Abstract

Introduction: A series of enzymes deliver protection from harmful injury by toxic chemicals. Among these, Glutathione-S-transferase (GST) is most imperative for detoxifying exogenous and endogenous substances to protect cells from the toxic effects of ROS. Reactive oxygen free radicals are implicated in the pathogenesis of a multistage process of head and neck carcinogenesis, which is proposed to cause DNA base alterations, strand breaks, damage to tumor suppressor genes and an enhanced expression of proto-oncogenes.

Materials and Methods: This study was conducted in COMSATS institute of Information Technology Islamabad supported by a grant from Higher Education Commission, Islamabad (Pakistan).

Results: In the present study, alterations of Glutathione S-transferase (GST) enzyme activity were investigated in 500 samples (cohort 1 containing 200 head and neck cancer blood samples along with 200 healthy controls and cohort II with 50 head and neck squamous cell carcinoma tissue samples along with 50 control tissues) by high performance liquid chromatography and ELISA techniques. The results specified that mean blood GSH levels were significantly reduced in head and neck squamous cell carcinoma patients (p<0.001) blood samples as compared to respective controls. In contrast, the levels of GSH (p<0.05) were significantly elevated in head and neck squamous cell carcinoma tissues compared with adjacent cancer free control tissues. The Glutathione S-transferase (GST) enzyme activity, (p<0.05) were significantly reduced in head and neck squamous cell carcinoma patient’s compared to adjacent cancer-free control tissues.

Conclusion: Our study suggests that dysregulation of glutathione (GHS) levels and Glutathione S-transferase (GST) enzyme activity in head and neck cancer may have potential to contribute to the pathogenesis of HNSCC malignancies. This investigation of the expression of GST-activity and GSH levels may provide information for prediction of individual cancer risk and the anticipation of cancer.

Keywords: Glutathione; Glutathione S-transferase; HNSCC malignancies; ROS

Introduction

Head and neck cancer include cancer of oral cavity and is a heterogeneous group of malignancies [1]. Head and neck cancers have been studied frequently in relation to genetic abnormalities and changes have been reported frequently. The genetic variation for genes and pathways involved in pathogenesis of head and neck cancer are DNA repair genes, cell cycle genes, growth factor pathways, xenobiotic metabolism, oxidative free radicals (ROS). To protect cells and organ systems of body against reactive oxygen species (ROS), humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin that function interactively and synergistically to neutralize free radicals [2].

Glutathione is an endogenous antioxidant (Y-L-glutamyl-L-cysteinyl glycine) tri-peptide of L-glutamate, L-cysteine and glycine. Glutathione directly quenches reactive oxygen species (ROS) such as lipid peroxides (hydrogen peroxidase and thioredoxin peroxidase). Glutathione is associated with protection against head and neck cancer pathogenesis because it is the major intracellular antioxidant [3] detoxifies many carcinogens through Phase II conjugation, and maintains immune function by regulating mitogenic response and lymphocytic proliferation [4].

There are many enzymes that provide protection from damaging by toxic chemicals and carcinogens [5]. Among them, Glutathione-S-transferase (GST) is significant for detoxifying exogenous and endogenous substances and protecting the cells from toxic effects of ROS [6,7]. The key role of GST is to catalyze the formation of glutathione-S-conjugates with electrophiles that is decisive for inactivation and consequent excretion of other molecules [8,9]. The elevated expression of GST has been implicated in resistance to apoptosis initiated by a variety of stimuli. In addition, GST thought to play an important function in detoxification and protecting DNA from oxidative damage [6]. In our present study we performed ELISA analysis for the determination of expression of specific activity of GSTs in HNSCC, which is involved in carcinogen detoxification.
Materials and Methods

Samples collection and patient identifications

The present study was carried out with an earlier approval from ethical committees of both COMSATS Institute of Information Technology and collaborating hospitals. Two groups of HNSCC patient were drafted in this study. The cohort 1 consisted of blood of 200 HNSCC patients and 200 respective controls. The mean age of patients and respective controls was 45 (±16.35) and 43 (±32.23) years respectively. Non-significant difference was observed in case of age (p=0.9). In addition to this, a non-significant difference was seen in HNSCC patients for area of cancer (p=0.08). Patients and controls suffering from any other familial disease like blood pressure, diabetes and cardiovascular impairment were omitted from present study.

Cohort 2 included tumors of 50 HNSCC patients who had undergone surgery at Military Hospital (Rawalpindi, Pakistan) between 2009 and 2013. Demographic and clinical characteristics of study cohort 2 showed that median age of HNSCC cases and controls was 60 years with a range of 20-80. 50 tumors included 12 (24%) cases of the pharynx, 24 (48%) cases of SCC of the larynx and 14 (28%) cases of SCC of the oral cavity. Most of the patients were in stage III of clinical and T staging with stage I of N staging. Most of the HNSCC patients had well differentiated tumors. All tumors were histologically confirmed as squamous cell carcinoma of head and neck and were graded as well differentiated, moderately differentiated and poorly differentiated.

Blood and tissue samples preparation

The blood and tissue samples were prepared by an assayed for (GSH) [10]. For GST-activity total of 200 HNSCC patient’s blood samples along with 200 respective controls and 50 HNSCC patient’s tissues along with respective controls 50 tissues were prepared for the measurement of blood GST activity. Solutions and reagents were prepared according to the protocol of ELISA kit (Sigma Cat# CS0410). About 200 mM solution of L-glutathione was prepared by adding 246 mg L-glutathione (Sigma cat # G4251) in water 17MΩ (Sigma cat # W4502) making a final volume of 4 ml. Solution was then kept in ice. GST samples were diluted with sample buffer. CDNB solution and Dulbecco’s phosphate buffer saline were warmed at 250 C before starting the assay. Assay was performed in a 96-well plate. 2 μl of GST+18μl sample buffer was used. Control GST (2 μl) was added with 198 μl substrate solution; 18 μl patient protein was added with 182μl substrate solution and for blank 200 μl substrate solution was added to reaction mixture plate. Absorbance was then read on ELISA reader.

Statistical analysis

The results for groups are expressed as mean ± S.E. Multivariate analysis was performed to determine influence of the gender, age, and area of cancer on HNSCC blood samples and tissue glutathione levels. For statistical analysis, the experimental values were compared with their respective controls. P-values were calculated by chi-square test and ANOVA. Pearson correlation coefficient was used to calculate correlation among glutathione levels and clinical and histo-pathological parameters (*Indicates the level of significance of pears on correlations as calculated by SPSS).

Results

In present study, blood glutathione levels (GSH) and GST activity was observed in blood samples of 200 HNSCC patients and 200 control samples. Mean blood GSH [823.35±4.50; p<0.01] levels were observed significantly lower in HNSCC samples as compared to respective control, as shown in our recent work (my paper). The mean blood GST [7.5 ±11.5; p<0.01] activities were observed statistically significant as compared with respective controls (Table 1).

Table 1: Blood GSH levels and GST activity in HNSCC patients VS respective controls.

| Variables | Controls Blood | HNSCC Blood |
|-----------|----------------|-------------|
| GSH       | 848.55±3.54    | 823.35±4.50 ** |
| GST       | 11.2±7.5 U/L   | 7.5±11.5*    |

*p<0.05; **p<0.01

The multiple worsening analysis discovered that significantly lower level of GSH [p<0.01 below 45 years and p<0.007 above age 45 years] and GST [p<0.1 below 45 years and p<0.08 above age 45 years] was concrete in HNSCC in all age groups when compared with respective control. In case of gender, significant lower GSH- levels and GST-activity was observed in male and female HNSCC patients compared to male and female respective control groups. Furthermore, significantly lower GSH-levels (p<0.001) and GST-activity (p<0.01) was observed in different areas of cancer. In case of stages of cancer, no significant correlation was observed in blood glutathione and GST activity in cancer patients (Table 2).

Table 2: Comparison of Blood GSH and GST activity in Relation to Age, Gender, and area of cancer in HNSCC Patients and Controls.

| Variables | Control (Mean ± SEM) | Patients (Mean±SEM) |
|-----------|----------------------|---------------------|
| GSH       |                      |                     |
| Age       | ≤45 years 93±5.33    | 903±4.34            |
|           | ≥45 years 82±4.13    | 804±3.23            |
| Gender    | Male 848.55±3.54     | 823.35±4.50         |
|           | Female 823.40±4.1    | 804.70±2.40         |
| Area of HNSCC |             |                     |
|           | Oral Cavity SCC 930.89±2.29 | 895.42±2.34 |
|           | Laryngeal SCC 920.56±2.18 | 870.29±1.38 |
|           | Pharyngeal SCC 925.72±2.50 | 901.72±1.98 |
| GST-Activity |             |                     |
| Age       | ≤45 years 12.4±6.1   | 7.11±5.2            |
|           | ≥45 years 10.0±5.7   | 6.34±4.7            |
| Gender    | Male 11.3±5.2       | 6.0±4.9             |
|           | Female 10.7±4.1     | 7.1±5.4             |
| Area of HNSCC |             |                     |
|           | Oral Cavity SCC 10.4±5.1 | 6.3±4.9     |
|           | Laryngeal SCC 11.01±5.5 | 7.1±4.8   |
|           | Pharyngeal SCC 10.1±6.2 | 6.4±6.7  |

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In second part of the study, GSH levels and GST activity were observed in 50 head and neck squamous cell carcinomatises samples and compared with 50 control samples. The mean tissue GSH (29.67±0.85; p<0.05) levels were observed significantly higher in HNSCC compared to controls as shown in our recent work [10]. The mean specific GST activity in HNSCC patients was 0.28 (±0.03) U/L and control was 0.33 (±0.04) U/L. The specific GST activity was significantly reduced in HNSCC patients compared to respective adjacent cancer free tissues (p<0.01) (Figure 1). The multiple worsening demonstrated that GSH and GST activity were statistically significant when compared to respective control tissues (Table 3).

![Figure 1: Specific GST activity in HNSCC patients compared to respective adjacent cancer free tissues.](image)

**Table 3:** Comparison of tissue GSH and GST activity in Relation to Age, Gender, and area of cancer in HNSCC Patients and respective Controls.

| Variables       | Control (Mean ± SEM) | Patients (Mean±SEM) |
|-----------------|----------------------|---------------------|
| **GSH**         | GSH                  |                     |
| Age             | ≤45 years            | 11.35±0.75          | 14.17±0.25          |
|                 | ≥45 years            | 9.86 ± 0.36         | 15.50±0.57          |
| Gender          | Male                 | 9.97±3.66           | 11.16±4.29          |
|                 | Female               | 10.23±0.01          | 13.32±0.25          |
| Area of HNSCC   | Oral Cavity SCC      | 9.27±2.15           | 13.24±2.44          |
|                 | Laryngeal SCC        | 9.11±2.41           | 15.99±1.76          |
|                 | Pharyngeal SCC       | 10.57±1.89          | 14.92±2.09          |
| **GST-Activity**| Age                  |                      |
| ≤45 years       | .33 ± .05            | .28 ± .02           |
| ≥45 years       | .32 ± .07            | .26 ± .07           |
| Gender          | Male                 | .30±.01             | .27 ± .04           |
|                 | Female               | .34 ± .09           | .27 ± .09           |
| Area of HNSCC   | Oral Cavity SCC      | .31 ± .05           | .26 ± .08           |
|                 | Laryngeal SCC        | .31 ± .02           | .27 ± .09           |
|                 | Pharyngeal SCC       | 1.01 ±6.2           | 6.4 ± 6.7           |

**Discussion**

Humans have well defined defense system to protect the body against stress and reactive oxygen species, among them the most important are GST and GSH that directly quenches reactive oxygen species and also plays a major role in xenobiotic metabolism [11]. The glutathione regulation is controlled by glutathione transferases and glutathione trans-peptidases. This present results demonstrated that glutathione (GSH) and GST specific activity in HNSCC and compared with controls both in blood as well as in tissue samples. In our recent works, it was observed that mean blood glutathione levels are significantly reduced in head and neck cancer compared with respective controls [10]. Our results are in agreement with earlier studies which have demonstrated that glutathione levels differ in patients of head and neck cancers and this depletion could be due to imbalance in free-radical and antioxidant homeostasis in body fluids during carcinogenesis [10-14]. The other plausible explanation for decreased levels of glutathione observed in head and neck cancer patients is possibly GSH, being exhausted as a main low molecular weight antioxidant in cellular protection [15,16]. Our results are in agreement with earlier studies which showed that levels of glutathione decrease in blood of the breast cancer when compared to respective controls [17].

In current study, GST specific activity was measured in HNSCC patients along with control samples by ELISA. The results indicated that specific GST activity is significantly lower in HNSCC when compared to respective controls. Our results are in agreement with earlier reported studied in different cancer [18]. Our present
study demonstrated that due to decrease in GST expression and activity the levels of GST are increase which may result in HNSCC pathogenesis. Our present results are also in agreement of previous studies by Masood [19] demonstrated that increase levels of glutathione-s-transeptidase can result in pathogenesis of head and neck cancer. The increased levels of glutathione-s-transpeptidase and genotypic polymorphisms expression may be an important mechanism involved in the carcinogenesis and mutagenesis which possibly can result in cancer of head and neck.

Variant genotypes and reactive oxygen species results in diminish activity of glutathione-S-transferase enzyme that increase or up regulate Y-glutamyl tranpeptidase enzyme and decrease the ability to detoxify carcinogens and mutagens that may confer an increase in cancer pathogenesis. The underlying processes that regulate expression and antioxidant defense mechanisms deserve further investigations.

Conclusion

In conclusion our study suggested that dysregulation of glutathione (GHS) levels and Glutathione S-transferase (GST) enzyme activity in head and neck cancer may have an association with pathogenesis of HNSCC malignancies, which may provide information for prediction of individual cancers of HNSCC risk and the anticipation of cancer.

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