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Clinical Significance of Hypermethylation Status in NSCLC: Evaluation of a 30-Gene Panel in Patients with Advanced Disease

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Abstract. *Background: DNA methylation is one of major factors in cancer progression. We observed multiple genes involved in cancer-related signaling and focused on patients with advanced non-small cell lung cancer (NSCLC) and evaluated methylation in relation to various clinical parameters. Patients and Methods: Thirty genes were examined in 121 NSCLC patients using the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) method. Correlations to gender, smoking status, tumor subtype, disease stage and EGFR/KRAS mutation status were performed by chi-square test. Results: 90% of tumors exhibited methylation of at least one gene. Most frequently methylated were cadherin-13 (CDH13), Ras associated domain-containing protein (RASSF1A), Wilms' tumor protein (WT1), adenomatous polyposis coli protein (APC), paired box protein Pax-5 (PAX5), estrogen receptor (ESR1), an inhibitor of cyclin-dependent kinase p15 (CDKN2B), paired box protein Pax-6 (PAX6), transcription factor GATA-5 (GATA5) and cell adhesion molecule 4 (IGSF4). Overall methylation (any gene) was increased in adenocarcinomas (p=0.0329), unrelated to gender or disease stage. Several genes exhibited variable methylation with gender (CDH13, p<0.001; GATA5, p=0.02; PAX6, p=0.01 and ESR1, p=0.03), smoking (CDH13, p=0.002), or epidermal growth factor receptor (EGFR) mutation status [Von Hippel-Lindau disease tumor supresor (VHL), p=0.001; CDKN2B, p=0.02; CDH13, p=0.02; APC, p=0.04 and ESR1, p=0.04]. Conclusion: Differences in gene methylation associated with gender, smoking and EGFR mutation suggest potential for prediction in relation to management of tyrosine kinase inhibitor therapy.*

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DNA hypermethylation is a key epigenetic mechanism regulating gene transcription in living cells. DNA of cells in normal tissue exhibits methylation of all cytosines adjacent to glycines. The methylated cytosine-guanine sequences (termed CpG islands) prevent binding of most transcription factors resulting in effective repression of the transcription of most genes (1-3). In tumor cells, methylation-associated silencing of vital tumor-supressor genes results in facilitation of tumor development. Methylation-associated silencing may play a role in cellular systems responsible for (i) cell cycle control, (ii) tumor cell proliferation and differentiation, (iii) cell adhesion, invasion, metastasis and (iv) regulation of apoptosis, as well as (v) DNA repair gene transcription and detoxification of DNA adducts, such as those induced by cancer chemotherapy.

Studies of DNA methylation in lung cancer are aiming at several clinical applications including a) screening for cancer predisposition and primary diagnostics, b) monitoring of disease progression and early detection of recurrence, and c) prediction of response to anticancer therapy. Several reports of methylation of various genes in lung carcinomas have previously been reported (4-6). The most frequently reported genes include cadherin-13 (*CDH13*), retinoic acid receptor (*RARB*), Ras associated domain-containing protein (*RASSF1A*), adenomatous polyposis coli protein (*APC*), tumor protein p16 (*CDKN2A*), O^6-methyltransferase-DNA methylguanin (*MGMT*), and E3 ubiquitin-protein ligase (*CHFR*) (7). Studies investigating various genes have proven that the methylation of more than three genes is associated with a 6.5-fold greater likelihood of carcinoma presentation (8). In addition, patients with concurrent methylation of more genes have greater risk of relapse (9). Aberrant methylation of *14-3-3σ* (*SFN*) gene was found to increase sensitivity to cisplatin plus gemicitabine therapy in patients with advanced-stage non-small cell lung cancer (NSCLC) (10). Recently, the methylation status of selected genes has also been examined on cell-free tumor DNA, circulating in peripheral blood of lung cancer patients (11).

The objective of this study was to analyze DNA methylation of an extensive panel of genes in cytology samples from NSCLC tumors in patients with locally advanced disease. In addition to investigating the spectrum of methylation, we sought for correlation between the methylation status and clinically relevant parameters of tumor subtype, disease stage, smoking and epidermal growth factor receptor (*EGFR*)/c-Kirsten-ras protein (*KRAS*) mutation status.

Patients and Methods

We investigated patients with morphologically proven progressive NSCLC who underwent cancer treatment in the course of their disease. The cohort consisted of 121 patients (71 males and 50 females) aged between 28 and 83 years with a median age of 63 years. Of these, 99 were smokers and 22 non-smokers. There were 75 adenocarcinomas, 35 squamous cell carcinomas, 6 anaplastic carcinomas and 5 unspecified/non-differentiated carcinomas of stage III (47) and IV (76). Eleven tumors (9.7%) were *EGFR* mutation positive and 17 tumors (14%) were *KRAS* positive.

Tissue samples from patients with clinically confirmed NSCLC having progressed on chemotherapy and targeted therapy were collected and processed as either cytology slides or formalin fixed paraffin embedded (FFPE) sections. Genomic DNA for methylation analysis was extracted by standard spin-column method using JetQuick tissue isolation kit (Genomed, G.m.b.H, Loehne, Germany). Hypermethylation was evaluated by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) using a combination two commercial kits (SALSA ME001-C1 and ME002-A1, MRC Holland, Amsterdam, the Netherlands). The evaluated panel consisted of 30 genes listed in Table I.

Mutual relations between methylation of genes and sex, cancer type and stage, smoking status and response to therapy were studied in detail. Statistical analysis was performed using contingency tables, chi-square test, Wilcoxon test and Kruskal-Wallis analysis. In addition, we evaluated the effect of hypermethylation in regard to the above factors after segregating the tumors according to *EGFR* and *KRAS* mutation status.

Results

The typical result from methylation-specific restriction approach by MS-MLPA approach is shown in Figure 1. The upper trace (A) shows a sample without hypermethylation, displaying uncleaved intact fragments for all tested genes. The botton trace (B) shows the same set of tested fragments, but in addition, several extra peaks (*CDH13*, Wilms' tumor protein (*WT1*) and paired box protein Pax-6 (*PAX6*) in this case), can be observed as products of methylation-specific restriction. The intensity of the extra peaks is directly related to the methylation level – *i.e.* the content of cells bearing methylated DNA in the studied sample.

Overall methylation rates. A total of 109 out of 121 (90%) tumor samples exhibited methylation of at least one gene. In adenocarcinomas, the number of samples with methylation was 93% (70 out of 75), which is significantly higher compared to squamous cell carcinomas, with 77% of methylated samples (27 out of 35; chi-square=4.549, p=0.0329). *EGFR*-mutated tumors exhibited methylation in al cases (11 out of 11) and *KRAS*-mutated samples in 88.2% cases (15 out of 17). When comparing males *vs.* females, no significant difference in overall methylation rates was found, with 64% *vs.* 65% (males *vs.* females, respectively). Similarly, overall methylation rates were not related to the disease stage, with 96% in stage III and 87.5% in stage IV.

Methylation of specific genes. As shown in Figure 2, the most frequently methylated genes, with frequency of at least 10%, were *CDH13* (48%, 58/120), *RASSF1A* (32%, 36/114), *WT1* (31%, 28/89), *APC* (30%, 35/115), paired box protein Pax-5 (*PAX5*) (27%, 24/89), *ESR1* (25%, 28/111), *CDKN2B* (25%, 27/107), *PAX6* (19%, 17/90), transcription factor GATA-5 (*GATA5*) (14%, 13/91) and *IGSF4* (12%, 14/119). In the majority of tumors, the methylation affected more than a single gene with the most common combinations of *CDH13* with *WT1*, *APC* with *WT1, CDH13* with *PAX6* and *APC* with *CDH13*.

Methylation of *CDH13* was more frequent in females compared to males (67% *vs.* 36%; 33/49 *vs.* 25/69; chi-square=0.98, p<0.001), in non-smokers compared to smokers (80% *vs.* 43%; 16/20 *vs.* 42/98; chi-square=0.96, p=0.002) and in *EGFR*-positive tumors compared to *EGFR*-negative ones (82% *vs.* 46%; 9/11 *vs.* 49/107; chi-square=0.88, p=0.02). Several other genes were also more frequently methylated in females compared to males, including *GATA5* (25% *vs.* 7%; 9/36 *vs.* 4/55; chi-square=0.89, p=0.02), *PAX6* (32% *vs.* 11%; 11/34 *vs.* 6/55; chi-square=0.91, p=0.01) and *ESR1* (36% *vs.* 18%; 16/44 *vs.* 12/67; chi-square=0.87, p=0.03). *EGFR*-mutated tumors also had higher methylation rates of *APC* (7/11 *vs.* 34/103; chi-square=0.83, p=0.04), *CDKN2B* (6/11 *vs.* 21/98; chi-square=0.90, p=0.02), *ESR1* (5/10 *vs.* 23/111; chi-square=0.85, p=0.04) and *VHL* (1/11 *vs.* 0/110; chi-square=0.87, p=0.001) compared to tumors without *EGFR* mutation (64% *vs.* 33%, 55% *vs.* 21%, 50% *vs.* 23% and 9% *vs.* 0%, respectively).

Discussion

Our observations of the most hypermethylated genes in NSCLC may be correlated with similar results which were described by other authors (4, 12-16). On the studied gene set, the average cumulative methylation rate was 90%, leaving only 10% of lung tumors with none of the 30 studied genes methylated. In a separate project, we only found methylation of *CDKN2B* gene in normal tissue (data not shown). It is known that methylations rarely occur in normal non-cancerous lung tissue (14), and is therefore seen as an early sign of malignant transformation, especially in smokers

Table I. *List of genes evaluated for methylation status in non-small cell lung cancer samples*.

Gene	Chromosome	Name of protein	Protein localization	Function
APC	5q21-q22	Adenomatous polyposis coli protein Apc	Cell membrane	Signal transduction–Wnt, cell cycle regulation, cell adhesion and migration
CDKN2A	9p21	Tumor protein p16(INK4A)	Cytoplasm, nucleus	Regulation of cell cycle (stop in G_1 and G_2 phase), involved in proliferation and apoptosis
RASSF1	3p21.3	Ras associated domain-containing protein	Cytoplasm, nucleus	Inhibition of proliferation, apoptosis
CDH13	16q23.3	Cadherin-13	Cell membrane	Cell adhesion (vascular control functions)
MGMT	10q26	O^6-Methyltransferase-DNA methylguanin	Nucleus	DNA repair (removing of alkyl-groups from the guanine rest)
CHFR	12q24.33	E3 ubiquitin-protein ligase	Nucleus	Cell cycle regulation (checkpoint for entry into the metaphase of cell division)
RARB	3p24	Retinoic acid receptor	Cytoplasm, nucleus	Regulation of cell cycle and growth
CDKN2B	9p21	p15, an inhibitor of cyclin-dependent kinase	Cytoplasm, nucleus	Regulation of cell cycle (stop in G1 phase), participation in cell differentiation, senescence and hematopoiesis
WT1	11p13	Wilms' tumor protein	Cytoplasm, nucleus	Transcription factor-Wnt signaling pathway
IGSF4 (CADM4)	19q13.31	Cell adhesion molecule 4	Cell membrane	Cell adhesion
GATA5	20q13.33	Transcription factor GATA-5	Nucleus	Transcription factor–VegT signaling pathway
PAX5	9p13	Paired box protein Pax-5	Nucleus	Transcription factor–Notch signaling pathway, B-cells
PAX6	11p13	Paired box protein Pax-6	Nucleus	Transcription factor–Shh, Notch and EGFR signaling pathway
TP73	1p36.3	Tumor protein p73	Nucleus	Transcription factor, cell development, apoptosis
TP53	17p13.1	Tumor protein p53	Cytoplasm, nucleus, endoplasmatic reticulum	p53 signaling pathway, regulation of cell cycle, apoptosis
MSH6	2p16	DNA mismatch repair protein Msh6	Nucleus	Mismatch repair
TIMP3	22q12.1-q13.2;q12.3	Inhibitor of metalloproteinase	Extracellular matrix	Antiapoptic function, promote of proliferation
THBS1	15q15	Thrombospondin-1	Cell membrane	Cell adhesion (interactions of cell–cell and cell–matrix)
ESR1	6q25.1	Estrogen receptor	Nucleus	Regulation of cell proliferation
CASP8	2q33-q34	Caspase-8	Cytoplasm	Apoptosis (caspase activation)
RB1	13q14.2	Retinoblastoma-associated protein Rb1	Nucleus	Regulation of cell cycle, cell differentiation
MLH1	3p21.3	DNA mismatch repair protein Mlh1	Nucleus	Mismatch repair
ATM	11q22-q23	Serin-protein kinase	Nucleus, cytoplasmatic vesicles	Regulation of cell cycle (inhibition as a response to double-stranded DNA breaks)
VHL	3p25.3	Von Hippel-Lindau disease tumor supresor	Cytoplasm, nucleus, cell membrane	Regulation of cell cycle (repression of transcription, protein degradation)
DAPK1	9q34.1	Death-associated protein kinase 1	Cytoplasma	Apoptosis
BRCA1	17q21	Breast cancer typ 1 susceptibility protein	Cytoplasm, nucleus	Repair of damaged DNA
PTEN	10q23.3	Phosphatidylinositol-3,4,5-triphosphate-3-phosphatase and dual-specific protein phosphatase	Cytoplasm, nucleus	Signal pathway–PTEN/PI3K/AKT, cell cycle regulation
CD44	11p13	CD44 antigen	Cell membrane	Cell adhesion, migration, activation of lymphocytes, hematopoiesis
GSTP1	11q13	Glutathione S-transferase P		Detoxification of endo- and exogenous substances
STK11	19p13.3	Serin/threonin-protein kinase11	Cytoplasm, nucleus	Regulation of cell cycle (stop in G_1 phase)

(17). The high frequency of methylated genes found in our study only further supports such a concept. We also correlated the results of methylation with clinical parameters such as sex, smoking status, disease stage, duration of overall survival and disease-free period. Several conclusions arose from these correlations. We confirmed previous reports that there are differences in the frequency of methylation among the particular histological types of lung cancer (18, 19) and

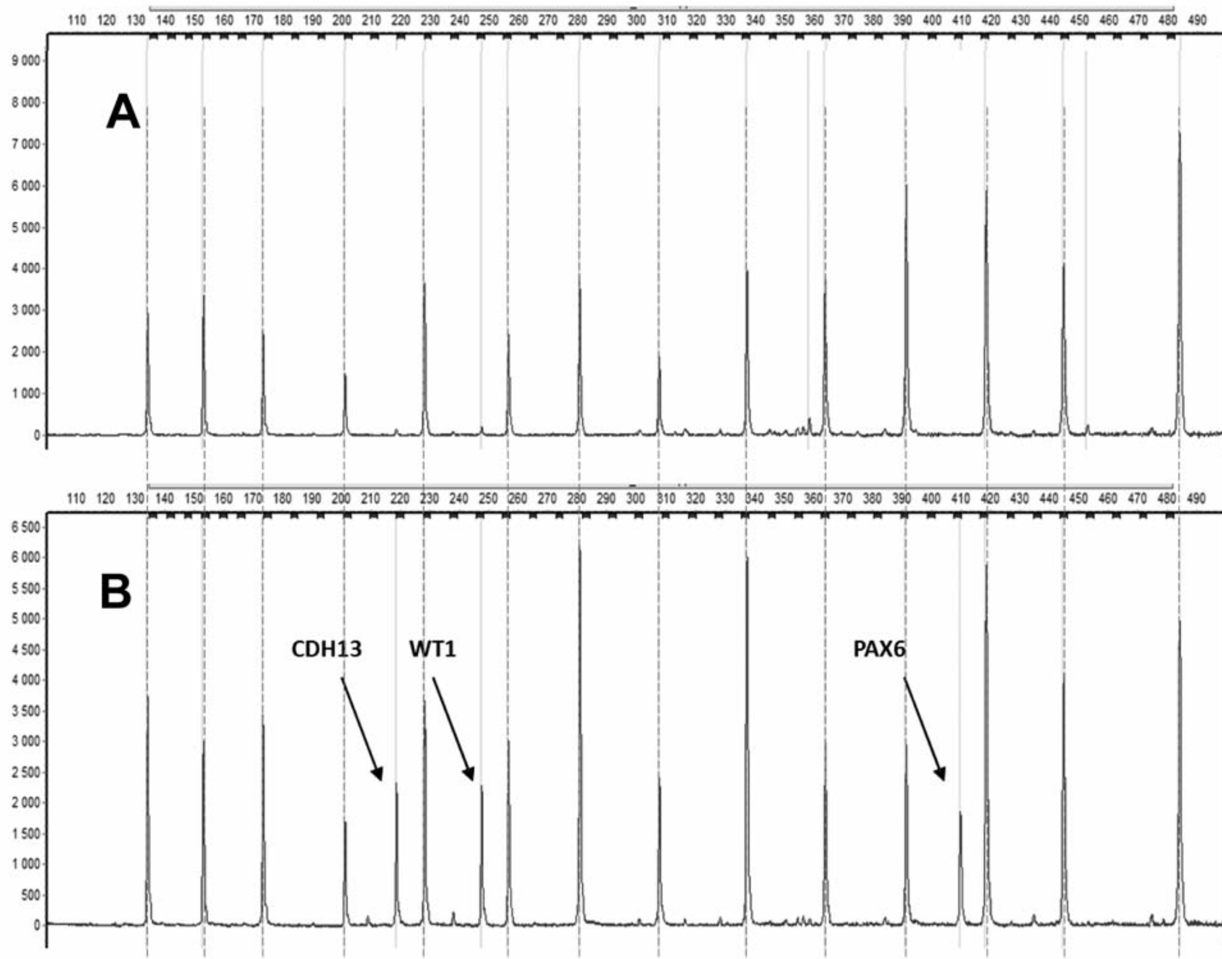

Figure 1. *Result of methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) analysis showing a sample without methylation (A) and a sample with hypermethylation of cadherin-13 (CDH13), Wilms' tumor protein (WT1) and paired box protein PAX-6 (PAX6) genes. The uncleaved fragments can be observed in both samples (connected by dotted lines), but only a sample with methylation reveals the additional fragments as a result of the methylation-specific cleavage.*

between specific subtypes of NSCLC. In our samples, we detected both the individual and combined gene methylation more frequently in adenocarcinomas, which correlates with results of other authors (20).

Differences in frequency of gene methylation were also found depending on the gender of patients. In our case, however, other genes were methylated [more frequent methylation of *CDH13, GATA5, PAX6* and *ESR1* in women and caspase-8 (*CASP8*) in men] unlike in literature, where more frequent methylation of *RASSF1A* (21) and *RARB* (22) are described in men and death-associated protein kinase 1 (*DAPK1*) gene methylation in women (23).

The occurence of methylation changes and the presence of *EGFR* and *KRAS* mutations are interesting from the perspective of the now widely used biological therapy in locally advanced and metastatic non-small cell carcinomas (24). Despite the significant efficacy of therapy, there is recurrence or progression of originally tyrosine kinase inhibitor (TKI) sensitive *EGFR*-mutated tumors within months or even years. In addition to the TKI-resistant *EGFR* and *KRAS* mutations (25, 26), it can be assumed that the failure of treatment may also be caused by epigenetic factors, such as DNA methylation. In our study, we related methylation analysis of 30 selected genes with the analysis of *EGFR* and *KRAS* mutations, which may also have a negative prognostic significance under certain circumstances (27). Methylation was more frequent in carcinomas containing mutations in the *EGFR* gene. Patients with *EGFR* mutations had a greater percentage of methylation of *APC, CDKN2B, ESR1* and *VHL* genes.

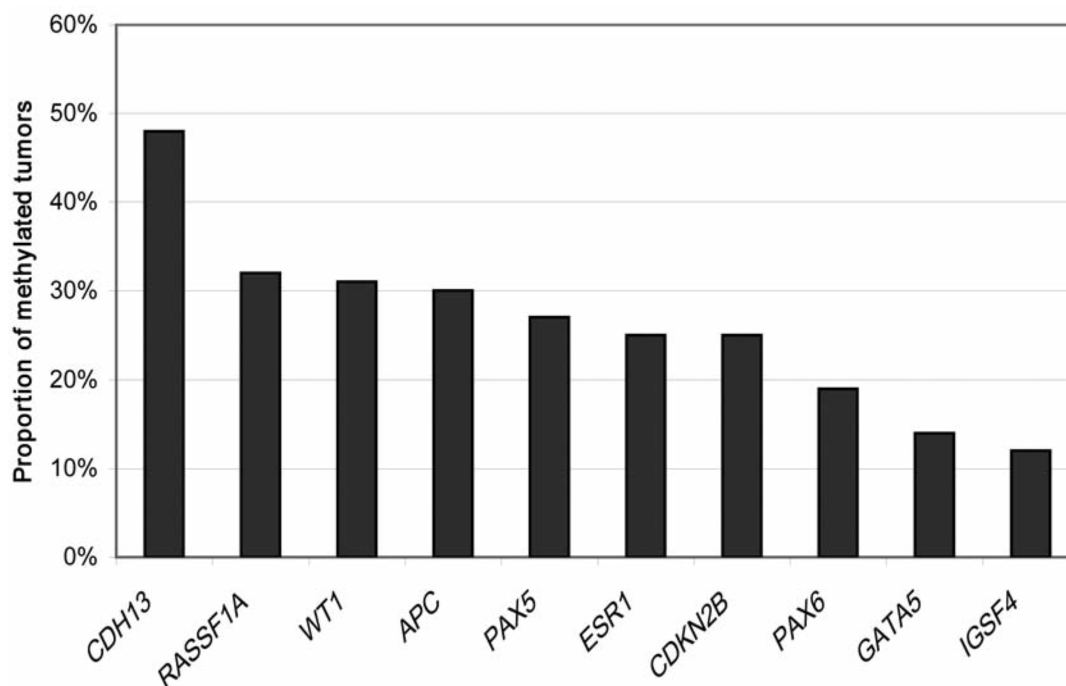

Figure 2. *Genes with the most frequent methylation found in a set of 111 non-small cell lung cancer samples (See Table I for gene names).*

We observed methylation of several genes to be linked to factors such as female gender, smoking status and *EGFR* mutation, which have positive predictive and prognostic value in relation to therapy by TKIs. Among them, methylation of *CDH13* was found to be associated with all three factors. This gene was found to be more frequently methylated in nonsmokers ($p<0.01$).

The above findings only further suggest a complex interaction between genetic and epigenetic changes in the process of lung adenocarcinoma tumorigenesis. In view of the fact that methylation is, as opposed to DNA mutation, a reversible event, it is possible to consider the use of demethylating agents in the treatment for patients with methylation-positive tumors. For example, 5-aza-2'-deoxycytidine (Decitabine) or hydralazine represent such demethylators. Properly chosen inhibitors would therefore represent a complementary form of chemotherapy in the future in the effective treatment of solid tumors.

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