Medicine Updates Faculty of medicine
January 2023,volume 12, issue 12
https://muj.journals.ekb.egdean@med.psu.edu.eg
vice_dean_postgraduate@med.psu.edu.eg
DOI: 10.21608/muj.2022.144129.1109

ISSN : 2682-2741

Submitted: 29 /6/2022
Accepted 18/10/2022
Pages: 21 - 39

"Role of Methylated RASSF1A Gene as a potential biomarker in diagnosis of lung cancer"

Authors

Eman M Mahmoud [1]; Ebtsam sayd Abdulkareem[2]; Suzan Sayed[3]; Ragga Salama[4]; Maha Elkholy[5]

[1] Department of Chest diseases Faculty of Medicine port said University

[2]Department of Chest diseases Faculty of Medicine, Aswan University

[3]Department of Chest diseases Faculty of Medicine, Assiut University

[4]Department of medical Biochemistry & Molecular Biology, Faculty of Medicine, Assiut University

[5]Department of Chest diseases Faculty of Medicine,Assiut University

Abstract :

Background: The leading reason of cancer-related mortality in the world is Lung cancer .The human RASSF1A has been extensively recognized as a indispensable goal suppressor tumour gene.

Aims: Verify the significance of DNA methylation of RASSF1A gene as early detection biomarker for lung cancers.

Settings and Design: it is a case control study.

Methods and Material: This study was carried in Aswan and Assiut University Hospitals patients were classified into two corporations along with 30 sufferers with lung cancer and 20 aberrantly wholesome heavy people who smoke as a manage group. Both corporations were subjected to clinical examination , imaging modalities, laboratory investigation and blood pattern for methylation specific PCR and DNA extraction in addition bronchoscopy and histopathological examination have been executed to the group of lung cancer patients.

Statistical analysis used: SPSS 20.0 for home windows and MedCalc IBM_SPSS. Statistical Package for Social Science. Ver.21. Standard version.

Results: RASSF1A gene was hyper methylated by 64.8 folds in lung cancer patients more than the control group. There used to be statistical considerable difference between those with methylated shape and these with un-methylated form of RASSF1A gene in the two studied groups, the frequency of methylation used to be greater in patients with lung cancer more than the control crew.

Conclusions identifying the methylation fame of RASSF1A gene may be beneficial as a molecular biomarker in analysis and screening for lung cancers.

Key-words: Lung cancer, ***RASSF1A***, tumor-suppressor gene.

Key Messages: identifying the methylation status of *RASSF1A* gene may be useful as a molecular biomarker in diagnosis and screening for lung cancer

Introduction:

One of the leading cause of cancer deaths in both women and men worldwide is Lung cancer, with over a million deaths annually [1] [2].

The interaction between genetic and epigenetic modifications results in lung cancer initiation and progression [3]. Observed Epigenetic changes in carcinogenesis of lung consist of aberrant methylation DNA patterns, regulation by noncoding RNAs (ncRNA) and modifications of histone [4]. At early stage of carcinogenesis Methylation occurs, and has become an attractive screening and early detection biomarker for cancer. DNA methylation of carbon-5 position of cytosine within CpG dinucleotides occurs that scattered in human genome [5]. One of the most powerful environmental modifiers of DNA methylation is Cigarette smoke [6]. Carcinogens in cigarette smoke [7] can injury DNA by causing double-stranded breaks smoke condensate [8]. *RASSF1A* (Ras association domain family 1 isoform) may cause apoptosis, tumourigenesis associated with the regulation of the cell cycle, adhesion and migration [9, 10].

Patients and Methods

This is a case control study conducted on 50 persons, 30 patients of lung cancer (diagnosis depending on Imaging studies , bronchoscopy and histopathological studies) and 20 persons aberrantly healthy heavy smokers 10 of them are cigarette and the other 10 are goza smokers.

Inclusion criteria:- 1- Patients with lung cancer. 2- Age more than 18 years. 3- Aberrantly healthy smokers whether cigarette or goza.

Exclusion criteria: Metastatizing lung cancer and Patients with any organ cancer other than lung cancer.

patients were subjected to: history and examination and 2 ml blood sample was collected on EDTA , mixed well and kept on -20 C, DNA extraction of all samples by Qiagen kit(Bioline, USA)

- The primers are :

  F : tgttgtttagtttggattttgg and

  R : tacaacccttcccaacacac
- DNA methylation of all samples by DNA methylation kit
- The Methylated primers are :

  F: GTGTTAACGCGTTGCGTTGCGTATC

  R: AACCCCGCGAACTAAAAACGA

Real time PCR of ***RASSF1A*** using Syber green master mix and standard kit for methylation.

DNA purification from Whole Blood (Spin Protocol):-

1. Twenty ul QIAGEN Protease (or proteinase K) was pipeted into the bottom of a 1.5 ml microcenrifuge tube.

2. Two hundred ul sample was added from whole blood to the tube of microcentrifuge.

3. 200 ul Buffer AL was added . Mix by pulse-vartexing for 15s.

4. Incubation at 56 C for 10 min

5. Briefly centrifuged the 1.5 ml tube of microcentrifuge to remove drops from the inside of the lid.

6. We added 200 ul ethanol (96-100%) to the sample, and mixed by pulse-vortexing or 1.5 s. after mixing, briefly centrifuged the 1.5 ml microcentrifuge tube.

7. We applied the mixture carefully to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Then we centrifuged at 6000 xg(8000 rpm) for 1 min. We placed the QIAamp Mini spin column in a clean 2 ml collection tube, and discarded the tube containing the filtrate .

8. 500 ul washing Buffer (washing buffer I) was added to the QIAamp Mini spin column without wetting the rim. and and centrifuged at 6000 x g (8000 rpm) for 1 min, and placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded.

9. Carefully we added 500 ul washing Buffer (washing buffer II) centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.

10. The QIAamp Mini spin column was placed in a new 2 ml collection tube and the add collection tube with the filtrate was discarded. Centrifuged at full speed for 1 min.

11. Then placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. Carefully the QIAamp Mini spin column was opened and 200 ul elusion Buffer was added.

At room temperature (15-25 C) Incubated for 1 min, and then centrifuged for 1 min at 6000 x g(8000 rpm).

DNA Methylation using (EZ DNA Methylation Kits):-

I- Blood purified DNA is used for the EZ DNA Methylation- Direct TM Kit as the starting material.

II- Bisulfide Conversion of DNA:

1. In a PCR tube 20 ul of purified DNA added to 130 ul of CT Conversion Reagent solution. Then mixed and centrifuged briefly.
2. In a thermal cycler the PCR tubes were placed and the following steps performed:

    1 for 8 minutes at 98°C

    2. for 3.5 hours at 64°C

    3. 4°C storage for up to 20 hours

3. Six hundred µl of M-Binding Buffer was added into Column of Zymo-Spin™ IC and the then placed into a Collection Tube.
4. The sample (from Step 2) was loaded into the Column of Zymo-Spin™ IC containing the M-Binding Buffer. Then mixed several times by inverting the column.
5. Centrifuged at speed (≥10,000 x *g*) for 30 seconds. And discarded the flow-through.
6. A hundred µl of M-Wash Buffer was added . Centrifuged at full speed for 30 seconds.
7. Two hundred µl of M-Desulphonation Buffer was added to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuged for 30 seconds at full speed.
8. Two hundred µl of M-Wash Buffer was added . Centrifuged at full speed for 30 seconds. Another 200 µl was added and centrifuged for 30 seconds.
9. The column was placed into a 1.5 ml microcentrifuge tube. Ten µl of M-Elution Buffer was added . Centrifuged for 30 seconds at full speed to elute the DNA then stored at or below -20°C for later use.

Methylation specific polymerase chain reaction (MSP) :

In order to analyze the CpG Island methylation status of this promoter First PCR amplification using methylation specific primers was performed in 50 subjects in blood samples.

- PCR reaction for the methylated *RASF1A* gene: set and carried out in f 25µl total volum containing 12.5 Ampliq on master mix, 3.5 µ1 DDW, lmM of each primer , 3 µl (100 ng)

DNA and 4 µl magnesium chloride. initial denaturation at 95°C for 5 min, and 35 cycles of the following profile: 15 s at 94°C, 30 s at 59°C, and 30 s at 72°C, and a final extension step at 72°C for 10 min.

- PCR reaction for the unmethylated *RASF1A* gene: PCR condition is the same as methylated primer.

Consents & Ethics: Faculty's ethics committee approved this study and permission was obtained assure confidentiality. A written consent was taken from each participant.

Statistical analysis:

We used SPSS version 21* to analyze Data . Means, percentages, medians and standard deviations were calculated. Test of significances: chi-square or Fisher's Exact test was used to compare the difference in distribution of frequencies among the studied groups. For continuous variables; independent t-test analysis was carried out. ANOVA test the mean differences of the data that follow normal distribution. Gene expression profile modulations were evaluated comparing ct values between cells, using the $2^{-\Delta\Delta Ct}$ method**. Correlation analysis was used to test the association between variables (Pearson correlation). To determine the best cutoff values for the lung cancer biomarkers, the receiver operating characteristic (ROC) curve technique for the parameters was used. The area under curve (AUC) was examined to assess performance for each test. Accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) at the best cut-off values for the parameters were also calculated. A p-value ≤ 0.05 was considered significance

Results:

In our study, the mean age in patients (59.33 ± 8.6) years had no significant difference compared to the control group (62.55 ± 11.9) years with ($p = 0.271$). About gender, the patients were 23 (76.7%) males and 7 (23.3%) females compared to control group 20 (100%) males and no (0%) females without significant statistically difference ($p = 0.056$).

Smoking conditions of the groups are shown in table 1.

Table1: Smoking status Differences between patients and control group

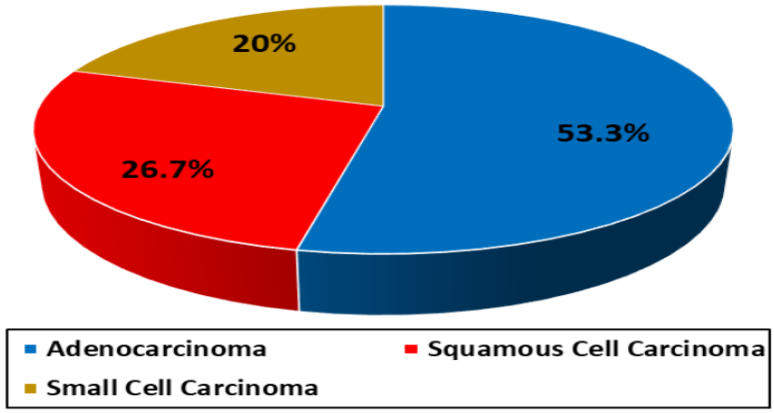
Parameter	control (No.=20)	Cases (No.=30)	P-value	OR (95% CI) ***
Smoking Type				
• **Non**	0 (0%)	7 (100%)		**1**
• **Cigarette**	10 (33.3%)	20 (66.7%)	**= 0.001****	------
• **Goza**	10 (76.9%)	3 (23.1%)		**6.67**
Smoking State				
• **Current**	9 (36%)	16 (64%)	**= 0.005****	------
• **Ex-smoker**	11 (61.1%)	7 (38.9%)		**0.385**
Smoking Index				
• **Mild**	0 (0%)	0 (0%)		------
• **Moderate**	0 (0%)	2 (100%)		-------
• **Heavy**	20 (48.8%)	21 (51.2%)		**8.53**

***T-test comparing the mean difference between groups**

**** To compare the percentages between groups Fisher's Exact test was used**

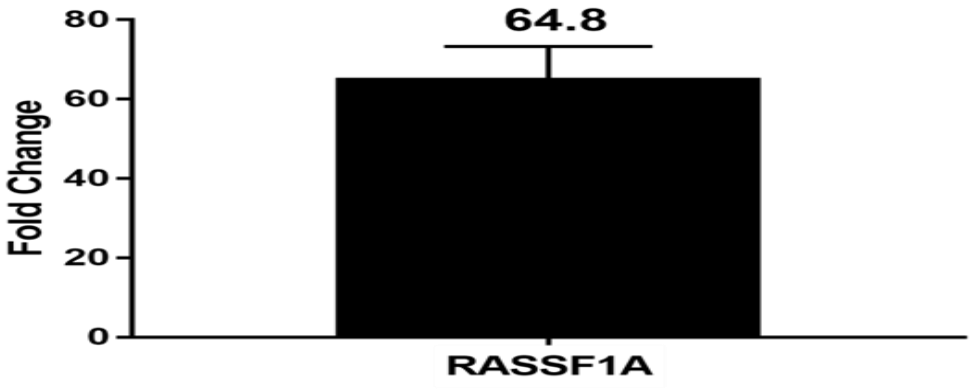
*****OR=Odds Ratio, CI=Confidence Interval**

Fig 1: Pathological Types of Tumour of the studied patients



Based on pathological type of tumour, the adenocarcinoma was detected in 16(53.3%) of cases where squamous cell carcinoma was present in 8(26.7%), 6(20 %) of cases were found as small cell carcinoma Figure1.

Figure2: Gene expression (Fold Change) of methylated RASSF1A gene among the studied groups



As regard the gene expression, RASSF1A gene was hyper methylated by 64.8 folds in lung cancer patients more than the control group as shown in figure 2.

Table 2: Frequency of aberrant methylation of RASSF1A biomarker in lung cancer cases vs control

Parameter	**Total no. (n=50)**	**RASSF-1A (%)**
Disease Status		
• **Control**	20	8 (40%)
• **Case**	30	20 (66.7%)
OR (95% CI) *		**3.00 (1.01 – 9.70)**

*** CI=Confidence Interval ,OR=Odds Ratio**

Table 2 RASSF1A hyper methylation was detected in 20(66.7%) of lung cancer patients versus 8(40%) in controls with Odds ratio 3 indicating that hyper methylation was 3 times in cases than controls.

Table 3: Frequency of hyper methylation of RASSF1A biomarker in association with Smoking characteristics in the studied groups

Parameter	Cases No.20	Control No.8
Smoking Type		
• **Cigarette**	14 (70%)	4 (50%)
• **Goza**	2 (10%)	4 (50%)
OR (95% CI) *	2.86 (0.07 – 11.36)	1.00 (0.17 – 5.99)
Smokers		
• **Ex-smoker**	6 (30%)	3 (37.5%)
• **Current**	10 (50%)	5 (62.5%)
OR (95% CI) *	1.29 (0.03 – 3.90)	3.33 (0.52 – 11.60)
Smoking Index		
• **Moderate**	1 (5%)	------
• **Heavy**	15 (75%)	8(100%)
OR (95% CI) *	2.50 (0.13 – 26.45)	

***OR=Odds Ratio, CI=Confidence Interval**

Table 3: hyper methylation of RASSF1A was higher in smokers 16(80%), while in non-smokers it was 4(20%). Furthermore, 14(70%) of cigarette smokers showed hyper methylation in RASSF1A while in goza smokers it was 2(10%). Ex-smokers showed less RASSF1A hyper methylation in comparison to current smokers 6(30%) and 10(50%) respectively.

Table 4: Frequency of hpermethylation of RASSF1A biomarker in Lung Cancer patients in association with Tumour Histopathological Type

Parameter	Total no. (n=30)	RASSF-1A (%)
Histopathological Type		
• **Adenocarcinoma**	16	9 (56.3%)
• **Squamous CC**	8	6 (75%)
OR (95% CI) *		**2.33 (0.36 – 15.3)**
• **Small CC**	6	5 (83.3%)
OR (95% CI) *		3.89 (0.37 – 21.3)

***OR=Odds Ratio, CI=Confidence Interval**

****OR not calculated due to 0 cell**

Table 4 : shows that the highest frequency was among small cell carcinoma 5(83.3%) followed by squamous cell carcinoma6 (75%) and lastly adenocarcinoma 9(56.3%).

Table5: Relationship between % methylation change in RASSF1A biomarker and smoking parameters and tumour types among lung cancer patients

Parameter	RASSF1A % methylation change
	Mean± sd
Smoking Type	
• **Non**	47.5 ± 5.1
• **Smoker**	169.1 ± 13.9
P-value*	**= 0.021**
Smoking Type	
• **Cigarette**	149.8% 14.6
• **Goza**	3.8% 0.7
P-value*	**= 0.033**
Smoking State	
• **Current**	261.2 ± 22.2
• **Ex-smoker**	15.4 ± 1.5
P-value**	**= 0.014**
Tumour Type	
• **Adeno-**	280.6 ± 24.8
• **SCC**	24.3 ± 0.7
• **Small CC**	44.8 ± 4.1
P-value**	**= 0.004**

***T-test was used to compare the mean difference between groups**

****ANOVA test compare the mean difference among groups**

Table5: shows that mean methylation change in RASSF1A in relation to smoking was higher in smokers than non-smokers 169.1%± 13.9 and 47.5%±5.1 respectively with statistically significant difference p= 0.021, also significant difference was detected between Cigarette (149.8% ± 14.6) and Goza (3.8% ± 0.7), while depending upon the smoking status, we found that the mean methylation change for RASSF1A was much higher in current than ex-smokers (261.2% ± 22.2), (15.4% ± 1.5), Moreover, the relation between RASSF1A hyper

methylation change and the different histopathological diagnosis we found that the highest methylation change occurred in adenocarcinoma followed by small cell carcinoma then squamous cell carcinoma (280.6 ± 24.8), (44.8 ± 4.1),(24.3 ± 0.7) respectively with significant difference p=0.004.

Figure 3 : ROC curve

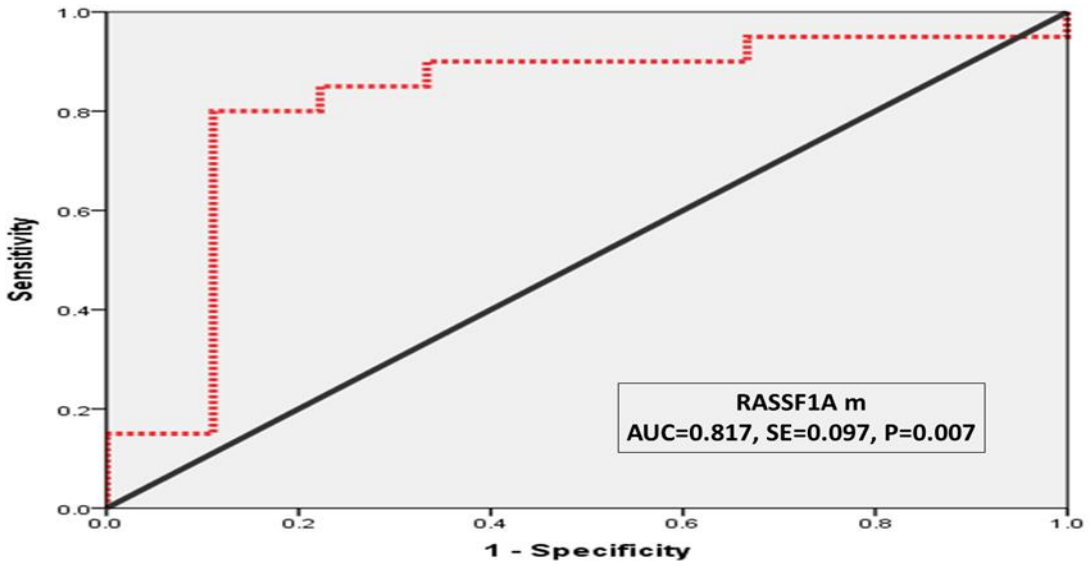


Figure 3: ROC curve for RASSF1A biomarker for the studied patients

Figure 4: Real time PCR results:

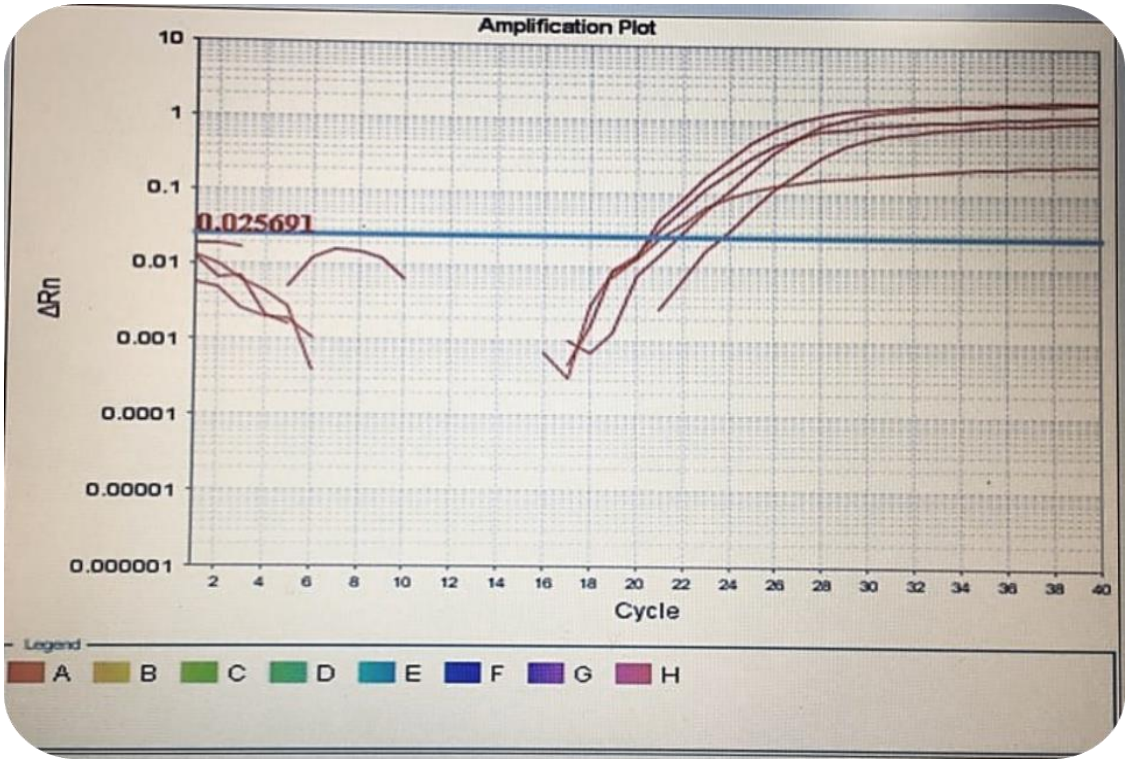


Figure 4: 7500 fast real time PCR amplification plot with red fluorescence of methylated samples

[X axis = fluorescence intensity, Yaxis = no. of cycles]

Cut of point in each sample indicate ct value (cycle threshold) which used in the analysis of the results.

The red fluorescence is for methylated samples, while the green fluorescence is for unmethylated sample.

Cycle threshold (ct) is the maximum fluorescence intensity at which numbers of copies of DNA of the studied gene can be detected by 7500 RT-PCR.

Table 6: Goodness criteria of Biomarkers for diagnosis of Lung Cancer in the studied sample

	RASSF1A-m
AUC	**0.817**
Cut-off	**25.26**
Accuracy	**82.1%**
Sensitivity, %	**91.7%**
Specificity, %	**72.4%**
PPV, %	**76.2%**
NPV, %	**89.7%**

***Sensitivity (true positives/all diseased); specificity (true negatives/all non-diseased); PPV (true positives/all test positives); NPV (true negatives/all test negatives).**

Table (6) RASSF1A biomarker Goodness criteria for diagnosis of Lung Cancer where AUC in RASSF1A-m was (0.833). Cut off point was 25.26 with accuracy 82.1%. The percentage of Sensitivity was 91.7 % and Specificity 72.4% while PPV was 76.2%, and NPV was 89.7%.

Photo 1:

Photo 1: Sagital CT chest showing lt. hilar mass of Male pt. 59ys old current heavy smoker complained of progressive exertional dyspnea, productive cough and hemoptysis of 2 months duration .

Photo 2:

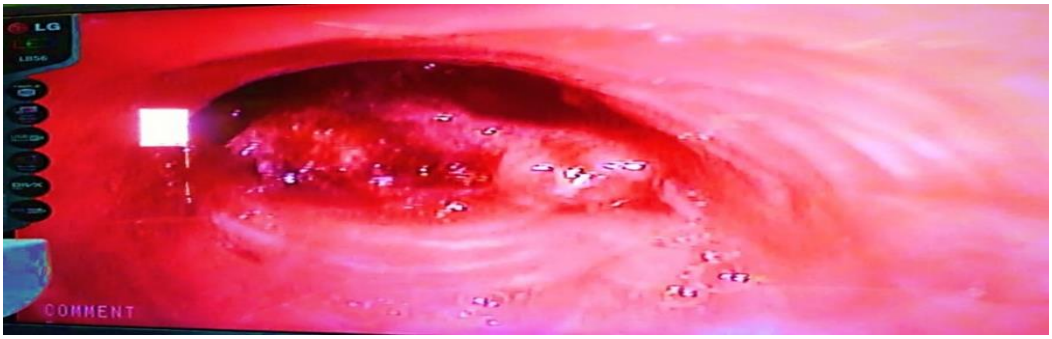
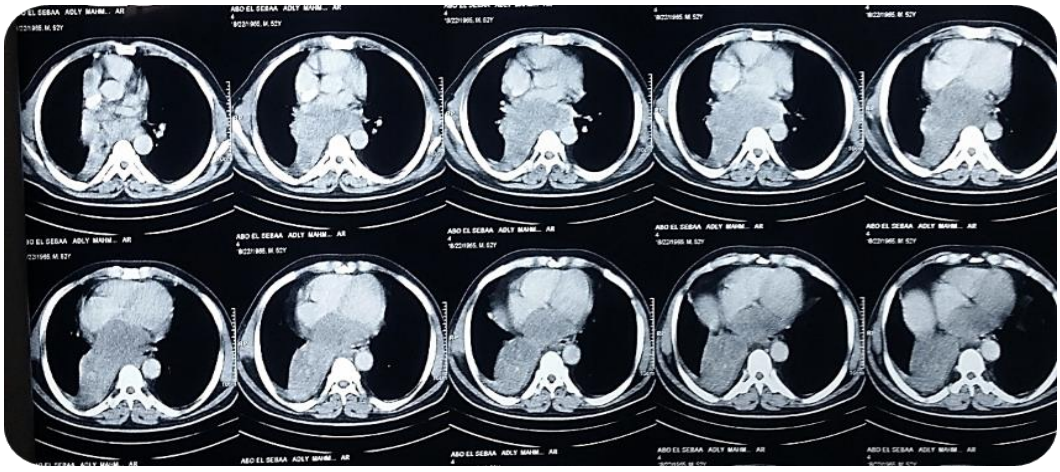


Photo 2: Bronchoscopy showing mass in the lt. main bronchus of the same patient in photo 1

Photo 3:



Axial CT chest showing rt. Lower lobe with mediastinal involvement of Male pt. 52ys old current heavy goza smoker, complained of productive cough and progressive dyspnea of 1 month duration, presented with signs of rt. Lower lobe collapse, photo (3) and (4) show CT chest and bronchoscopic finding of the pt. biopsy from the mass was examined histopathologically and was squamous cell carcinoma, RASSF1A methylation was 17%.

Photo 4: Bronchoscopy showing mass in the rt. Lower lobe bronchus

Discussion:

This case control study included 50 subjects, 30 of them were patients of lung cancer (16were adenocarcinoma, 8 were squamous cell carcinoma and 6 were small cell carcinoma) while 20 of them were heavy smokers as control group.

In our study**,** *RASSF1A* hyper methylation detected at a frequency of 66.7% (20/30) of lung cancer patients versus 40% (4/ 20) in controls with Odds ratio 3 indicating that the risk of hypermethylation was 3 times in cases than controls.

The aberrant methylation of RASSF1A gene reported by Topaloglu et al. [12] in the tumor samples with frequency of (45%). Different studies mentioned promoter hyper methylation of *RASSF1A* (30–40 %) in lung cancer [13, 14].

Aberrant methylation of *RASSF1A* gene frequency has been investigating in body fluids of lung cancer patients was 52.5% while it was 7.7% for benign diseases patients **[15].**

Minpu Ren et al. [16] found that the frequency of abnormal methylation *RASSF1A* gene was 50.4% (62/123) in group of lung cancer, 3.8% (5/130) in control group which is Closer to our figures .

In disagreement to our results hypermethylation of *RASSF1A* gene at a frequency of 31.1% (14/ 45) in lung cancer patients was detected by **El-Sherif et al.** [17] while none of the controls were having gene methylation

Regarding the methylation in relation to the smoking pattern among cases related results founded by **Gao et al.** [15] the frequency of methylation of RASSF1A in smoker lung cancer patients was 55.6%, while it was 46.2% in non- smoker.

Scesnaite et al. [18] investigated in a study of 165 ever-smokers (109 current smokers and 56 former smokers) and 47 never-smokers Promoter hyper methylation of *RASSF1A* gene was detected at frequency of 30.3% in ever smoker and 23.9% in never smokers. Moreover it was 32.1% in current smokers and 26.8% in former smokers and this was inconsistent with our study results, the same was with **El-Sherif et al.** [17] found that hypermethylation of *RASSF1A* in non smoker lung cancer patients was 25% while it was 34.5% in smokers.

Tobacco - specific nitrosamines (TSNAs) are a class of carcinogens that identified in cigarette tobacco[19 20], environmental tobacco smoke [21], and other tobacco products [22]. The formation of TSNAs is influenced by the tobacco blend and the curing processes [20] this can explain the reason of disagreement on the frequencies of hyper methylation for *RASSF1A* gene between smokers and non-smokers between different studies and also may die to the smaller non-smokers number involved in these studies, due to the smaller incidence of lung cancer occurrence among non-smokers compared to smokers.

As regard the frequency of methylation in relation to the histopathological type, related to our results **Minpu Ren et al.** [16] **observed** abnormal methylation of RASSF1A in 46.3% of adenocarcinoma patients, 75% of small cell carcinoma and, 58.8% squamous cell carcinoma.

While **Gao et al.** [15] detected that promoter hyper methylation of RASSF1A gene was 38.5% in adenocarcinoma, and 53.8% in squamous cell cancer, and 50% in small cell which is not consistent with our results, also **El-Sherif et al.** [17] showed that hyper methylation of *RASSF1A* was detected in 33.3% of adenocarcinoma, 25% of squamous cell carcinoma, and 50% in small cell carcinoma.

With regard to methylation change, *RASSF1A* methylation change occurred in 47.5%±5.1 of non-smokers and 169.1%± 13.9 of smokers with statistically significant difference p= 0.021,

Overall from the observed results in *RASSF1A* biomarkers, a significant difference between Cigarette (149.8% ± 14.6) and Goza (3.8% ± 0.7), Depending upon the smoking status, we identified (47.5% ± 5.1) non-smokers, (261.2% ± 22.2) were in current smokers and the least methylation percentage (15.4% ± 1.5) with ex-smoker in *RASSF1A*.

As regard the gene expression of RASSF1A, hypermethylation was 64.8 folds in cases greater than controls.

The methylation of the *RASSF1A* gene had a sensitivity value of 91.7%, a specificity value of 72.4%, and an AUC value of 0.817.

Inconsistent with our results**,** in a study of **Shumin Ni et al.** [23] When patients of lung cancer were compared in bronchial aspirates to non-tumor controls, Methylated *RASSF1A* gene had a sensitivity value of 0.40 (95% CI: 0.34-0.46), a specificity value of 0.99 (95% CI: 0.95- 1.00), and an AUC value of 0.66 (95% CI: 0.61-0.70).

El-Sherif et al. [17] noted that diagnostic sensitivity of methylated *RASSF1A* is 31.1% and specificity of 100% with PPV 100% and NPV 39.2% which is different from our results.

In agreement to our results **Gao et al.** [15] found that *RASSF1A* assay of methylation had an area of 0.81 under the ROC curve. By using the optimal cutoff value of150 copies/mL, they got a specificity of 90.3% and a sensitivity of 56.9%

This variation can explained by the variation in methods of detection and analysis of methylation and different laboratory facilitations

Conclusions

These results support the notion that identifying the methylation status of *RASSF1A* gene may be useful as a molecular biomarker in diagnosis and screening for lung cancer.

References

1- Siegel R, Naishadham D, Jemal A. Cancer statistics 2013. CA.

2- World Health Organization - World Cancer Report 2014; Chapter 1.1. ISBN 9283204298.

3- Mehta A, Dobersch S, Romero-Olmedo A.J. et al. Cancer Metastasis Rev 2015; 34: 229.

4- Brzezianska E, Dutkowska A, & Antczak A. The significance of epigenetic alterations in lung carcinogenesis. Molecular Biology Reports 2013; 40(1):309-325.

5- Powrózek T, Krawczyk P, Nicoś M, et al. Methylation of the DCLK1 Promoter Region in Circulating Free DNA and its Prognostic Value in Lung Cancer Patients. Clin Transl Oncol. 2016; 18:398–404.

6- Breitling L.P, Yang R, Korn B, et al. Tobacco-smoking-related differential DNAmethylation:27K discovery and replication. Am.J.Hum. Genet 2011; 88:450–457.

7- Suter M, Abramovici A, Showalter L, et al. In utero tobacco exposure epigenetically modifies placental CYP1A1expression. Metabolism 2010; 59:1481–1490.

8- Huang J, Okuka M, Lu W, etal. Telomere shortening and DNA damage of embryonic stem cells induced by cigarette smoke. Reprod. Toxicol 2012; 35:89–95.

9- Richter AM, Pfeifer GP, Dammann RH. The RASSF proteins in cancer; from epigenetic silencing to functional characterization. Biochim Biophys Acta. 2009; 1796:114 128.

10- van der Weyden L, Adams DJ. The Ras-association domain family (RASSF) members and their role in human tumourigenesis. Biochim Biophys Acta. 2007; 1776:58-85.

11- El-Hefni A.M, Shouman W, ElGammal, M.S. *et al.* Clinical and Epidemiological study of lung cancer cases in Sharkia Governorate, Egypt. Egyptian Journal of CHEST 2010; 52 (2, 3):236-250.

12- Topaloglu O, Hoque MO, Tokumaru Y, et al. Detection of promoter hypermethylation of multiple genes in the tumor and bronchoalveolar lavage of patients with lung cancer. Clin Cancer Res. 2004; 10:2284-2288.

13- Toyooka S, Maruyama R, Toyooka KO, et al. Smoke exposure, histologic type and geography-related difference in the methylation profiles of non-small cell lung cancer. Int. J. Cancer 2003; 103:153-160.

14- Zochbauer-Muller S, John D. Minn, Adi F. Gazdar. Aberrant DNA Methylation in Lung Cancer: Biological and Clinical Implications. The Oncologist 2002; 7:451-457.

15- Gao L, Erfu Xie, Tongfu Yu, et al. Methylated APC and RASSF1A in multiple specimens contribute to the differential diagnosis of patients with undetermined solitary pulmonary nodules. J Thorac Dis 2015; 7(3):422-432.

16- Minpu Ren, Chunhua Wang, Danli Sheng, et al. Methylation analysis of SHOX2 and RASSF1A in bronchoalveolar lavage fluid for early lung cancer diagnosis. Annals of Diagnostic Pathology 2017; 27: 57–61.

17- El-Sherif WT, Sayed SK, Makhlouf HA, et al. Diagnostic role of RASSF1A and p16INK4a promoter gene hypermethylation in serum DNA of lung cancer patients: clinicopathological significance. Egypt J immunol. 2016 Jun; 23(2):1-16.

18- Scesnaite A, Jarmalaite S, Mutanen P, et al. Similar DNA methylation pattern in lung tumours from smokers and never-smokers with second-hand tobacco smoke exposure. Mutagenesis 2012; 27 (4): 423–429.

19- Hoffmann D, Brunnemann K. D, Prokopczyk B, et al. Tobacco-specific N-nitrosamines and Areca-derived N-nitrosamines: Chemistry, biochemistry, carcinogenicity, and relevance to humans. Journal of Toxicology and Environmental Health 1994; 41:1 – 52.

20- Ashley D. L, Beeson M. D. Johnson D. R, et al. Tobacco-specifi c nitrosamines in tobacco from U.S. brand and non-U.S. brand cigarettes. Nicotine & Tobacco Research 2003; 5:323–331.

21- Brunnemann K. D, Cox J. E, & Hoffmann D. Analysis of tobacco-specifi c N-nitrosamines in indoor air. Carcinogenesis 1992; 13: 2415–2418.

22- Idris A. M, Prokopczyk B, & Hoffmann D. Toombak: A major risk factor for cancer of the oral cavity in Sudan. Preventive Medicine 1994; 23:832–839.

23- Shumin Ni, Meng Ye and Tao Huang. Short stature homeobox 2 methylation as a potential noninvasive biomarker in bronchial aspirates for lung cancer diagnosis. Oncotarget 2017; 8 (37):61253-61263.