Tobacco Smoking as A Risk Factor in DNA Methylation of Repair Gene (MLH1) Using Cytobbrush from Lateral Border of the Tongue

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Abstract

Objective: The aim of this study was to evaluate the epigenetic effect in the process of oral carcinogenesis by screening the methylation of repair gene in chronic tobacco smokers.

Material and Method: Study design: One hundred male volunteers, divided into two groups: the 1st group consisted of 58 smokers, each consumed 20 cigarettes/day for at least 10 years; and the 2nd group consisted of 48non-smokers who were consider as a control group.

The samples were taking from lateral border of the tongue by exfoliative cytology, and the extracted DNA was treated with phenol-chloroform gDNA,Screening of methylation was done by Methyl Specific PCR (MSP).

Results :- The results of this studyshowed significant effect of tobacco smoking in methylation of MLH1 gene in site 1in comparison to non-smoker group,($P > 0.05$), with Odds ratio = 4.957 CI ().

Keywords:*This study concluded that smoking considered as a risk factor predisposed to process of oral carcinogenesis.*

Introduction

The DNA methylation mechanism has a significant regulatory role in gene expression. The epigenetic modification induced by the hyper methylation act to suppress anti-oncogene expression by inhibition of transcription of its promotor region [(1)]. In addition, the direct effect of DNA methylation on DNA repair gene which inhibits transcription of its promotor region that induce cancer. Methylation of DNA is reversible so, the early detection as early as will help correct the error and avoid the serious hazardous consequences[(1,2)].

Oral mucosa mutagenesis takes place by multiple factors over several steps induced by hereditary and environmental factors affected the genetic material in which, the smoking considered as one of the most important risk factors [(3)].

Cellular genomic stability rely on the efficiency of DNA repair accordingly, the modification in DNA repair genes can cause cancer [(4)].

MutL Homolog 1 (MLH1) gene act as a DNA repair gene that can correct of error of base pairing along with other repair genes as MSH2-gene. The hyper methylation of these genes cause repress of their expression and predispose oral carcinogenesis [(5)].

Smoking considered one of the main hazardous risk habits that is associated with many diseases especially that are related with oral mucosa, smoking

can act initiator and/or inducer of squamous cell carcinoma as a result of DNA damage [6,7,8].

Materials and Methods

Cross-sectional study performed on 100 males (Age 20-40 years) who were divided into two group's (smokers) and control group (nonsmokers). The study done in Babylon city (The blood bank), Ethical approval for the study and informed patients consent obtained, and each patient will filled a case sheet questionnaire:

Including age, name, systemic diseases ……..

All the cases that included in this study had to fitful the exclusion criteria which

1. History of malignant neoplasia, vesicobullous and ulceration.

2. Absence of any visibleoral tissue changes alterations in the normal oralmucosa.

3. Alcohol drinking current and X.

Inclusion criteria include male with age between 20-40 years and heavy smoker (20 cigarette/ day) at least and duration ≥ 10 years.

Thecytobrush samples were collected from the lateral border of the tongue from the deep layer using a Rovers® Orcellex® Brush Soft Oral Cell Samplex (Rovers Medical Devices, NL, Netherlands). As such a procedure is only minimally painful, it is not necessary to use local anesthesia when performing it,All participants were refrained from drinking or eating for about 30 minutes, and then they asked to rinse their mouth with water before sample collection.

DNA extraction from whole tissues. The protocol begins with phenol-chloroform gDNAextraction **(10).**

Screening of MethylationbyMethyl Specific PCR (MSP):

The DNA was bisulfite-treated conversion was used and cleaned up by**EZ DNA Methylation™ Kit Catalog No.D5002** from (**ZYMO RESEARCH**, **USA**) MSP was performed primers as ;.

First site which employ the following primer according to[11].

M3f TATATCGTTCGTAGTATTCGTGT

M3r TCCGACCCGAATAAACCCAA 154bp

U 3 f TTTTGATGTAGATGTTTTATTAGGGTTGT

U3r ACCACCTCATCATAACTACCCACA 128 bp

Second site employ as primer site according to [12]

M2f gatagcgatttttaacgc 93bp

M2r tctataaattactaaatctcttcg

U2f agagtggatagtgatttttaatgt 100bp

U2r actctataaattactaaatctcttca

The PCR(Biometra TRIO Thermocycler)were conducted for two sites in **promoter region of MLH1 gen** .by employ the following added 8 μ**1** of (2.5X) master mix (Cyntol, Russian), 1 μ**1**of (10 PM) of each primer and the total volume completed to 20 μ**1** grade water and PCR conditions according to table 1.

Product then refer by **Agros 2**□ PCR product resolved by 2 prestained (ethmoidepromide) the presence of corresponding band with dedicate molecular weight assumed as positive result[13][14].

Table 1: Thermocyclingcondition for PCR

Stage	Steps	Tempreature	Time	No. of Cycling
1	Intial denaturation	94	5 min	1
2	DNA DENATURATION	94	30 sec	35
	Primer annealing	60	30 sec	
	Extension	72	50 sec	
3	Final extention	72	5 min	1

Statistical Analysis

All statistical calculation were performed using SPSS Version 21.0. USA) and Microsoft Excel (2010, Microsoft Corp. USA). All the results were expressed A $p < 0.05$ was considered statistically significant.. Chi-Square Test, and odds ratio were employed to evaluate the association between smoking and MLH1 gene methylation.

Results

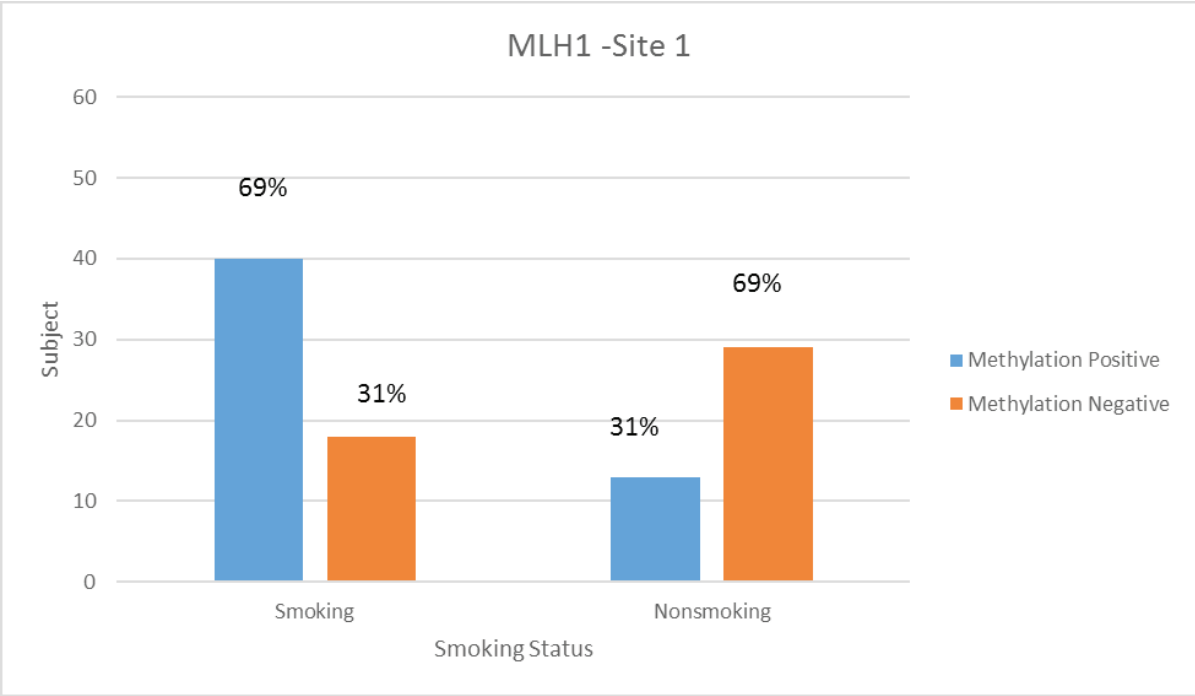
Smoking significantly hypermethylate MLH1 gene site- 1 with ($P > 0.05$) and non-significant relation on site -2 with (P.0.160) Table.2.

Table 2:The association between smoking and MLH1-gene promoter site 1and 2methylation.

Subject		MLH1 site 1				MLH1 site 2			
		Negative	Positive	Chi-Sequare	P-value	negative	positive	Chi-Square	P-value
	Nonsmoking	29	13	14.131	0.000	36	6	2.470	0.160
	Smoking	18	40			55	3		
Total		47	53			91	9		

Figure.1&2 shows that 69% of smoker have MLH1 gene methylation in the site 1, and 31% have not affected with methylation in site 1, considering non-smokers only 31% of them have methylation in nonsmokers group.

In the site 2, there is only 5% of smokerswere found with methylation and 95% have not affected.



Figure(1):The effect of smoking as a risk exposure factor on the methylation of repair

Gene site 1 MLH1.

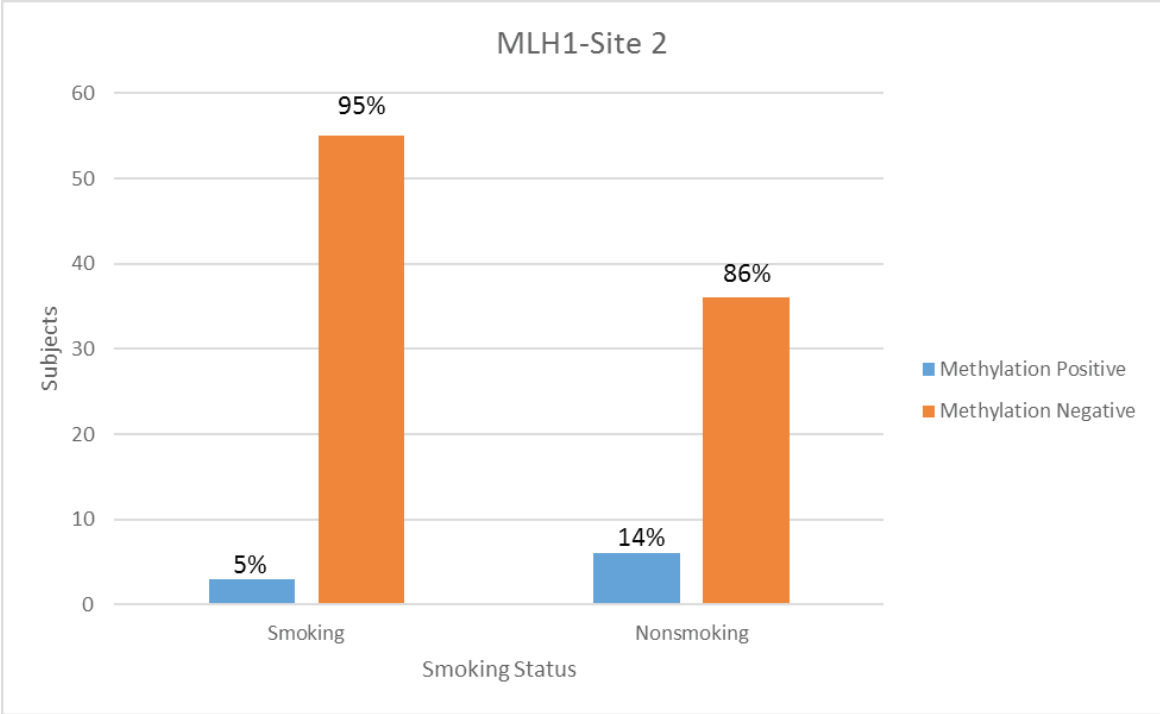


Figure (2): The effect of smoking as a risk exposure factor on the methylation of repair gene MLH1 site 2.

The hypermethylation of MLH1 gene induced by smoking can cause repressing of gene expression and inhibit transcription causing gene silencing and so impair MLH1 gene repair function. The odds ratio of this association was 4.957 with C/I (2.1-11.7), that illustrated the risk exposure to smoking.

Discussion

Exposure to tobacco smoke is considered to be the most important etiological factors for the development of squamous cell carcinoma of the head and neck (SCCHN)**.**

THE MLH1 gene is plays critical role in different types of cancer. Epigenetic silencing of MLH1 promoter methylation can cause mismatch repair (MMR) deficiency. Which may cause insertion or deletion mutations in repeated sequences[15].

The MLH1 promoter methylation has been reported as a well-established biomarker in several types of cancer, such as esophageal cancer, colorectal cancer, non-small cell lung cancer, gastric cancer, papillary thyroid cancer, and bladder cancer [16].

Carcinogens and activated procarcinogens in tobacco smoke may react with the DNA of exposed human tissues, such as the epithelial cells of the upper aero digestive tract. This can lead to the formation of DNA adducts and subsequently to mutations in crucial genes such as oncogenes and tumor suppressor genes, ultimately resulting in the development of cancer [17].

An important epigenetic mechanism of gene inactivation during carcinogenesis is gene silencing caused by hypermethylation of the promoter region[18,17].

The present study was showed that chronic smoking has direct effect on oral mucosa leads to the methylation of repair gene (MLH1), this result provide evidence that smoking causes methylation repair gene. The table (1) was showed that there was a significant effect of smoking on methylation of MLH1 gene compare to nonsmoking group $P > 0.001$. This result agree with[8,19,20].

It concludes that smoking present risk factor with an Odds ratio of(4.957)(confidenceintervals) as shown in figure (1.1).

difference function of DNA methylation comprises, other than the regulation of gene expression, the protection of the integrity of the genome .

Conflict of Interest: No

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Ethical Clearance: Not Required

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