Analysis of Methylation Patterns of Some Tumor Suppressor Genes in Non-Small Cell Lung Cancer Using the Multiplex Ligation-Dependent Probe Amplification [MLPA] Method

Küçük Hücreli Olmayan Akciğer Kanserleriyle İlişkilendirilmiş Tumor Süpressör Genlerin Multiplex Ligation-Dependent Probe Amplification (MLPA) Yöntemi ile Metilasyon Paternlerinin İncelenmesi

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Abstract

Lung cancer has the leading mortality rate among all cancer types and is the second most common cause of death after cardiovascular diseases. Apart from being an epigenetic mechanism, methylation is also a molecular mechanism which inhibits cancer-related gene expression although there is no mutation at DNA level. The aim of this study was to analyze a probe panel interrogated DNA for methylation patterns in 24 tumor suppressor genes in non-small cell lung cancer using the Methylation Specific- Multiplex Ligation-dependent Probe Amplification [MS-MLPA] method the Multiplex Ligation-dependent Probe Amplification [MLPA] method. Previously examined clinically and histopathologically diagnosed a hundred cases with “non-small cell lung cancer”were included into the study. The DNAs were extracted samples from both cancerous tissues of the cases and their corresponding control tissues. The relations of the methylation profile to clinicopathological factors in NSCLC were evaluated. The genes frequently methylated in NSCLC including *CDKN2B, BRCA1, CDH13* and *HIC1* were also hypermethylated in surrounding nontumorous lung tissues. However, hypermethylated *APC, CDKN2A, MLH1, RARB, CHFR* and *GSTP1* probe regions were only specific to the tumorous tissues of the cases. The aberrant methylation profile of the *CDH13* probe region was only detected in the surrounding normal tissues and the difference was statistically significant. Methylation rates for ATM, RARB, CDKN2B, HIC1, CDKN1B, PTEN, VHL and APC were different between squamous and adenocarcinomas. Almost all hypermethylated probe regions were detected in higher grade tumors but CDKN2B hypermethylation seemed to be an early event in NSCLCs. In conclusion, MS-MLPA can be used to determine aberrant methylation patterns of specific genes in lung tumors. However, since MS-MLPA is a screening test, the confirmation and expression analysis by using different approaches are necessary.

Key Words: Lung cancer,tumor-cuppressor gene, methylation, MS-MLPA.

Özet

Akciğer kanseri, tüm dünyada mortalitesi en yüksek kanser türüdür ve kardiyovasküler hastalıklardan sonra ölüm nedenleri arasında 2. sırada yer almaktadır. Metilasyon bir epigenetik mekanizma olup DNA düzeyinde herhangi bir mutasyon olmamasına rağmen ilgili genin eksprese olmasını engelleyen bir moleküler mekanizmadır. Çalışmada henüz çok yeni bir yöntem olan Multiplex Ligation-dependent Probe Amplification (MLPA) tekniği ile akciğer kanserleriyle daha önce ilişkilendirilmiş 24 ayrı tümör süpressör genin promoter bölgelerinin metilasyon paternlerinin SALSA MS-MLPA ME001 tumorsuppressor probemix kiti kullanılarak incelenmesi amaçlanmıştır. Bu çalışmaya İstanbul Yedikule Göğüs Hastalıkları Hastanesinde, histopatolojik olarak incelenerek “küçük hücreli olmayan akciğer kanseri” tanısı almış 100 olgu dahil edildi..Her hastaya ait doku örneklerinden kanser dokusu içeren 5 mikronluk kesitlerden elde edilen DNA örnekleri çalışma grubu olarak, aynı hastaya ait kanser dokusu içermeyen kesitlerden elde edilen DNA örnekleri de kontrol grubu olarak çalışmaya alındı. Çalışmada, akciğer kanserli tümöral ve çevre akciğer dokularında birbirlerine yakın oranlarda en sık CDKN2B, BRCA1, CDH13 ve HIC1 prob bölgelerinde metilasyon görüldü. Yine tümöral ve çevre dokularda PTEN, TIMP3, ATM, VHL, CD44, CDKN1B, RASSF1, IGSF4 ve ESR1 prob bölgelerinde de metilasyon saptandı. APC, CDKN2A, MLH1, RARB, CHFR ve GSTP1 prob bölgelerinde farklı oranlarda, tümöral dokularda metilasyon görülürken, çevre akciğer dokularında bu prob bölgelerinde metilasyon saptanmadı. Sonuç olarak MS MLPA yönteminin akciğer kanserlerinin metilasyon profillerinin taramasında kullanılabilecek, ucuz ve hızlı sonuç verebilen bir teknik olduğu görülmüştür. MS MLPA yönteminin diğer metilasyon tarama yöntemleriyle karşılaştırılarak bir an önce senstivite ve spesivitesinin belirlenmesi ve akciğer kanserinin erken tanısında rutin kullanıma geçirilmesi gerektiğini düşünmekteyiz.

Anahtar Kelimeler: Akciğer Kanseri, Tümör Süpressör Gen, Metilasyon, MS-MLPA

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Introduction

Lung cancer is the most common and major cause of cancer-related death and it has one of the lowest survival outcomes of any cancer because over two-thirds of patients are diagnosed at a late stage when curative treatment is not possible [1].

It is clear from previous studies that lung cancer development is characterized by sequential accumulation of genetic abnormalities [2,3]. Previously, it had been implicated that not only genetic mutations and polymorphisms, but also epigenetic alterations play important roles in lung cancer etiology. Recently, several studies have emphasized the potential of hypomethylation for unmasking the expression of putative oncogenes in tumor development [4,5]. In contrast to hypomethylation, focal hypermethylation contributes to cancer initiation and progression by transcriptional inactivation of tumor suppressor genes (TSGs) [6]. The promoter-specific hypermethylation silences the normal functions of TSGs during tumorigenesis and this epigenetic process is considered to be the major gene-silencing mechanism for TSGs along with genetic mutations.

Different methods and various types of samples such as sputum, blood, bronchial lavage, tumor tissues etc. have been used to determine the methylation status of genes in NSCLCs and therefore there is huge information related to the methylation patterns of genes in lung cancer. The methylation specific polymerase chain reaction (MS-PCR) and bisulfite genomic sequencing are the most frequently used methods whereas Vaissiere et al. and Hawes et al. have analysed the methylation status of genes quantitatively [7,8]. However, the question which method is optimal is still unanswered. Moreover, it has been shown that the sensitivity and specifity of the method are higher if the panel of markers is used instead of single gene analysis. However, a new question is arising: Which genes does the panel include for the highest sensitivity and specifity for NSCLCs? The multicandidate gene approaches specific to NSCLC are desirable in order to find new targets of CpG methylation.

Recently, MLPA has shown to be a reliable approach for the diagnosis of genetic diseases characterized by genomic copy aberrations. A recent extension of this approach is the methylation specific MLPA [MS-MLPA]. It allows simultaneous assessment of aberrant promoter methylation of 24 genes. The method has the ability to detect aberrant methylation at the specific CpG sites on the basis of digestion with methylation specific restriction enzymes. It is an easy and rapid method for detection of 24 genes at a time by using small amounts of DNA. Although several studies have evaluated the question of the sensitivity of the MS-MLPA in different tumor samples such as meningiomas, breast cancers and colon cancers, concordant results with conventional methods (MS-PCR, bisulphite sequencing, MethylLight) have been obtained for the specific CpG sites.

The present study is one of the first to apply genome-wide MS-MLPA in primary NSCLCs. The study was addressed to determine methylated TSGs in NSCLCs with different grades and to help further studies for determining of a panel of markers specific to NSCLC.

Materials and Methods

Study population

Methylation profiles of 24 genes were analysed in a hundred archival samples of tumors and their corresponding normal tissues. The demographic variables including age, gender, family history and smoking habit were obtained from case files. The study was evaluated by the Ethics Committee of the University.

Pathology

Genomic DNAs were extracted from 100 formalin-fixed, paraffin-embedded tumors and their corresponding normal tissue specimens held in the archives of the Department of Pathology of the İstanbul Yedikule Hospital of Chest Medicine. Four serial sections were obtained from each specimen and the first and last sections were stained with hematoxylin and eosin stain and then reassessed by two pathologists who were both blinded to the previous clinicopathological findings. The remaining two sections including at least 70% tumor cells from each specimen and corresponding tumor-free sections were used for MS-MLPA analysis. The high-molecular-weight DNAs from 15- m tissue sections were isolated by using Pure Compact Nucleic Acid Isolation Kit (Roche) in Manga Pure Compact DNA extraction Robot (Roche) according to manufacturer's protocol. The cancerous tissues were admitted to the study as the sample group whereas surrounding normal tissues were regarded as control group.

MLPA analyses

Gene methylation status of the research and control groups were evaluated by Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) using the ME001 Tumor Suppressor Kit (MRC Holland). The probe mixed contained 26 probe sequences that correspond to a set of 24 tumor suppressor genes frequently silenced by methylation and also 15 control genes that lack a site during the methylation sensitive HhaI restriction digestion. Since the sequences of the probes designed to recognize HhaI sites in unmethylated regions have become cut by HhaI, unmethylated

probe recognition sequences in the sample do not generate a signal. Conversely, clear amplification signal is formed if the target site is methylated, because the MLPA probe binds completely to intact site. Fragments were separated and quantified by electrophoresis on an ABI 310 capillary sequencer and the Peak. Gene Mapper analysis software (both Applied Biosystems) is Peak identification and values corresponding to peak size in base pairs (bp), and peak areas were used for further data processing. Automated fragment and data analysis was performed by exporting the peak areas to an excel-based analysis program (Ezersoft). For hypermethylation analysis the 'relative peak value' or the so-called 'probe fraction' of the ligation-digestion sample is divided by the 'relative peak value' of the corresponding ligation (undigested) sample, resulting in a so-called 'methylation-ratio' (M-ratio). Aberrant methylation was scored when the calculated M-ratio was ≥0.30, corresponding to 30% of methylated DNA. The methylated ratios were interpreted as absence of hypermethylation (0.00-0.29), mild hypermethylation (0.30-0.49), moderate hypermethylation (0.50-0.69) and extensive hypermethylation (>0.70). In genes with more than one probe, their ratios were calculated independently for methylation analysis.

The methylation profiles of 24 tumor-suppressor genes were determined in 32 females and 68 males, totally a hundred cases by MS-MLPA technique. The mean age of the patients was 58,98± 3,4. Of the patients 90% were smokers and 26% had positive cancer history in their families.
In the histopathologic examinations, 46% were squamous cells (SCCs) and 54% were adenocarcinomas (ADCs). The grades and TNM classifications of the tumors are given in Table 1.

Table 1: Demographic and clinicopathologic information of the lung cases analyzed

	Clinical Parameters	Cases n (%)	
Age	<60	50 (50%)	
	>60	50 (50%)	
Gender	Male	68 (68%)	
	Female	32 (32%)	
Family History	Yes	26 (26%)	
	No	74 (74%)	
Smoking History	Yes	90 (90%)	
	No	10 (10%)	
Histology	Squamous Cell Carcinoma	46 (46%)	
	Adenocarcinoma	54	(54%)
Grade	I-II	14	(14%)
	III	86	(86%)

Tumorous and their corresponding normal tissues without hypermethylated genes
The peak images obtained from the case samples treated with Hhal methylation-specific enzyme were examined. The samples without loss or excess of peak were interpreted normal methylation profiles from the point of probe regions. Of all analysed cases, 20 (20%) showed normal methylation profiles for all 24 probe regions in both tumorous and nontumorous tissue samples. The peak images obtained from the patients having normal methylation profiles are given in Figure 1A and Figure 1B.

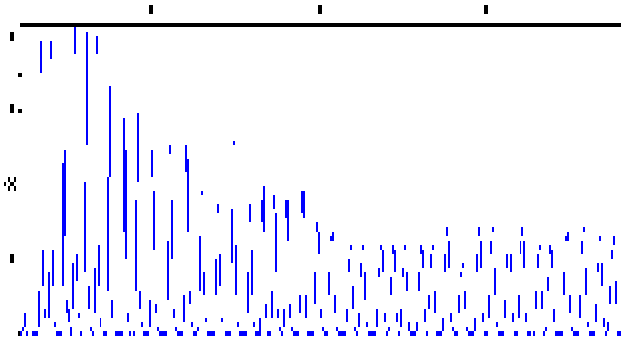


Figure 1A. MLPA image (before enzyme restriction) of the tumorous tissue of a patient having normal methylation profile

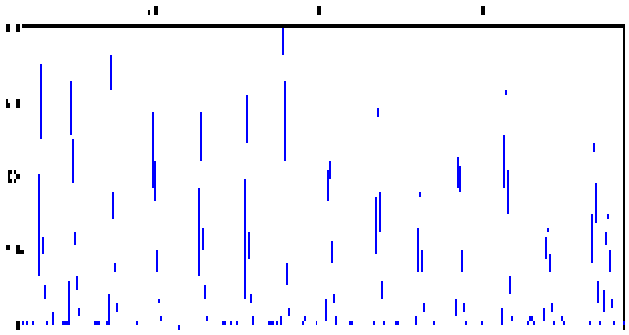


Figure 1B. The enzyme-restricted MLPA image of the tumorous tissue of a patient with a normal methylation profile.

Cases with hypermethylated genes in both tumorous and their corresponding normal tissues
The MS-MLPA analysis were revealed aberrantly methylated probe regions of the tumor suppressor genes including *CDKN2B, BRCA1, CDH13* and *HIC1* in both tumorous and their surrounding normal tissues of 44 cases Peak images of the the hypermethylated *CDKN2B* gene specific probe regions detected in both types of tissues of a case are given in Figure 2A and Figure 2B.

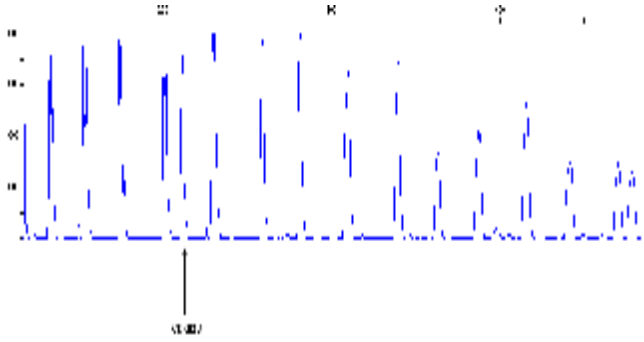


Figure 2A. Peak image of a patient found to have aberrant methylation in *CDKN2B* gene of the tumorous lung tissue.

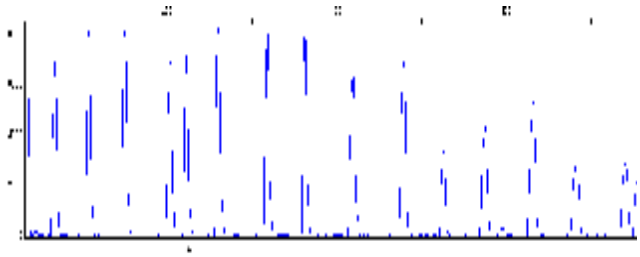


Figure 2B. Peak image of the surrounding lung tissue of the patient in Figure 2A.

Cases with hypermethylated genes in only tumorous tissues but not in their corresponding normal tissues

Of all analysed samples, hypermethylated profiles in *APC, CDKN2A, MLH1, RARB, CHFR* and *GSTP1* probe regions genes were revealed in only the tumorous tissues of 23 (23%) cases. The surrounding tumor-free tissues of these cases showed normal methylation specific peaks for above mentioned genes

Cases with normally methylated genes in tumorous tissues in contrast to aberrantly methylated tumor suppressor genes in their tumor-free tissues.

The frequencies of hypermethylated *CDH13* (%15, %7), *VHL* (%7, %4), *PTEN* (%9, %4), *CDKN1B* (%5, %4), *CASP8* (%2, %0), *BRCA1* (%17, %16) and *HIC1* (%11, %7) probe regions detected in the tumor-free normal tissues of 13 cases (13%) were higher than their corresponding tumor tissues.. The statistically significant difference was seen for the *CDH13* gene.

As seen in Table 2, the most frequently seen aberrantly methylated genes in tumorous and their surrounding tumor-free tissues were *CDKN2B* (64%), *BRCA1* (33%) and *CDH13 (22%).* When the hypermethylated gene frequencies were compared in respect to histopathological types of the NSLC samples, no difference was seen in the frequencies of hypermethylated *CDKN2B* and *BRCA1* genes. Their detection rates in both squamous and adenocarcinomas were similar (28.26%, 37.04% for *CDKN2B* and 14,8%,17.39% for *BRCA1*, respectively). However, hypermethylated *CDH13* gene frequency was higher in SCC tumors. Although no aberrant hypermethylation of APC gene was seen in SCCs, it was detected in four adenocarcinomas (7,4%). In contrast, abnormal *ATM, RARB, HIC1, VHL, CDKN1B* and *PTEN* gene methylations were determined in SCC tumors.

Unfortunately, of a hundred analysed samples, only 14 were Grade I-II tumors, the remainings were higher grade tumors. It was interesting that, although gene silencing of TSGs by hypermethylation is known as early events in the carcinogenesis, we could only determine *CDKN2B* gene methylation with higher frequency (42.86%). The rest of the genes were significantly seen in higher grade tumors.

Table 2: Methylation frequencies were observed in tumorous and tumor-free tissues.

Adenocarsinomas(N=54)				Squamous Cell Carsinomas (N=46)		
	Tumor	Normal	Total	Tumor	Normal	Total
TIMP3	4	2	6	5	2	7
APC	4	0	4	0	0	0
CDKN2A	1	0	1	2	0	2
MLH1	1	1	2	2	0	2
ATM	0	1	2	6	2	9
RARB	0	0	0	6	0	6
CDKN2B	20	16	36	13	15	28
HIC1	0	2	4	7	7	14
CHFR	0	0	0	2	0	2
BRCA1	8	9	17	8	8	16
CASP8	0	1	2	0	0	0
CDKN1B	0	0	0	4	5	9
PTEN	0	5	5	4	4	8
BRCA2	0	0	0	0	0	0
CD44	2	1	3	4	2	6
RASSF1	2	4	6	2	0	2
DAPK1	0	0	0	0	0	0
VHL	0	1	1	4	6	10
ESR1	0	2	2	2	0	2
TP73	0	0	0	0	0	0
FHIT	0	0	0	0	0	0
IGSF4	1	0	1	3	2	5
CDH13	2	10	12	5	5	10
GSTP1	0	0	0	2	0	2

Discussion

The DNA sequence of the NSCLC cell has acquired changes from an early progenitor cell which confer a growth advantage [9]. These changes include single base substitutions, insertions, deletions or translocations of DNA segments, copy number aberrations and epigenetic changes [10].

In comparison to normal cells, in cancer cells, hypomethylation is observed along with the genome and hypermethylation and loss of genomic imprinting is observed in some genes such as tumor-suppressor genes [11, 12, 13]. Promoter methylation was also detected in many genes of NSCLC tumorous tissues [14, 15].

$P16^{INK4a}$, $p15^{INK4b}$, Rb, $p14^{ARF}$ in cell cycle, BRCA1, MGMT, hMLH1 in DNA repair, *GSTP1* and glutathion S-transferase P1 in carcinogen metabolism, *CDH1* and *CDH13* in cell adherence and *DAPK* and *TMS1* in apopotosis could serve as examples to some of the genes exposed to abnormal DNA methylation [13, 16].

It is now well-known that hypermethylation at CpG sites within the promoters of tumor-suppressor genes has been verified as an important epigenetic mechanism of gene inactivation in cancer. Detection of aberrant hypermethylation at the earliest stages of carcinogenesis and presence of tumor-type specifity make think the possibility of cancer biomarker for early diagnosis and therapy. The presented study evaluated the usability of MS-MLPA approach to determine hypermethylated tumor suppressor genes in NSCLC and their surrounding tumor-free tissue samples. Our results showed that aberrant methylation is a frequently seen event in not only tumor tissues but also tumor-free surrounding normal tissues. Of all tumor tissues, 59% exhibited methylation of at least one gene tumor-free normal lung tissues were hypermethylated. Previous studies have reported that, inactivation of these genes due to methylation is likely permissive for the acquisition of additional genetic and epigenetic changes leading. or methylation in nonneoplastic epithelia may be different from the tumor or even occur in patients without malignancy [17-20].

Paluzczak et al have reported that methylation rate was found for FHIT, MGMT, DAPK, RARbeta, RASSF1A is common in laryngeal cancer cells and normal epithelial cells from epiglottis or trachea [21].

There is a lot of literature describing the existence of promoter hypermethylation in lung cancer [22-27]. A well studied example of lung cancer is the abnormal promoter methylation of the tumor suppressor gene *p16* (*CDKN2A*). This event is correlated to gene silencing and is an early occurring event in tumorigenesis. Methylation was observed in the probe region of this gene in three of the tissue samples tested in our study. The ratio we found is a considerably low ratio when compared to the literature. The methylation ratios of this gene in the literature vary between 25-41% [28]. It has been thought that these differences in the publications are related to gene-oriented specificities of methods used. Besides, Kim et al. has showed that methylation of *BRCA1* gene is closely related to homozygous deletion of *CDKN2A* gene and this makes clear how *CDKN2A* methylation ratio was found low in our study [29]. *BRCA1* gene methylatıon was the second frequently methylated gene seen in our study although not frequently reported gene in NSCLC tumor tissues.

The highest methylation rate was found for *CDKN2B*, *BRCA1* and *CDH13* in both tumorous and tumor-free surrounding tissues. Although Zhang et al. have reported 12 genes (*APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RARb, p16INK4A, RUNX3, hMLH1 and DAPK*) with high sensitivity and cancerous specificity, we could not detect abnormal hypermethylation patterns of *APC, RASSF1A, hMLH1, DAPK, CDKN2A* and *RARb* at higher frequencies [30]. However, when two different histopathological NSCLC tumor types were compared, *VHL, HIC1, CDKN1B, PTEN and RARB* were predominantly seen in squamous type tumors. Previous reports have the genes more frequently methylated in adenocarcinomas were: RARB, TWIST1, and CACNA1A, while the most commonly methylated genes in squamous tumors were SCGB3A1, ID4, SFRP4, SFRP5, DCL1, BNIP3, H2AFX, CACNA1G, TGIF, TIMP3 and BCL2. Statistically significantly different methylation rates were observed for ID4-2 ($p = 0.011$), DCL1 ($p = 0.019$), BNIP3 ($p = 0.003$), H2AFX ($p= 0.001$), H2AFX-2 ($p = 0.005$), CACNA1G ($p = 0.007$) and TIMP3 ($p = 0.021$) when comparing squamous versus adenocarcinoma cases [31]. Kontic et al have reported that methylation in tumors compared with normal lung tissue identified higher methylation of *CDH13, RASSF1A,* and *DAPK* genes, whereas *ESR1* and *MGMT* methylation did not differ significantly between these tissue types [32].

Another gene regulating the G1 phase in the cell cycle is $p15^{INK4b}$ (CDKN2B). In our study, we found the highest ratio of methylation in the promoter region of this gene. There are not so many studies in the literature oriented to this gene. In the study of Fronaka et al. [24], the methylation frequency of this gene in the tumorous tissues of NSCLC was reported to be 20%. Arvind et al. [33], on the other hand, found this ratio to be 13%. In our study, we found that this ratio was 33% in tumorous tissues and 31% in surrounding lung tissues.

The other examples are H-cadherin, death-associated protein (DAP), kinase 1 (DAPK1), 14-3-3 sigma and the candidate tumor suppressor gene RASSF1A. Although there is a description of methylation in the single cancer gene of many reports, there is no measurement which could determine the total distribution of promoter methylation existing in the lung carcinogenesis. As a reply, Zochbauer-Müller et al. showed methylation with varying degrees in their study on 107 patients of NSCLC including the genes of retinoic acid receptor β -2 (RAR β), tissue inhibitor of metalloproteinase 3 (TIMP3), p16, O^6-methylguanine-DNA methyl transferase (MGMT), DAPK1, E-cadherin (ECAD), p14ARF and glutathione S-transferase P1 (GSTP1) (34). According to the reports, the DNA methylation event is a diagnostic marker for some stages or types of tumors and the DNA methylation was determined to be a molecular marker. In parallel to these studies, methylation was observed with different ratios in the common genes examined in our study. Promoter methylation and gene inactivation do not occur classically only in the cell proliferation

of the tumor suppressor genes. For instance, silencing of RASSF1A, FHIT, RIZ1, FUS1, SEMA3B and C/EBPα genes is reported in lung tumors; however, tumor suppressor function is still taken into account. Silencing of the genes by promoter methylation may result in weak or no tumor suppressing activity. This includes target genes which have cellular function in DNA repair or drug metabolism such as MLH1, MGMT and GSTP1. That being said, silencing of these genes or gene groups could make contributions to the overall malignant phenotypes. In our study, no methylation was observed in DAPK1 and FHIT genes examined in this group.

Another gene in which we detected a high ratio of methylation is the BRCA1 gene. The mutations observed in this gene or especially the epigenetic changes occurred as a result of methylation will result in the inactivation of the gene and contribute to the development of cancer especially because of loss of DNA repair functions. This relationship is brought into view in many types of cancer, especially the breast cancer. The number of studies on lung cancer is quite low. In the study of Kim et al., the methylation frequency was stated as 6% [19]. According to the results we obtained, the methylation ratio of this gene was observed to be higher when compared to other genes. This ratio is 16% in tumorous tissues and 17% in surrounding lung tissues. The results indicate that the *BRCA1* gene has also influence on the lung cancer.

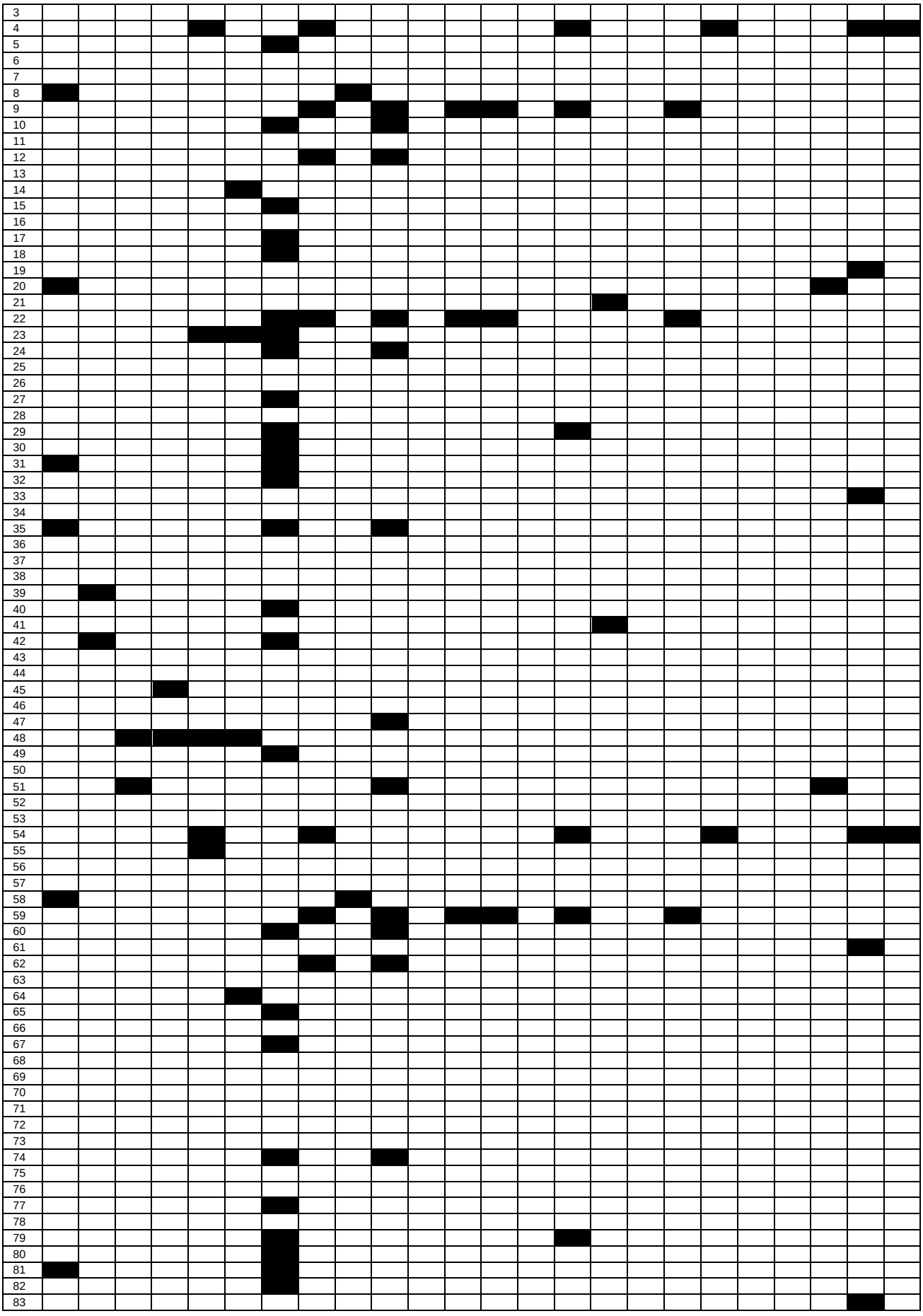
When methylation frequencies of tumorous and surrounding tissues were evaluated together, it was observed that the frequency was also high in the surrounding tissues which were histopathologically normal. Apart from bringing forward the question whether the resection area should be narrow or wide, this high frequency is also important for molecular mechanisms in tumor formation. The question whether tumor suppressor gene hypermethylation is an early-occurring event in cells prone to tumor development is still unanswered. As the number of cases relating to different grades included in the study is not enough for each of the 24 genes examined, no evaluation was made on the comparison of methylation frequency of each gene and the place of methylation mechanism in tumor progression during the NSCLC carcinogenesis process. However, when we classified GI and GII cases as low grade and GIII cases as high grade, we found that the genes we examined both in tumorous tissues and surrounding lung tissues of the high grade cases were more frequently methylated. Methylation was detected in neither tumorous tissues nor surrounding lung tissues of the *TIMP3, APC, CDKN2A, MLH1, ATM, RARB, CHFR, CASP8, CDKN1B, VHL1, RASSF1, ESR1, IGSF4* and *GSTP1* genes of low grade cases. However, methylation was observed with different ratios in high grade cases.

In conclusion, the studies examining the methylation ptofiles of some tumor suppressor genes in the DNA samples obtained from both blood and pituitary result in significant findings at this point. The association between methylation of these interrogated genes in NSCLC and clinicopathologic parameters in these patients was important for understanding the pathogenesis of the disease. Different methods and sensitivities should be taken into consideration while examining the methylation profiles of genes. We are of the thought that the utility of the MS-MLPA method is also indicated by this study.

Table 3. Correlation between Clinical Data and DNA Methylation of the Different Genes

Clinical Parameter		*TIMP3*	*APC*	*CDKN2A*	*MLH1*	*ATM*	*RARB*	*CDKN2B*	*HIC1*	*CHFR*	*BRCA1*	*CASP8*	*CDKN1B*	*PTEN*	*BRCA2*	*CD44*	*RASSF1*	*DAPK1*	*VHL*	*ESR1*	*TP73*	*FHIT*	*IGSF4*	*CDH13*	*GSTP1*
Sex	Male (n)	6	2	3	3	6	5	21	7	2	11	0	3	3	0	5	2	0	3	2	0	0	2	5	2
	Female (n)	3	2	0	1	1	1	12	0	0	5	0	1	1	0	1	2	0	1	0	0	0	2	2	0
Age	<60 (n)	2	2	1	2	2	0	13	4	0	7	0	2	2	0	6	2	0	2	0	0	0	1	7	2
	>60 (n)	7	2	2	2	5	6	23	3	2	9	0	2	2	0	0	2	0	2	2	0	0	3	0	0
Family History	Yes (n)	2	2	1	2	0	0	6	0	0	6	0	0	0	0	0	2	0	0	0	0	0	2	0	0
	No (n)	7	2	2	2	7	6	27	7	2	10	0	4	4	0	6	2	0	4	2	0	0	2	7	2
Smoking History	Yes (n)	7	4	3	4	5	6	28	7	2	14	0	4	4	0	5	4	0	4	2	0	0	4	6	2
	No (n)	2	0	0	0	2	0	5	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Histology	SCC (n=46)	5	0	2	2	7	6	13	7	2	8	0	4	4	0	4	2	0	4	2	0	0	3	5	2
	AC (n=54)	4	4	1	2	0	0	20	0	0	8	0	0	0	0	2	2	0	0	0	0	0	1	2	0
Grade	I-II (n=14)	0	0	0	0	0	0	6	0	0	2	0	0	0	0	2	0	0	0	0	0	0	0	1	0
	III (n=86)	7	4	3	7	7	6	27	7	2	14	0	4	4	0	4	4	0	4	2	0	0	4	6	2

protocol	*TIMP3*	*APC*	*CDKN2A*	*MLH1*	*ATM*	*RARB*	*CDKN2B*	*HIC1*	*CHFR*	*BRCA1*	*CASP8*	*CDKN1B*	*PTEN*	*BRCA2*	*CD44*	*RASSF1*	*DAPK1*	*VHL*	*ESR1*	*TP73*	*FHIT*	*IGSF4*	*CDH13*	*GSTP1*
1	■		■	■	■	■																		
2																								



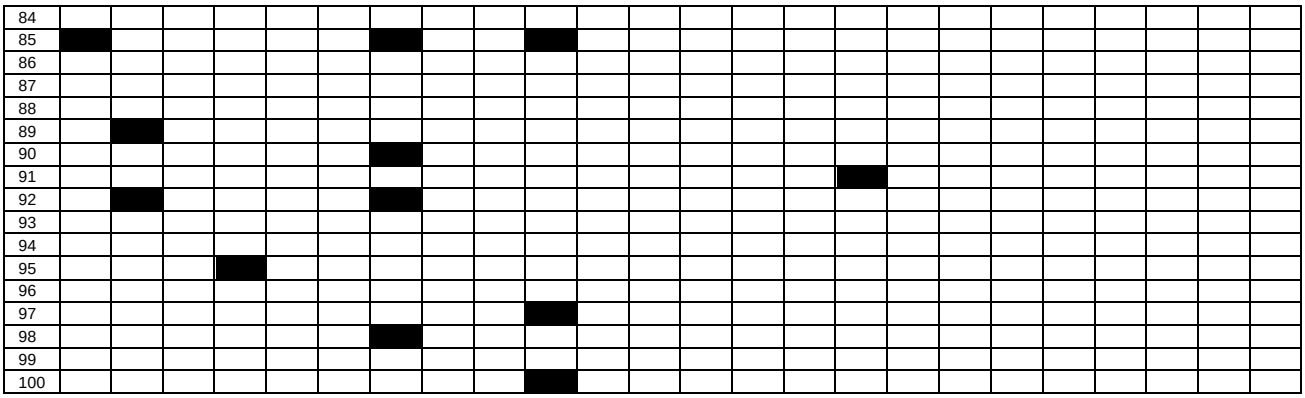


Figure 3. Summary of the methylation status of the 24 genes that were studied in resected primary nonsmall cell lung cancers.

References

1. The Information Centre for Health and Social Care and RCP. National Lung Cancer Audit, Report for the audit period 2005 December 2006
2. Liloglou T, Bediaga NG, Brown BRB, Field JK, Davies MPA. Epigenetic biomarkers in lung cancer. Cancer Letters (2012), article in press.
3. A. Portela, M. Esteller. Epigenetic modifications and human disease, Nat. Biotechnol. 28 2010 1057–1068.
4. Kondo Y, Issa JPJ. Epigenetic changes in colorectal cancer. Cancer Metastasis Rev. 2004; 23: 29–39.
5. Lin CH, Hsieh SY, Sheen I. Genome-wide hypomethylation in hepatocellular carcinogenesis. Cancer Res. 2001, 61: 4238–4243.
6. Zhu ZZ, Sparrow D, Hou L, Tarantini L, Bollati V, et al. Repetitive element hypomethylation in blood leukocyte DNA and cancer incidence, prevalence, and mortality in elderly individuals: the Normative Aging Study.Cancer Causes Control 2011, 22: 437–447.
7. Vaissiere T, Hung RJ, Zaridze D, Moukeria A, Cuenin C, Fasolo V, Ferro G, Paliwal A, Hainaut P, Brennan P, Tost J, Boffetta P, Herceg Z. Quantitative analysis of DNA methylation profiles in lung cancer identifies aberrant DNA methylation of specific genes and its association with gender and cancer risk factors. Cancer Res 69(1):243-252, 2009.
8. Hawes SE, Stern JE, Feng Q, et al. DNA hypermethylation of tumors from non-small cell lung cancer (NSCLC) patients is associated with gender and histologic type. Lung Cancer. 2010;69(2):172–179.
9. Sriram KB, Larsen JE, Yang IA, Bowman RV, Fong KM. Genomic medicine in non-small cell lung cancer: paving the path to personalized care. Respirology. 2011 Feb;16(2):257-63.
10. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N. Engl. J.Med. 2008; 359: 1367–80.
11. Esteller M. Epigenetics provides a new generation of oncogenes and tumour- supressor genes. Br. J Cancer,2006, 94(2):179-83.
12. Esteller, M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nav. Rev Genet. 2007, 8: 286-98.
13. Esteller M. Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum. Mol.Genet. 2007, 6:1, R50-R59.
14. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. Nature Reviews Cancer. 2004, 4: 707-717.
15. Shames DS, Girard L, Gao B, Sato M, Lewis CM, Shivapurkar N, Jiang A, Perou CM,Kim YH, Pollack JR, Fong KM, Lam CL, Wong M, Shyr Y, Nanda R, Olopade OI, Gerald
16. W, Euhus DM, Shay JW, Gazdar AF, Minna JD. A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies. Plos Med. 2006, 3(12):e486.
17. Jacinto FV, Esteller M. Mutator pathways unleashed by epigenetic silencing in human cancer. Mutagenesis. 2007, vol. 22 no. 4 pp. 247–253.
18. Grote HJ, Schmiemann V, Gedder t H, et al. Aberrant promoter methylation of p16INK4a, RARB2 and SEMA3B in bronchial aspirates from patients with suspected lung cancer. Int JCancer 2005;116:720^ 5.
19. Guo M, House MG, Hooker C, et al. Promoter hypermethylation of resected bronchial margins: a field defect of changes ? Clin Cancer Res 2004; 10:5131 ^ 6.
20. Belinsky SA, Palmisano WA, Gilliland FD, et al. Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. Cancer Res 2002;62:2370^ 7.
21. Zochbauer-Muller S, Fong KM, A.K. Virmani AK, Geradts J, A.F. Gazdar A.F,
22. Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res. 61 (2001) 249–255.
23. Paluzczak J, Misiak P. et al. Frequent hypermethylation of DAPK, RARbeta, MGMT, RASSF1A and FHIT in larengeal squamous cell carsinomas and adjacent normal mucoza. Oral Oncology 47 (2011) 104–107.
24. Bowman RV, Yang IA, Semmler ABT, Fong KM. Invited review series: lung cancer, Respirology. 2006, 11, 355-365.
25. Eunice CC, Lam SY, Fu KH, Kwong YL. Polymorphisms of the GSTM1, GSTP1, MPO, XRCC1 and NQO1 genes in Chinese patients with non small cell lung cancers: relationship with aberrant promoter methylation of the CDKN2A and RARB genes, Cancer Genetics and Cytogenetics. 2005, 162, 10-20 p.
26. Fronaka O, Takeshima Y, Awaya H, et al. Aberrant methylation of p14, p15 and p16 genes and location of the primary site in pulmonary squamous cell and carcinoma, Pathology International. 2004,54, 549-555.
27. Kim SD, Cha SI, Lee JH, et al. Aberrant DNA Methylation profiles of non small cell lung cancer in a Korean population, Lung Cancer. 2007, 2749, 1-6.
28. Sato K, Morishita Y, Fukasawa M, et al. Anthracotic index and DNA methylation status of sputum contents can be used for identifying the population at risk of lung carcinoma, Cancer Cytopathology. 2004, 102, 6, 348-354.
29. Ulivi P, Zoli W, Calistri D, Fabbri F, et al. p16 and CDH13 hypermethylation in tumor and serum of non small cell lung cancer patients, Journal of Cellular Physiology. 2006, 206, 611-615.
30. Heller G, Zielinski CC, Müler SZ. Lung cancer: From single-gene methylation to methylome profiling. Cancer Metastasis. 2010, 29:95–107.
31. Kim SD, Cha SI, Lee JH, et al. Aberrant DNA Methylation profiles of non small cell lung cancer in a Korean population, Lung Cancer, 2007, 2749, 1-6.
32. Zhang Y,Wang R, Song H, Huang G, Yi J, Zheng Y, Wang J, Chen L. Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer . Cancer Letters. 2011, 303, 21–28.
33. Castro M, Grau L, Puerta P, Gimenez L, Venditti J, Quadrelli S, Sánchez-Carbayo M. Multiplexed methylation profiles of tumor suppressor genes and clinical outcome in lung Cancer. Journal of Translational Medicine 2010, 8:86.
34. Kontic M, Stojsic J, Jovanovic D, Bunjevacki V, Ognjanovic S, Kuriger J, Puumala S, Nelson HH. Aberrant Promoter Methylation of

CDH13 and *MGMT* Genes is Associated With Clinicopathologic Characteristics of Primary Non–Small-Cell Lung Carcinoma Clinical Lung Cancer, 2011,Dec, 12.

35. Arvind, K, Tsou, JA, Siegmund KD, Shen YC, et al. Hierarchical clustering of lun cancer cell lines using NA methylation marker. Cancer Epidemiology, Biomarkers and prevention. 2002, 11, 291-297.
36. Zöchbauer-Müller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res, 2001,1; 61(1): 249-55.