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Review

The Role of Glutathione S-Transferases in Head and Neck Squamous Cell Carcinogenesis

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INTRODUCTION

HEAD AND NECK carcinogenesis is thought to be a multistep process [1, 2]. In the first instance, carcinogen-induced genetic events are the driving force in susceptible cells, when cells have gained a selective growth advantage and have undergone clonal expansion, leading to the life-threatening malignancy [3]. In epithelial lining tissues, like the mucosa of the head and neck, the first step in carcinogenesis is thought to be generated by the covalent binding of exogenous electrophilic xenobiotics to DNA [3]. More and more evidence exists that glutathione S-transferases (GST) are important enzymes involved in detoxification processes of potential carcinogenic compounds, including electrophilic agents which are potentially harmful to DNA.

Presently, the availability of antibodies directed against the many molecular forms of GSTs, molecular techniques, e.g. the polymerase chain reaction, and specific enzyme activity measurements, give factual knowledge about this matter. In particular, antibody research is being investigated on their value in predicting cancer prognosis. In addition, serum GST levels are currently being tested as potential markers for cancer detection. With this background, we review recent progress in the knowledge of GSTs in relation to head and neck squamous cell carcinoma (HNSCC).

GLUTATHIONE

Glutathione (GSH) is the most ubiquitous and abundant intracellular thiol acting in concert with a wide spectrum of anti-oxidative agents, such as vitamins C and E, and carotenoids as a universal protectant against electrophilic attacks. GSH plays a prominent part in antimutagenesis and anti-carcinogenesis by donating a hydrogen atom to certain free radicals. Trapping of electrophiles is achieved by direct interaction with GSH or by enzymatic conjugation with GSTs [4]. GSH is synthesised within many cells from its constituent non-essential amino acids, glutamate, cysteine and glycine, which are synthesised by the body or obtained via the diet. Under conditions of GSH depletion the electrophilic load for DNA damage will increase. However, since GST isoenzymes catalyse GSH detoxification pathways, the geno- and cytotoxicity of electrophilic substrates is greatly reduced, even when GSH is scarcely available.

Tumour cells exhibit the (sometimes high) capacity to synthesise GSH, and exhibit elevated cellular GSH concentrations when compared with non-tumoral adjacent tissues [5]. The knowledge that notably drug- and radiation-resistant tumour cells exhibit high cellular GSH levels makes modulators of the GSH metabolism and GSH synthesis inhibitors of interest in increasing the efficacy of treatment-resistant tumours. In summary, high cellular GSH levels could arrest effective cancer treatment by electrophilic drugs, but could, in a precancerous stage, protect against tumour development.

Based on the knowledge that *N*-acetylcysteine has the ability to scavenge noxious free radicals by itself, and supports GSH biosynthesis, cancer chemoprevention research has directed its attention to the application of this antioxidant. At present, *N*-acetylcysteine is tested in the prospective Euroscan trial, a chemoprevention study in curatively treated patients with early stage oral, laryngeal and lung cancer. The aim of this trial is to prevent or delay the occurrence of second primary tumours [6]. Recently, we showed in a relatively small group of patients, participating in the Euroscan trial, that treatment with *N*-acetylcysteine leads to increased thiol concentrations in blood plasma and erythrocytes [7].

GLUTATHIONE S-TRANSFERASES

GSTs are multifunctional intracellular, soluble or membrane-bound enzymes, which catalyse the conjugation of many electrophilic, hydrophobic toxins and carcinogens with the sulphur atom of the tripeptide GSH. The K_m values for most of the GST-catalysed conjugations of alkylating agents with GSH are in the low micromolar range. Therefore, these enzymes are still very good catalysts under conditions of considerable GSH depletion. Besides this function, they also act as binding proteins in various detoxification processes [4]. Removal of these xenobiotic GSH-conjugates from the interior of the cell into vesicles has recently been shown by studies concerning multiple drug resistance proteins to occur by energy dependent membrane transporters. The precise

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identity of these pumps is as yet unknown, but membrane proteins concerned with the efflux of GSH-conjugates are being identified [8]. Export from the cell of GSH-conjugates is sometimes, and only in some organs (e.g. the kidney), preceded by degradation along the mercapturic pathway, although this is not necessary for adequate excretion.

The cytosolic GSTs form a multigene family of dimeric proteins divided into four classes, on the basis of distinctive isoelectric focusing properties, amino acid sequence and immunoreactivity [9, 10]. Although separate classes have been identified, they share homology and probably have evolved from a common ancestral gene. Separate classes exist of the multigene α, μ, π and θ classes. Class α are basic, class π acidic and class μ near neutral proteins [11]. Until now, most attention has been focused on the cytosolic GSTs, and less is known about the function of the membrane-bound GSTs. Although little structural homology between membrane-bound and cytosolic GSTs exists, any common functionality is not precluded [9]. A known function of the membrane-bound GSTs is the protection of membranes against lipid peroxidation by their glutathione peroxidase activity of hydroperoxides, thus protecting cells from oxidative stress [12]. GSTs detoxify not only exogenous metabolites, but also endogenous chemical compounds, such as heme, bilirubin, polycyclic aromatic hydrocarbons and metabolites of arachidonic acid by direct binding [13, 14].

Most of the exploratory work has been done in the rat, but as more has become known about human GST, it is apparent that much information obtained from the rat is relevant to man. The same multigene families are seen and there is considerable homology in the primary structures across the two species [15]. Within a species, homology between classes is 25–30%, and that between members of the same class is 75–95%. GSTs have catalytic activity either as homo- or as heteromers, which can be formed between the subunits belonging to the same subclass. Each subunit of a dimer exhibits catalytic activity independently of the other, possessing a hydrophobic binding site for electrophilic substrates (H-site) and a binding site for GSH (G-site). The H-site is presumed to be specific for each subunit, whereas the G-site may be similar for all GSTs [16]. Xenobiotics, including carcinogens and anticancer drugs, can influence the transcriptional regulation of GST genes. Enhanced expression of GSTs has been found in many tumours. Discrepancy between GST-expression, as measured at the protein and RNA level, and enzyme activity studies has, to our knowledge, until now not been described. These results suggest that increased enzyme expression correlates with increased detoxification potential. However, the anticipated role of GSTs in cancer prevention provides potential overlap with anticancer drug resistance, since toxic chemicals with electrophilic centres are used in cancer chemotherapy [17].

GLUTATHIONE S-TRANSFERASE π

GST-π appears to be the most ubiquitous of the human GST isoenzymes. The regulatory mechanism involved in the expression of GST-π, isolated from both rat (referred to as GST-P) and human placentas, is of particular interest in carcinogenesis-associated processes. It is present in many tissues with the highest concentations in cells of the alimentary tract, lung, and erythrocytes [18, 19]. The expression of GST-π is not only tissue-specific, but also depends on the stage of development. GST-π is inducible and frequently over-expressed in a wide variety of human (pre)neoplastic tissues when compared with their normal counterparts ([9], for colon cancer see [20], for renal carcinoma see [21], and for lung cancer see [22]). Developmental changes in enzyme expression, which may be re-activated by induction mechanisms, make altered GST-π expression in carcinogenesis an interesting regulatory mechanism and possible tumour marker. Early studies revealed that GST-P in the rat increases 30- to 50-fold during chemical hepatocarcinogenesis [23]. Furthermore, GST-P has been found to be a good and early tumour marker in the hamster buccal pouch mucosa model, appearing in carcinogen-induced preneoplastic and neoplastic lesions as positive foci [24].

Whereas GST-π is supposed to have a protective role in carcinogenesis, more and more indications exist that this same isoenzyme is involved in multiple drug resistance. There is presently little direct evidence that any anticancer drug is a direct substrate for GST-π. The involvement of GST-π in multiple drug resistance is most likely a consequence of a pleiotropic stress response [17].

Immunohistochemical staining, using antibodies directed against GST-π can successfully be applied to detect early precancerous states, dysplasia, or differentiated carcinomas. Indeed GST-π is a tumour marker for many tumours [25]. GST-π is usually the most common of the GSTs in tumour tissues and, therefore, the easiest to detect experimentally. The predominance of GST-π in the literature reflects this practical fact. However, this does not preclude a major role for other GSTs. Qualitative and quantitative increased immunohistochemical GST-π staining has already been reported in moderate and severe dysplasia and carcinoma *in situ* of the oral cavity and the oesophagus when compared to normal tissue (see Table 1) [26]. Also, in "normal" tissue surrounding an oesophageal carcinoma, elevated GST-π levels have been detected, reflecting possibly a (pre)cancerous field effect [27]. GST-π expression has been reported to be increased and to be the predominant isoenzyme among three classes of GSTs in non-small cell lung cancer tumour tissue [28]. In HNSCC the higher activity of GST-π might have a genetic basis, because the GST-π gene is located on band q13 of chromosome 11, which is often amplified in this tumour type [29].

GST-π levels in serum are considered to be a useful aid for early diagnosis, predicting tumour extent and as a follow-up marker. Investigations of GST-π content in sera of 61 patients

Table 1. Quantitative tissue distribution of glutathione S-transferase subclasses in squamous cell carcinomas of the respiratory and upper digestive tract in comparison with the corresponding healthy mucosa based on cellular protein expression as visualised by immunohistochemical studies

	GST-class			
Tissue	α	μ	π	References
Oral cavity	?	=/−*	++	[26]
Larynx	=	=/−*	+	[58]
Oesophagus	++	+/−*	=	[63]
Lungs	++	=/−*	++	[28, 42, 22]

?, To our best knowledge, not reported in the literature; =, comparable amounts in tumour and healthy tissue; +, moderately higher levels in tumours; ++, strongly elevated levels in tumour; −, absent; *, 40–50% of the individuals express the M0 allele.

with oral cancer revealed significant elevated serum levels in 50% of cases with stage I and II disease and in 70% of cases with stage III and IV disease [30]. Furthermore, elevated levels were also discovered in most patients with tumour relapse before recurrence was detected clinically [30]. However, more studies with more patients have to be carried out to prove the definitive value of this marker. Also, in oesophageal carcinoma, elevated levels of GST-π have been shown to be a tumour marker [30] and in non-small cell lung cancer, GST-π alone (sensitivity 41.3%) or in combination with carcinoembryonic antigen (CEA) (sensitivity 64.5%) appears to be a superior tumour marker over other tested markers [31].

GLUTATHIONE S-TRANSFERASE-μ

To date, five members of the μ class of GST have been identified in human tissues, designated GSTM1–5 [32, 33]. The μ form shows higher efficiency for conjugating epoxides such as benzo[*a*]-4,5-oxide and styrene-7,8-oxide and other aromatic hydrocarbon epoxides and diolepoxides than either α or π forms [9, 34, 35]. Specific GSTM1 activity can be detected by measurements with the substrate *trans*-stilbene oxide [36]. GSTM1 displays different functional alleles, GSTM1*a and GSTM1*b, and GSTM1*0, a null allelotype, expressed by a significant number of people, who are lacking a constitutive expression of the isoenzyme. The numbers vary from 40 to 50%, depending on the race and the methodology used, namely measurements with *trans*-stilbene oxide or genotyping using PCR [37, 38]. Carcinogenesis could be related to the insufficient capacity to detoxify tobacco related carcinogens, like benzo(*a*)pyrene, by a GSTM1*0 carrier. Indeed, several studies have indicated that individuals homozygous for this null allele, have an increased risk of smoking-related cancers, such as laryngeal [39], lung [40] and bladder cancer [39]. Odds ratio analysis indicates that smokers with this polymorphic variant have an approximately 2-fold higher risk of developing these cancers. Lack of expression of GST-μ is related to increased DNA damage by mutagens in smoke [41]. It has even been shown that increasing GST-μ activity was associated with decreasing risk in a dose–response pattern [42]. The gene loci for GSTM1 and GSTM2 are closely linked on chromosome 1p. It has been suggested that the GSTM1*0 allele has resulted from an unequal crossover between these two loci [32]. Genetic polymorphism has not been described for GSTM2 or for any of the other μ class GST enzymes. Correlations between genetic characteristics and elevated susceptibility for environmental toxicities are a promising way of identifying patients at high risk of developing cancer in the future. Another useful approach of markers like GSTs is the identification of high risk groups of treatment failure, as recently shown in a study of 71 children with acute lymphoblastic leukaemia. They showed inferior relapse-free survival when lymphoblasts were positive for GST-μ [43]. In such high risk groups more aggressive therapy with greater side effects is justified.

GLUTATHIONE S-TRANSFERASE-α

Originally, GST-α was called ligandin, because its first known function was the transport of bilirubin within the hepatocyte [44]. Cytosolic class α isoenzymes generally exhibit high peroxidase activity. Characteristic properties of the class α GSTs include the catalysis of the isomerisation of ketosteroids and the selenium-independent glutathione peroxidase activity towards C19 and C21 Δ5-3-ketosteroids [45].

Class α consists of two different monomers, encoded by two different genes on chromosome 6, identified as B1 and B2 [46]. Developmental and age-dependent changes in the amounts of these isoenzymes have been observed [47]. In certain pathological conditions an increase of GST-α has been shown. The association of drug resistance between GST-α and nitrogen mustards has been described [17]. In contrast, various tumour types exhibited a dramatic decrease in the level of GST-α enzymes [48].

GLUTATHIONE S-TRANSFERASE-θ

GST-θ has been identified as a GST enzyme concerned in the detoxification of monohalomethanes, dichloromethane and ethylene oxide. Approximately 60–70% of the human population is able to carry out the conjugation reaction of monohalothanes with GSH. The others show a null phenotype, and are unable to execute this conjugation reaction of naturally occurring halothanes. The GSTM1 locus has attracted interest because of its absence in approximately 50% of the population and the observed elevated lung cancer risk. However, a positive GST-θ conjugator status is not necessarily beneficial because conjugation of monohalomethanes and ethylene oxide is detoxifying, whereas conjugation of dichloromethane yields a mutagenic metabolite [49]. Dichloromethane metabolites can induce liver and lung tumours in mice and, therefore, GST-θ polymorphism is thought to be associated with a greater risk of cancer [50].

GLUTATHIONE S-TRANSFERASES AND CARCINOGENESIS

It is conceivable that the increase of GST in (pre)neoplastic tissue of the head and neck is a response to ongoing carcinogenic damage during the multistep carcinogenic process. However, the crucial significance of elevated GST expression in preneoplastic tissues has not been known until now. An increase in GST activity may be a response to exposure to noxious, potentially carcinogenic compounds. According to this hypothesis, the progression of preneoplastic alterations into more advanced neoplastic lesions could be prevented by elevated GST levels. However, the opposite may also be true. It has been noted that GSTs can convert substrates via conjugation with GSH, into either cytotoxic, genotoxic or mutagenic metabolites [47]. Another theory, also overshadowing the benefit of elevated GST tissue levels is the nonsubstrate ligand binding theory [51]. Usually the binding of substrates to GSTs ends in detoxification. However, a non-competitive inhibitor binds to a site distinct to the binding of GSH and the electrophilic second substrate and diminishes the rate of catalysis. At that time the turnover rate of the enzyme decreases, while the proportion of enzyme molecules that have a bound substrate does not [51]. On the other hand, binding of substrates to a GST-site distinct of the GSH binding site can even enhance GST-enzyme activity [52]. Such findings require confirmation in additional experiments, comparing cellular GST expression patterns with its corresponding activity levels.

An increase of GST enzymes in the serum of cancer patients, as has also been described for HNSCC patients, can either be a result of the release by cancer cells or an acute-phase response of the host against cancer cell proliferation. An

observation that elevated serum GST-π levels revert to the normal range after surgical removal of the tumour, is suggestive that the enzymes were produced by the cancer cells [30]. In general, whatever the mechanism underlying elevated serum GST levels might be, when GST serum levels follow the clinical outcome, return to normal with successful therapy and re-appear prior to clinical recurrence, they might be of value in clinical cancer care. The lack of tumour and organ specificity makes the determination of serum GSTs unsuitable as a screening tool for primary respiratory and upper digestive tract cancer detection, but could be of great value in monitoring therapy efficiency and the discovery of recurrent disease at a very early stage. Also, its application in the prediction of the development of second primary tumours in the respiratory and upper digestive tract, another major cause of treatment failure in early stage HNSCC [53], could be of great value. However, to establish the applicability of GSTs as tumour markers, a large prospective trial has to be conducted.

Other non-invasive screening methods for early cancer detection consist of the analysis of broncho-alveolar lavage fluid and cytology specimens obtained by brushing the mucosa. Both screening methods might be of value for especially early detection of second primary tumours. Broncho-alveolar lavage fluids are easily obtainable during bronchoscopy, but bronchoscopies are very incriminating. Radio-immunoassays for four subfractions of GST (π, α (B1 and B2) and μ) on broncho-alveolar lavage fluid showed statistically significant elevated monomer fractions of the α class GST (GST-B1 and B2) in the lavage fluid. Elevation of GST-B1 and GST-B2 levels in the lavage fluid were found before a tumour was visualised or detected by cytology [54]. Therefore, these parameters could be of value in the early diagnosis of lung cancer. In summary, extensive validation of this technique might lead to a new modality to detect and monitor cancer.

A non-invasive screening procedure executed in our own laboratory concerns the application of GST antibodies to exfoliated cytology [55, 56]. This non-invasive technique can easily be repeated during follow-up. The great need for such screening methods in long term cancer follow-up, screening for second primary tumours at an early stage and monitoring of chemoprevention drugs makes exfoliated cytology an attractive approach. Therefore, we tested GST antibodies on cell scrapes of six different sites of the upper aerodigestive tract from HNSCC patients and comparable non-cancer individuals. We observed a significantly higher expression of GST-π and GST-μ in cell scrapes of the healthy mucosa of HNSCC patients at high risk of developing a second primary tumour [57]. For GST-μ we found a more heterogeneous expression pattern in exfoliated cells and a nonsignificant higher expression in patients [57]. Moreover, these findings support the field cancerisation theory, such as the findings of Janot *et al.*, who found that tumours and adjacent mucosa of HNSCC patients had similar enhanced GST-levels and activity [58]. However, further research must be carried out before the value of GST expression in exfoliated cells as a predictive biomarker for early second primary tumour detection in clinical follow-up studies and during chemoprevention trials can be assessed.

CONCLUSIONS

Smoking and alcohol consumption are well-known risk factors for the development of HNSCC [59]. However, the fact that only a proportion of smokers develop cancer suggests that interindividual differences exist in dealing with carcinogens. The ultimate amount of carcinogen-induced damage results from activation, detoxification and DNA repair pathways. These intrinsic factors are now thought to have a genetic basis that may underlie differences in cancer predisposition. Recently, epidemiological data have provided evidence that a genetic predisposition to HNSCC exists [60]. Also the fact that HNSCC patients have an intrinsic relatively low ability to deal with bleomycin-induced damage supports the concept of cancer predisposition [61]. Most of the evidence for a genetic predisposition for cancer is found at the level of xenobiotic metabolism since genetic polymorphism has not only been described for GSTs, but also for certain P-450 subtypes, e.g. CYP1A1. The combination of a certain cytochrome P-450 enzyme genotype, which activates environmental carcinogens to their electrophilic intermediates, and GSTM1 deficiency, which detoxifies reactive metabolites, has been associated with a synergistically increased risk for squamous cell lung carcinoma [62]. This correlation has been demonstrated for lung carcinoma [62] and looks also to be of value in HNSCC with respect to the corresponding aetiological carcinogenic processes for lung and HNSCC. The need for complementary research in such interacting metabolising pathways still exists. A better understanding of the interaction of known single risk factors could contribute to better individual risk assessment [63]. Besides risk assessment by determining GSTM1 presence, upregulated GST-subtypes in HNSCC could be of additional help in early detection. In particular, GST-π looks to be of value as a tumour serum marker or as a tumour marker in exfoliated cells.

In conclusion, the findings of GST research have led to a better understanding of the many events involved in head and neck carcinogenesis. On the basis of the present results, further studies to assess the practical value of GSTs as markers for the early detection and/or efficacy of chemopreventive agents of second primary tumours in HNSCC patients are warranted.

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