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RESEARCH

Expression Patterns of Carcinogen Detoxifying Genes (CYP1A1, GSTP1 & GSTT1) in HNC Patients

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Abstract Carcinogen detoxifying genes may be involved in pathogenesis of head and neck cancer (HNC). CYP1A1 is phase I enzyme that converts carcinogens into water soluble compounds which are easily excreted from body. GSTs constitute phase II detoxification enzymes that recognize these highly electrophilic compounds and detoxify them. Abnormal expression of these genes can potentially lead to cancer initiation. In present study, we analyzed protein expression of these genes in a total of 192 HNC patients and noncancerous healthy control serum samples screened for GSTs specific activity by ELISA. Furthermore, expression of these molecules was also determined in 49 HNC tissues/adjacent control tissue by immunohistochemistry with specific antibodies. Mean serum GSTs specific activity was found to be 7.7 (±11.5)U/L in HNC patients and 11.4 (±7.5)U/L in controls. Significant decrease ($P<0.05$) in GSTs specific activity was observed in HNC patients compared with controls ($P<0.001$). Data for immunohistochemistry showed that CYP1A1 and GSTT1 was down expressed whereas GSTP1 was over expressed in HNC tissues compared with adjacent normal control tissues. Results of immunohistochemistry revealed 63 % HNC tissues had weak, 27 % moderate and 10 % strong staining for CYP1A1. For GSTT1, 27 % HNC tissues had no staining, 49 % weak staining, 16 % moderate and 8 % strong staining. Similarly for GSTP1, percentages for weak, moderate and strong staining were 6 %, 12 % and 82 % respectively. These reduced proteins observed in cancer patients highlight a potential breach on DNA repair mechanism when compared with control. Thus altered expression of these detoxifying molecules may collectively contribute to HNC development in Pakistani population.

Keywords Immunohistochemistry · GSTP1 · CYP1A1 · GSTT1

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Introduction

Head and neck cancers, include lip, oral cavity, pharynx and larynx, are placed amongst the top ten malignancies globally [1]. Geographical variations in the incidence of head and neck cancer indicate global differences in the prevalence of risk factors associated with the cancer. Head and neck cancer is the sixth most frequent cancer worldwide and seventh leading cause of cancer related deaths [2]. In Pakistan, prevalence of HNC is also very high and during the year 2000–2008 the most frequent cancer among men was head and neck cancer accounting approximately 32.6 % of all cancers. During the same time period, in females head and neck cancer was the second most common with 15.1 % incidence among all cancers [3].

The salient enzymes included in phase I detoxification process are multigene family Cytochrome P450 (CYP). Phase I enzymes function by adding an oxygen atom into the xenobiotic compound (toxic or lipophilic chemical) [4]. The resulting product from CYP1A1 detoxification is highly hydrophilic and thus excreted from the body. Few chemicals, though being highly hydrophilic, are not excreted but are activated to their more toxic form. The phase II enzymes are activated and this intermediate carcinogenic form is made more hydrophilic through membrane bound cytosolic enzymes such as glutathione S transferases (GST). They attach highly water soluble moieties to the polar groups introduced by the phase I reactions [4]. Thus the coordinated expression and regulation of phase I and phase II metabolizing

enzymes and their metabolic balance are important factors in determining the outcome of exposure to carcinogens.

The previous studies by same research group have found novel mutations in CYP1A1, GSTP1 [5–7] and polymorphisms in GSTM1 and GSTT1 genes [8] in germline screening. Similarly at RNA screening, significant variations in expression have been reported in CYP1A1, GSTM1, GSTT1 and GSTP1 genes [9]. From review of literature it was found that CYP1A1 shows differences in enzymatic activities [10] and GSTs are expressed in tissue specific manner [11–15] in HNC. Specific activity of GSTs and expression of CYP1A1, GSTT1 and GSTP1 in head and neck cancer tumor and control tissue is an important area to explore. Therefore in continuation of the previous germline screening and somatic screening at mRNA level of these genes in head and neck cancer patients the current case control study was designed to screen these genes protein expression.

Materials and Methods

Tissue Collection

A total of 96 head and neck cancer patients and 96 controls serum were screened for GSTs specific activity. For CYP1A, GSTT1 and GSTP1 expressional analysis immunostaining was carried out on 49 HNC tumors collected along with adjacent normal tissue used as control. Patients were recruited from different Hospitals (Military Hospital & Allied Hospital) and controls from individuals who were healthy with informed consents. Study was prior approved from ethical committees of both university and hospitals.

ELISA

Reagents were prepared according to the manufacturer's protocol provided with ELISA kit (Sigma Cat# CS0410). Solutions were kept on ice and used freshly prepared. The assay was performed in a 96-well plate in duplex. Control GST was run to check the sensitivity of ELISA and absorbance was read in the plate reader at 340 nm immediately after preparing the reaction tests and every minute thereafter to obtain 6 time points. The increase in absorbance is directly proportional to GST activity. Specific activity was determined by formula given by the manufacturer's protocol.

Immunohistochemistry

Immunohistochemistry was performed on 49 tissues of head and neck cancer and 49 control tissues. The procedure was as followed by Niitsu [16] with minor modifications. Immunohistochemical analysis was made on the basis of weak moderate and strong staining in oral, pharyngeal and laryngeal cancer. Glass slides were precoated with adhesive so that the sections do not wipe out during the procedure. Specific antibodies for CYP1A1, GSTT1 and GSTP1 were obtained for IHC and DAB chromogen was used for staining. Section of slides were dipped in xylene and then descending strength of alcohol and washed in phosphate buffer saline (PBS) followed by heating in citrate buffer. After washing in PBS, 2 drops of blocking solution (Reagent 1A) were added. After 2 min blocking solution was blotted off and primary antibody was added to the slides and kept at room temperature for 1 h. Slides were then washed with PBS three times, 2 drops of second antibody (1B) was added and kept at room temperature for about 30 min. Slides were again washed in PBS three times and 2 drops of reagent 2 conjugate were added for approximately 30 min. This was followed by rinsing in PBS solution three times. One drop of reagent A, two drops of reagent B and 1 drop reagent C were added in 1 ml of distilled water. It was mixed and protected from light and used with in 1 h. Two drops of DAB chromogen were added to slides and left for 10–15 min. The slides were washed in distilled water and counter stained with haemotoxyline for approximately 10 min. Again the slides were washed in differentiated acid alcohol and then in running tap water for 10 min for blueing. At the end the slides were air dried and mounted with Canada balsam before observing them under photographic microscope with 40x and 100x magnification. The staining intensity was calculated as weak, moderate or strong according to previously defined protocol [37]. Analysis was made for expressional variations of markers.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism (5) software.

Results

ELISA

The mean age of head and neck cancer patients was 57.8 ($\pm$16.9) years and control was 56 ($\pm$15.5) years. GST specific activity and absorbance was found to be directly correlated with each other in controls and head and neck cancer patients. The mean serum GST specific activity in patients was 7.7 ($\pm$11.5) U/L and control was 11.4 ($\pm$7.5) U/L (Fig. 1). The specific activity of GST was found to be significantly reduced in head and neck cancer patients compared to normal healthy controls ($P<0.001$). GST Specific activity in oral cancer patients was found to be 6.5 ($\pm$ 5.2) U/L, in laryngeal cancer patients was 7.3

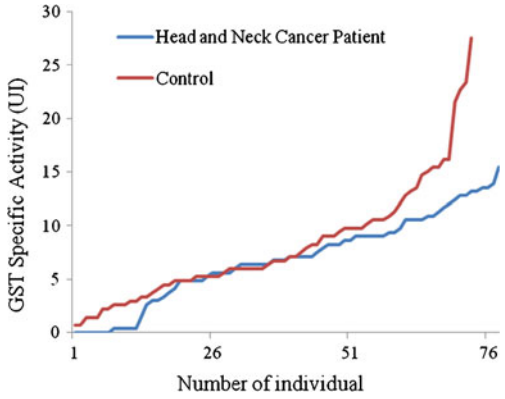

Fig. 1 Graphical representation of GSTs specific activity in patients as well as controls showing increased GSTs level in patients compared to controls

(±4.9) U/L and of pharyngeal cancer patients was 6.6 (±6.9) U/L. Nonsignificant association was observed between GST specific activity and different stages of head and neck cancer ($P>0.05$).

Immunohistochemistry

CYP1A1 was expressed in cancer tissues as well as controls; however HNC tissues had mild expression compared to controls (Fig. 2). Regarding the levels of CYP1A1 expression; 31 cases (63 %) demonstrated weak staining, 13 cases (27 %) were moderately stained and 5 (10 %) were strongly stained. There was also a significant association between the overall CYP1A1 expression and mRNA levels as studied previously [9] where reduced levels of CYP1A1 mRNA in tissues showed weak staining compared to normal mRNA tissues.

In the case of GSTT1 expression; 13 (27 %) cases demonstrated negative staining, 24 cases (49 %) were weakly stained, 8 cases (16 %) were moderately stained and 4 cases (8 %) were strongly stained (Fig. 3). Tissues with reduced GSTT1 mRNA showed less staining and loss of mRNA in tissues resulted in no staining. It was found that GSTT1 expression was directly correlated with genotypic status [6, 8, 9]. Homozygous deleted GSTT1 gene resulted in loss of mRNA and protein expression whereas heterozygous deletion resulted in reduced mRNA and protein expression.

GSTP1 was overexpressed in most of the tissues compared with controls (Fig. 4). A total of 3 cases (6 %) were weakly stained, 6 cases (12 %) were moderately stained and 40 cases (82 %) were strongly stained. Regarding the subcellular localization of GSTP1 expression nuclei staining was seen in 35 cases (71 %) and cytoplasm staining in 30 (61 %) of cases. Nuclear GSTP1 over-expression (moderate or strong staining) was seen in 32 (65 %) of sections and cytoplasmic GSTP1 over-expression seen in 22 (45 %) of the sections. Significant association between the overall level of GSTP1 expression and GSTP1 mRNA was observed when related with previous study [9]. Over expressed mRNA of GSTP1 resulted in strong staining compared to control.

Discussion

The current study is in continuation with our previous studies regarding screening of CYP1A1, GSTT1, GSTM1 and GSTP1 for their potential role in head and neck carcinogenesis. Earlier germline screening was performed for these genes and novel mutations were found in CYP1A1 and GSTP1 genes [5–7]. Mapping of GSTM1 and GSTT1 gene deletion was also done with a novel technique locating the exact point of deletion [8]. As a next step moving from central dogma, we screened at somatic level for mRNA expression of these genes [9]. Therefore as a proceeding step from DNA to mRNA we screened these genes at protein levels in head and neck cancer.

Hereditary differences in the expression and activity of human GSTs have been reported and low enzymatic activity of GSTs found associated with lung cancer [17]. GSTT1 reduced expression is due to null genotype of these genes [18, 19]. Previously, reduced as well as increases GST activity has been reported head and neck cancer tissue [20, 21]. However we found a decreased activity that may

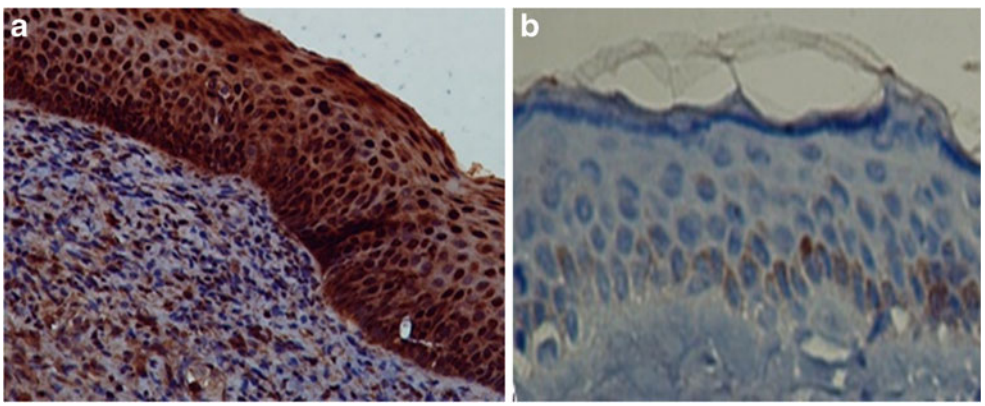

Fig. 2 Representative immunostaining for CYP1A1 in paraffin sections of head and neck cancer tissue. Representative positive immunostaining (*brown* signal) in the epithelial layer is shown in **a** (40×), less immunostaining in most cells is shown in **b** (100×), a kidney section was used as the positive control

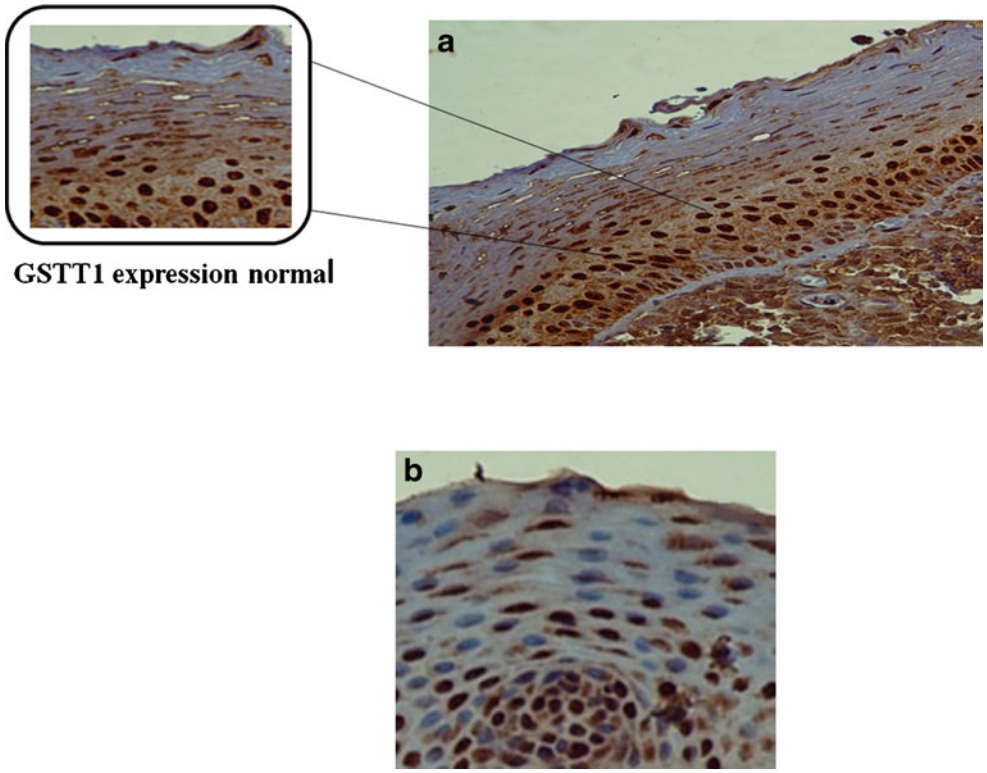

Fig. 3 Representative immunostaining for GSTT1 in paraffin sections of head and neck cancer tissue. Representative positive immunostaining (*brown* signal) in the cells is shown in **a** (40×), less immunostaining in most cells is shown in **b** (100×), a kidney section was used as the positive control

be attributed to null polymorphism of GSTM1 and GSTT1 in our study group. We were unable to find any correlation between stages of head and neck cancer and GST activity. Patel et al [20] also demonstrated that GST activity was not correlated with stages of head and neck cancer and similar results were also reported in ovarian cancer in which Van Der Zee et al. [22] failed to find any correlation between the GST activities and histopathological types, differentiation and grades of the tumor tissues. Similarly, Sprem et al [23] reported that the increase of GST activity in malignant tumor tissue was not directly related with the histopathological or clinical prognostic factors. Our results support these finding these findings; we did not observe any significant difference in serum GST activities between the patient groups which were designated with respect to their histopathological differentiation and tumor grades.

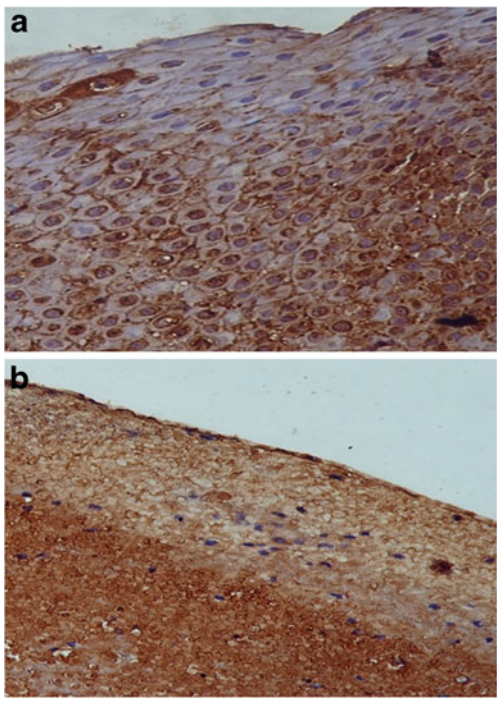

Fig. 4 Representative immunostaining for GSTP1 in paraffin sections of head and neck cancer tissue. Representative less immunostaining (*brown* signal) is shown in **a** (100×), strong immunostaining in most cells is shown in **b** (100×), a kidney section was used as the positive control

Expressional variations in the protein expression of CYP1A1, GSTT1 and GSTP1 are dependent on genetic as well as post transcriptional factors that may be tissue specific [24–26]. Therefore the most accurate method to determine the effects of CYP1A1, GSTT1 and GSTP1 on carcinogen detoxification is to evaluate the expression in the tissues of interest associated with head and neck carcinoma. It was observed that the expression of CYP1A1 was more weakly stained in tumor tissues than in the adjacent control tissues. Similar findings have also been reported previously in breast cancer [27]. CYP1A1 enzyme expressed variation in esophageal carcinomas and control tissue [28]. CYP1A1 has also been detected as down regulated in 68 % of the urinary bladder tumors [29]. Expressional levels for GSTT1 were lower in head and neck cancer tumor tissue compared

to control tissue. Under expression of GSTT1 had also been reported previously in head and neck cancer [30]. Loss of GSTT1 expression had also been found in breast and prostate cancer [31, 32]. In our study, GSTP1 was strongly stained in tumor tissues compared to adjacent control tissue in the current study. Similar results in head and neck cancer have been reported elsewhere [33–35] where GSTP1 was found over expressed in many cancers such as stomach, bladder, colorectal, lung, ovarian, skin and breast [12, 33, 36]. GSTP1 over expression is not related to genotype but probably transcriptionally regulated. GSTP1 over-expression may be due to a number of different mechanisms including gene amplification, transcriptional activation, protein stabilization, and genetic abnormalities [15]. Increased levels of GSTP1 may be occasionally involved in the intrinsic drug resistance of head and neck cancers. However, silencing of this gene has been shown to increase tumor sensitivity to same drug. Lower expression of GSTP1 may be associated with better response to chemotherapy and improved prognosis [31].

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