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Original Article

Assessment of glutathione peroxidase 1 and 4 expression in oral squamous cell carcinoma patients in a tertiary care center: A comparative cross-sectional study

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الملخص

أهداف البحث: "جي بي اكس ١" و "جي بي اكس ٤" إنزيمات مضادة للأكسدة، تتواجد بكثرة في سيتوسول أنسجة الثدييات. تتمتع هذه الإنزيمات بالقدرة على التخلص من الجذور الحرة، وتلعب دورا في أنواع مختلفة من السرطان، وخاصة سرطان الخلايا الحرشفية الفموي.

طريقة البحث: أولاً: تقييم التعبير الجيني لكل من "جي بي اكس ١" و "جي بي اكس ٤" ومقارنته بالمراحل المرضية للورم الأولي لسرطان الخلايا الحرشفية الفموي. ثانياً: تقييم ارتباط التعبير الجيني لكل من "جي بي اكس ١" و "جي بي اكس ٤" بمتغيرات مستقلة أخرى (العمر، والجنس، وموقع الآفة، والجانبية، والدرجات النسيجية المرضية، وإصابة العقد الليمفاوية في سرطان الخلايا الحرشفية الفموي). تألفت الدراسة من ١٣٣ عينة نسيجية مستقبلية لسرطان الخلايا الحرشفية الفموي، تم اختيارها من مختبر داو للأبحاث التشخيصية والإحالة التابع لجامعة داو. تم تقييم التعبير الجيني لكل من "جي بي اكس ١" و "جي بي اكس ٤" باستخدام تقنية الكيمياء المناعية. لاحقا، تم إجراء التقييم المناعي التفاعلي باستخدام برنامج "اميج جيه". ثم قورنت درجات التقييم المناعي التفاعلي لجين "جي بي اكس ١" و "جي بي اكس ٤" وفقا للمراحل المرضية للسرطان ومع متغيرات مستقلة أخرى.

النتائج: من بين ١٣٣عينة، وجد أن "جي بي اكس ٤" كان موجودا في جميع مراحل سرطان الفم، وأظهر في الغالب درجة تقييم مناعية عالية تتوافق مع شدة تلطيخ متوسطة إلى قوية. بينما لم يعثر على "جي بي اكس ١" في جميع مراحل سرطان الفم، أظهرت معظم الحالات درجة تقييم مناعية منخفضة تتوافق مع شدة تلطيخ ضعيفة. ولكن لم يتم العثور على أي علاقة مع معايير أخرى.

الاستنتاجات: كان "جي بي اكس ٤" موجودا في جميع مراحل سرطان الفم وفي جميع عينات الأنسجة، بينما لم يعثر على "جي بي اكس ١" في جميع الحالات. كان لدرجات التقييم المناعي التفاعلي لجين "جي بي اكس ١" و "جي بي اكس ٤" علاقة مهمة بالمرحلة المرضية لسرطان الفم.

الكلمات المفتاحية: جسم مضاد؛ إنزيم مضاد للأكسدة؛ الجلوتاثيون بيروكسيديز؛ الكيمياء المناعية النسيجية

Abstract

Background: Glutathione peroxidase 1 (GPX1) and GPX4 are abundant antioxidant enzymes within the cytosol in mammalian tissues. These enzymes have the capacity to scavenge free radicals, and they play roles in various cancers, especially oral squamous cell carcinoma (OSCC).

Objectives: 1. To compare the expression levels of GPX1 and GPX4 among different pathological stages for OSCC primary tumors. 2. To assess the associations of GPX1 and GPX4 expression with other independent variables (age, gender, site of lesion, laterality, histopathological grades, and lymph node involvement of OSCC tumor).

Methods: In total, 133 prospective OSCC tissue specimens were selected from Dow Diagnostic Research and

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Referral Laboratory of Dow University. Expression levels of GPX1 and GPX4 were evaluated by immunohistochemistry. Immunoreactive score (IRS) values were obtained using ImageJ software. The IRS values for GPX1 and GPX4 were compared according to the pathological stages of cancer and other independent variables. Data were statistically analyzed by using SPSS version 21 and STATA.

Results: Among the 133 samples, GPX4 was present in all stages of oral cancer and high IRS values mostly corresponded to moderate to strong staining intensity. By contrast, GPX1 was not found in all stages of oral cancer, and low IRS values mostly corresponded to weak staining intensity. No relationships were found with other variables.

Conclusion: GPX4 was present in all stages of oral cancer and in all tissue samples, whereas GPX1 was not found in all cases. The IRS values for GPX1 and GPX4 had significant relationships with pathological stages of oral cancer.

Keywords: Antibody; Antioxidant enzyme; Glutathione peroxidase; Immunohistochemistry

Introduction

Cancer is the one of the most frequent causes of death, and oral carcinoma is the sixth most common type in humans.[1] In particular, oral carcinoma is very common in South Asian countries such as India, Pakistan, Sri Lanka, and Bangladesh.[2] According to GLOBOCAN 2020, there are 377,173 new cases of oral carcinoma every year and they account for 177,757 deaths worldwide.[3] About 90 % of oral malignancies are due to oral squamous cell carcinoma (OSCC), which has a 50 % mortality rate of around five years.[4] OSCC is one of the most aggressive tumors of the head and neck region.[5]

Cancer development is a multistep process characterized by uncontrolled cell growth and division,[6] and it is divided into three phases. The first is the initiation phase when oxidative stress induced DNA damage is sustained. The second is the promotion phase when clonal expansion of previously altered cells and inhibition of apoptosis occur. The third is the progression phase when the generation of large amounts of reactive oxygen species (ROS) contributes to mutations, inhibition of antiproteases, upregulation of matrix metalloproteinases, and progression of local tissue injury.[7] Reactive and non-reactive radicals are collectively known as ROS, and they have significant roles in the development of cancer.[7] ROS molecules have one or more unbound electrons, which make them highly active. ROS are produced endogenously by the mitochondrial respiratory chain, and they have beneficial as well as deleterious effects on living beings, although their harmful effects are initially counteracted by antioxidant enzymes.[8] An imbalance between oxidant and antioxidant levels leads to oxidative stress, which eventually causes DNA modification and damage to cellular macromolecules, eventually resulting in the development of cancer.[7]

Glutathione peroxidase (GPX) is an abundant antioxidant enzyme within the cytosol that catalyzes the reduction of peroxide radicals to alcohols and oxygen, as well as reducing hydrogen peroxide to water and oxygen, and thus it has the capacity to scavenge free radicals.[9] There are eight different isotypes of GPX (GPX1−8), where GPX1 is present in higher quantities within the cytosol of nearly all mammalian tissues including humans, and GPX4 is also present in the cytosol of the membrane fraction. GPX is known to have an association with OSCC, and high GPX expression is considered a useful prognostic biomarker for OSCC patients. The levels of GPX1 and GPX4 fluctuate as the tumor grade and stage progress, and these fluctuating levels can be used as prognostic biomarkers to help predict the disease prognosis and facilitate treatment planning.[10]

In the present cross-sectional study, we investigated the associations of GPX1 and GPX4 with OSCC. Very few studies have assessed the associations of GPX1 and GPX4 expression with OSCC, so this type of investigation is necessary as OSCC is among the most prevalent cancers. We hypothesized that the levels of GPX1 and GPX4 would increase with the progress of the pathological staging of primary tumors. We examined paraffin-embedded tissue specimens because most previous studies focused on the levels of GPX1 and GPX4 in blood, serum, and saliva. Moreover, no previous comparative study has been conducted in Pakistan to assess GPX1 and GPX4 expression levels in OSCC patients.

Materials and Methods

This comparative cross-sectional study was conducted in the histopathology department of Dow Diagnostic Research and Reference Laboratory, Dow University of Health Sciences. We included all known cases of excisional biopsy of OSCC cases confirmed after histopathological evaluation of OSCC, regardless of gender, age, histological grade, and pathological stage. Formalin fixed, paraffin-embedded tissue blocks containing at least 70 % or more tumor tissue were included in our study for staging, grading, and immunohistochemical analysis. However, we excluded recurrent cases or those who had already received radiotherapy and chemotherapy treatment, tumor slides containing less than 70 % tumor tissue, inadequately fixed slides, and degraded or autolyzed tissue. This study was conducted after Institutional Review Board (IRB) approval from Dow University of Health Sciences (**IRB Number: IRB-2472/DUHS/Approval/2022 /847**).

In total, 133 tumor samples comprising 45 well differentiated tumors, 48 moderately differentiated tumors, and 40 poorly differentiated tumors were obtained using the purposive sampling technique. Specimens were fixed immediately in 10 % neutral buffered formalin. Verbal consent was obtained from the patients by contacting them via the phone number mentioned on the request card. Subsequently, patients signed consent forms when they came back to collect their reports. The study was completed over a period of 14 months from January 2022 to February 2023.

Microscopic evaluation of pathological stages was conducted after gross assessment. Full thickness sections of tumors were selected and GPX1 and GPX4 antibodies were applied using the immunohistochemistry technique. Immunohistochemistry slides were evaluated by a semi-quantitative method to check the staining intensity and percentage of positive cells according to negative, mild, moderate, and strong categories. The immunoreactive score (IRS) values were calculated to confirm the expression levels of GPX1 and GPX4. Data collation and analyses of results were performed using SPSS version 21.0 and STATA.

The primary outcome variable was GPX1 or GPX4 IRS value categorized as negative, weak, moderate, or strong. The main predictor variable was the pathological stage (TNM I to IV). Other independent variables (covariates) included age, gender, province, city, site of lesion, laterality, lymph node involvement, and histopathological grades of OSCC tumor (G1, G2, and G3).

Means and standard deviations were calculated for continuous variables such as age (years), whereas categorical variables were represented as frequencies and proportions. GPX1 and GPX4 IRS values were assigned to four categories (0−1, negative; 2−3, mild; 4−8, moderate; and 9−12, strong) for analysis.[12] Chi square tests were conducted to assess the associations of GPX1 and GPX4 IRS values with other categorical variables (gender, laterality, and lymph node involvement). In cases with expected frequencies less than five, Fisher's exact tests were performed for variables such as site of lesion, histopathological grade, and tumor staging. All tests were two-sided and a p-value <0.05 was considered to indicate a significant difference.

Tissue sampling protocol

Hematoxylin and eosin (H&E) stained slides were evaluated to determine the histological grade and stage for tumor tissues. Expression levels of GPX1 and GPX4 were assessed by immunohistochemistry. The principal investigator performed the initial assessments of slides to evaluate the staining intensity score. The slides were re-evaluated by a consultant histopathologist. The percentage of positive cells was categorized by a semi-quantitative method as mild, moderate, or strong intensity by using Fiji ImageJ software. IRS values were evaluated by multiplying the two values above.

Immunohistochemical staining of GPX1 and GPX4

Tissue sections of OSCC specimens for immunohistochemical staining were cut into sections with a thickness of 4 μm, mounted on histological adhesive coated slides, and then dried for 1 h. Deparaffinization of tissue sections was conducted using xylene, and rehydration with a graded series of water:ethanol solutions (serial dilution by 100 %, 90 %, 70 %, and 50 %). Subsequently, slides were washed using deionized water. Target retrieval for antigenic sites was performed using target retrieval solution in a water bath, which was pre-heated to 90−95 °C for 20 min. A Coplin jar filled with target retrieval solution was kept in a steamer at a temperature of 90−95 °C. A preheated solution was used for immersing sections on slides, which were incubated for approximately 40 min. The slides were removed from the Coplin jar and allowed to cool to room temperature for 20 min. After retrieving the antigen, the slides were placed in a tank of buffer (20 × concentrated) to cool for 8−10 min. Excess buffer was removed by wiping around the tissue so the reagents were restricted to their designated areas. Dako 500 immunostain was applied to the slides, which were then kept at room temperature for 30 min. Hydrogen peroxide was applied as a blocking agent for 10−12 min, before washing with buffer solution. Excess buffer solution was removed as described above. After blocking, primary antibodies against GPX1 (product name: PAA295Hu01, catalog no. SAA544Rb19; 100 μL) and GPX4 (product name: PAC994Hu01, catalog: SAA544Rb19; 100 μL) were applied, followed by incubation for 45 min and carefully rinsing the slides with Tris buffer solution (pH 9.0). Secondary antibody (HRP linked Caprine Anti-Rabbit IgG Polyclonal Antibody) was applied to the tissue section and incubated for 45 min, before rinsing the slides with buffer solution and removing any excess. 3,3′-Diaminobenzidine substrate chromogen solution was applied to the slides, which were then incubated for 10 min and washed with buffer solution to allow antigen−antibody color development.

Tissue analysis and immunohistochemistry scoring

Histological grading and pathological staging of tumors was performed using the College of American Pathologists (CAP) protocol proposed by the American Joint Committee on Cancer (AJCC), as follows:

Grade 1: Well differentiated.
Grade 2: Moderately differentiated.
Grade 3: Poorly differentiated[11]

A semi-quantitative scoring method was used to assess the positive expression of GPX1 and GPX4 antibodies in OSCC specimens using ImageJ software (see Tables 1−3). The semi-quantitative staining scores for cells are shown in Table 4.

IRS method

The IRS values shown in Table 6 were the final results. The IRS value was calculated by multiplying the staining intensity score (calculated manually, as shown in Table 5) and the percentage of positive cells (semi-quantitative method shown in Table 4) obtained by using ImageJ software.

$$\text{IRS} = \text{staining intensity} \times \text{percentage of positive cells}$$

Abbreviations: GPX1, Glutathione Peroxidase 1; GPX4, Glutathione Peroxidase 4; IRS, Immunoreactive Score; OSCC, Oral Squamous Cell Carcinoma; TNM, Tumor Node Metastasis; H&E, Hematoxylin and Eosin.

Results

Our study sample consisted of 133 specimens. The demographic and clinicopathological details are summarized in Table 7. We observed that 22−36 years was the most common age group for cancer occurrence (26.3 %) and 78.2 % cases were in males. Among the lesion sites, most

Table 1: T Staging of Oral Cancer.[11]

T staging	Cancer of Oral Cavity Stages
TX	Primary tumor cannot be evaluated
Tis	Carcinoma in situ
T1	Tumor size ≤2 cm with depth of invasion ≤5 mm
T2	Tumor size ≤2 cm with depth of invasion between 5 and 10 mm, or, tumor size >2 cm but not larger than 4 cm with depth of invasion ≤10 mm
T3	Tumor size > 4 cm and depth of invasion >10 mm
T4	Moderately advanced or very advanced local disease T4a (lip) T4a (oral cavity) T4b (invading bone, muscle, skull base)

Table 2: N Staging of Oral Cancer.[11]

N staging	Cancer of oral cavity stages
NX	Cannot be evaluated
N0	Zero positive
N1	Single positive L/N on ipsilateral side involved, and size 3 cm or smaller. ENE negative
N2a	Single L/N is 3 cm or smaller, ENE positive, or cancer has spread to a single ipsilateral L/N, measuring >3 cm but not larger than 6 cm and ENE negative
N2b	>1 L/N involved on the same side, and none measures >6 cm. ENE negative
N2c	>1 L/N involved on either side of the body is involved and none measures >6 cm. ENE negative
N3a	L/N involved is > 6 cm. ENE negative
N3b	ENE in a single L/N on the same side as the primary tumor >3 cm, or cancer has spread to many lymph nodes and at least one has ENE, or ENE in a single lymph node on the opposite side of the primary tumor that is 3 cm or smaller

(L/N, lymph node; ENE, extranodal extension).

Table 3: M Staging of Oral Cancer.[11]

M Staging	Cancer of oral cavity stages
M0	No metastasis
M1	Metastasis present

Table 4: Semi-quantitative staining scores for cells.[12]

Score	Percentage of positive cells
0	0 %
1	<10 %
2	10 %–50 %
3	51 %–80 %
4	>80 %

The percentage of positive cells was scored from 0 to 4 as: Score 0 = 0 % staining, Score 1 ≤ 10 % staining, Score 2 = 10–50 % staining, Score 3 = 51–80 % staining, and Score 4 ≥ 80 % staining.

Table 5: Staining intensity of cells.[12]

Staining intensity score	Type of reaction
0	Negative/no color reaction
1	Mild reaction
2	Moderate reaction
3	Intense reaction

Table 6: Immunoreactive score (IRS) method.[12]

Percentage of positive cells	(multiplied by) Intensity of staining	= IRS (0–12)
0 = no positive cells	0 = no color reaction	0–1 = negative
1 ≤ 10 % positive cells	1 = mild reaction	2–3 = mild
2 = 10–50 % positive cells	2 = moderate reaction	4–8 = moderate
3 = 51–80 % positive cells	3 = intense reaction	9–12 = strongly positive
4 ≥ 80 % positive cells	—	—

Table 7: Demographic and clinicopathological details.

Demographic and clinicopathological details		Total no. of samples (n = 133)	
		n	%
Age (years)	22–36	35	26.3
	37–45	34	25.6
	46–58	31	23.3
	59–84	33	24.8
Gender	Male	104	78.2
	Female	29	21.8
Site	Buccal mucosa	77	57.9
	Floor, lip, tongue, alveolus mandible	48	36.1
	Other	8	6.0
Laterality	Right	43	32.3
	Left	64	48.1
	Other	26	19.5
Grade	Well-differentiated tumor	45	33.8
	Moderately differentiated tumor	48	36.1
	Poorly differentiated tumor	40	30.1
Stage	T1	37	27.8
	T2	29	21.8
	T3	34	25.6
	T4	33	24.8
Lymph node involvement	Tumor without lymph node involvement	112	84.2
	Tumor with lymph node involvement	21	15.8

Table 8: Frequency of GPX1 and GPX4 expression in different tumor stages.

Tumor stage	GPX-4 only		GPX-1 and GPX-4 (both)		Total	
	n	%	N	%	n	%
T1	14	37.8	23	62.2	37	100.0
T2	16	55.2	13	44.8	29	100.0
T3	8	23.5	26	76.5	34	100.0
T4	1	3.0	32	97.0	33	100.0
Total	39	29.3	94	70.7	133	100.0

Table 9: Relationships between GPX1 and GPX4 IRS values.

		GPX4 IRS value								
		2–3 = Mild		4–8 = Moderate		9–12 = Strong		Total		*p*-value[a]
		N	%	N	%	n	%	N	%	
GPX1 IRS value	**0–1 = Negative**	13	33.3	15	38.5	11	28.2	39	100.0	<0.001
	2–3 = Mild	0	0.0	12	26.7	33	73.3	45	100.0	
	4–8 = Moderate	0	0.0	9	18.4	40	81.6	49	100.0	
	Total	13	9.8	36	27.1	84	63.2	133	100.0	

[a] Fisher's exact test result.

cases were reported in the buccal mucosa (59.7 %) and about 27.8 % were in stage T1.

We also determined the relationships between GPX1 and GPX4 and their expression levels with tumor stages. Table 8 shows that GPX4 was present in all cases of OSCC, whereas GPX1 was not observed in all cases. GPX4 was present in all stages of oral cancer either individually or also with GPX1in some cases, whereas GPX1 was not observed individually in any case. GPX1 was always co-expressed with GPX4. The combined expression of GPX1 and GPX4 was observed in higher proportions of severe pathological stages (T3 and T4), and isolated GPX4 expression was observed in higher proportions of less severe (initial) pathological stages (T1 and T2).

The relationships between the GPX1 IRS values and GPX4 IRS values are shown in Table 9. The IRS values for GPX1 and GPX4 were assigned to the following categories: negative (0–1), mild,[2,3] moderate,[4–8] and strong.[9–12] The statistically significant *p*-value of <0.001 showed that the strength of GPX4 expression (IRS value) increased exponentially as the intensity of GPX1 expression increased.

The relationships between the GPX1 IRS values and clinicopathological parameters are shown in Table 10 (see Figure 1–6). Highly significant relationships were observed

Table 10: Relationships between GPX1 IRS values and clinicopathological parameters.

	Total	GPX-1 IRS value								
		0–1 = Negative		2–3 = Mild		4–8 = Moderate		Total		*p*-value
		n	%	n	%	n	%	N	%	
		39	29.3	45	33.8	48	36.1	133	100.0	
Age (years)	**22–36**	8	22.9	12	34.3	15	42.9	35	100.0	0.65[a]
	37–45	9	26.5	11	32.4	14	41.2	34	100.0	
	46–58	12	38.7	8	25.8	11	32.3	31	100.0	
	59–84	10	30.3	14	42.4	9	27.3	33	100.0	
Gender	**Male**	31	29.8	32	30.8	41	39.4	104	100.0	0.33[a]
	Female	8	27.6	13	44.8	8	27.6	29	100.0	
Site	**Buccal mucosa**	21	27.3	29	37.7	27	35.1	77	100.0	0.79[b]
	Floor, lip, tongue, alveolus mandible	16	33.3	14	29.2	18	37.5	48	100.0	
	Other	2	25.0	2	25.0	4	50.0	8	100.0	
Laterality	**Right**	13	30.2	19	44.2	11	25.6	43	100.0	0.31[a]
	Left	19	29.7	17	26.6	28	43.8	64	100.0	
	Other	7	26.9	9	34.6	10	38.5	26	100.0	
Grade	**Well-differentiated tumor**	27	60.0	16	35.6	2	4.4	45	100.0	<0.001[b]
	Moderately differentiated tumor	12	25.0	20	41.7	16	33.3	48	100.0	
	Poorly differentiated tumor	0	0.0	9	22.5	31	77.5	40	100.0	
Stage	**T1**	14	37.8	20	54.1	3	8.1	37	100.0	<0.001[b]
	T2	16	55.2	8	27.6	5	17.2	29	100.0	
	T3	8	23.5	6	17.7	20	58.8	34	100.0	
	T4	1	3.0	11	33.3	21	63.6	33	100.0	
Lymph node involvement	**Tumor without lymph node involvement**	34	30.4	37	33.0	41	36.6	112	100.0	0.82[a]
	Tumor with lymph node involvement	5	23.8	8	38.1	8	38.1	21	100.0	

[a] Chi-square test result.
[b] Fisher's exact test result.

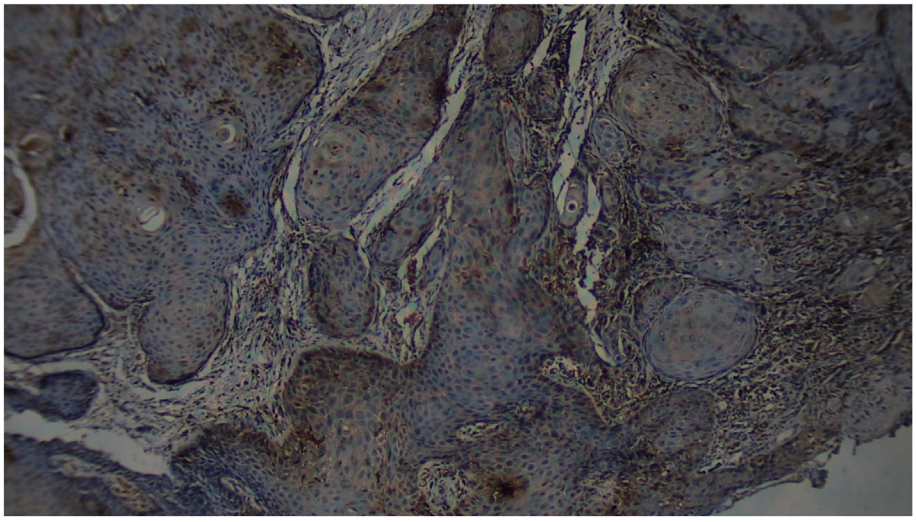

Figure 1: Images showing immunohistochemistry results for GPX1 for well differentiated tumor.

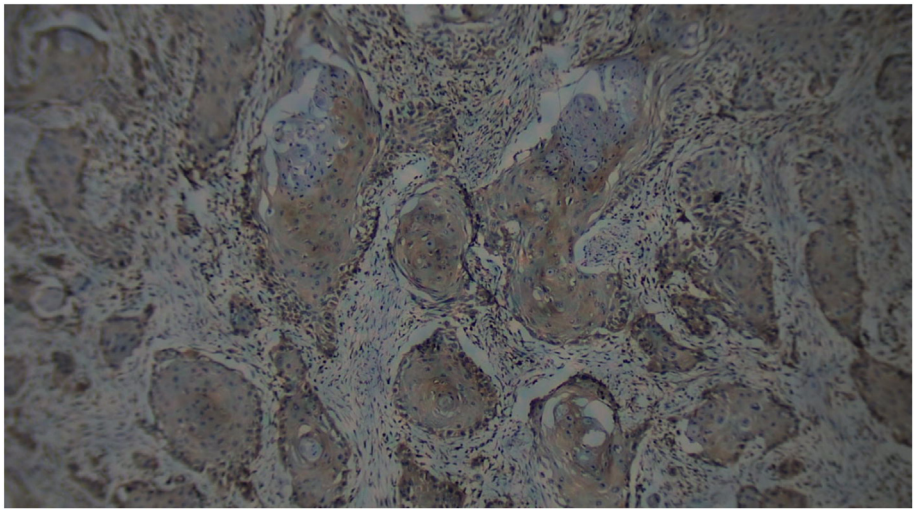

Figure 2: Images showing immunohistochemistry results for GPX1 for moderately differentiated tumor.

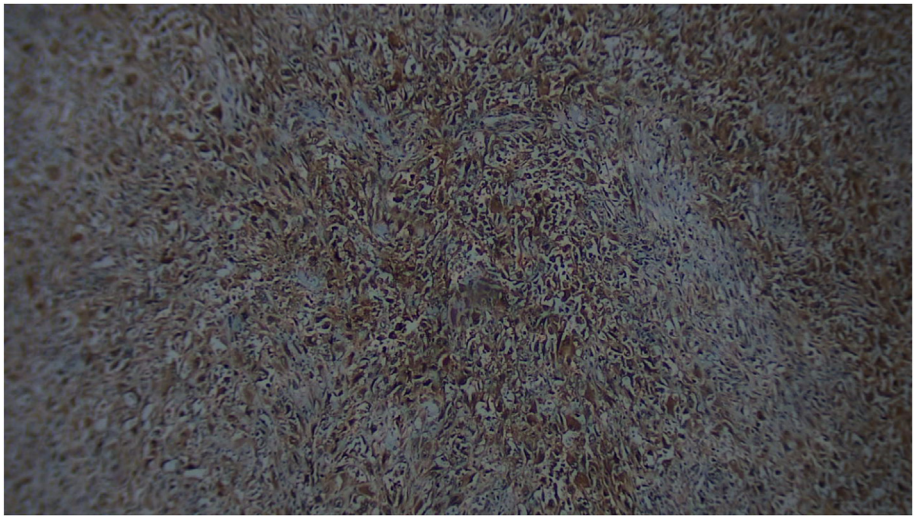

Figure 3: Images showing immunohistochemistry results for GPX1 for poorly differentiated tumor.

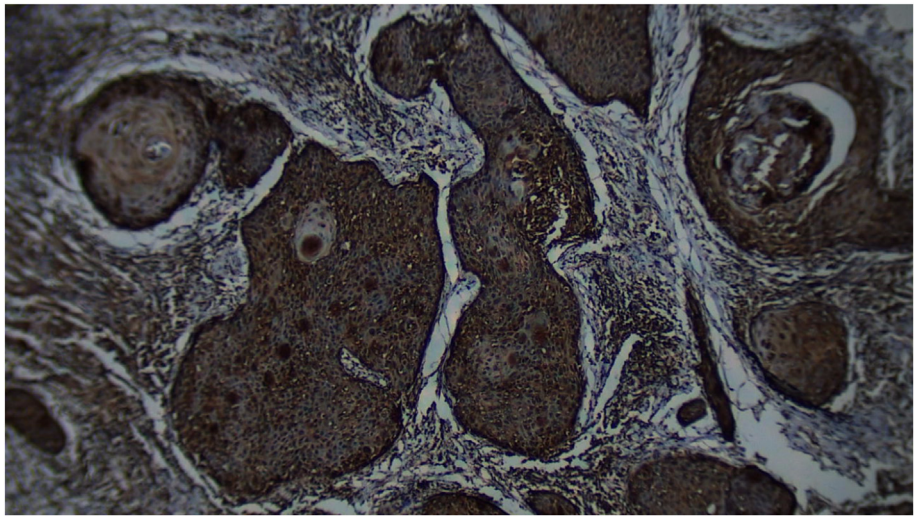

Figure 4: Images showing immunohistochemistry results for GPX4 for well differentiated tumor.

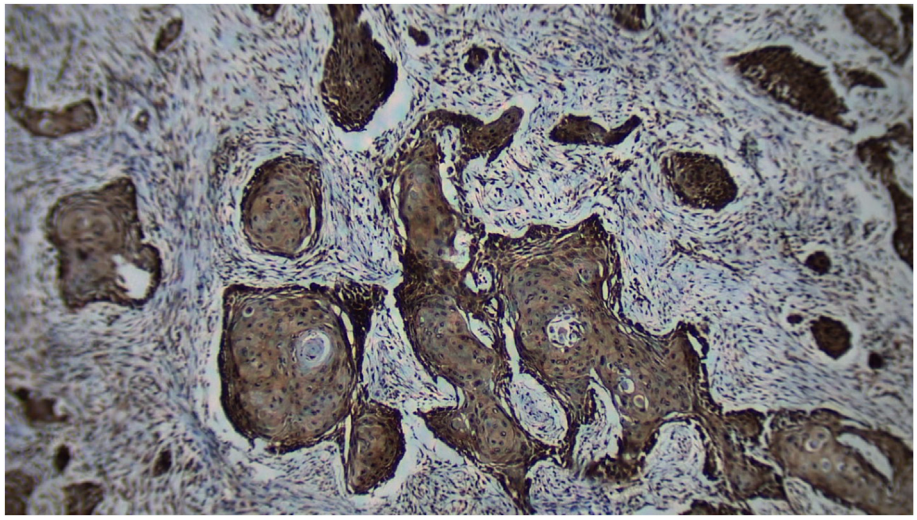

Figure 5: Images showing immunohistochemistry results for GPX4 for moderately differentiated tumor.

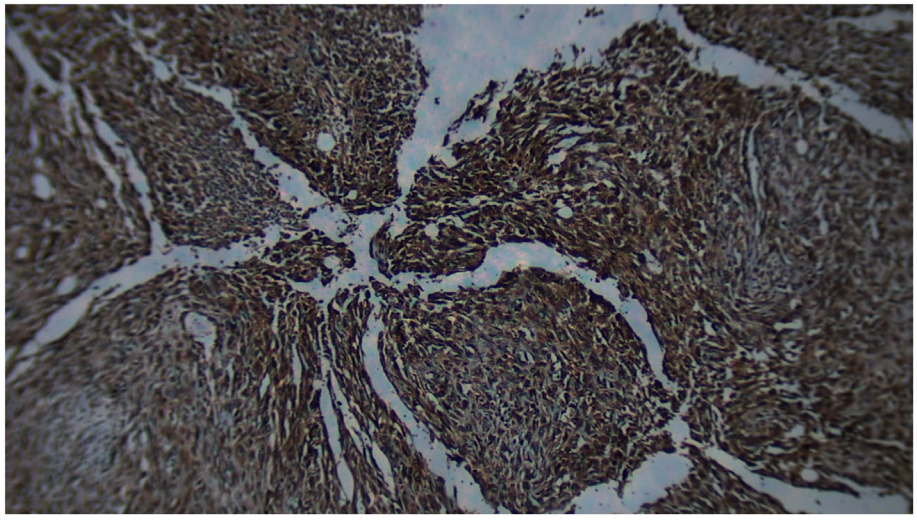

Figure 6: Images showing immunohistochemistry results for GPX4 for poorly differentiated tumors

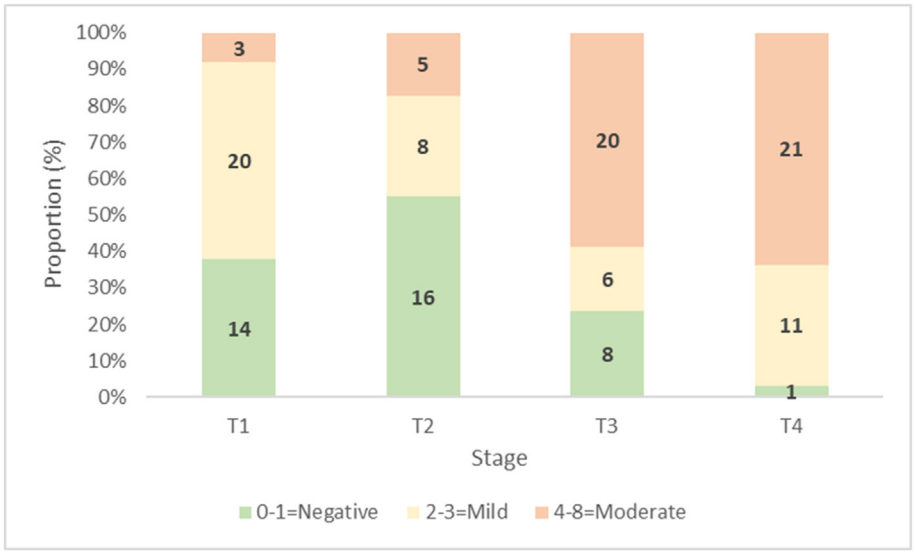

Figure 7: Associations of pathological stages with immunostaining of GPX1.

between the tumor grade, tumor stage, and GPX1 IRS values. Among the well differentiated tumors, 60 % had IRS values of 0−1 = negative GPX-1, 41.7 % of the moderately differentiated tumors had IRS values of 2−3 = mild GPX-1, and 77.5 % of the poorly differentiated tumors had IRS values of 4−8 = moderate GPX-1. Similarly, more than half of the stage T1 and T2 tumors had GPX-1 IRS values ranging between 0 and 3 (negative to mild), whereas the vast majority of stage T3 and T4 tumors had GPX-1 IRS values ranging from 4 to 8 (moderate). Therefore, we observed that stronger expression of GPX-1 was observed as the tumor grade progressed from well differentiated to poorly differentiated, and as the pathological stage advanced from T1 to T4 (Figure 7 and 8).

The GPX4 IRS values were also compared with the clinicopathological parameters, as shown in Table 11. Highly significant relationships were found between the tumor grade, tumor stage, and GPX4 IRS values. Among the well differentiated tumors, 46.7 % had IRS values of 9−12 = strong GPX-4, 50 % of the moderately differentiated

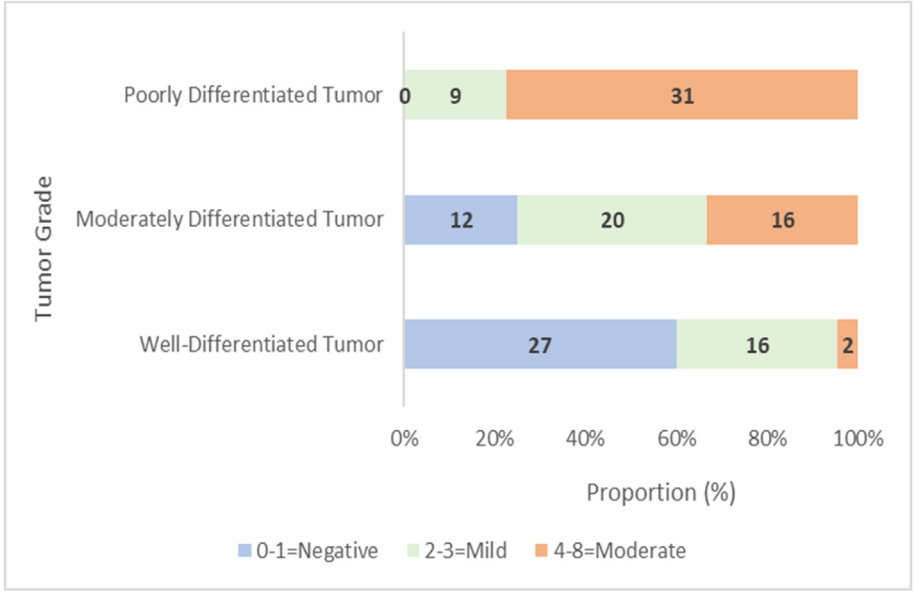

Figure 8: Associations of histological grades with immunostaining of GPX1.

Table 11: Relationships of GPX4 IRS values with clinicopathological parameters.

	Total	GPX4 IRS value								
		2–3 = Mild		4–8 = Moderate		9–12 = Strong		Total		*p*-value
		N	%	n	%	n	%	n	%	
		13	9.8	36	27.1	84	63.2	133	100.0	
Age (years)	**22–36**	3	8.6	10	28.6	22	62.9	35	100.0	0.22[b]
	37–45	3	8.8	10	29.4	21	61.8	34	100.0	
	46–58	5	16.1	3	9.7	23	74.2	31	100.0	
	59–84	2	6.1	13	39.4	18	54.6	33	100.0	
Gender	**Male**	11	10.6	26	25.0	67	64.4	104	100.0	0.55[b]
	Female	2	6.9	10	34.5	17	58.6	29	100.0	
Site	**Buccal mucosa**	8	10.4	16	20.8	53	68.8	77	100.0	0.12[a]
	Floor, lip, tongue, alveolus mandible	5	10.4	15	31.3	28	58.3	48	100.0	
	Other	0	0.0	5	62.5	3	37.5	8	100.0	
Laterality	**Right**	5	11.6	11	25.6	27	62.8	43	100.0	0.99[b]
	Left	6	9.4	18	28.1	40	62.5	64	100.0	
	Other	2	7.7	7	26.9	17	65.4	26	100.0	
Grade	**Well-differentiated tumor**	12	26.7	12	26.7	21	46.7	45	100.0	<0.001
	Moderately differentiated tumor	1	2.1	24	50.0	23	47.9	48	100.0	
	Poorly differentiated tumor	0	0.0	0	0.0	40	100.0	40	100.0	
Stage	**T1**	1	2.7	14	37.8	22	59.5	37	100.0	<0.001[b]
	T2	11	37.9	8	27.6	10	34.5	29	100.0	
	T3	1	2.9	7	20.6	26	76.5	34	100.0	
	T4	0	0.0	7	21.2	26	78.8	33	100.0	
Lymph node involvement	**Tumor without lymph node involvement**	13	11.6	28	25.0	71	63.4	112	100.0	0.17[b]
	Tumor with lymph node involvement	0	0.0	8	38.1	13	61.9	21	100.0	

[a] Chi-square test result.
[b] Fisher's exact test result.

tumors had IRS values of 4–8 = moderate GPX-4, and 48 % had IRS values of 9–12 = strong GPX-4, whereas 100 % of the poorly differentiated tumors had IRS values of 9–12 = strong GPX-4. Similarly, most stage T1, T3, and T4 tumors had GPX-4 IRS values ranging between 9 and 12 (strong), whereas 37.9 % of stage T2 tumors had GPX-4 IRS values ranging from 2 to 3 (mild) and 34.5 % had GPX-4 IRS values ranging from 9 to 12 (strong). Hence, we found that stronger expression of GPX-4

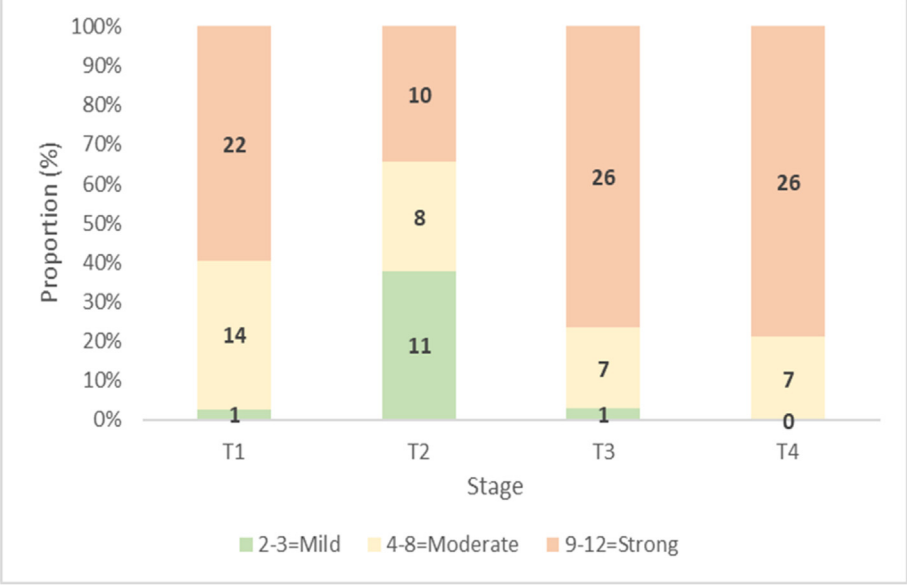

Figure 9: Associations of pathological stages with immunostaining of GPX4.

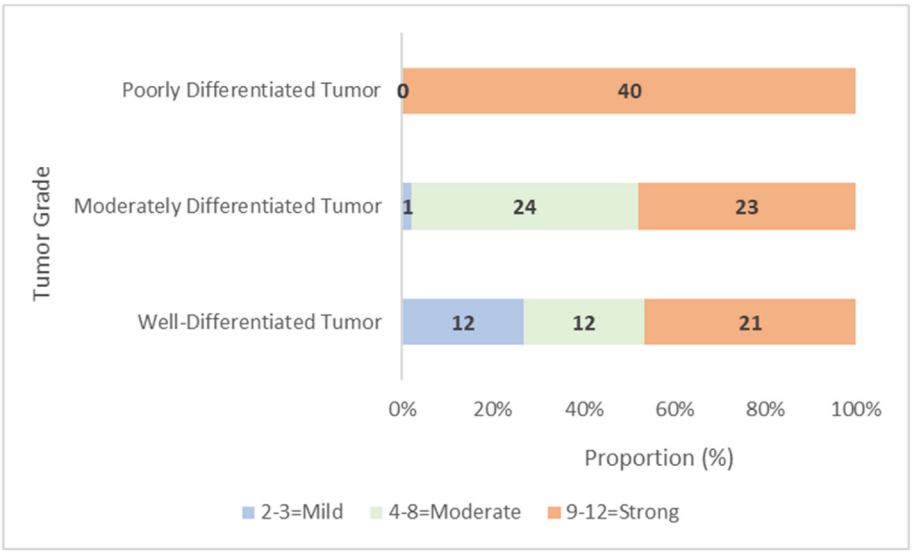

Figure 10: Associations of histological grades with immunostaining of GPX4.

was observed as the tumor grade progressed from well differentiated to poorly differentiated, and as the pathological stage advanced from T1 to T4 (Figures 9 and 10).

Discussion

The main predisposing factors associated with oral cancer are excessive smoking and the consumption of smokeless tobacco. Hence, chewing tobacco and smoking cause imbalanced pro-oxidant and antioxidant levels, which lead to oxidative stress within cells. The heat that is generated during smoking and the pH changes that occur while chewing tobacco result in the formation and stabilization of free radicals, and eventually the development of cancer.[1]

Therefore, diagnosing oral cancer at a later stage leads to a lower survival rate due to the lack of access to clinical diagnosis and treatment, and lack of awareness regarding the pathology of this disease.[13] Antioxidants such as GPX1 and GPX4 play important roles in counteracting the effects of tumor cells.[10] However, the roles of antioxidants in tumor progression and prognosis have been controversial for several years.[10] GPX1 and GPX4 are considered important biomarkers that may help in the early diagnosis of OSCC.[10] Therefore, in the present study, we investigated the associations of GPX1 and GPX4 with the pathological stages of primary tumors. We hypothesized that the GPX levels would increase with the tumor stage. Indeed, all of the poorly differentiated tumors had high GPX4 IRS values in our study. As the stage increased from T1 to T4, the proportion of samples that stained strongly due to GPX4 expression also increased. Among the samples in stages T3 and T4, the highest proportion of samples had moderate GPX1 IRS values in our study. However, GPX1 expression increased as the stage increased as poorly differentiated tumors had the highest proportion of moderate GPX1 IRS values. Hence, as reported previously by Ryung Lee et al., we conclude that ROS production is higher in cancer cells than normal cells, thereby leading the body to activate the antioxidant mechanism but regardless of decreasing oxidant levels, this antioxidant system also enables the survival of transformed or mutated cells in other cancers by limiting the apoptotic mechanism. This can be explained by antioxidants reducing the harmful effects of ROS but also protecting cancer cells from the oxidative stress that might otherwise kill them. Thus, even though they are abnormal, mutated or cancerous cells can survive better because antioxidants shield them.[10]

Hence, our results are similar to those obtained in a study of GPX4 levels in oral cancer conducted by Fatura et al. who concluded that GPX4 was immunopositive in the membranes of human OSCC cells and also correlated with p53 immunoreactivity. They also concluded that GPX4 was associated with oral cancer proliferation.[14] Similarly, Ryung Lee et al. found that high expression of GPX1 was associated with aggressive type OSCC cases with a greater likelihood of recurrence and poor survival, thereby indicating its usefulness as an important biomarker for OSCC.[10] GPX4 was also associated with high tumor grades and stages.[10] A study conducted by Bagul et al. based on the serum levels of antioxidants in OSCC patients showed that GPX1 expression was higher in patients suffering from OSCC compared with control subjects due to increased oxidative stress and high levels of circulating free radicals in OSCC patients.[15] Hence, all of the studies mentioned above demonstrated the poor survival of patients as the levels of GPX1 and GPX4 increased with tumor progression. By contrast, Fu et al. found that manganese superoxide dismutase, GPX, catalase, and myeloperoxidase were expressed in the buccal mucosa of OSCC patients and correlated with higher survival rates. They showed that GPX expression was related to better survival in stage IV cancer patients because of its high affinity for hydrogen peroxide and ability to react through lipid peroxidation. GPX scavenges free radicals and has a protective effect

against carcinogenic agents.[16] In 2018, Strycharz-Dudziak et al. showed that the GPX and SOD levels were lower in the tumor tissues of oropharyngeal cancer patients compared with the control group. They found that the antioxidant enzyme activity decreased as the stage of tumor development advanced.[17] Similarly, Dequanter et al. showed that GPX1 expression decreased in stages T3 and T4, and found a negative correlation between the tumor stage and GPX expression.[18] Salezman et al. studied the oxidative stress levels in patients with a high risk of head and neck cancer recurrence and found that the serum GPX1 activity was low in advanced tumor stages (T3 and T4) but high in stages T1 and T2.[19] Banerjee et al. also demonstrated that the expression levels of GPX1, GPX4, and catalase decreased with the progression of OSCC. Hence, the expression levels of both GPX1 and GPX4 decreased with tumor progression in OSCC because they have anti-tumorigenic properties, as also found in breast and pancreatic cancers.[20] Therefore, these studies indicated the better survival of patients as the levels of GPX1 and GPX4 decreased with tumor progression.

Other parameters such as age, gender, and tumor site were also assessed in the present study. Our results are similar to those obtained in a preliminary study conducted by Bagul et al. as well as a study of head and neck cancer by Dequanter et al. who found that OSCC was more common in males.[15,18] Previous studies also found that the most common stage was T1 and most patients had no lymph node involvement, which are similar to our results.[16] Ryung Lee et al. found that the tongue was the most common site followed by the buccal mucosa, which do not agree with our results because we showed that the buccal mucosa was the most common site followed by the tongue.[10]

The involvements of GPX1 and GPX4 in OSCC are not known in Pakistan because no relevant studies have been conducted previously in this country. However, in the studies mentioned above conducted in other countries, the expression levels of GPX1 and GPX4 were evaluated separately but they were not compared according to the pathological stages. Thus, in our study, we compared the expression levels of GPX1 and GPX4 according to the pathological stages to address the lack of previous comparative studies. However, our study also had some limitations. In particular, only tumor tissues were used to evaluate the GPX1 and GPX4 levels. Serum or blood levels of antioxidant enzymes were not detected and most samples were obtained from Sindh, specifically Karachi. A larger sample size from multiple centers should be evaluated in future research.

Our study provides useful insights into the status of GPX1 and GPX4 in tumor tissues from OSCC patients, but further studies should be conducted with larger sample sizes from multiple centers in Pakistan, and the blood and serum levels of antioxidant enzyme levels should also be assessed. Other techniques such as western blotting and spectrophotometry can also be used to determine the expression levels of GPX1 and GPX4 in tumor tissues.

Conclusion

In the present study, we found positive relationships between OSCC and the expression levels of GPX1 and GPX4. GPX4 was consistently expressed in al OSCC cases, whereas GPX1 was always co-expressed with GPX4, suggesting that co-expression is a key feature in OSCC pathology. However, isolated GPX4 expression was more common in the early stages of tumors (T1 and T2), and the combined expression of both enzymes was observed in the advanced stages (T3 and T4). In addition, the IRS values for both enzymes increased as the tumor stage progressed from T1 to T4, and from well to poorly differentiated cases. Hence, these findings suggest that GPX1 and GPX4 co-expression may be valuable biomarkers for determining the OSCC prognosis and tumor aggressiveness.

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Conflict of interest

The authors have no conflicts of interest to disclose.

Ethical approval

In this study, all techniques were performed using human samples after approval from the ethical committee institutional review board (IRB) of Dow University of Health Sciences. **IRB Number: IRB-2472/DUHS/Approval/2022 /847**.

Consent

The purpose of the study was thoroughly explained to the patients. Written consent was obtained from patients who were willing to participate.

Authors contributions

SF contributed to performing the procedures, acquiring data, and drafting the manuscript. SAA and FD contributed to analyzing and interpreting the data. RFK helped with sample collection and drafting the manuscript. AAM contributed to statistical analysis of data and interpretation of results. AZ also helped with manuscript drafting. The final text was thoroughly examined and authorized by all authors, who are also responsible for the manuscript's content and similarity index. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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