ORIGINAL ARTICLE

The prognostic role of hypoxia and the microenvironmental acidity in chemo-radio resistance in oral squamous cell carcinoma patients

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Abstract. *Background:* In many tumors including oral cancer, hypoxia is a frequent occurrence that is linked to a poor prognosis, malignant transformation, and therapeutic resistance. Hypoxic tumor cells could increase the production of hypoxia-inducible factor 1alpha (HIF-1) which is a very important chemical mediator that enables tumor cells to react to hypoxia. Likewise, the vacuolar ATPase subunit C (ATPaseV1C1) has a role in regulating the acidity within the OSCC microenvironment. This study evaluated the expression of HIF-1α and ATPaseV1C1 concerning the clinicopathological characteristics, overall survival (OS), and the disease-free survival (DFS) of patients with OSCC. Methods: The retrospective study was conducted on 50 cases of OSCC. Immunohistochemical expression of both HIF-1α and ATPaseV1C1 was analyzed quantitatively and qualitatively. Clinico-pathologic correlations including different treatment modalities, response to treatment, and the 3-year-survival rates (OS/ DFS) were performed. Results: The tumor size was substantially correlated with high ATPaseV1C1 expression. (P=0.018), advanced stage IV (64.7%; P=0.004), histopathological high-grade tumors (41.2% of grade 2; P=0.021), presence of recurrence (94.1%; P=0.002) and the progressive response (76.5%; P<0.001) as well as decrease OS and DFS (1.73, 0.814 years respectively). While HIF-1α high expression was significantly associated with histopathologically high-grade tumors (50% of grade 2; P=0.007), the presence of recurrence (87.5%; P=0.01), the progressive response (68.8%; P=0.002) and decrease OS and DFS (1.81, 1.09 years respectively). Conclusions: Hypoxic and acidic microenvironment had an adverse impact on the prognostic parameters of OSCC. HIF-1α and V-ATPase immunohistochemical expression are associated with poor OS and DFS. Both markers could be employed as prognostic indicators in OSCC patients.

Keywords: Oral squamous cell carcinoma, HIF-1α, ATPaseV1C1, Micro-environment, Therapy, Prognosis.

Introduction

In the world, head and neck squamous cell carcinoma (HNSCC) is the seventh most prevalent cancer, with an estimated 890,000 new cases (or 4.5% of all cancer diagnoses worldwide) and 450,000 deaths annually (or 4.6% of all cancer deaths worldwide) (Sung et al., 2021). Despite the recent advancement in the eradication or control of OSCC and treatment modalities, resistance to chemotherapeutic drugs significantly reduced their efficacy and frequently resulted in treatment failure in these patients (Varun et al., 2020).

Therefore, it's important to identify suitable markers that could provide a prognostic assessment of the disease with the ability to predict and identify tumor responses to the treatment (Huma Khan, 2021).

Hypoxia is one of the key components that affect the cellular expression program and contribute to therapy resistance (Jing et al., 2019). Oxygen is typically reduced in solid tumors, including OSCC, because of irregular blood flow brought on by the aberrant tumor microvasculature. Hypoxia-inducible (HI) genes, which have functions related to pro-survival, anti-apoptosis, angiogenesis, DNA repair, and metabolic signaling pathways, can be activated by oxygen deprivation in hypoxic tumor cells. (Nijkamp et al., 2013). Cancer cells must be able to adapt to survive, whether the hypoxia is temporary or persistent. The main adaptive mechanism to such low tissue oxygen concentrations is the control of Hypoxia-Inducible Factor-1 (HIF-1) which is a heterodimeric transcriptional complex that regulates cellular and systemic oxygen homeostasis, can promote the transcription of over 60 genes when activated to prevent hypoxia-mediated cell death. (Cassavaugh and Lounsbury, 2011).

Vacuolar-type ATPases subunit C (ATPaseV1C1) are ATP-powered proton pumps that can be found in the membranes of intracellular structures like lysosomes, endosomes, and secretory vesicles. They do this by establishing the acidic milieu needed for acid-dependent proteases to break down proteins.(Pamarthy et al., 2018).

As the exact molecular mechanisms of chemoresistance are still not fully understood, further studies are needed urgently to develop tailored treatments for oral cancer, it is important to comprehend more precise and sensitive markers that can be used to help with tumor selection of a therapeutic approach and monitoring of therapeutic intervention response. So, the current study was maintained to assess the immunohistochemical expression of HIF-1α and ATPase V1C1 in OSCC cases and investigate their actual roles in the hypoxia and acidity of OSCC tumor microenvironment.

And its impact upon the clinical response of treatment as well as the survival analysis (OS and DFS) of OSCC patients.

2. Materials and Methods

2.1. Design and data collection

This retrospective cross-sectional study included 50 patients who had tumor masses surgically removed at the Oncology Centre, Mansoura University (OCMU), Faculty of Medicine, Mansoura University between January 2012, and December 2019. All patients had a confirmed histopathological diagnosis of OSCC.

Ethical approval was obtained before the study from the Ethics Committee of the Faculty of Dentistry, Mansoura University, by code no. M03040521. The 1964 Helsinki Declaration and its later amendments or equivalent ethical standards were followed during all procedures carried out for this investigation. These standards were also followed by institutional and national research committees.

From OCMU archives and computerized databases, clinical, gross pathology, and data laboratory that were pertinent were gathered. To review the microscopic pathology results, two pathologists re-evaluated the Hematoxylin and Eosin (H&E)-stained slides representative of tumor sections from the archives of Pathology Laboratories at OCMU. The Tumor-Node-Metastasis (TNM) approach was used for the staging. Table 1 displays the clinicopathological and laboratory information that was gathered. The inclusion criteria for OSCC cases were the accessibility of preserved paraffin tissue blocks from tumor excision samples and clinical, laboratory, and follow-up data (three years from the surgery date to the conclusion of the follow-up period or passing away). Patients who did not receive follow-up care or those who had preoperative therapy were not included in the study. The term "disease-free survival" (DFS) was used to describe the period between the date of surgery and the first confirmed radiological or pathological indication of tumor recurrence.

Table1. The clinicopathological information of the 50 oral squamous cell carcinomas

	Total number =50	**%**
Age /years mean± SD (Min-Max)	54.14±12.38 (25-76)	
Age group		
25-	11	22.0
45-	29	58.0
65-	10	20.0
Gender		
Male	25	50.0
Female	25	50.0
Site		
Tongue	24	48.0
Palate	3	6.0
Lip	9	18.0
Floor of mouth	2	4.0
Cheek	5	10.0
Alveolar margin	7	14.0
Size (T)		
T1	12	24.0
T2	20	40.0
T3	10	20.0
T4	8	16.0
Nodal metastasis (N)		
N0	36	72.0
N1	5	10.0
N2	8	16.0
N3	1	2.0

Continued

TNM clinical Stage		
I	9	18.0
II	13	26.0
III	11	22.0
IV	17	34.0
WHO grading system.		
Well	20	40.0
Moderate	24	48.0
Poorly	6	12.0
Neural invasion		
+ve	13	26.0
-ve	37	74.0
Lymph vascular		
invasion	5	10.0
+ve	45	90.0
-ve		
treatment types		
Chemotherapy	5	10.0
CCRTH	18	36.0
Radiotherapy	27	54.0
response		
progressive	19	38.0
Complete response	31	62.0

SD; standard deviation, %; percentage, CCRTH; concurrent chemo-radio therapy

2.2 Hematoxylin and eosin evaluation

For each paraffin block, five microns thick sections were cut and stained with hematoxylin and eosin stain to confirm the diagnosis of each case. The criteria of the latest WHO classification were used to grade the conventional SCC cases into well, moderately, and poorly differentiated SCCs. The current cases also were examined for the following pathological parameters; presence or absence of lymph vascular invasion and perineural invasion.

2.3 Immunohistochemical staining.

Two five-micron thick sections were cut from each paraffin block for immunostaining using the following primary antibodies: anti- HIF-1α (from Abclonal; Rabbit polyclonal antibody, Catalog No.: A16873, diluted at 1:100, anti-human IgG, Normal human kidney tissue is a positive control:) and anti-V-ATPase (from Fine Test; Rabbit polyclonal antibody, Catalog No.: FNab00719, diluted at 1:50, anti-human IgG, Human esophagus tissue is a positive control:). Then, The Bio GEMEX laboratory (4600 Caryon Road, San Ramon, UAS, 2011) provided sections mounted on Opti Plus slides.

To promote adhesion between the tissue sections and the slide surfaces, these slides are electrically charged. Using the Avidin-Biotin complex approach by hand and in accordance with the manufacturer's instructions, immunostaining was carried out.

2.4 Computer-assisted imaging

Examination with a light microscope [BX60, Olympus, Japan] under low magnification [×40] was done to determine hot spots of positive expression of HIF1α and AtpaseV1C1. Photomicrographs were captured by a digital camera [Olympus, C5060, Japan] mounted on the microscope. Images were then digitally manipulated by Microsoft office 2010 software for brightness and contrast on Intel [R] Core i5 computer system for further analysis.

2.5 Evaluation of the IHC staining

Two independent pathologists used a light microscope to semi-quantitatively evaluate the immunohistochemistry results. A score of 1 was given when fewer than 10% of tumor cells were stained positively, a score of 2 was given when between 10% and 50% of cells were stained positively, and a score of 3 was given when more than 50% of tumor cells were stained positively. Negative= 0, weak= 1, moderate= 2, and strong=3 was used to rate the strength of the signal. Both percentage and intensity scores were multiplied to classify the HIF-1α expression as negative (0 or 1), weak (2 to 6), or strong (more than 6) (Santos et al., 2012). The following criteria were used to score the results for ATPaseV1C1, the percentage of positive cells are as follows: (0=5%), (1=6–25%), (2=26–50%), (3=51–75%), and (4= >75%). The intensity (Yellow=1), (Brown=2), (Tan=3). Third, the integral of the cell positive rate times the staining intensity is: 0 = negative (-), 1-4 = weak positive (+), 5-8 = moderate positive (++), while 9–12 = strongly positive (+++) (Lu et al., 2013).

2.6. Statistical Analysis

The data were analyzed using Windows' Standard version 25 of the SPSS statistical software package. The first step was to perform a Kolmogorov-Smirnov test to see if the data was normal. The qualitative data were described in terms of numbers and percentages. The Fischer exact test was used when the projected cell count was less than 5, and the Chi-square test was used to evaluate relationships between categorical variables.

Continuous variables were presented as mean SD (standard deviation) for correctly distributed data and as median (min-max) for non-normally distributed data. For survival analysis, the Kaplan-Meier test was applied, and the Log-Rank test was employed to assess the statistical significance of changes between curves. The 5% level (P-value) is the set threshold of significance for all the statistical tests. The P value of 0.05 was used to determine whether the results are significant. The results are more significant the lower the P -value that was attained.

3.Results

3.1 Hematoxylin and eosin staining:

The hematoxylin and eosin-stained sections of the studied cases were examined and graded according to WHO classification into well-differentiated SCC (Fig1. A), moderately differentiated SCC (Fig1. B), and poorly differentiated SCC (Fig1. C). Cases of moderately differentiated SCC or grade II represented the highest percentage (n=24, 48%) followed by the well-differentiated SCC (n=20, 40%). The poorly differentiated SCC showed only 12% (n=6) among cases.

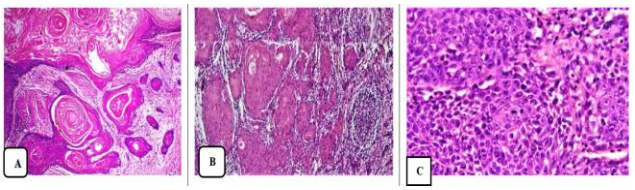

Figure 1. Hematoxylin and eosin sections for SCC. A Well-differentiated SCC with keratin pearls and epithelial Pearls X200. B Moderately differentiated SCC cases were characterized by the formation of abundant cell nests of polyhedral epithelial cells X200. C poorly differentiated SCC with prominent signs of malignancy and anaplastic epithelial cells X400.

3.2 Distribution of the studied cases according to immunohistochemical markers.

As regards ATPaseV1C1 immunohistochemical marker, there were 18 cases (36%) that showed a weak reaction, 15 cases (30%) with moderate reaction, and 17 cases (34%) with a strong reaction. On the other hand, most of the instances in the present study regards the HIF1α had a weak expression (n=34, 68%), whereas the remaining cases (n=16, 32%) had strong expressions (Table 2).

Table 2. Distribution of the studied cases according to ATPaseV1C1 and HIF1 α.

	Total number =50	%
V-ATPase		
Weak	18	36.0
Moderate	15	30.0
Strong	17	34.0
HIF		
Weak	34	68.0
Strong	16	32.0

3.3 Pattern of expression of the immunohistochemical markers in the studied cases of oral SCC.

Regards the ATPaseV1C1, the expression appeared with different pattern where there was a weak cytoplasmic expression of V-ATPase that localized mostly at the periphery of the tumor nest (Fig2.A) , moderate cytoplasmic expression of V-ATPase in case of moderate differentiated SCC in (Fig2.B), also a diffuse cytoplasmic strong expression (Fig2.C), the Cell nests of SCC showed expression of ATPase at the periphery of nests more than the center and some of these cells showed nuclear expression and cytoplasmic expression of ATPase (Fig2.D), the membranous reaction of the ATPase was also seen in some of the cases of SCC (Fig2.E) and (Fig2.F).

While the HIF1α immunohistochemical marker appears as a brown nuclear staining in all cases of SCC distributed as weak expression either focally (Fig3.A) or diffuse distribution (Fig3. B) or the expression was strong (Fig3.C).

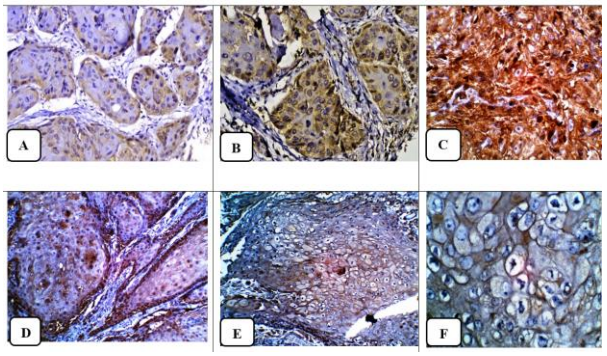

Figure 2. Immunocytochemical localization of ATPaseV1C1 protein in the OSCC A. Case of SCC with weak expression of V-ATPase mostly localized at the periphery of the tumor nests X200 B. Moderately differentiated SCC cases with diffuse moderate expression of V-ATPase X200 C. Poorly differentiated SCC with diffuse strong expression X400 D. Cell nests of SCC showed expression of ATPase at the periphery of nests more than the center and some cells showed nuclear expression X200 E. Membranous reaction of the ATPase X200 F. High power of cytoplasmic and membranous reaction of V-atpase X400.

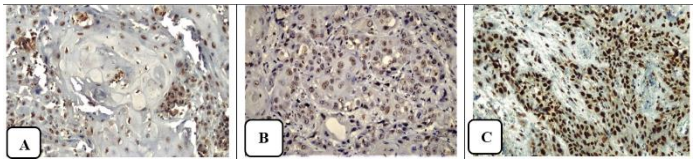

Figure 3. Immunocytochemical localization of HIF1α protein in the OSCC A. Case of well differentiated SCC showed focal weak expression of nuclear HIF1α X200 B. moderate differentiated SCC showed weak diffuse nuclear expression of HIF1α X200 C. Strong nuclear expression of HIF1α in case of poorly differentiated SCC X400.

3.4 Distribution of the studied cases according to tumor recurrence.

The recurrence was observed in 31 cases (62%) divided into local (n=14, 28%) which is the most common type followed by the nodal (n=10, 20%), and 7 cases (14%) showed mixed types of recurrence: local and nodal recurrence in four cases; distant and nodal recurrence in 2 cases; local, nodal, and distant recurrence one case (Table 3).

Table 3. Distribution of the studied cases according to tumor recurrence.

Recurrence Types		**Recurrence n=31,62%**			**No recurrence**
		N	%		
Local only		14	28%		
Nodal only		10	20%		
Mixed	Local and nodal	4	7	14%	n= 19 38%
	Nodal and distant	2			
	Local, nodal, and distant	1			

n; number, %; percentage

3.5. Associations between HIF-1α and V-ATPase immunohistochemical expression and the prognostic variables in OSSC.

High V-ATPase expression strongly correlated with tumor size (P=0.018) when compared to prognostic factors (Table 4). advanced stage IV (64.7%; P=0.004), histopathological high-grade tumors (41.2% of grade 2; P=0.021), presence of recurrence (94.1%; P=0.002) and the progressive response (76.5%; P<0.001).

While HIF1α high expression was significantly associated with high-grade tumors (50% of grade 2; P= 0.007), the presence of recurrence (87.5%; P=0.01), and the progressive response (68.8%; P=0.002).

3.6. Survival analysis

The Kaplan-Meier method was used in this study to plot the survival curves for the patients, and the Log-Rank test was used to evaluate differences between the curves. OSSC cases with either V-ATPase and HIF1 α strong expression had shorter median OS times (1.73 and 1.81 years, respectively) in comparison to individuals with SCC that express weakly HIF1 or A-ATPase (2.84 and 2.63 years, respectively).

The median DFS time was also shorter for patients with either V-ATPase and HIF1 α strong expression compared to patients with V-ATPase and HIF1α weak expression (0.814 vs 1.09 and 2.42 vs 2.04 years, respectively). But Kaplan-Meier survival analysis using Log-Rank (Mantel-Cox) demonstrated a statistically significant difference in shorter OS in poorly differentiated tumors (1.77 years, P=0.035), advanced stages (P<0.001), presence of recurrence (P <0.001), and progressive response (1.56 years, P <0.001). likely, a significant statistical difference in decreased DFS in either advanced tumor stages IV (0.985 years, P <0.001), using adjuvant chemotherapy (0.933, P =0.016), and progressive response (0.575 years, P <0.001). Cox regression analysis for predictors of OS and DFS among studied cases revealed that the tumors' response to therapy remains a predictive factor for OS (p=0.019), while the types of treatments used, and the tumors' responses persisted as separate prognostic variables in DFS (Table 5 and 6).

In the current study, V-ATPase expression was found in the cytoplasm in most cases. However, a membranous reaction was seen in some cases similar to the studies by (Garcia-Garcia et al., 2012). This may be attributed to the V-ATPase's structure, in which the V1 subunit resides in the cytoplasm of the cell but binds to the V0 subunit that exists in the cell membrane; the reaction is primarily cytoplasmic. The V 1 complex dissociates from the membrane during starvation or molts when glucose is removed in cases of tumors, but the membranous reaction appears due to some type of dysfunction of the normal cell's constituents, these constituents include the cytoskeleton (Vitavska et al., 2003) or due to changes in the gene that codes for the V-ATPase (Garcia-Garcia et al., 2012).

Table 4. shows associations between the prognostic factors in squamous cell carcinoma and the immunohistochemistry expression of V-ATPase and HIF1α.

	V-ATPase				**HIF1**		
	Weak n=18(%)	**Moderate n=15(%)**	**Strong n=17(%)**	**Test of significance**	**Weak n=34 (%)**	**Strong n=16(%)**	**Test of significance**
Age group							
25-	2(11.1)	3(20.0)	6(35.3)	χ^2=3.59	6(17.6)	5(31.2)	χ^2=1.193
45-	11(61.1)	9(60.0)	9(52.9)	p=0.463	21(61.8)	8(50)	p=0.551
65-	5(27.8)	3(20.0)	2(11.8)		7(20.6)	3(18.8)	
Gender							
Male	9(50)	7(46.7)	9(52.9)	χ^2=0.125	17(50)	8(50)	χ^2=0.0
Female	9(50)	8(53.3)	8(47.1)	p=0.939	17(50)	8(50)	p=1.0
Site							
Tongue	10(55.6)	7(46.7)	7(41.2)		18(52.9)	6(37.5)	
Palate	1(5.6)	1(6.7)	1(5.9)		1(2.9)	2(12.5)	
Lip	2(11.1)	4(26.7)	3(17.6)	χ^2=6.94	6(17.6)	3(18.8)	χ^2=3.67
Floor of mouth	1(5.6)	1(6.7)	0	p=0.731	2(5.9)	0	p=0.598
Cheek	3(16.7)	0	2(11.8)		3(8.8)	2(12.5)	
Alveolar margin	1(5.6)	2(13.3)	4(23.5)		4(11.8)	3(18.8)	
Tumor Size							
T1	4(22.2)	8(53.3)	0	**χ^2=15.33**	11(32.4)	1(6.2)	χ^2=4.77
T2	9(50)	3(20)	8(47.1)	**p=0.018***	11(32.4)	9(56.2)	p=0.189
T3	4(22.2)	2(13.3)	4(23.5)		7(20.6)	3(18.8)	
T4	1(5.6)	2(13.3)	5(29.4)		5(14.7)	3(18.8)	
Nodal metastasis	14(77.8)	11(73.3)	11(64.7)		26(76.5)	10(62.5)	
N0	3(16.7)	2(13.3)	0	χ^2=8.03	4(11.8)	1(6.2)	χ^2=3.94
N1	1(5.6)	2(13.3)	5(29.4)	p=0.236	4(11.8)	4(25)	p=0.268
N2	0	0	1(5.9)		0	1(6.2)	
N3							

Continued

TNM Stage							
I	3(16.7)	6(40.0)	0	**χ^2=18.84**	9(26.5)	0	χ^2=6.37
II	7(38.9)	2(13.3)	4(23.5)	**p=0.004***	8(23.5)	5(31.2)	p=0.095
III	6(33.3)	3(20.0)	2(11.8)		8(23.5)	3(18.8)	
IV	2(11.1)	4(26.7)	11(64.7)		9(26.5)	8(50)	
Recurrence							
no	11(61.1)	7(46.7)	1(5.9)	**χ^2=12.0**	17(50)	2(12.5)	**χ^2=6.49**
yes	7(38.9)	8(53.3)	16(94.1)	**p=0.002***	17(50)	14(87.5)	**p=0.01***
Treatment modalities							
Chemotherapy	1(5.6)	2(13.3)	2(11.8)	χ^2=3.23	4(11.8)	1(6.2)	χ^2=0.368
CCRTH	7(38.9)	3(20)	8(47.1)	p=0.520	12(35.3)	6(37.5)	p=0.832
Radiotherapy	10(55.6)	10(66.7)	7(41.2)		18(52.9)	9(56.2)	
Response							
progressive	3(16.7)	3(20)	13(76.5)	**χ^2=16.22**	8(23.5)	11(68.8)	**χ^2=9.44**
Complete	15(83.3)	12(80)	4(23.5)	**p<0.001***	26(76.5)	5(31.2)	**p=0.002***
WHO grading system.							
Well	11(61.1)	4(26.7)	5(29.4)	**χ^2=11.52**	17(50)	3(18.8)	**χ^2=994**
Moderate	7(38.9)	10(66.7)	7(41.2)	**p=0.021***	16(47.1)	8(50)	**p=0.007***
Poor	0	1(6.7)	5(29.4)		1(2.9)	5(31.2)	
Neural invasion							
-ve	16(88.9)	10(66.7)	11(64.7)	χ^2=3.26	26(76.5)	11(68.8)	χ^2=0.337
+ve	2(11.1)	5(33.3)	6(35.3)	p=0.196	8(23.5)	5(31.2)	p=0.562
Lymph vascular invasion							
-ve	18(100)	12(80)	15(88.2)	χ^2=3.73	32(94.1)	13(81.2)	χ^2=2.0
+ve	0	3(20)	2(11.8)	p=0.155	2(5.9)	3(18.8)	p=0.157

*Significant p≤0.05, X^2, Chi square test, -ve; negative, +ve; positive, n; number, CCRTH; Concurrent chemo-radio therapy, V-ATPase; vacuolar ATPase, HIF1α; hypoxia inducible factor1α

Table 5. Factors affecting OS and DFS in 50 SCC patients

Overall survival			
	Median (95% CI)	Log-rank χ^2	p-value
Age group(years) 25- 45- 65-	 2.21(1.63-2.78) 2.35(2.05-2.65) 2.58(2.07-3.1)	1.58	0.454
Gender Male Female	 2.46(2.15-2.78) 2.27(1.89-2.64)	0.064	0.800
WHO grading system. Poor Moderate Well	 1.77(0.995-2.54) 2.24(1.87-2.59) 2.7(2.42-2.98)	6.71	**0.035***
Neural invasion -ve +ve	 2.38(2.09-2.66) 2.33(1.87-2.79)	0.169	0.681
Lymph vascular invasion -ve +ve	 2.42(2.18-2.66) 1.85(0.916-2.78)	1.58	0.209
TNM Stage I II III IV	 no statistics computed#	22.88	**<0.001***
Recurrence no yes	no statistics computed#	18.74	**<0.001***

Continued

Treatment types			
Chemotherapy	1.79(0.858-2.72)	2.10	0.350
CCRTH	2.27(1.83-2.71)		
Radiotherapy	2.54(2.27-2.80)		
Response to therapy			
Progressive	1.56(1.18-1.93)	33.38	**<0.001***
Complete response	2.86(2.73-2.99)		
HIF1α			
Weak	2.63(2.38-2.87)	13.58	**<0.001***
Strong	1.81(1.37-2.25)		
V-ATPase			
Weak	2.84(2.67-3.01)	14.46	**0.001***
Moderate	2.52(2.12-2.92)		
Strong	1.73(1.28-2.17)		
Disease free survival			
Age group (years)			
25-	1.44(0.72-2.17)	5.89	0.053
45-	1.60(1.22-1.99)		
65-	2.45(1.77-3.13)		
Gender			
Male	1.67(1.17-2.17)	0.045	0.833
Female	1.81(1.37-2.25)		
WHO grading system.			
Poor	1.06(0.269-1.84)	4.13	0.127
Moderate	1.60(1.13-2.08)		
Well	2.11(1.61-2.61)		
Neural invasion		0.154	0.694
-ve	1.81(1.44-2.18)		
+ve	1.53(0.887-2.18)		

Continued

Lymph vascular invasion		3.64	0.056
-ve	1.83(1.49-2.17)		
+ve	0.917(0.002-1.84)		
TNM Stage			
I	2.49(1.89-3.08)	12.39	**0.006***
II	1.87(1.14-2.59)		
III	2.14(1.49-2.78)		
IV	0.985(0.581-1.39)		
treatment types			
Chemotherapy	0.933(0.002-1.99)	8.23	**0.016***
CCRTH	1.95(1.40-2.51)		
Radiotherapy	1.74(1.33-2.16)		
response			
progressive	0.575(0.445-0.704)	47.37	**<0.001***
Complete response	2.45(2.13-2.77)		
HIF1α			
Weak	2.04(1.66-2.43)	9.21	**0.002***
Strong	1.09(0.595-1.59)		
V-ATPase			
Weak	2.42(1.94-2.89)	20.66	**<0.001***
Moderate	1.97(1.39-2.54)		
Strong	0.814(0.496-1.13)		

X^2, Chi-square test, CI: Confidence interval, *significant p≤0.05; # If one or more categories are censored, -ve; negative, +ve; positive, OS; overall survival, n; number, DFS; disease-free survival, CCRTH; Concurrent chemo-radio therapy, V-ATPase; vacuolar ATPase, HIF1α; hypoxia-inducible factor1α

Table 6. Cox regression for predictors of OS and DFS among studied cases

Overall survival			
	B	**p-value**	**Hazard ratio (95%CI)**
WHO grading system.			
Poor (r)			
Moderate	r		1
Well	0.527	0.417	1.69(0.474-6.06)
	0.003	0.997	1.003(0.169-5.94)
TNM Stage			
I (r)	r		
II	8.15	0.935	Undefined
III	9.39	0.926	Undefined
IV	9.94	0.921	Undefined
Recurrence			
No (r)			1
Yes	10.11	0.897	24.56(0.002-36.58)
response			
progressive	1.48	**0.019***	4.39(1.27-15.17)
Complete response (r)			1
HIF1α			
Weak (r)	0.333	0.552	1
Strong			1.39(0.466-4.18)
V-ATPase			
Weak (r)	r		1
Moderate	0.100	0.905	1.105(0.214-5.71)
Strong	0.167	0.824	1.18(0.270-5.18)

Continued

Disease free survival			
TNM Stage			
I (r)			1
II	0.481	0.540	1.62(0.346-7.56)
III	0.316	0.692	1.37(0.287-6.55)
IV	0.813	0.323	2.25(0.450-11.29)
treatment types			
Chemotherapy	1.68	**.01***	5.42(1.48-19.88)
CCRTH	-1.25	**0.017***	0.287(0.103-.796)
Radiotherapy (r)			1
response			
progressive	3.34	**<0.001***	28.19(6.01-132.26)
Complete response (r)			1
V-ATPase			
Weak (r)			1
Moderate	0.717	0.214	5.42(1.48-19.88)
Strong	0.557	0.385	0..287(0.103-0.796)

X^2, Chi-square test, CI: Confidence interval, *significant p≤0.05, # If one or more categories are censored, r: reference group, -ve negative, +ve; positive, OS; overall survival, DFS; disease-free survival, n; number, CCRTH; Concurrent chemo-radio therapy, V-ATPase; vacuolar ATPase, HIF1α; hypoxia-inducible factor1α

In this work, there was a significant association between the V-ATPase expression and TNM stage where the expression rises with the progression of the stage, comparable to the research of (Huang et al., 2012) who discovered that the level of ATP6 V1C1 expression increased as tumor stage progressed, which is consistent with the notion that tumors with lower levels of differentiation have lower pH values, which forces cells to produce larger levels of V-ATPase to live in the acidic milieu. microenvironment (Pérez-Sayáns et al., 2010).

We had a significant association between the V-ATPase expression and the recurrence of OSCCs. With the strong expression of the V-ATPase, there was a great chance for recurrence and metastasis of the tumors similar to the study of (Cotter et al., 2015) that demonstrated a higher expression of V-ATPase in proliferating cancer cells of breast, lung, prostate, liver, ovarian, melanoma, pancreatic, and esophageal cancers. (Sennoune et al., 2004) verified the relationship between higher plasma membrane V-ATPase expression and higher invasiveness and metastatic capacity of breast cancer cell lines and (Huang et al., 2012) detected a much greater expression of V-ATPase which was higher in the lymphatic metastasis group than it was in the non-lymphatic metastatic group.

This might be accounted for by the fact that there is a large amount of lysosomal enzyme release from tumor cells that contributes to the destruction of the extracellular matrix required for metastatic invasion. These enzymes' optimum pH is low, and V-ATPases are in charge of making the microenvironment more acidic (Nishi and Forgac, 2002). Cells must develop motility and an invasive phenotype to be metastatically competent (Sennoune et al., 2007). Reducing extracellular pH in the tumor microenvironment increases tumor cell motility by causing the development of pseudopodia, which promotes more invasive cell movement. Additionally, extracellular acidification in metastatic cells causes a rise in the quantity and size of pseudopodia, which extend in the direction of the capillary capillaries, in which the tumor cells move (Condeelis and Segall, 2003).

A statistically significant correlation existed between the V-ATPase expression and the tumor cells' response to the treatment; the strong expression of the V-ATPase was associated with the tumor cells' progression, whereas the weak expression was associated with the tissues' full recovery. This may be because OSCC's extracellular pH is significantly more acidic than that of normal tissue, which may explain the association and this acidity disrupts the absorption of chemotherapeutic drugs (Pérez-Sayáns et al., 2009). V-ATPases are responsible for mediating these pH shifts, and it has been discovered that cells that express large quantities of the C subunit are more resistant to chemotherapy. This study is according to the research of (Kulshrestha et al., 2015) that discovered the use of V-ATPase inhibitors is an effective method for treating chemo-resistant ovarian cancer.

In the current work, HIF 1α-positive tumor tissues revealed nuclear immunostaining, and this nuclear expression is due to the dimerization of the HIF1α with the HIF1ß in the nucleus to form HIF1. There was a cytoplasmic expression in some cases of the current study, but this cytoplasmic staining is a relatively uncommon occurrence and had no bearing on the statistical outcomes following (Beasley et al., 2002).

This cytoplasmic staining may be caused by the inhibition of prolyl hydroxylases during hypoxia, which causes an accumulation of HIF1 in the cytoplasm of the cell; that is similar to the study of (Raju et al., 2021). There was a statistically significant relationship between HIF expression and the recurrence of OSCCs. This relationship was similar to the study of (González-González et al., 2021) who revealed that the overexpression of HIF1α in cancer tissues was associated with recurrence. This is because the HIF and hypoxia promote epithelial-mesenchymal transition (EMT) by interacting with EMT regulators such as Slug, Snail, β-catenin, and TWIST and by enhancing the Wnt/β-catenin signaling pathways and NF-κB in hypoxia leading to degradation of local basement membranes and loosening of cell-cell junctions thus accelerating the migration, invasion, and recurrence. In the current study, there was a statistically significant association between the expression of HIF and the tumor cells' response to the treatment; a strong expression of HIF1α is predicted to indicate that the tumor will not respond to the treatment and will progress, whereas a weak expression indicates that the tumor will completely respond to the treatment (Sowa et al., 2017) and this could be due to activation of the ABC transporter protein, a drug efflux pump that reduces the concentration of chemotherapy medicines inside cancer cells in the hypoxic tumor microenvironment leading to chemoresistance. Additional HIF-related mechanisms of resistance include a reduction in cell metabolism, suppression of senescence, and an increase in apoptosis (Fallah and Rini, 2019). The present study confirmed an association between the median OS and DFS with the V--ATPase expression in OSCC where the mortality rate increased with the strong expression of the marker similar to the study of (Song et al., 2017) and this could be due to poor perfusion of the solid tumors that have a dense tumor tissue (Robova et al., 2013, Pc Yee et al., 2013). For instance, in hypoxic tumors, where the extracellular space is ineffectively cleared of protons and lactic acid, an acidic extracellular milieu is created. (Reshkin et al., 2014) Proton pumps, including V-ATPase, are therefore overexpressed in the bulky hypoxic tumor's acidic microenvironment to make up for the cytoplasm's alkalization. (Boedtkjer et al., 2012).

In the current study, the OS and DFS were significantly associated with the HIF1α expression, where the high levels of marker's expression result in a decline in the median OS, and this accords to several studies (Sumera et al., 2023), as they showed that HIF-1 overexpression in OSCCs has prognostic importance because the HIF-1 is one of the most significant transcription factors in mediating cellular adaptation to hypoxia and has been associated with poor prognosis in a variety of tumors because it correlated with an advanced TNM stage, nodal involvement, increased tumor size, tumor recurrence, and a poor prognosis. (Shamis et al., 2021) but on the other hand, (Fillies et al., 2005) revealed no association between the HIF1 α and the OS and DFS.

Conclusion

The development of tumorous aggression and metastasis is facilitated by the presence of hypoxia and acidity, two prevalent microenvironmental variables, in OSCC. As a result, OSCC's expression of HIF-1α and V-ATPase is anticipated to be very valuable in predicting the disease's prognosis and may offer crucial information for a focused therapy plan. Additionally, it may be inferred that HIF-1α and V-ATPase could be employed as prognostic indicators in OSCC patients.

Conflict of Interest Statement: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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References

1. Beasley, N. J., Leek, R., Alam, M., Turley, H., Cox, G. J., Gatter, K., Millard, P., Fuggle, S. & Harris, A. L. J. C. R. 2002. Hypoxia-inducible factors HIF-1α and HIF-2α in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. 62, 2493-2497.
2. Boedtkjer, E., Bunch, L. & F Pedersen, S. J. C. P. D. 2012. Physiology, pharmacology and pathophysiology of the pH regulatory transport proteins NHE1 and NBCn1: similarities, differences, and implications for cancer therapy. 18, 1345-1371.
3. Cassavaugh, J. & Lounsbury, K. M. J. J. O. C. B. 2011. Hypoxia-mediated biological control. 112, 735-744.
4. Condeelis, J. & Segall, J. E. J. N. R. C. 2003. Intravital imaging of cell movement in tumours. 3, 921-930.
5. Cotter, K., Stransky, L., Mcguire, C. & Forgac, M. J. T. I. B. S. 2015. Recent insights into the structure, regulation, and function of the V-ATPases. 40, 611-622.
6. Fallah, J. & Rini, B. I. 2019. HIF Inhibitors: Status of Current Clinical Development. Current Oncology Reports, 21, 6.
7. Fillies, T., Werkmeister, R., Van Diest, P. J., Brandt, B., Joos, U. & Buerger, H. J. B. C. 2005. HIF1-alpha overexpression indicates a good prognosis in early stage squamous cell carcinomas of the oral floor. 5, 1-9.
8. Garcia-Garcia, A., Pérez-Sayáns, M., Rodriguez, M., Antúnez-López, J., Barros-Angueira, F., Somoza-Martín, M., Gándara-Rey, J., Aguirre-Urízar, J. J. B. & Histochemistry 2012. Immunohistochemical localization of C1 subunit of V-ATPase (ATPase C1) in oral squamous cell cancer and normal oral mucosa. 87, 133-139.
9. González-González, R., Ortiz-Sarabia, G., Molina-Frechero, N., Salas-Pacheco, J. M., Salas-Pacheco, S. M., Lavalle-Carrasco, J., López-Verdín, S., Tremillo-Maldonado, O. & Bologna-Molina, R. J. C. 2021. Epithelial–Mesenchymal Transition Associated with Head and Neck Squamous Cell Carcinomas: A Review. 13, 3027.
10. Huang, L., Lu, Q., Han, Y., Li, Z., Zhang, Z. & Li, X. J. D. P. 2012. ABCG2/V-ATPase was associated with the drug resistance and tumor metastasis of esophageal squamous cancer cells. 7, 1-7.
11. Huma Khan, S. G., Nuzhat Husain, Sanjeev Misra, Navin Singh, M.P.S. Negi 2021. Prognostics factors for evaluation of chemoradiation response in oral squamous cell carcinoma patients. cancer journal.net.
12. Jing, X., Yang, F., Shao, C., Wei, K., Xie, M., Shen, H. & Shu, Y. 2019. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. Molecular Cancer, 18, 157.
13. Kulshrestha, A., Katara, G. K., Ibrahim, S., Pamarthy, S., Jaiswal, M. K., Sachs, A. G. & Beaman, K. D. J. O. 2015. Vacuolar ATPase 'a2'isoform exhibits distinct cell surface accumulation and modulates matrix metalloproteinase activity in ovarian cancer. 6, 3797.

14. Lu, Q., Lu, S., Huang, L., Wang, T., Wan, Y., Zhou, C. X., Zhang, C., Zhang, Z. & Li, X. J. D. P. 2013. The expression of V-ATPase is associated with drug resistance and pathology of non-small-cell lung cancer. 8, 1-7.
15. Nijkamp, M. M., Span, P. N., Bussink, J. & Kaanders, J. H. a. M. 2013. Interaction of EGFR with the tumour microenvironment: Implications for radiation treatment. Radiotherapy and Oncology, 108, 17-23.
16. Pamarthy, S., Kulshrestha, A., Katara, G. K. & Beaman, K. D. J. M. C. 2018. The curious case of vacuolar ATPase: regulation of signaling pathways. 17, 1-9.
17. Pc Yee, G., De Souza, P. & M Khachigian, L. J. C. C. D. T. 2013. Current and potential treatments for cervical cancer. 13, 205-220.
18. Pérez-Sayáns, M., García-García, A., Reboiras-López, M. D. & Gándara-Vila, P. J. I. J. O. O. 2009. Role of V-ATPases in solid tumors: importance of the subunit C. 34, 1513-1520.
19. Pérez-Sayáns, M., Reboiras-López, M. D., Somoza-Martín, J. M., Barros-Angueira, F., Diz, P. G., Gándara Rey, J. M. & García-García, A. 2010. Measurement of ATP6V1C1 expression in brush cytology samples as a diagnostic and prognostic marker in oral squamous cell carcinoma. Cancer Biology & Therapy, 9, 1057-1064.
20. Raju, P. R., Raj Kumar, M. D. N. G., Nagaraj Anand, M. J. E. J. O. M. & Medicine, C. 2021. An Immunohistochemical assessment of Hypoxia Inducing Factor 1 alfa expression in Oral Epithelial Dysplasias and Oral Squamous Cell Carcinomas. 8, 2021.
21. Reshkin, S. J., Greco, M. R. & Cardone, R. a. J. P. T. O. T. R. S. B. B. S. 2014. Role of pHi, and proton transporters in oncogene-driven neoplastic transformation. 369, 20130100.
22. Robova, H., Rob, L., Halaska, M. J., Pluta, M., Skapa, P., Strnad, P., Lisy, J. & Komar, M. J. G. O. 2013. High-dose density neoadjuvant chemotherapy in bulky IB cervical cancer. 128, 49-53.
23. Santos, M. D., Mercante, A. M. D. C., Louro, I. D., Goncalves, A. J., Carvalho, M. B. D., Da Silva, E. H. T. & Da Silva, A. M. Á. 2012. HIF1-alpha expression predicts survival of patients with squamous cell carcinoma of the oral cavity.
24. Sennoune, S. R., Bakunts, K., Martínez, G. M., Chua-Tuan, J. L., Kebir, Y., Attaya, M. N. & Martínez-Zaguilán, R. J. a. J. O. P.-C. P. 2004. Vacuolar H+-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. 286, C1443-C1452.
25. Sennoune, S. R., Martinez-Zaguilan, R. J. J. O. B. & Biomembranes 2007. Plasmalemmal vacuolar H+-ATPases in angiogenesis, diabetes and cancer. 39, 427-433.
26. Shamis, S. a. K., Mcmillan, D. C. & Edwards, J. 2021. The relationship between hypoxia-inducible factor 1α (HIF-1α) and patient survival in breast cancer: Systematic review and meta-analysis. Critical Reviews in Oncology/Hematology, 159, 103231.
27. Song, T., Jeon, H.-K., Hong, J. E., Choi, J.-J., Kim, T.-J., Choi, C. H., Bae, D.-S., Kim, B.-G., Lee, J.-W. J. C. R. & Association, T. O. J. O. K. C. 2017. Proton pump inhibition enhances the cytotoxicity of paclitaxel in cervical cancer. 49, 595-606.
28. Sowa, T., Menju, T., Chen-Yoshikawa, T. F., Takahashi, K., Nishikawa, S., Nakanishi, T., Shikuma, K., Motoyama, H., Hijiya, K. & Aoyama, A. J. C. M. 2017. Hypoxia-inducible factor 1 promotes chemoresistance of lung cancer by inducing carbonic anhydrase IX expression. 6, 288-297.
29. Sumera, S., Ali, A., Yousafzai, Y. M., Durrani, Z., Alorini, M., Aleem, B. & Zahir, R. J. D. 2023. Overexpression of Hypoxia-Inducible Factor-1α and Its Relation with Aggressiveness and Grade of Oral Squamous Cell Carcinoma. 13, 451.
30. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. J. C. a. C. J. F. C. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. 71, 209-249.
31. Varun, B., Jayanthi, P., Ramani, P. J. J. O. O. & Jomfp, M. P. 2020. Cancer stem cells: A comprehensive review on identification and therapeutic implications. 24, 190.
32. Vitavska, O., Wieczorek, H. & Merzendorfer, H. J. J. O. B. C. 2003. A novel role for subunit C in mediating binding of the H+-V-ATPase to the actin cytoskeleton. 278, 18499-18505.

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