Int. J. Oral Maxillofac. Surg. 1997; 26: 205–209
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International Journal of
Oral & Maxillofacial Surgery
ISSN 0901-5027

Evaluation of glutathione S-transferase activity in human buccal epithelial dysplasias and squamous cell carcinomas

Y. K. Chen, L.-M. Lin
Oral Pathology & Diagnosis Department, School of Dentistry, Kaohsiung Medical College, Kaohsiung, Taiwan

Y. K. Chen, L.-M. Lin: Evaluation of glutathione S-transferase activity in human buccal epithelial dysplasias and squamous cell carcinomas. Int. J. Oral Maxillofac. Surg. 1997; 26: 205–209. © Munksgaard, 1997

Abstract. Glutathione S-transferase (GST) activity and amount of GST α, μ and π isoforms were measured in 40 patients with histopathologically confirmed oral epithelial dysplasia (OED) and squamous cell carcinoma of buccal mucosa. The results were compared with those of normal mucosa in an equal number of age- and sex-matched healthy controls. Mean total GST activities were significantly elevated from normal buccal mucosa for mild OED, moderate OED, severe OED and squamous cell carcinoma. GST activity of value approximating 100 nmol/min/mg distinguished between normal and dysplasia, and of value about 400 nmol/min/mg delineated between dysplasia and squamous cell carcinoma were observed. GST π was the predominant class in both the diseased and normal buccal mucosa examined. This class π GST was present at an intracellular concentration, which was significantly higher in diseased buccal mucosa than in normal buccal mucosa. These results indicated that π class GST was the major form of this enzyme in the cytosolic fraction of oral mucosa. The severity of OED related to squamous cell carcinoma development seemed to increase concomitantly with an increase in the level of this enzyme. Further studies will validate the role of GST π estimation in predicting the potential malignancy of OED.

Key words: glutathione S-transferase; oral squamous cell carcinoma; oral epithelial dysplasia.

Accepted for publication 6 November 1996

Cytosolic glutathione S-transferases (GSTs) are a family of enzymes divided into four classes, called α, μ, π and θ, on the bases of structural, physiochemical, enzymatic and immunological properties[11,12]. They function as detoxification enzymes by catalysing the conjugation of glutathione to various electrophilic substrates including carcinogens and cytotoxic drugs. This reaction is the first step in the formation of mercapturic acids, a water soluble product resulting in the elimination of potentially toxic compounds[3]. Total GST activity has been found to be significantly elevated in head and neck squamous cell carcinomas when compared to normal tissues[15]. Also, the total GST activity as well as the GST π level were higher in the malignant than in the normal oral/oropharyngeal tissues[14]. Although GST π isoform was demonstrated immunohistochemically in human premalignant and malignant oral epithelial lesions[3,15,25], there are no previous quantitative studies regarding GST isoenzymes' total activity and the amount of GST α, μ and π isoforms in human OED. Therefore, the present study is designed to assay the GST activity: levels of GST α, μ and π proteins in patients with histopathologically confirmed OED with various degrees of severity and squamous cell carcinomas. The results are compared with those from normal buccal mucosa in an equal number of age- and sex-matched healthy controls.

Material and methods

Patients

Samples of the diseased buccal mucosa were obtained from 40 male patients aged between 32 and 80 (mean age 51 years), who visited the Oral Pathology Department of Kaohsiung Medical College Hospital. The diseased buccal mucosa involved in this study comprised mild OED (10 samples), moderate

OED (10 samples), severe OED (10 samples), and well-differentiated squamous cell carcinoma (10 samples). The TNM staging (T – primary tumor; N – regional lymph nodes; M – distant metastases) of the ten samples of squamous cell carcinoma was also recorded (Table 1). Ten normal buccal mucosal tissues, taken from sex- and age-matched healthy individuals (mean age: 47 years; range: 22–65) without the habits of betel quid chewing and cigarette smoking, were included as controls. In all cases, no previous treatments had been given before the removal of tissues.

Histopathological classification of the samples

A representative part of each specimen was fixed in 10% neutral buffered formalin solution and routinely processed for hematoxylin and eosin staining for light microscopic examination to enable the histological diagnosis of various degrees of severity of OED and squamous cell carcinoma. The histopathologic characteristics of OED include: 1) basal layer hyperplasia; 2) nuclear enlargement and hyperchromatism; 3) loss of intercellular adhesion and normal polarization; 4) abnormal mitoses above the basal cell layer; 5) individual cell keratinization within the spinous layer; 6) cellular pleomorphism; 7) drop-shaped epithelial ridges; 8) irregular stratification; and 9) altered nuclear-cytoplasmic ratio[23]. Among these histologic changes, the presence of basal cell hyperplasia, nuclear enlargement and hyperchromatism and drop-shaped rete ridges are regarded as the minimal criteria for the histological diagnosis of OED[24]. Diagnosis was successfully achieved and the degrees of dysplasia were graded with reference to the following criteria[9]: 1) mild OED-dysplastic alterations confined to the lower third of the buccal epithelium; 2) moderate OED-dysplastic changes observed for up to two-thirds of the thickness of the buccal epithelium; and 3) severe dysplasia – more than two-thirds but less than the whole thickness – of the buccal epithelium contains the dysplastic cells.

Extraction and assay of GST isoenzymes

The freshly excised tissues, cleaned in ice-cold 0.9% NaCl solution, were trimmed to remove the connective tissues with the dissecting microscope (Bauch & Lomb Stereo Zoom® 7, zoom range 1.0×–7.0×). They were either immediately processed or frozen in liquid nitrogen and stored at −80°C. The GST isoenzymes were extracted by the method of PETERS et al.[17] with some modifications. All procedures were performed at 4°C, unless otherwise stated. Tissues (~100 mg) were homogenized in ~5 volumes of ice-cold 20 mM tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1.4 mM dithiothreitol with 10 strokes in glass/glass tissue grinders. The homogenates were centrifuged at 10000×g at 4°C for 10 min and the cytosolic fractions were obtained after recentrifugation at 150000×g for 1 h. Supernatants were frozen in liquid nitrogen and stored at −80°C in small portions. Protein contents were determined using the method of LOWRY et al.[8] with percentage of coefficient of variation (% CV) of intra- and inter-assay precision determined to be 3.1 and 2.3, respectively. GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate was assayed by the method of HABIG et al.[5] with % CV of intra- and inter-assay established to be 8.6 and 7.2, respectively.

Statistical analysis

The Mann-Whitney U tests were used to evaluate differences of activities between tissues of normal mucosa, OED and squamous cell carcinoma. Differences were considered significant if the P value was less than 0.05.

Immunoblot analysis of GST π

For additional identification of GST π, immunoblotting of the tissue extracts was performed as described by TOWBIN et al.[22] with antisera raised against GST π[6] (Novocastra Lab. Ltd., Newcastle upon Tyne, UK).

Results

Total GST activity

GST activity appeared to increase with the increased size of squamous cell carcinoma, with the two T_4 cases having the highest values (Table 1). The mean total GST activity was significantly el-

Table 1. TNM staging and GST activities in patients with squamous cell carcinomas

Patient No.	TNM staging	GST activity (nmol/min/mg protein)	GSTπ activity (μg/mg protein)
1.	$T_4N_2M_0$	601	28.3
2.	$T_4N_1M_0$	732	31.0
3.	$T_3N_2M_0$	560	26.6
4.	$T_3N_1M_0$	506	22.9
5.	$T_3N_0M_0$	432	23.1
6.	$T_3N_0M_0$	381	21.8
7.	$T_2N_0M_0$	414	17.8
8.	$T_1M_0M_0$	431	15.3
9.	$T_1N_0M_0$	384	15.2
10.	$T_1M_0M_0$	391	16.3

Table 2. GST activities in normal and diseased human buccal mucosa

	Total GST activity (nmol/min/mg protein)	GST π activity (μg/mg protein)
Squamous cell carcinoma	[a]483.1±36.7	21.83±1.78
Severe OED[b]	308.2±14.6	14.90±0.38
Moderate OED	245.0±13.4	13.12±0.39
Mild OED	153.2±7.7	9.46±0.41
Normal mucosa	94.7±5.3	4.73±0.38

[a] mean±SEM.
[b] OED: oral epithelial dysplasia.

Table 3. Mann-Whitney U test for GST activities in normal & diseased human buccal mucosa

	Squamous cell carcinoma	Severe OED	Moderate OED	Mild OED	Normal mucosa
Squamous cell carcinoma		[a]7.795*	7.795*	7.795*	7.795*
		[b]5.006*	7.873*	7.683*	7.795*
Severe OED[c]			3.624*	8.012*	7.795*
			3.955*	7.683*	8.012*
Moderate OED				7.795*	7.795*
				7.065*	8.012*
Mild OED					5.939*
					8.012*

[a] critical values for total GST activity.
[b] critical values for GST π activity.
[c] OED: oral epithelial dysplasia.
* Significance at 95% confident level with degrees of freedom: 18 ($P<0.05$)

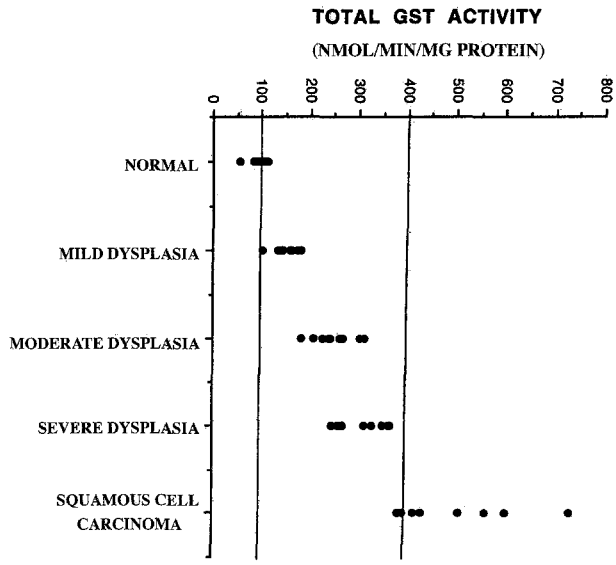

Fig. 1. GST activity with value of about 100 nmol/min/mg protein delineated between normal & oral epithelial dysplasia (OED), as well as with value of approx. 400 nmol/min/mg protein distinguished between OED & squamous cell carcinoma.

evated from normal buccal mucosa for mild OED, moderate OED, severe OED and squamous cell carcinoma (Tables 2 and 3). GST activity was observed of approximately 100 nmol/min/mg protein, namely 105.3 nmol/min/mg protein, as determined by adding the mean of GST activity of normal mucosa with 2 standard errors, delineated between the normal and OED (specificity: 100%; sensitivity: 70%) (Fig. 1). Also, GST activity was observed of about 400 nmol/min/mg protein, namely 408.7 nmol/min/mg protein, as obtained by substracting the mean of GST activity of squamous cell carcinoma with 2 standard errors, distinguished between OED and squamous cell carcinomas (specificity: 93.3%; sensitivity: 60%) (Fig. 1).

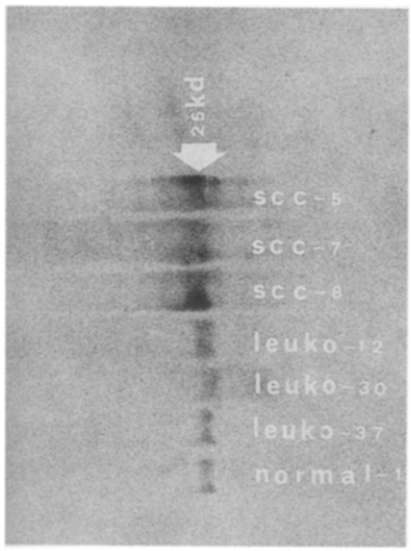

Fig. 2. Immunoblotting of representative normal and diseased human buccal mucosal tissue extracts, stained for GST π with the specific antibody, expressed the GST π polypeptide of molecular weight 25 kd (arrow). Normal-1: sample no. 1 of normal control; leuko-37: sample no. 37 of mild oral epithelial dysplasia (OED); leuko-30: sample no. 30 of moderate OED; leuko-12: sample no. 12 of severe OED; SCC-8: sample no. 8 of squamous cell carcinoma (T_1); SCC-7: sample no. 7 of squamous cell carcinoma (T_2); SCC-5: sample no. 5 of squamous cell carcinoma (T_3).

Activities of GST α, μ and π

GST π activity appeared to be elevated in relation to the size of squamous cell carcinoma, with the highest values in the two T_4 cases (Table 1). GST π was the predominant class in normal and diseased human buccal mucosa examined (Table 2). This class π GST is present at an intracellular concentration which is significantly higher in diseased than it is in normal buccal mucosa (Table 3). A significant increase in the level of this enzyme seems to be conclusively concomitant with the increase in severity of epithelial dysplasia related to oral squamous cell carcinoma development (Tables 2 and 3). A strong correlation was observed ($r=0.92$, $P<0.0001$) between GST activity and GST π levels in all the samples. No μ class and only low levels of α class GST could be detected in any of the samples.

Immunoblot analysis of GST π

Immunoblotting with specific antisera demonstrated GST π as a single band with molecular weight approximately 25 kd in the samples of normal and diseased buccal mucosa examined (Fig. 2).

Discussion

In some studies on GST activity, such as MULDER et al.[14] and PARISE et al.[16], the control tissues were taken adjacent to the diseased mucosae. Despite the advantage of the direct comparison of the GST activities between the experimental and control tissues within the same individual, these values may not really represent the true disease-free, normal GST activities. For this reason, healthy volunteers were employed in the present study.

An epidemiological study[7] has demonstrated that betel quid chewing alone or in combination with cigarette smoking, are associated with an increased risk of oral cancer in Taiwan. Therefore, it may at least partially explain why patients in the present report had a higher GST activity than patients in the previous study conducted by MULDER et al.[14] (483.1±36.7 nmol/min/mg protein vs 291±66 nmol/mg protein[14]). However, a more confirmative conclusion should only be achieved by measuring the GST activity in individuals who had betel quid chewing and cigarette smoking habits but without diseases in buccal mucosa. On the other hand, the normal GST activity in the present study is lower than the report of MULDER et al.[14] (94.7±5.3 nmol/min/mg protein vs 195±23 nmol/min/mg protein[14]). This may be due to the different sources of the normal tissues in these two reports. The present study comprised control tissues taken from disease-free, healthy subjects, whereas adjacent non-malignant, but not necessarily normal, tissues were used in the investigation of MULDER et al.[14].

The number of squamous cell carcinomas of various stages assayed in this study is small, and it would be premature to obtain a definite conclusion concerning the apparently higher GST activity in the two T_4 cases. However, a similar finding of larger GST activity in

T_3/T_4 patients was also reported by PARISE et al.[16] in head and neck squamous cell carcinomas.

GST activity was found to be increased in head and neck[16] and oral/oropharyngeal[14] squamous cell carcinoma, but, as far as we know, no previous quantitative data of GST isoenzyme concerning oral premalignant epithelial lesions are available. The present results reveal a significant increase of total GST activity in mild OED, moderate OED, severe OED and squamous cell carcinoma, when compared to normal buccal mucosa. The finding of a higher total GST activity in carcinomas compared with normal tissues, has also been found in the cases of other human tumors[13].

The results of the present study indicate that certain levels of GST activity delineated between the normal and OED, as well as distinguishing between OED and squamous cell carcinoma. These are in agreement with the study of BONGERS et al.[2] who reported on the usefulness of GST immunohistochemical activity detected in exfoliated cells, in predicting the development of a second primary tumor in patients with resected oral squamous cell carcinomas. However, considering the rather small group of patients in this report, further studies with more subjects and follow-up studies on the dysplastic lesions may validate the role of GST π estimation to predict the potential malignancy of OED with various degrees of severity. These are crucial not only in understanding the mechanism of oral carcinogenesis, but also in the establishment of an early prevention of oral squamous cell carcinomas.

Because essentially all activities are attributed to the class π GST in this experiment, the cytosolic activities may be a good measure of the concentration of this enzyme in the cytosol. Furthermore, the π class GST activity appears to be a concomitant increase in the severity of epithelial dysplasia related to oral squamous cell carcinoma development. The increased GST expression may be linked to carcinogen inactivation in oral carcinogenesis. The appearance of GST π could potentially enhance the ability of keratinocytes to resist the burden of carcinogens, therefore decreasing the risk of carcinoma development. However, it has been suggested that the role of placental GST in models of chemical carcinogenesis may be its permissive influence on cell cycle activity and down regulation of apoptosis, thereby permitting expansion of a population of initiated cells[10]. Epithelial GST π may alternatively confer an anticarcinogenic defense mechanism on normal keratinocytes on exposure to carcinogens and provide protection against the development of cancer. However, the same isoenzyme, epithelial GST π, induced in oral carcinogenesis may, at least partially, render the putative atypical keratinocytes more susceptible to clonal expansion[10].

A seven-fold elevation in GST π messenger ribonucleic acid (mRNA) levels in five head and neck squamous cell carcinomas as compared with the adjacent normal tissues, was demonstrated by MOSCOW et al.[13]. A significantly higher GST π expression in cutaneous squamous cell carcinoma than in normal skin with Northern blot and immunohistochemical analyses, was also reported by SHIMIZU et al.[20]. In this paper, class π GST is the most abundant isoform and is significantly higher in diseased human buccal mucosa than in normal buccal mucosa. This corresponds with the strong positive correlation of GST π content with CDNB activity ($r=0.92$, $P<0.0001$). This paper demonstrates quantitatively that the predominant isoform of the enzyme in OED is π class GST. Indeed, using the Northern blot technique, RIOU et al.[18] found an enhanced GST π mRNA in dysplasias and in carcinomas of the uterine cervix. In addition, enhanced expression of GST π human cutaneous premalignant lesions compared with normal human skin was also reported by SHIMIZU et al.[21]. Furthermore, the quantitative results obtained in the present study are consistent with the recent immunohistochemical studies of GST π in human oral mucosal epithelium[4,25]. The substantial isoform investigated, class π GST, has also been found in human oral/orpharyngeal tissues[14], epidermis[1] and other human tumors and human cell lines[19].

We are unaware of previously published results concerning electrophoretic identification of GST π isoform in human oral mucosal epithelium. Immunoblotting in the present study, conducted in oral mucosal tissues, indicated that, as in other human malignant tumors[19], the presence of GST π class of molecular weight was approximately 25 kd in both the normal and diseased buccal mucosa.

Acknowledgments. The authors wish to acknowledge the technical assistance of Ms N.Y. Dai. This research was supported by a National Science Council of Republic of China grant (N.S.C. 82-0412-B-037-034).

References

1. AKIRA K, IZUMI K, WANDA TS, et al. Placental glutathione S-transferase π mRNA is abundantly expressed in human skin. J Invest Dermatol 1990: **95**: 119–26.
2. BONGERS V, SNOW GB, VRIES NDE, et al. Second primary head and neck squamous cell carcinoma predicted by the glutathione S-transferase expression in healthy tissue in the direct vicinity of the first tumor. Lab Invest 1995: **73**: 503–10.
3. BOYER TD, KENNEY WC. Preparation, characterization and properties of glutathione S-transferases. In ZAKIM D, VESSEY D, eds.: Biochemical Pharmacology and Toxicology. New York: John Wiley & Sons, 1985: 297–364.
4. CHEN YK, LIN LM. Immunohistochemical demonstration of epithelial glutathione S-transferase isoenzymes in normal, benign, premalignant and malignant human oral mucosa. J Oral Pathol Med 1995: **24**: 316–21.
5. HABIG WH, PABST MJ, JAKOBY WB. Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974: **249**: 7310–9.
6. HALL A, FOSTER S, PROCTOR SJ, CATTAN AR. Purification and characterization of a π class glutathione S-transferase from human leukemic cells. Br J Haematol 1990: **75**: 494–500.
7. KO YC, HUANG YL, LEE CH, CHEN MJ, LIN LM, TSAI CC. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. J Oral Pathol Med 1995: **24**: 450–3.
8. LOWRY OG, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951: **193**: 265–75.
9. LUMERMAN H, FREEDMAN P, KERPEL S. Oral epithelial dysplasia and the development of invasive squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995: **79**: 321–9.
10. MACAUGHAN FM, BROWN AL, HARRISON DJ. The effect of inhibition of glutathione S-transferase P on the growth of the Jurkat human T cell line. J Pathol 1994: **172**: 357–62.
11. MANNERVIK B, ALIN P, GUTHERBERG C, et al. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc Natl Acad Sci USA 1985: **82**: 7202–6.
12. MEYER DJ, COLES B, PEMBLE SE, GILMORE KS, FRASER GM, KETTER B. Theta, a new class of GSH transferases purified from rat and man. Biochem J 1991: **274**: 409–14.

13. Moscow JA, Fairchild CR, Madden MJ, et al. Expression of anionic glutathione S-transferases and P-glycoprotein genes in human tissues and tumors. Cancer Res 1989: **49**: 1422–8.
14. Mulder TPJ, Manni JJ, Roelofs JMJ, Peters WHM, Wiersma A. Glutathione S-transferases and glutathione in human head and neck cancer. Carcinogenesis 1995: **16**: 619–24.
15. Odajima T, Hirata S, Yoko T, et al. Glutathione S- transferase-π activity in benign and malignant human oral epithelial lesions. Tumor Res 1989: **24**: 49–56.
16. Parise O, Janot F, Luboinski B, et al. Thymidylate synthase activity, folates and glutathione system in head and neck carcinoma and adjacent tissues. Head & Neck 1994: **16**: 158–64.
17. Peters WHM, Boon CEW, Roelofs HMJ, Wobbes TH, Nagengast FM, Kremers PG. Expression of drug metabolizing enzymes and P-170 glycoprotein in colorectal carcinoma and normal mucosa. Gastroenterology 1992: **103**: 448–55.
18. Riou G, Barrois M, Zhou D. Expression of anionic glutathione S- transferases (GST π) gene in carcinomas of the uterine cervix and in normal cervices. Br J Cancer 1991: **63**: 191–4.
19. Shea TC, Kelley SL, Henner WD. Identification of an anionic form of glutathione S-transferase present in many human tumors and human tumor cell lines. Cancer Res 1988: **48**: 527–33.
20. Shimizu K, Toriyama F, Yoshida H. The expression of placental-type glutathione S-transferase (GST-π) in human cutaneous squamous cell carcinoma and normal skin. Virchows Arch 1995: **425**: 589–92.
21. Shimizu K, Toriyama F, Zhang HM, Yoshida H. The expression of placental-type glutathione S-transferase (GST-π) in human cutaneous carcinoma *in situ*, that is, actinic keratosis and Bowen's disease, compared with normal human skin. Carcinogenesis 1995: **16**: 2327–30.
22. Towbin H, Stachelin T, Gordon L. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979: **76**: 4350–4.
23. Van der Waal I. Diagnostic and therapeutic problems of oral precancerous lesions. Int J Oral Maxillofac Surg 1986: **15**: 790–8.
24. Wright A, Shear M. Epithelial dysplasia immediately adjacent to oral squamous cell carcinoma. J Oral Pathol 1985: **14**: 559–64.
25. Zhang L, Xiao Y, Priddy R. Increase in placental glutathione S-transferase in human oral epithelial dysplastic lesions and squamous cell carcinomas. J Oral Pathol Med 1994: **23**: 75–9.

Address:
Dr Li-Min Lin
Oral Pathology & Diagnosis Department
School of Dentistry
Kaohsiung Medical College
100 Shih-Chuan 1st Rd
Kaohsiung
Taiwan