

## ***SHOX2* and *WT1* Promoter Methylation Correlates with the Lung Cancer in Iranian Patients**

M. T. Khatibi<sup>1</sup>, Sh. Mohammad Ganji\*<sup>2</sup>, A. Kiani<sup>3</sup>, S. Chavoshei<sup>1</sup>,  
M. Negrini<sup>4</sup>, M. Tavallaie\*<sup>1</sup>

<sup>1</sup> Human Genetic Research Center, Baqiyatallah University of Medical Sciences, Tehran, Islamic Republic of Iran

<sup>2</sup> Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Islamic Republic of Iran

<sup>3</sup> Tracheal Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, Islamic Republic of Iran

<sup>4</sup> Dipartimento di Medicina Sperimentale e Diagnostica, Università di Ferrara, Ferrara, Italy

Received: 16 December 2017 / Revised: 26 February 2018 / Accepted: 17 June 2018

### **Abstract**

Lung cancer is among the most common cause of cancer death in the world. Since the disease is diagnosed in the middle or late stages of the cancer development a more efficient method for an early diagnosis of the disease is required. The main goal of this study was to investigate the correlation between aberrant promoter methylation of the two genes: *SHOX2* (Short stature homeobox 2) and *WT1* (Wilms' tumor gene1), in the plasma samples of the Iranian lung cancer patients. Following filling questionnaires and obtaining the consent of all participants, 70 cases including 45 patients and 25 healthy controls who underwent bronchoscopy procedure in the Masih-Daneshvari Hospital in Tehran, the blood samples were collected. DNA extraction was done and treatment was performed by sodium bisulfite applying Qiagen DNA methylation kit and Methylation specific PCR (MSP) was performed. The results showed a frequency of 82.2% and 55.55% of the hypermethylation for the *SHOX2* and *WT1* gene promoters in the caces samples, respectively. Our study demonstrated that hypermethylation of *SHOX2* and *WT1* genes promoter regions might be involved in lung cancer. The methylation of *SHOX2* and *WT1* in the blood plasma could be considered as the competent biomarkers for an early diagnosis of the lung cancer as a noninvasive method.

**Keywords:** Lung cancer; MSP; *SHOX2*; *WT1*; Plasma.

### **Introduction**

Lung cancer is the leading cause of cancer-related death worldwide. It has become the second leading cause of new cancer cases and the first leading cause of

death in the USA in 2015 [1].

Lung cancer is the fifth leading cause of tumors in Iran. While its incidence is very low, however, investigations have indicated a steady growth in the incidence rate of this type of cancer both in men and

\* Corresponding authors: Tel: +982144787466; Fax: +982144787399; Email: shahla@nigeb.ac.ir, Tel: +982186035680; Fax: +982186034943; Email: tavalla.mah@gmail.com

women during the recent years in this country. The low incidence of lung cancer is surprising, since, the prevalence of smoking among Iranian males over the age of 15 is 12.9%. The low incidence in part could be explained to be due to under-reporting and difficulty in tissue diagnosis [2-4]. Lung cancer is classified as non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [5]. The NSCLC accounts for about 80% of the global lung cancer cases and as a result the leading cause of worldwide lung cancer-related death [5]. Histologically, NSCLC is classified into adenocarcinoma (AC), squamous cell carcinoma (SCC), and large cell carcinoma [6].

Cigarette smoking is the foremost cause of lung cancer [7, 8]. However, additional significant risk factors including passive smoking [9], occupational exposure to the carcinogens, ambient air pollutants [10, 11], dietary factors, and cooking fuels can independently cause lung cancer [7]. The significance of each of these factors varies with gender, country, and region within a given country.

A major problem associated with the lung cancer is the late diagnosis as the disease could be diagnosed most often in the advanced stages of cancer development. However, it is suggested that early detection of cancer could be possible through regular screening [12]. The major reasons for the late diagnosis are the late appearance of the symptoms and a lack of reliable biomarkers for an early detection [13]. Overall the 5-year survival rate for the lung cancer varies globally, but, is consistently low. The 5-year survival rate of the lung cancer in the USA is lower than most other cancers [14]. Therefore, early detection of the lung cancer can improve 5-year survival rate and lower the rate of mortality [15].

Epigenetics has proven to have a role in the etiology of the lung cancer. Esteller, *et al* [16] have reviewed the important advances on the epigenetic biomarkers for the lung cancer. Among all epigenetic modifications, DNA methylation is the best characterized and methylation of cancer-related genes has been suggested to be the most common mechanism of their inactivation [17], and is commonly found in the lung cancer [18]. Hypermethylation of the critical genes involved in the important cellular functions such as cell cycle regulation, differentiation, adhesion, and apoptosis was reported to be involved in the downregulation of the related genes and as a result affecting the associated pathways in the lung cancer [19, 20]. Such a gene function downregulation mechanism has been suggested to contribute to the development of chemotherapy resistance [21]. Abnormal DNA methylation is a key factor in carcinogenesis [22]. Methylation patterns in

different cancer-related genes exhibit distinct features, which can be applied in the early cancer detection. DNA methylation occurs mainly at the C-phosphate-G (CpG) dinucleotides in the promoter region. It has been shown that between 60 and 90 % of the CpGs are methylated in mammals [23, 24]. Gene transcriptional silencing inhibits the expression of the downstream genes and often leads to the abnormal cell function, which, in turn, increases the risk of cancer [25-27, 45].

The *SHOX2* gene; also called OG12, OG12X or SHOT gene, is a member of the homeobox genes family and is localized on the chromosome 3 (3q25.q26.1) [28]. The *SHOX2* gene has 10 kb in size and consists of 7 exons encoding a 319 amino-acid protein. The protein encoded by this gene comprises a 60-amino acid DNA binding domain. The splicing variants of the *SHOX2* transcripts consist of the two variants (A (993 bp) and B (570 bp) [29-30]. The *SHOX2* gene is mostly expressed in the heart, gill arches, nose, limb bud, nervous system, and reproductive nodules of the human embryos [31]. Studies have shown that *SHOX2* is mostly expressed in the connective tissues and muscles at the proximal and middle part of the limbs, while *SHOX* gene expression is more toward distal [32, 33].

The *SHOX2* gene includes two large CpG islands, one at the 5' end, covering a region of about 1 kb, the other at the 3' end, covering about 500 bp [34]. Current studies have revealed that the level of *SHOX2* gene methylation at CpG islands significantly increases in the tissues and cells of the lung cancer compared to that of normal tissues and cells [34-36]. Since *SHOX2* gene encodes an intranuclear transcription factor, hypermethylation of the *SHOX2* gene may have a major influence on its gene expression, which, in turn, affects the level of expression of the multiple genes regulated by *SHOX2*, thus promoting cellular carcinogenesis [15]. Wilms' tumor gene 1 (*WT1*) was first reported as a tumor suppressor gene in Wilms' tumor, a childhood kidney neoplasm [37]. *WT1* was found to have oncogenic properties in the other malignancies, including breast [38], lung [39], ovarian [40], and brain [41] cancers. *WT1* is expressed in a tissue-specific manner. In the developing embryo, the *WT1* expression is found primarily in the urogenital system. In adult tissues, the *WT1* expression is found in the urogenital system, the central nervous system, and in the tissues involved in the hematopoiesis, including bone marrow and lymph nodes [42]. The *WT1* gene is located in the chromosomal locus 11p13. It encodes a 3 kb mRNA consisting of 10 exons [43]. This gene encodes a zinc finger transcription factor that plays an important role in the cell growth and differentiation [44]. The *WT1* overexpression has been demonstrated in the

carcinomas originated from a variety of tissues, for example, lung cancer [39]. Methylation of the *WT1* gene has been studied in different types of cancers, including colorectal cancer [46], breast cancer [47], ovarian clear cell adenocarcinoma [48], AML [49], and lung cancer [50]. *WT1* was identified to be a modulator of the oncogenic K Ras signaling in the lung cancer. Both in the mouse and human lung cells *WT1* regulates the proliferative potential of the oncogenic K Ras as loss of *WT1* drives cells expressing oncogenic K Ras toward a senescence program [51]. It was previously approved that Methylation Specific PCR (MSP) is a simple, rapid and inexpensive method for determining the methylation pattern of a gene [52, 53]. The aim of this study was to evaluate the hypermethylation of *SHOX2*, *WT1* promoter in plasma specimens for an early detection of the Lung cancer in Iranian patients using MSP method.

### Materials and Methods

**Study population:** A total of 70 patients who underwent bronchoscopy procedure during 2015 were included in the present study. Patients were admitted to the Masih Daneshvari Hospital, Tehran, Iran. None of the patients had received irradiation or chemotherapy preoperation. Tumor stage and histology reports were obtained from the department of pathology. Questionnaires were prepared with regard to patients' history, demographic status, as well as other information by the trained interviewers. According to the details presented in Table 2, the histopathological features indicate that 88.9% (40 out of 45 samples) of the patients were affected by NSCLC and 11.1% of the (5 out of 45) were patients with SCLC. Blood samples were collected from patients in K3EDTA vacuoliner tubes (Gold vac™China) and samples were divided into two groups, Lung cancer patients, and normal healthy individuals. The patient who had a previous therapeutic intervention such as chemotherapy, other types of

cancers and lung cancer disease were excluded from the study.

**DNA extraction:** The plasma was immediately separated from the cellular fraction by centrifugation at 2,500 rpm for 10 min and was frozen at -80 °C. DNA isolation from 500 µL plasma was conducted using salting out modified method, dissolved in 100 µL of the sterile distilled water and was stored at -80 °C. The concentration of the isolated DNA was quantified using a Nanodrop spectrophotometer (Maestrogen-USA).

### Methylation-specific PCR

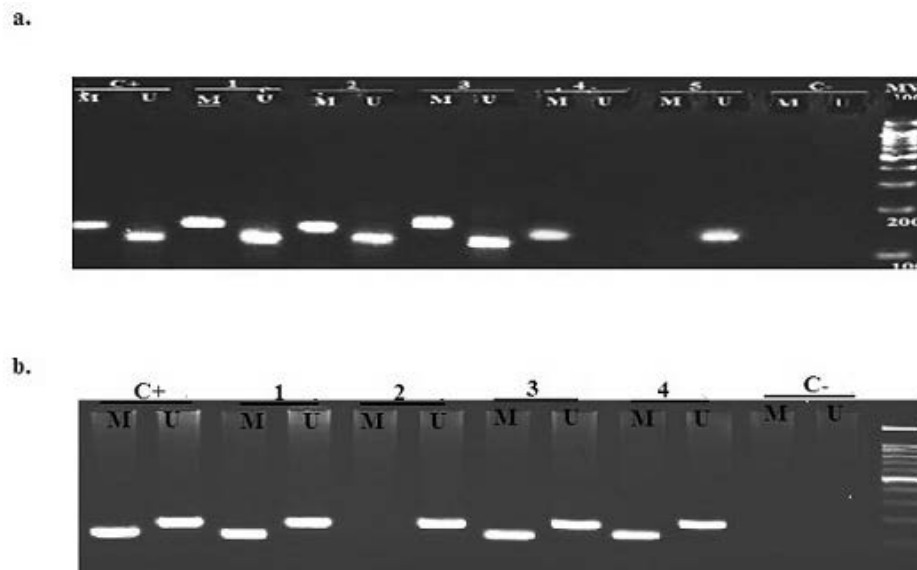
**Bisulfite modification:** 20 µL of the extracted DNA was treated by sodium bisulfite to convert all unmethylated cytosines to uracils using the EZDNA Methylation – Lightning™ Kit (Zymo Research-USA) according to the manufacturer instruction. To confirm bisulfite DNA conversions, unmethylated and methylated human control DNA (Qiagen-Germany) was used as a control.

**Methylation Specific PCR:** The methylation status of the target genes was determined using MSP method using specific primers for *TPEF* (a transmembrane protein containing epidermal growth factor and follistatin domains), as well as methylated and unmethylated *SHOX2*, *WT1* promoter, respectively. The bisulfate-modified DNA was used as the template for PCR. The primer sequences for the methylated and unmethylated templates are shown in Table 1. All amplifications were carried out in 12.5 µL reaction volume containing 1 mM dNTPs “(Fermentase Co., Burlington, Ontario, Canada)”, 0.2 µM of each specific oligonucleotide primers, 100 ng modified DNA, and 1 U/µL Taq polymerase “(Hot start, Qiagen,Valencia, CA, USA)”. After 15 min initial denaturation at 95 °C, the first amplification was carried out for 35 cycles of 95 °C denaturation and annealing at 54 °C, 56 °C, and 53 °C for *TPEF*, Meth and UnMeth *SHOX2* , *WT1* primers, respectively. Elongation was carried out at 72

**Table 1.** Primers and the target genes used in the present study.

Oligo name	Primer Sequences 5'--> 3'	Product Length (bp)
<i>SHOX2</i> _M*	GTTTTTGGATAGTTAGGTAAT(sence) CCTCCTACCTTCTAACCC(antisense)	122
<i>SHOX2</i> _U*	TAATTTTAGGTTAGAGGGTTATAC(sence) CACAACCAATCAACAACAGT(antisense)	112
<i>WT1</i> _M*	TTTTCGGGCGTTTCGGTTTAC(sence) ACTCCTCGTACGACTCCG(antisense)	149
<i>WT1</i> _U*	GGGTGTTTTATTGGGTCTATTCT(sence) AAAAAACTAACACAAACAAATAGAAAAG(antisense)	151
TPEF	TTAGTGGATGATTTTTTTGTTTTG(sence) ACAACAACAATAACAATAA(antisense)	122

\*M: Methylated-specific primers; \*U: Unmethylated-specific primers



**Figure 1.** Aberrant DNA methylation of *SHOX2* (a) and *WT1* (b) genes' promoter in lung cancer affected patients. (Panel A): the status of methylated and unmethylated *SHOX2* promoter. From left to right: C+/M, C+/U, lanes 1, 2, and 3: (M+/U+) represents three plasma samples displaying heterozygote for the *SHOX2* methylation, lane 4: M+, lane 5: U+, C-/M, C-/U, (MW): Molecular weight marker (100 bp ladder). (Fig B): the status of methylated and unmethylated *WT1* gene promoter. From left to right: C+/M, C+/U, Lanes 1, 3, and 4: (M+/U+), lane 2: U+, C-/M, C-/U, (MW): Molecular weight marker (50 bp ladder). C+: positive control, C-: negative control, M: methylation status, U: unmethylation status, M+: methylated positive, U+: unmethylated positive, M+/U+: heterozygote which displaying both methylated and unmethylated lanes are positive.

°C for 30 s, followed by the final extension at 72 °C for 10 min, universal gradient, applying thermal cycler (Thermal cycler, Camlab, UK). The PCR products were run in a 1.5% agarose gel (BioReagents, Fisher Scientific, UK). Positive control (methylated normal DNA, from Qiagen Co.) and Negative control (unmethylated normal DNA) was also included in each PCR reaction set (Figure 1).

**Statistical Modeling:** Statistical analysis was conducted using the SPSS22 software (version 22.0, SPSS Inc, Chicago, USA). The Fisher's exact test and  $\chi^2$  test was used to study the statistical relationships

between either MSP status, pathological, as well as demographical results evaluation of the hypermethylation in patients. The association between methylation status of these genes and lung cancer was determined using the logistic regression method in order to assess the odds ratio (ORs) and 95% confidence intervals (95%CI). The P-values less than 0.05 were considered as statistically significant.

## Results

Sample collection: During 2015 seventy patients

**Table 2.** Personal, demographic, and histopathological features correlated with the lung cancer

Information of patients	Cases(n=45)%	Controls(n=25)%
Age(mean,SD), years	64± 24.04	70±18.38
<50 yr, n (%)	3 (6.7)	9 (36.0)
51–60 yr, n (%)	14 (31.1)	3 (12)
>60 yr, n (%)	28(62.2)	13(52)
Range of age (year)	31–87	45–81
Gender, n (%)		
Male	36(80)	18 (72)
Female	9 (20)	7 (28)
Smoking status		
Nonsmokers, n (%)	17(37.8)	16(34)
Smokers, n (%)	27(60)	9(36)
Former smokers, n (%)	1(2.2)	0
Consumption opium, n (%)	14(31.1)	0

Table 2. Cntd

Family history of cancer	4(8.9)	0
Histology subtype, n (%)		
NSCLC, n (%)	40(88.9)	0
<i>Squamous cell carcinoma</i>	18(40)	0
<i>Adenocarcinoma,</i>	22(48.9)	0
SCLC, n (%)	5(11.1)	0
Benign lung disease and healthy, n (%)	0	25(100)
Stage, n (%)		
I& II	11(24.4)	0
III	17 (37.8)	0
IV	9 (20)	0
Unknown	4 (8.9)	-
Air born occupational Exposures, n (%)		
Yes	10(22.2)	1(2.9)
No	35(77.8)	24(97.1)

Table 3. The frequency of the methylation of the SHOX2, WT1 in patients based on clinical and Demographical Characteristics of the patients

Clinical characteristics	NO of Cases=45	Methylated n(%)		Unmethylated n(%)		P value	
		WT1	SHOX2	WT1	SHOX2	WT1	SHOX2
Gender, n(%)							
Male	36(80)	21(58.4)	28(77.8)	15(41.6)	8(22.3)	0.48	0.17
Female	9(20)	4(44.5)	9(100)	5(55.6)	0(0)		
Age distribution							
≤50 yr, n(%)	17(37.8)	7(41.17)	14(82.35)	10(100)	3(17.65)	0.21	>0.5
>50 yr, n(%)	28(62.23)	18(64.3)	23(82.2)	10(35.7)	5(17.8)		
Smoking status							
Nonsmokers, n(%)	17(37.8)	11(64.7)	16(94.1)	6(35.3)	1(5.88)	>0.5	0.12
Smokers, n(%)	27(60)	14(51.85)	20(74.07)	13(48.15)	7(25.93)		
Former smokers, n(%)	1(2.2)	0(0)	1(100)	1(100)	0(0)		
Histology subtype							
Adenoma carcinoma, n(%)	22(48.88)	11(50)	18(81.81)	10(45.55)	3(13.63)	*0.0088	*0.0001
Squamous cell carcinoma, n(%)	18(40)	13(72.22)	14(77.7)	4(22.22)	3(16.7)	*0.0001	*0.0001
SCLC, n (%)	5(11.11)	1(20)	5(100)	2(40)	0	>0.05	*0.0021
Stage							
I+II, n(%)	11(24.44)	8(72.7)	9(81.8)	3(27.3)	2(18.2)	*0.032	*0.002
III, n(%)	17(37.77)	9(52.9)	15(87.3)	6(35.3)	1(5.9)	*0.002	*0.0001
IV, n(%)	9(20)	5(55.5)	7(77.8)	4(44.4)	2(22.8)	*0.003	*0.0046
Unknown, n(%)	4(8.88)	2(50)	2(50)	1(25)	1(25)	>0.05	>0.05

were admitted to the Masih Daneshvari Hospital for cancer treatment and were underwent bronchoscopy. The demographic features, as well as pathology data, of the patients are summarized in Table 2.

#### SHOX2 and WT1 promoter methylation by MSP

We analyzed the methylation pattern of the SHOX2, WT1 promoter region in lung cancer samples and controls the results of which are listed in Tables 3, 4. The aberrant promoter methylation of the SHOX2 gene was detected in 82.2% (37 of 45) of the cases; for WT1 gene 55.55% (25 of 45) of cases were detected to be methylated. Moreover, the frequency of methylation was 48.88% (22 of the 45 studied samples) between SHOX2 and WT1 genes, while in case of healthy individuals the unmethylated promoter was observed in

19 out of 25 (76%) of the cases. There was a significant statistical association between promoter methylation of the separate genes with the lung cancer compared to the controls (p<0.01) (Table 4). As well, the combined methylation in the two genes demonstrated a significant association with the lung cancer when it was compared to the healthy controls (p<0.01).

#### Correlation of the SHOX2 and WT1 methylation with clinicopathological features

The relationship between methylation status of the SHOX2 and WT1 genes and the clinicopathological characteristics of the lung cancer is shown in Tables 3 and 4. There w'sn't a significant difference in the clinicopathological factors such as gender, age, site of disease, TNM stages, and other habitual risk factors

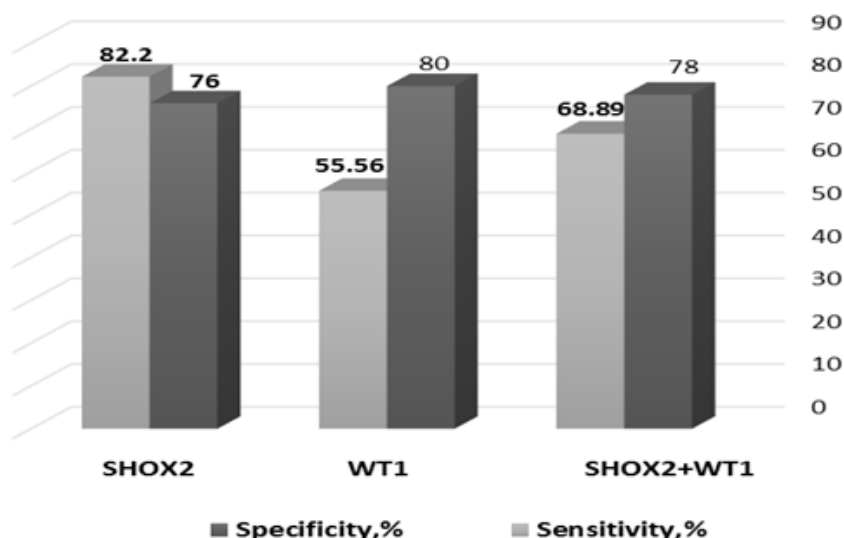
**Table 4.** The sensitivity and specificity of the different biomarkers and biomarker are presented. All data for *SHOX2*, *WT1* methylation values are presented as percentage, numbers, and a confidence of 95% intervals, respectively.

Statistical analysis	<i>WT1</i>	<i>SHOX2</i>	<i>WT1+SHOX2</i>
Sensitivity,% ,OR(95% CI)	55.56(40-70.36)	82.22 ( 67.95-92)	68.89(58.26-78.23)
Specificity,% , OR95% CI)	80(59.3-93.17)	76(54.87-90.64)	78(64.04-88.47)
PLR,% , OR(95% CI)	2.78( 1.22-6.35)	3.43(1.68-6.97 )	3.13(1.82-5.37)
NLR,% , OR(95% CI)	0.56 ( 0.38-0.81)	0.23(0.12-0.46)	0.40( 0.28-0.56)
PPV,% , OR(95% CI)	83.33(5.28-94.36)	86.05(72.07-94.7)	84.93 (74.64-92.23)
NPV,% , OR(95% CI)	50(33.80-66.20)	70.37( 49.8-86.25)	58.21(45.52-70.15)

\* PLR:Positive Likelihood Ratio,NLR: Negative Likelihood Ratio,PPV: Positive Predictive Value, NPV:Negative Predictive Value.

(Table 3) with methylation status of these genes. We studied the frequency of *SHOX2*, *WT1* genes promoter (hypermethylation in this group of patients with samples (adenocarcinomas, squamous cell carcinomas, small cell carcinoma) representing all stages of the disease using MSP. Statistical analysis revealed that *SHOX2* gene was hypermethylated in 37 of the 45 patients (82.2%), in which 6 individuals were homozygous (13.3%) and 31 other were heterozygous (68.9%). On the others hand, *WT1* gene was hypermethylated in 25 of 45 patients (55.55%), in which 1 individual was homozygous (2.2%) and 24 were heterozygous (53.33%). While in the case of healthy individuals the unmethylated status of *WT1* gene was observed in 19 of the 25 (76%) of the cases (Table 4). The invalid methylation was observed in 2 cases (1 case of adenocarcinoma and the other case of a healthy individual). These results indicated 82.22% sensitivity and 76% specificity for detecting lung cancer by using the plasma samples as a noninvasive method (Table 4). Patients under study had the mean age of 64 years.

Among the patient 20% of cases were female and the other 80% were male. Therefore, analysis of *SHOX2*, *WT1* methylation in the peripheral blood plasma revealed the sensitivity of 82.2% and 55.56% , as well as the specificity of 76% and 80%, the PPV of 86.05% and 83.33%, in addition to NPV of 70.37% and 50%, respectively (Table 4, and Figure 2). The present study shows that methylation levels were significantly higher in patients with lung cancer compared to their normal counterparts ( $p < 0.005$ ). The greatest amount of methylation of the *SHOX2* was observed in patients with small cell lung cancer (100%) and the lowest methylation was seen in squamous cell carcinoma (77.7%). This result confirmed the previous studies [34, 35]. The greatest amount of methylation of the *WT1* was observed in patients with squamous cell carcinoma (72.2%) and the lowest methylation was seen in small cell lung cancer (20%). The methylation of *SHOX2* was frequent in adenocarcinoma (81.81%) somewhat higher than that of squamous cell carcinoma (77.7%), ( $p < 0.05$ ), Table 3, Figure 2), while methylation of *WT1* was more



**Figure 2.** The specificity and Sensitivity of the *SHOX2* and *WT1* genes promoter methylation based on the Combine Cross Tabulation applying SPSS vrsion 22.

frequent in the squamous cell carcinoma (72.2%) when compared to adenocarcinoma (50%), ( $p < 0.05$ ). There were 33 cases of non-smokers (47.14%), 17 cases in the positive group, and 16 cases in the negative group. The rest of the patients (52.85%) were active or former smokers ( $n=37$ ), 28 cases with detected malignancy, and 9 cases without lung cancer (Table 4). The relationship between methylation of these genes and clinicopathological features of the lung cancer was analyzed and the results were listed in Table 3. As result shows 81.8% (9/11) of the *SHOX2* methylated lung cancer patients were in stage I+II, whereas 87.3% (15/17) were in stage III, and 77.8% (7/9) in stage IV of the lung cancer, respectively (Table 2). For *WT1* methylated lung cancer, it was seen that 72.7% (8/11) of the patients were in stage I+II, 52.9% (9/17) in stage III, and 55.5% (5/9) were in stage IV of lung cancer development (Table 3). The largest evaluation of the *SHOX2* gene methylation was observed at high levels in stage III. As well, the greatest amount of methylation of the *WT1* was observed in patients in the stage I+II (Table 4).

### Discussion

DNA methylation detection has shown to be as a promising potential biomarker identification technology for implementation in the cancer medicine [25, 26]. DNA is the most stable biological macromolecule, and being as a covalent modification, DNA methylation represents a chemical change to the native DNA that remains stable upon the removal of clinical specimens from the body. The pattern of gene methylation affects long sequences of the DNA rather than a single nucleotide enabling more efficient and robust assay design [26, 27]. The promoter hypermethylation occurs frequently in human cancer and is an early event in the cancer development [12]. The aim of this study was an evaluation of the two genes epigenetic marker *SHOX2*, *WT1* for an early detection of the patients with lung cancer, NSCLC subtype. Since hypermethylation of these genes occurs at the early stages of the tumor formation, they are detectable in plasma and tissue samples. Within these years, several groups and research centers had reported a large number of clinical results about the sensitivity and specificity of the DNA tests by using different markers in lung cancer patients. The *SHOX2*, *WT1* genes methylation may represent the potential improvement in the lung cancer management. There are several techniques for the detection of DNA methylation and each technology has its advantages and disadvantages [52, 53]. A suited method to screen a large number of samples with respect to the methylation

status of the specific genes is methylation-specific PCR (MSP) as it is rapid and cost-effective [23]. MSP is very sensitive and allows detection of a methylated gene among 1000 unmethylated copies of the genomic DNA [24]. We used methylation-specific PCR (MSP) for analysis of the methylation status of the *SHOX2* and *WT1* genes, while the previous studies have used quantitative MSP in these genes [34, 36, 49, 50]. Bernd Schmidt *et al.* introduced *SHOX2* DNA methylation as a tumor marker in patients suspected of lung cancer in using bronchial fluid samples obtained after bronchoscopy by QMSP [35]. Kneip *et al.* have evaluated methylation status of the *SHOX2* gene in patients with lung cancer applying their plasma samples with a sensitivity of 60% and a specificity of the 90% [34]. In 2016, M. Konecny *et al.* reported methylation detection of the *SHOX2* in plasma samples with a sensitivity of 64.5% and specificity of 78.57 % [36]. We could detect the hypermethylation frequency of *SHOX2* gene in plasma samples of the non-small cell lung cancer (NSCLC) patients, as well as the healthy individuals. The results indicated a sensitivity of 82.2% and specificity of 76%; estimations which are higher and lower than the previously reported studies, respectively [34, 36, 54]. Moreover, based on the statistically significant results, the frequency of the simultaneous methylation in patients was lower than single gene methylation. It could be argued the use of larger sample size plus application of the quantitative method in the similar studies would increase the reliability and performance of the Q-MSP method in the analysis of the methylation and will lead to the more accurate results.

Our result shows *SHOX2* methylation in patients with stages III is detectable at a higher sensitivity rate when compared with the stage II+I patients (Table 3). Both small cell lung cancer and squamous cell carcinoma were detected at the highest sensitivity when compared with adenocarcinomas [34, 36]. We detected a hypermethylated *WT1* gene in the plasma samples of NSCLC patients as well as healthy individuals. The estimated sensitivity of 55.56 % is higher than that reported by G. Nikolaidis *et al.* and the specificity of 80% is lower than their report, respectively [49]. Our result shows that the rate of *WT1* methylation in NSCLC patients in stages II&I is detectable at a higher sensitivity than stage III (Table 3). Up to now few studies have been done on the methylation of the *WT1* gene in the lung cancer in the world, this study was the first study on this gene in plasma samples. One of the limitations in the present research was the size of the sample under study, so authors propose an increase in the sample size both for blood and lung tissue will

provide us with a more accurate evaluation on the role of these two genes' promoter methylation in the tumorigenesis of the lung cancer.

### Conclusion

Considering the results obtained in the present study, several important outcomes could be achieved. As an example, focusing on the results obtained for the frequency of the simultaneous hypermethylation of the *SHOX2* and *WT1* genes' promoter in combination to the pathology reports of the patients it could be expressed that these genes could be introduced as the specific and sensitive biomarkers for diagnosis of the patients at risk for lung cancer through bronchoscopy. Briefly, the results of numerous studies on the use of biomarkers, especially by noninvasive sampling methods, are expected to envisage a better landscape of the lung cancer screening, resulting in a reduction the mortality caused by lung cancer through an early detection of the lung cancer. The results of this study suggest that methylation status of the *SHOX2* and *WT1* genes may be competent biomarkers for the early diagnosis of the lung cancer as a noninvasive method in the patients with negative cytology.

### Acknowledgments

This work was supported by the Center for International Scientific Studies & Collaboration (CISSC). Also, the Human Genetic Research Center, Baqiyatallah University of Medical Sciences supported this work by providing equipment and reagents. The authors would like to express their appreciation to the Baqiyatallah Hospital and Masih Daneshvari Hospital, Tehran, for providing the Blood and tissue samples.

### References

- Siegel R., Fedewa S., Miller K., Goding-Sauer A., Pinheiro P., Martinez-Tyson D., Jemal A. Cancer statistics for Hispanics/Latinos. *CA. Cancer J. Clin.*, **65**(6):457-480(2015).
- Abedifar H. The most common cancers in Iranian women. *Iranian J. Publ. Health*, **38**(1): 109-112(2009).
- Hosseini M., Naghan P.A., Karimi S., SeyedAlinaghi S., Bahadori M., Khodadad K., Mohammadi F., Kaynama K., Masjedi M.R. Environmental risk factors for lung cancer in Iran: a case-control study. *Int. J. Epidemiol.*, **38**(4):989-996(2009).
- Meysamie A., Ghaletaki R., Zhand N., Abbasi, M. Cigarette smoking in Iran. *Iranian J. Publ. Health*, **41**(2):1-14(2012).
- Brambilla E., Travis W., Colby T., Corrin B., Shimosato Y. The new World Health Organization classification of lung tumours. *Eur Respir J*, **18**(6):1059-1068(2001).
- Jemal A., Bray F., Center M., Ferlay J., Ward E., Forman D. Global cancer statistics. *CA. Cancer J. Clin.*, **61**(2):69-90(2011).
- Biesalski H.K., Bueno de Mesquita B., Chesson A., Chytil F., Grimble R., Hermus R.J., Köhrle J., Lotan R., Norpoth K., Pastorino U., Thurnham D. European consensus statement on lung cancer: risk factors and prevention. Lung cancer panel. *CA. Cancer J. Clin.*, **48**(3):167-176(1998).
- Hoffmann D. H. I. The changing cigarette, 1950-1995. *J. Toxicol. Environ. Health*, **50**(4): 307-364(1997).
- Jaakkola M., Jaakkola J. Impact of smoke-free workplace legislation on exposures and health: possibilities for prevention. *Eur. Respira. J.*, **28**(2): 397-408(2006).
- Alberg A., Ford J., Samet J. Epidemiology of lung cancer: ACCP evidence-based clinical practice guidelines. *Chest J.*, **132**(3): 29S-55S(2007).
- Goldberg M. A systematic review of the relation between long-term exposure to ambient air pollution and chronic diseases. *Rev. Environ. Health*, **23**(4): 243-298(2008).
- Schiller J., Harrington D., Belani C., Langer C., Sandler A., Krook J., Zhu J. and Johnson D.H. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N. Engl. J. Med.*, **346**(2): 92-98(2002).
- Lokk K., Vooder T., Kolde R., Väik K., Vösa U., Roosipuu R., Milani L., Fischer K., Koltsina M., Urgard E. and Annilo T. Methylation markers of early-stage non-small cell lung cancer. *PloS one*, **7**(6), e39813 (2012).
- Howlander N., Noone A.M., Krapcho M., Neyman N., Aminou R., Waldron W., Altekruse S.F., Kosary C.L., Ruhl J., Tatalovich Z., Cho H., Mariotto A., Eisner M.P., Lewis D.R., Chen H.S., Feuer E.J., Cronin K.A. and Edwards B.K., SEER cancer statistics review, 1975-2008. *J. Nat. Cancer Inst. Monogr.*, **19**(2011).
- Song L., Yu H., Li Y. Diagnosis of lung cancer by *SHOX2* gene methylation assay. *Mol. Diag. Ther.*, **19**(3):159-167(2015).
- Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Gen.*, **8**(4): 286-298(2007).
- Zöchbauer-Müller S., Fong K., Virmani A., Geradts J., Gazdar A., Minna J. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res.*, **61**(1): 249-255(2001).
- Tsou J., Hagen J., Carpenter C., Laird-Offringa I. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. *Oncogene*, **21**(35): 5450-5461(2002).
- Walter K., Holcomb T., Januario T., Yauch R.L., Du P., Bourgon R., Seshagiri S., Amler L.C., Hampton G.M., S Shames D.. *Discovery and development of DNA methylation-based biomarkers for lung cancer*, **6**(1):59-72 (2014).
- Belinsky S. Silencing of genes by promoter hypermethylation: key event in rodent and human lung cancer. *J. Carcinog.*, **26**(9): 1481-1487(2005).
- Zhang X., Li W., Li H., Ma Y., He G., Tan G. Genomic methylation profiling combined with gene expression microarray reveals the aberrant methylation mechanism



- involved in nasopharyngeal carcinoma taxol resistance. *Anticancer Drugs*, **23**(8): 856-864(2012).
22. Das P. M., Singal R. DNA methylation and cancer. *J. Clin. Oncol.*, **22**(22): 4632-4642(2004).
  23. Dahl C., Guldberg P. DNA methylation analysis techniques. *Biogerontology*, **4**(4): 233-250(2003).
  24. Herman J. G., Graff J., Myöhänen S., Nelkin B., Baylin S. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Nat. Acad. Sci.*, **93**(18): 9821-9826(1996).
  25. Sharma S., Kelly T., Jones P. Epigenetics in cancer. *Carcinog*, **31**(1): 27-36(2010).
  26. Hatziapostolou M., Iliopoulos D. Epigenetic aberrations during oncogenesis. *Cell Mol. Life Sci.*, **68**(10): 1681-1702(2011).
  27. Ducasse M., Brown M. Epigenetic aberrations and cancer. *Mol. cancer*, **5**(1): 1(2006).
  28. De Baere E., Speleman F., Van Roy N., De Paep A., Messiaen L. Assignment1 of *SHOX2* (alias OG12X and SHOT) to human chromosome bands 3q25→q26. 1 by in situ hybridization. *Cytogenet. Genome Res.*, **82**(3-4): 228-229(1998).
  29. Blaschke R.J., Monaghan A., Schiller S., Schechinger B., Rao E., Padilla-Nash H., Ried T., Rappold G.A. SHOT, a SHOX-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. *Proc. Nat. Acad. Sci.*, **95**(5): 2406-2411(1998).
  30. Semina E., Reiter R., Murray J. A new human homeobox gene OG12X is a member of the most conserved homeobox gene family and is expressed during heart development in mouse. *Human Mol. Genet.*, **7**(3): 415-422(1998).
  31. Clement-Jones M., Schiller S., Rao E., Blaschke R.J., Zuniga A., Zeller, R., Robson S., Binder G., Glass I., Strachan T., Lindsay, S. The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. *Human Mol. Genet.*, **9** (5): 695-702(2000).
  32. Yu L., Liu H., Yan M., Yang J., Long F., Muneoka K., Chen Y. *SHOX2* is required for chondrocyte proliferation and maturation in proximal limb skeleton. *Dev. Biol.*, **306**(2): 549-559(2007).
  33. Oliveira C., Alves C. The role of the SHOX gene in the pathophysiology of Turner syndrome. *Endocrinol. Nutr.*, **58**(8): 433-442(2011).
  34. Kneip C., Schmidt B., Seegebarth A., Weickmann S., Fleischhacker M., Liebenberg V., Field J.K., Dietrich D.. *SHOX2* DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *Thorac. Oncol.*, **6**(10): 1632-1638(2011).
  35. Schmidt B., Liebenberg V., Dietrich D., Schlegel T., Kneip C., Seegebarth A., Flemming N., Seemann S., Distler J., Lewin J., Tetzner R., Weickmann S., Wille U., Liloglou T., Raji O., Walshaw M., Fleischhacker M., Witt Ch. and Field J.K. *SHOX2* DNA methylation is a biomarker for the diagnosis of lung cancer based on bronchial aspirates. *BMC cancer*, **10**(1): 1(2010).
  36. Konecny M., Markus J., Waczulikova I., Dolesova L., Kozlova R., Repiska V., Novosadova H., Majer I. The value of *SHOX2* methylation test in peripheral blood samples used for the differential diagnosis of lung cancer and other lung disorders. *Neoplasms*, **63**(2):246-253 (2016).
  37. Haber D., Sohn R., Buckler A., Pelletier J., Call K., Housman D. Alternative splicing and genomic structure of the Wilms tumor gene *WT1*. *Proc. Nat. Acad. Sci.*, **88**(21): 9618-9622(1991).
  38. Loeb D., Evron E., Patel C., Sharma P., Niranjani B., Buluwela L., Weitzman S., Korz D., Sukumar S. Wilms' tumor suppressor gene (*WT1*) is expressed in primary breast tumors despite tumor-specific promoter methylation. *Cancer Res.*, **61**(3): 921-925(2001).
  39. Oji Y., Miyoshi S., Maeda H., Hayashi S., Tamaki H., Nakatsuka S., Yao M., Takahashi E., Nakano Y., Hirabayashi H., Shintani Y. Overexpression of the Wilms' tumor gene *WT1* in de novo lung cancers. *Int. Cancer*, **100**(3): 297-303(2002).
  40. Shimizu M., Toki T., Takagi Y., Konishi I., Fujii S. Immunohistochemical detection of the Wilms' tumor gene (*WT1*) in epithelial ovarian tumors. *Int. Gynecol. pathol.*, **19**(2): 158-163(2000).
  41. Oji Y., Suzuki T., Nakano Y., Maruno M., Nakatsuka S.I., Jomgeow T., Abeno S., Tatsumi N., Yokota A., Aoyagi S., Nakazawa T. Overexpression of the Wilms' tumor gene *WT1* in primary astrocytic tumors. *Cancer Sci.*, **95**(10): 822-827(2004).
  42. Scharnhorst V., van der Eb A., Jochemsen A. *WT1* proteins: functions in growth and differentiation. *Gene*, **273**(2): 141-161(2001).
  43. Call K., Glaser T., Ito C., Buckler A., Pelletier J., Haber D., Rose E., Kral A., Yeger H., Lewis W., Jones C. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell*, **60**(3): 509-520(1990).
  44. Sugiyama H. Wilms' tumor gene *WT1*: its oncogenic function and clinical application. *Int. Hematol.*, **73**(2): 177-187(2001).
  45. Gerd P. Pfeifer. Defining Driver DNA Methylation Changes in Human Cancer. *Int. J. Mol. Sci.* **19**: 1166-1178 (2018).
  46. Hiltunen M. O., Koistinaho J., Alhonen L., MyrÄ, S. Hypermethylation of the *WT1* and calcitonin gene promoter regions at chromosome 11p in human colorectal cancer. *Brit. Cancer*, **76**(9): 1124(1997).
  47. Laux D., Curran E., Welshons W., Lubahn D., Huang T. Hypermethylation of the Wilms' tumor suppressor gene CpG Island in human breast carcinomas. *Breast Cancer Res. Treat.*, **56**(1): 35-43(1999).
  48. Kaneuchi M., Sasaki M., Tanaka Y., Shiina H., Yamamoto R., Sakuragi N., Enokida H., Verma M., Dahiya R. *WT1* and *WT1-AS* genes are inactivated by promoter methylation in ovarian clear cell adenocarcinoma. *Cancer*, **104**(9): 1924-1930(2005).
  49. Guillaumet-Adkins A., Richter J., Odero M., Sandoval J., Agirre X., Catala A., Esteller M., Prósper F., Calasanz M., Buño I., Kwon M. Hypermethylation of the alternative *AWT1* promoter in hematological malignancies is a highly specific marker for acute myeloid leukemias despite high expression levels. *J. Hematol. Oncol.*, **7**(1): 1(2014).
  50. Bruno P., Gentile G., Mancini R., De Vitis C., Esposito M.C., Scozzi D., Mastrangelo M., Ricci A., Mohsen I.,

- Ciliberto G., Simmaco M. *WT1* CpG islands methylation in human lung cancer: a pilot study. *Biochem. Biophys. Res. Commun.*, **426**(3): 306-309.
51. Vicent S., Chen R., Sayles L., Lin C., Walker R., Gillespie A., Subramanian A., Hinkle G., Yang X., Saif S., Root D. Wilms tumor 1 (*WT1*) regulates KRAS-driven oncogenesis and senescence in mouse and human models. *J. Clin. Invest.*, **120**(11): 3940-3952(2010).
52. Shames D., Minna J., Gazdar A. Methods for detecting DNA methylation in tumors: from bench to bedside. *Cancer lett.*, **251**(2): 187-198(2007).
53. Shen L., Waterland R. Methods of DNA methylation analysis. *Cur. Opin. Clin. Nutr. Metabol. Care.*, **10**(5): 576-581(2007).
54. Yan L., Shulin L., Shiguo S., Yabin G., Jun S., Ling L. Methylated DNA/RNA in Body Fluids as Biomarkers for Lung Cancer. *Biol. Proc. Online.* 19: 2-9(2017).