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Original Article

Evaluation of Methylation Panel in the Promoter Region of *p16^{INK4a}*, *RASSF1A,* and *MGMT* as a Biomarker in Sputum for Lung Cancer

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Abstract

Lung cancer is the most common cause of cancer death in the world. Effective early detection and appropriate medications can help treat this deadly cancer. Therefore, early detection of lung cancer is of utmost importance, especially in screening high-risk populations (such as smokers) with an urgent need to identify new biomarkers. The present study aimed to demonstrate the potential of using the panel of DNA methylation as a biomarker for the early diagnosis of lung cancer from sputum samples. The methylated promoter of *p16^{INK4a}*, *RASSF1A,* and *MGMT* genes was estimated by the methylation-specific polymerase chain reaction in a sample of 84 lung cancer patients (65 smokers and 19 non-smokers). Based on the results, *p16^{INK4a}* promoter methylation was significantly associated with smoking habit and lung cancer progression in terms of histological grading and patient staging. The sensitivity and specificity of the p16^{INK4a} gene as a biomarker for lung cancer were 71% and 90%, respectively. The methylated promoter of RASSF1A was less sensitive (48%) as a biomarker for lung cancer with 83%. The results demonstrated a strong association between promoter methylation of RASSF1A and late stages of lung cancer (*P=0.0007*). The sensitivity of the *MGMT* gene as a biomarker for lung cancer was 61% with high specificity (92%), compared to other candidate genes in this study. The epigenetic alteration in the promoter region of *p16^{INK4a}*, *RASSF1A,* and *MGMT* genes is highly associated with cancer cell development. It is suggested that the use of these candidate biomarkers can be used as an adjunct to computed tomography screening to diagnose patients at high risk for lung cancer after validation.

Keywords: Biomarker, DNA methylation, Lung cancer, Tumor suppressor gene

1. Introduction

Lung cancer is an invasive disease leading to the highest number of cancer deaths across the globe (1). According to the GLOBOCAN database, there were 2.1 million new cancer cases and 1.8 million deaths in 2018 (2). Currently, screening by low-dose computed tomography (LDCT) can diminish lung cancer mortality by 20%, as compared to chest X-rays; nonetheless, there are many disadvantages, including over-diagnosis and radiation exposure (3). Moreover, biopsies need to be taken from patients to further diagnose the regions identified with LDCT. Several complications, such as hemorrhage and infection, may develop by bronchoscopy, transthoracic needle aspiration, and surgery (4). In addition, tumor size and location influence the sensitivity of LDCT in a significant manner (5). However, LDCT is associated with a high rate of false-positive for smokers, 25% of whom have indeterminate pulmonary nodules (PNs), and 95 % of these patients are not diagnosed with lung cancer later (6).

In the non-invasive approach, sputum is useful for the detection of aberrant epigenetic alteration in respiratory epithelial cells exfoliated from the bronchial airways. It can provide a noninvasive diagnosis of lung cancer in the early stages of progression (7). Several studies have sought to diagnose lung cancer with the cytological approach of sputum by the diagnosis of cell morphological abnormalities. However, these studies revealed poor sensitivity for lung cancer diagnosis at an early stage (6). The detection of CpG island hypermethylation in promoter sequences of tumor suppressor genes (TSGs) revealed great promise as biomarkers for the diagnosis of lung cancer at an early stage since these genes have an epigenetic inactivation at early stages of cancer progression by hypermethylation (8).

Several studies elucidated the possibility of using hypermethylation of many tumor suppressor genes as biomarkers (9-12). To improve prognosis, candidate biomarkers must be identified in the early stages of cancer progression with high sensitivity and specificity (11). The present study focused on the evaluation of the individual and combined analysis of three candidate tumor suppressor genes as sputum molecular biomarkers, including *p16*^{INK4a}, *RASSF1A*, and *MGMT*.

The *p16*^{INK4a}is an important tumor suppressor gene which plays an essential role in the regulation of the cell cycle. The protein encoded by this gene is engaged in *p16*/cyclin-dependent kinase/retinoblastoma pathway, in which *p16* protein has a negative regulation that prevents G1 to S phase progression (12). The Ras association domain family 1 A (*RASSF1A*) gene is the major target tumor suppressor that plays a major role in cell apoptosis. Moreover, *RASSF1A* induces microtubule stability implying cell adhesion and motility (13).

The O-6-methylguanine-DNA methyltransferase (MGMT) gene encodes a nuclear protein with a repairing activity to correct the mismatches during DNA replication (14). Numerous studies have indicated the frequent methylation and silence of these candidate genes in the early stages of carcinogenesis in different types of cancer, including lung cancer (15). In light of the aforementioned issues, the present study aimed to assess the methylation panel in the promoter region of *p16*^{INK4a}, *RASSF1A*, and *MGMT* as biomarkers for the diagnosis of lung cancer using sputum secretion.

2. Materials and Methods

2.1. Study Population

The present study was conducted on 84 patients who were diagnosed with primary lung cancer in Al Hillah Teaching Hospital in Babil, Iraq, and Hospital in Bagdad, Iraq, from June 2019 to April 2021. All diagnoses were based on pathological analysis and/or cytological approach. The medical information of patients, pathological staging of the tumor, and histopathological classification of the tumor grade were obtained from their pathology reports. The cases were staged according to revised TNM guidelines classification criteria (16). Moreover, 42 healthy participants were adopted as control.

2.2. Collection of Sputum Samples

The samples of sputum secretions were collected from the participants as described previously (17-19). In brief, participants were provided with two cups containing Saccomanno's fixative solution (50% ethyl alcohol and 2% carbowax) and received detailed verbal instructions to collect spontaneous cough sputum specimens in early mornings for six consecutive days: three days into the first container and three days into the second. Sputum samples were centrifuged at 1500 rpm for 15 min for the next DNA extraction step. The pathologist prepared two slide smears from the re-suspended cell pellets for further histologic classification of the tumor grade (Table 1).

Table 1. Baseline Characteristics of the samples obtained from patients and healthy controls

Demographic characteristics		Patients	Healthy controls
No.		84	42
Gender	Male	71	27
	Female	13	15
Age	Mean age	61	57
	<55	31	19
	>55	53	23
Smoking	Non-smokers	19	24
Number of years in use	< 15	26	11
	>15	39	7
Differentiation	Well	21	
	Moderately	40	
	Poorly/undifferentiated	23	
Stage	I	11	
	II	20	
	III	35	
	IV	18	

2.3. DNA Isolation and Bisulfite Conversion

The Genomic DNA was extracted by Promega Wizard® Genomic DNA Purification Kit, according to the manufacturer's protocol. Thereafter, all the genomic DNA was treated by DNA methylation modification using spin-column DNA Methylation™ Kit (ZYMO RESEARCH), according to the manufacturer's instructions. The kit has a "99% conversion efficiency of converting non-methylated C residues into U, and the modified DNA recovery from the kit is >80%", according to the information provided by the manufacturer. The modified DNA was then used as a template for Methylation Specific PCR(MSP).

2.4. Methylation Specific Polymerase Chain Reaction PCR (MSP)

Methylation-specific PCR (MSP) was performed as described previously (11) using PCR thermal cyclers system (Techne® Prime). The nested PCR protocol was achieved to improve primers' sensitivity in discrimination between methylated and unmethylated alleles by two PCR stages. The MSP primer sequences used in the two PCR stages for three genes: *p16*^{INK4a}, *RASSF1A*, and *MGMT* are displayed in table 2 (20, 21). The stage I PCR products were diluted 50-fold and then subjected to a stage II PCR in which primers specific to the methylated or unmethylated template were used. The PCR amplification procedure for stage I was: 95°C for 10 min, followed by denaturation at 95°C for 30 sec, annealing at 60°C (*p16*), 52°C (*MGMT*), or 58°C (RASSF1A) for 30 sec, extension at 72°C for 30 sec for 40 cycles, and a final 10-min extension. In stage II, the primers used to selectively amplify unmethylated or methylated, along with their annealing temperatures, are presented in table 2. The autopsy samples of nonsmoker normal human tissue were used as unmethylated control. Seven microliters of each PCR product of stage-II were separated on an 8% polyacrylamide gel. The gel was stained with ethidium bromide and the bands were analyzed by UV gel documentation system (Bio-Rad).

Table 2. Primers used for Methylation-specific polymerase chain reaction

Step.	Gene of promoter	Forward primer	Reverse primer	annealing temperature	PCR product
Stage I	*P16*	GAAGAAAGAGGAGGGGTTGG	CTACAAACCCTCTACCCACC	60°C	280
	MGMT	GGATATGTTGGGATAGTT	CCAAAAACCCCAAACCC	52°C	289
	RASSF1A	GGAGGGAAGGAAGGGTAAGG	CAACTCAATAAACTCAAACTCCC	58°C	260
Stage II	*P16 M*	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65°C	150
	P16 U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	65°C	151
	MGMT M	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	59°C	81
	MGMT U	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	59°C	93
	RASSF1A M	GGGGGTTTTGCGAGAGCGC	CCCGATTAAACCCGTACTTCG	65°C	204
	RASSF1A U	GGTTTTGTGAGAGTGTGTTTAG	ACACTAACAAACACAAACCAAAC	65°C	170

2.5. Statistical Analysis

The relationships were assessed by Fisher's exact test, and p-values were calculated by Two-tailed Fisher's exact contingency table. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of Samples and Sputum Samples

A total of 84 lung cancer patients and 42 healthy control subjects were included in the present study. Demographic variables for cases and controls are summarized in table 1. The sputum samples showed that lung cancer incidence was not associated with age and gender. On the other hand, the recorded samples illustrated that there was a significant relationship between lung cancer and the smoking habits of patients who had smoked for more than 15 years (*P=0.0002*).

3.2. Methylation Status *of p16^{INK4a}* Promoter

Methylation-specific PCR revealed that out of 84 patients, the *p16^{INK4a}* promoter region of 60 cases was methylated, while 24 samples of controls were unmethylated. (Table 3). Based on the results, *p16^{INK4a}* promoter methylation was significantly associated with smoking habit and lung cancer progression in terms of histological grading and patient staging (Table 4). Moreover, 90% of cases in well grade were methylated, 17 methylated vs 4 unmethylated. In the present study, the sensitivity and specificity of this gene as a biomarker for lung cancer were 71% and 90%, respectively.

3.3. Methylation Status of *RASSF1A* Promoter

The results suggested that the promoter region of *RASSF1A* was methylated in approximately half of the patients, while seven control cases had methylated promoter for this gene (Table 3). This finding revealed low sensitivity (48%) of this gene as a biomarker for lung cancer with 83% specificity. The MSP technique elucidated that the association between methylation of *RASSF1A* promoter and the smoking habit was considered to be not statistically significant. However, there was a strong association between promoter methylation of this gene and late stages of lung cancer (*P=0.0003*) (Table 4).

Table 3. Methylation status of candidate genes

Gene of Promoter	Patients (n=84)		Healthy controls (n=42)		Sensitivity	Specificity
	M	U	M	U		
p16^{INK4a}	60	24	4	38	71%	90%
RASSF1A	41	43	7	35	48%	83%
MGMT	52	32	3	39	62%	92%

Table 4. Association between methylation and subjects' demographic characteristics

Demographic characteristics		*p16^{INK4a}*		*P-value*	*RASSF1A*		*P-value*	*MGMT*		*P-value*
		M	**U**		**M**	**U**		**M**	**U**	
		60	**24**		**41**	**43**		**52**	**32**	
Gender	Male	51	20	1.0000	33	38	0.3758	41	30	1.0000
	Female	9	4		8	5		7	6	
Age	<55	24	7	0.4552	15	16	1.0000	19	12	1.0000
	>55	36	17		26	27		33	20	
Smoking	Non-smokers	10	9		9	10		9	10	
Number of years in use	< 15	17	9	0.029	16	10	0.265	13	13	0.03
	>15	33	6		16	23		30	9	
Differentiation	Well	17	4		8	13		9	12	
	Moderately	22	18	0.004	14	26	0.0007	25	15	0.0536
	Poorly/undifferentiated	21	2		19	4		18	5	
Stage	I/ II	16	15	0.0029	7	24	0.0001	14	17	0.0206
	III/ IV	44	9		34	19		38	15	

3.4. Methylation Status of *MGMT* Promoter

The promoter region of *MGMT* was methylated in 52 patients, whereas only three control cases were methylated. As illustrated by the results, the association between methylation of *MGMT* promoter and the smoking habit was considered to be statistically significant. The sensitivity of this gene as a biomarker for lung cancer was 61% with high specificity (92%), compared to other candidate genes in this study.

4. Discussion

The present study evaluated the aberrant methylation in the promotor region of three genes, including *p16*^{INK4a}, *RASSF1A*, and *MGMT*, as a biomarker for lung cancer in its early stages. The candidate biomarker that is used in the medical approach must have a high sensitivity and specificity (22). The *p16*^{INK4a}gene displayed the highest sensitivity (71%) for distinguishing incident cases from controls. Moreover, a strong association between well-graded samples and aberrant methylation of this gene supported that the inactivation of this gene occurs in the early stages of lung cancer (23, 24). Both *p16*^{INK4a} and *MGMT* demonstrated high specificity (90% and 92% respectively), suggesting that these genes are normally activated in healthy controls (12, 14). On the contrary, *RASSF1A* illustrated low sensitivity (48%) without any association with a smoking habit. The early histological grade (well and moderately) indicated that the *RASSF1A* promoter was unmethylated and methylated in 39 and 22 patients, respectively. Based on some other studies, a low prevalence of aberrant methylation of RASSF1A promoter observed in sputum supports that the inactivation of this gene occurs in the late stages of cell cancer progression (20, 23).

Several studies used quantitative methods, including pyrosequencing technology, to estimate methylation levels at individual CpG loci. This technique may overcome the limitation of methylation-specific PCR analyses (25). To overcome the low sensitivity and specificity of candidate biomarkers, the present study emphasizes the need for validating other promising genes as a marker panel for screening high-risk populations in the early stages of lung cancer from sputum.

The present study highlighted the potential of using the panel of DNA methylation as a biomarker for the early diagnosis of lung cancer with high sensitivity and specificity. This epigenetic alteration in the promoter region of our candidate genes is highly associated with cancer cell development. If the candidate biomarkers are validated, they can be used, along with CT screening, to diagnose patients at a high risk of lung cancer, signifying that the early detection of cancer may allow treatment with various therapeutic techniques, such as nanoparticles (26).

Authors' Contribution

Study concept and design: A. B. A. H.
Acquisition of data: F. M.
Analysis and interpretation of data: A. B. A. H.
Drafting of the manuscript: T. A.
Critical revision of the manuscript for important intellectual content: F. M.
Statistical analysis: T. A.
Administrative, technical, and material support: F. M. and A. B. A. H.

Ethics

The study protocol was approved by Medical Sciences, Department of Pharmacy, Maysan, Iraq. Written informed consent was obtained from all participants.

Conflict of Interest

The authors declare that they have no conflict of interest.

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