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Immunohistochemical demonstration of epithelial glutathione S-transferase isoenzymes in normal, benign, premalignant and malignant human oral mucosa

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The expression and localization of glutathione S-transferase (GST) isoenzymes in the epithelium of normal oral mucosa (n=9), overlying reactive fibrous hyperplasia (n=9), and of potentially malignant [leukoplakia (n=25), submucous fibrosis (n=12), verrucous hyperplasia (n=16)] and malignant [squamous cell carcinoma (n=36), verrucous carcinoma (n=13)] oral lesions were examined immunohistochemically using polyclonal antibodies raised against GST isoenzymes (alpha, mu and pi) with the standard avidin-biotin-peroxidase complex (ABC) method. GST alpha, mu and pi were almost completely absent in the epithelium of normal oral mucosa and overlying benign fibrous tissues. GST alpha staining was cytoplasmic and focally positive, while GST mu staining was similar to but weaker than that seen for GST alpha. GST pi showed both cytoplasmic and nuclear staining and was expressed in 60% of leukoplakias with mild dysplasia (n=15), 80% of leukoplakias with moderate to severe dysplasia (n=10). 75% of submucous fibrosis samples (n=12), 75% of verrucous hyperplasias (n=16), 77% of verrucous carcinomas (n=13), 81% of well-differentiated squamous cell carcinomas (n=26) and 70% of moderate- to poorly-differentiated squamous cell carcinomas (n=10). In addition, GST pi expression was independent of the state of differentiation of oral cancers. Since GST pi was significantly over-expressed in the oral premalignant and malignant lesions, the kinetics of GST pi-positive cells and the value of GST pi as a tumor marker in oral carcinogenesis need further investigation.

Key words: glutathione S-transferase isoenzymes; oral cancer; oral premalignancy; tumor markers

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Glutathione S-transferases (GST) (E.C. 2.5.1.18) were first identified in 1961 (1, 2) and are a complex family of multifunctional proteins. They function as detoxification enzymes by catalyzing the nucleophilic attack of glutathione on electrophilic substrates including carcinogens and cytotoxic and therapeutic drugs (3–5). In humans, there are three distinct cytosolic forms of GST (alpha, mu and pi) (6) and one membrane-bound form known as microsomal GST (7). The cytosolic GSTs, homo and hetero dimeric, of molecular weight between 22,500–27,700 (4, 5, 8), are classified according to their isoelectric points into basic (alpha), neutral (mu) and acidic (pi) forms. The classes are also immunologically distinct and differ in gene locus, amino acid sequence and substrate specificity (9, 10).

Studies have reported the over-expression of GST pi in both rodent (11–13) and human (14–21) cancerous tissues. GST P (a placental form of GST) has been identified as a marker for premalignant lesions during hepatocarcinogenesis in rats (11–13) and hamsters (22), as well as for pancreatic carcinogenesis in hamsters (22). Moreover, human GST pi has been regarded as a potential marker of transformation in cervical (21), colonic (23, 24) and he-

Table 1. The sites and number of samples used for GST isoenzymes staining

	Buccal mucosa	Tongue	Lip	Mouth floor	Palate	Alveolar mucosa	Total
Normal tissue	5	1	3	0	1	0	9
Benign fibrous tissues*	9	0	0	0	0	0	9
Leukoplakia	21	2	1	1	0	0	25
Submucous fibrosis	10	1	1	0	0	0	12
Verrucous hyperplasia	11	2	2	0	0	1	16
Verrucous carcinoma	10	0	3	0	0	0	13
Squamous cell carcinoma (well-differentiated)	12	8	3	2	0	1	26
Squamous cell carcinoma (moderate to poorly differentiated)	7	0	0	3	0	0	10

* Cases include reactive fibrous hyperplasia, fibrous polyp and irritation fibroma

patic (25) malignant tumors. However, little is known about GST isoenzymes in the oral mucosa.

Head and neck cancer has become the sixth most common cancer in the world (26), comprising about 4% of all cancers and 2% of all cancer deaths (27). Despite improvements in surgery, radiotherapy and chemotherapy over the past few decades, no remarkable advances in the prognosis for oral cancer have been obtained (28); in general the survival rate after 5 years is approximately 30% (29, 30). Earlier diagnosis using appropriate tumor markers should improve this statistic.

Therefore, cell markers associated with malignant transformation within oral mucosa have been investigated with the purpose of diagnosing the disease at an earlier stage (31, 32). GST isoenzymes appear to be such potential markers in oral mucosa (33). Thus, assessments of the expression and localization of the GST isoenzymes in epithelium of biopsies from normal oral mucosa, reactive fibrous hyperplasias, potentially malignant [leukoplakia (34, 35), submucous fibrosis (36–38), verrucous hyperplasia (39)] and malignant (squamous cell carcinoma and verrucous carcinoma) oral lesions were made immunohistochemically with specific polyclonal antibodies raised against GST isoenzymes (alpha, mu and pi).

Material and methods

Tissues

The tissues examined were obtained from biopsies of 120 patients sent to the Oral Pathology Department of Kaohsiung Medical College Hospital in 1992 (Table 1). The histological diagnoses of leukoplakia with epithelial dysplasia (34, 40), submucous fibrosis (41), verrucous hyperplasia (39), verrucous carcinoma (39) and squamous cell carcinoma were established independently and then agreed by both authors. All samples were fixed in 10% buffered formalin; blocks were taken and routinely processed into paraffin wax. Histological sections (5 μm thick) were prepared, stained with hematoxylin and eosin, and examined by light microscopy.

Immunohistochemistry for GST isoenzymes

Serial sections from lesions of interest were used for the immunohistochemical staining of GST isoenzymes (alpha, mu and pi) by the standard avidin-biotin peroxidase complex technique (ABC) according to Hsu *et al.* (42). Polyclonal antibodies (Novocastra Lab, Ltd. Newcastle upon Tyne, U.K.) were raised in rabbits using enzymes extracted from human spleen (pi class) and liver (alpha, mu) obtained post-mortem (43). Enzymes were purified using affinity chromatography and fast protein liquid chromatography (8, 44). Antibody specificity was tested using ELISA to purified GSTs and by Western blotting using both purified GSTs and crude cytosolic preparations from leukemic cells, placenta and liver. In all cases the antibodies reacted only to the homologous protein and did not cross-react with any other proteins under the conditions of the various assays. The antisera were known to be effective on both frozen and paraffin-embedded tissues (manufacturer's instructions). The specificity of the antisera had been established in a previous study in which GST isoenzyme expression was found not to be significantly altered by formalin fixation and paraffin embedding (45). Paraffin sections were passed through xylene and a series of graded alcohols (99% to 70%) and then treated sequentially with 3% H_2O_2 (Sigma), normal goat serum (Dakopatts, 1:20), anti-GST alpha (1:500), mu (1:50), and pi (1:500) rabbit polyclonal antibodies and, after storing overnight at 4 °C, with biotin-bound anti-rabbit IgG antibody (Vectastain ABC kit, PK4001, 1:400) and avidin-biotin-peroxidase complex (Vectastain ABC kit, PK4001). The binding sites of peroxidase were determined using diaminobenzenes (DAB) as the substrates. Sections were then lightly counterstained with Mayer's hematoxylin before mounting. Negative control reactions were performed by substituting non-immune serum for the GST antibodies (alpha, mu and pi). Positive control sections of human kidney (alpha antibody) and human liver (pi and mu antibodies) were also included. The intensity of staining was classified as follows: (−) negative; (+) positive, light brown color and (++) strongly positive, dark brown color. The grades were assessed independently and then agreed by both authors.

Table 2. Intensity of epithelial GST pi staining in normal and diseased human oral mucosa

	Intensity of GST pi staining
Normal mucosa	−(−)
Benign fibrous tissues	−(−)
Leukoplakia with mild dysplasia	+(+)
Leukoplakia with moderate to severe dysplasia	++(++)
Submucous fibrosis	+(+)
Verrucous hyperplasia	+(+)
Verrucous carcinoma	++(++)
Squamous cell carcinoma (well-differentiated)	++(++)
Squamous cell carcinoma (moderate to poorly differentiated)	++(++)

−: negative staining
+: positive staining, light brown color
++: strongly positive staining, dark brown color
In parentheses, intensity of nuclear staining

Results

Results of GST staining are summarized in Tables 2 & 3 and Figs. 1–4, while the statistical analysis is shown in Table 4.

GST alpha and mu

Both normal oral epithelium and that overlying benign fibrous tissue showed negative GST alpha staining except for one case (Table 3). GST alpha reactions were generally cytoplasmic and focally positive. The pattern of GST mu stain-

Table 3. Percentage expression of epithelial GST isoenzymes in specimens from normal and diseased human oral mucosa

	GST pi	GST alpha	GST mu
Normal mucosa	*1/9 (11%)	1/9 (11%)	1/9 (11%)
Benign fibrous tissues	0/9 (0%)	0/9 (0%)	0/9 (0%)
Leukoplakia with mild dysplasia	9/15 (60%)	5/15 (33%)	4/15 (27%)
Leukoplakia with moderate to severe dysplasia	8/10 (80%)	4/10 (40%)	2/10 (20%)
Submucous fibrosis	9/12 (75%)	5/12 (41%)	4/12 (33%)
Verrucous hyperplasia	12/16 (75%)	7/16 (44%)	5/16 (38%)
Verrucous carcinoma	10/13 (77%)	6/13 (46%)	5/13 (38%)
Squamous cell carcinoma (well-differentiated)	21/26 (81%)	10/26 (39%)	11/26 (42%)
Squamous cell carcinoma (moderate to poorly differentiated)	7/10 (70%)	3/10 (30%)	2/10 (20%)

* number of positive staining cases/total number of cases
Number in parentheses, percentage of expression

Table 4. Statistical results (Chi-squared test) of epithelial GST pi expression in the premalignant and malignant oral lesions compared to normal tissue

	P value	χ^2
Leukoplakia with mild dysplasia	$P<0.05$	5.53
Leukoplakia with moderate to severe dysplasia	$P<0.005$	9.29
Submucous fibrosis	$P<0.005$	8.43
Verrucous hyperplasia	$P<0.005$	9.41
Verrucous carcinoma	$P<0.005$	9.20
Squamous cell carcinoma	$P<0.0001$	13.96

ing was similar but weaker than that for GST alpha.

GST pi

Normal squamous epithelial cells were nearly all negative for GST pi binding (Tables 2, 3, Fig. 1) but occasionally the cytoplasm of a few cells in the intermediate layer showed a weak positive reaction. All epithelium overlying benign fibrous tissue lesions was negative for GST pi staining (Tables 2 & 3). Sixty percent of leukoplakias with mild dysplasia showed weak positive staining in the intermediate layers of the epithelium, with focally positive areas in the superficial and parabasal layers (Tables 2, 3, Fig. 2). Eighty percent of moderate to severe dysplastic leukoplakias showed strongly positive staining in the intermediate layer of focally positive staining in the superficial and parabasal layers (Tables 2, 3, Fig. 3). Seventy-five percent of oral submucous fibrosis samples and verrucous hyperplasias showed positive staining in the intermediate layer and focally positive staining in both the superficial and parabasal layers (Tables 2 & 3). Cytoplasmic staining was observed in oral verrucous carcinomas (77%) and squamous cell carcinomas (70% for moderate to poorly differentiated and 81% for well differentiated) (Tables 2 & 3, Fig. 4). Furthermore, GST pi staining of the nucleus was also noted (Table 2, Figs. 3 & 4). No obvious differences in GST pi expression were found with regard to histological differentiation ($P>0.05$, $\chi^2=0.49$). The control stainings using non-immune serum proved negative, while the positive control sections showed positive reactions.

Discussion

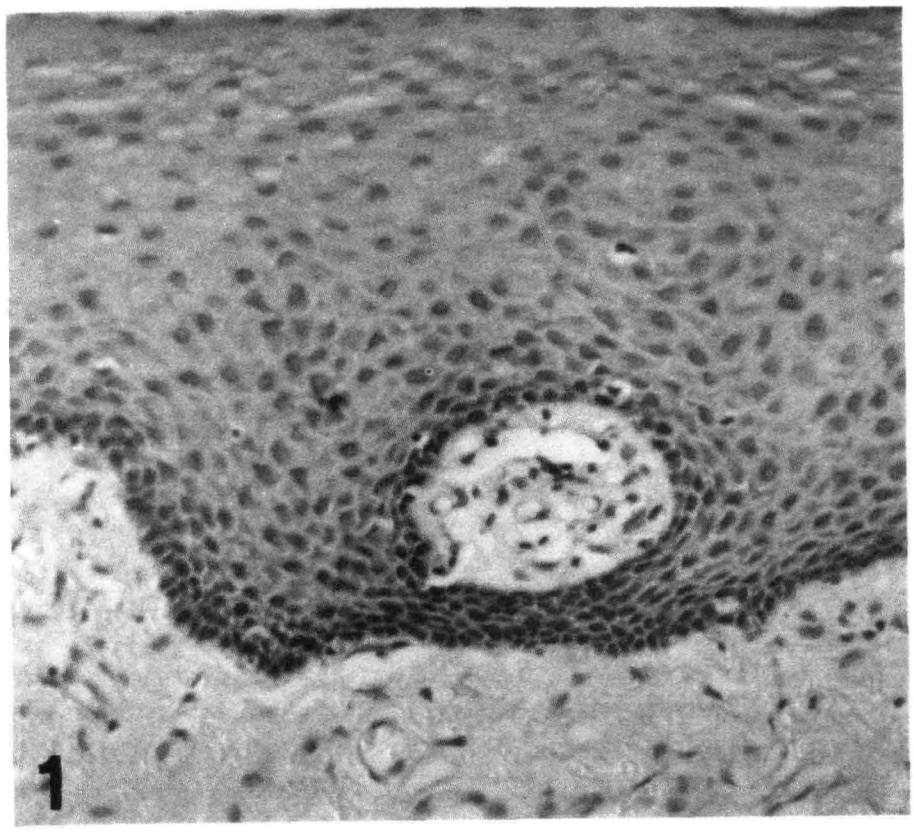
Thus far, a number of tissue markers such as cell surface carbohydrates (46, 47), squamous cell carcinoma antigens (48, 49), cytokeratins (50, 51), silver-binding nucleolar organizer regions (52), ras oncogenes (53), p53 tumor suppressor genes (54, 55) and gamma-glutamyl transpeptidase (56) have been applied to oral cancers with some success, but are not yet consistently helpful clinically (57). However, this study has demonstrated that GST pi is expressed in oral premalignant lesions and is almost completely absent in normal oral epithelium and the epithelium covering benign fibrous lesions of the oral mucosa. This suggests that GST pi may prove to be another useful marker of potentially malignant lesions. Similar results were found in premalignant lesions of other organs (21, 23–25). GST pi is immunologically related to GST P, which has been shown to be a marker for early detection of premalignant lesions in rat chemical hepatocarcinogenesis (11–13). GST pi has also proved useful for the immunohistochemical detection of human premalignant lesions in the colon (23, 24) and uterine cervix (21). The results of the present study support the hypothesis that GST pi may similarly be of assistance in the immunohistochemical diagnosis of premalignant lesions in human oral mucosa.

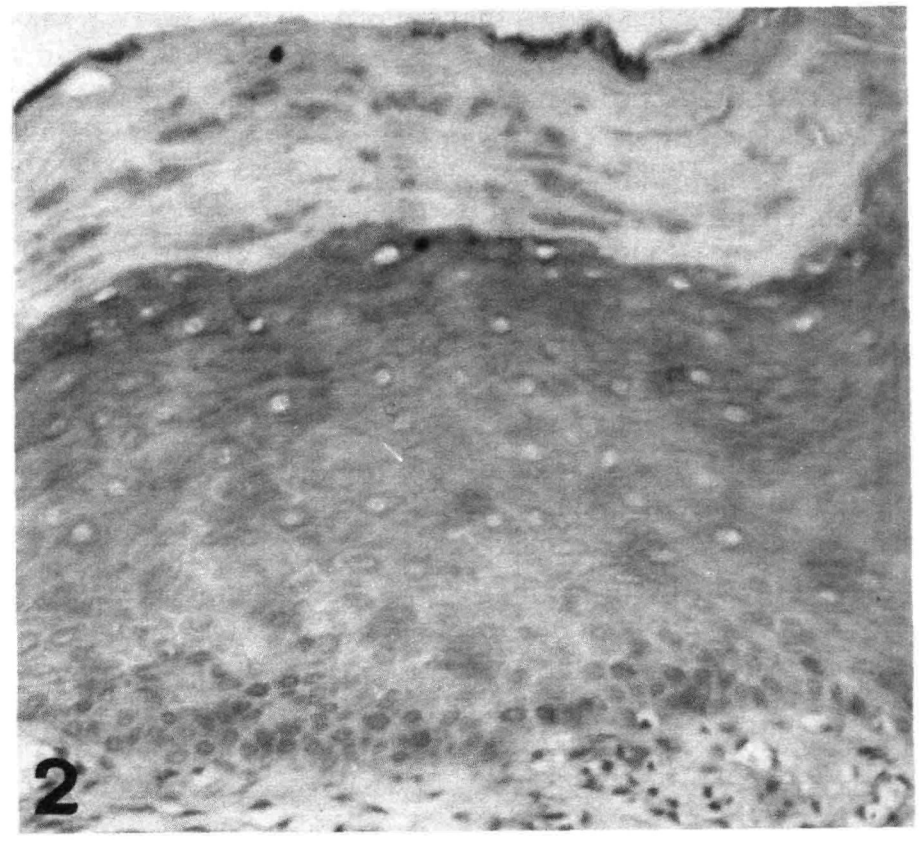
Increased expression of GST pi was found in more than 75% of oral cancers (verrucous carcinomas and squamous cell carcinomas). This finding supports a number of immunohistochemical studies which have identified over-expression of GST pi in both rodent (11–13, 22) and human (21, 23, 24) malignant tumors.

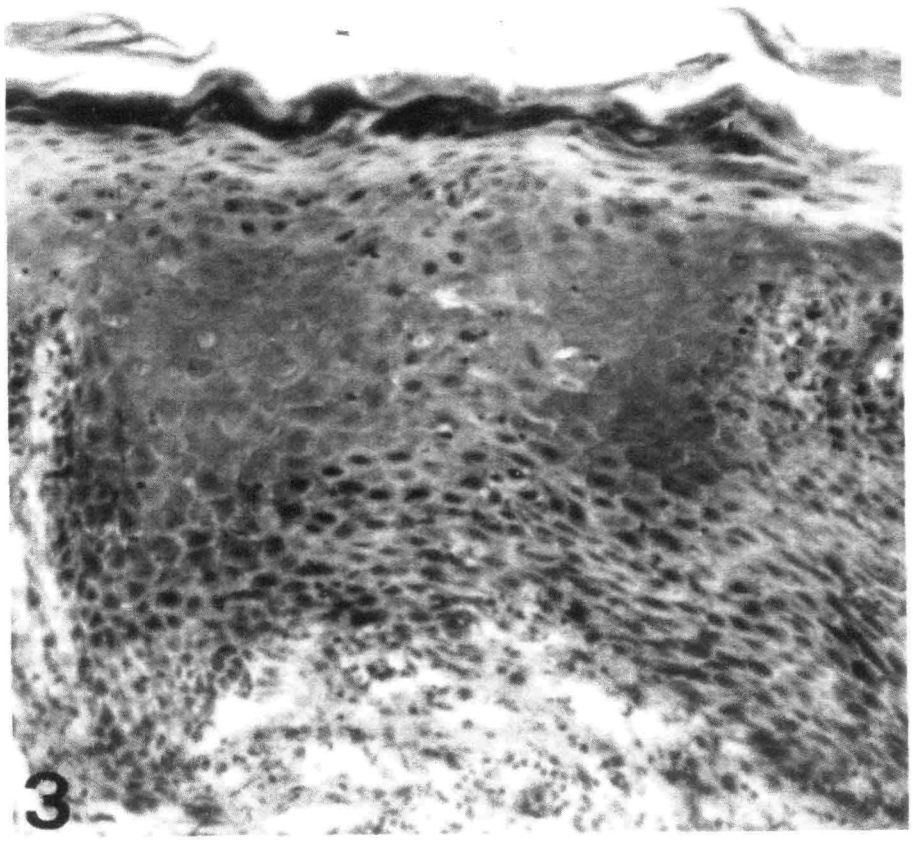
CAIRNS *et al.* reported an association between GST pi and tumor grade in breast carcinoma; high grade, poorly differentiated tumors were less likely to express this enzyme (45). TSUCHIDA *et al.* noted significantly higher levels of GST pi in well-differentiated oseophageal carcinomas compared with poorly differentiated tumors (58). However, in the present study GST pi expression was independent of the state of differentiation of the tumor ($P>0.05$, $\chi^2=0.49$). This may imply that GST pi expression is associated with a subset containing both well and less differentiated tumors.

After about two years follow-up, two out of eight cases (25%) of leukoplakia with mild to moderate dysplasia and four of the twelve cases (33%) of verrucous hyperplasia showing GST pi positive staining have undergone malignant transformation to develop squamous cell carcinomas. On the other hand, about 30% of oral cancers (verrucous carcinomas and squamous cell carcinomas) showing GST pi positivity had recurred after treatment. These preliminary data further suggest that GST pi expression may be a useful predictor of the prognosis of oral cancer (33). However, further studies on the characteristics of GST pi-positive cells and more long-term clinical follow-up data are necessary before a confirmative conclusion and possible clinical application can be made.

The results from this study concerning the elevated expression of GST pi are compatible with the work of HIRATA *et al.*, who found that GST pi levels of patients with oral cancers were enhanced in plasma using the sandwich







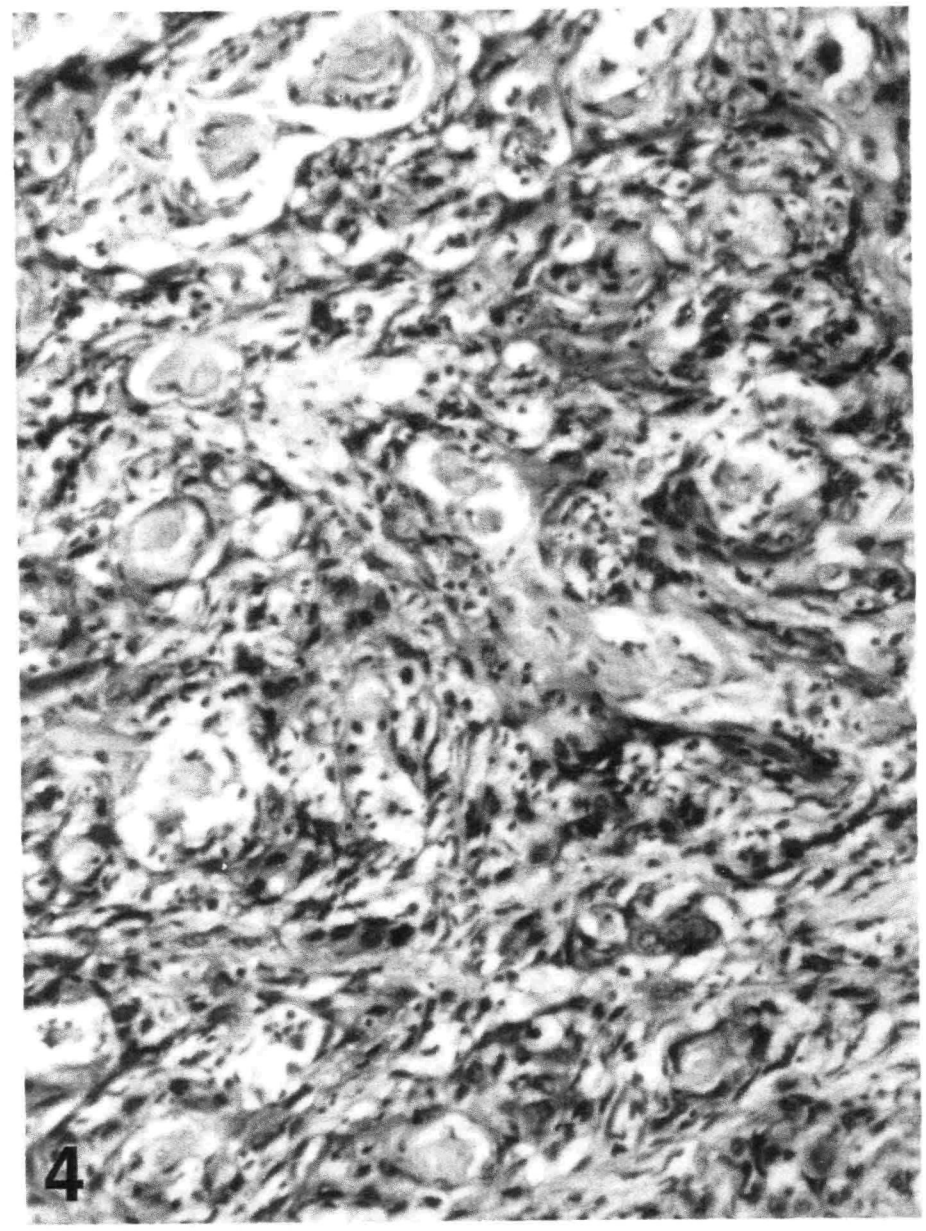


Fig. 1. Absence of GST pi staining in normal oral mucosa (×100).

Fig. 2. GST pi staining in leukoplakia with mild dysplasia (×100).

Fig. 3. GST pi staining in leukoplakia with moderate dysplasia (×100).

Fig. 4. GST pi staining in poorly differentiated squamous cell carcinoma (×50).

enzyme-immunoassay (EIA) technique (33). However, this technique does not allow ready assessment of the distribution of GST isoenzymes within tissues at a cellular or subcellular level.

Although the exact causes of the enhancement of GST pi in premalignant and malignant oral lesions remain to be clarified, it is reasonable to speculate that GST pi may be increased in response to exposure to carcinogens such as betel quid, tobacco and viruses in order to provide protection from the mutagens, as oral epithelium is a conspicuous barrier to a broad range of toxins and carcinogens. Furthermore, GST pi has been found to be involved in the process of carcinogenesis, perhaps by carcinogen activation or inactivation or in response to viral infection (59–61).

In another study (62), ras p21 oncogene product was found to be immunohistochemically associated with oral squamous cell carcinoma, especially in less differentiated ones. The expression of ras p21 has also been detected in parts of tumors with a high proliferative and infiltrative character, which may have a bearing on the progression of oral squamous cell carcinomas (63).

Recent evidence has shown that the promotor sequences of the GST pi gene contain a ras-responsive element (64). Indeed, elevation of GST pi in human cervical carcinogenesis is suggested to be related to the expression of the ras oncogene (21). Therefore, further investigation of the possible correlation between GST pi and ras oncogene expression are indicated.

The significance of nuclear and cytoplasmic staining of GST pi in oral premalignant and malignant lesions may have implications for clinical diagnosis, reducing both the time and cost of assessment. Correlation of smears from tumors showing GST pi-positive areas proven by biopsy is worthy of further study.

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