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Full Length Research Paper

Tissue specific epigenetic silencing of the distinct tumor suppressor *genes* in lung cancer

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The role of aberrant methylation of the cytosine-guanine dinucleotide islands in the promoter region of tumor suppressor *genes* in lung cancer development is increasingly recognized. DNAs extracted from cancer tissue biopsies of 40 patients with lung cancer were used. Methylated sites of tumor suppressor *genes* were examined by bisulfate treatment and methylation-specific PCR method. Strip Assay was used to access the promoter profiles of cytosine-guanine dinucleotide islands. Some of the tumor specimens evaluated showed fully inactive methylated pattern profiles for the tumor suppressor genes that were studied and some showed partially inactive methylated patterns. In lung cancers, the prevalence of SFRP2, p16, DAPK1, HIC1, MGMT promoter methylation status were 11/40(27.5), 8/40(20), 30/40(75), 16/40(40%) and 10/40(25%), respectively. DAPK1 gene inactivation was seen in all (5/5, 100%) the adenocarcinoma type of tumors and was fully hypermethylated in 13/19 squamous cell type (68.4%), 6/9 SCLC (66.7%), and 4/7 malign epithelial (57.1%) tumors. Our results confirm the importance of methylation in the molecular pathogenesis of lung cancer with the majority of tumors having one or more tumor suppressor *genes* islands methylated for some tumor suppressor genes promoters.

Key words: Lung carcinoma, tumor suppressor *genes*, epigenetic alterations.

INTRODUCTION

Inactivation of tumor suppressor *genes* by DNA methyltion in promoter region plays an important role in carcinogenesis. The tumor suppressor *genes* that undergo aberrant cytosine-guanine dinucleotid (CpG) island methylation in human cancer can affect important cellular pathways including cell cycle regulation and proliferation (Paz et al., 2003; Jemal et al., 2006; Zaridze et al., 2004; Dammann et al., 2005).

The underlying basis of cancer is a cumulative series of genetic and epigenetic alterations leading to deregulated cell growth, including point mutations of regulatory and coding *gene* regions, micro-satellite instability, allele losses, changes in the levels of *gene* transcription and translation, epigenetic modification in promoter regions of tumor supressor *genes*.

For the most common tumors, mutations have been identified as early events (Demoly et al., 1994). Several oncogenes have been identified in lung cancer. Dominant oncogenes of the c-myc family are frequently over

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Abbreviations: PCR, Polymerase chain reaction; **MSP,** methylation specific; **TSG,** tumor suppressor *gene*; **DAPK1,** death-associated protein kinase 1; **p16,** a tumour suppressor *gene* contributing in cell cycle arrest as cyclin dependent kinase (DAPK2) inhibitor; **SFRP2,** secreted frizzle-related protein 2; **HIC1,** hypermethylated in cancer-1; **MGMT,** O(6)-methylguanine-DNA methyltransferase; **5-mC,** epicytosine, 5-methyl cytosine; **Ca,** cancer.

expressed in both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), while the K-ras oncogene is never mutated in SCLC, but it is in 30% of NSCLCs (Paz et al., 2003). Point mutations of the oncogene K-ras is found in 15 - 30% of adeno- carcinomas, especially in smokers (Esteller and Herman, 2002).

In the last decade, aberrations in DNA methylation patterns have been considered to be a common feature of human cancer (Esteller and Herman, 2002). Aberrations in the DNA methylation patterns such as global hypomethyla-tion in proto-oncogenes and/or region-specific hyper-methylation in TSGs are recognized as common hallmarks of all human cancers.

These aberrations include hypomethylation leading to oncogene activation and chromosomal instability, hyper-methylation and tumor suppressor *gene* silencing, and chromatin modification acting directly, and cooperatively with methylation chances, to modify *gene* expression (Demoly et al., 1994; Esteller and Herman, 2002).

The Ts SFRP2 promoter hyper-methylation has been reported for other cancer types such as breast cancer, gastric cancer, hepato-cellular carcinoma, lung and renal cell carcinomas (Suzuki et al., 2008; Nojima et al., 2007; Shih et al., 2007; Urakami et al., 2006).

P16, the cell cycle inhibitor, is hypermethylated in a variety of human tumors and cell lines allowing the cancer cell to escape senescence and start proliferating (Herman et al., 1995). The promoter hypermethylation of MGMT, which prevents the removal of groups at the O6 position of guanine, leads to appearance of K-ras and P53 mutations (Esteller et al., 1999). CpG islands are 0.5 - 2.5 kb in size of the target bases that are mostly methylated by DNA methyltransferase. If tumor suppressor (Ts) and other housekeeping *genes* that harbor CpG islands in their promoter sequences remain hypermethylated, they cannot be regulated by transcription factors, which results in *gene* silencing (Ozdemir et al., 2000).

Hypermethylation of promoter CpG islands in tumor suppressor *genes* or tumor-related *genes* is a common finding in human cancers, regardless of tissue type (Arslan et al., 2008; Tellez et al., 2009). Death-associated protein kinase (DAPK) is a calmoduline-regulated serine/ threonine kinase and possesses apoptotic and tumor-suppressive functions (Kuo et al., 2006).

DAPK 1 is an important tumor suppressor *gene* for variety of human cancer types including lung cancer (Fujiwara et al., 2005). The tumor suppressor hypermethylated in cancer 1 (HIC1) is a transcriptional repressor, which is epigenetically silenced in solid cancers (Porebska et al., 2009).

Lung cancer is the phenotypic consequence of an accumulation of genetic changes in airway epithelial cells that result in unrestrained cellular proliferation. In the current study, we used the reverse hybridization strip-assay multiplex *gene* amplification technique to assay the hypermethylated CpG islands of some distinct TSGs *gene* promoters in cases of different type lung carcinomas.

METHODS

Patient and biological specimens

Forty consecutive eligible patients with endobronchial malign tumor were studied. Tissue collection and analysis in this study were approved by the Research Ethics Committees of Faculty of Medicine University of Cumhuriyet, and written informed consent was obtained from all the patients. Endobronchial tumor tissue biopsies were collected for epigenetical analysis during the fiber-optic bronchoscopy (FOB) application. We examined the methylation status of the promoter region in 5 cancer-related Ts *genes*: SFRP2, p16, DAPK1, HIC1, MGMT.

Tumoral tissue specimens were used for histopathological, epigenetic analyses and the total genomic DNA isolated from these specimens. Tumor tissue chosen for analysis was routinely processed and microscopically examined for the regions enriched in transformed cells. For the correlation study of methyl patterns, normal solid and peripheric blood tissues from healthy controls were used for histopathological, immuno-histochemical and epigenetical analyses. The tumoral tissue lysates (20 µl) were aliquoted into different tubes and stored at -20°C until sodium bisulfite modification was performed.

Direct *in vitro* amplification of the tumor supressor *genes* of SFRP2, p16, DAPK1, p53 and MGMT were performed by bisulfite treatment and methylation-specific PCR (MSP) method. Strip-Assay which is based on the reverse-hybridization technique was used to access the promoter profiles of CpG islands.

Histopathological examination of the tumors

Fresh tumoral tissue samples from 40 patients with lung tumors of 3 types were studied. Tumoral biopsies were taken under fiberoptic endobronchial technique at the Department of Chest Diseases and sent to two different departments for pathogenetic diagnosis. The samples were fixed in 10% buffered formalin (pH = 7.0) and embedded in paraffin wax. Sections of 5 µm were used in hematoxylin and eosin (H and E) staining for histological evaluation and for immunostaining procedure. A part of the specimens were directly used and/or immediately frozen and stored at -20ºC until analyzed for total genomic DNA analysis.

Bisulphate treatment of DNA and MSP

DNA methylation patterns in the promoter CpG islands were determined in tumoral tissue samples by MSP following the bisulfite modification of isolated genomic DNA.

45 µl of isolated DNA were denatured by alkalizer (finally, 0.3 mmol/L) at 55°C for 10 min and modified by sodium bisulfite (5.20 - 5.69 mol/L, pH 5.0, Viennalab, Austria) for 4 h at 55°C in the dark. After incubation, binding buffer was added (300 µl per sample) and lysate was transferred into receiver tube with spin filter, centrifuged at 13 000 rpm for 30 s.

Filtrate was discarded; wash buffer (600 µl per sample) was added in spin filter and centrifuged at 13 000 rpm for 30 s. In the mean time, a mixture of alkalizer ethanol (1:10) was prepared. The mixture was added into spin filter 300 µl per sample, and then incubated at room temperature for 30 min. After incubation, spin filter was washed again with same procedure.

Elution buffer (30 µl) was added into spin filter, incubated for 3 min at room temperature, centrifuged at 13000 rpm for 1 min and spin filter was discarded. Resulting filtrate was kept at -20°C. Aliquots of 5 µl of bisulfate modified DNA were used for MSP reactions. Primers for a methylated and unmethylated promoter of the different Ts *genes* were used, and multiplex PCR based amplification procedure (Viennalab, Austria) was performed for the

Table 1. Clinical features of the patients with different types of lung carcinomas.

Parameter	n %
Age (Mean)	59.9 ± 9.1 Range 34 – 77
Gender	
Female	3 (7.5)
Male	37(92.5)
Smoke	
Active smoker	33(82.5)
Exsmoker	6(15.0)
Nonsmoker	1(2.5)
(pocket/year)	33.2 ± 12.7
Chemical expose	
Asbestos	12(30)
Biomass	8(20)
Asbestos+Biomas	10(25)
Metastases	17(42.5) (7 liver, 4 bone and 6 brain)
Familial cancer history	16(34.8)

in vitro gene amplification. Amplification was carried out in a Bioer XP thermal cycler after 15 min at 94°C for Pre-PCR, 45 seconds at 94°C for denaturation, 45 s at 66°C for annealing and 45 s at 72°C for polymerization of 45 cycles of with final extension for 7 min at 72°C.

Modified DNA from tumoral tissues (10 µL of each PCR reaction) was compared in reverse hybridization strip-Assay technique (ViennaLab, Austria). One signal was evaluated as heterozygous inactive (inactivation of one allele); double signals were homozygous hypermethylated fully inactive, and a sample without signals was evaluated as hypomethylated fully active *gene*.

For statistical evaluation, SPSS 14.0 for Windows was used. The parameters of the patients were presented as mean ± standard deviation. $p < 0.05$ was considered statistically significant.

RESULTS

The cohort was composed of 40 patients. The clinical findings of the patients are showed in Table 1. In the histopathological analysis, the tumors were diagnosed as: squamous cell type (n = 19, 47.5%), SCLC (n = 9, 22.5%), adenocarcinomas (n = 5, 12.5%), (Figure 1 and 2) and malign epithelial tm (unclassified) (n = 7, 17.5%). Some of the tumor specimens evaluated showed completely inactive methylated pattern profiles for the TSGs that were studied and some showed partially inactive methylated patterns. The prevalence rates of SFRP2, p16, DAPK1, HIC1, MGMT promoter methylation status were 11/40 (27.5), 8/40 (20), 30/40 (75), 16/40 (40) and 10/40 (25%), respectively, in the tumors with metastatic profile. The same inactive profiles were seen in different tumor types (Table 2). Figure 3 showed epigenetically modified profiles (fully methylated,partially and unmethylated) of the TS genes of SFRP2, p16, DAPK1, p53, MGMT in some blood and tumoral tissue samples of the current cases.

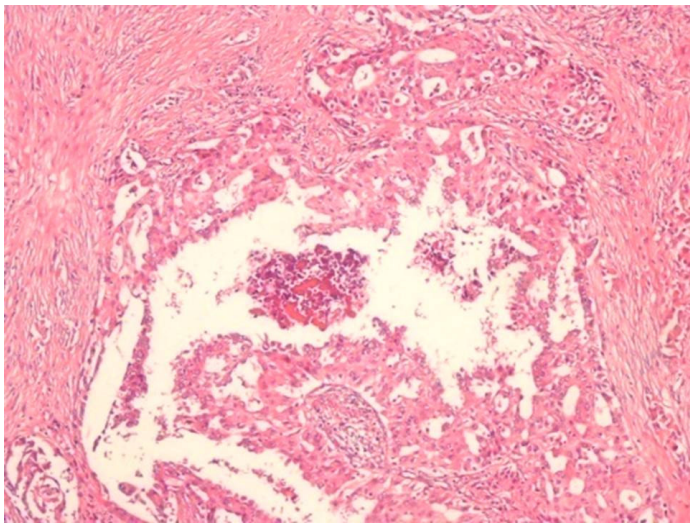

Figure 1. Histopathological appearance of case with NSCLC.

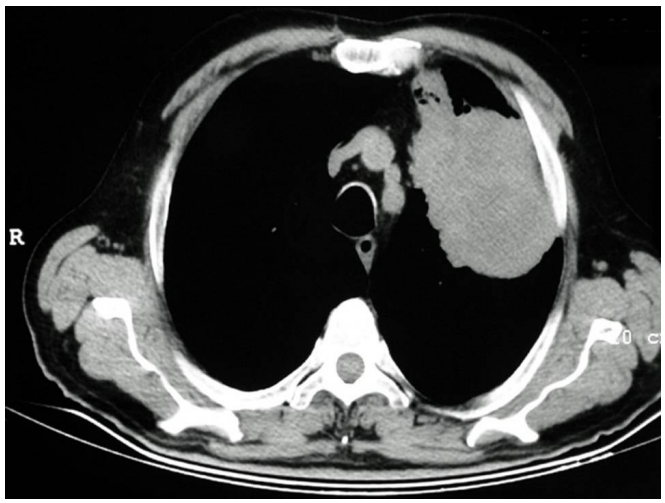

Figure 2. Thoracic computed tomography shows the tumor (NSCLC).

The prevalence rates of SFRP2, p16, DAPK1, HIC1, MGMT promoter methylation status were 6/19 (21.6.5), 2/19 (10.5), 14/19 (73.7), 7/19 (36.9) and 6/19 (31.6%), respectively, in squamous cell type of tumors (Table 2). DAPK1 *gene* inactivation was seen in all (5/5, 100%) adenocarcinoma type of tumors. *Gene* was fully inactive in 13/19 squamous cell type tumors (68.4), 6/9 SCLC (66.7), and 4/7 malign epithelial (57.1%) tumors (MET).

Table 2. Types of lung cancer and methylation profiles of tumour suppressor *genes.*

TSGs	Epigenetic profile	Tumour type								Total = 40	
		SCLC*n = 9		Squamous cell type n:19		Adenocarcinoma n:5		Non differentiated n:7			
		n	%	n	%	n	%	n	%	N	%
SFRP2	Unmethylated, active gene	8	88.9	13	68.4	3	60.0	5	71.4	29	72.5
	Partially methylated-heterozygous inactive gene	-	-	2	10.5	-	-	1	14.3	3	7.5
	Hypermethylated, fully inactive gene	1	11.1	4	21.1	2	40.0	1	14.3	8	20.0
p16	Unmethylated, active gene	7	77.8	17	89.5	2	40.0	6	85.7	32	80.0
	Partially methylated-heterozygous inactive gene	-	-	-	-	-	-	-	-	-	-
	Hypermethylated, fully inactive gene	2	22.2	2	10.5	3	60.0	1	14.3	8	20.0
DAPK1	Unmethylated, active gene	3	33.3	5	26.3	-	-	2	28.4	10	25.0
	Partially methylated-heterozygous inactive gene	-	-	1	5.3	-	-	1	14.3	2	5.0
	Hypermethylated, fully inactive gene	6	66.7	13	68.4	5	100	4	57.1	28	70.0
HIC1	Unmethylated, active gene	4	44.4	12	63.2	3	60.0	5	71.4	24	60.0
	Partially methylated-heterozygous inactive gene	2	22.2	3	15.8	-	-	1	14.3	6	15.0
	Hypermethylated, fully inactive gene	3	33.3	4	21.1	2	40.0	1	14.3	10	25.0
MGMT	Unmethylated, active gene	8	88.9	13	68.4	3	60.0	6	85.7	30	75.0
	Partially methylated-heterozygous inactive gene	-	-	1	5.3	2	40	-	-	3	7.5
	Hypermethylated, fully inactive gene	1	11.1	5	26.3	-	-	1	14.3	7	17.5

* SCLC = small cell lung cancer

High frequency of methylation was found in DAPK1 *gene* in nearly all tumor types. Distant organ metastases (liver, bone, and brain) and methylation status of tumor suppressor genes are showed in Table 3.

DISCUSSION

Epigenetic modifications, such as DNA methylation, histone acetylation/deacetylation, and histone methyltion, are currently believed to play a major role in human cancers (Yamada et al., 2009). Recently, the *gene* inactivation by the hypermethylation of CpG islands in the promoter regions of cancer-related tumor suppressor *genes* is a popular research area in the oncogenesis. There is limited knowledge regarding the hypermethylation of tumor suppressor *genes* by epigenetical modification in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) in human. This study was designed to investigate the promoter hypermethylation of a wide

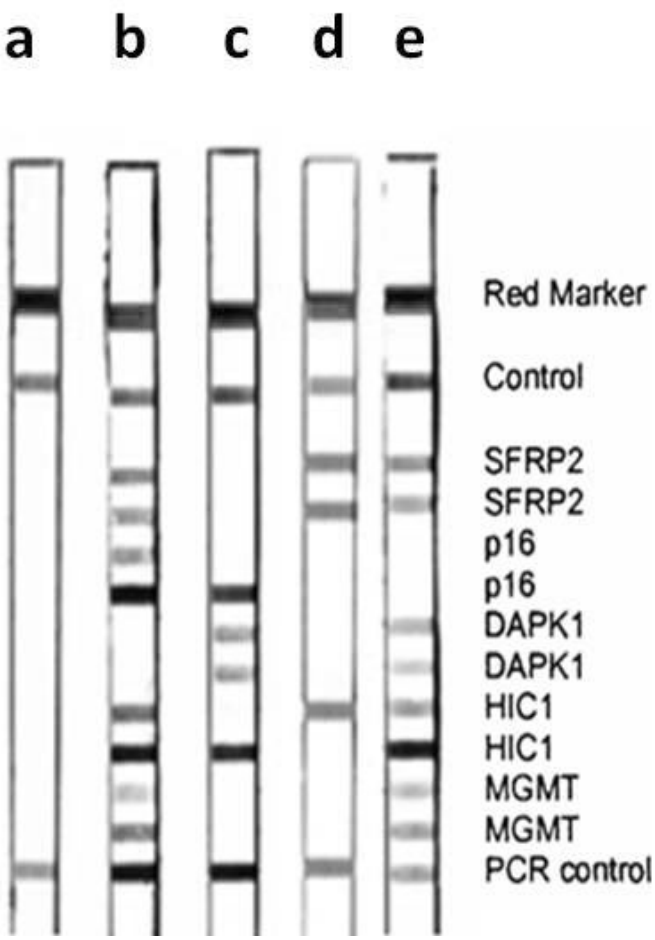

Figure 3. Shows epigenetically modified profiles (fully methylated, partially and unmethylated) of the TS *genes* of SFRP2, p16, DAPK1, p53, MGMT in some blood and tumoral tissue samples of the current cases.
(Bands show the methylated promoters, control, PCR test control and positive amplification as a red marker).
Lanes a - b: Shows epigenetically modified promoter region of the distinct tumor suppressor genes in two different tissues belonging to the case 7. Fully hypomethylated - active *gene* profiles from peripheral blood (a) and fully active DAPK1 but fully inactive (hypermethylated) profiles of SFRP2, p16, HIC1 and MGMT genes in tumors tissue of the same case (b).
Lane c: The endobronchial tumor tissue biopsy shows partially hypermethylated of p16 and HIC1 and fully hypermethylated – inactive *gene* profiles of DAPK1 in case 22 with NSCLC. The SFRP2 and MGMT were in normal appearance of active *gene* profiles.
Lane d: Tumor tissue sample shows fully methylated – inactive gene profiles of SFRP2 and partially methylated HIC1 *genes*. The p16, DAPK1 and MGMT *genes* were in hypomethylated active gene profiles.
Lane e: Only p16 gene had an active profile but the rest of the target genes were fully inactive in tumor tissue of the case 29.

range of cancer-related *genes* in lung Ca. All the cases had various hypermethylation status of the cancer-related TGS *genes* that were studied. Among them, the most frequently methylated *gene* was DAPK1 (100%). DAPK1 is a 160 kDa protein that is involved in regulation of the cell cycle and induction of apoptosis. Exposures to biomass, smoke and/or asbestos are the main environmental risk factors for lung carcinomas in the current population. Exposing to the chemicals such as smoke and or coal dust, biomass and asbest may initiate the tissue specific tumor genesis in the current cases. These results suggest that, full inactivation of DAPK1 and full and/or partial inactivation of some other TSGs such as SFRP2 and HIC1 represent a major effect on pathogenic importance for carcinogenesis in lung carcinoma and metastasis. The Ts MGMT is a DNA repair *gene* critical for the removal of mutagenic adducts from the O6 position of guanine.

The inactivation of TSG or tumor-related *genes* by hypermethylation of promoter CpG islands is a common finding in human cancers, regardless of tissue type. Full promoter hypermethylation of DAPK1 was detected in 5/5 (100%) adenocarcinoma type of tumors in the current results means; *gene* is a positive mediator of apoptosis induced by interferon-γ and may cause late type tumor genesis in lung Ca. Lung tissue carcinomas, especially recurrent NSCLC, are the most frequent reason of cancer related deaths all over the world (Jemal et al., 2007). As indicated by some researchers, while genetic alterations contribute to cancer initiation, epigenetic alterations have important role in carcinogenesis, tumour invasion and metastasis.

Tumoral specimens showed fully methyl pattern profiles for the DAPK1, SFRP2, p16, and partially hypermethylated profile for the HIC1 and MGMT *genes* Heterogeneous expressivity of tumor supressor of p53, bcl-2 and nm23 were reported in squamous-cell lung cancer (Porebska et al., 2009; Zöchbauer-Müller et al., 2001). Our data also suggest that DAPK1 and other TS *genes* have an important role of fullly and /or partially silencing through promoter CpG island hypermethylation in the development of lung Ca and that the detection of aberrant hypermethylation on DAPK1 promoter from tumoral samples have potential clinical implications as an NSCLC and adenocarcinomas. The inactivation of the p16 *gene* in murine cancers induced by NNK most likely arises as a late event via homozygous deletion as claimed by Belinsky (1998). All of those changes of NSCLC metastasis, which occur early in neo-plastic evolution, may be induced by DAPK1 and other tumor supressor *genes*.

These findings verify that DNA hypermethylation are critical for the initiation and progression of malignant lung carcinomas. Lung carcinoma was one of the most common cancers that occurred in males and resulted in recurrence or metastasis in the studied population of many genetic (activation of proto-oncogenes and/or epigenetical inactivation of different tumor suppressor *genes*) and environmental factors such as biomass, asbest, smoke, and exposure to ultraviolet (UV) radiation have led to tumor formation (Tellez et al., 1998; Wang et al., 2009). In addition, Tellez et al. found the cumulative

Table 3. Distant organ metastases (liver, bone, and brain) and methylation status of tumor suppressor genes.

TSGs	Epigenetic profile	Distant organ metastases n = 17
	Unmethylated, active gene	14
SFRP2*	Partially methylated-heterozygous inactive gene	3
	Hypermethylated, fully inactive gene	-
	Unmethylated, active gene	12
p16 §	Partially methylated-heterozygous inactive gene	5
	Hypermethylated, fully inactive gene	-
	Unmethylated, active gene	3
DAPK1º	Partially methylated-heterozygous inactive gene	14
	Hypermethylated, fully inactive gene	-
	Unmethylated, active gene	8
HIC1¤	Partially methylated-heterozygous inactive gene	5
	Hypermethylated, fully inactive gene	4
	Unmethylated, active gene	13
MGMT^	Partially methylated-heterozygous inactive gene	4
	Hypermethylated, fully inactive gene	-

*SFRP2 **=** The secreted frizzled-related protein 2.
§P16 = A tumour suppressor gene contributing in cell cycle arrest as cyclin dependent kinase (DAPK2) inhibitor.
ºDAPK1 = The death-associated protein kinase 1.
¤HIC1 = Hypermethylated In Cancer 1.
^MGMT **=** Methyl guanine DNA methyltransferase.

aberrant hypermethylation of the MGMT and p16 *gene* promoters in melanoma cell lines (Tellez et al., 1998). Tumor suppressor *genes* whose CpG islands in the promoter regions are hypermethylated can be transcriptionally silenced as indicated in the current results of different types of lung carcinomas.

Conclusion

The methylation of the CpG islands, which are concentrated within *gene* promoter regions, prevents *gene* transcription and causes inactivation of the tumor suppressor *gene*. Our results confirm the importance of methylation in the molecular pathogenesis of lung Ca with the majority of tumors having one or more CpG islands methylated for some TS *gene* promoters (Table 2).

These findings verify that DNA hypermethylation is critical for the initiation and progression of malignant cells within adenocarcinomas. The hypermethylation in DAPK1 and other TSG promoters of tumoral samples has potential clinical implications of tumor progression. In addition, the results of this study showed that, epigenetic changes occur at late and metastatic stage of lung carcinomas in human.

In conclusion, we analyzed the methylation status of 5 TS *genes* in 40 cases of lung carcinomas and have shown that each tissue type has its own *gene*-specific and tumor type-specific methylation pattern. According to the above findings, it can be concluded that, different tumoral epigenetic profiles need alternative therapy for a good life quality and prognosis.

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