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The Role of Glutathione S-Transferases in Pleomorphic Adenomas of the Salivary Glands

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Abstract

The aim of this study is to determine the expression levels of GST isoenzymes in salivary gland pleomorphic adenoma from 26 patients. In this study, we investigated the immunohistochemical staining characteristics of the Glutathione-S-transferase alpha (GSTA-1), sigma (GSTS-1), theta (GSTT-1), kappa (GSTK-1), mu (GSTM-1), omega (GSTO-1) isoenzymes in tumor and surrounding tumor-free (normal) salivary gland tissues from 26 patients. For immunohistochemical studies, tissues were obtained from 26 patients with salivary gland pleomorphic adenoma (PA). Tumor and control tissues of patients were compared according to their staining intensity. The correlations between GST expressions in PA tissue were analyzed by Spearman's rho and the clinicopathological data were examined by Mann Whitney-U test and Spearman's rho. Considering the differences in the expressions of GST isoenzymes between tumor tissues and normal tissues of the patients; No differences in GSTT1 and GSTS1 isoenzymes expression were found between tumor and normal tissues ($p>0.05$). GSTM1 and GSTO1 expression were significantly higher in tumor tissues than in normal tissue.

However, GSTK1 and GSTA1 expressions were found to be significantly higher in the normal tissues of the patients compared to the tumor tissues. GSTM1 and GSTO1 expressions were increased in salivary gland PAs. These results should be confirmed with a larger series and different enzyme subtypes.

Key words: Salivary gland, Pleomorphic adenoma, Glutathione-S-Transferase, Immunohistochemistry

1. Introduction

The most prevalent neoplasm of the salivary glands is pleomorphic adenoma (PA), which accounts for 50 to 74 percent of all salivary gland malignancies (Tarakji et al., 2010; Khandker et al., 2019). It's a non-cancerous tumor made up of epithelial and myoepithelial cells (Khandker et al., 2019). The parotid and submandibular glands are the most prevalent sites of PA (Polat et al., 2013). PA can affect people of any age, however, it is more common in people between the ages of 30 and 50, with a small female predominance (Alves et al., 2002; Fatah and Khaleel, 2016). Despite the fact that PA is a benign tumor, it has a 20-45 percent recurrence rate after a simple tumor enucleation operation (Stennert et al., 2001).

Due to overlapping clinicopathologic features, differential diagnosis and prognostic assessment of salivary gland cancers based on histomorphologic criteria alone might be problematic. Tumors with the same categorization system have varying clinical outcomes (Skalova and Leivo, 1996). In the majority of equivocal tumor cases, immunohistochemistry is an effective adjuvant to histopathological diagnosis and is based on hematoxylin and eosin (H&E)-stained sections. It aids in the confirmation of H&E-stained sections or the formation of a definitive diagnosis (Stenner and Klussmann, 2009; Jordan et al., 2002).

Salivary gland tumors have unknown etiological factors. On the other hand, therapeutic radiation for various head and neck malignancies, occupational exposure in rubber manufacture and woodworking, as well as employment at hairdressers or beauty shops, are all possible risk factors (Guzzo et al., 2010; Horn-Ross et al., 1997).

Many of the harmful compounds, from which people suffer, are found in nature. In addition to these exogenous chemicals, reactive oxygen species (ROS) such as superoxide and hydroxyl radicals, and hydrogen peroxide, which are produced as a result of aerobic respiration, ionizing irradiation, and inflammation, can interact with membrane lipids and DNA to produce a wide range of harmful carbonyl-containing compounds. Foreign chemical metabolism normally occurs in two stages, known as phases I and II. The initial oxidation of the xenobiotics by cytochrome P450 (CYP) monooxygenases is a phase I metabolism (Hayes and Pulford, 1995).

Glutathione S-transferases (GST) are members of phase II enzymes that catalyze the conjugation of electrophilic xenobiotic reactive intermediates with glutathione (Parl, 2005). In mammalian tissues, seven kinds of cytosolic GSTs have been identified: zeta (GSTZ), theta (GSTT), omega (GSTO), sigma (GSTS), pi (GSTP), mu (GSTM), and alpha (GSTA). Individual differences in GST expression exist, and these differences are tissue and gender-specific (Flanagan and Jowsey, 2005). The GSTs play critical roles in the metabolism of chemical carcinogens, particularly those found in tobacco smoke.

The amount of carcinogen that reaches the upper aerodigestive tract depends on whether the carcinogen activates phase I enzymes and is detoxified by glutathione S-transferases (GSTs) of phase II enzymes (Aydın et al., 2010). The aim of this study is to determine the expression levels of GST isoenzymes in salivary gland pleomorphic adenoma from 26 patients.

2. Material and Methods

2.1. Patients

The retrospective study was approved by the Ethics Committee of the University of Health Sciences, Kartal Dr. Lütfi Kırdar City Hospital, Istanbul, (Date: 10.11.2021/ Decision No: 2021/514/213/1). This study was supported by Kırıkkale University Scientific Research Projects Unit with project code number 2020/035.

This study included 26 patients who had been histopathologically diagnosed as PA in the University of Health Sciences, Kartal Dr. Lütfi Kırdar City Hospital, Department of Pathology Laboratory between the years 2010 and 2020. The histopathology reports of the archived paraffin-embedded tissue samples were reviewed. Demographic data of the patients were obtained from electronic media records and from relevant clinicians. Data have also collected the age, gender, tumor localization, histopathological diagnosis, and tumor diameter. The hematoxylin and eosin slides of the selected cases were examined microscopically by a pathologist to confirm the previous diagnosis.

The median patients' age 43.19 (15.64) (minimum:18, maximum:77), of which 14 (53.8%) were male and 12 (46.2%) were female. The tumor diameter is the largest 7cm, the smallest 0.1cm, and the mean tumor diameter is 2.17 (0.86). Patients' demographic data are shown in Table 1.

Table 1. Patients' demographic data.

	n=26	Mean (Standard Deviation)	%
Gender			
Female	12		46.2
Male	14		53.8
Age	26	43.19 (15.64)	100
Mean Tumor Diameter (MTD)	26	2.17 (0.86)	100
Tumor Localization			
Left Parotid	7		26.9
Right Parotid	11		42.3
Unknown origin of the tumor	8		30.8

2.2. Immunohistochemical staining

The Glutathione-S-transferase alpha (GSTA-1), sigma (GSTS-1), theta (GSTT-1), kappa (GSTK-1), mu (GSTM-1), omega (GSTO-1) isoenzymes were studied by immunohistochemical staining in the tumor tissues of the patients. The formalin-fixed tissue blocks were cut into 4µm sections and mounted onto poly-L-lysine-coated slides. For immunohistochemistry, dewaxed in xylene and rehydrated in ethanol sections were washed with distilled water for 3 min. The sections were peroxidase-incubated for 10 minutes using 3% H_2O_2 in methanol (v/v).

Subsequently, the sections were washed with distilled water for 3 min and antigen retrieval was performed for 3 min using a 0.01M citrate buffer, pH 6.0 in a domestic pressure cooker.

Following washing with water, sections were incubated at room temperature for 10 min with superblock (SHP125; Scy Tek laboratories, west logan, UT) to block non-specific background staining. After sections were incubated with the primary antibody for anti-GSTS1 (Sc-30,067; Santa Cruz Biotechnology, Inc) diluted 1:200, anti-GSTK1 (ERP 1939; Origen Technologies Inc, Rockville, MD) diluted 1:250, anti-GSTT1 (anti GST-Theta1 from Cloud Clone Corp. TX, USA) diluted 1:200, anti-GSTO1 (ab88604; Abcam Inc., Cambridge, UK) diluted 1:150, anti-GSTA1 (bs-13396 R; Bioss Inc) diluted 1:50, anti-GSTM1 (Sc-517262; Santa Cruz Biotechnology, Inc) diluted 1:50. After washing for 15 minutes in TBS, the sections were incubated at room temperature with a biotinylated link antibody (SHP125; ScyTek Laboratories) followed by streptavidin/HRP complex (SHP125; ScyTek laboratories). After washing with TBS for 15 min, the sections were incubated at room temperature with biotinylated link antibody (SHP125; ScyTek Laboratories) then diaminobenzidine was used to visualize peroxidase activity in tissues. The sections were counterstained with hematoxylin, and then the sections were dehydrated and mounted. Scoring of immunohistochemically stained sections were performed for each enzyme was: -, negative (no staining); 1, weak staining; 2, moderate staining; 3, strong staining.

2.3. Statistical analysis

Analyzes were made using RStudio version 1.4.1103. GST isoenzymes expression in normal and tumor tissues was investigated using the Mann Whitney U test according to gender groups. The relationships between GST expression and age were examined using Spearman's rho correlation coefficient. GST isoenzymes expression according to tumor localization in normal and tumor tissues was compared using the Kruskall Wallis H test and post-hoc pair-wise tests were used in case of statistical significance between localizations as a result of the test. The relationships between normal and tumor tissues GST expression and mean tumor diameter (MTD) were analyzed using Spearman's rho correlation coefficient. GST isoenzymes expression in normal and tumor tissues was compared using the Wilcoxon signed-rank test. The results were considered significant for $p < 0.05$.

3. Results

Protein expressions of GSTT-1, GSTM-1, GSTS-1, GSTK-1, GSTO-1, and GSTA-1 were evaluated in tumors and normal tissues by immunohistochemical method. Demographic data of the patients with PAs of the salivary glands was represented in Figure 1. The expression of GST isoenzymes according to gender, age, tumor localization, and mean tumor diameter was shown in Table 2 and Figure 2.

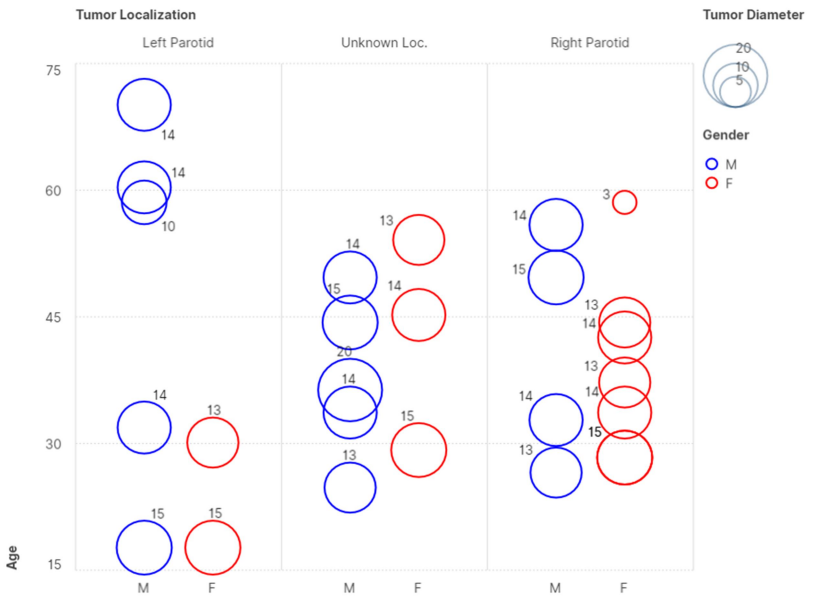

Figure 1. Demographic data of the patients with PAs.

Table 2. The expression of GST isoenzymes according to gender, age, tumor localization, and mean tumor diameter.

Variable		GSTA1		GSTT1		GSTK1	
		N	T	N	T	N	T
	Median	1	0	2	2	2	1
	Min-Max	1-2	0-2	0-3	0-3	0-3	0-2
	p	0.001*		0.116		0.003*	
Age	Sperman's rho	-0.143	0.194	-0.274	0.115	-0.338	0.111
	p	0.485	0.363	0.175	0.576	0.091	0.589
MTD	Sperman's rho	-0.338	0.130	0.106	-0.092	0.055	0.146
	p	0.091	0.546	0.607	0.653	0.790	0.476
Gender							
Female	Mean Rank	14.25	10.14	14	15.38	11.75	12.63
Male	Mean Rank	12.86	14.5	13.07	11.89	15	14.25
	p	0.498	0.086	0.742	0.155	0.215	0.532
Localization							
Left Parotid	Mean Rank	12.86	10.83	16.71	13.21	15.93	13.43
Right Parotid	Mean Rank	13.36	13.27	15.82	15.36	13.32	13.59
Unknown origin of the tumor	Mean Rank	14.25	12.71	7.5	11.19	11.63	13.44
	p	0.870	0.736	0.017*	0.349	0.456	0.998
		GSTM1		**GSTO1**		**GSTS1**	
		N	T	N	T	N	T
	Median	2	2.5	2	3	1	1.5
	Min-Max	0-3	1-3	0-3	1-3	0-3	0-3
	p	0.048*		0.018*		0.247	
Age	Sperman's rho	-0.075	0.033	-0.519*	-0.091	-0.115	0.571*
	p	0.716	0.874	0.007	0.659	0.577	0.002
MTD	Sperman's rho	-0.051	0.127	-0.158	-0.204	0.325	0.044
	p	0.803	0.537	0.44	0.318	0.105	0.832
Gender							

Female	Mean Rank	11.79	12.13	14.46	13.79	11.33	13.08
Male	Mean Rank	14.96	14.68	12.68	13.25	15.36	13.86
	p	0.265	0.352	0.529	0.836	0.149	0.786
Localization							
Left Parotid	Mean Rank	18.14	12.79	14.64	14.64	16.29	15.29
Right Parotid	Mean Rank	12.73	16	14.91	16.73	12.45	13.5
Unknown origin of the tumor	Mean Rank	10.5	10.69	10.56	8.06	12.5	11.94
	p	0.112	0.248	0.380	0.017*	0.477	0.671

N: normal T: tumor

There was not a statistically significant difference between the gender groups with respect to GST expression for both normal and tumor tissues (p-values>0.05). For normal tissues; There was a negative significant correlation between age and GSTO1 expression (Spearman's rho=-0.519, p-value=0.007 <0.05). For tumor tissues; GSTS1 expression and age were found to be positively and significantly correlated (Spearman's rho= 0.571, p-value=0.002 <0.05) (Table 2).

In normal tissues, the test results indicate that only GSTT1 expression differed over tumor localizations, with a mean rank of 16.71 for left parotid, 15.82 for right parotid, and 7.50 for unknown localization (H = 8.176, p = 0.017 <0.05). In tumor tissues, on the other hand, there was a statistically significant difference between tumor localizations and GSTO1 expression, with a mean rank of 14.64 for left parotid, 16.73 for right parotid, and 8.06 for the unknown origin of the tumor (H = 8.187, p = 0.017) (Table 2).

For both normal and tumor tissues, GST expressions and MTD were not significantly correlated (p-values>0.05) (Table 2).

GSTA1 expression significantly was different between normal and tumor tissues. Accordingly, the GSTA1 expression in normal tissues (median=1) is significantly higher than in tumor tissues (median=0). The difference between GSTT1 expression of normal and tumor tissues was not statistically significant (z=-1.57, p-value=0.116> 0.05) (Table 2).

GSTK1 expression in normal tissues were significantly higher (median=2) than tumor tissues (median=1) (z=-3.01, p-value=0.003 <0.05). There was statistically significant difference between normal and tumor tissues in terms of GSTM1 expression (z=-1.98, p-value=0.048 <0.05) Table 2. Accordingly, the GSTM1 expression in tumor tissues (median=2.5) was significantly higher than in normal tissues (median=2) (Table 2).

GSTO1 expression in tumor tissues (median=3) were significantly higher than in normal tissues (median=2) (z=-2.36, p-value=0.018 <0.05). The difference between GSTS1 expression of normal and tumor tissues was not statistically significant (z=-1.16, p-value=0.247> 0.05) (Table 2).

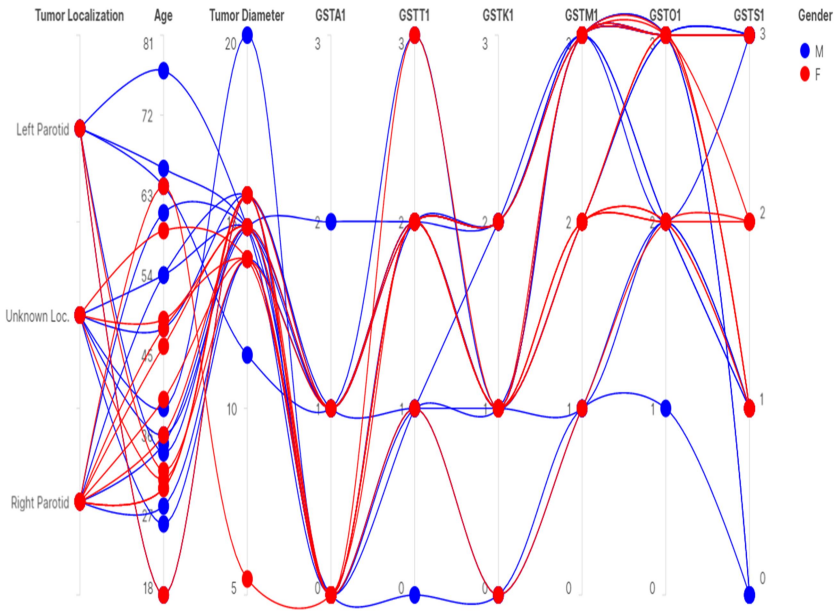

Figure 2. The expression of GST isoenzymes according to gender, age, tumor localization, and tumor diameter.

In normal tissues, there were positive statistically significant correlations between GSTT1 and GSTK1 (Spearman's rho=0.466, p-value=0.017 <0.05), GSTT1 and GSTO1 (Spearman's rho=0.529, p-value=0.005 <0.05) GSTT1 and GSTS1 (Spearman's rho=0.421, p-value=0.032 <0.05). Similarly, the correlations of GSTK1 with GSTM1(Spearman's rho=0.436, p-value=0.026 <0.05) and of GSTK1 with GSTS1(Spearman's rho=0.701, p-value=0.000 <0.05) were significant and positive (Table 3).

Pairs of expressions with significant correlations in tumor tissues are GSTA1- GSTK1 (Spearman's rho=0.447, p-value=0.028 <0.05), GSTA1- GSTO1 (Spearman's rho=0.453, p-value=0.026 <0.05). In addition, GSTT1- GSTO1 (Spearman's rho=0.533, p-value=0.005 <0.05), GSTK1- GSTM1 (Spearman's rho=0.726, p-value=0.000 <0.05), GSTM1- GSTO1(Spearman's rho=0.512, p -value=0.008 <0.05) expressions were found to be significantly positive correlated. The correlations between GST expressions for normal and tumor tisues are shown in Table 3.

Table 3. Relationship between GST expression of normal and tumor tissues (Spearman's rho).

		GSTT1	GSTK1	GSTM1	GSTO1	GSTS1
Normal	GSTA1	-0.090 (0.661)	0.075 (0.717)	0.062 (0.764)	0.228 (0.262)	0.091 (0.658)
	GSTT1		0.466* (0.017)	0.315 (0.117)	0.529* (0.005)	0.421* (0.032)
	GSTK1			0.436* (0.026)	0.241 (0.235)	0.701* (0.000)
	GSTM1				0.379 (0.056)	0.269 (0.184)
	GSTO1					0.195 (0.340)
Tumor	GSTA1	0.293 (0.164)	0.447* (0.028)	0.285 (0.177)	0.453* (0.026)	0.385 (0.063)
	GSTT1		0.319 (0.112)	0.378 (0.057)	0.533* (0.005)	0.291 (0.149)
	GSTK1			0.726* (0.000)	0.240 (0.237)	0.365 (0.067)
	GSTM1				0.512* (0.008)	0.305 (0.130)
	GSTO1					0.333 (0.096)

4. Discussion

Pleomorphic adenoma is a benign tumor that most usually develops in the parotid gland and is painless and fast-growing. It accounts for half to seventy percent of parotid gland tumors (Tarakji et al., 2010). Pleomorphic adenoma has a wide range of histopathological variants. Different biological activities can be detected even in the same tumor type (Khandker et al., 2019). The pathophysiology and origin of pleomorphic adenomas remain unknown, despite the fact that their clinical and microscopic aspects are thoroughly documented in the literature. The use of immunohistochemistry in the diagnosis of salivary gland cancers is crucial (Zhu et al., 2015). PA is divided into two categories based on histology: cell poor and cell-rich. There are several stromal patterns in cell-poor types, including hyaline, myxoid, and chondromyxoid. On the one hand, cellular and ductal tumor cells show strong staining, whereas pleomorphic adenoma with mesenchymal tumor cells shows modest staining (Zieper et al., 1994). In terms of GST enzyme activity and staining, acinus and ductal cells behave differently. When a tumor has more than one kind of tumor cells, such as in pleomorphic adenoma and adenoid cystic carcinoma, the strongest staining reaction was shown in cells with ductal differentiation, while the lowest staining was seen in myoepithelial tumor cells, much as it is in normal glands (Zieper et al., 1994).

GSTP1 isoenzyme activity was investigated in the bile duct, salivary duct, epididymis, and renal collecting tube. Although the pi class of enzymes has sparked a lot of attention, the other types of these detoxication enzymes and their putative function in carcinogenesis in the salivary gland should not be overlooked (Campbell et al., 1991). The expression of glutathione S-transferase isoenzymes such as GST- theta, sigma, mu, alpha, omega, and kappa in the salivary gland has not been observed. Both GSTA1 and GSTK1 expression

levels were greater in normal parotid tissue than in tumor tissues in our investigation, however, GSTM1 and GSTO1 expression levels were higher in tumor tissue than in normal parotid tissue. There was no significant change in GSTS1 and GSTT1 expression between normal and tumor cells (p-value=0.247>0.05, p-value=0.116> 0.05, respectively). We postulate that normal tissue has higher GSTA1 and GSTK1 expression than tumor tissue, that these isoenzymes protect normal tissue against tumor growth, and that high GSTM1 and GSTO1 expression in tumor tissue contributes to tumor development.

Usarek et al. In 2004, found that cancer tissue had higher expression of GSTP (the main isoform in the larynx) and decreased expression of GSTM4 and GSTA1 (Usaerk et al., 2004). Aydın and his colleagues When researchers evaluated the staining intensities and percentages of positive staining in normal larynx and laryngeal carcinoma cells, they discovered that GSTA1 expression in normal cells was substantially greater than in tumor cells. Normal cells have greater levels of GSTM1 and GSTT1. GSTA1 expression was elevated in poorly differentiated laryngeal tumors whereas GSTM4 and GSTT1 expression were decreased ($p<0.05$) when the same authors linked the immunohistochemistry results of GST isoenzymes with patients' clinical characteristics (Aydın et al., 2010).

GSTA1 expression in hepatocellular carcinoma was examined by immunohistochemical staining, and analysis revealed that GSTA1 abundances were lower in HCC tissues than in neighboring para-tumor liver tissues. Higher GSTA1 was linked to prolonged overall survival and disease-free survival, while lower GSTA1 was linked to a worse prognosis (Liu et al., 2020). They discovered that patients with greater GSTA1 levels had a better prognosis and that GSTA1 overexpression can inhibit liver cancer cell growth and metastasis (Larasati et al., 2018; Liu et al., 2020). The expression of GSTA1 and GSTK1 was greater in normal cells than in malignant parotid cells, according to our findings. These findings corroborate those of Hayes PC and Campbell JA, who discovered lower GSTA1 activity in HCC tissue (Campbell et al., 1991; Hayes et al., 1991).

During the malignant transformation process, tumor cells may modify some of their activities (for example, protein expression). It's possible that this change resulted in a greater amount of GST expression in the tumor cells.

High GSTM3 expression has been shown to be a bad prognosis factor in some studies, whereas low GSTM3 expression has been found to be a poor prognostic factor in others. GSTM3 expression in lymphoblasts was related to a favorable prognosis in children with acute lymphoblastic leukemia (Kearnes et al., 2003). High GSTM3 expression was linked to lymph node metastases and advanced stage of colon cancer, whereas low GSTM3 expression was linked to better survival. Patients with low GSTM3 expression had the best survival rate in bladder cancer, while those with normal or high GSTM3 expression had a poorer survival rate. As a result, GSTM3 function appears to be context-dependent and may change depending on the kind of cancer (Meding et al., 2012).

Low GSTM3 expression in esophageal squamous cell carcinoma specimens was linked with aggressive tumor characteristics and predicted lower disease-free survival (Yang et al., 2021). Our findings, on the other hand, revealed that tumor tissue had greater levels of GSTM1 than normal tissue. However, no correlations were

discovered between GSTM3 expression levels and the patients' age, gender, tumor location, or tumor diameter in this investigation (Yang et al., 2021).

GSTO1 expression levels have previously been discovered to be greater in cancers such as lymphoma, melanoma, and colorectal cancer (Wang et al., 2021; Bulus et al., 2019). GSTO1 has been found to be increased in tumor tissue when compared to normal tissue (Li et al., 2014). GSTO1 expression has previously been found to be elevated in a variety of cancers, including lymphoma, melanoma, and colorectal cancer (Kearnes et al., 2003; Meding et al., 2012; Bulus et al., 2018). Apart from comparable findings in esophageal squamous cell carcinoma, GSTO1 overexpression has also been demonstrated to have prognostic relevance in Barrett's esophagus, a premalignant lesion (Li et al., 2014; Piaggi et al., 2009). In this investigation, we found that tumor tissues have greater GSTO1 expression than normal tissues.

The expression of GST isoenzymes rises as cancer progresses. GSTA1, GSTM1, and GSTZ1 have been shown to be downregulated in esophageal squamous cell carcinoma, hepatocellular carcinoma (HCC), and Barrett's esophagus, and have been linked to a poor prognosis (Kearns et al., 2003; Campbell et al., 1991; Lu et al., 2019; Li et al., 2019), whereas GSTT1, GSTO1, However, we found that GSTK1 expression was greater in normal cells than in PA cells in our investigation**.**

5. Conclusion

Tumor cells reveal multiple genetic alterations resulting in morphologic and functional differences from normal cells. Tumour cells may lose some of their functions (eg, expression of some proteins) in the tumour transformation process. Hence, GST enzyme expression would be different in normal and tumour tissue from different patients, but in this study, we studied normal and tumour tissues from the same patients. GSTA1 and GSTK1 were observed higher in normal tisues than in tumours tisues. However GSTM1 and GSTO1 were observed in higher tumour tisues than in normal tisues. GSTs are a kind of toxicit that can expel harmful compounds for cells. Free oxygen radicals (ROS) accumulated at the beginning of tumor formation can be eliminated by enzymatic and nonenzymatic antioxidants. However, ROS, which increase with the progression of the tumor, can suppress antioxidants in the tumor and thus may contribute to the development of the tumor.

In this study, it was found that GSTA1, GSTK1, GSTO1, and GSTM1 isoenzymes would be useful in the diagnosis of pleomorphic adenoma. We think that these findings will reduce the confusion that may be encountered in the differentiation of normal and benign salivary gland tumors.The number of pleomorphic adenoma patients in this analysis is small. Future studies with substantially larger numbers of pleomorphic adenoma patients will be needed to examine prospectively for the possible relationship between GST expression and prognostic factors.

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Conflicts of Interests

The authors declare that they have no conflicting interests

Ethics Committee Approval

This study was approved by the University of Health Sciences, Kartal Dr. Lütfi Kirdar City Hospital Ethics Committee (Date: 10.11.2021, Decision No: 2021/514/213/1).

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