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Glutathione S-transferases and glutathione in human head and neck cancer

Theo P.J.Mulder³, Johannes J.Manni¹,
Hennie M.J.Roelofs, Wilbert H.M.Peters and
Anne Wiersma²

Departments of Gastroenterology, ¹Otorhinolaryngology and ²Pathology,
University Hospital St Radboud, PO Box 9101, Nijmegen, The Netherlands

³To whom correspondence should be addressed

Glutathione S-transferase (GST) enzyme activity, GST isoenzyme composition and glutathione (GSH) concentration were assessed in normal and squamous cell carcinoma specimens of 14 patients with oral or oropharyngeal cancer and 11 patients with laryngeal cancer. Comparing malignant with normal oral/oropharyngeal tissues, no significant differences in GSH content, GST enzyme activity or isoenzyme composition were found. However, some tumours had up to 3-fold increased GST enzyme activities and 11 malignant samples over-expressed GST- π . GST- π was present in all normal and malignant oral/oropharyngeal specimens investigated, whereas class α and class μ were detected in only a few samples. GST- μ was present in 28% of the patients with oral/oropharyngeal tumours as compared with ~60% in the normal population. GST- α , - μ and - π were detected in 91, 64 and 100% of the normal laryngeal tissues respectively. In laryngeal tumours significantly higher levels of GST- π and GSH but significantly lower amounts of GST- α were detected. Levels of class μ GST were generally lower in cancerous tissues, but differences were not significant. In comparison with normal oral/oropharyngeal tissues, normal laryngeal tissues contained almost twice the amount of GST enzyme activity due to higher class α enzyme levels. It is concluded that GST- π is elevated in 11 out of 14 tumours of the oral cavity and values are significantly increased in tumours of the larynx, which may contribute to the inherent anti-cancer drug resistance of these malignancies. In laryngeal tumours the increased GSH levels may confer additional resistance to radiation therapy.

Introduction

Oral/oropharyngeal and laryngeal cancers account for 2.1 and 1.3 cancer deaths per 100 000 population/year respectively in The Netherlands (1). Alcohol consumption and smoking have been implicated as causative factors in the development of these tumours. Early involvement of regional lymph nodes tends to occur in patients with these types of cancers. Chemotherapy, usually added for the treatment of extensive disease, is of limited use in oral/oropharyngeal and laryngeal cancer due to either intrinsic resistance or development of (multi-drug) resistance during treatment with chemotherapeutic drugs (2,3).

The mechanisms involved in drug resistance are poorly understood. *In vitro* studies with tumour cell lines selected for resistance to commonly used chemotherapeutics demonstrated that several interrelated mechanisms may be involved in

*Abbreviations: GST, glutathione S-transferase; GSH, glutathione.

the acquisition of the drug resistant phenotype. Decreased intracellular drug accumulation by increased expression of the P170 glycoprotein drug efflux pump (4), increased repair or tolerance to drug-induced damage by over-expression of DNA topoisomerases (5) and high metabolic drug inactivation by conjugation with glutathione (GSH*) (6) are all considered to contribute to the observed resistance.

Glutathione S-transferases (GSTs) are enzymes which catalyse the nucleophilic addition of GSH to electrophilic centres of a wide variety of compounds. This reaction is the first step in the formation of mercapturic acids, a pathway mostly resulting in the elimination of potentially toxic compounds (7,8). GSTs are also involved in the metabolism of several types of anti-cancer drugs and are over-expressed in many human refractory tumours (9). In cell lines resistant to anti-cancer drugs as diverse as alkylating agents, anthracyclines and *cis*-platinum, increased GST and GSH concentrations have been implicated as resistance mechanisms [see Tsuchida and Sato (9) for review and references]. High GSH and/or GST levels in tumours may therefore be a barrier to an effective treatment with chemotherapeutics. Based on structural, physicochemical, enzymatic and immunological properties, cytosolic GSTs are divided into three classes: α , μ and π (8). Recently a fourth class of GST (θ) has been described (10). The predominant form present in most investigated human tumours is class π GST and comparison of matched pairs of normal and malignant tissues revealed elevated levels in stomach, colon, bladder, cervix and lung tumours (9,11-13).

Since different classes of GSTs have distinct substrate specificities, GST subclass composition may influence the detoxifying ability of tissues (8). Therefore we examined GST activity, GST isoenzyme composition and GSH levels in squamous cell carcinoma and corresponding normal epithelium of 14 patients with oral/oropharyngeal carcinoma and in 11 patients with laryngeal carcinoma.

Materials and methods

Tissue samples

Twenty-five patients with oral/oropharyngeal or laryngeal squamous cell carcinoma were operated upon at the Department of Otorhinolaryngology and Maxillofacial Surgery of the University Hospital St Radboud. All patients received primary surgical treatment. Patient data are given in Table I. Representative parts of tumour and adjacent non-malignant mucosal tissue were excised by a pathologist. From six patients samples of neck lymph node metastases were obtained from the same resection specimen. The neck metastasis of patient no. 4 occurred 5 months after resection of the primary tumour and was removed by radical neck dissection.

All tissue samples obtained were frozen within 20 min after resection of the tumour and stored at -70°C . Tissue fragments (10-100 mg) were thawed and homogenized on ice in 5 volumes of homogenizing buffer (250 mM sucrose, 20 mM Tris-HCl, 1 mM dithiothreitol, pH 7.4) using small glass-glass homogenizers. The homogenates were centrifuged at $150\,000\times g$ at 4°C for 1 h. Supernatants (cytosolic fractions) were divided into portions and stored at -70°C .

Assays

Protein was assayed by the method of Lowry *et al.* (15). GST enzyme activity with 1-chloro-2,4-dinitrobenzene as substrate by the method of Habig *et al.*

Table I. Patient and tumour characteristics

Patient number	Age	Gender	Site of primary tumour	Classification ^a	Differentiation grade tumour	Metastatic tissue available
Patients with oral/oropharyngeal tumours						
1	54	M	Tonsil	pT ₃ N ₀ M ₀	Moderate	-
2	66	M	Tongue	pT ₂ N ₀ M ₀	Moderate	-
3	64	M	Floor of mouth	pT ₂ N ₀ M ₀	Moderate	-
4	60	F	Tongue	pT ₂ -M ₀	Well	+ ^b
5	70	M	Floor of mouth	pT ₄ N ₂ aM ₀	Moderate	-
6	61	M	Base of tongue	pT ₂ N ₁ M ₀	Moderate	+
7	61	M	Base of tongue	pT ₂ N ₁ M ₀	Moderate	-
8	74	M	Base of tongue	pT ₃ N ₂ bM ₀	Moderate	-
9	66	M	Tongue	pT ₂ N ₁ M ₀	Moderate	-
10	47	M	Lateral pharynx wall	pT ₂ N ₀ M ₀	Moderate	-
11	59	M	Base of tongue	pT ₂ N ₀ M ₀	Moderate	-
12	60	M	Floor of mouth	pT ₃ N ₀ M ₀	Moderate	-
13	65	M	Lateral pharynx wall	pT ₄ N ₀ M ₀	moderate	-
14	65	M	Lateral pharynx wall	pT ₂ N ₀ M ₀	Poor	-
Patients with laryngeal tumours						
15	57	M	Transglottic	pT ₄ N ₂ aM ₀	Poor	+
16	84	M	Supraglottic	pT ₄ N ₀ M ₀	Poor	-
17	61	M	Supraglottic	pT ₄ N ₀ M ₀	Poor	-
18	66	M	Supraglottic	pT ₄ N ₂ aM ₀	Moderate	+
19	65	M	Glottic	pT ₄ N ₂ aM ₀	Moderate	+
20	62	M	Supraglottic	pT ₄ N ₀ M ₀	Moderate	-
21	63	M	Supraglottic	pT ₄ N ₁ M ₀	Moderate	-
22	72	M	Transglottic	pT ₄ N ₀ M ₀	Moderate	-
23	69	M	Supraglottic	pT ₃ N ₂ aM ₀	Moderate	+
24	60	M	Supraglottic	pT ₄ N ₂ cM ₀	Moderate	+
25	49	M	Supraglottic	pT ₄ N ₀ M ₀	Moderate	-

^aStaging according to the International Union Against Cancer Classification (14).

^bNeck metastasis was removed 5 months after resection of the primary tumour.

(16). Total GSH was quantified by high performance liquid chromatography as described by us recently (17). GSTs class α , μ and π were quantified in cytosolic fractions by densitometric analyses of immunoblots, essentially as described before (12). The coefficient of variation of this method is 10–15%. Class α antibodies react against GST A1-1, GST A1-2 and GST A2-2 (12), class μ antibodies recognize GST M1a-1a, GST M1a-1b and GST M1b-1b (18,19) and class π antibodies are directed against GST P1-1 (20). Cross-reactivity of the above-mentioned monoclonal antibodies with recently discovered (i.e. GST M2-M5) or yet unknown GST isoforms cannot be excluded.

Calculations and statistical analyses

Total GSH was calculated as nmol/mg protein. Concentrations of GST subclasses were calculated as μ g/mg total cytosolic protein. Values below the detection limit of the immunoblot assays (~ 0.02 μ g/mg protein) were evaluated as zero.

Wilcoxon's matched-pairs signed-ranks test was used to evaluate differences between pairs of normal and malignant tissue. Differences between non-paired samples were analyzed using the Mann-Whitney *U*-test. Correlations between the parameters were tested with Pearson's linear correlation procedure. Differences were considered significant if the *P* value was < 0.05 .

Results

Oral cavity and oropharynx

In most normal and squamous cell carcinoma specimens GST- α levels were below the detection limit (Table II).

Four patients (29%) expressed GST- μ in normal as well as in malignant tissue and these tumours contained less GST- μ than the surrounding normal tissue.

GST- π was by far the major subclass present and accounts for 92 and 97% of the cytosolic GST protein in normal and malignant tissues respectively. GST- π content was higher in the tumours of 11 out of 14 pairs of samples and the mean tumour value was also higher (3.57 ± 0.61 versus 2.78 ± 0.37 μ g/mg protein in normal tissue), but the difference was not significant (*P* = 0.12).

Mean GST activity was higher in the malignant tissues (291 ± 66 versus 195 ± 23 nmol/min/mg protein in normal tissues) and tumour GST activities were higher in the same 11 patients with increased tumour GST- π levels. However, differences were not significant (*P* = 0.08).

Most tumours (10/14) contained less GSH than the matched normal tissue, resulting in a slightly lower mean GSH concentration in the tumours (26.9 ± 1.6 versus 30.5 ± 3.0 nmol/mg protein in normal epithelium), but differences were not significant (*P* = 0.25).

The two lymph node metastases investigated were similar to the corresponding primary tumour in all aspects analyzed.

Larynx

Mean GST- α levels were lower in tumours (0.26 ± 0.17 versus 1.38 ± 0.43 μ g/mg protein in normal mucosa) and in nine of the 11 pairs the tumour contained less GST- α than the normal mucosa (Table III, *P* < 0.05).

GST- μ was expressed in six patients (55%) and in five of these patients the tumour contained less GST- μ than the surrounding normal tissue, but the difference was not significant (*P* = 0.18).

GST- π was the predominant GST subclass detected in all samples and accounts for 63 and 89% of the total GST protein in normal tissues and tumours respectively. All except one carcinoma contained higher GST- π concentrations than the matched normal tissue (*P* < 0.01) and mean GST- π level was higher in tumour tissue (4.67 ± 0.47 versus 3.31 ± 0.39 μ g/mg protein in normal mucosa).

Mean GST activity in laryngeal tumours was higher than in surrounding normal tissues (495 ± 73 versus 382 ± 73 nmol/min/mg protein respectively), but no significance was reached (*P* = 0.47).

Table II. GST activity, GST isoenzyme level and GSH concentration in cytosols of oral/oropharyngeal tumours, corresponding normal tissues and metastases

Patient number	Tissue	GST Activity (nmol/min/ mg protein)	Content ($\mu\text{g}/\text{mg}$ protein)			GSH (nmol/mg protein)
			α	μ	π	
1	N	320	—	—	3.68	36.0
	T	131	—	—	1.66	23.5
2	N	84	—	—	1.25	17.4
	T	135	—	—	1.71	30.2
3	N	182	0.07	—	1.81	29.8
	T	245	0.05	—	2.70	23.6
4	N	168	—	0.61	2.46	29.2
	T	191	0.09	0.17	3.26	24.4
	M	166	—	0.43	2.87	32.7
5	N	195	0.03	—	2.64	27.3
	T	438	0.05	—	4.20	31.7
6	N	228	—	1.05	1.27	32.9
	T	307	—	0.93	2.94	24.2
	M	183	—	0.44	2.03	20.6
7	N	98	—	—	1.84	11.0
	T	143	—	—	2.06	28.0
8	N	204	—	0.56	2.58	23.3
	T	211	—	0.15	2.79	35.2
9	N	166	—	1.12	1.02	23.0
	T	226	—	0.25	2.32	19.1
10	N	79	—	—	2.81	40.7
	T	150	—	—	3.32	26.4
11	N	181	—	—	4.29	48.6
	T	511	—	—	8.26	32.5
12	N	322	—	—	5.44	48.9
	T	233	—	—	3.97	32.9
13	N	154	—	—	2.87	20.9
	T	104	—	—	1.80	13.1
14	N	355	—	—	5.00	38.2
	T	1053	—	—	8.97	31.7
Mean \pm SEM	N	195 \pm 23	0.01 \pm 0.01	0.24 \pm 0.11	2.78 \pm 0.37	30.5 \pm 3.0
	T	291 \pm 66	0.01 \pm 0.01	0.11 \pm 0.07	3.57 \pm 0.61	26.9 \pm 1.6

N, normal tissue; T, tumour; M, metastasis; —, not detectable.
Values are given as mean \pm standard error of the mean.

Mean GSH concentration in laryngeal tumours was almost twice as high as in normal tissues (40.9 ± 4.9 versus 23.6 ± 5.2 nmol/mg protein respectively) and in nine of the 10 matched pairs the tumour contained more GSH ($P < 0.05$).

GST subclass composition as well as GST activity and GSH concentration in four of the five lymph node metastases were similar to primary tumours. However, one metastasis (patient no. 15) contained very high levels of GSH, GST- μ and GST- π and consequently had a high GST enzyme activity.

Comparison of normal laryngeal with normal oral/oropharyngeal epithelium

In contrast to normal oral/oropharyngeal mucosa samples, GST- α was detected in all samples of normal laryngeal mucosa ($P < 0.001$, Tables II and III). In addition, laryngeal mucosae contained significantly more GST enzyme activity than oral/oropharyngeal tissues ($P < 0.001$). GST- μ , GST- π and GSH concentrations in normal laryngeal mucosae were not significantly different from those in normal oral/oropharyngeal mucosae.

GST activity measured in all samples correlated well with calculated GST activities (Figure 1). Calculations were based on the isoenzyme levels determined by immunoblot and assuming specific activities (with 1-chloro-2,4-dinitrobenzene as substrate) of 187, 105 and 64 $\mu\text{mol}/\text{min}/\text{mg}$ protein for class μ , π and α respectively (8).

Discussion

Calculated GST enzyme activities, based on GST subclass levels quantified on immunoblot, demonstrated a good linear correlation with the measured GST enzyme activities. Moreover, the slope of the regression line was ~ 1 , indicating that almost all GST activity towards 1-chloro-2,4-dinitrobenzene in the cytosolic fractions was accounted for by GST subclass immunoreactivity on Western blots. The failure to pass the origin may be due to underestimation of the highest calculated GST activities.

Normal laryngeal mucosae had significantly higher GST- α concentrations and GST enzyme activities than normal oral/oropharyngeal samples. Mean GST- μ and GST- π levels were also higher in normal laryngeal mucosae, whereas GSH levels were higher in normal oral/oropharyngeal samples, but differences were not significant.

Teicher *et al.* (21) developed a *cis*-platinum-resistant head and neck squamous cell carcinoma cell line which, among other changes, displayed an increased GST activity. Numerous cell lines selected for resistance towards common chemotherapeutic drugs over-express GSTs. In addition, many refractory tumours have higher GST activities than the normal tissues they originated from (9). Since GSTs are involved in metabolizing several clinically relevant anti-cancer drugs (6), it has been suggested that increased GST concentrations may account for

Table III. GST activity, GST isoenzyme level and GSH concentration in cytosols of laryngeal tumours, corresponding normal tissues and metastases

Patient number	Tissue	GST			GSH (nmol/mg protein)	
		Activity (nmol/min/mg protein)	Content (µg/mg protein)			
			α	μ		π
15	N	279	1.37	0.60	2.08	17.6
	T	354	—	0.31	3.67	35.3
	M	1752	—	2.94	12.77	140.8
16	N	405	1.58	0.30	3.53	ND
	T	397	0.06	0.07	3.92	39.0
17	N	596	1.40	2.27	4.14	28.6
	T	570	0.40	1.43	4.89	32.2
18	N	540	2.55	—	5.68	22.8
	T	381	—	—	6.75	78.1
	M	339	—	—	6.22	64.7
19	N	389	0.16	1.46	1.75	13.8
	T	178	—	0.10	2.44	24.2
	M	260	—	1.50	2.88	40.8
20	N	536	4.99	—	4.80	55.6
	T	512	—	—	5.43	35.9
21	N	381	1.47	—	2.81	23.1
	T	322	—	—	2.42	26.4
22	N	274	1.16	0.13	2.90	6.3
	T	765	0.36	1.04	4.52	41.6
23	N	233	—	0.44	2.62	17.0
	T	1038	1.88	—	7.20	39.5
	M	276	—	—	3.95	0.0
24	N	207	0.38	—	1.90	5.2
	T	332	—	—	4.30	43.7
	M	199	—	—	2.36	42.0
25	N	371	0.16	1.17	4.25	46.4
	T	600	0.17	0.39	5.88	52.0
Mean ± SEM	N	382 ± 39	1.38 ± 0.43	0.58 ± 0.23	3.31 ± 0.39	23.6 ± 5.2
	T	495 ± 73	0.26 ± 0.17 ^a	0.30 ± 0.15	4.67 ± 0.47 ^a	40.9 ± 4.9 ^a
	M	565 ± 298	0.00	0.89 ± 0.59	5.64 ± 1.90	57.7 ± 23.3

N, normal tissue; T, tumour; M, metastasis; —, not detectable; ND, not determined.

^a*P* < 0.05 versus normal laryngeal mucosa. Values are given as mean ± standard error of the mean.

part of the inherent drug resistance observed in various human tumours (9).

Moscow *et al.* (22) reported a 7-fold increase in GST-π mRNA levels in five squamous head and neck cancers as compared with adjacent normal tissue. In addition, Riou *et al.* (23) found increased levels of GST-π mRNA in squamous cell carcinomas of the cervix and Eimoto *et al.* (24) observed higher GST-π immunoreactivity in squamous cell carcinomas of the lung when compared with other histological types of lung cancer. In contrast, oesophageal squamous cell carcinomas had lower mean GST-π concentrations than the surrounding normal tissue, but tumours with much higher GST-π levels were also found (25). The head and neck squamous cell carcinomas analysed in the present study contained more GST-π (~30% in oral/oropharyngeal and 40% in laryngeal tumours) than the surrounding normal tissue in 21 out of 25 pairs of samples. In laryngeal samples the differences in GST-π concentrations were significant. Previously, Janot *et al.* (26) reported a slightly higher mean GST activity and GST-π concentration in a limited number of normal and malignant specimens from the pyriform sinus of the larynx, however, differences did not reach significance. In a more extended study, Parise *et al.* (27) reported significantly higher GSH and GST enzyme activity levels in head and neck tumours as compared with paired control tissues.

Since GST-π is by far the most prominent GST isoenzyme

in both normal and malignant head and neck tissue, GST enzyme activities are largely determined by GST-π levels. Analogous to GST-π levels, most tumours therefore contain more GST enzyme activity than the tissues they originated from (mean GST activity was almost 50% higher in oral/oropharyngeal and 30% higher in laryngeal tumours), but differences were not significant.

Several refractory human cancers investigated contained elevated levels of GST-π, suggesting a special role in drug resistance for this isoform (9). Studies by Lewis *et al.* (28) and Berhane *et al.* (29) indicate that class α and μ GSTs may also be involved in anti-cancer drug resistance. However, GST-α and GST-μ levels were either undetectable or lower in the tumour tissues investigated here and it seems unlikely that these two GST subclasses contribute to intrinsic drug resistance in head and neck tumours. In contrast, GST-π concentrations were higher in more than 80% of the head and neck tumours when compared with the adjacent normal mucosa and therefore this isoenzyme may mediate part of the inherent anti-cancer drug resistance that is frequently encountered in these squamous cell carcinomas.

Due to a genetic polymorphism, only ~55% of the Caucasian population has one or two functional GSTM 1 genes. Presence of GSTM 1-1 in smokers has been associated with a lower risk of squamous cell carcinoma of the lung (30,31), and bladder or larynx cancer (32). We did not assess smoking

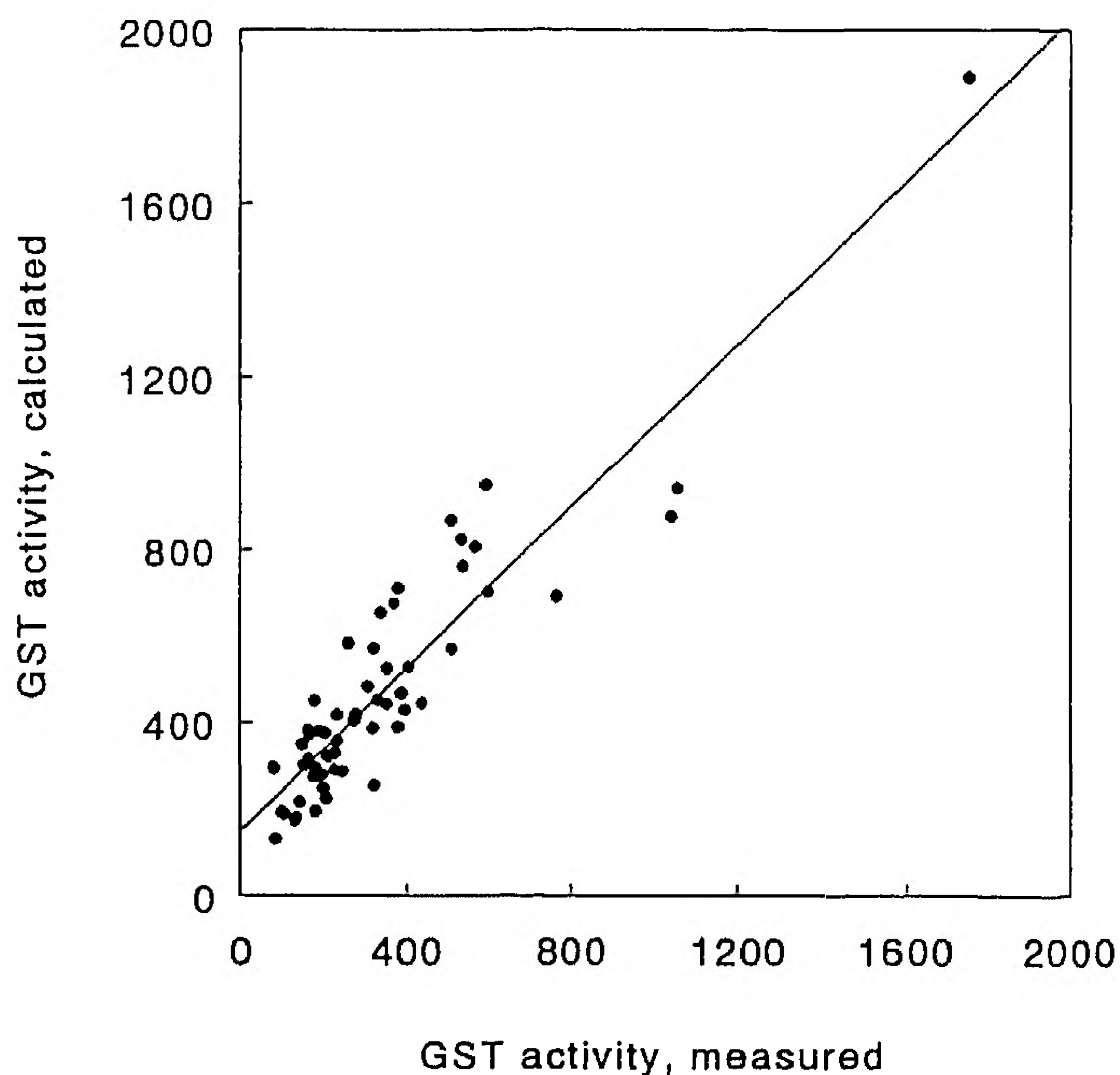


Fig. 1. Plot of calculated versus measured GST enzyme activities. Values are given as nmol/min/mg protein. GST enzyme activities (GST activity measured) was determined in all 57 samples with 1-chloro-2,4-dinitrobenzene as substrate. GST activities were calculated (GST activity calculated) from the sum of the concentrations of GST subclasses as determined by immunoblot, multiplied by their specific activities with 1-chloro-2,4-dinitrobenzene (187, 105 and 64 $\mu\text{mol}/\text{min}/\text{mg}$ protein for class μ , π and α respectively; 8). Calculated GST activities = $0.94 \times (\text{measured GST activities}) + 150$; correlation coefficient 0.92.

habits of our patients, but 29% of the patients with oral/oropharyngeal tumours and 55% of the patients with laryngeal tumours were GSTM 1-1-positive. Overall, 10 of the 25 patients (40%) did express GSTM 1-1, which is not significantly different from the 55% value reported for the general population (31). However, the characteristics of laryngeal and oral/oropharyngeal tissue with respect to GST subclass composition are rather different and the latter group especially needs further study with respect to GSTM 1-1 expression.

High GSH levels may mediate resistance towards both radiation therapy and treatment with some anti-cancer drug regimens (33–35). Buthionine sulfoximine, a specific inhibitor of GSH synthesis, circumvents cisplatin resistance in human head and neck squamous cell carcinomas grown in nude mice (36). The high GSH levels found in most laryngeal tumours may therefore contribute to inherent drug and radiation resistance in such tumours.

Recently, Fountzilias *et al.* (37) reported more complete responses during platinum-based chemotherapy for oral carcinomas than for laryngeal carcinomas; 46 and 10% respectively. In the present study, laryngeal tumours were found to contain higher GST and GSH levels than oral/oropharyngeal squamous cell carcinomas, which might also be indicative of a role for the GST/GSH system in anti-cancer drug resistance. However, data on tumours which were treated with chemotherapeutics are required before definite conclusions can be drawn.

Regional neck lymph node metastases are frequently encountered in head and neck cancer. To our knowledge no data on GST composition of such metastases have been published.

The few metastases we were able to analyse had similar GST and GSH levels to their primary tumours, with the exception of one lymph node metastasis from a laryngeal tumour, which contained both a very high GST activity and a very high GSH concentration. If GST and GSH contribute to drug and/or radiotherapy resistance in primary tumours, regional metastases with very high levels may be even less responsive to such therapies.

In conclusion, GST- π is the predominant GST subclass detected in both normal tissues and in human oral/oropharyngeal and laryngeal squamous cell carcinomas. The malignant tissues generally contain more GST- π than the surrounding normal tissues. In addition, GSH levels were higher in laryngeal tumours when compared with adjacent normal tissue. These high GST- π and GSH levels may occasionally be involved in the intrinsic drug resistance of head and neck cancers.

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