Introduction

Brain tumor is a solid neoplasm that arises within the brain, inside the cranium or in the central spinal canal. Among children it is the second most common cancer, ranging from 15-25% of all pediatric malignancy (Davis and McCarthy, 2012). Brain is highly vulnerable to damage by toxic compounds due to the limited regenerative capability of the neurons, the major cell type involved in specialized functions of the brain (Ravindranath, 1995). The distinctive features of the capillary endothelial cells surrounding the cerebral blood vessels render protection to the brain by preventing entry of circulating molecules (Joan et al., 2010). However, certain relatively more lipophilic xenobiotics can diffuse through the endothelial cells of the brain capillaries and thus penetrate into the brain (Liu et al., 2004). Improper metabolism of these lipophilic xenobiotics ultimately results in carcinogenesis in brain region (Chang et al., 2005).

A wide variety of enzymes provide protection from harmful injury by toxic chemicals (Thomas and Manfred, 2005). Among these enzymes, Glutathione-S-transferase (GST) is important for detoxifying exogenous and endogenous substances and protecting cells from the toxic effects of ROS (Narasimhan et al., 2011; Yao et al., 2012). The main function of these enzymes is to catalyze the formation of glutathione-S-conjugates with electrophiles, which is crucial for inactivation and subsequent excretion of these molecules (Koh et al., 2011; Nosheen et al., 2011). In the brain, most GSTs are located in glial cells, which are rich in GSH and may protect neurons with low GSH against oxidative insults. Elevated expression of GSTs has been implicated in resistance to apoptosis initiated by a variety of stimuli (Francis et al., 1995). In addition, these enzymes are thought to play a role in detoxification and protecting DNA from oxidative damage (Yao et al., 2012).

In this study, we aimed to test the association between deregulation of metabolizing genes and brain tumorigenesis. The expression profile of metabolizing genes CYP1A1 and GSTP1 was therefore studied in a cohort of 36 brain tumor patients and controls using Western blotting. In a second part of the study we analyzed protein expression of GSTs in the same study cohort by ELISA. CYP1A1 expression was found to be significantly high (p<0.001) in brain tumor as compared to the normal tissues, with ~4 fold (OR=4, 95%CI=0.43-37) increase in some cases. In contrast, the expression of GSTP1 was found to be significantly low in brain tumor tissues as compared to the controls (p<0.02). This down regulation was significantly higher (OR=0.05, 95%CI=0.006-0.51; p<0.007) in certain grades of lesions. Furthermore, GSTs levels were significantly down-regulated (p<0.014) in brain tumor patients compared to controls. Statistically significant decrease in GST levels was observed in the more advanced lesions (III-IV, p<0.005) as compared to the early tissue grades (I-II). Thus, altered expression of these xenobiotic metabolizing genes may be involved in brain tumor development in Pakistani population. Investigation of expression of these genes may provide information not only for the prediction of individual cancer risk but also for the prevention of cancer.

Keywords: CYP1A1 - GSTP1 - gene expression - brain tumors - Pakistan

Expression of CYP1A1 and GSTP1 in Human Brain Tumor Tissues in Pakistan

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Abstract

Most of the exogenous and endogenous chemical compounds are metabolized by enzymes of xenobiotic processing pathways, including the phase I cytochrome p450 species. Carcinogens and their metabolites are generally detoxified by phase II enzymes like glutathione-S-transferases (GST). The balance of enzymes determines whether metabolic activation of pro-carcinogens or inactivation of carcinogens occurs. Under certain conditions, deregulated expression of xenobiotic enzymes may also convert endogenous substrates to metabolites that can facilitate DNA adduct formation and ultimately lead to cancer development. In this study, we aimed to test the association between deregulation of metabolizing genes and brain tumorigenesis. The expression profile of metabolizing genes CYP1A1 and GSTP1 was therefore studied in a cohort of 36 brain tumor patients and controls using Western blotting. In a second part of the study we analyzed protein expression of GSTs in the same study cohort by ELISA. CYP1A1 expression was found to be significantly high (p<0.001) in brain tumor as compared to the normal tissues, with ~4 fold (OR=4, 95%CI=0.43-37) increase in some cases. In contrast, the expression of GSTP1 was found to be significantly low in brain tumor tissues as compared to the controls (p<0.02). This down regulation was significantly higher (OR=0.05, 95%CI=0.006-0.51; p<0.007) in certain grades of lesions. Furthermore, GSTs levels were significantly down-regulated (p<0.014) in brain tumor patients compared to controls. Statistically significant decrease in GST levels was observed in the more advanced lesions (III-IV, p<0.005) as compared to the early tissue grades (I-II). Thus, altered expression of these xenobiotic metabolizing genes may be involved in brain tumor development in Pakistani population. Investigation of expression of these genes may provide information not only for the prediction of individual cancer risk but also for the prevention of cancer.

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2012). Other important enzyme involved in detoxification is the cytochrome P450 1A (CYP1A1) family of proteins which are involved in the bioactivation and detoxification of environmental toxins to the generation of chemical carcinogens (Shukla et al., 2013). CYP1A1 contributes notably to the toxicity of many carcinogens, especially polycyclic aromatic hydrocarbons (PAHs), as it is the principal enzyme that bio-activates inert hydrocarbons into DNA-binding reactive metabolites (Tsutomu, 2006). The presence of CYP1A1 in brain could potentially play a role in the initiation of carcinogenesis in situ, as PAHs are lipophilic and can cross the blood brain barrier. Several CYP1A1 polymorphisms resulted in increased risk of brain tumor (Salnicova et al., 2010). Epidemiological studies carried out on workers in petroleum industry (Peters et al., 2013) and on smokers (Milne et al., 2013) have shown association with brain tumor incidence. Several studies have been reported the specific activity and expression levels of CYP1A1 and GSTs in different cancers. Specific activity of GSTs and expression of CYP1A1 and GSTs in brain tumors and control tissue is an important area to explore. We have, therefore, utilized western blot analysis for the determination of expression levels of these two genes (CYP1A1, GSTP1) which are involved carcionogen detoxification. Furthermore, we have also determined the specific activity of GSTs in brain tumors.

Materials and Methods

Human tissue

Brain tumor tissues were collected from both males and females patients with brain tumor. These specimens were taken from the department of neurosurgery Lady Reading Hospital Peshawar. 36 samples were collected. The samples with no evidence of malignancy in their histopathological reports were used as control samples.

Protein extraction and SDS-PAGE

RIPA buffer (NP40, PMSF, 1mM EDTA, 20% SDS) was used for protein extraction from the tumor tissues. The tissues were fractionated in a homogenizer placed on ice. The homogenate was centrifuged at 4000rpm for 10min and the supernatant was collected and stored at -80°C. The extracted proteins were then separated according to their size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and run at 100 volts until the dye front reached at the bottom.

Western blotting

PAGE samples were transferred from gel to PVDF membrane using Tris-glycine buffer prepared from 20% methanol, for 90 min at 100 volts. The blot was then blocked with 5% TBST-BSA for 1 hour on a shaker. Blot was incubated with primary antibody with a dilution of 1:500 overnight at 4°C. Blots were washed with TBST solution for five minutes and this procedure was repeated five times. Secondary antibody with a dilution of 1:1000 was applied for 2 hours on a shaker. Blots were then washed again with TBST solution for five minutes and it was repeated five times. Color was developed using chromogen substrate, the blot was then washed with distilled water and stored.

ELISA

Reagent was prepared according to the ELISA kit. 200mM solution of L-glutathione was prepared by adding 246mg L-glutathione (Sigma cat#G4251) in water 17MΩ (Sigma#W4502) making a 4ml final volume of solution. The solution was then kept in ice. GST samples were diluted with sample buffer. Dulbecco’s phosphate buffer saline and CDNB solution were warmed at 25°C before starting the assay. The assay was performed in a 96-well plate. 2µl GST+18µl sample buffer was used. 2µl control GST was added with 198µl substrate solution; 18µl patient protein was added with 182µl substrate solution and for blank 200 µl substrate solution was added to the reaction mixture plate. Absorbance was then read on ELISA reader.

Results

36 tissues were collected to analyze the expression of CYP1A1 and GSTP1 genes by western blotting and for determination of GSTs specific activity by ELISA. Analysis of the patient data was carried out in correlation to different parameters e.g. age, gender, location of tumor and grade. Both male and female patients were encountered during the sample collection. Age of brain tumor patients ranged from 1 to 75 years. Brain tumor was found significantly high (p<0.01) in patients with <25 years of age as compared to patients >25. In this study more males (p<0.03) were affected as compared to females. All the data collected, represent tumors of different portions of the brain, meningioma was significantly higher (p<0.04) as compared to other type of brain tumor. Most of patients were significantly higher in grade I-II when compared with patients in grade III-IV (Figure 1).

Western blotting

Protein profiling of brain tumor was performed to analyze variations between cancerous and normal tissues from patients. SDS PAGE technique was used to separate proteins present in tumor samples according to their molecular weight along with protein ladder. To investigate the differential expression of CYP1A1 and GSTP1 protein in brain tumor, western blotting was performed. CYP1A1 protein was found up regulated (75%, p<0.001) in tumor tissue samples as compared to control tissue samples (Figure 2). ~0.64 folds (OR=0.64, 95%CI=0.14-2.92) and ~2 folds (OR=2.4, 95%CI=0.42-13.83) increased up regulation of CYP1A1 was observed.

Figure 1. Column Plot Demographic and Clinical Characterization of Study Cohort
in gender and age respectively while in case of different types of brain tumors, statistically significant ($p<0.027$) up regulation was observed. ~4 folds (OR=4.1, 95% CI=0.43-37.00) increase in up-regulation was observed in case of different grades of brain tumors (Table 1). The expression of GSTP1 in tumor and control samples is shown in Figure 2. GSTP1 protein level was observed to be down regulated (78%, $p<0.02$) in brain tumor samples compared to levels in normal tissues. ~0.2 folds (OR=0.19, 95% CI=0.02-1.76) more decrease in expression was observed in GSTP1 in case of gender and age respectively. This down regulation was significantly higher ($p<0.007$) in different grades of brain tumors (Table 1).

ELISA
ELISA technique was performed to determine the specific activity of GST in brain tumor and control samples in order to confirm our western blotting results. The mean GST specific activity in patients was 0.21 (±0.03) U/L and control was 0.26 (±0.02) U/L. The specific activity of GST was found to be significantly reduced in brain tumor patients compared to normal healthy controls ($p<0.01$) (Figure 3). The GST level was significantly ($p<0.004$) more down regulated in males when compared to females. Similar trend in down regulation was observed in meginnia (tumor type, $p<0.02$) and glioma (tumor type, $p<0.02$) compared to other types of brain tumors. Statistical significant decrease in GSTs level was observed in the more advance tissues grade (III-IV, $p<0.005$) as compared to those with early tissue grade (I-II) (Figure 4).

**Discussion**

Carcinogenic detoxification is one of the most important areas of research in brain tumor (Dutheil et al., 2009). In the environment pro carcinogens are present, and enter into the human body through a variety of sources such as diet, industrial dust, tobacco and smoking. Initially they are metabolically inactive and are harmless but are activated in vivo and results in cancer (Curran et al., 2000). Carcinogens are detoxified by phase I and phase II enzymes. Phase I enzymes like CYP1A1 either detoxify the carcinogens or convert them into more electrophilic compounds. Among all the phase I xenobiotic metabolizing enzymes CYP comprises 70-80%. Phase II metabolizing enzymes such as GSTP1 play an important role in biotransformation of endogenous compounds and xenobiotics to more easily excretable forms as well as in the metabolic inactivation of active compounds making them water soluble (Jancova et al., 2010; Nosheen et al., 2011).

Variations in expression of CYP1A1 and GSTP1 could potentially explain the differences in susceptibility to the carcinogenic effects leading to brain carcinoma. Very few studies have investigated the GSTs enzymes and risk of developing brain cancer with inconsistent results (Schwartzbaum et al., 2007; Salnikova et al., 2010). In this study we evaluated possible associations between expression pattern of CYP1A1, GSTP1 and the risk of developing brain tumors. First, we observed expression profile of these two enzymes by western blotting from a subset of 36 brain tumor samples and 36 control samples. Our study found significant up regulation of CYP1A1 (phase 1 enzyme) in brain tumor samples compared to controls. Similar results have also been observed in lung cancer and human endothelial cell (Shah et al., 2009; Conway et al., 2009). Whereas conflicting results for

<table>
<thead>
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<th>No. of individual</th>
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**Table 1. Expressional Analysis of CYP1A1 and GSTP1 in Brain Tumor Samples**

<table>
<thead>
<tr>
<th>CYP1A1</th>
<th>GSTP1</th>
</tr>
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<tbody>
<tr>
<td>Down-regulation (27%)</td>
<td>Up-regulation (7%)</td>
</tr>
<tr>
<td>Down-regulation (28%)</td>
<td>Up-regulation (8%)</td>
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<table>
<thead>
<tr>
<th>Gender</th>
<th>Male</th>
<th>Female</th>
<th>aOR(95%CI)</th>
<th>p value</th>
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<tbody>
<tr>
<td>Male</td>
<td>4(11%)</td>
<td>3(9%)</td>
<td>0.64 (0.14-2.92)</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>Female</td>
<td>5(14%)</td>
<td>5(14%)</td>
<td>2.22 (0.04-11.18)</td>
<td>&lt;0.4</td>
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<table>
<thead>
<tr>
<th>Age</th>
<th>aOR(95%CI)</th>
<th>p value</th>
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<tbody>
<tr>
<td>&lt;25</td>
<td>2.4 (0.42-13.8)</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>&gt;25</td>
<td>0.06 (0.006-0.51)</td>
<td>&lt;0.22</td>
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<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>Grade</th>
<th>aOR(95%CI)</th>
<th>p value</th>
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<tbody>
<tr>
<td>Grade I and II</td>
<td>0.64 (0.14-2.92)</td>
<td>&lt;0.7</td>
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<tr>
<td>Grade III and IV</td>
<td>0.06 (0.006-0.51)</td>
<td>&lt;0.22</td>
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<table>
<thead>
<tr>
<th>p value</th>
<th>aOR(95%CI)</th>
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<tbody>
<tr>
<td>&lt;0.4</td>
<td>2.22 (0.44-11.18)</td>
</tr>
<tr>
<td>&lt;0.007</td>
<td>0.05 (0.006-0.51)</td>
</tr>
</tbody>
</table>

**Figure 2. Lysates were Subjected to Western Blotting Analysis Using anti CYP1A1 Antibody and anti GSTP1 Antibody.** [A] shows expression level of CYP1A1 in control tissue samples, [B] shows up regulation of CYP1A1 in tumor tissue samples, [C] shows expression level of GSTP1 in control samples [D] shows down regulation of GSTP1 in control samples

**Figure 3. Graphical Representation of GSTs Specific Activity in Patients as Well as Controls Showing Decreased GSTs Level in Patients Compared to Controls**

**Figure 4. GST Level in Brain Tumor Samples.** Column plot comparing the GST levels of brain tumors in males and females, in different age limits, in different types of brain tumors and in different brain tumor grades


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CYP1A1 expression pattern have been reported in some of other studies in different cancers (Chang et al., 2007; Masood et al., 2011; Hafeez et al., 2012). Significant up-regulation of CYP1A1 was observed in different types of brain tumors in current study. Accumulating evidence indicates CYP1A1 expression is controlled via AhR pathway when benzo pyrene activates CYP1A1 (Kasai et al., 2013). The increase in CYP1A1 activity leads to H$_2$O$_2$ production. An increase in cellular levels of H$_2$O$_2$ has been linked to several key alternations in cells leading to cancer including DNA alternation, cell proliferation, apoptosis resistance, metastasis, angiogenesis and hypoxia-inducible factor 1 (Androutsopoulos et al., 2009; Wincent et al., 2012).

In current study statistically significant down-regulation of GSTP1 was observed in brain tumor samples compared to control samples. Similar results have also been observed in breast cancer (Eralp et al, 2013), prostate cancer (Okino et al, 2007; Re et al., 2011) and colon cancer (Ritchie et al., 2009). Whereas conflicting results for GSTP1 expression pattern have been reported in some of other studies (Masood et al., 2011; Uchida et al., 2013). In present study, significant down-regulation of GSTP1 was observed in more advance grade of brain tumor samples when compared with early grade of brain tumor. The exact mechanism of this down regulation of GSTP1 expression and the role that decreased GSTP1 expression plays in brain tumorigenesis is as yet unclear. GSTP1 is an enzyme containing four selenium-cofactors that protects tissues from damage by catalyzing the breakdown of hydrogen peroxide and organic hydroperoxides into water (Briqelius-Flohe et al., 2009). Decreased level of GSTP1 can induce an ROS burden in the brain that cannot be reversed by chemotherapy or any other drug. This accumulation of ROS would provide a plausible biochemical mechanism to explain a role of down regulation GSTP1 in carcinogenesis (Shen et al., 2011).

In second part of current study, ELISA is done to detect GSTS specific activity in 36 brain tumors along with 36 control samples. Specific activity of GSTS found to be significantly low in tumor samples as compare to controls. Similar trends in GSTS level has also been reported in different studies in different cancer (Nosheen et al., 2010). When samples were analyzed according to histopathological and clinical parameters, lowest GSTS activity was found in males compared to female. Down regulation in GSTS was more pronounced in advance grade of brain tumors when compared with early grade. The main reason of lower level of GSTS in brain tumor in Pakistani population is unknown. However, previous studies have reported that GSTS down regulation may be attributed to null polymorphism of GSTS in Pakistani population in HNC (Nosheen et al., 2010: Nosheen et al., 2010).

This is the first study on the prognostic value of xenobiotic metabolizing genes (CYP1A1 and GSTP1) in patients with brain tumor in Pakistani population. Our data suggested that up-regulation of xenobiotic metabolizing genes such as CYP1A1, combined with decreased levels of GSTP1 and GSTs, may contribute to the initiation and progression of brain cancer in Pakistani population. Nevertheless, further validation studies with larger, independent sample sets and additional stratification to control potential confounding factors are needed to validate our findings.

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