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Glutathione *S*-transferase π-class as a tumour marker in lingual preneoplastic and neoplastic lesions of rats and humans

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Abstract Immunocytochemical expression of the π class glutathione *S*-transferase (GST) was investigated in preneoplastic and neoplastic lingual lesions in a 4-nitroquinoline 1-oxide (4NQO)-induced rat genetic model [Wistar/Furth rats (WF) and Dark-Agouti rats (DA)] and in human surgical material [fibrous polyp, mild to moderate dysplasia, severe dysplasia, carcinoma in situ (CIS), squamous cell carcinoma (SCC)]. Two polyclonal antibodies raised against rat (GST-P) and human (GST-π) antigens were used. In the rat model, DA and WF rats showed contrasting susceptibility to 4NQO, DA rats having a much higher tumour incidence and a significantly shorter survival time than WF rats. While the established lingual SCC in DA and WF rats all expressed GST-P, the number of GST-P$^+$ foci in the preneoplastic lingual epithelium was significantly higher in DA (14.5 ± 6.5) than in WF rats (5.5 ± 2.6; $P < 0.0001$). In contrast, GST-π epithelial staining in human specimens was more variable and the results overlapped in different groups. More frequent nuclear and/or basal cell staining was detected in severe dysplasia, CIS and SCC than in benign and mild to moderate dysplastic lesions. Although the π class GST may be a useful marker for rat lingual carcinogenesis, its value in clinical applications is unclear. GST-π staining patterns and their distribution may be helpful in identifying high-risk lingual lesions in humans.

Key words Placental glutathione *S*-transferases · Lingual carcinogenesis · 4NQO · Squamous cell carcinoma · Epithelial dysplasia

Introduction

Glutathione *S*-transferases (GSTs) belong to a family of isoenzymes that have an important role in protecting cells from cytotoxic and carcinogenic agents [1, 5]. Their activity is responsible for catalysing the conjugation of glutathione to a large spectrum of lipophilic electrophiles, which is the first step in the formation of mercapturic acids, a pathway that provides an important means of eliminating compounds that are potentially cytotoxic or mutagenic from the body. The GSTs are widely distributed in the animal kingdom, and their activity has been detected in almost all human tissues [11]. The major isoenzymes of cytosolic GSTs from rat, mouse and man have been shown to share structural and catalytic properties and can be separated, by a species-independent classification, into three distinct classes named α, μ and π [12]. Several studies in rat models of chemical liver carcinogenesis have showed that the π-class GST (the placental form) is induced in preneoplastic hepatocytic foci and may be used as a marker for preneoplastic and neoplastic lesions [6, 14, 19, 20]. It has thus been proposed that the π-class might be a useful marker for the detection of preneoplastic and neoplastic cells in humans, and increased GST-π expression has been found in a wide variety of human tumours [4, 10, 16, 17, 22, 23, 31].

Recently, both enzyme activity analysis [15] and immunocytochemical studies [2, 34] have shown that GST-π is elevated in human oral premalignant and malignant lesions compared with normal tissues or benign lesions. However, these studies have either discussed 'head and neck' cancers as a group, or included such disease sites as lip, floor of the mouth, palate, tongue, and buccal and alveolar mucosa. The distinctive structures of oral mucosa in different functional regions makes it desirable to look into GST-π expression in various lesions at a specific oral site. The most common site of intraoral carcinoma involvement is the tongue (25–50% of all intraoral cancer [21]). The aim of this study was to explore the potential role of the π-class GST as a marker for lingual carcinogenesis, and we initially characterized the expression of GST-P in a rat genetic model. Two different strains of rats [Dark-Agouti (DA) and Wistar/Furth (WF)] were used and showed contrasting susceptibility to 4-nitroquinoline 1-oxide (4NQO) during lingual carcinogenesis

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[8, 9]. The diagnostic value of GST-π in human premalignant and malignant lingual lesions was then examined in surgical material.

Materials and methods

Animal details and experimental procedures have been described previously [8, 9]. Six-week-old DA (n = 20, male 10; female 10) and WF (n = 19, male 10; female 9) rats were supplied with drinking water containing 0.001% 4NQO (approximately 7 mg/kg per day), whereas control groups of DA (n = 5) and WF (n = 5) rats were given only drinking water. All rats were provided with the experimental and basic diets and weighed weekly. Administration of 4NQO was lasted until the rats became moribund and were killed. This period varied from 17 to 41 weeks. The control animals were killed at 30 weeks. Following decapitation under ethyl ether anaesthesia, the whole tongue specimens taken from the vallecula epiglottica to the anterior border of the mouth floor were fixed in 10% buffered formalin for 24–48 h. Through routine processing and paraffin-embedding, tongue tissue blocks of sagittal direction were prepared. Detailed macro- and microscopic examination of the tongue was performed, and the survival time of each animal was recorded.

Biopsy and/or surgery tongue specimens from a total number of 49 patients with benign, premalignant and malignant lesions were collected from the files of the Department of Oral Pathology, Kagoshima University Dental School for the period of 1988–1996. After reviewing the patient details and histology, the diagnoses of fibrous polyp (n = 10), mild to moderate epithelial dysplasia (n = 10), severe epithelial dysplasia (n = 8), carcinoma in situ (CIS, n = 8) and squamous cell carcinoma (SCC, n = 13) were established independently and then agreed by the three authors. Lesions showing the presence of *Candida* hyphae in the superficial epithelial layers, as visualized by periodic acid–Schiff (PAS) staining, were excluded from the study to minimize possible inconsistencies in immunocytochemistry due to *Candida* infection. For the 13 cases of SCC, both initial biopsies and surgery specimens after chemo- and/or radio-therapy (1.6 ± 0.9 months) were available for study. Lymph nodes with metastatic tumour deposits were found in 4 cases. All tissue specimens were fixed in 10% neutral buffered formalin and routinely processed and embedded in paraffin.

A sensitive biotin-streptavidin immunoperoxidase technique and polyclonal antibodies of GSTs raised against rat (GST-P, 1/1000; MBL, Japan) and human (GST-π, 1/500; GST-μ, 1/100; GST-α, 1/100; Novocastra, UK) antigens were used on serial 4-μm paraffin sections of rat and human specimens respectively. Sections were deparaffinized and rehydrated through xylene and graded alcohols. After blocking endogenous peroxidase activity with 3% hydrogen peroxide in buffer (5 min), antibodies diluted in 0.05 M Tris-HCL-buffered saline (TBS) containing 1% bovine serum albumin, pH 7.6, were applied to the sections at 4°C overnight. After washing, sections were then overlaid with biotinylated multilink (10 min; Dako, prediluted). Unbound conjugate was removed by washing, and the sections were overlaid with peroxidase-labelled streptavidin (10 min; Dako, prediluted). Excess streptavidin was removed by washing, and bound peroxidase was visualized using diaminobenzidine (DAB) reagent. After washing in water, the reaction product was darkened by treatment with 0.5% copper sulphate (W/V in saline) for 5 min. Stained sections were lightly counterstained in Mayer's haematoxylin and mounted in Xam. All washings were performed with TBS. Negative staining controls consisted of omission of the primary antibody and one or both second layers. Optimum dilutions, incubation times and temperatures were determined in preliminary experiments on a variety of paraffin-embedded rat (kidney, liver and spleen) and human (SCC) tissues.

For the rat model, GST-P staining intensity in the established lingual SCC was recorded as strong (++), weak (+) and negative (–). Because varying numbers of well-defined GST-P$^+$ foci were seen in the lingual epithelium of all carcinogen-treated rats, quantification of these foci was performed by manual counting along the whole length of non-neoplastic mucosa covering both dorsal and ventral sides of the tongue. The quantitative data was analysed statistically using the Mann-Whitney U-test. Due to the often weak and patchy staining of GST-μ and GST-α, detailed description of GST immunocytochemistry in different human lingual lesion groups was made only for GST-π-stained sections, in which staining intensity (strong, ++; weak, +; negative, –), cellular staining pattern (cytoplasm, C; cytoplasm and nuclear, C&N; nuclear, N) and the epithelial distribution of the staining (granular and prickle layers, G&P; prickle layer, P; prickle and basal layers, P&B) were scored and recorded for each case. All scoring was performed in a blind fashion without prior knowledge of the diagnosis.

Results

Macroscopic and microscopic examinations of the whole tongue specimens from DA and WF experimental groups, together with the recorded animal survival times were summarized in Table 1. Whilst administration of 4NQO p.o. was carcinogenic in both strains of rats, there were marked differences in oncogenicity between the two groups (Table 1, Fig. 1). Although varying degrees of lingual epithelial dysplasia were induced in all experimental animals, the number and size of the induced SCC varied between DA and WF rats. While all DA rats developed tumours, frequently of large mass-type (> 7 mm in diameter, 78.6%) with 8 cases being multiple, the majority of WF rats either were tumour free (68.4%) or only developed single, smaller tumours. Furthermore, WF rats had a significantly longer survival time than DA rats (Table 1). Neither tumour nor dysplastic lesions were detected in the tongues of control animals.

Table 1 Summary of macro- and microscopic findings of 4-nitroquinoline 1-oxide-induced lingual lesions in rats and animal survival times (*DA* Dark-Agouti, *WF* Wistar-Furth)

Strain	No. (M/F)	Epithelial dysplasia[a]	No. of SCC induced			Tumour size[b]		Survival time[c] (mean days±SD)
			Single	Multiple	Total	>7 mm	<7 mm	
DA	20 (10/10)	20	12	8	28	22	6	174.1±29.4
WF	19 (9/10)	19	6	0	6	3	3	245.2±23.5

[a] Number of rats showing the presence of dysplastic lesions in lingual mucosa
[b] Maximum diameter of the tumour
[c] Significant difference between WF and DA groups (P<0.0001, Mann-Whitney U-test)

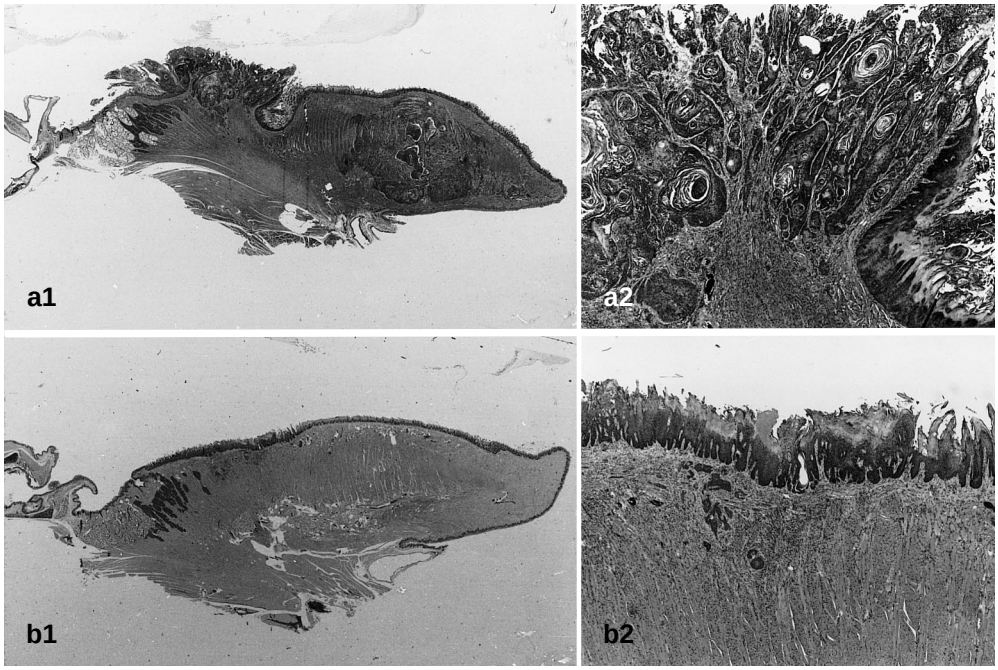

Fig. 1 Histological sections of the tongue taken from **a** a Dark-Agouti (DA; **a**1, ×2.5; **a**2, ×16 and **b** a Wistar-Furth (WF; **b**1, ×2.5; **b**2, ×16) rat on the 127th and 246th experimental days following 4-nitroquinoline 1-oxide (4NQO) administration, respectively. While two massive squamous cell carcinomas were induced in the tongue of the DA rat (**a**1, **a**2), only a smaller, early invasive carcinoma was detected in the WF rat (**b**1, **b**2)

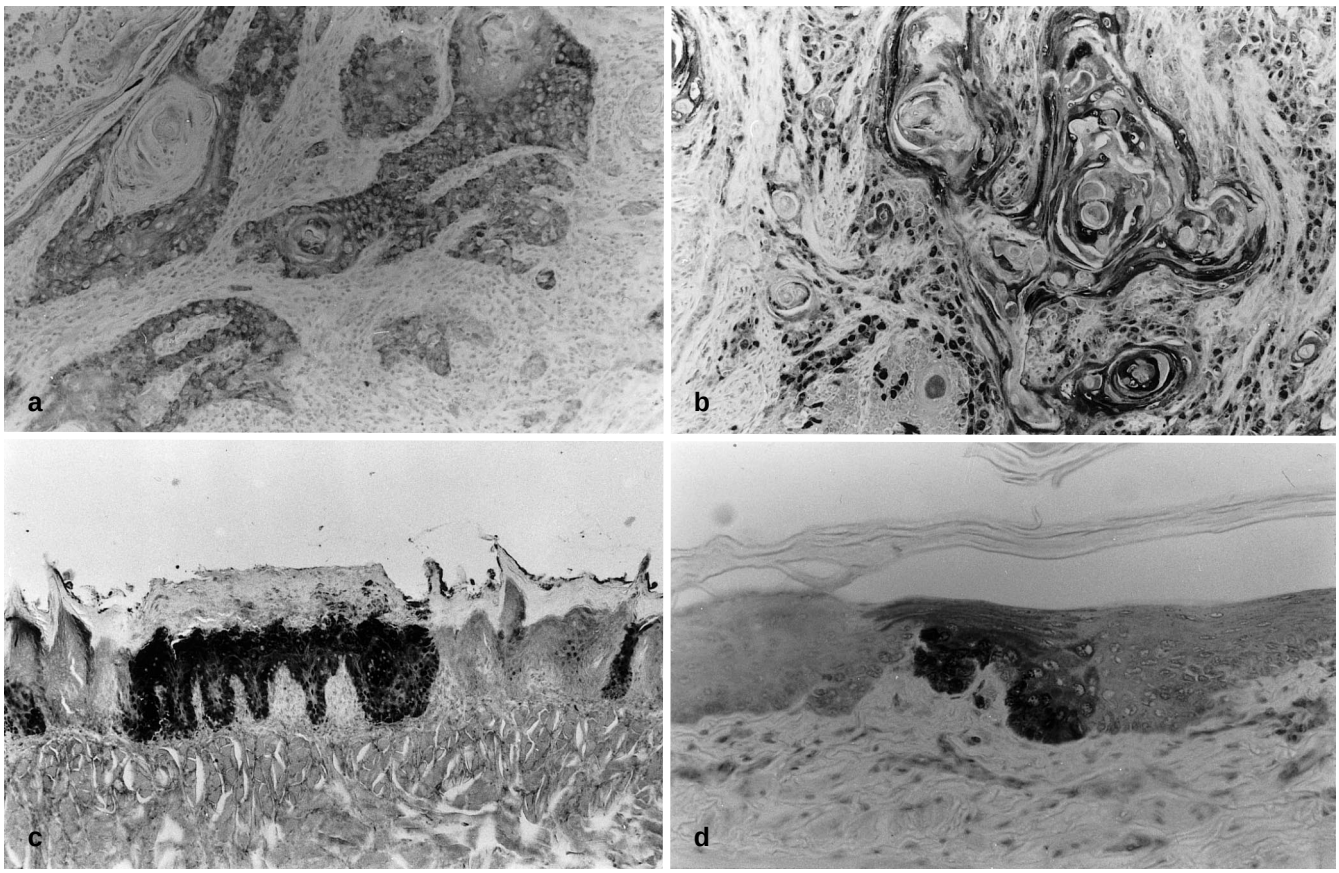

Fig. 2 Immunocytochemical staining of glutathione *S*-transferase (GST)-P in **a**, **b** lingual squamous cell carcinomas (×100) and **c**, **d** non-neoplastic lingual epithelium (**c** ×50, **d** ×160) of 4NQO-treated rats. Both cytoplastic (**a**) and nuclear staining (**b**) patterns are noted in the carcinomas. Well-defined epithelial GST-P$^+$ foci are detectable in both dorsal (**c**) and ventral (**d**) aspects of the tongue, **c** with and **d** without histological evidence of epithelial dysplasia. Note the varying sizes of the foci and the combined cytoplasm and nuclear staining pattern

GST-P expression was detected in all 4NQO-induced tongue SCC, with 11 tumours (39.3%) from DA and 2 (33.3%) from WF showing strong reactivity. The staining of the tumour cells was mainly located in cytoplasm, but nuclear staining particularly in the peripheral basal cell layers of the tumour nests were also noted (Fig. 2a, b). No apparent difference in GST-P staining was detected between tumours induced in DA and WF rats. While tongue specimens from all the non-4NQO-treated control animals were completely negative for GST-P, numerous GST-P$^+$ foci were found in both dysplastic (Fig. 2c) and non-dysplastic (Fig. 2d) lingual epithelium of carcinogen-treated rats. These foci were made up of a cluster of GST-P$^+$ cells, which were usually well-demarcated from the adjacent negative areas. The positive staining was mainly cytoplastic, but often accompanied by strong nuclear staining in the basal and suprabasal cell layers (Fig. 2c, d). The size of the foci varied from a group of a few cells, mostly located in basal and/or suprabasal layers, to a large focus involving the full thickness of the epithelium (Fig. 2c, d). Subjectively, there appeared to be more large GST-P$^+$ foci in DA lingual mucosa than in WF. Quantitative study revealed that the number of epithelial GST-P$^+$ foci in DA rats (14.45 ± 6.52) was significantly higher than that in WF (5.47 ± 2.59; Fig. 3).

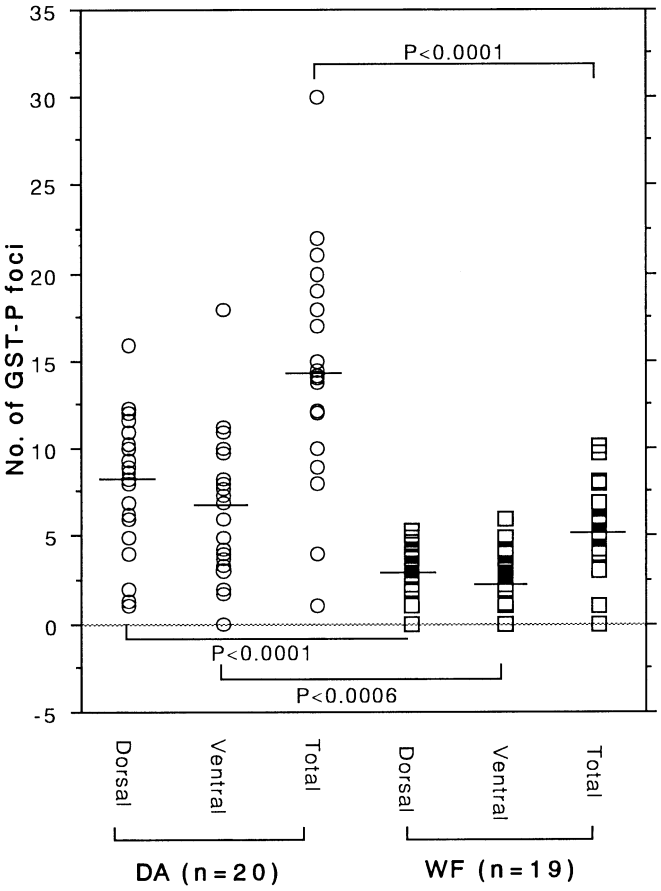

Fig. 3 GST-P$^+$ foci counts of non-neoplastic lingual epithelium (dorsal, ventral and total) in DA and WF experimental groups. Significant differences were determined using Mann-Whitney U-test

Of the three antibodies raised against human GSTs (GST-π, μ and α), GST-π appeared to give more consistent and darker staining in various human lingual lesions. The μ and α staining was usually weak, patchy and more variable. The results for GST-π staining in various human lingual lesions are summarized in Table 2. All lesions except 1 from the fibrous polyp group showed reactivity for GST-π. The staining was localized in cytoplasm, or nuclei, or both, and was mainly detected in epithelial cells. Well-defined positive foci, typical of the epithelial GST-P$^+$ foci in the rat model, were not seen in any of the human lesions, although focal GST-π strong staining was seen in areas (Fig. 4). In terms of GST-π staining intensity, overlying epithelium of fibrous polyp generally showed a weaker reactivity than epithelial dysplasia and carcinomas, but a considerable overlap was found among the latter groups, strong staining being detected in 60% of cases with mild to moderate dysplasia and 87.5% with severe dysplasia, in 75% cases of CIS and in 76.9% cases of SCC. Interestingly, GST-π staining pattern and epithelial distribution appeared to vary between different lesion

Table 2 Expression of GST-π in human benign, premalignant and malignant lingual lesions (*C* cytoplasm, *N* nuclear, *G* granular, *P* prickle, *B* basal)

Lesion	No.	Staining intensity (%)[a]			Staining pattern (%)[b]			Staining epithelial layer (%)[c]		
		–	+	++	C	C & N	N	G & P	P	P & B
Fibrous polyp	10	1 (10)	9 (90)	0	7 (77.8)	2 (22.2)	0	3 (33.3)	6 (66.7)	0
Mild to moderate dysplasia	10	0	4 (40)	6 (60)	9 (90)	1 (10)	0	5 (50)	4 (40)	1 (10)
Severe dysplasia	8	0	1 (12.5)	7 (87.5)	1 (12.5)	6 (75)	1 (12.5)	0	3 (37.5)	5 (62.5)
Carcinoma in situ	8	0	2 (25)	6 (75)	1 (12.5)	5 (62.5)	2 (25)	0	2 (25)	6 (75)
Squamous cell carcinoma	13	0	3 (23.1)	10 (76.9)	3 (23.1)	10 (76.9)	0	0	9 (69.2)	4 (30.8)

[a] Number of cases (percentage) showing the intensity of positive reaction; ++: strong, +: weak, –: no staining
[b] Number of cases (percentage) showing cytoplasm and/or nuclear staining
[c] Number of cases (percentage) showing staining within the various layers

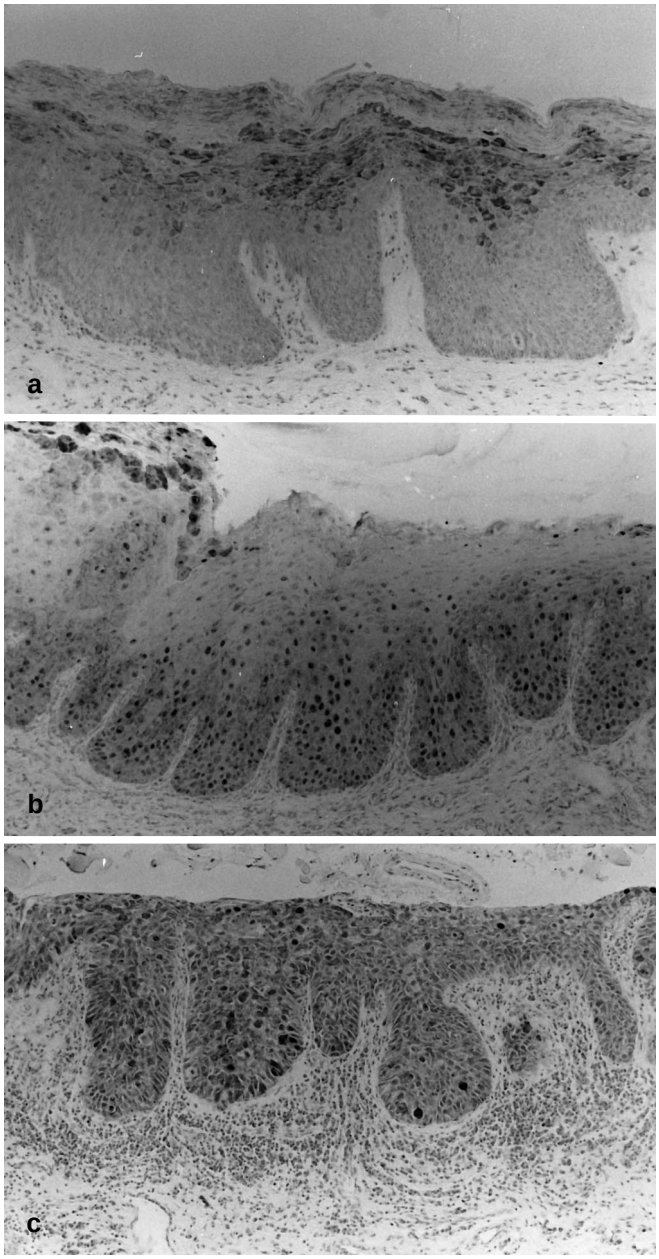

Fig. 4 **a** Mild epithelial dysplasia (×50), **b** severe epithelial dysplasia (×50) and **c** carcinoma in situ (×50) of human lingual biopsies stained for GST-π. Note the strong nuclear and basal/suprabasal reactivity in severe dysplasia and carcinoma in situ

groups. While fibrous polyp and mild to moderate dysplasia showed predominantly cytoplasm staining in the prickle and/or granular cell layers of the epithelium, the severe dysplasia, CIS and SCC groups tended to have more cases showing nuclear and/or basal cell reactivity. This trend of change was even more apparent in severe dysplasia and CIS lesions than in the SCC group (Table 2, Fig. 4). Consistent GST-π staining intensity, pattern and distribution were found within the SCC biopsies and their post-therapy surgical specimens. Staining of the metastatic tumour deposits within the lymph nodes was also identical to that of the initial biopsies.

Discussion

Administration of 4NQO dissolved in drinking water is known to induce a high incidence of squamous cell carcinoma in the upper digestive tract, including the tongue [32]. Our previous studies have demonstrated that there are significant differences in susceptibility to 4NQO-induced tongue carcinoma among seven strains of rats [8, 9]. Among those strains, DA and WF appear to represent the two ends of the spectrum of variation. While DA rats frequently developed multiple, large tongue carcinomas and had the highest tumour incidence and the shortest survival time, WF rats showed the lowest susceptibility to 4NQO. This contrasting strain-specific difference in oncogenicity between DA and WF rats was confirmed in the present study, and this genetic model was seemingly suitable for clarifying the sensitivity and reliability of the presumptive tumour marker, namely the π-class of GST.

GST-P has been found to be one of the best tumour markers in rat hepatocarcinogenesis, as it detects early alterations to hepatic foci by most hepatocarcinogens [6, 18–20, 29]. Recently, GST-P expression was detected in hamster buccal pouch mucosa treated by 7,12-dimethylbenz[a]anthracene (DMBA), and increased levels were described in the later stages of carcinogenesis [3, 34]. This study is the first to demonstrate that 4NQO treatment induces lingual epithelial expression of GST-P; specimens from normal control animals were negative for GST-P, but all 4NQO-induced tongue carcinomas invariably stained for GST-P. Well-defined epithelial GST-P^+ foci in the preneoplastic lingual epithelium varied quantitatively between DA and WF rats, reflecting their susceptibility and survival time. It has been suggested that in chemical carcinogenesis there are two opposing pathways in the initial metabolism of 4NQO, catalysed by cytosolic enzymes (including GSTs) either to promote the detoxification of 4NQO by its conversion to a glutathione conjugate [24] or via nitroreductive activation to form 4-hydroxyaminoquinoline 1-oxide, a proximate carcinogenic metabolite [13, 28]. The balance between 4NQO activation and detoxification is considered to be crucial in determining the oncogenicity [25]. Thus, the genetic variation associated with GST in different strains of rat may be an important factor in determining their susceptibility to 4NQO.

It is interesting to note that rat lingual epithelial GST-P^+ foci were often seen in the histologically non-dysplastic areas as well as in dysplastic areas, suggesting that GST-P may be used as a marker for the altered preneoplastic cell population in the absence of histological evidence of dysplasia. However, it is not known whether all or only a portion of the GST-P^+ epithelial cells are precursors of the progressive malignant lesions. In rat chemical hepatocarcinogenesis, for example, only a small portion of GST-P^+ hepatic cells undergo subsequent neoplastic transformation [7]. It is essential, therefore, to understand the mechanism(s) underlying the malignant potential/transformation of the GST-P altered cells. Recent studies in models of chemical liver carcinogenesis have

suggested that the role of GST-P may be to exert a permissive effect on cell cycle activity and the down-regulation of apoptosis, allowing expansion of an initiated cell population [26, 27]. Experiments are currently underway in our laboratory to examine whether p53 abnormality is related to 4NQO-induced epithelial GST-P$^+$ foci during rat lingual carcinogenesis.

The predominant form of GSTs present in most human tumours investigated is the π-class, and comparisons of matched pairs of normal and malignant tissues have revealed elevated levels in stomach, colon, bladder, cervix, skin, and lung as well as in head and neck tumours [2, 4, 15–17, 22, 31, 34]. Our results on human benign, premalignant and malignant lingual lesions are in general agreement with these previous studies. Overall, GST-π staining intensity was weaker in benign lesions than in dysplastic or neoplastic lesions. While staining intensity showed a considerable overlap between the varying degrees of dysplasia, CIS and SCC, some tendencies in staining pattern were indicated. In contrast to benign lesion and mild to moderate dysplasia, the cases of severe dysplasia, CIS and SCC showed a tendency to nuclear and/or basal cell staining. Nuclear staining of GST-π has already been previously reported in premalignant lesions of human oral mucosa [34] and skin [22]. Its presence has been related to the malignant transformation and progression in transitional cell carcinoma [30]. The increased occurrence of basal cell GST-π reactivity in severe dysplasia and CIS has not been described in oral premalignant lesions. However, observations of the present 4NQO rat lingual model and the DMBA-induced hamster buccal pouch models [3, 33] all demonstrated that small epithelial GST-P$^+$ foci initiated early seemed to involve only basal and suprabasal cells. With time, these foci could expand to involve the full thickness of the epithelium [33]. These data suggest that the basal cells of oral epithelium may be the target cells in oral carcinogenesis, and we speculate that relocalization of GST-π from the cytoplasm to the nucleus and/or a basal cell reactivity may be an early event in malignant transformation in human lingual lesions.

The data presented here in experimental and human conditions are not directly comparable. Under controlled experimental condition, expression of GST-P may well reflect the carcinogenic effect of 4NQO and the genetic susceptibility of animals. The well-defined epithelial GST-P$^+$ foci were analysed quantitatively and may be used as a variable in the follow-up of the formation of preneoplastic lesions. In contrast, the expression of GST-π in human lingual lesions was more complex, and the results were often overlapping between different lesion groups. The oral mucosa is subject to a multitude of physiological and pathological variables, such as dietary and oral micro-organisms, which we cannot control but which may influence the expression of GST-π in lingual epithelial cells. The demonstration of a more frequent GST-π nuclear and/or basal cell reactivity in severe dysplasia and CIS may nevertheless be useful in identifying high-risk lingual lesions in humans.

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