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Research Article

ABNORMAL PROMOTER METHYLATION OF THE *ADENOMATOUS POLYPOSIS COLI* (APC) GENE IN IRANIAN LUNG CANCER PATIENTS

Malek Rahmani-khalili[1,3], *Mastaneh Zohri[1], Seyed Kazem Bidoki[3], Mehdi Fazlalipour[4], Reza Sheikhnejad[1], Mohammad Behgam Shadmehr[2] and Neda Doozandeh[2]

[1]*Molecular and Cancer Biology, Tofigh Darau, Research and Drug Engineering Company, Tehran, Iran*
[2] *Tracheal Diseases Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[3] *Payam Noor University, Basic Science and Agriculture, Tehran branch, Tehran, Iran*
[4] *Department of Medical Virology, Iran University of Medical Sciences, Tehran, Iran*
**Author for Correspondence*

ABSTRACT

Lung cancer is the main cause of cancer mortality throughout the world and leads more than 1.3 million annual deaths. Epigenetic inactivation of certain tumor suppressor genes by irregular promoter methylation is frequently reported in lung cancer. Genetic abnormalities of tumor suppressor genes are famous changes which are frequently involved in lung cancer pathogenesis. Hypermethylation of CpG islands in adenomatous polyposis coli (*APC*) gene has been shown in different human tumors, which has roles in the pathogenesis of lung tumors. The real-time PCR-based method MethyLight used to detect DNA methylation of the *APC* gene in 97 primary lung cancers (50 surgery and 47 biopsy), 20 lung samples from non-cancer patients (other lung diseases) and 43 healthy controls. *APC* promoter methylation was detected in 47 (48.4%) of 97 tumor samples (76% in surgery samples as compared to 19.1% in biopsy samples), but also in five of 20 (25%) lung samples from non-cancer patients, and in only three (6.9%) of 43 healthy controls. The median level of *APC* promoter methylation was 24.29 in human lung cancer, 9.85 in non-cancer patients, and 1.69 in healthy controls. QMSP analysis demonstrated a statistically significant difference in methylation level (P=0.00009 Mann Whitney Test) in tumor samples as compared with normal leukocyte. In this prospective study, the aberrant *APC* gene methylation in lung cancer patients is significantly correlated to primary tumor. However, we observed there is a ratio of difference in APC's methylation which is significant among the biopsy and surgery groups.

***Keywords**: Lung Cancer, APC, Hypermethylation, Methylight, Tumor Suppressor Genes*

INTRODUCTION

Lung cancer is the leading reason of cancer-related deaths and the most common cancer in the world. In 2008, 1.6 million new cases of primary lung cancer were diagnosed, and 1.3 million people died of this disease in worldwide (Ferlay *et al.,* 2010). Clinically, Lung cancer is classified into two classes: small non-small cell lung cancer (NSCLC) and cell lung cancer (SCLC). SCLC is the more aggressive subtype, and responsible for 10–15% of all cases. The remaining 85–90% of cases classified as NSCLC, which divided into four categories; adenocarcinoma (AD), squamous cell carcinoma (SCC), large cell carcinoma (LC) and "other" [comprising neuroendocrine cancers, carcinoids etc.] (Moran *et al.,* 2012; Urgard *et al.,* 2011). The most effective treatment for lung cancer is Surgery, but this method is limited because more than 60% of patients are in advanced stages of the disease at the time of diagnosis. So lung cancer's detection in earlier stages could increase survival rates by 10- to 50-fold (Begum *et al.,* 2011). Although lung cancer screening by chest X-ray and sputum cytology are ineffective in increasing patient survival, leading to the search for more sensitive and specific tests (Jantus-Lewintre, 2012). One favorable incoming is the recognition of lung cancer-specific biomarkers and noninvasive methods for the detection of these biomarkers in early stages. More recently, epigenetic changes appeared as a source for biomarker development (Ma *et al.,* 2013). Epigenetic changes, such as DNA methylation, are one of the most common molecular modifications in human neoplasia (Sajan *et al.,* 2013). DNA hypermethylation is the

addition of a methyl group to the cytosine ring of those cytosines which are ahead of guanosine (called CpG islands) to form methyl cytosine (5-methylcytosine). CpG islands are detected in the promoter region of many genes, and methylation in the promoter region is related to "gene silencing" (Auwera *et al.*, 2009). Silencing of tumor suppressor or other cancer-related genes by methylation of CpG islands is a common feature of human cancer (Ibragimova *et al.*, 2010). There for some genes such as *APC*, *CDKN2A*, *CDH1*, *RAR*-β2, and *RASSF1A* have been reported to be hypermethylated in over 30% of lung tumors (Li *et al.*, 2012; Sasaki *et al.*, 2010; Pan *et al.*, 2009). The adenomatous polyposis coli (*APC*) tumor suppressor gene is located on chromosome 5q21-q22 and encodes a homodimeric protein which has especial functions in the cytoplasm and nucleus of the cells and has an important role in cell cycle arrest and apoptosis (Rigi-Ladiz *et al.*, 2011).The wild type *APC* protein plays a role as a main controller in the Wnt signaling pathway (Roberts *et al.*, 2012). In different studies, *APC* promoter methylation also occurs in a significant number of primary lung tumors (ranging from 20 to 95% of cases) (Pan *et al.*, 2009; Anglim *et al.*, 2008; Zhu *et al.*, 2012). Based on huge investigations across the worldwide, it is clear that APC gene methylation has been reported in different places with different cultures in varied population. This can prove the theory of environment and lifestyle in different countries leading methylation of a certain gene in cancer, which is a good reason for more investigations on lung cancer cases in countries. Iran is located in Middle East and the World Health Organization (WHO) estimated the fraction of 11% for males and 3% for females in the regions of western Asia and northern Africa suffering from lung cancer. The incidence of lung cancer in Iran is reported 12 per 100,000 population and 12% of the lung cancers are males and 1% among females (Driscoll *et al.*, 2005; Mohagheghi *et al.*, 2009). This study is intended to determine the valid relationship between hypermethylation of *APC* 1A gene promoter and occurrence of lung cancer in Iran. One of the latest techniques of QMSP, methylight has been used together with Real time PCR to determine methylation level in *APC* gene.

MATERIALS AND METHODS

Population

In our study, 117 tissue samples of patients evaluated with undergoing bronchoscopy and surgical resection of lung diseases and 97 primary lung tumors and 20 non-cancerous lung tissues reported in 2010 to 2012 at Tracheal Diseases Research Center, Shahid Beheshti University of Medical Sciences, Tehran/Iran. Obtained tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C until use. Histological examination was performed by pathology department to determine cancer type and their stage. Methylation analysis was done in Molecular and Cancer Biology, Tofigh Darau, Research and Drug Engineering Company, Tehran/Iran. DNAs from peripheral blood lymphocytes (n=43) from healthy volunteers were used as negative controls for Quantitative methylation specific assays.

DNA Isolation and Bisulfite Treatment

DNA isolation was done by QIAamp DNA Mini Kit (Cat. No. 51304; Qiagen, Hilden, Germany). According to the manufacturer's instructions, our samples were digested with protease K and DNAs were extracted from 200 µL protease K digested samples, following the manufacturer's protocol. DNA concentration was measured using a spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE) at 260 nm absorbance. Bisulfite treatment was performed by using EpiTect Bisulfite kit (Cat. No. 59104; Qiagen, Hilden, Germany) following the guidelines of the manufacturer. Bisulfite-treated DNA was eluted in 40 µL of elution buffer and stored at −80°C.

Quantitative Real-Time PCR (MethyLight)

Bisulfite-modified DNAs were used as template for fluorescence-based real-time PCR (Taqman), as previously described (Carvalho *et al.*, 2011). In brief, primers and probe were designed to specifically amplify the bisulfite-converted APC promoter 1A gene. In addition, primers and a probe were designed to amplify an internal reference gene, *ACTB*. These were located in areas without CpG nucleotides, thus amplifying the modified *ACTB* gene independently of the methylation status of CpG nucleotides.

The sequences are the following: (*a*) *APC promoter* (74-bp amplicon; position 761–834; GenBank accession number U020509), forward primer 5′-GAA CCA AAA CGC TCC CCA T-3′; reverse primer 5′-TTA TAT GTC GGT TAC GTG CGT TTA TAT-3′; TaqMan probe 6FAM5′-CCC GTC GAA AAC CCG CCG ATT A-3′TAMRA; and (*b*) *ACTB* (133-bp amplicon; position 390–522; deposited at GenBank accession number Y00474), forward primer 5′- TGG TGA TGG AGG AGG TTT AGT AAG T-3′; reverse primer 5′- AAC CAA TAA AAC CTA CTC CT -3′; TaqMan probe 6FAM5′- ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA -3′TAMRA (Harder *et al.*, 2008).

Amplification reactions were carried out in triplicate in a reaction volume of 20 µL containing 600 nmol/L of each primer; 200 nmol/L probe; 5 unit of Taq polymerase (Cinnagen, Iran); 200 µmol/L of each dATP, dCTP, dGTP, and dTTP; 16.6 mmol/L of ammonium sulfate; 6.7 mmol/L of magnesium chloride; 10 mmol/L of mercaptoethanol; and 0.1% dimethylsulfoxide. Three microliters of treated DNA solution were used in each real-time QMSP reaction. Amplifications were done in 96-well plates in a StepOne plus Detector System (Applied Biosystems, ABI, USA). Thermal cycling was initiated with a first denaturation step at 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each plate included patient DNA samples, positive (in vitro methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls, and multiple water blanks. Leukocyte DNA from a healthy individual was methylated in vitro with excess SssI methyltransferase (Zymoresearch, USA) to generate completely methylated DNA, and serial dilutions of this DNA were used to construct a calibration curve for *APC* and *ACTB* genes (Figure 1). The ratio between the values is calculated in these two TaqMan analyses. The extent of methylation at a specific locus is determined by the following PMR (percentage methylated reference) formula: $[(\text{gene/actb})^{\text{sample}}: (\text{gene/actb})^{\text{SssI-treated genomic DNA}}] \times 100$.

Statistical Methods

The quantitative MethyLight data were classified in two different ways: the first class is a specific gene positive for any hypermethylation and the second is positive for high levels of hypermethylation. The Fisher's exact test was used to test the significant differences between the incidence of positivity for methylation in tumor and normal samples. The quantitative ratios of different groups were compared using the Mann-Whitney *U* nonparametric test. Nonparametric analysis was executed because results of Kolmogorov-Smirnov and Shapiro tests rejected the Gaussian distribution theory. The level of significance was set to $P < 0.05$ and analyses were carried out by using the IBM's SPSS statistics software (Versions 19.0).

RESULTS AND DISCUSSION

From total 117 tissue samples of patients, 97 cases have primary lung tumors and 20 cases are non-cancerous lung tissues and 43 samples from healthy volunteers were used as negative controls. The demographic features of the patient population and healthy cases are described in Table 1 and age, sex, stage of cancer and histological characteristics were shown. The median age of the control population was 37.6 years (range: 35–65 years), 60.3 years (range: 24–80 years) in non-cancerous lung patient and 56.2 years (range: 23–80 years) in the lung cancer patient population.

The real-time PCR based MethyLight method used to examine DNA methylation of the *APC promoter 1A* gene in 97 primary lung cancer, 20 lung samples from non-cancer patients (other lung diseases) and normal leukocyte (n=43) for healthy controls (Figure 1).

Hypermethylation of the APC gene promoter was observed in 47 of 97 (48.4%) lung tumors. A low frequency of methylation was also present in 5 of 20 (25%) non-cancer patients tissues and 3 of 43 (6.9%) in healthy controls. QMSP analysis demonstrated a statistically significant difference in methylation level (P=0.00009; Mann- Whitney Test) in tumor samples as compared with normal leukocyte. The median level of APC promoter methylation was 24.29 in human lung cancer, 9.85 in non-cancer patients, and 1.69 in healthy controls (normal leukocyte). No significant association was found between in non-cancer patients samples as compared with tumor samples (P=0.368; Mann- Whitney Test) and healthy controls (P=0.055; Mann- Whitney Test) (Figure 2).

Table 1: Demographic characteristics of the study population

	Primary lung cancer (*n*=97)	Non-cancerous patients (*n*=20)	Normal leukocyte (*n*=43)
Age, mean (range)	56.2 (23-80)	60.3 (24-80)	37.6 (35-65)
50>	22 (22.7)	3 (15)	40 (93)
50-65	50 (51.5)	8 (40)	3 (7)
65<	25 (25.8)	9 (45)	
			Gender, no. (%)
Female	32 (33)	10 (50)	20 (46.5)
Male	65 (67)	10 (50)	23 (53.5)
			Stage, no. (%)
I	16 (22.2)		
II	28 (38.9)		
III	28 (38.9)		
Limited	11 (44)		
Extensive	14 (56)		
			Histology, no. (%)
AC	29 (29.9)		
SCC	28 (28.9)		
Carcinoid	17 (17.5)		
SCLC	16 (16.5)		
Other	7 (7.2)		

AC, adenocarcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer

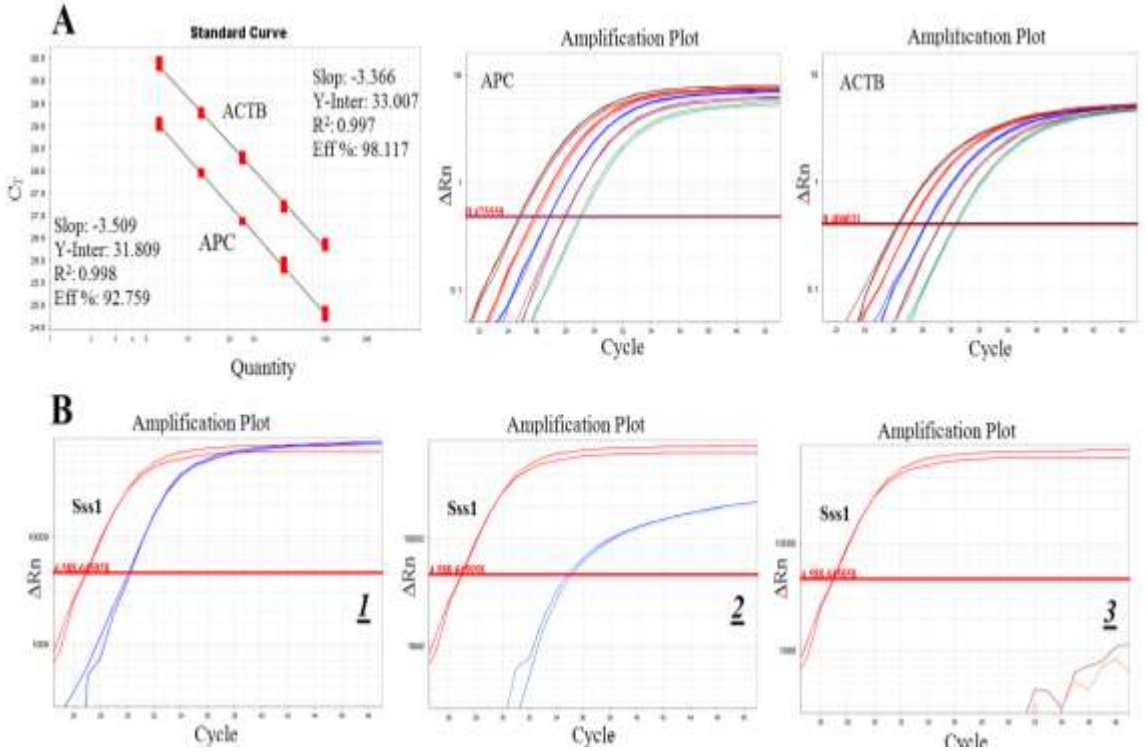

Figure 1: (A) standard curves obtained with *APC* and *ACTB* Taqman assays using five serial dilution of the positive control DNA after bisulphite conversion. (B) MethyLight results in a methylated *APC* (1), *APC* with a negligible percentage of methylation (2) and an unmethylated *APC* (3)

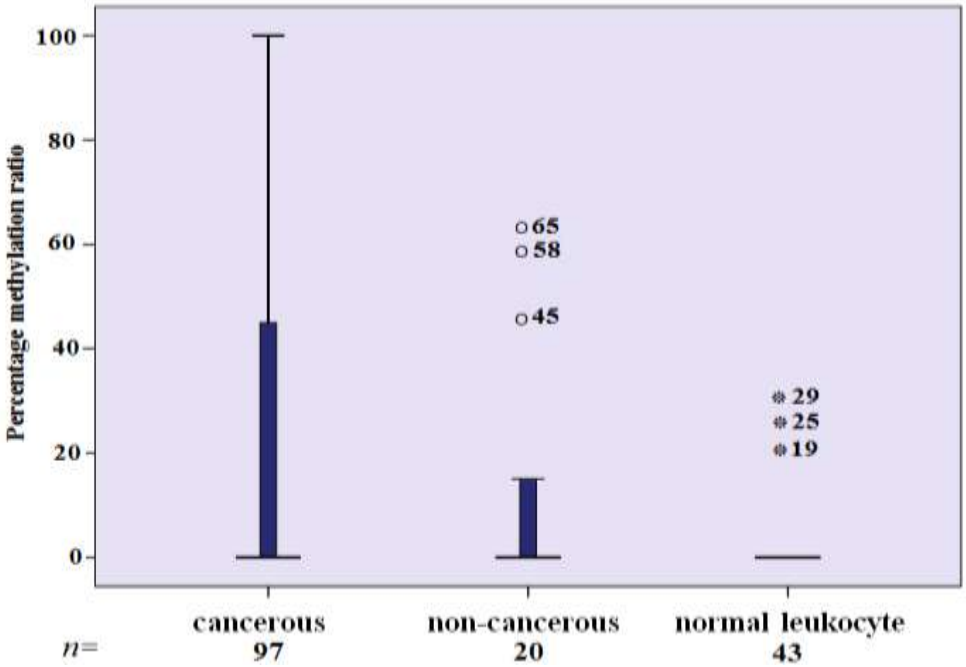

Figure 2: *Box plot* of methylation lists for the *APC* gene *APC/ACTB ratios* determined by Quantitative Methylatiom Specific PCR in all samples

Among all of the lung cancer samples, The *APC* gene methylation frequency by tumor subtype were 20 of 29 (69%) in adenocarcinoma (AD), 13 of 28 (46.4%) in squamous cell carcinoma (SCC), 6 of 17 (35.3%) in carcinoid, 3 of 16 (18.7%) in small cell lung carcinoma (SCLC) and 5 of 7 (71.4%) in other lung tumors of APC's promoter methylation and also for different levels of lung cancer (staging), 7 of 16 (43.7%) were in stage I, 17 of 28 (60.7%) in stage II, 15 of 28 (53.5%) in stage III, 3 of 11 (27.2%) in the limited stage and 5 of 14 (35.7%) in the extensive stage (Figure 3).

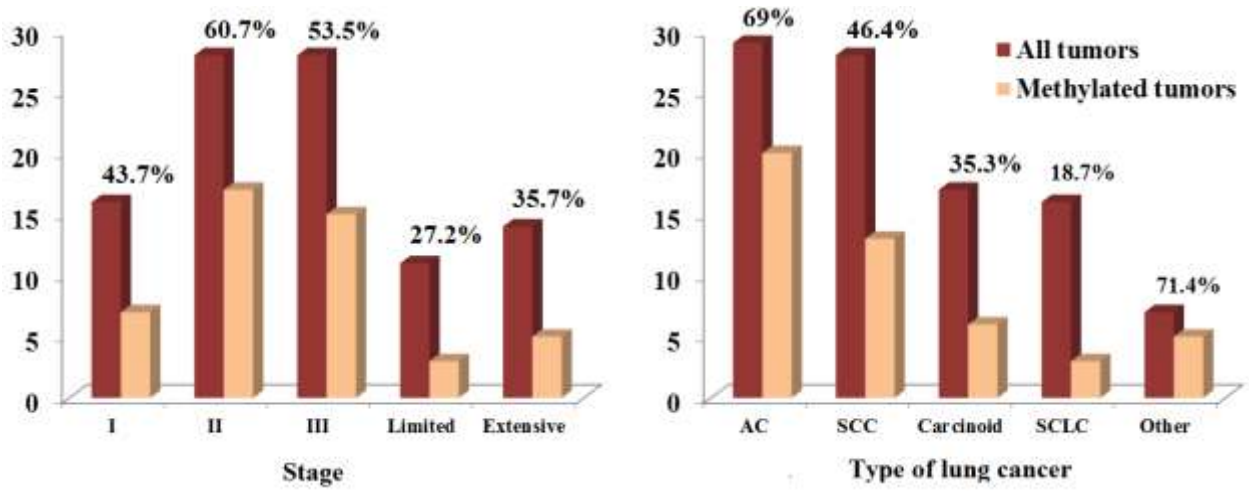

Figure 3: The *APC* promoter methylation frequency by tumor subtype and stage of lung cancer

The most important aim in the current research is the study of APC's methylation in lung cancer tissues in tow sorts of sampling (surgery or biopsy). Among 97 samples suffering from cancer, 50 samples were obtained by surgery and 47 samples were obtained as biopsy. Methylation promoter *APC* occurred in 38 of 50 (76%) samples of surgery and 9 of 47 (19.1%) samples of biopsy. The methylation status of the

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APC gene was compared with the clinicopathologic features (type and staging) in surgery and biopsy sample of lung tumor (Figure 4). As it shows in surgery samples, 100% of AC samples are methylated and for SCC and SCLC, 72.7% and 75% reported but 40% of Carcinoids are methylated. Although in biopsy cases, 18.8% of AC samples and 29.4% (SCC) and 28.5% (carcinoid) are methylated. In biopsy only in stage II 35.7% and in stage III 28.5% of cases are methylated but in surgery cases, in stage II 85.7% of samples and in stage III 78.5% of samples are methylated.

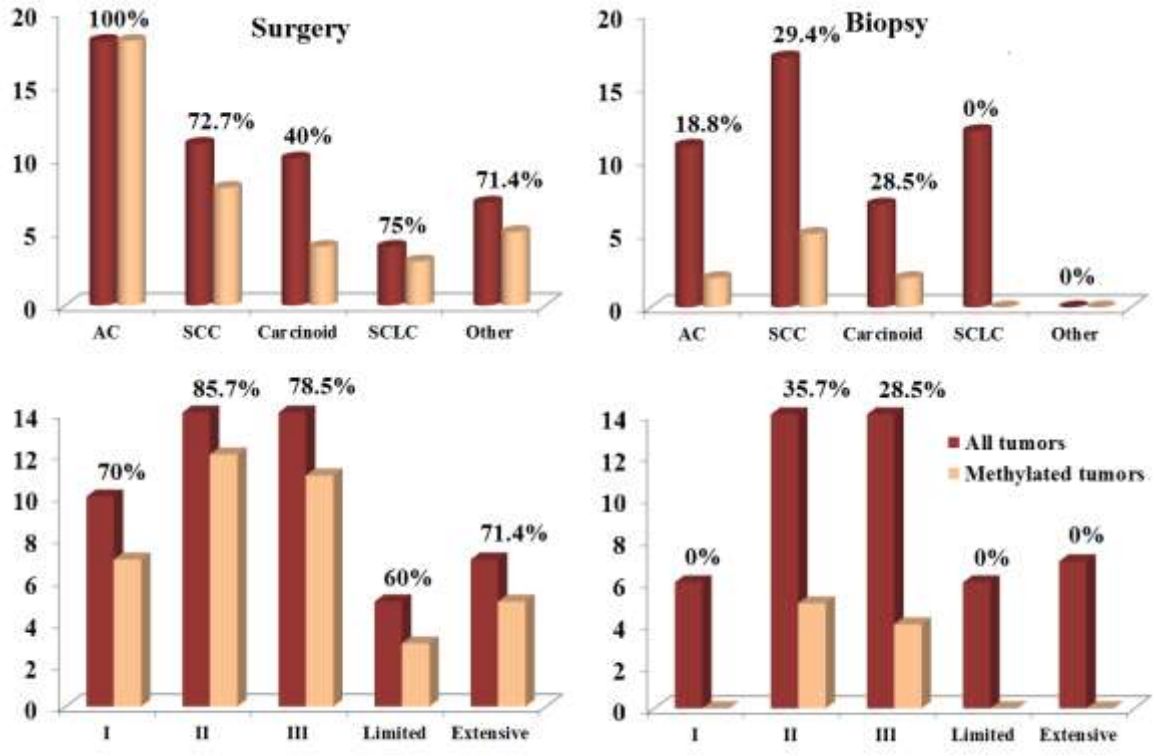

Figure 4: Methylation status of *APC* promoter region in surgery and biopsy sample of lung tumors

Conclusion

Lung cancer is one of main causes of cancer in the world, and surgery is the most effective treatment for lung cancer, but dramatically there is limitation in this method because most of patients have advanced stages of the disease at the time of diagnosis and many of the patients will die because of cancer depending on the stage of the disease. Thus, earlier diagnosis of lung cancer would be helpful for treatment and management of this disease. Real-time PCR as a reliable and fast molecular test has been done in our study to detect methylation sites of APC 1A gene and it was concluded that there is a positive and significant relationship between methylation of APC and emergence of lung cancer in Iranian population. We detected methylation of the APC promoter in lung cancer tissues. Our observation supports a role for APC promoter methylation in the development and progression of lung cancer. As a higher average of methylation ratio has been observed in the group with cancer comparing with the healthy group, approved the main hypothesis of the research.

The fact that the highest percentage of APC's methylation of the lung cancer is in NSCLCs (adenocarcinoma and squamous cell carcinoma) was approved because we reported that 33 out of 57 samples (57.8%) have been methylated. In fact, it could be concluded that NSCLCs are more likely to be identified through APC's methylation as compared to other lung tumors.

In contrast, SCLC with an 18.7% methylation is less likely to be identified among these tumors. Generally, it could be explained that APC's methylation is more effective in NCSLCs and our data is very

similar to other past reports (Lu and Zhang, 2011; Hawes *et al.*, 2011; Anglim *et al.*, 2008). However, it cannot involve all types of lung tumors. Thus, we recommend that future investigation shall consider APC gene together with other genes in order to identify other lung tumors like Carcinoid and small cell carcinoma.

The most important aim of this research is the study of APC's methylation in cancer samples in terms of sampling methods (surgery and biopsy).

It is found, while the numbers of biopsy and surgery samples are close to each other, the ratio of APC's methylation differ significantly (76% in surgery samples as compared to 19.1% in biopsy samples). This difference in some kinds of tumors like NSCLCs (adenocarcinoma and squamous cell carcinoma) was so much significant that methylation occurred about 90% in surgery and 25% in biopsy samples.

It seems although there is relatively similar distribution for type of lung tumors and different staging among the biopsy and surgery groups, this difference for APC's methylation ratio could be discussed. And also comparing between non-cancer and cancer samples, no significant association was found between in non-cancer patients samples as compared with tumor samples (P=0.368; Mann- Whitney Test) and healthy controls (P=0.055; Mann- Whitney Test).

Having studied the quantities of the extracted DNA in biopsy and surgery samples, we observed a significant relationship between APC's methylation percentage and average concentration of the extracted DNA in cancer samples. The average extracted DNA in surgery samples was 0.376 µg/µl while it was less than 0.104 µg/µl in biopsy samples. Even when we consider on the stage of DNA treatment with sodium Bisulfate, equal quantities of DNA were used in all cancer samples (both of biopsy and surgery) [2 µg/µl]. It could be concluded that the difference in the quality of the extracted DNA can be one of the difference factor for methylation ratio of the biopsy and surgery samples. Therefore, we recommend that due to the importance of sampling, investigations shall be done on improving the quality of the extracted DNA from the biopsy samples so that it could be expected that together with improving the quality of DNA in biopsy samples, methylation can be better identified.

As the matter of fact, using more accessible samples is more valuable for diagnosis. Blood sample seems to be more appropriate than the biopsy sample which itself is more appropriate than surgery sample. Also, blood serum or plasma sample is considered as one of the most important role for cancer diagnosis (Levenson and Melnikov, 2012; Jin *et al.*, 2012). The genomic substance of the cells ruined by apoptosis is released in to the serum when cancer occurs and this makes serum as an appropriate basis for tracing special biomarkers of cancer prognosis. In addition, it should be mentioned that a low quantity of DNA has been released in serum and it is not simple to extract comparing with tissues.

This is a basic challenge for diagnosis of cancer biomarkers in serum. It is recommended that the plasma samples can be used together with testing surgery samples of those affected with lung cancer in the same investigations.

Earlier studies have reported that the 4% cutoff distinguishes between normal and cancerous tissues. However, other researchers have evaluated three levels of methylation based on PMR values, assigning PMR=0 as no methylation, and using two levels of methylation (low and high) based on PMR values above and below the median value for a given gene (Ogino *et al.*, 2006). In our unpublished data, we confirmed the presence of low levels of methylation when PMR values were positive but < 4%, but the significance of this level of methylation is not clear. Because of the uncertainty regarding an appropriate cutoff for PMR values, we have presented our data in two ways: (a) a specific gene was considered positive for any hypermethylation if the PMR > 0% and (b) a specific gene was considered positive for high levels of hypermethylation if the PMR ≥ 4% in the sample.

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