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Immunohistochemical expression of hypoxia inducible factor-1 alpha (Hif-1 α) in patients of head and neck squamous cell carcinoma

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ABSTRACT

BACKGROUND & OBJECTIVE: Hypoxia-inducible factor-1 alpha (HIF-1 α) could serve as a molecular target for therapy in head and neck squamous cell carcinoma (HNSCC). Limited research has been conducted on HIF-1 α expression in the Pakistani population, and global studies have shown conflicting results. This study aims to measure the expression of Hypoxia-inducible factor-1 alpha (HIF-1 α) in patients with HNSCC through immunohistochemistry.

METHODOLOGY: Sixty biopsy specimens from HNSCC patients were analyzed for HIF-1 α expression using immunohistochemistry with a standard immunoperoxidase technique. The final immunolabeling score was derived by multiplying the proportion and intensity scores.

RESULTS: The age range in our study sample varied greatly between 25 to 82 years, with the mean age being 55.4 $\pm$ 7.4 years. The gender distribution revealed 43 male patients (72%) and 17 female patients (28%). The highest number of cases involved the oral cavity (n=25), followed by the laryngeal region (n=23), the nasopharynx (n=8) and the head and neck skin (n=4). Results revealed that the highest frequency of cases was of intermediate grade (n=28), followed by low-grade tumor (n=17) and high-grade tumors (n=15). A total of 36 tumor showed positive HIF-1 α staining (60% of the cases). However, no significant associations were found between HIF-1 α expression and other clinicopathological parameters.

CONCLUSION: Our population recorded a significantly elevated expression of HIF-1 α in HNSCC patients. Therefore, HIF-1 α may be an invaluable marker for early diagnosis and a potential target for molecular therapy against HNSCC.

KEYWORDS: Squamous Cell Carcinoma of Head and Neck, Hypoxia-Inducible Factor 1 alpha, Subunit, Immunohistochemistry, Prognosis.

INTRODUCTION

Head and neck squamous cell carcinoma is one of the most prevalent cancers globally, especially in the Indo-Pak subcontinent. Head and neck carcinomas account for the second most common malignancies in Pakistan, following pulmonary cancer, with an incidence of 21% in males and 11% in females^[1], along with a very common practice of chewing betel nut^[2]. Hypoxia is a regular occurrence in malignant solid tumors that creates a favourable environment for the cancer cells to proliferate, infiltrate and invade the surrounding tissues. It also renders the tumour cells more resistant to radiation and chemotherapy^[3].

Intratumoral cellular hypoxia can cause an elevated expression of Hypoxia-inducible factor-1 alpha (HIF-1 α), an oxygen-dependent master transcriptional heterodimeric consisting of an alpha and beta subunit. The expression of HIF-1 α is an initial step in oral carcinomas. Research has shown that the nuclear HIF-1 α expression is negligible in normal epithelium, mild in dysplastic oral epithelium and shows a drastic increase in cells of Oral squamous cell carcinoma (OSCC)^[4]. It has been documented that high expression of HIF-1 α was linked with metastasis of lymphatics and peritumoral lymphangiogenesis^[5].

HIF-1 α is also reported to be correlated with T stage, histologic differentiation and micro-vessel density^[6].

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Thus, HIF-1 α may be a vital indicator of lymphatic regrowth and lymph node metastases in OSCC. Patients who expressed HIF-1 α staining revealed a drastically poor survival period [7]. Strangely, research shows HIF-1 α overexpression is associated with worse prognosis in the Asian population but not in patients of European descent [8].

Research directed towards hypoxia in Head and Neck Squamous Cell Carcinoma (HNSCC) to enhance the effectiveness of the treatment is the need of the hour. Tumors that express HIF-1 α are predicted to exhibit resistance towards radiation therapy due to their indication of tumor hypoxia and a higher turnover of genes that will promote the replication of malignant cells. On the contrary, inhibiting the activity of HIF-1 α could be beneficial in preventing cancer progression since it would deprive the developing tumors of their oxygen and nutrients, thereby starving them [9].

Studies show the variation in the expression of HIF 1- α among different populations [10]. However, very few studies have been conducted to explore the expression of HIF-1 α levels in the Pakistani population. Therefore, this study aims to measure the expression of Hypoxia-inducible factor-1 alpha (HIF-1 α) in patients with head and neck squamous cell carcinoma through immunohistochemistry.

METHODOLOGY

All procedures followed in this study were according to the ethical standards of the University of Health Sciences, Lahore, Pakistan, and the Ethical Review Committee under approval letter number UHS/REG-16/ERC/251. This descriptive study was conducted in the Department of Oral Pathology, University of Health Sciences (UHS), Lahore, Pakistan, from January to July 2020. The sample size was calculated to be 60 with a 95% confidence rate, and the sampling technique chosen was convenient non-probability sampling [11]. All previously confirmed cases of head and neck squamous cell malignancies of all ages and both genders reporting to the Department of Histopathology in Sheikh Zayed Hospital, Lahore, from 2011 to 2017 were taken as a study population, and 60 cases were withdrawn from it on a random basis. Relevant clinical data, including the patient's name, age, gender and site of the primary tumor were recorded. Pathological data with the tumor grades of differentiation were also retrieved. Previously diagnosed cases of all age groups and genders were included in the study. Patients who had undergone chemotherapy or radiotherapy before biopsy or surgery were excluded from the study. Any damaged blocks were also excluded from the sample.

Two slides were prepared from each block. One slide was kept for routine H&E staining, while the second one was designated for IHC processing, and universal staining techniques were followed for the procedure (Bancroft and Gamble) [12]. The primary antibody, monoclonal anti-HIF1 α antibody [IA3] (code ab113642; Abcam), was diluted (1:200) as per the manufacturer's suggestion before incubation at 90 °C for 90 minutes, followed by the application of Biotinylated Secondary antibody reagent. Then, the slides were covered with Streptavidin peroxidase

reagent for 30 minutes, and a detection kit, Peroxidase DAB, was used. Counterstaining with hematoxylin was also done.

HIF-1 α expression was evaluated semi-quantitatively by calculating the percentage of positively marked cells and the intensity of reactivity in the tumour cell cytoplasm with an immunoreactive score [11]. The final score for all the cases was obtained by multiplying the proportion score (PS) and intensity score (IS) (IRS, according to Remmele and Stegner) [11]. Proportion Score (PS) was taken as '0' when none of the tumor cells showed positive stain, '1' When 1-10% of the tumor cells were positively stained, '2' when 11-50% of the tumor cells were positively stained, '3' when 50-80% of the tumor cells were positively stained, '4' when 80% of the tumor cells were positively stained. Intensity Scores (IS) were measured as 0 (no staining), 1 (mild staining), 2 (moderate staining) and 3 (strong staining). Total Score (TS) was taken by multiplying both the scores (TS= PS x IS) as 0-2 for negative, 3-5 weak positive, 6-8 for moderate positive, and 9-12 strong positive [11].

Data was assessed by using SPSS Version 24.0. Mean and standard deviation were evaluated for age. Frequency and percentage were analyzed to determine the intensity of the marker's immunostaining. The association between HIF-1 α expression and the rest of the variables was evaluated using the chi-square test of independence and Fisher's Exact test, and $p < 0.05$ was marked as significant.

RESULTS

The mean age was 55.4 $\pm$ 14.5 years, ranging from 25 to 82 years. Gender distribution revealed 43 male (72%) and 17 (28%) female patients. The highest number of cases involved the oral cavity 25(42%), followed by larynx 23(38%), nasopharynx 8(13%) and lip 4 (7%). Results also showed that the highest frequency of cases was of intermediate grade (46.7%), followed by low grade (28.3%) and high grade (25%). 36 (60%) cases showed positive HIF-1 α staining. Among the HIF-1 α positive cases, the highest frequency showed moderate positivity (28.3%), closely followed by weak positive cases (23.3%). The results of HIF-1 α expression among our population are summarized in Table-I.

Table-I: Interpretation, frequency and percentage of HIF-1 α expression in the present study.

Expression Score	Interpretation	n(%)
Score 0-2	Negative	24(40.0)
Score 3-5	Weak positive	14(23.3)
Score 6-8	Moderate positive	17(28.3)
Score 9-12	Strong positive	5(8.3)

For statistical analysis purposes, some categories were pooled, where appropriate, to ensure that each group had a sufficient sample size for accurate analysis and statistical power. We pooled the data for age to categorize them into two groups (25-60 years & >60 years). Less frequent sites (the Nasopharynx and lip) were pooled together for the site. For intensity scores, no intensity and mild intensity scores were added up in a single category, as 'low intensity' and moderate intensity scores were combined with strong

intensity scores under the ‘high intensity’ category. Finally, to analyze the association of grades of tumours with final HIF-1 α expression, we combined the scores of negative expression with weak expression and moderate expression

with strong expression to get statistically non significant results. The association between HIF-1 α and other variables is summarized in the Tables below.

Table-II: Association between HIF-1 α and other clinicopathological parameters.

Variables	Categories	Negative HIF-1 α expression n(%)	Positive HIF-1 α expression n(%)	P-value
Age(years)	25-60	17(42.5)	23(57.5)	0.576
	≥ 61	7(35)	13(65)	
Gender	Male	17(39.5)	26(60.5)	0.906
	Female	7(41.2)	10(59.9)	
Primary site	Oral cavity	13 (52)	10(59.9)	0.272
	Lip+Nasopharynx	4(36.4)	8(63.6)	
	Larynx	7(30.4)	16(69.6)	
Grade	Grade 1(low)	36(60)	11(64.7)	0.802
	Grade2 (intermediate)	11(39.3)	17(60.0)	
	Grade 3(high)	7(46.7)	8(53.4)	

Table-III: Association between histological grade and intensity of HIF-1 α expression.

Histological Grade	Low intensity (No intensity + Mild) n(%)	High intensity (Moderate + Strong) n(%)	Total	P-value
Low Grade	8 (13.3)	9 (15.0)	17 (28.3)	0.29
Intermediate Grade	13 (21.6)	15 (25)	28 (46.6)	
High Grade	8 (13.3)	7 (11.6)	15 (25.1)	
Total	29 (48.3)	31 (51.6)	60 (100)	

Table-IV: Association of grade of tumor with proportion score and total score.

Proportion Score	Low Grade n(%)	Intermediate Grade n(%)	High Grade n(%)	Total n(%)	P-value
No staining	2(14.3)	8(57.1)	4(28.6)	14(100)	0.253*
1-10%	0 (0)	4 (100.0)	0 (0)	4(100)	
11-50%	7(43.8)	4(25.0)	5(31.3)	16(100)	
51-80%	4(25.0)	9(56.3)	3(18.8)	16(100)	
$\geq 80\%$	4(40.0)	3(30.0)	3(30.0)	10(100)	

*P-value is calculated using fishers exact test.

Table-V: Association of histological grades of tumor with HIF-1 α expression.

HIF-1 α Expression	Low Grade n(%)	Intermediate Grade n(%)	High Grade n(%)	Total score n(%)	P-value
Mild Expression (Negative + Weak HIF-1 α) Expression	11 (28.9)	17 (44.7)	10(26.3)	38 (100)	0.919
Strong Expression (Moderate + Strong HIF-1 α) Expression	6 (27.2)	11 (50.0)	5 (22.7)	22 (100)	
Grade-wise HIF-1 α Expression	17 (28.3)	28 (46.7)	15(25.0)	60 (100)	

DISCUSSION

The mean age of the present group was 55.4 years. Many local and international studies also show that the same age group is the most highly affected^[13]. This can be due to higher chances of mutations in cells in the older population, in addition to an inefficient DNA repair mechanism^[14]. The male prevalence observed in this study coincides with the trend shown by local studies^[8]. Various international studies have also reported male predominance of HNSCC cases^[4]. This might be due to increased usage of tobacco products or alcohol and more

chances of sun exposure due to the outdoor occupation of men^[15]. Site involvement in this study also showed similar results to local and international studies, where oral cavities are the most commonly involved regions^[4]. This can be due to very low standards of oral hygiene maintenance and the practice of tobacco/betel nut chewing, which places the oral cavity in direct contact with these carcinogens. Also, dental checkups are not mandatory in our country, and regular appointments are not enforced. Many pathologies are ignored in their early stages and are only addressed when they become serious. The most common tumor grade found in our study was moderately

differentiated tumor (MDSCC), which was in concordance with numerous local and international studies [4].

In contrast to these findings, some studies conducted in Pakistan established grade 1 (Well-differentiated tumor) as the most prevalent tumor grade [16]. This contradiction can be blamed on the differences in sample size, sample selection, and awareness of the sample population. Worldwide studies have reported erratic behaviour in the expression of HIF-1 α among different populations and even regions of the same population. A systematic review shows that the overall expression of HIF-1 α has shown immense inconsistency, ranging from 30% positivity to a full-blown 100% expression [17].

In this study, we analyzed the cells for cytoplasmic HIF-1 α staining. Our results showed positive staining in 60.0% of the cases, while 40% showed no staining. A Maximum number of cases (n=25) showed tumor cell staining of moderate intensity, while only 6 showed intense staining (10%). In his study, Eckert reported similar results, with only seven cases showing strong expression of HIF-1 α and 48.8% with moderate expression, but the inconsistency of results requires further studies [18]. The present research could not observe any significant association between age and HIF-1 α expression, as reported by Sumera et al. [8]. Several studies carried out in Japan, China, Germany, the USA, and Brazil showed that HIF-1 α expression had no link with the sex of patients [19]. Similar results were observed in our study. It was also observed that HIF-1 α expression was not contingent on the site affected by HNSCC. Similar results were reported by a local study [8].

On the other hand, some studies have reported a significant association between HIF-1 α expression and cancer localization, which could be due to different tumors subjected to different tumor microenvironments [20]. In our study, the association between tumor grade and HIF-1 α expression was found to be insignificant. This is in agreement with the local and international studies [8,20]. We gained similar results in the present study by achieving no significant link between high HIF-1 α expression and poor histological grading of the tumor. However, this finding conflicts with a few studies where a significant association between the two parameters was found [4].

These studies claim that strong or intense expression of HIF-1 α was correlated with a high cancer grade. Our sample size included only 60 cases, so generalization of the results as a representative of the target population might not be fair, and larger studies need to be conducted to obtain more accurate data.

CONCLUSION

The present study revealed 60% expression of HIF-1 α in HNSCC with zero expression in histologically normal cells. This expression was independent of the age, gender, size and grade of the tumour, making it an independent biomarker for HNSCC. Furthermore, there is an imperative need for future studies that should be carried out on a larger scale to extend these findings and find a correlation of HIF-1 α expression with tumor stage, treatment modalities and

patient outcome. A better understanding of the central role of HIF-1 α in carcinogenesis could offer promising new avenues to researchers and aid in clarifying numerous issues regarding using HIF-1 α as an anticancer therapeutic target.

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Authors' Contribution:

Syeda Sadaf Kazmi: Substantial contributions to the conception and design of the work.

Rabia Anjum: The acquisition and analysis of data for the work.

Waqar Ali: Interpretation of data for the work.

Aman ur Rahman: Drafting the work.

Zia Qazi: Reviewing it critically for important intellectual content.

Saima Chaudhry: Final approval of the version to be published.

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