

RESEARCH ARTICLE

Assessment of the Prognostic Value of Methylation Status and Expression Levels of FHIT, GSTP1 and p16 in Non-Small Cell Lung Cancer in Egyptian Patients

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Abstract

Background: Methylation of tumor suppressor genes has been investigated in all kinds of cancer. Tumor specific epigenetic alterations can be used as a molecular markers of malignancy, which can lead to better diagnosis, prognosis and therapy. Therefore, the aim of this study was to evaluate the association between gene hypermethylation and expression of fragile histidine triad (FHIT), glutathione S-transferase P1 (GSTP1) and p16 genes and various clinicopathologic characteristics in primary non-small cell lung carcinomas (NSCLC). **Materials and Methods:** The study included 28 primary non-small cell lung carcinomas, where an additional 28 tissue samples taken from apparently normal safety margin surrounding the tumors served as controls. Methylation-specific polymerase chain reaction (MSP) was performed to analyze the methylation status of FHIT, GSTP1 and p16 while their mRNA expression levels were measured using a real-time PCR assay with SYBR Green I. **Results:** The methylation frequencies of the genes tested in NSCLC specimens were 53.6% for FHIT, 25% for GSTP1, and 0% for p16, and the risk of FHIT hypermethylation increased among patients with NSCLC by 2.88, while the risk of GSTP1 hypermethylation increased by 2.33. Hypermethylation of FHIT gene showed a highly significant correlation with pathologic stage ($p < 0.01$) and a significant correlation with smoking habit and FHIT mRNA expression level ($p < 0.05$). In contrast, no correlation was observed between the methylation of GSTP1 or p16 and smoking habit or any other parameter investigated ($p > 0.05$). **Conclusions:** Results of the present study suggest that methylation of FHIT is a useful biomarker of biologically aggressive disease in patients with NSCLC. FHIT methylation may play a role in lung cancer later metastatic stages while GSTP1 methylation may rather play a role in the early pathogenesis.

Keywords: Fragile histidine triad - glutathione S-transferase P1 - p16 - promoter methylation

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Introduction

Lung cancer is one of the most common malignances, of which the occurrence and mortality is increasing every year due to air pollution, environmental breakdown and cigarette abuse (Xie et al., 2014). While the prognosis of lung cancer is generally grim, with 5-years survival rates of only 15%, there is hope and evidence that early detection of lung cancer can reduce mortality (Subramaniam et al., 2013). Lung cancer can be classified into two major groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter group is further subdivided into squamous cell carcinoma, large cell carcinoma and adenocarcinoma. Among these three, adenocarcinoma is the most common subtype and has a high mortality rate (Liu et al., 2013).

Lung carcinogenesis is a complex process requiring the acquisition of genetic mutations that confer the malignant phenotype as well as epigenetic alterations (Bhat et al., 2013).

Epigenetic silencing of genes plays an important role in the inactivation of tumor suppressor genes in carcinogenesis. Aberrant DNA methylation in the CpG islands at the promoter region begins early in tumorigenesis and is an important epigenetic mechanism underlying the inactivation of tumor suppressor. Three tumor suppressor genes were chosen for assessment of their roles in lung carcinogenesis: FHIT, GSTP1 and p16 genes (Sinha et al., 2013).

The fragile histidine triad (FHIT) gene, located on chromosome 3 at band p14.2 (3p14.2) and encompassing the FRA3B fragile site, comprises 10 exons that encode a

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1.1 kb transcript and a protein of 147 amino acids (Li et al., 2010). The chromosomal localization of FHIT in the common fragile region of the human genome suggests a positive correlation between the loss or inactivation of the FHIT gene and carcinogenesis. As predicted for a tumor suppressor, the Fhit protein is absent or markedly reduced in most human cancers. The role of FHIT in tumor suppression is perhaps best exemplified by studies performed with FHIT-deficient mice. Transgenic mice carrying one or two inactivated Fhit alleles are viable and long-lived, but they show increased rates of spontaneous and carcinogen-induced cancers. Encouragingly, the development of carcinogen induced tumors in these mice can be prevented by administration of Fhit-expressing viral vectors. Moreover, Fhit overexpression enhances the susceptibility of many types of cancer cells to exogenous inducers of apoptosis (Zuo et al., 2013).

Glutathione S-transferases (GSTs) present a family of soluble isoenzymes that play an important role in detoxification process. They catalyze the nucleophilic addition of glutathione to lipophilic electrophiles produced by phase I enzymes and mark the first step of carcinogen elimination. GSTs are expressed in tissue specific manner most of the GSTs are in liver, muscles, brain, testes, heart, blood and upper aerodigestive mucosa. The fact that most of the xenobiotic enzymes are expressed in a tissue specific manner leads to great differences in the activation and inactivation of xenobiotics in different tissues. Hereditary differences in the expression and activity of human GSTs have been reported and low enzymatic activity of GSTs was associated with lung cancer (Masood et al., 2011). The 17 human cytosolic GST subunits are classified as seven gene families according to their biochemical characteristics and amino acid sequence similarities: GSTA, GSTM, GSTT, GSTP, GSTO, GSTZ, and GSTS. Human GSTs are nearly ubiquitously expressed, and GSTP is the most abundant subunit in lung and brain. GSTP1 is widely expressed in different human epithelial tissue and is the most abundant GST isoform in the lung (Sun et al., 2010). Aberrant expression of GSTP1 is associated with carcinogenesis and development of multidrug resistance (MDR) (Slonchak et al., 2009).

The p16^{INK4a} gene is one of the negative regulators of cell cycle. The p16 INK4a protein binds to the cyclin-dependent kinases (CDKs) CDK4 and CDK6 and then inhibits their activities, which results in hypophosphorylation of retinoblastoma protein (pRb) as well as inhibition of cell cycle progression (Sargolzaei et al., 2014). Furthermore, it has also been demonstrated that an elevated level of P16 expression induced by oncogenes, DNA damage response or aging can trigger and accelerate cellular senescence. In addition, genetic inactivation of the p16 gene by deletion, methylation, and point mutation has been found in nearly 50% of all human cancers. The overexpression of P16 at both mRNA and protein levels is also associated with poor prognosis for cancers, including neuroblastoma, cervical, ovarian and breast cancers, prostate tumors, and oral cancers (Li et al., 2011).

Identification of tumor specific epigenetic alterations can be used as a molecular marker of malignancy, which can lead to better diagnosis, prognosis and

therapy. Therefore, the aim of this study was to evaluate the association between gene hypermethylation and expression of FHIT, GSTP1 and p16 tumor suppressor genes and various clinicopathologic characteristics in primary non-small cell lung carcinomas.

Materials and Methods

Patients and samples

The present study was conducted on 28 primary untreated NSCLC patients who were under surgical treatment in the National Cancer Institute, University of Cairo, Egypt, between July 2011 and May 2013. The sample included 28 primary non-small cell lung carcinomas and 28 tissue samples taken from apparently normal safety margin surrounding the tumor (used as controls). The clinicopathologic characteristics of the NSCLC patients are shown in Table 1.

An institutional approval of the study protocol by the Institutional Review Board Decision of the National Cancer Institute, University of Cairo, Egypt was taken on 10 July 2011 (IRB number: IRB00004025). At the time of sample collection, all subjects provided written informed consent to participate in this study. Also information on sociodemographic characteristics were obtained using an interviewer-administered questionnaire (age, gender, history of disease, smoking habit, occupational held). The patients were 20 males and 8 females with age ranging from 32-68 years (median, 53 years) at the time of diagnosis.

All tissue specimens were collected on RNAlater RNA stabilization reagent (cat # 76104) (Qiagen, Germany) and incubated overnight in the reagent at 2-8°C, then transferred for storage at -70°C in the reagent, until extraction of DNA or RNA.

DNA extraction and sodium bisulfite modification

Genomic DNA was extracted from NSCLC tissues and adjacent normal lung tissues (control) using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Two

Table 1. Clinicopathologic Characteristics of the NSCLC Patients

Parameter	No.	%
Median age, yrs (range)	53 (32-68)	
Gender		
Male	20	71.4
Female	8	28.6
Smoking habit		
Smoker	19	67.9
Non-smoker	9	32.1
Histological type		
Adenocarcinoma	16	57.1
Squamous cell carcinoma	9	32.1
Large cell carcinoma	3	10.7
Pathologic TNM stage		
I	5	17.9
II	11	39.3
III	12	42.9

*TNM; tumor, node and metastasis classification of tumors

μg gDNA from each sample were subjected to sodium bisulfite modification using an EpiTect Bisulfite Kit (Qiagen, Germany) according to the manufacturer's instructions.

Methylation-specific PCR (MSP)

The methylation status of the FHIT, GSTP1 and p16 promoters was analyzed by methylation-specific PCR (MSP) as previously described by Herman et al., (1996). Two sets of primers were designed for each gene, one specific for DNA methylated at the promoter region and the other specific for unmethylated DNA. The primer sequences and annealing temperatures used for MSP are shown in Table 2. PCR reactions were carried out in a volume of 25 μl with 12.5 μl of DreamTaq™ Green PCR Master Mix (2X) (Thermo Scientific, USA), 100 ng of gDNA and 400 nmol of each primer. PCR reactions were overlaid with mineral oil (Sigma Chemical Co.). The cycling parameters included an initial denaturing step at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, different annealing temperatures of the primers (Table 2) for 45 s, and 72°C for 1 min. This was followed by a final extension for 5 min at 72°C. Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gels and stained with ethidium bromide.

RNA extraction and reverse transcription

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse transcription (RT) was done on a scale of 20 μl using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) with random hexamer primer. Briefly, RNA (1 μg), 1 μl of 0.2 $\mu\text{g}/\mu\text{l}$ random hexamer primer, and RNase/DNase-free water was added to make up the final volume to 12 μl . The RNA was denatured at 65°C for 5 min and then chilled on ice. Then 8 μl of a solution containing 4 μl of 5×Reaction

buffer, 1 μl of RiboLock™ RNase Inhibitor (20 $\mu\text{g}/\mu\text{l}$), 2 μl of 10 mM dNTP Mix and 1 μl of RevertAid™ M-MuLV Reverse Transcriptase (200 $\mu\text{g}/\mu\text{l}$) were added followed by incubation 5 min at 25°C then at 42°C for 60 min and finally at 70°C for 5min.

Quantitative RT-PCR analysis

FHIT, GSTP1 and p16 mRNA expression levels were measured using a real-time PCR assay with SYBR Green I and Mx3000P real-time PCR thermal cycler (Stratagene, La Jolla, CA, U.S.A.). Primer pairs are listed in Table 3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize FHIT, GSTP1 and p16 expression levels. Specifically, 1 μg of total RNA was used for the cDNA synthesis using the Thermo Scientific RevertAid™ First Strand cDNA synthesis kit (#K1621) and 1 μl of the resultant cDNA was amplified in a PCR reaction containing the Thermo Scientific 2X Maxima® SYBR Green/ROX qPCR Master Mix (#K0221), 400 nmol of each primer and 10 μmol ROX passive reference dye in a final volume of 20 μl . The resulting 20 μl reaction mixture was subjected to the following cycling conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Fluorescence data were automatically collected and analyzed by Mx3000P Optical Software version 3.0a (Stratagene, La Jolla, CA, U.S.A.). Dynamic melt-curve analysis and agarose-gel electrophoresis were used for all post-PCR reaction tubes to confirm the size of detected cDNA. Each gene expression ratio was determined by the comparative method (Livak's $2^{-\Delta\Delta C_t}$ equation) (Livak and Schmittgen, 2001) in relation to the GAPDH housekeeping gene. A 2-fold increased (≥ 2) or decreased (≤ 0.5) value was considered mRNA overexpression or downregulation, respectively, in that NSCLC sample.

Statistical analysis

Statistical analyses were carried out by the aid of a digital computer, using both Excel and the Statistical Package for Social Science (SPSS software package, version 16.0; SPSS Inc., Chicago, IL, USA) programs. The following statistical analyses were performed:

Fisher's exact test was used to assess the association between methylation and patient characteristics and the association between pairs of genes.

Statistical significance was evaluated using Spearman correlation coefficient (r). The variables on the association analyses included: gender, age, smoking status, histological subgroup and stage. The difference was considered to be statistically highly significant if the p value was <0.01 , while it was significant if the p value was <0.05 and it was non-significant if the p value was >0.05 .

Calculated Relative Risk Ratio (RRR), which measures how many times the risk is present among the target or diseased group relative to that of non-diseased group.

Results

Gene promoter methylation profile

The frequencies of FHIT, GSTP1 and p16 promoter methylation among NSCLC and control patient groups are

Table 2. Primer Sequences for MSP

Target Gene	Primer sequence	Annealing Temp. (oC)	Product size (bp)
FHIT-M	F: 5'-TTGGGGCGCGGGTTTGGGTTTTTCGC-3' R: 5'-CGTAAACGACGCCGACCCCACTA-3'	65	74
FHIT-U	F: 5'-TTGGGGGTGTGGGTTTGGGTTTTATG-3' R: 5'-CATAAACAAACACCAACCCCACTA-3'	65	74
GSTP1-M	F: 5'-TTCGGGGTGTAGCGCTCGTC-3' R: 5'-GCCCAATACTAAATCACGACG-3'	59	92
GSTP1-U	F: 5'-GATGTTTGGGGTGTAGTGGTTGTT-3' R: 5'-CCACCCCAATACTAAATCACAACA-3'	59	99
P16-M	F: 5'-TTATTAGAGGGTGGGGCGGATCGC-3' R: 5'-GACCCCAACCGCGACCGTAA-3'	60	150
P16-U	F: 5'-TTATTAGAGGGTGGGGTGGATTGT-3' R: 5'-CAACCCCAACCAACCAACATAA-3'	60	151

Table 3. Primer Sequences for Real-Time PCR

Target Gene	GenBank Accession #	Primer sequence	Product size (bp)
FHIT	NM_002012.2	F: 5'-AAACATTTCCATGGGACCTCTCT-3' R: 5'-GAGCTCCTCATAGATGCTGTCACTC-3'	141
GSTP1	NM_000852.3	F: 5'-AGGTCCTAGCCCCTGGCTGC-3' R: 5'-TCAGGGGAGGCCAGGAAGGC-3'	103
P16	NM_000077.4	F: 5'-GTGGACCTGGCTGAGGAG-3' R: 5'-CTTCAATCGGGGATGCTG-3'	132
GAPDH	NM_002046.4	F: 5'-GCTCTGTCTCCTCCTGTTC-3' R: 5'-ACGACCAAAATCCGTTGACTC-3'	115

shown in Table (4). It was found that promoter methylation of the FHIT gene was 53.57% (15/28) and 3.57% (1/28) in NSCLC and control groups; respectively, while that for the GSTP1 gene was 25% (7/28) and 0% (0/28) in NSCLC and control groups; respectively, however, the frequency for p16 gene promoter methylation was 0% (0/28) in both groups.

There were a significant statistical association between promoter methylation of the FHIT and GSTP1 genes and lung cancer risk as results of Relative Risk Ratio (RRR) showed that the risk of FHIT hypermethylation increased among patients with NSCLC by 2.88 while the risk of GSTP1 hypermethylation increased among patients with NSCLC by 2.33. A representative agarose gel electrophoresis image of the MSP results is shown in Figure 1.

Clinicopathological characteristics and FHIT & GSTP1 methylation

Table (5) depicts the relationship between the promoter methylation of the FHIT & GSTP1 genes and the clinicopathological characteristics of NSCLC patients. FHIT methylation occurred in 13 out of 20 men (65%) and 2 out of 8 women (25%) while GSTP1 promoter

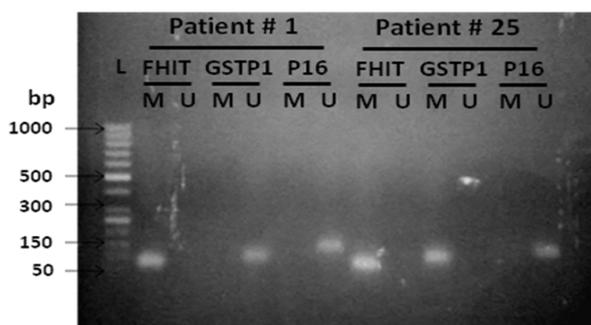


Figure 1. Methylation Analysis of FHIT, GSTP1 & p16 Gene Promoters. L: GeneRuler™ 100 bp Plus DNA Ladder; M: methylated locus; U: unmethylated locus; bp, base pair

Table 4. Frequency of FHIT, GSTP1 and p16 Promoter Methylation among NSCLC and Control Patient Groups

	NSCLC group			Control group		
	FHIT	GSTP1	p16	FHIT	GSTP1	p16
Methylated (n) %	(15) 53.6	(7) 25	(0) 0	(1) 3.6	(0) 0	(0) 0
Unmethylated (n) %	(13) 46.4	(21) 75	(28) 100	(27) 96.4	(28) 100	(28) 100
Total (n)	28	28	28	28	28	28

Table 5. Clinicopathological Characteristics and FHIT & GSTP1 Methylations in NSCLC Patients

Parameter	No.	FHIT			GSTP1			
		Methylated (n)%	Unmethylated (n)%	p value	Methylated (n)%	Unmethylated (n)%	p value	
Gender	Male	20	(13) 65	(7) 35	0.06	(5) 25	(15) 75	1
	Female	8	(2) 25	(6) 75		(2) 25	(6) 75	
Smoking	Smoker	19	(13) 68.4	(6) 31.6	0.02*	(5) 26.3	(14) 73.7	0.82
	Non-smoker	9	(2) 22.2	(7) 77.8		(2) 22.2	(7) 77.8	
Histological type	Adenocarcinoma	16	(7) 43.8	(9) 56.2	0.61	(4) 25	(12) 75	0.88
	Squamous cell carcinoma	9	(7) 77.8	(2) 22.2		(2) 22.2	(7) 77.8	
	Large cell carcinoma	3	(1) 33.3	(2) 66.7		(1) 33.3	(2) 66.7	
Pathologic TNM stage	I	5	(0) 0	(5) 100	0.00**	(1) 20	(4) 80	0.67
	II	11	(3) 27.3	(8) 72.7		(4) 36.4	(7) 63.6	
	III	12	(12) 100	(0) 0		(2) 16.7	(10) 83.3	

**p<0.01=highly significant (HS); *p<0.05=significant (S)

methylation in both males and females was 25% (5/20 and 2/8; respectively). Hypermethylation of the FHIT & GSTP1 gene promoters had no correlation with gender (p>0.05), Table 5.

The percentage of smoking patients with FHIT promoter methylation was 68.42% (13/19) and that for nonsmokers was 22.22% (2/9), while the percentage of smoking patients with GSTP1 methylation was 26.32% (5/19), similar to nonsmoking patients (22.22%), i.e. percentages of nonsmoking patients with FHIT & GSTP1 methylation were the same. Hypermethylation of FHIT gene promoter had a significant correlation with smoking habit (p<0.05) but there was no correlation between GSTP1 promoter hypermethylation and smoking habit (p>0.05), Table 5.

FHIT methylation occurred more frequently in squamous cell carcinomas (77.78%) than in adenocarcinomas (43.75%) and large cell carcinoma (33.33%) while GSTP1 methylation occurred more frequently in large cell carcinoma (33.33%) than in adenocarcinomas (25%) and squamous cell carcinomas (22.22%), however, these differing results were not statistically significant (p>0.05), Table 5.

FHIT promoter methylation in stage I was 0% (0/5), in stage II was 27.27% (3/11), and in stage III was 100% (12/12) while GSTP1 methylation in stage I was 20% (1/5), in stage II was 36.36% (4/11) and in stage III was 16.67% (2/12). Hypermethylation of FHIT gene had a highly significant correlation with pathologic staging (p<0.01) but there was no correlation between GSTP1 hypermethylation and pathologic staging (p>0.05), Table 5.

The association between promoter methylation of the FHIT and GSTP1 genes among NSCLC patients is indicated in Table 6. Results show that about 10.71%

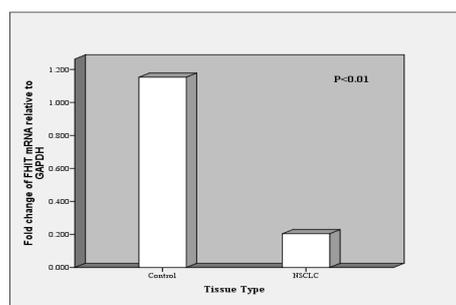
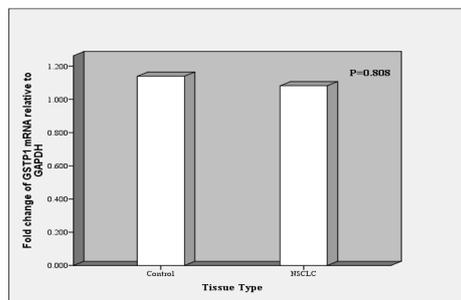
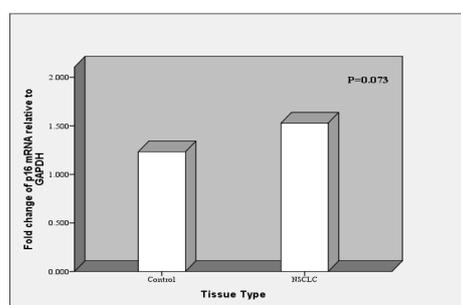


Figure 2. Fold Change of FHIT mRNA Relative to GAPDH in NSCLC and Control Samples

Table 6. Association of FHIT Methylation with GSTP1 Methylation

GSTP1	FHIT		Total (n)
	Methylated (n)	Unmethylated (n)	
Methylated	3	4	7
Unmethylated	12	9	21
Total (n)	15	13	28

**Figure 3. Fold Change of GSTP1 mRNA Relative to GAPDH in NSCLC and Control Samples****Figure 4. Fold Change of p16 mRNA Relative to GAPDH in NSCLC and Control Samples**

(3/28) of the NSCLC patients had methylation of both gene promoters, while 57.14% (16/28) of NSCLC patients had methylation of at least one of the two gene promoters, and 32.14% (9/28) of NSCLC patients had no methylation of any of the two gene promoters.

FHIT, GSTP1 and p16 mRNA expression in NSCLC patients

Data obtained from qPCR analysis revealed that the expression level of FHIT mRNA was downregulated in 53.57% (15/28) of NSCLC tissues and that of GSTP1 mRNA was downregulated in 21.43% (6/28) of NSCLC tissues while that of p16 mRNA was upregulated in 75% (21/28) of NSCLC tissues.

As shown in Figures (2-4), the mean FHIT mRNA level was at least 5 times lower in NSCLC (0.204) than in control (1.153) tissues ($p < 0.01$; Figure 2), while the mean GSTP1 mRNA level was nearly the same in both tissues ($p = 0.808$; Figure 3). In addition, the mean p16 mRNA level was increased in NSCLC (1.98) than in control (1.35) tissues, however, these differing results were not statistically significant ($p = 0.073$; Figure 4).

Discussion

Methylation is the main epigenetic modification in human and plays a significant role in cancer. Methylation

of tumor suppressor genes has been investigated in all kinds of cancer (Li et al., 2010).

Various studies reported separately that methylation of CPG islands of FHIT and GSTP1 has a significant part in the development of human cancers. In this study, the frequencies of FHIT promoter methylation among NSCLC and control groups were 53.57% and 3.57%, while for GSTP1 gene were 25% and 0% in NSCLC and control groups, respectively. In addition, the risk of FHIT hypermethylation increased among patients with NSCLC by 2.88, while the risk of GSTP1 hypermethylation increased among patients with NSCLC by 2.33.

The human FHIT gene, a tumor suppressor gene, is a member of the histidine triad gene family. Methylation of the FHIT gene has been observed in several solid tumors and an abridged or complete loss of expression of FHIT protein has been shown to be due to gene methylation leading to its transcriptional inactivation and disease progression (Sinha et al., 2013). Thus, detection of aberrant methylation of FHIT gene promoter would be useful for lung cancer diagnoses.

The CpG island in the GSTP1 promoter has been reported to be hypermethylated in prostate, hepatocellular, breast, renal, lung and colon cancer, as well as some lymphomas. Hypermethylation results in reduced GSTP1 expression, which has been speculated to make cells more susceptible to mutation and damage as a result of exposure to electrophiles and oxidative stress (Moyer et al., 2008). It is reported that CpG islands in GSTP1 are hypermethylated in >80% of hepatocellular carcinomas, ~30% of breast cancers, and >90% of prostate cancers (Maxwell et al., 2009).

In the present study, the results on the association analysis between FHIT and GSTP1 gene methylation and clinical pathological characteristics revealed that hypermethylation of FHIT gene had no correlation to gender ($p > 0.05$) but showed a significant correlation to smoking habit ($p < 0.05$). In contrast, no correlation between GSTP1 hypermethylation and smoking habit or any other tested parameter ($p > 0.05$).

Tobacco smoking is a major risk factor in the etiology of lung cancer which has been reported in many researches. There is evidence that smoking affects the mechanism of lung carcinogenesis (Zhou et al., 2014). A number of researchers have reported that the FHIT gene is specifically targeted by carcinogens in cigarette smoke and the frequency of aberrant methylation of FHIT gene increases in smokers (Stein et al., 2002; Mascaux et al., 2003; D'Agostini et al., 2006). The FHIT gene is sensitive to several chemical carcinogens such as benzo[a]pyrene (BP), benzo[a]pyrene diol epoxide (BPDE, the metabolic product of BP), dimethyl sulfate (DMS), and dimethylnitrosamine (DMN), found abundantly in cigarette smoke. The FHIT gene is inactivated by exposure to these carcinogens. So, abnormalities of FHIT are expected in tumor samples from lung cancer patients who have a history of smoking (Cecener et al., 2008).

GSTs appeared to be modestly tobacco responsive in the lung cancer. Gene expression of GSTP1 in alveoli, alveolar macrophages and respiratory bronchioles is more abundant than that of GSTM and other GSTs (Cantlay et

al., 1994; Spivack et al., 2003). It is therefore possible that GSTP1 may play an important role in local detoxification of xenobiotics in the lung, but is mostly noninducible to tobacco exposure, which was observed in multiple in vitro studies (Spivack et al., 2004; Han et al., 2005; Cauchi et al., 2006). If true, this is biologically problematic, as this phase I versus phase II imbalance potentially favors mutagenesis, rather than mutagen quenching. An animal GSTP1 knockout study indicates that GSTP1 plays a key role in vivo in determining susceptibility to lung cancer following exposure to chemical carcinogens (Ritchie et al., 2007).

Results obtained from this study indicated that FHIT methylation occurred more frequently in squamous cell carcinomas (77.78%) than other subtype of NSCLC while GSTP1 methylation occurred more frequently in large cell carcinoma (33.33%) but these differing results were not statistically significant ($p>0.05$).

Squamous cell carcinoma is usually present at central masses with endobronchial growth, while adenocarcinoma tends to be present as peripheral nodules or masses with pleural involvement. Thus, the more centrally located cells of the airways giving rise to squamous cell carcinoma are exposed to a greater concentration of tobacco carcinogens. The effect on carcinogen exposure depends on carcinogen deposition that is influenced by lipid solubility and diffusion between the air interface and the capillary bed. Thus, the profile of carcinogens and perhaps concentration would differ between the central and peripheral lung. Therefore, it is possible that FHIT of the centrally located cells of airways are more easily methylated by more deposition of carcinogens in smokers than in never-smokers and that this contributes to the development of squamous cell carcinoma in individuals exposed to tobacco smoke (Kim et al., 2004). To the best of our knowledge, there is no published data on the association between GSTP1 hypermethylation and the large cell carcinoma.

Several reports have clearly summarized data in favor of FHIT functioning as a TSG, including the frequent presence of homozygous deletions and inactivation at a relatively early stage of multistage pathogenesis (Lin et al., 2008). The loss of FHIT is an early event in the pathogenesis of lung cancer, showing that FHIT methylation plays a role in the early pathogenesis of lung cancer rather than in the progression of lung cancer (Tseng et al., 1999; Ki et al., 2008). However, in this study, a highly significant correlation was found between FHIT methylation and the pathologic stage, indicating that FHIT methylation plays a role in lung cancer's later metastatic stage rather than in its early pathogenesis. In contrast, no correlation between GSTP1 hypermethylation and pathologic staging ($p>0.05$) was observed, indicating that GSTP1 methylation plays a role in the early pathogenesis of lung cancer rather than in its later metastatic stage.

Aberrant FHIT transcripts were detected in 80-100% of small cell lung cancer (SCLC) and in 40-80% of non-small cell lung cancer (NSCLC) specimens (Zochbauer-Muller et al., 2000). In addition, FHIT expression was lost or reduced in NSCLC (Sozzi et al., 1998; Geradts et al., 2000; Mascaux et al., 2003). In the current study the level

of FHIT mRNA was reduced in 53.57% of NSCLC tissues and the mean FHIT mRNA level was lower in NSCLC (0.204) than in control (1.153) tissues.

Variation in the expression and activity of GSTP1 has been associated with a variety of human cancers. GSTP1 has been shown to function not only as a phase II drug-metabolizing enzyme, but also as a regulator of mitogen-activated protein kinases (MAPK) as a result of nonenzymatic, ligand-binding activity. Specifically, GSTP1 is an inhibitor of c-Jun-NH2 kinase (JNK) and TRAF2, resulting in alterations in downstream processes, such as cell cycle control and apoptosis (Moyer et al., 2008). Data obtained from this study revealed that the level of GSTP1 mRNA was reduced in 21.43% (6/28) of NSCLC tissues, however, the mean GSTP1 mRNA level was nearly the same in both tissues ($p=0.808$).

Transcriptional inactivation by cytosine methylation at promoter CpG islands of tumor suppressor genes is an important mechanism contributing to the development of human cancer (Loh et al., 2014). Aberrant methylation of the FHIT gene was reported in primary NSCLCs, and the methylation was associated with loss of FHIT mRNA and protein expression (Zochbauer-Muller et al., 2001). In this study there is a significant correlation between FHIT methylation and its mRNA expression ($p=0.001$).

Aberrant methylation of GSTP1 allows methyl binding domain (MBD) family proteins to interact with the promoter and makes it inaccessible to transcription factors, which inhibits gene expression. However, results of this study showed no significant correlation between GSTP1 methylation and its mRNA expression ($p=0.082$).

High p16 expression level has been associated with disease progression and poor prognosis in several tumor types, as it was noted in early-stage ovarian cancer (Henshall et al., 2001). In this study, no p16 promoter methylation was found among NSCLC and control groups and the level of p16 mRNA was elevated in 75% of NSCLC tissues.

p16INK4a is usually inactivated in many cancers through mutation, deletion or hypermethylation of the gene, resulting in reduced or loss of expression. But in situation of cellular transformation, in which pRB is directly inactivated, cells are released from growth-suppressive stimuli mediated by the p16INK4a. This leads to the conclusion that reduced or lost pRB function results in enhanced p16INK4a levels, as a result of a negative feedback control (Prakash et al., 2013).

In conclusion, the results of the current study suggest that methylation of FHIT is a useful biomarker of biologically aggressive disease in patients with NSCLC. FHIT methylation may play a role in lung cancer's later metastatic stage while GSTP1 methylation may play a role in the early pathogenesis of lung cancer rather than in its later metastatic stage.

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