, and nature and severity of the conditions for which the active ingredient is being administered. The pharmaceutical compositions may be conveniently presented in unit dosage form and prepared by any of the methods well known in the art of pharmacy

[0132] In practice, the inhibitor compounds (including pharmaceutically acceptable salts thereof) of this invention can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g. oral or parenteral (including intravenous). Thus, the pharmaceutical compositions of the present invention can be presented as discrete units suitable for oral administration such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient. Further, the compositions can be presented as a powder, as granules, as a solution, as a suspension in an aqueous liquid, as a non-aqueous liquid, as an oil-in-water emulsion, or as a water-in-oil liquid emulsion. In addition to the common dosage forms set out above, an inhibitor compound (including pharmaceutically acceptable salts of each component thereof) may also be administered by controlled release means and/or delivery devices. The combination compositions may be prepared by any of the methods of pharmacy. In general, such methods include a step of bringing into association the active ingredients with the carrier that constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the active ingredient with liquid carriers or finely divided solid carriers or both. The product can then be conveniently shaped into the desired presentation.

[0133] An inhibitor compound (including pharmaceutically acceptable salts thereof) used in this invention, can also be included in pharmaceutical compositions in combination with one or more other therapeutically active compounds. Other therapeutically active compounds may include those cytotoxic, chemotherapeutic or anti-cancer agents, or agents which enhance the effects of such agents, as listed above.

[0134] Thus in one embodiment of this invention, the pharmaceutical composition can comprise an inhibitor compound in combination with an anticancer agent, wherein the anticancer agent is a member selected from the group consisting of alkylating drugs, antimetabolites, microtubule inhibitors, podophyllotoxins, antibiotics, nitrosoureas, hormone therapies, kinase inhibitors, activators of tumor cell apoptosis, and antiangiogenic agents.

[0135] The pharmaceutical carrier employed can be, for example, a solid, liquid, or gas. Examples of solid carriers include lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, and stearic acid. Examples of liquid carriers are sugar syrup, peanut oil, olive oil, and water. Examples of gaseous carriers include carbon dioxide and nitrogen.

[0136] In preparing the compositions for oral dosage form, any convenient pharmaceutical media may be employed. For example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, and the like may be used to form oral liquid preparations such as suspensions, elixirs and solutions; while carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like may be used to form oral solid preparations such as powders, capsules and tablets. Because of their ease of administration, tablets and capsules are the preferred oral dosage units whereby solid pharmaceutical carriers are employed. Optionally, tablets may be coated by standard aqueous or nonaqueous techniques.

[0137] A tablet containing the composition used for this invention may be prepared by compression or molding, optionally with one or more accessory ingredients or adjuvants. Compressed tablets may be prepared by compressing, in a suitable machine, the active ingredient in a free-flowing form such as powder or granules, optionally mixed with a binder, lubricant, inert diluent, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine, a mixture of the powdered compound moistened with an inert liquid diluent. Each tablet preferably contains from about 0.05 mg to about 5 g of the active ingredient and each cachet or capsule preferably contains from about 0.05 mg to about 5 g of the active ingredient.

[0138] For example, a formulation intended for the oral administration to humans may contain from about 0.5 mg to about 5 g of active agent, compounded with an appropriate and convenient amount of carrier material that may vary from about 5 to about 95 percent of the total composition. Unit dosage forms will generally contain between from about 1 mg to about 2 g of the active ingredient, typically 25 mg, 50 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg, 600 mg, 800 mg, or 1000 mg.

[0139] Pharmaceutical compositions used in the present invention suitable for parenteral administration may be pre-

pared as solutions or suspensions of the active compounds in water. A suitable surfactant can be included such as, for example, hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Further, a preservative can be included to prevent the detrimental growth of microorganisms.

[0140] Pharmaceutical compositions used in the present invention suitable for injectable use include sterile aqueous solutions or dispersions. Furthermore, the compositions can be in the form of sterile powders for the extemporaneous preparation of such sterile injectable solutions or dispersions. In all cases, the final injectable form must be sterile and must be effectively fluid for easy syringability. The pharmaceutical compositions must be stable under the conditions of manufacture and storage; thus, preferably should be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol and liquid polyethylene glycol), vegetable oils, and suitable mixtures thereof.

[0141] Pharmaceutical compositions for the present invention can be in a form suitable for topical sue such as, for example, an aerosol, cream, ointment, lotion, dusting powder, or the like. Further, the compositions can be in a form suitable for use in transdermal devices. These formulations may be prepared, utilizing an inhibitor compound (including pharmaceutically acceptable salts thereof), via conventional processing methods. As an example, a cream or ointment is prepared by admixing hydrophilic material and water, together with about 5 wt % to about 10 wt % of the compound, to produce a cream or ointment having a desired consistency.

[0142] Pharmaceutical compositions for this invention can be in a form suitable for rectal administration wherein the carrier is a solid. It is preferable that the mixture forms unit dose suppositories. Suitable carriers include cocoa butter and other materials commonly used in the art. The suppositories may be conveniently formed by first admixing the composition with the softened or melted carrier(s) followed by chilling and shaping in molds.

[0143] In addition to the aforementioned carrier ingredients, the pharmaceutical formulations described above may include, as appropriate, one or more additional carrier ingredients such as diluents, buffers, flavoring agents, binders, surface-active agents, thickeners, lubricants, preservatives (including anti-oxidants) and the like. Furthermore, other adjuvants can be included to render the formulation isotonic with the blood of the intended recipient. Compositions containing an inhibitor compound (including pharmaceutically acceptable salts thereof) may also be prepared in powder or liquid concentrate form.

[0144] Dosage levels for the compounds used for practicing this invention will be approximately as described herein, or as described in the art for these compounds. It is understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

[0145] Many alternative experimental methods known in the art may be successfully substituted for those specifically described herein in the practice of this invention, as for example described in many of the excellent manuals and textbooks available in the areas of technology relevant to this invention (e.g. Using Antibodies, A Laboratory Manual, edited by Harlow, E. and Lane, D., 1999, Cold Spring Harbor Laboratory Press, (e.g. ISBN 0-87969-544-7); Roe B. A. et. al. 1996, DNA Isolation and Sequencing (Essential Techniques Series), John Wiley & Sons. (e.g. ISBN 0-471-97324-0); Methods in Enzymology: Chimeric Genes and Proteins", 2000, ed. J. Abelson, M. Simon, S. Emr, J. Thorner. Academic Press; Molecular Cloning: a Laboratory Manual, 2001, 3rd Edition, by Joseph Sambrook and Peter MacCallum, (the former Maniatis Cloning manual) (e.g. ISBN 0-87969-577-3); Current Protocols in Molecular Biology, Ed. Fred M. Ausubel, et. al. John Wiley & Sons (e.g. ISBN 0-471-50338-X); Current Protocols in Protein Science, Ed. John E. Coligan, John Wiley & Sons (e.g. ISBN 0-471-11184-8); and Methods in Enzymology: Guide to protein Purification, 1990, Vol. 182, Ed. Deutscher, M. P., Acedemic Press, Inc. (e.g. ISBN 0-12-213585-7)), or as described in the many university and commercial websites devoted to describing experimental methods in molecular biology.

[0146] It will be appreciated by one of skill in the medical arts that the exact manner of administering to the patient of a therapeutically effective amount of an inhibitor as described herein (for example an EGFR kinase inhibitor, bispecific EGFR kinase inhibitor, or HER2 inhibitor) following a diagnosis of a patient's likely responsiveness to the inhibitor will be at the discretion of the attending physician. The mode of administration, including dosage, combination with other anti-cancer agents, timing and frequency of administration, and the like, may be affected by the diagnosis of a patient's likely responsiveness to the inhibitor, as well as the patient's condition and history. Thus, even patients diagnosed with tumors predicted to be relatively insensitive to the type of inhibitor may still benefit from treatment with such inhibitor, particularly in combination with other anti-cancer agents, or agents that may alter a tumor's sensitivity to the inhibitor.

[0147] For purposes of the present invention, "co-administration of" and "co-administering" an inhibitor with an additional anti-cancer agent (both components referred to hereinafter as the "two active agents") refer to any administration of the two active agents, either separately or together, where the two active agents are administered as part of an appropriate dose regimen designed to obtain the benefit of the combination therapy. Thus, the two active agents can be administered either as part of the same pharmaceutical composition or in separate pharmaceutical compositions. The additional agent can be administered prior to, at the same time as, or subsequent to administration of the inhibitor, or in some combination thereof. Where the inhibitor is administered to the patient at repeated intervals, e.g., during a standard course of treatment, the additional agent can be administered prior to, at the same time as, or subsequent to, each administration of the inhibitor, or some combination thereof, or at different intervals in relation to the inhibitor treatment, or in a single dose prior to, at any time during, or subsequent to the course of treatment with the inhibitor.

[0148] The inhibitor will typically be administered to the patient in a dose regimen that provides for the most effective treatment of the cancer (from both efficacy and safety perspectives) for which the patient is being treated, as known in the art, and as disclosed, e.g. in International Patent Publication No. WO 01/34574. In conducting the treatment method of the present invention, the inhibitor can be administered in any effective manner known in the art, such as by oral, topical, intravenous, intra-peritoneal, intramuscular, intra-articular, subcutaneous, intranasal, intra-ocular, vaginal, rectal, or

intradermal routes, depending upon the type of cancer being treated, the type of inhibitor being used (for example, small molecule, antibody, RNAi, ribozyme or antisense construct), and the medical judgement of the prescribing physician as based, e.g., on the results of published clinical studies.

[0149] The amount of inhibitor administered and the timing of inhibitor administration will depend on the type (species, gender, age, weight, etc.) and condition of the patient being treated, the severity of the disease or condition being treated, and on the route of administration. For example, small molecule inhibitors can be administered to a patient in doses ranging from 0.001 to 100 mg/kg of body weight per day or per week in single or divided doses, or by continuous infusion (see for example, International Patent Publication No. WO 01/34574). In particular, erlotinib HCl can be administered to a patient in doses ranging from 5-200 mg per day, or 100-1600 mg per week, in single or divided doses, or by continuous infusion. A preferred dose is 150 mg/day. Antibody-based inhibitors, or antisense, RNAi or ribozyme constructs, can be administered to a patient in doses ranging from 0.1 to 100 mg/kg of body weight per day or per week in single or divided doses, or by continuous infusion. In some instances, dosage levels below the lower limit of the aforethe range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect, provided that such larger doses are first divided into several small doses for administration throughout the day.

[0150] The inhibitors and other additional agents can be administered either separately or together by the same or different routes, and in a wide variety of different dosage forms. For example, the inhibitor is preferably administered orally or parenterally. Where the inhibitor is erlotinib HCl (TARCEVA™), oral administration is preferable. Both the inhibitor and other additional agents can be administered in single or multiple doses.

[0151] The inhibitor can be administered with various pharmaceutically acceptable inert carriers in the form of tablets, capsules, lozenges, troches, hard candies, powders, sprays, creams, salves, suppositories, jellies, gels, pastes, lotions, ointments, elixirs, syrups, and the like. Administration of such dosage forms can be carried out in single or multiple doses. Carriers include solid diluents or fillers, sterile aqueous media and various non-toxic organic solvents, etc. Oral pharmaceutical compositions can be suitably sweetened and/or flavored.

[0152] The inhibitor can be combined together with various pharmaceutically acceptable inert carriers in the form of sprays, creams, salves, suppositories, jellies, gels, pastes, lotions, ointments, and the like. Administration of such dosage forms can be carried out in single or multiple doses. Carriers include solid diluents or fillers, sterile aqueous media, and various non-toxic organic solvents, etc.

[0153] All formulations comprising proteinaceous inhibitors should be selected so as to avoid denaturation and/or degradation and loss of biological activity of the inhibitor.

[0154] Methods of preparing pharmaceutical compositions comprising an inhibitor are known in the art, and are described, e.g. in International Patent Publication No. WO 01/34574. In view of the teaching of the present invention, methods of preparing pharmaceutical compositions comprising an inhibitor will be apparent from the above-cited publications and from other known references, such as Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 18th edition (1990).

[0155] For oral administration of inhibitors, tablets containing one or both of the active agents are combined with any of various excipients such as, for example, micro-crystalline cellulose, sodium citrate, calcium carbonate, dicalcium phosphate and glycine, along with various disintegrants such as starch (and preferably corn, potato or tapioca starch), alginic acid and certain complex silicates, together with granulation binders like polyvinyl pyrrolidone, sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc are often very useful for tableting purposes. Solid compositions of a similar type may also be employed as fillers in gelatin capsules; preferred materials in this connection also include lactose or milk sugar as well as high molecular weight polyethylene glycols. When aqueous suspensions and/or elixirs are desired for oral administration, the inhibitor may be combined with various sweetening or flavoring agents, coloring matter or dyes, and, if so desired, emulsifying and/or suspending agents as well, together with such diluents as water, ethanol, propylene glycol, glycerin and various like combinations thereof.

[0156] For parenteral administration of either or both of the active agents, solutions in either sesame or peanut oil or in aqueous propylene glycol may be employed, as well as sterile aqueous solutions comprising the active agent or a corresponding water-soluble salt thereof. Such sterile aqueous solutions are preferably suitably buffered, and are also preferably rendered isotonic, e.g., with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal injection purposes. The oily solutions are suitable for intra-articular, intramuscular and subcutaneous injection purposes. The preparation of all these solutions under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art. Any parenteral formulation selected for administration of proteinaceous inhibitors should be selected so as to avoid denaturation and loss of biological activity of the inhibitor.

[0157] Additionally, it is possible to topically administer either or both of the active agents, by way of, for example, creams, lotions, jellies, gels, pastes, ointments, salves and the like, in accordance with standard pharmaceutical practice. For example, a topical formulation comprising an inhibitor in about 0.1% (w/v) to about 5% (w/v) concentration can be prepared.

[0158] For veterinary purposes, the active agents can be administered separately or together to animals using any of the forms and by any of the routes described above. In a preferred embodiment, the inhibitor is administered in the form of a capsule, bolus, tablet, liquid drench, by injection or as an implant. As an alternative, the inhibitor can be administered with the animal feedstuff, and for this purpose a concentrated feed additive or premix may be prepared for a normal animal feed. Such formulations are prepared in a conventional manner in accordance with standard veterinary practice.

[0159] One of skill in the medical arts, particularly pertaining to the application of diagnostic tests and treatment with therapeutics, will recognize that biological systems may exhibit variability and may not always be entirely predictable, and thus many good diagnostic tests or therapeutics are occasionally ineffective. Thus, it is ultimately up to the judgement of the attending physician to determine the most appropriate course of treatment for an individual patient, based upon test results, patient condition and history, and his own experience.

There may even be occasions, for example, when a physician will choose to treat a patient with an EGFR inhibitor even when a tumor is not predicted to be particularly sensitive to EGFR kinase inhibitors, based on data from diagnostic tests or from other criteria, particularly if all or most of the other obvious treatment options have failed, or if some synergy is anticipated when given with another treatment. The fact that the EGFR inhibitors as a class of drugs are relatively well tolerated compared to many other anti-cancer drugs, such as more traditional chemotherapy or cytotoxic agents used in the treatment of cancer, makes this a more viable option.

Methods of Advertising

[0160] The invention herein also encompasses a method for advertising an EGFR, or a pharmaceutically acceptable composition thereof, comprising promoting, to a target audience, the use of the inhibitor or pharmaceutical composition thereof for treating a patient population with a type of cancer which is characterized by a methylation pattern indicative of a epithethial-like tumor, or promoting, to a target audience, the non-use of the inhibitor or pharmaceutical composition thereof for treating a patient population with a type of cancer which is characterized by a methylation pattern indicative of a mesenchymal-like tumor.

[0161] Advertising is generally paid communication through a non-personal medium in which the sponsor is identified and the message is controlled. Advertising for purposes herein includes publicity, public relations, product placement, sponsorship, underwriting, and sales promotion. This term also includes sponsored informational public notices appearing in any of the print communications media designed to appeal to a mass audience to persuade, inform, promote, motivate, or otherwise modify behavior toward a favorable pattern of purchasing, supporting, or approving the invention herein.

[0162] The advertising and promotion of the diagnostic method herein may be accomplished by any means. Examples of advertising media used to deliver these messages include television, radio, movies, magazines, newspapers, the internet, and billboards, including commercials, which are messages appearing in the broadcast media. Advertisements also include those on the seats of grocery carts, on the walls of an airport walkway, and on the sides of buses, or heard in telephone hold messages or in-store PA systems, or anywhere a visual or audible communication can be placed.

[0163] More specific examples of promotion or advertising means include television, radio, movies, the internet such as webcasts and webinars, interactive computer networks intended to reach simultaneous users, fixed or electronic billboards and other public signs, posters, traditional or electronic literature such as magazines and newspapers, other media outlets, presentations or individual contacts by, e.g., e-mail, phone, instant message, postal, courier, mass, or carrier mail, in-person visits, etc.

[0164] The type of advertising used will depend on many factors, for example, on the nature of the target audience to be reached, e.g., hospitals, insurance companies, clinics, doctors, nurses, and patients, as well as cost considerations and the relevant jurisdictional laws and regulations governing advertising of medicaments and diagnostics. The advertising may be individualized or customized based on user characterizations defined by service interaction and/or other data such as user demographics and geographical location.

TABLES

[0165]

TABLE 1

methylated cytosine nucleotides associated with mesenchymal phenotype

gene	chrom	position	gene	chrom	position
PON2	7	94888497	TBCD	17	78440559
	1	113544125	TBCD	17	78440498
BET1	7	93459766	TBCD	17	78440426
	X	48900705	MYST1, PRSS8	16	31050024
	X	48900845	ARHGEF38	4	106693255
	X	48900694		1	27023897
SCNN1A	12	6353969	LIMA1	12	48882614
SCNN1A	12	6354033		7	80389667
SCNN1A	12	6354000	KIAA0182	16	84236385
ELMO3	16	65791484		19	49971610
ELMO3	16	65791362		19	49971605
NRBP1, KRTCAP3	2	27519047	ITGB6	2	160822102
NRBP1, KRTCAP3	2	27519011	LOC643008,	17	71147845
KRTCAP3	2	27519215	RECQL5		
KRTCAP3	2	27519142	LOC643008,	17	71147779
NRBP1, KRTCAP3	2	27518810	RECQL5		
NRBP1, KRTCAP3	2	27518521	CCDC57	17	77655395
NRBP1, KRTCAP3	2	27518632		7	155407896
NRBP1, KRTCAP3	2	27518654		7	155407740
NRBP1, KRTCAP3	2	27518645		7	155407629
NRBP1, KRTCAP3	2	27518643		16	86850497
NRBP1, KRTCAP3	2	27518583		16	86850474
MST1R	3	49914707		16	29204205
SLC9A7	X	46499386		16	29204115
LYN	8	57066177		16	29204298
ACAP2	3	196640585		16	29204194
TBC1D14	4	7008013		7	2447019
PITPNM3	17	6396092		7	2447061
	10	11963508	TMEM79	1	154520773
	1	41738700	LOC254559	15	87723993

TABLE 1-continued

methylated cytosine nucleotides associated with mesenchymal phenotype

ARHGAP39	8	145777560	CCDC19	1	158137355
ARHGAP39	8	145777354	CCDC19	1	158137539
COX10	17	14050396		4	129368833
	7	27744012		1	24156244
COL18A1,	21	45757802		3	135552584
SLC19A1			CAMK2G	10	75302072
RAB25	1	154297806		2	74006703
CGN	1	149753111		2	74006825
TBCD	17	78440835		2	74006594
TBCD	17	78440951		2	74006721
TBCD	17	78440786	PPP1R13L	19	50595498
				3	49919159

gene	chromosome	position	gene	chromosome	position
VPS37C	11	60682632	FURIN	15	89213122
NA, RCC1	1	28726284	BRE	2	28261767
CTNND1	11	57305264		11	67106174
EPHB2	1	23025895		11	67106217
	6	134742250		11	67106166
FRMD6	14	51101498		11	67105913
GRHL2	8	102576558		11	67105885
P2RY6	11	72658514	SLC44A2	19	10596482
VTI1A	10	114516704	SLC44A2	19	10596548
S100A14	1	151855406	SLC44A2	19	10596578
S100A14	1	151855551	SLC44A2	19	10596594
PRSS8	16	31054183	RNF144A	2	7089748
THSD4	15	69416262		1	201755387
	2	189266325	QSOX1	1	178404541
SIPA1L1	14	71183551	CCDC85C	14	99114910
ARL13B, STX19	3	95230100	PLA2G4F	15	40236307
ARL13B, STX19	3	95230218	PLA2G4F	15	40236078
PVRL4	1	159326278	FTO	16	52372627
PVRL4	1	159326159		1	234153824
PVRL4	1	159326053	PPFIBP2	11	7578132
PVRL4	1	159326082	NINJ2	12	587066
ARHGAP32	11	128399061		2	30294748
ARHGAP32	11	128399150		4	189558130
	8	125219633		4	189558238
	15	76213522		1	206105036
PNKD	2	218868246	KRT8	12	51586560
CD44	11	35152089		4	185905387
ANKRD22	10	90600502	LIMK2	22	30001702
CEACAM19	19	49866511	BOLA2,	16	30023636
CEACAM19	19	49866752	GDPD3		
CEACAM19	19	49866521		3	129636934
	11	71134595		4	154136934
	11	71134808		9	131184926
SCYL3	1	168127429		19	1855554
CPA4	7	129749798		8	102519033
CLUAP1	16	3499552		1	100204254
CLUAP1	16	3499688	IMMP2L	7	110988180
CLUAP1	16	3499569		19	60699327
	8	28514725	PLEKHG6	12	6292029
ASAP2	2	9458210	PLEKHG6	12	6292067
			JMJD7-PLA2G4B	15	39918027

gene	chrom	position	gene	chrom	position
JMJD7-PLA2G4B	15	39917942		2	216504192
JMJD7-PLA2G4B	15	39917954	PLEKHF1	19	34854611
PNPLA8	7	107955947	PLEKHF1	19	34854406
PNPLA8	7	107955918		10	6202160
PNPLA8	7	107955957		10	6202194
HIVEP3	1	41753450		10	6202124
RAI1	17	17572988	SH3KBP1	X	19812050
DIXDC1	11	111337086		10	28996094
BOLA2, TBX6	16	30009181		11	73806817
SEMA3A	7	83655334		8	82806235
	2	27838190		11	354805
TNFAIP8	5	118637864		11	354809
SNX8	7	2267181		11	354623
JARID2	6	15564181		11	354752
AHRR	5	439883		10	31428642

TABLE 1-continued

methylated cytosine nucleotides associated with mesenchymal phenotype

CDH5	16	64970341		17	52465903
CDH5	16	64970599		12	15846291
	6	8381465	TESK2	1	45587219
SLC35B3	6	8381262		1	2456135
	6	8381295	TSEN54	17	71032445
DAGLA	11	61221452	TSEN54	17	71032354
	19	2105603	ACOT2	14	73109663
SVOPL	7	137999314	PDGFRA, LNX1	4	54153003
	17	8252319	PDGFRA, LNX1	4	54152866
	17	8252561	SLC40A1	2	190154739
	17	8252360	ATL1	14	50069808
	17	8252425	ZNF398	7	148472457
IGF1R	15	97074397		17	37862949
WDR82	3	52277292		17	37862906
WDR82	3	52277190		4	40328026
FBXO34	14	54834400		2	41940393
RAB11FIP1	8	37868570	AFF1	4	88113322
VPS37B	12	121944095	INPP5A	10	134254904
NAV2	11	19732081	INPP5A	10	134254935
C4orf36	4	88031692	MST1R	3	49913008
PLXNB2	22	49062415	PHGDH	1	120075342
PLXNB2	22	49062595	GLI2	2	121266304
C19orf46	19	41191166	GLI2	2	121266336
	2	70222288	GLI2	2	121266195
VTI1A	10	114492308	C2orf54	2	241484135
C2orf54	2	241484343	TBCD	17	78426815
	6	112413022	TBCD	17	78426927
	4	100956161	TBCD	17	78427378
CCNY	10	35716880	TBCD	17	78427517
MLPH	2	238063229		2	64687610
CDKAL1	6	21131464		16	52025113
GPR81	12	121777086	PPARD	6	35417845
	17	41697531		8	144892671
F11R	1	159258976		8	144892697
F11R	1	159258982		8	144892896
CDC42SE2	5	130692428		8	144892814
FTO	16	52472915	LRP5	11	67866681
	10	73752495	XAB2	19	7590468
MYO18A	17	24529971	RAP1GAP2	17	2815637
MYO18A	17	24529383	SLC37A1	21	42809566
DGAT1	8	145518703		13	109313445
SDCBP2	20	1258000		12	13179838
SDCBP2	20	1257800	OFCC1	6	10271555
SDCBP2	20	1257722	PTK7	6	43172438
TRAK1	3	42147101	TEAD3	6	35562412
SCNN1A	12	6354480	TEAD3	6	35562047
SCNN1A	12	6354974	TEAD3	6	35561916
SCNN1A	12	6354868	C16orf72	16	9097893
SCNN1A	12	6354990	ARID1A	1	26953185
SCNN1A	12	6354782	SGK223	8	8276184
ZCCHC14	16	86078911	GNA12	7	2739598
ZCCHC14	16	86078864	GNA12	7	2739653
GLIS1	1	53831204	GNA12	7	2739536
TSPAN1	1	46418811	PWWP2B	10	134072208
TSPAN1	1	46418555	PWWP2B	10	134072043
TSPAN1	1	46418745	SMARCD2	17	59270462
ST3GAL2	16	68973602	GPR56	16	56211203
ST3GAL2	16	68973365	GPR56	16	56211170
C10orf95	10	104201478	GPR56	16	56211418
C10orf95	10	104201378	GPR56	16	56211405
C10orf95	10	104201309	GPR110	6	47117696
C10orf95	10	104201286	GPR110	6	47118136
C10orf95	10	104201414	GPR110	6	47118050
C10orf95	10	104201318	EHF	11	34599461
TBCD	17	78426682		21	38521991
	14	64792338	CDS1	4	85724598
NSMCE2	8	126223268	GNAI3	1	109914827
PPCDC	15	73115889	NCOA2	8	71402682
WISP1	8	134293893		12	103938284
WISP1	8	134294072	CPEB3	10	93872825
WISP1	8	134293996	TACC2	10	123744125
	17	36932205		1	227296135
	8	144727414		6	7477665
CHD2	15	91266091		19	50356091
	1	8053252	LLGL2	17	71057739

TABLE 1-continued

methylated cytosine nucleotides associated with mesenchymal phenotype

DDR1	6	30959396	ANKFY1	17	4098222
DDR1	6	30958847	CLDN7	17	7106144
DDR1	6	30958892		1	59052878
DDR1	6	30958855		17	75403317
DDR1	6	30959065		17	75403479
DDR1	6	30959030		16	66828383
DDR1	6	30959048	ESRP2	16	66826542
DDR1	6	30958956	ESRP2	16	66826796
BAIAP2	17	76626137	OVOL1	11	65310618
BAIAP2	17	76625735		8	95720275
BAIAP2	17	76625947	FAM110A	20	770788
BAIAP2	17	76625872	SPINT1	15	38924311
MANF	3	51401417	GRHL2	8	102575162
PVRL4	1	159325891	SH3YL1	2	253559
PVRL4	1	159325951	SH3YL1	2	253656
RHOBTB3	5	95089583	TMEM159, DNAH3	16	21078740
	2	70221961	TMEM159, DNAH3	16	21078585
GPR56	16	56211848	TMEM159, DNAH3	16	21078568
RAB25	1	154297433	TMEM159, DNAH3	16	21078598
RAB25	1	154297468	C1orf210	1	43524150
	3	53164930	C1orf210	1	43523857
RAB24	5	176661226	C1orf210	1	43524084
SPINT1	15	38925452	C1orf210	1	43523963
RAB24	5	176661618	C1orf210	1	43523950
	8	8356184	C1orf210	1	43524056
	20	36661934	C1orf210	1	43524091
	1	113106832	C1orf210	1	43523957
CHD3	17	7732607	CLDN7	17	7105979
ABCF1	6	30667066	CLDN7	17	7105734
	16	83945057	CLDN7	17	7106573
CLDN7	17	7106633		1	1088914
CLDN7	17	7106571		1	1088855
CLDN7	17	7106564	AGAP3	7	150443215
CLDN7	17	7106566	ARHGEF1	19	47084177
CLDN7	17	7106555		4	100955681
GRHL2	8	102575727	ARHGAP39	8	145777081
GRHL2	8	102575565	STX2	12	129869431
GRHL2	8	102575811	STX2	12	129869200
GRHL2	8	102574732	STX2	12	129869047
GRHL2	8	102574469	STX2	12	129869147
GRHL2	8	102574689	STX2	12	129868969
TMEM30B	14	60817996		22	35136360
TMEM30B	14	60818107		22	35136601
TMEM30B	14	60818193		22	35136526
TMEM30B	14	60818089		22	35136389
PDGFRA, LNX1	4	54152685	CLDN15	7	100662856
PDGFRA, LNX1	4	54152402	E2F4, ELMO3	16	65790422
PDGFRA, LNX1	4	54152494	E2F4, ELMO3	16	65790778
PDGFRA, LNX1	4	54152599	ELMO3	16	65790933
PDGFRA, LNX1	4	54152503	PTPRF	1	43788610
GRHL2	8	102573922	PTPRF	1	43788636
GRHL2	8	102574035	PTPRF	1	43788601
GRHL2	8	102573658	PWWP2B	10	134071493
GRHL2	8	102573623	PWWP2B	10	134071845
GRHL2	8	102573655	PWWP2B	10	134071623
GRHL2	8	102573797		14	64239711
GRHL2	8	102573842		14	64239802
GRHL2	8	102573677	ETV6	12	11922571
GRHL2	8	102573740	SH3BP5	3	15344685
	4	124687980	GAS8	16	88638299
	4	124687986	SULT2B1	19	53747224
	4	124688290	SULT2B1	19	53747250
	1	117976728	SULT2B1	19	53747255
	1	1088243	SULT2B1	19	53747202
	1	1089514	SULT2B1	19	53747244
	1	1089426	LAMA3	18	19707129
	1	1089493	LAMA3	18	19706893
	1	1089446	LAMA3	18	19706728
	1	1088763	LAMA3	18	19706786
	1	1089029	LAMA3	18	19706817
LAMA3	18	19706827	APBB1	11	6375542
LAMA3	18	19706842	ABCA7	19	1016712
NCRNA00093, DNMBP	10	101680658	ABCA7	19	1016728
C1orf106	1	199130846	ABCA7	19	1016688
C1orf106	1	199130930		11	66580163

TABLE 1-continued

methylated cytosine nucleotides associated with mesenchymal phenotype

	12	6942075		11	66580192
	12	6941973		11	66580260
	12	6943440	ANK3	10	62162307
	12	6943501	ANK3	10	62161917
	12	6943503	ANK3	10	62162163
	12	6943508	ABLIM1	10	116269176
	12	6943525		14	53642609
	12	6942988	XDH	2	31491126
	12	6943026	XDH	2	31491353
	12	6943152	DAPP1	4	100957034
	12	6942957	DAPP1	4	100956844
TALDO1	11	753339	DAPP1	4	100956853
TALDO1	11	753485	TNS4	17	35911401
CNKSR1	1	26376210	TNS4	17	35911460
CNKSR1	1	26376363	TNS4	17	35911441
CNKSR1	1	26376365	TNS4	17	35911475
CNKSR1	1	26376449	PARD3	10	34756309
CNKSR1	1	26376445	RGL2	6	33373111
CNKSR1	1	26376434	RGL2	6	33373221
CNKSR1	1	26376520	RGL2	6	33373245
CNKSR1	1	26376566		19	17763242
CNKSR1	1	26376606		1	150076158
CNKSR1	1	26376578	PCCA	13	99941258
	3	37200270	RAP1GAP2	17	2855119
MERTK	2	112421048	EPHB3	3	185766002
RGS3	9	115383006	TNFRSF10C	8	23019312
PLXNB2	22	49062679	MICAL2	11	12226862
PLXNB2	22	49062940	SGSM2	17	2197812
	16	86381426	RABGAP1L	1	173111020
	10	75306867	RABGAP1L	1	173111113
FAM83A	8	124264314	ARHGEF10L	1	17750038
FAM83A	8	124264583	TBC1D1	4	37666838
FAM83A	8	124264373	CGN	1	149751930
TAF1B	2	9955012	ELF3	1	200243703
ERI3	1	44566745	PROM2	2	95304202
PROM2	2	95304432	EPN3	17	45966053
PROM2	2	95303758		2	128333798
PROM2	2	95303838	GJB3	1	35020553
PHEX	X	22046472	C10orf91	10	134111645
ADAP1	7	952365	C10orf91	10	134111403
ADAP1	7	952156	C10orf91	10	134111470
ADAP1	7	952310		20	30796655
ADAP1	7	952245	DLEU1	13	49829837
ADAP1	7	952140		8	101497819
VCL	10	75485630		22	28307949
	11	67206458		22	28308158
	11	67206243		16	86536340
	14	51288831	UNC5A	5	176181830
	14	51288704		4	154076297
	21	36592419		4	154075997
	14	34872148		4	154075953
PLA2G4F	15	40236052	USP43	17	9491021
	1	201096568	USP43	17	9490981
FAM46B	1	27207475	USP43	17	9490898
OPA3	19	50723356	USP43	17	9490862
	11	3454830	CXCL16	17	4588805
	6	36205670	CXCL16	17	4588796
CST6	11	65535543		7	139750442
FGGY	1	59989219		7	139750014
	15	72463284		7	139750140
FUT3	19	5802616		7	139750233
FUT3	19	5802465		7	139750195
FUT3	19	5802504		7	139750252
PLS3	X	114734137		7	139750206
WWC1	5	167725172		7	139750225
	8	15408729	CLDN4	7	72883980
RASA3	13	113862226	ARAP1, STARD10	11	72169819
ST3GAL4	11	125781207		9	131185398
ST3GAL4	11	125781216	CDKN1A	6	36758711
	12	104024772	MBP	18	72930014
IL17RE, CIDEC	3	9919512	ERBB2	17	35115639
IL17RE, CIDEC	3	9919537	C14orf43	14	73281541
SIGIRR, ANO9	11	407907	MED16	19	834879
SYT8	11	1812460		2	101234948
SYT8	11	1812427		2	101234788

TABLE 1-continued

methylated cytosine nucleotides associated with mesenchymal phenotype

IL10RB	21	33563377	MACC1	7	20223703
ESRP2	16	66825963	MACC1	7	20223521
ESRP2	16	66825753	MACC1	7	20223687
SPINT2	19	43448222		1	27160055
CCDC120	X	48803602	ST14	11	129535669
CCDC120	X	48803499	ST14	11	129535471
C19orf46, ALKBH6	19	41191679	SPINT1	15	38923139
C19orf46, ALKBH6	19	41191561	SPINT1	15	38923085
C19orf46, ALKBH6	19	41191506	SPINT1	15	38923161
CLDN7	17	7105010	SPINT1	15	38923192
PRSS8	16	31054518	C1orf172	1	27159869
PRSS8	16	31054678		2	74064398
PRSS8	16	31054500		2	74064468
PRSS8	16	31054545		2	74064365
PRSS8	16	31054555		8	102573146
PRSS8	16	31054700		8	102573120
	2	238165128		8	102573068
ANKRD22	10	90601891	POU6F2	7	39022925
ANKRD22	10	90601835	LAMB3	1	207892566
ITGB6	2	160764766	LAMB3	1	207892295
ITGB6	2	160764885	LAMB3	1	207892301
ITGB6	2	160764846	LAMB3	1	207892354
BOK	2	242150379	LAMB3	1	207892472
TMC8, TMC6	17	73640271	LAMB3	1	207892370
TMC8, TMC6	17	73640278	LAMB3	1	207892479
CRB3	19	6415885		3	129911695
EPS8L1	19	60279005		16	2999774
EPS8L1	19	60278851	BMF	15	38186296
	12	88144203	BMF	15	38186393
	7	64096077	BMF	15	38186423
KIAA0247	14	69194460	GALNT3	2	166357860
	14	64239962		8	144893629
	5	74369044		8	144893700
	16	11613936	C20orf151	20	60435990
NEURL1B	5	172048817	C20orf151	20	60436252
CLDN4	7	72882009	C20orf151	20	60436261
PAK4	19	44350154	C20orf151	20	60436106
P2RY2	11	72616798	C20orf151	20	60436134
	4	69806346	C20orf151	20	60436052
MACC1	7	20223945			

gene	chromosome position
ADAMTS16	hg19 hr5: 5139160-5139859
ANKRD34A	hg19 chr1: 145472863-145473562
ARID5A	hg19 chr2: 97215439-97216138
APC2	hg19 chr19: 1467602-1468301
BMP4	hg19 chr14: 54422575-54423274
CA12	hg19 chr15: 63673688-63674360
CCK	hg19 chr3: 42306174-42306873
CCNA1	hg19 chr13: 37005581-37006453
CDH4	hg19 chr20: 59826862-59827561
CLDN7	hg19 chr17: 7165943-7166642
DKK1	hg19 chr10: 54072931-54073630
SEPTIN9	hg19 chr17: 75404213-75404912
DLX1	hg19 chr2: 172950047-172950746
ERBB4	hg19 chr2: 213402181-213402880
ESRP1	hg19 chr8: 95651545-95652244
FGFR1	hg19 chr8: 38279279-38279921
FOXA1	hg19 chr14: 38061638-38062337
GATA2	hg19 chr3: 128202381-128203080
GNE	hg19 chr10: 54072931-54073630
GRHL2	hg19 chr8: 102504509-102505208
GLI3	hg19 chr7: 42267369-42268068
HDAC4	hg19 chr2: 240113948-240114647
HOXA10	hg19 chr7: 27213776-27214475
HS3TS3B1	hg19 chr17: 14202839-14203538
ID2	hg19 chr2: 8823406-8824105
ITIH4	hg19 chr3: 52854493-52855192
LAMA1	hg19 chr18: 7013604-7014303
LAD1	hg19 chr1: 201368681-201369380
LHX9	hg19 chr1: 197889343-197890042
MAP6	hg19 chr11: 75378150-75378849
MEOX1	hg19 chr17: 41738845-41739544
MGC45800	hg19 chr4: 183061951-183062650

TABLE 1-continued

methylated cytosine nucleotides associated with mesenchymal phenotype	
MSX1	hg19 chr4: 4859635-4860334
MTMR7	hg19 chr8: 17270755-17271454
PARD3	hg19 chr10: 35104748-35105447
PAX6	hg19 chr11: 31833994-31834693
PCDHGA8	hg19 chr5: 140807001-140807700
PI3KR5	hg19 chr17: 8798216-8798915
RNF220	hg19 chr1: 44883347-44884046
RNLS	hg19 chr10: 90342854-90343553
RPS6KA2	hg19 chr6: 167177930-167178629
SFRP1	hg19 chr8: 41167914-41168613
WNT5B	hg19 chr12: 1739567-1740266
MEOX2	hg19 chr7: 15727091-15727790
TP73	hg19 chr1: 3569053-3569719
RASGRF1	hg19 chr15: 79381517-79382216
TWIST	hg19 chr7: 19157773-19158472
AGAP3	hg18 chr7: 150442790-150443639
ANKRD33B	hg18 chr5: 10617913-10618612
ARHGEF1	hg18 chr19: 47083827-47084526
C10orf91	hg18 chr10: 134111053-134111752
CHD3	hg18 chr17: 7732182-7733031
CXCL16	hg18 chr17: 4588455-4589154
ESRP2	hg18 chr16: 66828033-66828732
KIAA1688	hg18 chr8: 145777004-145777703
TBC1D1	hg18 chr4: 37654711-37655410
SERPINB5	hg18 chr18: 59295387-59296621
STX2	hg18 chr12: 129868969-129869727
miR200C	hg18 chr12: 6942800-6943200
MST1R	hg18 chr3: 49916155-49916617
MACC1	hg18 chr7: 20223293-20224058
HOXC4/HOXC5	hg18 chr12: 52712961-52713967
CP2L3	hg19 chr8: 102504509-102505208
RON	hg18 chr3: 49916155-49916617
TBCD	hg18 chr17: 78440426-78440951
C20orf55	hg18 chr20: 770741-770860
ERBB2	hg19 chr17: 37861100-37863650

TABLE 2

methylated cytosine nucleotides associated with epithelial phenotype					
gene	chromosome	position	gene	chromosome	position
ALDH3B2	11	67204971	COLEC10	8	120175608
	2	62409583		5	147237412
AMICA1	11	117590946		5	147237649
TMPRSS13	11	117294776		5	147237518
	1	20440804		1	167067800
	20	1420914	DLG2	11	84558496
	1	20374821	RAB19	7	139760606
DAPP1	4	101009384	PRR5-ARHGAP8,	22	43564823
AMICA1	11	117590130	ARHGAP8		
	19	47895208		2	230797885
MYO1D	17	28170353	CHMP4C	8	82834065
AFAP1	4	7945103		7	21037502
SPINK5	5	147423445		7	21031624
SPINK5	5	147423477		20	36533851
SPINK5	5	147423260		14	74735436
ANO3, MUC15	11	26538572	MYCBPAP	17	45964331
	3	183702446	TMEM30B	14	60814048
	3	183767276		9	84869514
SYT16	14	61608563		10	100127021
SYT16	14	61532507		6	80178446
TC2N	14	91391048		3	106815813
TC2N	14	91375355	CNGA1	4	47710738
CEACAM6	19	46966764	SLAMF9	1	158190485
KIAA0040	1	173395004	CD180	5	66513564
KIAA0040	1	173396807	ESR1	6	152166508
SYK	9	92692359		12	72730512
SYK	9	92659288	MRVI1	11	10559098
SEMA6D	15	45522047	CYP4B1	1	47037188
ERP27	12	14982225	MFSD4	1	203816771
IVL	1	151148554	PLA2G2F	1	20338719

TABLE 2-continued

methylated cytosine nucleotides associated with epithelial phenotype

IVL	1	151148439	CYP4B1	1	47057214
KRTAP3-3	17	36403692	CYP4A22	1	47375597
KRTAP3-3	17	36403856		1	47036300
	5	55990316	SDR16C5	8	57375347
DHRS9	2	169653716		5	39796557
	4	55490421	SAMD12	8	119525751
SPAM1	7	123353161		1	190775347
	8	127777938	TAT	16	70168544
	8	120206280	SALL3	18	74858829
COLEC10	8	120187865		11	128964767
	11	2178652	PKHD1	6	51787037
IRF6	1	208029710	ZC4H2	X	64171340
UBXN10	1	20391491	TRAM2	6	52549027
	7	7359082	BVES	6	105690909
SCEL	13	77066219	BVES	6	105690842
TMC1	9	74639567	MLLT11	1	149299631
	8	127457153	MLLT11	1	149299586
	4	55742586	MLLT11	1	149299347
PHLDB2	3	113112565		2	42128114
HMHB1	5	143180348		2	42128123
	7	19927552		1	113301372
	16	68155671		12	95407994
LAMA2	6	129245818	TENC1	12	51729752
LAMA2	6	129245899	TENC1	12	51729851
	11	65020493		3	42088628
SHANK2	11	70217854	SPRY4	5	141675957
SHANK2	11	70350904	SPRY4	5	141679764
NFIC	19	3312374		19	13808229
NFIC	19	3312154		19	13808284
FLNB	3	58020380		19	13808262
TEAD4	12	2978486		19	13808473
ABCC3	17	46113650		19	13808469
TMEM120B	12	120670919	DGAT1	8	145510701
SCNN1A	12	6347262	NRM	6	30764049
	8	103890347	NRM	6	30764073
SAMD11,	1	869821	NRM	6	30764003
NOC2L			FLOT1	6	30817584
KIRREL	1	156231153	FLOT1	6	30817649
MYADM	19	59061828		19	52793249
INPP5B	1	38185106	LAMB3	1	207868102
INPP5B	1	38185405	LAMB3	1	207867974
INPP5B	1	38185271	AP1M2	19	10544470
INPP5B	1	38185275	MAP3K14	17	40747948
INPP5B	1	38185298	MAP3K14	17	40748115
INPP5B	1	38185331	ELOVL7	5	60094877
PDE4D	5	58457316	ADAP1	7	913183
	11	65013559		17	17470224
CADPS2	7	122024033	PTK2B	8	27325072
ITGA5	12	53098352		1	19211638
ZC4H2	X	64171392		17	54761551
ZC4H2	X	64171381	ITGB3	17	42685877

gene	chromosome	position	gene	chromosome position
ITGB3	17	42686081	INPP5B	hg18 chr1: 38184921-38185620
ITGB3	17	42685928	BVES	hg18 chr6: 105690492-105691191
ITGB3	17	42685861	ITGA5	hg18 chr12: 53098002-53098701
ITGB3	17	42686060	ITGB3	hg18 chr17: 42685578-42686277
C11orf70	11	101423920	JAKMIP2	hg18 chr5: 147142066-147142765
EPN3	17	45974036	MLLT11	hg18 chr1: 149299281-149299980
	1	20672639	NFIC	hg18 chr19: 3311804-3312503
LIX1L	1	144189643	NTNG2	hg18 chr9: 134026339-134027038
SIGIRR	11	403594	ZEB2	hg18 chr2: 144989568-144989952
	17	73861330	PCDH8	hg18 chr13: 52321009-52321560
	11	32068730	PEX5L	hg18 chr3: 181236933-181237780
KLF16	19	1810341	GALR1	hg18 chr18: 73090412-73090797
	1	28457982		
	10	129592716		
LY6G6C	6	31795616		
CDS1	4	85777370		
MRVI1	11	10562607		
	10	17309649		
	10	17309781		
	17	23722410		
	16	67990001		

TABLE 2-continued

methylated cytosine nucleotides associated with epithelial phenotype

ZEB2	2	144994583
	4	40953131
ANK3	10	62002852
	5	10618263
	5	66600290
NTNG2	9	134026689
JAKMIP2	5	147142568
JAKMIP2	5	147142416
JAKMIP2	5	147142654
JAKMIP2	5	147142625
	10	30178594
TBC1D1	4	37655153
TBC1D1	4	37655061
TBC1D1	4	37655126

TABLE 3

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
TSPAN14	10	82209390	81619	
LFNG	7	2530782	3955	CpG_35
PRKCH	14	61062573	5583	
SDC4	20	43406736	6385	
SCYL3	1	168127429	57147	
TNXB	6	32162072	7148	
ARHGAP39	8	145777560	80728	CpG_52
SPINT1	15	38937072	6692	
SLC9A7	X	46499386	84679	
	3	49919159		
TBCD	17	78440786	6904	
VTI1A	10	114492308	143187	
LDLRAP1	1	25767066	26119	
PLEKHG6	12	6291928	55200	
PNPLA8	7	107955918	50640	
PNPLA8	7	107955957	50640	
ARID1A	1	26953185	8289	
ABTB2	11	34241186	25841	
SLC9A3R1	17	70267242	9368	
	7	2447061		
GALNTL2	3	16220636	117248	
ZNF321	19	58139084	399669	
DIP2B	12	49261110	57609	
	3	178803691		
	2	242481901		
	7	6491550		
WDR82	3	52277292	80335	
TRAF5	1	209569842	7188	
PPARD	6	35417906	5467	CpG_65
LYN	8	57066177	4067	
LOC254559	15	87723993	254559	CpG_155
LOC254559	15	87723796	254559	CpG_155
	7	27744012		
TMEM79	1	154520773	84283	
	8	102520036		
JMJD7-PLA2G4B	15	39918027	8681	
FTO	16	52372627	79068	
	15	78857906		CpG_157
BAIAP2	17	76625735	10458	
	8	102520234		
	8	102520167		
NRBP1, KRTCAP3	2	27518521	29959, 200634	CpG_42
PVRL2	19	50073777	5819	
	7	6491523		
CSK	15	72868625	1445	
PITPNM3	17	6396092	83394	
GRHL2	8	102575811	79977	CpG_104
PVRL4	1	159325891	81607	
LAMA3	18	19706786	3909	

TABLE 3-continued

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
	8	144892697		
	8	144892671		
STX2	12	129869200	2054	CpG_56
STX2	12	129869147	2054	CpG_56
OBSCN	1	226625610	84033	CpG_30
GNA13	17	60466557	10672	
ACAP2	3	196640585	23527	
WDR82	3	52277190	80335	
NSMCE2	8	126223268	286053	
	10	73752495		
RAB24	5	176661226	53917	
ETV6	12	11922571	2120	
ENDOD1	11	94481032	23052	
	7	155407740		
LIMA1	12	48882614	51474	
TBCD	17	78426682	6904	
TBCD	17	78426927	6904	
TBCD	17	78426815	6904	
C10orf91	10	134111645	170393	
	2	64687934		
	2	64687784		
	2	64687610		
SPIRE1	18	12636025	56907	
STX2	12	129869047	2054	CpG_56
LRP5	11	67866681	4041	
OBSCN	1	226625713	84033	CpG_30
OBSCN	1	226625944	84033	CpG_30
OBSCN	1	226625706	84033	CpG_30
OBSCN	1	226625779	84033	CpG_30
CGN	1	149753111	57530	
	12	6943501		
RAB25	1	154297806	57111	
	12	6943503		
	12	6943508		
TBCD	17	78440951	6904	
MYST1, PRSS8	16	31050024	84148, 5652	
TBCD	17	78440835	6904	
TBCD	17	78440498	6904	
TBCD	17	78440559	6904	
TBCD	17	78440426	6904	
GRHL2	8	102574469	79977	CpG_104
GRHL2	8	102575727	79977	CpG_104
GPR110	6	47118050	266977	
	6	7477665		
THSD4	15	69416262	79875	
	3	53164930		
C20orf151	20	60435990	140893	
PWWP2B	10	134072208	170394	
	7	2447019		
	2	70221961		
LAMA3	18	19706842	3909	
LAMA3	18	19706817	3909	
RHOBTB3	5	95089583	22836	
GPR56	16	56211848	9289	
RAB25	1	154297468	57111	
RAB25	1	154297433	57111	
TMEM159, DNAH3	16	21078585	57146, 55567	
C1orf210	1	43524091	149466	
CCDC19	1	158136950	25790	
C1orf210	1	43524084	149466	
CLDN7	17	7105979	1366	CpG_159
GRHL2	8	102574035	79977	CpG_31
SPINT1	15	38925452	6692	
ADAP1	7	952140	11033	
	12	6943440		
	12	6943525		
RAP1GAP2	17	2815637	23108	
VPS37C	11	60682632	55048	
IGF1R	15	97074397	3480	
BOLA2, GDPD3	16	30023636	552900, 79153	
	22	28307742		
	22	28308158		

TABLE 3-continued

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
NA, RCC1	1	28726284	751867, 1104	
CTNND1	11	57305264	1500	
	2	101234788		
MPRIP	17	16907618	23164	
FRMD6	14	51101498	122786	
	16	86381426		
ARHGAP39	8	145777354	80728	CpG_52
MAPK13	6	36207101	5603	
	10	5583926		
	10	5583949		
	13	109313445		
F11R	1	159258982	50848	
SDCBP2	20	1257722	27111	
F11R	1	159258976	50848	
EHF	11	34599461	26298	
ABLIM1	10	116269176	3983	
MCCC2	5	70933152	64087	
COX10	17	14050396	1352	
SLC37A1	21	42809566	54020	
MYO18A	17	24529971	399687	
IL17RE, CIDEC	3	9919537	132014, 63924	
S100A14	1	151855406	57402	
IL17RE, CIDEC	3	9919512	132014, 63924	
TALDO1	11	753485	6888	
PHGDH	1	120075342	26227	
SIPA1L1	14	71183551	26037	
	2	189266325		
TMEM159, DNAH3	16	21078740	57146, 55567	
PPCDC	15	73115889	60490	
GPR56	16	56211418	9289	
LLGL2	17	71057739	3993	
SPINT1	15	38923139	6692	CpG_135
CLDN15	7	100662856	24146	CpG_54
CNKSR1	1	26376445	10256	
GRB7	17	35149701	2886	
NRBP1, KRTCAP3	2	27519047	29959, 200634	CpG_42
KRTCAP3	2	27519215	200634	CpG_42
	16	83945057		
GPR56	16	56211405	9289	
TACC2	10	123744125	10579	
ADAT3, SCAMP4	19	1858677	113179, 113178	CpG_34
CHD2	15	91266091	1106	
GRHL2	8	102575565	79977	CpG_104
	7	139750195		
	8	102573120		
	1	227296135		
PDGFRA, LNX1	4	54152503	5156, 84708	
PDGFRA, LNX1	4	54152494	5156, 84708	
	11	3454830		
ITGB6	2	160764885	3694	
PDGFRA, LNX1	4	54152866	5156, 84708	
	20	36661934		
	1	1088243		CpG_183
ST14	11	129535669	6768	CpG_64
	7	139750206		
C20orf151	20	60436134	140893	
	7	139750140		
LOC643008, RECQL5	17	71147779	643008, 9400	
GRB7	17	35147553	2886	
GRB7	17	35147540	2886	
C1orf210	1	43523857	149466	
CNKSR1	1	26376606	10256	
CNKSR1	1	26376566	10256	
CLDN7	17	7106571	1366	CpG_159
CLDN7	17	7106564	1366	CpG_159
CLDN7	17	7106566	1366	CpG_159
C1orf210	1	43523950	149466	
C1orf210	1	43523957	149466	
CLDN4	7	72883688	1364	CpG_46

TABLE 3-continued

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
CLDN7	17	7105734	1366	CpG_159
C1orf210	1	43523963	149466	
CLDN7	17	7106573	1366	CpG_159
KRTCAP3	2	27519142	200634	CpG_42
MST1R	3	49914707	4486	CpG_23
MST1R	3	49915923	4486	CpG_53
XAB2	19	7590468	56949	
KIAA0182	16	84236385	23199	
PWWP2B	10	134072043	170394	
CCDC57	17	77655395	284001	
NRBP1, KRTCAP3	2	27518810	29959, 200634	CpG_42
NRBP1, KRTCAP3	2	27518583	29959, 200634	CpG_42
NRBP1, KRTCAP3	2	27518645	29959, 200634	CpG_42
MOCOS	18	32022494	55034	CpG_141
PWWP2B	10	134071493	170394	
LAMA3	18	19706893	3909	
	12	6943152		
	12	6942988		
	12	6943026		
	12	6942957		
	14	64239711		
PRSS8	16	31054518	5652	
	17	75403479		CpG_427
C20orf151	20	60436252	140893	
GRHL2	8	102574732	79977	CpG_104
C20orf151	20	60436106	140893	
SULT2B1	19	53747255	6820	
SULT2B1	19	53747244	6820	
SULT2B1	19	53747224	6820	
SULT2B1	19	53747250	6820	
CBLC	19	49973124	23624	
NRBP1, KRTCAP3	2	27519011	29959, 200634	CpG_42
NRBP1, KRTCAP3	2	27518654	29959, 200634	CpG_42
GRHL2	8	102573658	79977	CpG_31
DOK7	4	3457234	285489	
FAM110A	20	770788	83541	CpG_71
NRBP1, KRTCAP3	2	27518643	29959, 200634	CpG_42
PWWP2B	10	134071623	170394	
TALDO1	11	753339	6888	
OVOL1	11	65310618	5017	CpG_204
SH3YL1	2	253656	26751	CpG_176
	7	139750225		
LAD1	1	199635571	3898	CpG_54
TMEM159, DNAH3	16	21078568	57146, 55567	
GRHL2	8	102573922	79977	CpG_31
PDGFRA, LNX1	4	54152402	5156, 84708	
LAD1	1	199635569	3898	CpG_54
LAD1	1	199635537	3898	CpG_54
KRT8	12	51586560	3856	
	3	135552584		
	19	49971605		
ITGB6	2	160822102	3694	
ADAP1	7	952310	11033	
ADAP1	7	952245	11033	
PROM2	2	95304202	150696	
PROM2	2	95304432	150696	
PROM2	2	95303758	150696	
SYT8	11	1811862	90019	
	16	70401148		
	17	15737821		
QSOX1	1	178404541	5768	
CCDC85C	14	99114910	317762	
C1orf116	1	205273070	79098	
GRHL2	8	102576558	79977	
C19orf46	19	41191166	163183	
CBLC	19	49973366	23624	

TABLE 3-continued

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
CAMK2G	10	75302072	818	
SCNN1A	12	6354990	6337	
SCNN1A	12	6354868	6337	
JUP	17	37182909	3728	
	19	60699327		
VCL	10	75485630	7414	
BOLA2, TBX6	16	30009181	552900, 6911	
IMMP2L	7	110988180	83943	
SLC44A2	19	10596548	57153	CpG_46
	8	144726627		
RAI1	17	17572988	10743	
SYT1	12	78333487	6857	
	8	28514725		
	6	134742250		
GPR56	16	56211203	9289	
EPN3	17	45967146	55040	
GPR56	16	56211170	9289	
C4orf36	4	88031692	132989	
ARL13B, STX19	3	95230218	200894, 415117	
	2	70222288		CpG_118
PVRL4	1	159326053	81607	
	1	27066922		
GPR110	6	47117696	266977	
EPHB2	1	23025895	2048	
ANKRD22	10	90601891	118932	
ZNF398	7	148472457	57541	
PWWP2B	10	134071845	170394	
ARHGAP32	11	128399061	9743	
	7	80389667		
	4	154136934		
	1	27023897		
	19	1855554		
BAIAP2	17	76626137	10458	
PLXNB2	22	49062595	23654	
ACAA1	3	38150460	30	
DNAJC17	15	38867650	55192	
	7	72795287		
COL18A1, SLC19A1	21	45757802	80781, 6573	
LOC643008, RECQL5	17	71147845	643008, 9400	
MANF	3	51401417	7873	
TRAK1	3	42147101	22906	
GRB7	17	35147329	2886	
C1orf210	1	43524150	149466	
RNF144A	2	7089548	9781	
GRB7	17	35147290	2886	
	19	58230499		
	1	234153824		
PPFIBP2	11	7578132	8495	
GPR81	12	121777086	27198	
	19	58230695		
	8	101497819		
CPEB3	10	93872825	22849	
RABGAP1L	1	173111113	9910	
RABGAP1L	1	173111020	9910	
RNF207	1	6202430	388591	
MUC1	1	153429495	4582	
	1	2456135		
PLEKHG6	12	6292029	55200	
PLEKHG6	12	6292067	55200	
PNPLA8	7	107955947	50640	
RASA3	13	113862226	22821	
ARL13B, STX19	3	95230100	200894, 415117	
VTI1A	10	114516704	143187	
COL21A1	6	56342813	81578	
	2	74064468		CpG_113
SDCBP2	20	1258000	27111	
FAM167A	8	11340393	83648	
S100A14	1	151855551	57402	
PRSS8	16	31054183	5652	
HIVEP3	1	41753450	59269	
PRSS8	16	31054700	5652	

TABLE 3-continued

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
SULT2B1	19	53747202	6820	
C19orf46, ALKBH6	19	41191679	163183, 84964	CpG_49
C19orf46, ALKBH6	19	41191506	163183, 84964	
C19orf46, ALKBH6	19	41191561	163183, 84964	CpG_49
	17	52465903		
RAP1GAP2	17	2855119	23108	
C10orf91	10	134110971	170393	
	8	144892814		
	9	131184926		CpG_71
BMF	15	38186423	90427	
RGS3	9	115383006	5998	
	19	17763242		
	19	50356091		
DLEU1	13	49829837	10301	
MBP	18	72930014	4155	
	1	150076158		
JMJD7-PLA2G4B	15	39917942	8681	
PARD3	10	34756309	56288	
MICAL2	11	12226862	9645	
ANKFY1	17	4098222	51479	
CDKN1A	6	36758711	1026	
	19	49971610		
JARID2	6	15564181	3720	
SGSM2	17	2197812	9905	
SMARCD2	17	59270462	6603	
PNKD	2	218868246	25953	
EVPLL	17	18221746	645027	
EVPLL	17	18221574	645027	
MED16	19	834879	10025	
RAB24	5	176661618	53917	
	7	155407629		
ERBB2	17	35115639	2064	
CGN	1	149751930	57530	
	8	8356184		
GNAI3	1	109914827	2773	
	8	37880723		
ANKRD22	10	90601835	118932	
	15	81670543		
PAK4	19	44350154	10298	
PRR15L	17	43390182	79170	
RAB17	2	238164820	64284	
P2RY2	11	72616798	5029	
	22	28307949		
	8	144893700		CpG_78
SPINT1	15	38923085	6692	CpG_135
PVRL4	1	159326159	81607	
	6	13981646		CpG_39
C1orf210	1	43524056	149466	
	7	139750233		
TBC1D1	4	37666838	23216	
	7	72795153		
	2	238165064		
ARHGAP32	11	128399150	9743	
	12	88144203		
TMC8	17	73650109	147138	
ABCF1	6	30667066	23	
ST3GAL4	11	125781216	6484	
ST3GAL4	11	125781207	6484	
STAP2	19	4289769	55620	
STAP2	19	4289932	55620	
LAMA3	18	19706827	3909	
	1	201096568		CpG_80
GSDMC	8	130868275	56169	
AFF1	4	88113322	4299	
	17	71380179		
	14	34872148		
ASB13	10	5742089	79754	
CLDN7	17	7106144	1366	CpG_159
CDC42BPG	11	64367663	55561	

TABLE 3-continued

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
FAM46B	1	27207475	115572	
EPS8L1	19	60278851	54869	
	16	70401060		CpG_91
ESRP2	16	66825963	80004	
IL10RB	21	33563377	3588	
C14orf43	14	73281541	91748	
CCDC120	X	48803602	90060	
CCDC120	X	48803499	90060	
ESRP2	16	66825753	80004	
CNKSR1	1	26377135	10256	
CLDN7	17	7107442	1366	CpG_159
SCNN1A	12	6354974	6337	
MUC1	1	153429380	4582	
PRSS8	16	31054500	5652	
SLC35B3	6	8381262	51000	CpG_68
	12	13179838		
EPS8L1	19	60279005	54869	
GPR110	6	47118136	266977	
LAMA3	18	19706728	3909	
PVRL4	1	159325951	81607	
PVRL4	1	159326082	81607	
RIPK4	21	42058454	54101	
NEURL1B	5	172048817	54492	
PROM2	2	95303838	150696	
FAM167A	8	11340449	83648	
CLDN4	7	72882009	1364	
	8	102573068		
CANT1	17	74513111	124583	
PRR15L	17	43390296	79170	
MICALL2	7	1461837	79778	
NCOA2	8	71402682	10499	
ITGB6	2	160764766	3694	
ITGB6	2	160764846	3694	
	14	64792338		
	8	102573146		
NRBP1, KRTCAP3	2	27518632	29959, 200634	CpG_42
TMEM159, DNAH3	16	21078598	57146, 55567	
ADAP1	7	952156	11033	
TMEM159, DNAH3	16	21078428	57146, 55567	
SH3YL1	2	253559	26751	CpG_176
	7	139750252		
PRSS22	16	2848212	64063	
PRSS22	16	2848220	64063	
SDCBP2	20	1257800	27111	
LAMA3	18	19707129	3909	
	2	74064398		CpG_113
	2	74064365		CpG_113
DAPP1	4	100956844	27071	
DAPP1	4	100956853	27071	
DAPP1	4	100957034	27071	
	1	999308		
ATG9B	7	150352451	285973	
CLDN7	17	7107017	1366	CpG_159
	9	131185398		CpG_71
STX2	12	129868969	2054	CpG_56
CNKSR1	1	26376578	10256	
E2F4, ELMO3	16	65790778	1874, 79767	
E2F4, ELMO3	16	65790422	1874, 79767	
CNKSR1	1	26376365	10256	
CNKSR1	1	26376363	10256	
ARAP1, STARD10	11	72169819	116985, 10809	CpG_41
CNKSR1	1	26376520	10256	
CNKSR1	1	26376434	10256	
CNKSR1	1	26376449	10256	
MUC1	1	153429376	4582	
PRSS8	16	31054545	5652	
PRSS8	16	31054555	5652	
	7	72795319		
PDGFRA, LNX1	4	54152685	5156, 84708	

TABLE 3-continued

methylated cytosine ucleotides associated with mesenchymal phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
C20orf151	20	60436261	140893	
LAD1	1	199635654	3898	CpG_54
PDGFRA, LNX1	4	54152599	5156, 84708	
	12	50912694		CpG_79
GRHL2	8	102573655	79977	CpG_31
GRHL2	8	102573677	79977	CpG_31
GRHL2	8	102574689	79977	CpG_104
GRHL2	8	102573797	79977	CpG_31
GRHL2	8	102573623	79977	CpG_31
RNF144A	2	7089414	9781	
NCRNA00093, DNMBP	10	101680658	100188954, 23268	
PRKCA	17	62088295	5578	
KIAA0247	14	69194460	9766	
ELF3	1	200246387	1999	
ELF3	1	200246469	1999	
ELF3	1	200246561	1999	
GAS8	16	88638299	2622	
HSH2D	19	16115489	84941	
C10orf91	10	134111403	170393	
	12	88143460		
SYT8	11	1812078	90019	
SYT8	11	1812322	90019	
	10	126879805		
	4	8587017		
ERGIC1	5	172264397	57222	
	12	50911753		
SYT8	11	1812236	90019	
	8	144727414		
	16	11613936		
CLDN7	17	7106555	1366	CpG_159
	5	74369044		
BAIAP2	17	76625947	10458	
BAIAP2	17	76625872	10458	
OPA3	19	50723356	80207	
GRHL2	8	102573740	79977	CpG_31
GRHL2	8	102573842	79977	CpG_31
	8	102085088		
CLDN7	17	7106633	1366	CpG_159
CLDN7	17	7107214	1366	CpG_159
ERBB3	12	54761038	2065	CpG_116
CLDN7	17	7105010	1366	CpG_159
	16	2999774		
	15	72610634		
	11	66579429		
ANKRD22	10	90601762	118932	
	14	64239802		
	14	64239962		
	8	144893629		CpG_78
SLC44A2	19	10596578	57153	CpG_46

TABLE 4

methylated cytosine nucleotides associated with epithelial phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
HBQ1	16	170343	3049	CpG_150
HBQ1	16	170341	3049	CpG_150
	10	118912877		CpG_110
	17	44427906		CpG_255
IGF2BP1	17	44430879	10642	CpG_255
	4	25120404		
TC2N	14	91391048	123036	
ALDH3B2	11	67204971	222	
MYO1D	17	28170353	4642	
SYK	9	92692359	6850	
SYK	9	92659288	6850	
AMICA1	11	117590130	120425	
MAL2	8	120326244	114569	

TABLE 4-continued

methylated cytosine nucleotides associated with epithelial phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
MACROD2	20	14267035	140733	
OVOL2	20	17972215	58495	
CAPN13	2	30821257	92291	
PLG	6	161094476	5340	
NCALD	8	102871791	83988	
	6	147353266		
	14	100245858		CpG_79
	14	100245905		CpG_79
	14	100246063		CpG_79
TRIM9	14	50630238	114088	CpG_199
KIAA0040	1	173395004	9674	
KIAA0040	1	173396807	9674	
	7	50601267		

TABLE 4-continued

methylated cytosine nucleotides associated with epithelial phenotype

Gene	CHROMOSOME	POSITION	EntrezID	CpG_island
	8	127777938		
	7	50601390		
	7	50601219		
SYDE1	19	15079713	85360	CpG_56
	11	65013559		
NUAK1	12	104998452	9891	
MMP2	16	54071026	4313	CpG_42
ZNF521	18	21184882	25925	
ZNF521	18	21185001	25925	
IRF6	1	208029710	3664	
SRD5A2	2	31656355	6716	
MMP2	16	54070981	4313	CpG_42
IGF2BP1	17	44430854	10642	CpG_255
ZC4H2	X	64171686	55906	CpG_71
	12	72730512		
IGF2BP1	17	44430757	10642	CpG_255
PAX7	1	18830954	5081	CpG_205
	17	44427856		CpG_255
MLLT11	1	149299347	10962	CpG_53
MLLT11	1	149299586	10962	
	6	114284192		
	6	114284228		
	6	114284034		
	6	114284022		
	10	118912831		CpG_110
	10	118912483		CpG_110
	10	118912726		CpG_110
	X	64171940		CpG_71

[0166] This invention will be better understood from the Examples that follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter, and are not to be considered in any way limited thereto.

EXAMPLES

Example 1

Materials and Methods

[0167] Fluidigm Expression Analysis:

[0168] EMT gene expression analysis was conducted on 82 NSCLC cell lines using the BioMark 96×96 gene expression platform (Fluidigm) and a 20-gene EMT expression panel (Supplementary Table S1 and Methods). The ΔCt values were used to cluster cell lines according to EMT gene expression levels using Cluster v.3.0 and Treeview v.1.60 software.

[0169] Illumina Infinium Analysis:

[0170] Microarray data were collected at Expression Analysis, Inc. (Durham, N.C.) using the Illumina Human Methylation 450 BeadChip (Illumina, San Diego, Calif.) as described below. Array data were analyzed and a methylation classifier was established using a "leave-one-out" cross-validation strategy (described below and in refs. 25, 26). Array data have been submitted to the Gene Expression Omnibus database (accession number GSE36216).

[0171] Cell Lines:

[0172] All of the NSCLC cell lines were purchased from the American Type Cell Culture Collection (ATCC) or were provided by Adi Gazdar and John Minna at UT Southwestern. The immortalized bronchial epithelial (gBECs) and small airway (gSACs) cell lines were created at Genentech using a tricistronic vector containing cdk4, hTERT, and G418 as a selection marker. The tricistronic vector was engineered from the pQCX1N backbone containing hTERT. The immortalization process was based on previously published protocols with some modification (Ramirez, Sheridan et al. 2004; Sato, Vaughan et al. 2006). The gBECs and gSACs have a diploid karyotype and are non-tumorigenic. Treatment of cell lines with 5-azadC, erlotinib, or TGFβ1 was performed as described.

[0173] NSCLC Normal Lung Tissue, Primary Tumor and Biopsy Tissue:

[0174] 31 NSCLC fresh-frozen primary tumor tissues (N=28 adenocarcinoma, 3 squamous cell carcinoma) representative of early stage, surgically resectable tumors and 60 formalin-fixed paraffin-embedded (FFPE) NSCLC biopsies from patients who went on to fail frontline chemotherapy. 35 fresh-frozen normal lung tissues (31 matched to primary tumor tissues were also part of this collection). All samples were obtained with informed consent under an IRB approved protocol. All samples were evaluated by a pathologist for tissue quality and tumor stage, grade, and tumor content. Peripheral blood mononuclear cells (N=20) were obtained from healthy volunteers at the Genentech clinic.

[0175] 5-azadC Treatment and TGFβ1 Treatment:

[0176] Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-Glutamine Cells were seeded on day 0 at 4000-9000 cells/cm2 and dosed with 1 μM 5-aza-2'-deoxycytidine (5-aza-dC) (SIGMA-ALDRICH Cat No. A3656) or DMSO control (Cat No. D2650) on days 1, 3, and 5. On day 6 cells were washed once in cold PBS and harvested by scraping in Trizol (Invitrogen, Cat No 15596018) and extracted for RNA or flash frozen for later RNA extraction. For induction of EMT, cells were plated at 20000-50000 cells/10 cm2 in complete medium and supplemented with 2 ng/mL human transforming growth factor beta 1 (TGFβ1) (R&D Systems, Cat No 100-B/CF) or PBS control. Media and TGFβ1 were replaced every 3 days, and RNA was extracted at 4-5 weeks following TGFβ1 induction of EMT. Gene expression changes were assessed using Taqman assays for the 20-gene EMT panel (FIG. **1**).

[0177] Erlotinib Treatment:

[0178] For erlotinib IC50 determination, cells were plated in quadruplicate at 3×102 cells per well in 384-well plates in RPMI containing 0.5% FBS (assay medium) and incubated overnight. 24 hours later, cells were treated with assay medium containing 3 nM TGFα and erlotinib at a dose range of 10 μM-1 pM final concentration. After 72 hrs, cell viability was measured using the Celltiter-Glo Luminescent Cell Viability Assay (Promega). The concentration of erlotinib resulting in 50% inhibition of cell viability was calculated from a 4-parameter curve analysis and was determined from a minimum of two experiments. Cell lines exhibiting an erlotinib IC50≦2.0 μM were defined as sensitive, 2.0-8.0 μMas intermediate, and ≧8.0 μM as resistant.

[0179] Fluidigm Gene Expression Analysis:

[0180] 2 μl of total RNA was reverse-transcribed to cDNA and pre-amplified in a single reaction using Superscript III/Platinum Taq (Invitrogen) and Pre-amplification reaction mix (Invitrogen). 20 Taqman primer/probe sets selected for the EMT expression panel (FIG. **1**) were included in the pre-amplification reaction at a final dilution of 0.05× original Taqman assay concentration (Applied Biosystems). The thermocycling conditions were as follows: 1 cycle of 50° C. for 15 min, 1 cycle of 70° C. for 2 min, then 14 cycles of 95° C. for 15 sec and 60° C. for 4 min.

[0181] Pre-amplified cDNA was diluted 1.94-fold and then amplified using Taqman Universal PCR MasterMix (Applied Biosystems) on the BioMark BMK-M-96.96 platform (Fluidigm) according to the manufacturer's instructions. All samples were assayed in triplicate. Two custom-designed reference genes that were previously evaluated for their expression stability across multiple cell lines, fresh-frozen tissue samples, and FFPE tissue samples, AL-1377271 and VPS-33B, were included in the expression panel. A mean of the Ct values for the two reference genes was calculated for each sample, and expression levels of EMT target genes were determined using the delta Ct (dCt) method as follows: Mean Ct (Target Gene)−Mean Ct (Reference Genes).

[0182] Illumina Infinium Analysis:

[0183] Microarray data was collected at Expression Analysis, Inc. (Durham, N.C.; www.expressionanalysis.com) using the IlluminaHumanMethylation450 BeadChip (Illumina) These arrays contain probes for approximately 450,000 CpG loci sites. Target was prepared and hybridized according to the "Illumina Infinium HD Methylation Assay, Manual Protocol" (Illumina Part #15019522 Rev. A).

[0184] Bisulfite Conversion:

[0185] A bisulfite conversion reaction was employed using 500 ng of genomic DNA according to the manufacturer's protocol for the Zymo EZ DNA Methylation kit (Zymo Research). DNA was added to Zymo M-Dilution buffer and incubated for 15 min at 37° C. CT-conversion reagent was then added and the mixture was denatured by heating to 95° C. for 30 s followed by incubation for 1 h at 50° C. This denature/incubation cycle was repeated for a total of 16 h. After bisulfite conversion, the DNA was bound to a Zymo spin column and desulfonated on the column using desulfonation reagent per manufacturer's protocol. The bisulfite-converted DNA was eluted from the column in 10 μl of elution buffer.

[0186] Infinium Methylation Assay:

[0187] 4 μl of bisulfite converted product was transferred to a new plate with an equal amount of 0.1N NaOH and 20 ul of MA1 reagent (Illumina) then allowed to incubate at RT for 10 min. Immediately following incubation, 68 ul of MA2 reagent and 75 ul of MSM reagent (both Illumina) were added and the plate was incubated at 37° C. overnight for amplification. After amplification, the DNA was fragmented enzymatically, precipitated and resuspended in RA1 hybridization buffer. Hybridization and Scanning: Fragmented DNA was dispensed onto the multichannel HumanMethylation BeadChips and hybridization performed in an Illumina Hybridization oven for 20 h. BeadChips were washed, primer extended, and stained per manufacturer protocols. BeadChips were coated and then imaged on an Illumina iScan Reader and images were processed with GenomeStudio software methylation module (version 1.8 or later).

[0188] Infinium Analysis:

[0189] Methylation data were processed using the Bioconductor lumi software package (Du, Kibbe et al. 2008). The Infinium 450K platform includes Infinium I and II assays on the same array. The Infinium I assay employs two bead types per CpG locus, with the methylated state reported by the red dye in some cases and the green dye in others (identical to the previous Infinium 27K platform). The Infinium II assay uses one bead type and always reports the methylated state with the same dye, making dye bias a concern. A two-stage normalization procedure was applied to the arrays: First, for each array, a color-bias correction curve was estimated from Infinium I data using a smooth quantile normalization method; this correction curve was then applied to all data from that array. Second, arrays were normalized to one another by applying standard quantile normalization to all color-corrected signals. After pre-processing, both methylation M-values (log 2 ratios of methylated to unmethylated probes) and -values (a rescaling of the M-values to the 0 and 1 range via logistic transform) were computed for each sample (Du, Zhang et al. 2010). For visualization, agglomerative hierarchical clustering of -values was performed using complete linkage and Euclidean distance.

[0190] Methylation Classifier:

[0191] A 10×10-fold cross validation strategy was used to select a set of differentially methylated CpG sites (DMRs) and to simultaneously evaluate the accuracy of a methylation-based EL vs. ML classifier. Cell lines were split into 10 evenly sized groups. Using 9 tenths of the lines (the training set), candidate DMRs were identified by first computing a moving average for each cell line's M-values (500 bp windows centered on interrogated CpG sites); then, a t-test was used to contrast the window scores associated epithelial-like vs. mesenchymal-like training lines. DMR p-values were adjusted to control the False Discovery rate (Benjamini and Hochberg 1995) and compared to a cutoff of 0.01. To enrich for more biologically relevant phenomena, candidates were required to have average window scores which (i) differed by at least 1 unit between the epithelial and mesenchymal lines, and (ii) had opposite sign in the two sets of cell lines. This process yielded both mesenchymal-associated (positive signal) and epithelial-associated (negative signal) candidate DMRs. To assess performance, the 1 tenth of lines held out for testing were scored by summing their signal for positive DMRs and subtracting off signal for negative DMRs and then dividing through by the total number of DMRs. The known epithelial vs. mesenchymal labels for the test lines were compared to the sign of the result. Finally, the cross-validation process was repeated with each tenth taking the test set role. Finally, the cross-validation process itself was repeated 9 more times, and the overall accuracy assessment was the average of the 100 different test set accuracy rates. To construct a final set of DMRs, we only retained candidates identified as relevant in 100% of the cross-validation splits. Contiguous DMRs which met this criterion were merged into a single DMR if they were separated by less than 2 kb.

[0192] Expression-Based EMT Score:

[0193] Behavior of some genes in our 20-gene Fluidigm expression panel was seen to differ between cell lines and tumor samples. To identify a more robust subset of this panel for purposes of EL vs. ML classification, we took CDH1 expression as an EMT anchor, and then selected genes (13 in total) whose correlation with CDH1 showed the same sign in both cell lines and tumor samples. To assign an EMT expression score to the tumor samples, -dCT values for each of the 13 genes were first centered to have mean 0 and scaled to have standard deviation 1. Next signs were flipped for those genes showing negative correlation with CDH1. Finally, individual tumor sample scores were computed by averaging the standardized and sign-adjusted results.

[0194] Bisulfite Sequencing and Analysis:

[0195] Genomic DNA was bisulfite-converted using the EZ DNA Methylation-Gold kit (Zymo Research). Primers specific to the converted DNA were designed using Methyl Primer Express software v1.0 (Applied Biosystems) (Sequences available upon request). PCR amplification was per-

formed with 1 μl of bisulfite-converted DNA in a 25-μl reaction using Platinum PCR supermix (Invitrogen). The PCR thermocycling conditions were as follows: 1 initial denaturation cycle of 95° C. for 10 minutes, followed by 10 cycles of 94° C. for 30 seconds, 65° C. for 1 minute and decreasing by 1° C. every cycle, and 72° C. for 1 minute, followed by 30 cycles of 94° C. for 30 seconds, 55° C. for 1.5 minutes, and 72° C. for 1 minute, followed by a final extension at 72° C. for 15 minutes. PCR products were resolved by electrophoresis using 2% agarose E-gels containing ethidium bromide (Invitrogen) and visualized using a Fluor Chem 8900 camera (Alpha Innotech).

[0196] PCR products were ligated into the pCR4-TOPO vector using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. 2 μl of ligated plasmid DNA were transformed into TOP10 competent bacteria (Invitrogen), and 100 μl transformed bacteria were plated on LB-agar plates containing 50 μg/ml carbenicillin (Teknova) and incubated overnight at 37° C. Twelve colonies per cell line for each candidate locus were inoculated into 1 ml of LB containing 50 μg/ml carbenicillin and grown overnight in a shaking incubator at 37° C. Plasmid DNA was isolated using a Qiaprep miniprep kit in 96-well format (Qiagen) and sequenced on a 3730×1 DNA Analyzer (Applied Biosystems).

[0197] Bisulfite Sequencing Analysis:

[0198] Sequencing data were analyzed using Sequencher v 4.5 software and BiQ Analyzer software (Bock, Reither et al. 2005).

[0199] Pyrosequencing:

[0200] Bisulfite-specific PCR (BSP) primers were designed using Methyl Primer Express software v 1.0 (Applied Biosystems) or PyroMark Assay Design software v 2.0 (Qiagen). PCR primers were synthesized with a 5' biotin label on either the forward or reverse primer to facilitate binding of the PCR product to Streptavidin Sepharose beads. Sequencing primers were designed in the reverse direction of the 5'-biotin-labeled PCR primer using PyroMark Assay Design software v 2.0 (Qiagen). Primer sequences are available upon request. 1 μl bisulfite modified DNA was amplified in a 25 μl reaction using Platinum PCR Supermix (Invitrogen) and 20 μl of PCR product was used for sequencing on the Pyromark Q24 (Qiagen). PCR products were incubated with Streptavidin Sepharose beads for 10 minutes followed by washes with 70% ethanol, Pyromark denaturation solution, and Pyromark wash buffer. Denatured PCR products were then sequenced using 0.3 μM sequencing primer. Pyrograms were visualized and evaluated for sequence quality, and percent methylation at individual CpG sites was determined using PyroMark software version 2.0.4 (Qiagen).

[0201] Quantitative Methylation Specific PCR:

[0202] A quantitative methylation specific PCR (qMSP) assays targeting DMRs identified by Infinium profiling was designed. Sodium bisulfite converted DNA was amplified with various 20× Custom Taqman Assays using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) with cycling conditions of 95° C. 10 min, then 50 cycles of 95° C. for 15 sec and 60° C. for 1 min. Amplification was done on a 7900HT and analyzed using SDS software (Applied Biosystems). DNA content was normalized using meRNaseP Taqman assay. qMSP of FFPE material was performed using a pre-amplification procedure.

[0203] Pre-amplification of FFPE Tumor Material:

[0204] Aa pre-amplification method for methylation analysis of pico gram amounts of DNA extracted from formalin-fixed paraffin embedded (FFPE) tissue was developed as follows. 2 μl (equivalent of 100 pg-1 ng) bisulfite converted DNA was first amplified in a 20 μl reaction with 0.1×qMSP primer-probe concentrations using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Cat No. 4324018) and cycling conditions of 95° C. 10 min, then 14 cycles of 95° C. for 15 sec and 60° C. for 1 min. 1 μl of the pre-amplified material was then amplified in a second PCR reaction with cycling conditions of 95° C. 10 min, then 50 cycles of 95° C. for 15 sec and 60° C. for 1 min. DNA content was confirmed using a pre-amplification with the reference meRNaseP Taqman assay and only samples that were positive for meRNaseP were included in further analysis of qMSP reactions. All reactions were performed in duplicate.

Example 2

Epithelial-Like and Mesenchymal-Like Expression Signatures Correlate With Erlotinib Sensitivity In Vitro

[0205] A gene expression signature that correlates with in vitro sensitivity of NSCLC cell lines to erlotinib was previously defined (11). This gene set was highly enriched for genes involved in EMT. A quantitative reverse transcriptase PCR-based EMT expression panel on the Fluidigm nanofluidic platform (FIG. **1**) was developed. A comparison of the 100-probe set from the study of Yauch, et al (11) and the 20-gene EMT Fluidigm panel for 42 of the lines profiled in the study of Yauch, et al showed that this 20-gene expression panel is a representative classifier of EMT (ref 11).

[0206] To further evaluate whether the 20-gene panel was representative of the phenotypic changes associated with an EMT, 2 cell lines were treated with TGFβ1. The results of this study showed that TGFβ1 induced morphologic changes associated with an EMT. The genes associated with an epithelial phenotype were downregulated and genes associated with a mesenchymal phenotype were upregulated in these cell lines.

[0207] To determine whether DNA methylation profiling could be used to classify NSCLC cell lines into epithelial-like and mesenchymal-like groups, the 20-gene expression panel was used to assign epithelial-like versus mesenchymal-like status to 82 cell lines. The NSCLC cell lines used in this study include most of the lines profiled in the study of Yauch, et al (11) and an additional 52 lines, which included 6 lines with EGFR mutations. Of the 82 cell lines, 36 were classified as epithelial-like and 34 were classified as mesenchymal-like on the basis of their expression of these markers (FIG. **2**). The expression data were normalized and median centered (samples and genes). Green indicates a low level or no mRNA expression for indicated genes; red indicates high expression. Twelve lines (indicated in the bottom cluster of FIG. **2**) were classified as epithelial-like but express a combination of epithelial and mesenchymal markers, indicating that these lines represent a distinct biology designated as intermediate. Thus, of the 82 NSCLC lines, 89% could be classified clearly as epithelial or mesenchymal. For the most part, this epithelial-like versus mesenchymal-like expression phenotype was mutually exclusive, possibly reflecting a distinct underlying biology, which may be linked to distinct DNA methylation profiles. A summary of cell line descriptions including histology is shown in FIG. **8**A-B.

Example 3

Genome-Wide Methylation Profiles Correlate with Fluidigm-Based EMT Signatures in NSCLC Cell Lines

[0208] The Illumina Infinium 450K array was analysed as a platform for high-throughput methylation profiling by comparing the β-values for 52 probes and sodium bisulfite sequencing data on a subset of cell lines (N=12). A highly significant, strong positive correlation between methylation calls by the Infinium array and direct bisulfite sequencing was observed (r=0.926).

[0209] To identify DMRs that distinguished between epithelial-like and mesenchymal-like cell lines, a cross-validation strategy which simultaneously constructed a methylation-based classifier was used and its prediction accuracy assessed, as described in Example 1. When applied to the 69 cell line training set, this analysis yielded 549 DMRs representing 915 individual CpG sites that were selected as defining epithelial-like versus mesenchymal-like NSCLC cell lines with a false discovery rate—adjusted P value below 0.01 in 100% of the cross-validation iterations. The cross-validation estimated accuracy of the methylation-based classifier was 88.0% (±2.4%, 95% confidence interval).

[0210] Next, the CpG sites included in our methylation-based EMT classifier were used to cluster the 69 NSCLC cell lines (including 6 EGFR-mutant, erlotinib-sensitive lines) and 2 primary normal lung cell strains and their immortalized counterparts. This analysis revealed a striking segregation of epithelial-like, mesenchymal-like, and normal lines (FIG. **3**). In this assay, seventy-two NSCLC cell lines and normal lung epithelial cells were profiled using the Illumina Infinium 450K Methylation array platform. Supervised hierarchical clustering was conducted using 915 probes that were significantly differentially methylated between epithelial-like and mesenchymal-like cell lines (false discovery rate=0.01; Example 1). Annotated probes sets used for the cluster analysis are listed. Each row represents an individual probe on the Infinium 450K array and each column represents a cell line. Regions shaded blue in the heat map represent unmethylated regions, regions shaded red represent methylated regions. The top color bar shows columns representing the epithelial-like or mesenchymal-like status of each cell line as determined by Fluidigm EMT gene expression analysis. Green indicates epithelial-like and black indicates mesenchymal-like cell lines. The bottom color bar indicates the erlotinib response phenotype of each cell line. Red indicates erlotinib-sensitive lines; black indicates erlotinib-resistant lines; gray indicates lines with intermediate sensitivity to erlotinib. A Euclidian distance metric was used for clustering without centering; the color scheme represents absolute methylation differences.

[0211] Notably, the methylation signal from these CpG sites clustered the epithelial-like and mesenchymal-like cell lines into their respective epithelial-like and mesenchymal-like groups with only 6 exceptions: the mesenchymal-like lines H1435, HCC4017, H647, H2228, H1755, and HCC15 clustered with the epithelial-like group. Interestingly, 5 of these 6 lines clustered closely together into a distinct subset of the mesenchymal-like lines by EMT gene expression analysis, suggesting that this gene expression phenotype associates with a somewhat distinct underlying methylation signature. Importantly, the mesenchymal-like phenotype harbors a larger proportion of hypermethylated sites than the epithelial phenotype. This suggests that changes in methylation may be required to stabilize the phenotypic alterations acquired during an EMT in NSCLCs.

[0212] EGFR-mutant NSCLCs typically present as well-differentiated adenocarcinomas in the peripheral lung. Based on their epithelial-like expression phenotype and their characteristic histology, the EGFR-mutant cell lines behaved more similarly to epithelial-like lines than to mesenchymal-like lines. A segregation pattern of the cell lines based on in vitro sensitivity to erlotinib was noted (FIG. **3**, indicated by Sensitivity in the middle). Nearly all erlotinib-sensitive lines were associated with an epithelial-like phenotype whereas nearly all mesenchymal-like lines were resistant to erlotinib. However, not all epithelial-like lines were sensitive to erlotinib. Ten of the erlotinib-resistant lines clustered with the epithelial-like lines, and 4 erlotinib-sensitive lines, H838, H2030, RERF-LC-MS, and SK-MES-1, clustered with the mesenchymal-like lines. Notably, H838 and SK-MES-1 behaved as outliers with regard to erlotinib sensitivity when clustered by gene expression using our previously defined EMT expression signature (11). Some of the other outliers with respect to erlotinib sensitivity have mutations that explain their apparent resistance. For example, the epithelial-like line H1975 harbors a T790M mutation in EGFR and H1993 harbors an MET amplification. These genetic alterations confer resistance to erlotinib specifically, suggesting that the epigenetic signatures observed are surrogates for the biologic state of the cell line rather than for erlotinib sensitivity, per se.

Example 4

Sodium Bisulfite Sequencing of Selected DMRs Validates Infinium Methylation Profiling

[0213] 17 DMRs identified by Infinium (FIG. **4**) that were spatially associated with genes (in the 5' CpG island or intragenic) were examined for their methylation status by direct sequencing of cloned fragments of sodium bisulfite-converted DNA. 5 epithelial-like lines, 4 mesenchymal-like lines, and one intermediate line were selected for sequencing validation. Bisulfite sequencing of approximately 10 clones per cell line for 10 loci revealed that nearly all of these markers were almost completely methylated in at least 4 of the mesenchymal-like cell lines and in the intermediate line H522. In contrast, these loci were completely unmethylated in all 5 of the epithelial-like lines. Four of 10 markers that were methylated in mesenchymal-like lines, ESRP1 and CP2L3/GRHL2, miR200C, and MST1R/RON, are involved in epithelial differentiation (2, 27, 28). ESRP1 is an epithelial-specific regulator of alternative splicing that is downregulated in mesenchymal cells and CP2L3/GRHL2 is a transcriptional regulator of the apical junctional complex (27, 28); miR200C is a known negative regulator of the EMT inducer ZEB1 (29). ESRP1 and GRHL2 expression was downregulated in a larger panel of mesenchymal-like lines relative to all of the epithelial-like lines, consistent with the known absence of ESRP proteins in mesenchymal cells and the ability of these proteins to regulate epithelial transcripts that switch splicing during EMT. Pyrosequencing analysis indicated that GRHL2 was also hypermethylated in this broader panel of mesenchymal-like lines relative to epithelial-like lines.

Example 5

Biologic Relevance of DMRs

[0214] To evaluate the role of methylation in regulating expression of the genes associated with select DMRs, quantitative PCR was carried out in a panel of 34 5-aza-2'-deoxycytidine (5-aza-dC) and dimethyl sulfoxide-treated NSCLC cell lines. Not all DMRs were associated with obvious gene expression changes following 5-aza-dC treatment but a significant induction of GRHL2, ESRP1, and CLDN7 transcripts in mesenchymal-like versus epithelial-like lines were noted. From this group of genes, CLDN7 was selected as a representative marker of EMT and its methylation status was quantified by pyrosequencing in an extended panel of 42 cell lines. Nearly all of the mesenchymal-like lines were methylated at the CLDN7 promoter region and exhibited dramatic induction of CLDN7 expression (>10-fold) in response to 5-aza-dC treatment (FIGS. **5**A and B). In contrast, CLDN7 was expressed in the majority of the epithelial-like cell lines and was not induced further by 5-aza-dC treatment. These data show a direct link between locus-specific DNA hypermethylation and transcriptional silencing in a subset of genes associated with epithelial-like and mesenchymal-like states in NSCLC cell lines.

[0215] In FIG. **5**A, quantitative methylation was determined at 7 CpG sites by PyroMark analysis software using the equation: % methylation=(C peak height×100/C peak height+T peak height). Data are represented as the mean±SD percentage of methylation at 7 CpG sites. In FIG. **5**B, relative expression of CLDN7 mRNA was determined using a standard ΔCt method in 42 (n=20 epithelial-like, 19 mesenchymal-like, 3 intermediate) DMSO-treated and 5-aza-dC-treated NSCLC cell lines. Expression values were calculated as a fold change in 5-aza-dC-treated relative to DMSO-treated control cells. Data are normalized to the housekeeping gene GAPDH and represented as the mean of 2 replicates. DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Example 6

Quantitative MSP Classifies NSCLC Cell Lines into Epithelial and Mesenchymal Subtypes and Predicts for Erlotinib Sensitivity

[0216] Following independent validation of the methylation status of 17 markers by direct sequencing analysis, 70 NSCLC cell lines were analyzed to determine whether these markers could correctly classify epithelial-like and mesenchymal-like phenotypes. On the basis of sodium bisulfite sequencing analyses, methylated regions were selected that best distinguished the epithelial-like lines from mesenchymal-like lines and quantitative methylation-specific PCR (qMSP) assays were designed based on TaqMan technology. qMSP was used as an assay platform because it has been shown to have use in detecting tumor-specific promoter hypermethylation in specimens obtained from patients with cancer. This method is highly sensitive and specific for quantifying methylated alleles and is readily adaptable to high-throughput formats, making it suitable for clinical applications (30-33). TaqMan technology is superior to SYBR-based designs for MSP due to the increased specificity of the assay imparted by the fluorescent probe, which does not act as a primer. To normalize samples for DNA input, a bisulfite-modified RNase P reference assay was designed to amplify input DNA independent of its methylation status. Titration curves were conducted using control methylated DNA, DNA derived from peripheral blood monocytes (N=20), and DNA from cell lines with known methylation status for each DMR. Of note, nearly all of the assays developed resulted in essentially binary outputs for the presence or absence of methylation, which obviates the need for defining cutoff points.

[0217] Thirteen candidate markers of epithelial (E) or mesenchymal (M) status were tested to determine if they differentiated epithelial-like from mesenchymal-like cell lines based on the EMT gene expression classification, including RON/MST1R (M), STX2 (M), HOXC5 (M), PEX5L (E), FAM110A (M), ZEB2 (E), ESRP1 (M), BCAR3 (E), CLDN7 (M), PCDH8 (E), NKX6.2 (M), ME3 (E), and GRHL2 (M). Ten of 13 markers were significantly associated with epithelial-like or mesenchymal-like status in using a $P<0.05$ cutoff value (FIG. **6**). In this assay, qMSP assays were used to determine methylation in epithelial-like (n=36) and mesenchymal-like (n=34) NSCLC cell lines. Total input DNA was normalized using a bisulfite-specific RNase P TaqMan probe. In FIG. **6**, methylation levels are plotted as $-\Delta C_t$ (indicated target gene-RNase P) for each sample on the y-axis. An increasing $-\Delta C_t$ value indicates increasing methylation. Cell lines are grouped by epithelial-like/mesenchymal-like status on the x-axis. P values were determined using a 2-tailed, unpaired Student t test. Receiver operating characteristic (ROC) plots for (B) RON, (D) FAM110A, (F) GRHL2, and (H) ESRP1 are presented. P values were determined using a Wilcoxon rank-sum test.

[0218] These same markers were examined to determine if they are predictive of erlotinib sensitivity in vitro. Seven of 13 DMRs were strongly predictive of erlotinib resistance (individual $P<0.005$; FIG. **7**) and 3 of 13 DMRs, PEX5L, ME3, and ZEB2, were significantly associated with an epithelial phenotype but were not statistically predictive of erlotinib sensitivity. In this assay, qMSP amplification of 58 NSCLC cell line DNA samples was performed using the indicated qMSP assays. ROC curves for erlotinib sensitive versus erlotinib resistant cell lines were generated using R statistical software. P-value was determined using a Student's t-test. FIG. **7**A-M and FIG. **8**A-B.

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INCORPORATION BY REFERENCE

[0267] All patents, published patent applications and other references disclosed herein are hereby expressly incorporated herein by reference.

EQUIVALENTS

[0268] Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims. The term "comprising" as used herein is non-limiting and includes the specified elements without limiting to inclusion of further elements.

What is claimed is:

1. A method of determining whether a tumor cell has a mesenchymal phenotype comprising detecting the presence or absence of methylation of DNA at a CpG site in at least one gene selected from the group consisting of CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, ERBB2, and C20orf55, wherein the presence of methylation at the CpG site indicates that the tumor cell has an mesenchymal phenotype.

2. A method of determining the sensitivity of tumor growth to inhibition by an EGFR kinase inhibitor, comprising detecting the presence or absence of methylation of DNA at a CpG site in at least one gene selected from the group consisting of CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, ERBB2, and C20orf55 in a sample tumor cell, wherein the presence of methylation at the CpG site indicates that the tumor growth is resistant to inhibition with the EGFR inhibitor.

3. A method of identifying a cancer patient who is likely to benefit from treatment with an EFGR inhibitor comprising detecting the presence or absence of methylation of DNA at a CpG site in at least one gene selected from the group consisting of CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, and C20orf55 in a sample from the patient's cancer, wherein the patient is identified as being likely to benefit from treatment with the EGFR inhibitor if the absence of DNA methylation the CpG site is detected.

4. The method of claim **3**, further comprising administering to the patient a therapeutically effective amount of an EGFR inhibitor if the patient is identified as one who will likely benefit from treatment with the EGFR inhibitor.

5. A method of treating a cancer in a patient comprising administering a therapeutically effective amount of an EGFR inhibitor to the patient, wherein the patient, prior to administration of the EGFR inhibitor, was diagnosed with a cancer which exhibits absence of methylation of DNA at a CpG site in at least one gene selected from the group consisting of CLDN7, HOXC4, P2L3, TBCD, ESPR1, GRHL2, and C20orf55.

6. The method of any one of claims **2-5**, wherein the EGFR inhibitor is erlotinib, cetuximab, or panitumumab.

7. A method of determining whether a tumor cell has an epithelial phenotype comprising detecting the presence or absence of methylation of DNA at a CpG site in at least one gene selected from the group consisting of the PCDH8, PEX5L, GALR1, and ZEB2, wherein the presence of methylation the CpG site indicates that the tumor cell has an epithelial phenotype.

8. The method of claim **1**, wherein the presence or absence of methylation is detected by pyrosequencing.

9. The method of claim **1**, wherein the DNA is isolated from a formalin-fixed paraffin embedded (FFPE) tissue or from fresh frozen tissue.

10. The method of claim **9**, wherein the DNA isolated from the tissue sample is preamplified before pyrosequencing.

11. The method of claim **1** or **2**, wherein the tumor cell is a NSCLC cell.

12. The method of claim **3**, wherein the cancer is NSCLC.

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