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Review

In Vivo Models for Measuring Placental Glutatione-S-transferase (GST-P 7-7) Levels: A Suitable Biomarker for Understanding Cancer Pathogenesis

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Abstract. *The Glutatione-S-transferases (GSTs) comprise a family of enzymes closely associated with the cell detoxification of xenobiotics. GSTs exist as homo- or heterodimers and have been grouped into at least seven distinct classes. The main function of GSTs is to catalyze the conjugation of reduced glutathione (GSH) to an electrophilic site of a broad range of potentially toxic and carcinogenic compounds, thereby making such compounds less dangerous and enabling their ready-excretion. Placental GST, known as GST-P 7-7, is the main isoform found in normal placental tissue and comprises 67% of the total GST concentration in this tissue. During development, GST-P 7-7 decreases in concentration and is absent in adult tissues. Interestingly, GST-P 7-7 expression has been detected in adult tissues after exposure to carcinogenic agents in several experimental test systems, being considered a reliable biomarker of exposure and susceptibility in early phases of carcinogenesis. In this article, we review a series of studies involving GST-P 7-7 expression as a suitable tool for understanding cancer pathogenesis, especially cancer risk.*

Placental Glutathione-S-transferase and Cancer

The glutatione S-transferases (GSTs) are a family of crucial enzymes involved in the detoxification of xenobiotics. GSTs exist as homo- or heterodimers and have been grouped into at least seven distinct classes (1). The main function of GSTs is to catalyze the conjugation of reduced glutathione (GSH) to an electrophilic site of a broad range of potentially toxic and carcinogenic compounds, thereby making such compounds less dangerous and enabling their ready-excretion (2).

Placental GST, called GST-P 7-7, is the main isoform found in normal placental tissue and comprises 67% of the total GST concentration in this tissue (3). During development, GST-P 7-7 decreases in concentration and is absent from adult tissues (4). Interestingly, GST-P 7-7 expression has been detected in adult tissues in rat during medium-term carcinogenesis assay, being regarded a suitable biomarker for early detection of neoplasms such as those of the liver and tongue (5-7).

Tsuda *et al.* (8) has purposed liver carcinogenesis and multi-organ carcinogenesis, that are often used in conjunction and constitute an efficient and rapid bioassay, for the identification of both genotoxic and nongenotoxic carcinogenic chemicals (8). This is an 8-week bioassay system that uses the number and size of foci of altered hepatocytes positive for GST-P 7-7-positive foci as the end-point marker. Forty-four compounds were tested using this multi organ model: 17 out of 17 liver carcinogens and 19 out of 22 (86%) non-liver carcinogens gave positive results. None of the five noncarcinogens tested led to positive findings (8). In particular, protein lysates from microdissected GST-P 7-7-positive foci and hepatocellular carcinomas (HCC) from livers of rats treated with *N*-diethylnitrosamine followed by phenobarbital at doses of 0 and 500 ppm were analyzed. Coordinated overexpression of the mitochondrial chaperons prohibitin (PHB) and prohibitin 2 (PHB2), septin 9 (SEPT9), neurabin 1, and other cytoskeletal and functional proteins in GST-P 7-7-positive foci during initiation and/or promotion stages of rat hepatocarcinogenesis, was associated with induction of cell proliferation and progression of these preneoplastic lesions. Newly discovered elevation of PHB,

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PHB2, and SEPT9 in GST-P 7-7-positive foci and HCC implies that these proteins might play an important role in the onset of liver carcinogenesis and be of potential value in the studies of hepatocarcinogenesis (9).

To further elucidate the role of metal-related molecules in hepatocarcinogenesis, immunolocalization of transferrin receptor (Tfrc), ceruloplasmin (Cp) and metallothionein (MT)-1/2 in GST-P 7-7-positive foci was examined during early stage tumor promotion by fenbendazole (FB), phenobarbital, piperonyl butoxide, and thioacetamide in a rat two-stage hepatocarcinogenesis model. To estimate the involvement of oxidative stress responses in the promoter's stage, immunolocalization of 4-hydroxy-2-nonenal, malondialdehyde and acrolein was similarly examined. The results suggested that facilitation of lipid peroxidation is involved in the induction of GST-P 7-7-positive lesions by tumor promoters, and up-regulation of transferrin receptor gene (*Tfrc*) and down-regulation of Cannabinoid receptor (Cp) may be a signature of enhanced oxidative cellular stress in these lesions (10). On the other hand, several chemopreventive agents have been studied in this context. For example, licorice flavonoid oil (LFO) is a new functional food ingredient consisting of hydrophobic licorice polyphenols in medium-chain triglycerides. Recently, it was reported that licorice and its derivatives have anticarcinogenic activity against some types of tumor cell lines. However, the anticarcinogenic activity has not been identified in the liver, which is a major target organ for carcinogenesis in humans. No increase in the number of GST-P 7-7-positive liver foci was observed in LFO-treated groups compared with the negative control (solvent alone) group, and the number of foci in the group treated with the 600 mg/kg LFO was significantly lower than that in the negative control group. These results indicate that LFO concentrate has a significant inhibitory effect on liver carcinogenesis at 600 mg/kg (11).

Accumulating evidence suggests that GST-P 7-7 is expressed in the cytoplasm, mitochondria, and nucleus in some tumour cells, and that the nuclear expression of GST-P 7-7 appears to correlate with resistance to anticancer drugs. Although the mitochondrial targeting signal of GST-P 7-7 was previously identified as being located in the amino-terminal region, the mechanism of nuclear translocation remains completely unknown. Some authors have investigated the region 195-208 of GST-P 7-7 that is critical for nuclear translocation, mediated by a novel and non-classical nuclear localization signal. In addition, using an *in vitro* transport assay, it was demonstrated that the nuclear translocation of GST-P 7-7 depends on cytosolic extraction and ATP. Although further experiments are needed to understand in depth the precise mechanism of the nuclear translocation of GST-P 7-7, these findings may help to establish more efficient anticancer therapy, especially with respect to resistance to anticancer drugs (12).

When a DNA sequencing method was used to investigate the *GSTP* gene, no significant associations between exon 5 and exon 6 *GSTP1* gene polymorphisms and response to therapy or overall survival was detected. Patients carrying both variant exon 5 (Ile/Val or Val/Val) and variant exon 6 (Ala/Val) genotypes had significantly shorter time to progression (TTP) (5 vs. 8 months, p=0.04). Moreover, patients with heterozygous exon 6 variant presented with extensive-stage disease. No individual effect of variant alleles was found in relation to chemotherapy response, median TTP and overall survival. Therefore, the carriage of both types of variant alleles may predict a worse outcome (13).

Increased expression of GST-P 7-7 and of P-glycoprotein (P-gp) in tissues from patients with non-small cell lung cancer (NSCLC) has been associated with poor antineoplastic drug sensitivity, response to treatment, and survival. However, the diagnosis of advanced NSCLC often is mainly based on cytology. GST-P 7-7 and P-gp expression levels were associated inversely with response to chemotherapy and survival. Cytologic evaluation of GST-P 7-7 and P-gp expression may predict the response to treatment and the survival of patients with advanced NSCLC (14).

It has well been established that colonic polyps examined were adenoma of low, mild and high-grade dysplasia as shown in the histopathological reports (15). Nevertheless, the examination of the above specimens by electron microscopy revealed that three out of nine cases of adenoma of mild dysplasia had ultrastuctural features similar to those of high-grade dysplastic adenoma. GST-P 7-7 was variably expressed in adenoma, with the lowest levels occurring in low-grade adenoma and the highest in high-grade adenoma (15). GST-pi was mainly located in undifferentiated colonic epithelial cells (15). GST-P 7-7 positive particles were found in the cytoplasm and especially in the nucleus adjacent to the nuclear membrane of these cells. The overexpression of GST-P 7-7 in mild-grade adenomas with significant subcellular changes and in the majority of high-grade dysplastic adenomas suggests that this might be related to the carcinogenic process (15). Immunohistochemical localization of GST-P 7-7 in combination with ultrastructural changes indicates that GST-P 7-7 might be a sensitive agent for the detection of pre-malignant changes in adenoma (15).

Taking into consideration that GST-P 7-7 and cellular proliferation play a crucial role during carcinogenesis, our research group has struggled to investigate the expression of GST-P 7-7, and proliferating cellular nuclear antigen (PCNA) by means of immunohistochemistry during rat tongue carcinogenesis induced by 4-nitroquinoline-1-oxide (4NQO) (6). GST-P 7-7-positive foci were detected in non-neoplastic oral epithelial cells at 4 weeks of 4NQO administration (6). In the same way, GST-P 7-7-positive cells were detected in pre-neoplastic lesions and squamous cell carcinomas induced after

12 and 20 weeks' treatment, respectively (6). None of the control animals expressed GST-P 7-7-positive cells. Regarding cellular proliferation, the frequency of PCNA positive nuclei was higher at 12 and 20 weeks following 4NQO exposure ($p<0.05$) when compared to the negative control (6). These results suggest that the expression of GST-P 7-7 is correlated with cellular proliferation, in which GST-P 7-7 is associated with risk and progression of oral cancer, whereas PCNA is closely involved in the neoplastic conversion. GSTP 7-7 may facilitate cell proliferation and inhibit apoptosis, hence allowing for the expansion of a population of initiated tumor cells. The enhanced expression of GST-P 7-7 at the protein level has been reported previously in chemically-induced oral carcinomas in hamster buccal-pouch mucosa, but the expression of *GST-P 7-7* at the mRNA level has not yet been demonstrated. Therefore, some authors have investigated the *GST-P 7-7* mRNA expression in 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal-pouch carcinomas using a reverse transcription-polymerase chain reaction (RT-PCR) (16). *GST-P 7-7* mRNA was demonstrated to be present amongst all the 12-week DMBA-treated hamsters, but neither in the untreated animals, nor in the animals in which the buccal pouch was treated with mineral oil. Multiple potential regulatory pathways including gene amplification, enhanced mRNA stability, chromosomal translocation/gene rearrangement, and hypomethylation of the promoter region can contribute to the overexpression of *GST-P 7-7* mRNA in DMBA-induced carcinoma (16). Further study is necessary to completely understand which candidate mechanism(s) contribute principally to the increased *GST-P 7-7* mRNA expression in chemically-induced oral carcinogenesis.

Study of the regulation of the *GST-P 7-7* gene demonstrated that the strong enhancer element GPE1 (GST-P 7-7 enhancer-1) specifically regulates the *GST-P 7-7* gene by interacting with specific transcription factors in normal liver and during hepatocarcinogenesis. In particular, C/EBPalpha was required for the suppression of *GST-P 7-7* gene in normal liver, whereas the NF-E2 p45-related factor 2/transcription factor MafK (Nrf2/MafK) heterodimer was required for the activation of this gene during hepatocarcinogenesis (17).

The rat GST-P 7-7 is not expressed in normal liver but is highly induced at an early stage of chemical hepatocarcinogenesis and in HCC. Recently, it has been postulated that the Nrf2/MafK heterodimer binds to GPE1, a strong enhancer of the *GST-P* gene, and activates this gene in pre-neoplastic lesions and HCC. In addition to the positive regulation during hepatocarcinogenesis, negative regulatory mechanisms might work to repress GST-P 7-7 in normal liver, but this remains to be clarified. A chromatin immunoprecipitation analysis showed that C/EBPalpha bound to GPE1 in the normal liver *in vivo* but did not bind in pre-neoplastic hepatocytes. Introduction of the *C/EBPalpha* gene fused with the estrogen receptor ligand-binding domain into HCC cells, and subsequent activation by β-estradiol led to the suppression of endogenous GST-P 7-7 expression. These results indicate that C/EBPalpha is a negative regulator of *GST-P 7-7* gene expression in the normal liver (18).

To explain the molecular mechanism underlying its specific expression concomitant with the malignant transformation, several analyses regarding the regulatory element of *GST-P 7-7* gene and the transcription factor that binds to this element are timely (19). From the extensive analyses by the establishment of the transgenic rat lines having various regions of the *GST-P 7-7* gene, it would be interesting to determine if the GPE1 is a specific essential enhancer element for GST-P 7-7 expression (19). Moreover, an examination of the transcription factor that binds and activates GPE1, specifically in the early stage of hepatocarcinogenesis and in the HCC, could contribute to a better understanding of the role of *GSTP* gene in the carcinogenesis process. Such analysis indicates that the Nrf2/MafK heterodimer binds and activates GPE1 element in pre-neoplastic lesions and HCC but not in the normal liver cells (18).

GST are also involved in cellular protection against xenobiotics and oxidative stress, as well as in resistance to chemotherapeutic compounds, such as doxorubicin. Levels of human GST are known to be increased in many tumor types and hematopoietic diseases. Transient transfection assays have been used to show that different GST-promoter reporter constructs generate cell-type specific levels of luciferase activity. In expressing cells, transcriptional activity is strongly dependent on (AP-1) binding elements within the -65 to -75 bp region of the *GSTP1* gene as shown by site-directed mutagenesis. Electrophoretic mobility shift assays show that DNA binding activity is exclusively observed in GSTP1-1-expressing cells and is increased after stimulation with hydrogen peroxide, (TPA), tert-butylhydroquinone and doxorubicin. Non-expressing cells present neither constitutive nor inducible AP-1 binding. Taken together, such findings provide evidence for the induction of the *GSTP1* gene via AP-1 binding activity in leukemia cells and contribute to a better understanding of the molecular events regulating genes involved in drug resistance mechanisms (20).

Vanadium has recently been found to possess potent anticarcinogenic activity in rat colon carcinogenesis. Some researchers have postulated the expression of aberrant crypt foci (ACF)-positive and GST-P 7-7 during 1,2-dimethylhydrazine (DMH)-induced rat colon carcinogenesis (21). Vanadium-treated groups showed a significantly reduced ($p<0.001$) expression of GST-P 7-7-positive ACF cells (by 71.13%) for the entire period of the study. Moreover, the histopathological examination also showed that vanadium minimized the development of ACF ($p<0.001$). Furthermore, vanadium supplementation also elevated superoxide dismutase (SOD) activities in both liver and colon when

compared to controls (21). The results confirm that vanadium is particularly effective in limiting the action of the DMH, thereby establishing its anticarcinogenicity in chemically-induced rat colon carcinogenesis (21).

Conclusion

In this review, we have highlighted recent advances in the study of GST-P 7-7 expression following experimental carcinogenesis. Herein, *in vivo* studies combined with diabetes, use of therapeutic drugs, or even immunological disorders. In addition, the role of GST-P 7-7 concerning its interference in cellular signaling pathways, gene expression profiles, and epigenetic mechanisms is fundamental to elucidate putative interactions with the cellular machinery. Therefore, this is an area that warrants investigation, since the estimation of GST-P 7-7 expression from using such important techniques following experimental carcinogenesis will be added to those already established in the literature as a way to a better understanding of cancer pathogenesis.

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