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Global DNA Methylation Profile of Head and Neck Squamous Carcinoma Patients at the University College Hospital, Ibadan, Nigeria

V. I. Akinmoladun[1*], F. V. Edem[2], O. A. Popoola[3] and O. G. Arinola[2]

[1]Department of Oral and Maxillofacial Surgery, College of Medicine, University of Ibadan, Nigeria.
[2]Department of Chemical Pathology, College of Medicine, University of Ibadan, Nigeria.
[3]Department of Community Medicine, College of Medicine, University of Ibadan, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author VIA designed the study. Author OAP performed the statistical analysis. Authors VIA and OGA wrote the protocol. Authors VIA and OGA wrote the first draft of the manuscript. Authors VIA and FVE did the laboratory work and authors VIA and OAP managed the analyses of the study. Author VIA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Head and neck squamous cell carcinoma (HNSCC) is commonly associated with tobacco and alcohol exposures among other risk factors, which are linked with epigenetics. Epigenetic alterations such as DNA methylation are increasingly implicated in the initiation and progression of cancer. This study quantitated global DNA methylation in peripheral blood of HNSCC patients and controls. The influence of environmental risk factors, age and gender on DNA methylation were also evaluated.
Methods: Venous blood samples were obtained from participants. DNA extraction was carried out using Qiagen kit according to the manufacturer's instructions. Level of global DNA methylation was obtained with an enzyme-linked immune sorbent assay (ELISA)-based technique (Cell Biolab, CA). Methylated DNA concentrations were obtained by interpolation of the standard curve. Select samples done in duplicates served as intra-assay controls. Data was analysed using IBM SPSS 20.

**Corresponding author: E-mail: viakinmoladun@gmail.com;*

Quantitative variables were summarised as means or medians as appropriate. An independent t-test was used to compare methylation values across cases and control groups and for various independent variable response categories. A multiple linear regression was fitted to explore the influence of study assignment, gender and age on DNA methylation values. All significance testing was conducted at α - 0.05.

Results: Global DNA methylation was higher in cases (10.24 ± 3.85) relative to controls (9.35 ± 3.34). This was however not statistically significant. Females (11.12 ± 4.49) were significantly hypermethylated relative to the males (9.03 ± 2.74). A linear regression analysis showed female gender as the only significant predictor of methylation. Females had values on average 0.28 units higher than males after correcting for age and study assignment. Tobacco and alcohol users among cases did not have significantly higher methylation values than non-users.

Conclusion: Relative hyper methylation was recorded among cases. However, only gender had a significant relationship with DNA methylation in HNSCC in this study.

Keywords: DNA; methylation; head; neck; carcinoma; blood.

1. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common type of cancer in the World [1]. Although survival rates can be high if detected early, majority of patients are diagnosed at advanced stage, reducing the chances of survival after 5 years to less than 50%, a figure which has not improved significantly in the last decade [2-4].

Tobacco and alcohol use are well established risk factors for HNSCC and are known to act synergistically [5,6]. High-risk human papillomavirus (HPV) types, particularly HPV16, has also been associated with increased risk. Additionally, dietary factors, including deficiencies in dietary single-carbon metabolism vitamins (B vitamins) are also plausible risk factors [7,8]. Changes in methylation patterns, are known to contribute to neoplasia and tumour growth [9,10]. Neoplastic growth is frequently preceded by aberrant DNA methylation, which leads to a loss of function that promotes cell proliferation [11]. Also, cancer-linked global DNA hypomethylation in tumour tissue is a common finding in a wide variety of malignancies, ranging from tumours such as breast, colon, oral, and lung cancers, to cancers of the blood [12,13]. A recent study by the authors found low levels of vitamin A, B12 and folate in HNSCC patients [14]. This study is a follow up to that on the likelihood of the use of these as surrogates and an indirect measure of the methylation status of HNSCC patients.

It is increasingly clear that epigenetics play a causal role in cancer development and HNSCC is a useful disease for studying DNA methylation; because the mechanisms of epigenetic maintenance are related to the risk factors linked to this disease [15]. The study of DNA methylation in development and disease has three major classes of measurement now routine, namely (i) locus-specific (ii) genome-scale/wide (iii) 'global' methylation approaches. Measures of global methylation refer to the level of 5-methylcytosine (5mC) content in a sample. This study is a global DNA methylation profiling of HNSCC in our environment.

2. MATERIALS AND METHODS

Sixty participants comprising of 20 cases of HNSCC and 40 individuals made up of members of staff and those accompanying patients to the hospital who were without clinical evidence of HNSCC served as controls. Study was explained to both groups and informed consent obtained before the commencement of sample collection. Five milliliters of venous blood was collected from the antecubital vein from participants. The blood samples were dispensed into EDTA bottles for DNA methylation study.

DNA extraction was carried out using a Qiagen DNA extraction kit according to the manufacturer's instructions. The samples were dissolved in DNA hydration buffer. DNA quality and quantity were measured by the Nano Drop ND-1000. Samples were stored at -20 degrees for later use.

Concentrations of methylated DNA were obtained with enzyme-linked immunosorbent assay (ELISA)-based technique, using a commercially available kit (Cell Biolab, CA) according to the manufacturer's instruction. Standard curves were generated and sample concentrations were calculated from the standard

curve. Methylated DNA concentrations were calculated by interpolation of the standard curve. Select samples were done in duplicates, thereby serving as intraassay controls. Descriptive statistics are presented as summary indices and in tables and charts. An independent t test was used to compare methylation values of sub groups and all cases and controls. A multiple linear regression model was fitted to explore the effect of study assignment, age and gender on DNA methylation values. Statistical significance set at $p < .05$.

3. RESULTS

Sixty participants comprising of 20 cases of HNSCC and 40 apparently healthy individuals were recruited. Cases were made up of 15 males and 5 females while controls were 25 and 15 males and females. Consequently, females constituted 25% of cases and 37.5% of controls. Cases were older than controls with a mean age of 49 years compared to 45 years for females (Table 1). There were 5 cases of laryngeal carcinoma and 4 mandibular tumours. The tongue accounted for 3 cases (Fig. 1).

This study found higher mean values of global DNA methylation in cases relative to controls (relative hypermethylation). This was however not statistically significant (Table 2). Methylation values for cases were higher than for controls at minimum, 25th, 50th, 75th and maximum value points. There was a difference in methylation values between males and females in the study group with females being hypermethylated relative to the males in both the case and control group. A similar pattern was seen among controls (Table 3).

Gender was the only variable identified to significantly influence methylation values, females on average had values 0.28 units higher than controls. Cases were 0.14 units higher than controls. Methylation values were also observed to increase with age, 0.1 unit increase on average from those under 40, through 10-year age bands to those older than 60 years. However, these age and study assignment differences were not statistically significant (Table 4).

All participants who used tobacco also consumed alcohol. However, six others used alcohol without tobacco. There were no statistically significant differences in the mean values of methylation between those with alcohol or tobacco exposure and those without (Table 5).

4. DISCUSSION

Head and neck squamous cell carcinoma include mainly squamous cell carcinomas of the sino-nasal area, or facial area, nasopharynx, pharynx, and larynx and less so, those of other contiguous structures in the region. This disease in the various sites has unique sets of epidemiologic, pathologic and treatment

Table 1. Patient characteristics of cases and controls

	Cases (n=20)	Control (n=40)	Total
Age Categories			
≤ 40 years	8 (40.0)	17 (42.5)	25 (41.7)
41 – 50 years	3 (15.0)	9 (22.5)	12 (20.0)
51 – 60 years	4 (20.0)	4 (10.0)	8 (13.3)
≥ 61 years	5 (25.0)	10 (25.0)	15 (25.0)
Age (Mean ± SD)	49.25 ± 20.27	45.00 ± 17.47	46.50 ± 18.25
Gender			
Male	15 (75.0)	25 (62.5)	40 (66.7)
Female	5 (25.0)	15 (37.5)	20 (33.3)

Table 2. DNA methylation values

Methylation value	Cases (n=20)	Control (n=40)	Total
Mean ± SD	10.24 ± 3.85	9.35 ± 3.34	9.70 ± 3.51
p-value 0.361			
Maximum	18.70	16.50	18.70
75th percentile	11.78	11.40	11.70
Median	10.15	9.05	9.30
25th percentile	8.08	6.83	7.60
Minimum	2.60	2.50	2.50

Table 3. DNA methylation comparison across age and gender groups

	Cases (Mean ± SD)	Control (Mean ± SD)	Total
Age categories			
<=39 years (n=24)	10.23 ± 2.93	8.64 ± 3.14	9.100 ± 3.10
	P 0.26		
40 years & above (n=36)	10.25 ± 4.38	9.92 ± 3.46	10.04 ± 3.78
	P 0.85		
Gender			
Male (n=40)	9.21 ± 2.07	8.92 ± 3.11	9.03 ± 2.74
	P 0.74		
Female (n=20)	13.32 ± 6.28	10.27 ± 3.53	11.12 ± 4.49
	P 0.19		

Table 4. Linear regression analysis of DNA methylation values

Variable	Beta	P value
Study assignment (Cases)	0.14	0.27
Gender (Female)	0.28	0.03
Age (<40, 41 – 50, 51 – 60, >60)	0.10	0.42

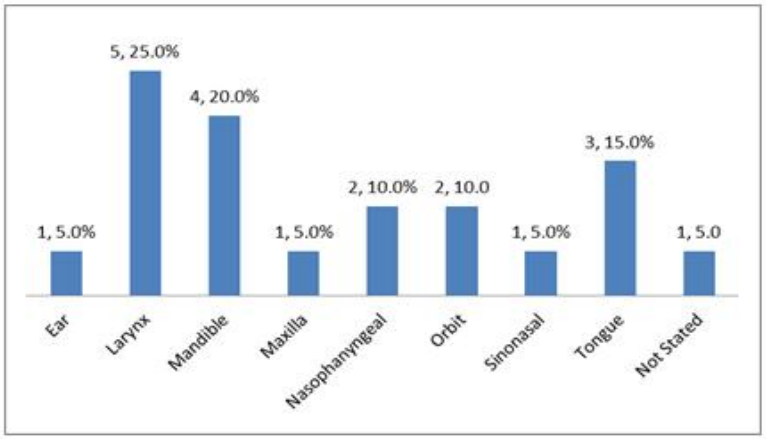

Fig. 1. Site distribution of tumours

Table 5. DNA methylation values by social risk behaviour and tumour stage of cases

	N	Mean	Std. deviation	t	p-value
Tobacco					
No	15	10.333	3.842	0.775	0.450
Yes	5	8.840	2.916		
Alcohol					
No	14	10.355	4.0285	0.708	0.490
Yes	6	9.050	2.6584		
Tumour					
Early	3	8.733	0.4041	0.727	0.447
Advanced	17	10.506	4.1297		

considerations [1]. Like most cancer types generally, incidence increases with age.

The mean age of HNSCC patients in this study was 49.3 (20.3) years. This finding is in consonance with previously reported studies [16,17].

The airway has the highest site distribution in this series closely followed by the oral environment. This is not different from reports from other local studies on HNSCC in the literature [16,18] which is often hospital based and reflects the setup and practices of the contributing units. The involvement of a site may be due to direct

contact with the particular environmental risk factor to which it is exposed.

Aberrant methylation is associated with cancers and reduced levels of global DNA methylation are known to result in genomic instability which is an independent predictor of cancer risk [19,20]. Also, malignant growth is frequently preceded by aberrant DNA methylation, with resultant loss of function that promotes cell proliferation [15]. One of the first alterations of DNA methylation to be recognized in neoplastic cells was a decrease in nonspecific (or global) DNA methylation, although more recently, attention is being directed toward loci or gene specific alterations. This study reports higher mean value of global DNA methylation in the blood samples of HNSCC patients relative to controls, although this is not statistically significant. This finding is in agreement with the report by Piyathilake [21] and Baylin [22] but contrary to hypomethylation reported more often with HNSCC [23-25]. This non concordance between studies may be related to tissues analysed, laboratory techniques employed, disease stage and mode of reporting among others in this growing field.

The impact of age and aging on DNA methylation has been well documented [7]. Aging cells show a progressive loss of 5 mc content [26,27]. Also, DNA methylation differs more in older than in younger monozygotic twins [28] and has been reported to longitudinally change over time [29,30]. The overall impact of aging on methylation is to reduce the global level of genomic DNA methylation, and this age-related decline in DNA methylation appears to be similar across tissue types [31]. However there was no age related difference in both groups involved in this study. This may not be unconnected with the narrow age groups involved, as disease of interest is age related and the relatively small sample size. A wider age group and a larger sample size may have improved power to detect a significant impact of age on global DNA methylation.

Sex differences at the DNA methylation level have previously been reported with the female gender being hypomethylated relative to their male counterpart [32-34]. The reason for this gender specific difference in global DNA methylation is not clear. In this study however, females recorded higher methylation level after controlling for case-control assignment.

Studies have explored the environmental or behavioural determinants of global DNA methylation in humans [35,36]. This, with regards to HNSCC, has to do with exposure to known risk factors of tobacco and alcohol. A growing number of studies have also shown that alcoholism may be associated with altered DNA methylation [36-38]. The additive effect of alcohol consumption and smoking on changes in DNA methylation has also been demonstrated [39].However, consistent with some previous studies [15,38], our study did not support an association between smoking, alcohol and DNA methylation. Although, Smith et al. [36] reported that levels of LINE-1 methylation were significantly associated with smoking and alcohol use in tissues of squamous cell head and neck cancer.

DNA hypomethylation is essentially a reduction in the level of 5-mC content in malignant tumours usually compared with the adjacent normal tissue or analogous normal tissue from healthy controls. [40,41]. However, because of the challenges associated obtaining autologous normal tissue, global DNA methylation is often evaluated in easily available tissue, such as peripheral blood, for epidemiological purposes and cancer screening [10,40]. With the result obtained in this study, peripheral blood DNA methylation measured by 5-mC content may not be used as a surrogate for actual methylation status in HNSCC.

Our findings may be due to the small sample size, method of analysis, or some yet unknown genetic and or environmental factors involved in global DNA methylation. This to be best of the author's knowledge is the first attempt on this subject matter in our environment.

5. CONCLUSION

HNSCC was associated with a relative but statistically insignificant DNA hypermethylation. However gender had a significant relationship with DNA methylation in HNSCC in this study with female being relatively hypermethylated.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

UI/UCH Ethical Review Committee approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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