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Detection of methylated gene (p16) in patients with head and neck squamous cell carcinoma using conventional PCR

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SUMMARY

Introduction: The role of detecting abnormal methylations in the genes and other epigenetic abnormalities in the patients with HNSCC, like p16 gene in the DNA of human cells could guide us to detect carcinoma early.

Aim: To detection abnormal methylation in p16 gene in HNSCC.

Patients and Methods: This is a case-control and cross sectional combine study conducted in the Otolaryngology Department at Al-Yarmouk Teaching Hospital. Forty one patients enrolled, they were divided into two groups. Group-A included 21 patients have HNSCC and group-B included 20 patients as controls. Saliva was collected from oral cavity. PCR was performed to identify the DNA. This is subjected to methylations looking for the presence or absence of gene methylation.

Results: 21 patients in group-A, 16 were males, and 5 were females. 7 patients were positive for methylation in EXON-1, 5 were smokers and 2 were alcoholic, whereas 12 were positive for methylation in EXON-2, 8 were smokers and 2 were alcoholic. In group B: 7 patients were positive for methylation in EXON-1, 6 were smokers and 3 were alcoholic, whereas 4 were positive for methylation in EXON-2, all were smokers and 1 was alcoholic.

Conclusion: Hyper-methylation in specific EXON of gene p16 is a useful method for the detection of abnormal epigenetic which could lead to the development of HNSCC in high risk people. The detection of methylation in p16 correlates well with nodal involvement. EXON-2 methylation of p16 gene was significantly detected more than EXON-1 and more with HNSCC.

Key words: squamous cell carcinoma, DNA, epigenetics, methylation

ABBREVIATIONS: PCR: Polymerase Chain Reaction; HNSCC: Head and Neck Squamous Cell Carcinoma; DNA: Deoxyribonucleic Acid; CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A; Rb: Tumour Suppressor Retinoblastoma; E2F: Transcription Factor; T: Tumour

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INTRODUCTION

HNSCC is a malignant tumours of the upper aero digestive tract. It is a disease with high incidence and mortality affecting mainly the oral cavity (lips, hard palate, tongue, gums and floor of mouth), pharynx (naso-, oro- and hypopharynx) and larynx. It is considered to be the fifth common cancer site worldwide [1]. Overall survival rate is around 50%, and the main reason for treatment failure is the frequent development of loco-regional recurrences [2, 3]. The primary risk factors for the development of HNSCC are two environmental toxins, tobacco and alcohol [4]. A third risk factor, which acts independently of the other two, is a history of an oral (HPV) infection and EB virus [2]. Approximately half of all HNSCC harbours mutations in TP53, making it the commonly mutated gene in this cancer type [5]. Functional loss of p53 is critical in malignant transformation, and mutations in TP53 are thought to arise early in HNSCC tumorigenesis [6]. Other alterations of cyclin-dependent kinase inhibitor 2A (CDKN2A), located on chromosome 9p21, have long been recognized in HNSCC. The CDKN2A locus encodes two proteins, p16 (INK4a) and p14ARF, which are alternative transcripts that differ in their first exons both proteins are thought to function as tumour suppressors [7]. The p16 protein play a critical role in cell-cycle regulation because of its ability to inactivate the proteins that phosphorylate the tumour suppressor Retinoblastoma (Rb), which bind to and inhibit the transcription factor E2F and there by prevent passage from G1 into S phase [7]. The p16 protein inhibits the catalytic activity of CDK4 and CDK6 and promotes the formation of the Rb-E2F complex, which causes G1 arrest [7, 8]. Inactivation of CDKN2A is common in HNSCC. Early studies demonstrated frequent loss of heterozygosity at 9p21 in dysplastic, pre-invasive, and malignant lesions, which suggest an event that occurs early in carcinogenesis figure [9].

PATIENTS AND METHODS

This is a cross sectional study was conducted in the Otolaryngology Department at Al-Yarmouk Teaching Hospital during the period from June 2017 to September 2018. It included 41 patients divided into two groups; group-A included 21 patients who had primary HNSCC (of the oral cavity, pharynx, larynx, and paranasal sinuses), and group-B included 20 patients who had no history of HNSCC as a control group. Group-A were subdivided into two subdivision early (T1-2) and

late (T3-4) and also classified into those with nodal involvement and those without.

Inclusion criteria

All age groups of both genders in any patient diagnosed histopathologically as having HNSCC before treatment and those participants after surgical treatment.

Exclusion criteria

1. Patients received radiotherapy and or chemotherapy
2. Patients with xerostomia
3. Patients with head and neck malignancy other than SCC

Saliva was collected from all the patients using special sterile tube (micro centrifuge tube), containing 1cc DW. The patients were asked not to eat or drink any fluid for at least 30 minutes and gurgle their mouth with 10 cc DW before the test. DNA extraction using PCR, to detect the presence of methylation at p16 gene using (PROMEGA kit). Gene methylation was detected through four steps (DNA extraction, DNA methylation, PCR to the methylated DNA, reading at electrophoresis).

1. Step one: DNA extraction obtaining a mixture of DNA, RNA, proteins
2. Step two: DNA methylation
3. Step three: Performing PCR
4. Step four: Reading the Electrophoresis

RESULTS

A total 21 patients with HNSCC, 16 males, and 5 females, their age ranged between 22 and 66 years old, with median age was 52 years. Most common site for HNSCC was at the larynx in 11 patients. The least common site for HNSCC was paranasal sinuses. Fifteen patients were smokers (71.4%) and 5 were alcoholic (23.8%) (All of them smoker), and only one of all patients had family history of HNSCC. Eleven patients of group-A have early stage (T1-2) and 7 of them had lymph node involvement. Twenty patients (with no history of HNSCC were included as control group, their age ranged between 19 and 68 years, with median age was (47.5 years), 12 were males and 8 were females. Ten patients were smokers (50%) and 4 were alcoholic (20%), and all alcoholic were smokers.

Results of methylation were as follows: 7 of group A patients with HNSCC (33.3%) were positive for methylation in EXON-1. 12 of group A patients with HNSCC (57.1%) were positive for methylation in EXON-2 and 4 patients (19%) were positive for both EXONs. Regarding control group 7 persons (35%) were positive for methylation in EXON-1, 6 (85%) were smokers and 3 (42%) were alcoholic, and 4 (20%) were positive for methylation in EXON-2, all of them were smokers and one was alcoholic (Tables 1-3).

DISCUSSION

HNSCC in males is frequent as 76.1%, while females were 23.8%. This goes with the study done by Righini et al. which showed that 77% of patients with HNSCC were males [10]. Most studies showed that HNSCC mostly presented at the 6th decade. Risk factors for developing HNSCC are smokers, which the present study showed (71.4%) of patients, which is less than what Ovachinnikov et al. showed, these due to the difference in patients sample [11]. Alcoholic with HNSCC were 23.8% in the present study, this is lower than what Rettori et al. showed which was 71.3% [12]. Larynx is the common HNSCC site in the present study in 62.5%, while the most common HNSCC

Tab. 1. Methylation in EXON-1,-2 in patients with HNSCC and controls

EXON		Patients (n=21)		Controls (n=20)		P value
		No	%	No	%	
EXON-1	Positive	7	33.3	7	35.0	0.910
	Negative	14	66.7	13	65.0	
EXON-2	Positive	12	57.1	4	20.0	0.015*
	Negative	9	42.9	16	80.0	

4 patients (19%) have both EXON-1 &-2

*Significant difference between proportions using Pearson Chi-square test at 0.05

Tab. 2. Correlation between methylation and HNSCC staging

Positive Methylated p 16 gene (HNSCC=15)		Early		Advanced		P value
		No	%	No	%	
Type	EXON 1	2	28.6	5	71.4	0.568
	EXON 2	5	41.7	7	58.3	

4 patients (19%) have both EXON-1 and -2 (all with advanced TNM)

*Significant difference between proportions using Pearson Chi-square test at 0.05

Tab. 3. Correlation between methylation, smoking and alcohol in controls positive methylation

Controls positive methylation (n=11)		Smoker		Non smoker		P value
		No	%	No	%	
Positive Methylated p 16 gene	EXON 1 (n=7)	6	85.7	1	14.3	-
	EXON 2 (n=4)	4	100	-	-	
		Alcoholic		Non alcoholic		
Positive Methylated p 16 gene	EXON 1 (n=7)	3	42.9	4	57.1	0.554
	EXON 2 (n=4)	1	25.0	3	75.0	

*Significant difference between proportions using Pearson Chi-square test at 0.05

in the study of Righini et al. was carcinoma of oropharynx [10]. Rettori et al. study showed that most common HNSCC was carcinoma oral cavity as 70% [12]. Stage of tumour and nodal involvement in our study, 52.3% of patient with HNSCC were early (T1-2) and 47.6% were late (T3-4) and 33.3% have positive nodal involvement this goes with the study of Righini et al. [10]. Regarding methylation, 8/11 patients having SCC in larynx, 72% have positive methylation in either EXON of p16 or both, which higher than what Pierini et al. found [13]. Regard the control group was positive for EXON-1, and EXON-2, these results agree with study by Ovachinnikov et al. [11]. Gene methylation correlated with nodal

involvement presented in all the patients either with EXON-1 or -2 or both, similar findings were shown in other studies [13]. Methylation in gene P16 occur more in smokers than non-smokers as most of the smokers in both groups (patients with HNSCC and the control group), showed positive methylation. This correlation between smoking and gene p16 methylation is similar to what was reported by and Ovachinnikov et al. and Pierini et al. studies [11, 13]. Sensitivity and specificity of the test was 71.4% and 70%, respectively. This is more sensitive and less specific than study done by Ovachinnikov et al. which showed sensitivity and specificity of 60% and 90 %, respectively [11]. In the present study methylation was more in patient with advanced HNSCC, this is going with study by Pierini et al. [13].

CONCLUSION

Hypermethylation in specific EXONs of gene p16 is a useful method to detect abnormal epigenetic, which could lead to development of HNSCC in high risk people. Smoking is major risk factor for developing of hypermethylation in p16 gene. Detection of methylation in p16 correlates well with nodal involvement. Detection of EXON-2 was more significant than detection of EXON-1. Detection of methylation correlate well with tumour advancement, the more advanced the tumour is the more methylation of p16 it contains.

AUTHOR'S CONTRIBUTION

Mohammed QM: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Visualization; Roles/ Writing-original draft; Writing-review and editing. Ehab Yassen: Conceptualization; Data curation; Project administration; Resources; Supervision; Validation; Visualization; Roles/ Writing-original draft.

COMPETING INTERESTS

The authors declare that they have no competing interestst.

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