**Glutathione S - transferase pi expression is down-regulated in patients with esophageal squamous carcinoma**

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Abstract

Background
Glutathione S–transferase pi (GST pi) is a subgroup of GST family, which provides cellular protection against free radical and carcinogenic compounds due to its detoxifying function. Expression pattern of GST pi has been studied in several carcinomas, and its down-regulation was implicated to be involved in malignant transformation in patients with Barrett’s esophagus. However, the exact role of GST pi in the pathogenesis of squamous esophageal carcinoma is not fully characterized, nor its prognostic impact.

Methods
Immunohistochemical staining for GST pi expression was used to investigate 153 archival squamous esophageal carcinoma specimens with a GST pi monoclonal antibody. Statistic analysis was performed to explore its association with clinicopathological factors and clinical outcome.

Results
The GST pi expression was greatly reduced in tissues of esophageal carcinomas compared to adjacent normal tissues and residual benign tissues. Absent of GST pi protein expression, in cytoplasm, nuclear and cytoplasm/nucleus was found in 51%, 64.7% and 48% of the carcinomas, respectively. GST pi deficiency in cytoplasm, nucleus and cytoplasm/nucleus was significantly correlated to poorer differentiation ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively). UICC stage and T stage were found significantly correlated to negative expression of GST pi in cytoplasm ($p < 0.001$ and $p = 0.004$, respectively) and cytoplasm/nucleus ($p = 0.017$ and $p = 0.031$, respectively). In univariate analysis, absent of GST pi protein expression, in cytoplasm, nucleus and cytoplasm/nucleus, was significantly associated with shorter overall survival ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively), whereas GST pi cytoplasmic staining retained independent prognostic significance ($p < 0.001$) in multivariate analysis.
Conclusions
Our results show that GST pi expression is down regulated in squamous esophageal carcinoma, and that lack of GST pi is associated with poor prognosis. Deficiency of GST pi protein expression might be an important mechanism involved in carcinogenesis of squamous esophageal carcinoma, and the underlying mechanisms leading to decreased GST pi expression deserve further investigation.
Background

Esophageal cancer (EC) is the third most common cancer of the digestive track and the seventh leading cause of cancer-related deaths worldwide [1,2]. China is among the most highest incidence areas, with new cases of EC per year accounting for nearly half new cases of the world [3]. With etiology still in dark and poor prognosis, EC is usually diagnosed in late stages with five-year survival rate of only 5-10 percent [3,4]. Surgery resection is still believed to offer the best chance to long-term survival, compared to other therapies including radiotherapy and chemotherapy, which are used alone or in combination as adjuvant treatments [3,5,6]. However, surgery resection is often followed by considerable morbidity which usually compromises with patients’ low life-quality. Individualized therapy, with minimized morbidity, therefore, is more and more stringent warranted for patients’ treatment, and this demands us in urge to identify markers and predictors of clinical response, as well as factors involved in tumorigenesis [7].

Glutathione S-transferases (GSTs), a supergene family with at least four distinct isoforms (α, µ, π, θ) identified in human, are involved in the metabolism of xenobiotic compounds in the phase II detoxification [8,9]. They could convert a variety of electrophilic and hydrophobic compounds into more soluble, more easily excretable compounds by catalyzing them in conjunction with glutathione [8,9]. As numerous potentially toxic carcinogenic compounds, being electrophilic and hydrophobic, are detoxified in this way, GST is believed playing an important role in cancer prevention [8-11]. Down-regulation of GSTs has been reported to increase the risk of developing gastric, colorectal, or lung cancer [12-14] and decreased GST enzyme activity in the gastrointestinal track is supposed to be associated with tumor incidence [2,15].
GST pi, the predominant isoform in the normal squamous esophagus epithelium[16], presents in a wide range of normal human tissues [16-23], as well as in various of malignant tumors of urinary, digestive, and respiratory tracks [18,24]. No consensus has been achieved as to the association between GST pi and malignant transformation. Some studies show the increased GST pi expression as an indicator for premalignant and malignant changes in tissues during tumor induction in several animal models [25-31]; However, in some other studies, GST pi expression is indicated to be a marker of carcinogen exposure in the upper aerodigestive track [29], and loss of GST pi expression is a phenotype associated with carcinogenesis [11].

As to esophageal carcinoma, several studies have been performed to find alternation of GST pi in Barrett’s metaplasia and adenocarcinoma, and the results suggest deficiency of GST pi may contribute to an increased cancer risk [2,32-35]. However, rare information is available about GST pi alternation in squamous esophageal carcinoma, nor is its connection with clinical parameters. Therefore, in this study, we report results of an immunohistochemical survey of GST pi in 153 squamous esophageal carcinoma cases with long term follow-up. Our study confirms the down-regulation of GST pi expression in this type of tumor, and the lack of GST pi protein expression is significantly associated with shorter overall survival.

**Methods**

**Patient materials**

One hundred and fifty-three patients, 93 men and 60 women, whom underwent potentially curative surgery with diagnosis of esophageal squamous cell carcinoma during the period of 1989-1994 at the Anyang Tumor Hospital, Henan, China, were enrolled in this retrospective study. The median age of diagnosis was 56.4 years (range 33-73 years). No preoperative chemotherapy and radiotherapy was given. All tumors were staged according to International Union against Cancer (UICC) 2003 Classification. One hundred (65.4%) cases were classified
as stage II and 53 (34.6%) cases as III. All patients were followed up until death or 31 May, 2004. Ninety-seven (63.4%) patients died of esophageal cancer. The median follow-up time for all patients was 90 months (range 1-155 months). The follow-up and data analysis were performed by researchers from both Anyang Tumor Hospital, China and The Norwegian Radium Hospital, Norway, as an international cooperation project. Patient information was available in tumor size, TNM staging, pathologic grade, demographic data and mortality. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

Specimens removed from surgery were fixed in formalin, processed and embedded in paraffin block for diagnosis and research use. Histological specimens were reviewed by two of the authors (Z.S and J.M.N) who had no access to clinical information. The detailed description of the tumor characteristics is provided in Table 1.

**Tissue microarray method**

Multi-tissue microarray blocks were produced by using MTA-1 manual tissue arrayer (Beecher Instruments Inc., Sun Prairie, WI, U.S.A). Firstly, Hematoxyline and Eosin (H&E) staining sections made form the paraffin blocks were used to define two representative tumor areas and one stroma area. Secondly, the defined regions on paraffin block were transferred by a hollow needle, with cores diameter of 0.6 mm, to a recipient paraffin block. Finally, sections from these recipient paraffin blocks were cut into 5 µm and mounted on charged Super-Frost Plus glass slides for immunohistochemistry analysis, after being dried at 60°C in an oven for 2-4 hours.
The whole tissue sections, from samples whose tissue microarray materials were either unrepresentative or unavailable, underwent immunohistochemical analysis as well.

**Immunohistochemical method**

Dako EnVision™ + System, Peroxidase (DAB) (K4007, Dako Corporation, CA, and U.S.A) was employed for immunostaining. The sections were deparaffinized in xylene, microwaved in 10mM citrate buffer pH 6.0 to unmask the epitopes, and treated with 0.3% hydrogen peroxidase (H₂O₂) for 5 min to block endogenous peroxidase. Monoclonal glutathione-S-transferanese pi antibody (clone 353-10, 1:50) from Acris Antibodies GmbH, Germany was applied on the sections for 30 min at room temperature. After incubation with the horzeradish peroxidase (HRP) labeled polymer conjugated to goat anti-mouse IgG for 30 min, the sections were incubated with 3’3-diaminobenzidine tetrahydrochloride (DAB) for 10 min before counterstained with hematoxylin, dehydrated and mounted in Diatex.

Immunostaining was scored for both intensity (1, absent/weak; 2, moderate; 3, strong) and extent of staining (percentage of positive tumor cells: 1, < 10%; 2, 10-50% ; 3, > 50%). The scoring results of intensity and extent were multiplied to give a composite score ranging from 1 to 9 for each section. Examination of immunostaining was performed by two independent observers (Z.W and Z.S) with no knowledge of patients outcome. All discordant scores were reviewed until final agreement was obtained.

**Statistical analyses**

The associations between GST pi protein expression and clinicopathologic variables were evaluated by the Person χ² test. The Kaplan - Meier method and the log - rank test were employed to estimate and compare survival rate. A Cox proportional hazards regression model was used to perform multivariate evaluation of survival rates. All calculation was
performed by usage of the SPSS 16.0 statistical software package (SPSS, Chicago, IL), and $p \leq 0.05$ was considered as statistical significance.

**Results**

**Frequency of GST pi protein expression**

The majority of cases contained adjacent normal tissues and residual benign tissues, on which strong positive staining was present and served as an internal control in both cytoplasm and nucleus. Immunostaining was found in parabasal, middle and top layers of the esophageal epithelium (Figure 1a and 1b).

In esophageal carcinomas, positive immunostaining in either cytoplasm (Figure 1c), or nuclear (Figure 1d), or cytoplasm/nucleus (Figure 1e) was presented in 75 of 153 (49%) cases, 54 of 153 (35.3%) cases, and 79 of 153 (51.6%) cases, respectively. Negative immunostaining for GST pi in either cytoplasm(Figure 1d), or nuclear (Figure 1c), or both cytoplasm and nucleus (Figure 1f) was found in 78 (51%) of 153 cases, 99 (64.7%) of 153 cases, and 74 (48.4%) of 153 cases, respectively. The detail of GST pi immunostaining score is shown in Table 2.

**GST pi immunostaining in relation to clinicopathological parameters and patients survival**

GST pi immunostaining status in relation to clinicopathological parameters is summerized in Table1. Negative expression of GST pi in cytoplasm, nucleus and cytoplasm/nucleus were significantly correlated to poorer differentiation ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively). UICC stage and T stage were found significantly correlated to negative expression of GST pi in cytoplasm ($p < 0.001$ and $p = 0.004$, respectively) and
cytoplasm/nucleus ($p = 0.017$ and $p = 0.031$, respectively). No significant association was found between GST pi expression levels and age, gender, location, tumor size and lymph node metastasis.

In the univariate analysis, high histological grade, high UICC stage, high T stage, and GST pi staining in cytoplasm, nucleus and cytoplasm/nucleus were found associated with poor overall survival (Figure 2), each of them with a significant $p < 0.001$. In multivariate analysis, only histological grade, T stage and GST pi cytoplasmic staining retained independent prognostic significance ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) (Table 2).

**Discussion**

Tissue and serum GST pi levels were suggested to serve as marker of cancers in several studies [30,31] with results showing inconsistent GST pi expression pattern in different carcinomas. In gastric cancer, increasing serum GST pi level was hypothesized being correlated to advanced stage, and its expression in tissue was inversely correlated to survival [36]. However, in prostate adenocarcinoma, down-regulation of GST pi was observed and the loss of GST pi expression was regarded as a phenotype associated with malignant transformation [11].

In esophageal carcinomas, many studies showed that down-regulation of GST pi expression was involved in malignant progression from Barrett’s esophagus to esophageal adenocarcinoma [2]. The decreased GST pi expression was observed in Barrett’s epithelium [2,10], and lower GST pi mRNA levels were detected by Northern blot analysis and measurements of enzymatic activity [37,38]. These studies anticipated that the absence of
such cell-protective enzymes may be involved in development and progression in esophageal carcinoma [10].

However, a study on limited samples (53 samples from 19 patients) by Chandra et al. [2] argued that high GST pi expression connoted a poor prognosis. In addition, their results failed to find statistical differences in the disease-free survival, nor to find GST pi expression in two well-differentiated adenocarcinoma cell lines [2].

In our present study, down-regulated GST pi was observed in squamous esophageal carcinomas, and loss of GST pi expression was demonstrated being associated with overall survival, indicating that GST pi down-regulation is associated with esophageal tumorigenesis, a process with a variety of genetic and epigenetic alterations [39-42].

GST pi polymorphism was suggested to be involved in alternation of GST pi enzyme activity. Van LIeshout et al. [43] showed that the reduced GST pi enzyme activity in Barrett’s esophagus was due to genotype of GST pi b, whose protein product showed less activity than that of GST pi a. Compton and coworkers [38] showed that down-regulation of GST pi might be second to the result of decreased GST pi mRNA expression, which occurred due to gene interaction at the transcriptional level [10]. In addition, gene transcriptional silencing might be followed by gene epigenetic alternation. Hypermethylation of CpG islands within promoter regions has been found in several genes and has been implied to be responsible for down-regulation of their protein products [44,45]. Hypermethylation of GST pi gene, although at a low frequency, has been reported in adenocarcinoma of the esophagus [39]. Although these may be useful to explain the down-regulation of GST pi in malignant transduction in Barrett’s esophagus, similar mechanisms have to be explored in squamous esophageal carcinoma. We
speculate that such epigenetic alterations exist in squamous esophageal carcinomas, attributing to decreased GST pi protein expression.

It is still not fully understood whether increased cancer development is secondary to GST pi down-regulation, but some studies on esophagus suggest that imbalance between redox and GST enzyme may be associated [2]. According to the study of Chandra et al. [2] redox molecular species may kill tumor cells by means of the induction of apoptosis and/or through other mechanisms in one hand, but they may also initiate a cascade of mutational events that promote the development or progression of malignancy through redox-mediated DNA damage in another hand.

The studies on NO·, a redox molecular, have shown that upper aerodigestive track malignancies strongly express the enzymatic machinery necessary to generate NO·, although evidence showed its physiologic roles in the regulation of vascular blood flow and aid in killing infectious and malignant cells [46]. The prevalence expression of NO· in tumor cells indicated a potential high concentration of NO· in the tumor microenvironment [1,33,34], and it was supposed to be involved in mutagenesis by means of the induction of double-stranded DNA breaks [35]. However, glutathione was suggested to counteract the effects of high NO· levels [47], and GST enzymes, by catalyzing nucleophilic addition of glutathione, providing a key biochemical sink for free radicals and highly reactive molecules [2]. Nevertheless, the lack of GST enzymes may lead to accumulation of redox-mediated DNA damage which attributes to tumorigenesis, due to imbalance between the production and neutralization of redox species.
As for development and progression of squamous esophageal carcinoma, we speculate that lack or loss of GST pi protection might predispose squamous esophagus cells to undergo further genetic alternation, and lead to cell malignancy ultimately.

Conclusions

This study of 153 cases, by using immunohistochemistry, confirms down-regulation of GST pi expression in esophageal epithelial carcinomas, and lack of GST pi protein expression is significantly associated with poorer overall survival. Deficiency of GST pi protein expression might be an important mechanism involved in the carcinogenesis of esophageal squamous carcinomas. Further studies are deserved to determine the underlying mechanisms behind decreased GST pi expression in this disease.
Competing interests
The authors declare that there are no competing interests.
Authors' contributions
ZW participated in the design of the study, carried out the immunohistochemical analysis, statistical and data analysis and draft the manuscript. WH participated in the design of the study, interpretation of data and manuscript revising. GY was involved in the project design, collection of clinical data, interpretation of data and manuscript revising. JMN revised the manuscript critically. RH participated in statistical and data analysis and helped to draft the manuscript. ZS participated in the design of the study, statistical and data analysis and helped to draft the manuscript. All authors read and approved the final manuscript.
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References


5. Siewert JR, Brucher BL, Stein HJ, Fink U: [Esophagus carcinoma--systemic or local risk of recurrence--which perioperative measures are successful?]. *Langenbecks Arch Chir Suppl Kongressbd* 1998, **115**: 290-294.


Figure legends

Figure 1. Expression of GST pi protein in squamous esophagus epithelium. Expression of GST pi protein in normal squamous esophagus epithelium (A) and (B). Expression of GST pi protein in esophageal squamous carcinoma with positive immunostaining in cytoplasm and negative in nucleus (C). Expression of GST pi protein in esophageal squamous carcinoma with negative immunostaining in cytoplasm and positive in nucleus (D). Expression of GST pi protein in esophageal squamous carcinoma with positive immunostaining in both cytoplasm and nucleus (E). Expression of GST pi protein in esophageal squamous carcinomas with negative immunostaining in both cytoplasm and nucleus (F).

Figure 2 - Survival curves using the Kaplan-Meier method. Kaplan-Meier curves drawn for the entire series (n = 153) based on GST pi protein expression levels in cytoplasm ($p = 0.001$) (A). Kaplan-Meier curves drawn for the entire series (n = 153) based on GST pi protein expression levels in nucleus ($p < 0.001$) (B). Kaplan-Meier curves drawn for the entire series (n = 153) based on GST pi protein expression levels in cytoplasm/nucleus ($p = 0.001$) (C).
Additional files provided with this submission:

Additional file 1: Coverletter.tif, 11653K
http://www.biomedcentral.com/imedia/8387135133290897/supp1.tif
Additional file 2: tables.doc, 151K
http://www.biomedcentral.com/imedia/1362985734329090/supp2.doc