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Original Contribution

EXPRESSION OF THE XENOBIOTIC- AND REACTIVE OXYGEN SPECIES-DETOXIFYING ENZYMES, GST-Pi, Cu/Zn-SOD AND Mn-SOD IN PRIMARY HEPATOCELLULAR CARCINOMA

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ABSTRACT

Pathology of liver carcinogenesis in HCC includes generation of oxidative stress and up-regulation of inflammatory cytokines or vice versa. Our current study assessed the expression levels of detoxifying enzymes of xenobiotics and oxygen reactive species (ROS), particularly GST-pi, Cu/Zn-SOD and Mn-SOD, and compared them with expression levels in the adjacent "normal" liver tissue. The expression of the enzymes was studied immunohistochemically in a small subset of 10 HCC biopsies obtained from 6 male and 4 female patients aged between 25 and 82 years. The expression level of GST-pi in tumour cells was significantly stronger than in the adjacent "normal" liver ($p<0.008$, Wilcoxon signed rank test). On the other hand, the expression of Cu/Zn-SOD was more prominent in 'normal" liver tissue than in the HCC ($p=0.008$, Wilcoxon signed rank test), whereas the immune signal for Mn-SOD was equally available and strong in both studied areas ($p>0.999$). Biopsies with stronger expression of Cu/Zn-SOD in "normal" liver was associated with lower serum levels of AlAT and AsAT ($p=0.030$, and $p=0.190$, respectively, ANOVA). We suggest that the changes in expression of xenobiotic- and ROS-detoxifying enzymes might be implicated in HCC via compromised response to injuring factors and their carcinogenic intermediators.

Key words: HCC, GST-pi, SOD1, SOD2, immunohistochemistry

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and appears currently to be the fifth most common solid tumour worldwide and the fourth leading cause of cancer-related death (1). Eighty percent of new cases occur in developing countries, but the incidence is increasing in economically developed regions, including Japan, The United States, and Western Europe (1). The cancer statistics in Bulgaria showed that there is no strong trend for raising the incidence of primary liver cancers: the age-adjusted rates/100,000 for males fluctuated from 4.9 in 1981, decreased to 3.7 in 1997 and went up to 5.5 in 2004, whereas for females these fluctuations were from 2.8 in 1981, down to 1.6 in 1992 and up to 2.1 in 2004 (2). According to a study on mortality from primary liver cancers in Europe, Bulgaria belongs to a group of 7 countries, for which the age-standardized mortality rates were approximately stable or showed no consistent trends (3). Though these are encouraging figures, the primary liver cancers, including the HCC, are serious medical problems because of the late diagnosis and short patient survival. The median survival in patients with HCC has been reported to be only 8.0 months (4).

Aetiologically HCC is a complex and multifactorial disease that is linked to both viral and chemical carcinogens. The main risk factors associated with HCC are chronic infections with hepatitis C and B viruses, alcoholic liver disease, idiopathic cirrhosis, and aflatoxine B1 (5). In the United States, hepatitis C virus (HCV), alcohol use, and non-alcoholic fatty liver disease are the most common cause of cirrhosis and consequently of HCC [1]. Bulgarian single institution studies reported a very high frequency of HCC associated with cirrhosis - 69.2% (6), and toxic factors, hepatitis B virus (HBV) infection and chronic alcoholism, were recognized as high risk aetiological factors (7).

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The pathogenetic processes underlying liver carcinogenesis by factors mentioned above are shown to involve the oxidative reactions mainly, leading to the generation of reactive electrophilic metabolic intermediates and reactive oxygen species (ROS)(8). The consequences of their effects in the cells are DNA damages and DNA adducts believed to be responsible for mutations leading to cancer (8, 9). Thus, like most solid tumours, the development and progression of HCC are believed to be caused by accumulation of genetic changes resulting in altered expression of either "liver" specific or "cancer" specific genes. The cancer-related gene products are proteins involved in different regulatory pathways, such as cell cycle control, apoptosis, adhesion and angiogenesis, whereas the hepatocyte-specific gene products participate in the metabolism of nutrient factors and those responsible for the liver-synthesized proteins and detoxification enzymes (10).

Glutathione-S-transferases (GSTs, EC. 2.5.1.18) are a family of phase II detoxification enzymes that catalyze the conjugation with glutathione of a wide variety of endogenous and exogenous toxic compounds, including aflatoxin B1, other dietary carcinogen (arylamines, polycyclic aromatic hydrocarbons, nitrosamines, etc.) several types of anticancer drugs, hydroperoxides and other ROS products (11-14). So far, in human 22 members of GST family have been identified and based on their protein structure they are classified into 8 classes (11, 15). The acidic GST isoenzyme, GST-pi, in addition to its catalytic activity to different substrates, including lipid peroxides, possesses ligand-binding properties for proteins and organic anions and carcinogens (16). Immunohistochemically this enzyme form is found to be hardly detectible in normal liver, but markedly increased in pre-neoplastic hepatic lesions, such as hyperplastic nodules and in HCC (17-19). However some discrepant results have also been reported (16, 20).

Superoxide dismutases (SODs, EC. 1.15.1.1) are a family of enzymes responsible for the first line of antioxidant defence against the highly reactive oxygen species (ROS) generated both by biochemical redox reactions of the normal cell metabolism and by exogenous factors, such as UV light, gamma radiation, environmental pollution, anticancer drugs and medications (21-25). There are three main SOD isoenzymes in mammalians, with different localization: the type I - Cu/Zn-SOD (SOD1), located primarily in the cytosol but also in the nucleus; type II- Mn-SOD (SOD2) sited in mitochondria; and type III - Cu/Zn-SOD (SOD3) is found as extracellular enzyme (24).

Cu/Zn-SOD (SOD1) is mainly a cytosolic enzyme, but also has been found in nucleus and peroxisomes (26). SOD1 levels have been detected in the liver, kidney, erythrocytes, and central nervous system. The mRNA of this enzyme has been reported to be increased in cultured cells by different cell stress factors as radiation, chemical oxidants and metal ions, suggesting a potential effect of this antioxidant on the growth and resistance of cancer cells (24).

Mn-SOD (SOD2) is located in the mitochondrial matrix near the electron transport chain (27). It is translated extramitochondrially as a precursor containing a mitochondrial targeting sequence to enable mitochondrial sequestration (24). SOD2 is induced by a wide variety of factors as hyperoxia, irradiation, cytokines (IL1, TNF-α), LPS, oxidized LDL and the cellular redox state, suggesting a role of this enzyme in protection against oxidant injury, hyperoxia and TNF-induced cytotoxicity (24, 28).

The type III SOD, Cu/Zn-SOD (SOD3), the extracellular secretory form of SOD (ECSOD) is a glycoprotein with a high affinity for heparin sulphate (24, 27). This enzyme is found in extracellular fluids and in the extracellular matrix of all human tissues, especially the heart, placenta, pancreas and lung (27, 29). Its main factions are confirmed to be the scavenging of superoxide generated from the cell surfaces and the regulation of NO bioavailability (24, 27).

The purpose of this study was to determine the ability of hepatocellular carcinoma and surrounding liver tissue to respond to the increased oxidative stress generated during the tumour transformation and metabolism via changes of the expression of some antioxidant enzymes, such as GST-pi, SOD1 and SOD2.

MATERIAL AND METHODS

The expression of the enzymes was studied in a small subset of 9 HCC biopsies obtained from 5 male and 4 female patients aged between 25 and 82 years (median of 59 years). All patients had no history of previous liver diseases, for example, diabetes mellitus, haemochromatosis, haemotransfusions or alcohol abuse. Informed consent was obtained from each patient.

Immunohistochemical detection of the expression of studied enzymes in non-involved liver tissue and in tumours was performed by applying the standard streptavidin-biotin method on formalin-fixed paraffin-embedded 10 μm thick sections as described earlier (30). The antibodies used were: monoclonal mouse antibody to human Glutathione-S-transferase pi (DAKO-GST Pi, 353-10, M0529); monoclonal mouse antibody to human Superoxide dismutase (SOD1) clone SD-G6 (Sigma, S-2147); Monoclonal mouse antibody to human Manganese Superoxide dismutase (MnSOD, clone MnS-1) (Bender MedSystems, BMS 122). The detection system was StreptABComplex/HRP Duet, Mouse/Rabbit kit (DAKO, Ko492) and 3.3'-diaminobenzidine (DAB) as a chromogene.

Sections incubated with non-immune sera instead of the primary antibodies were used as negative controls. The optimal dilutions of the primary antibodies was found out in a series of staining performed prior to this study and were as follows: 1:100 for GST-pi, 1:500 for SOD1, and 1:300 for SOD2.

For analyzing the results we used a package of statistical programs StatView for Windows v.4.53 (Abacus Concept Inc, USA). There were applied the standard descriptive analyses, ANOVA test, Wilcoxon signed rank test, $\chi 2$ test, and Fisher exact test. Factors with $p<0.05$ were considered statistically significant.

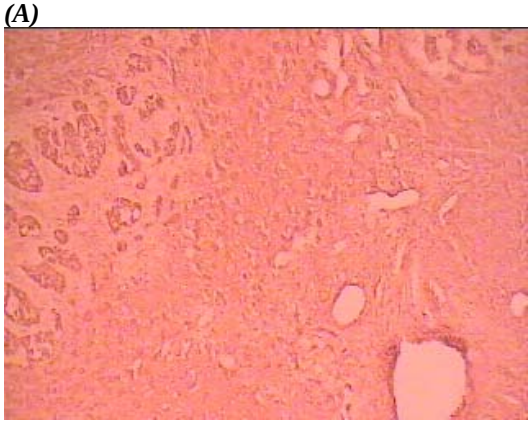
RESULTS

The immunohistochemical reaction for GST-pi was detected as brown staining in the cytoplasm of cells expressing this protein. A weak (+) GST-pi immunoreactivity was the main finding in non-affected liver tissues (**Figure 1a**), whereas there was a very strong immune reaction in epithelium of billiary ducts, which we considered as an internal positive control (**Figure 1b**). In all tumour specimens we observed cytoplasmic reaction for GST-pi (**Figure 1c**), as it was significantly stronger than in the adjacent "normal" liver ($p<0.008$, Wilcoxon signed rank test) (**Figure 2**).

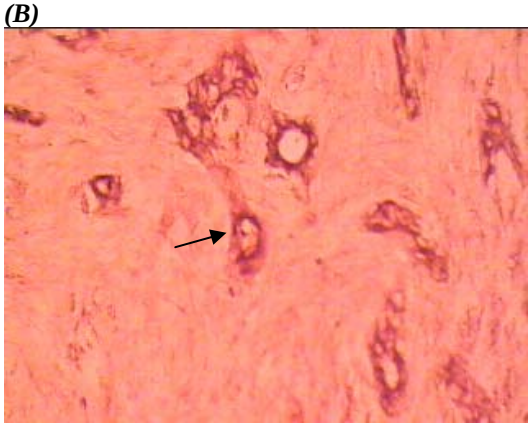
Immune deposits for SOD1 and SOD2 were predominantly localized in the cytoplasm of the cells, expressing these proteins. In contrast to GST-pi, the expression of SOD1 was more prominent in normal liver tissue than in the HCC (**Figures 3** and **4**) ($p=0.008$, Wilcoxon signed rank test), whereas the immune signal for SOD2 was almost equally weak to negative in both studied areas ($p>0.999$) (**Figures 4** and **5**).

The stronger expression of SOD1 in normal liver tissue was significantly associated with younger age compared to the moderate expression (43.2±12.8 years vs. 71.0±10.1 years, $p=0.009$, ANOVA test) (**Figure 6**). Similar tendency was observed for SOD2 expression in normal liver tissue (50.0±19.3 years for patients with SOD-2 weak or moderate expression vs. 62.5±16.7 years for patients with SOD-2 negative liver tissues, $p=0.340$, ANOVA test) (**Figure 6**).

(A)



(B)



(C)

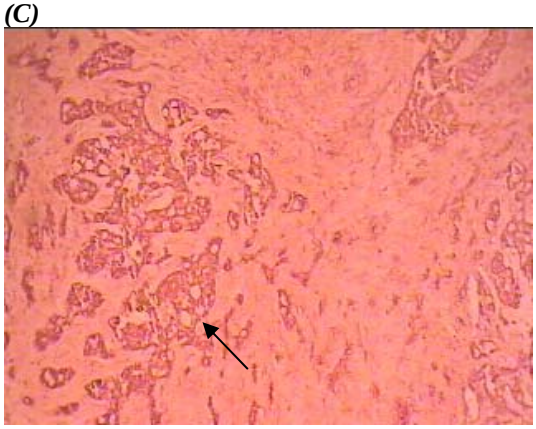


Figure 1: *Immunohistochemically detectable expression levels of GST-pi in non-affected liver tissues (A), in epithelium of billiary ducts (B, arrow) and in tumour tissue of HCC (C, arrow) (magnifications: A- 100x; B-400x; C-100x)*

When the serum concentrations of the transaminases, AlAT and AsAT were compared to the expression levels of SOD1

and SOD2 in normal liver tissues of the patients with HCC, we observed an interesting significant association: patients with stronger expression of SOD1 in normal liver had lower AlAT and AsAT serum concentrations (p=0.030, and p=0.190, respectively, ANOVA test) (**Figure 7**). Similar trend, although not significant, was observed between the expression of SOD2 in normal liver tissue and the levels of these transferases (p>0.05, ANOVA test) (**Figure 7**).

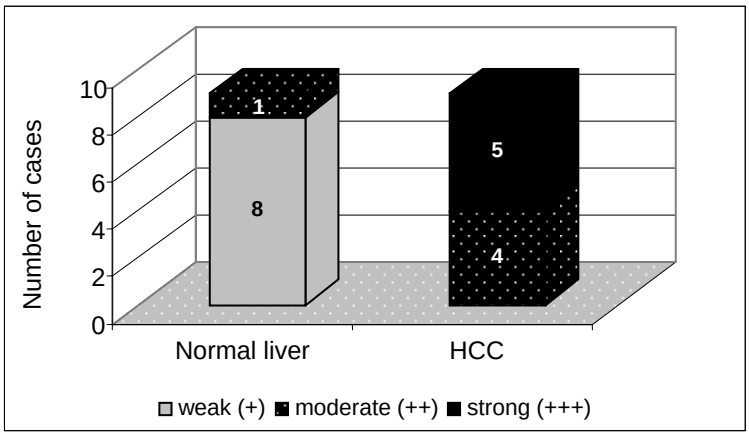


Figure 2: *Comparison of the frequency of immunohistochemically detectable expression levels of GST-pi in normal non-affected liver tissue and in HCC.*

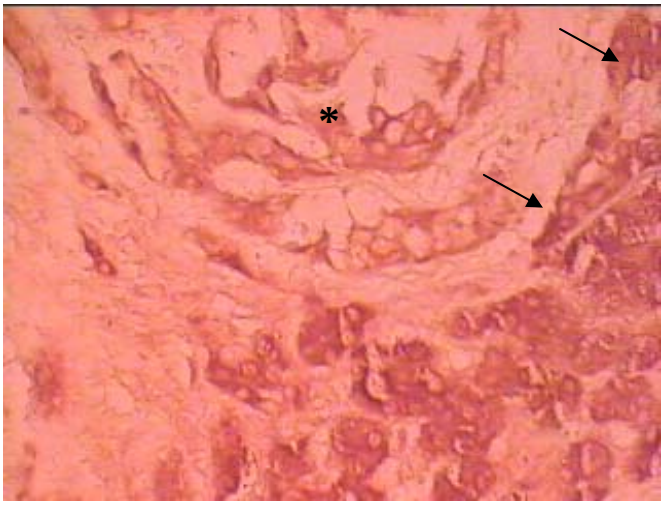


Figure 3: *Strong SOD1 immune signal in the cytoplasm of normal hepatocytes (arrows) and weak immune deposits in the tumour cells (*) (magnification: 400 x)*

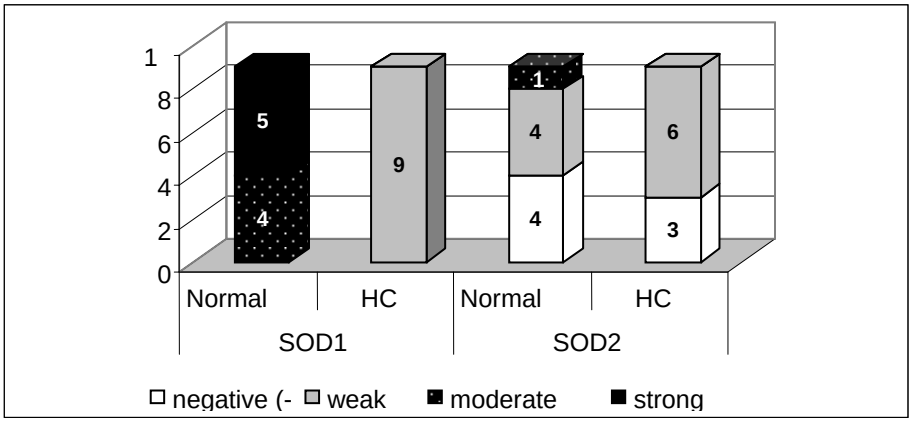


Figure 4: *Comparison of the frequency of immunohistochemically detectable expression of SOD1 and SOD2 in normal non-affected liver tissue and in HCC*

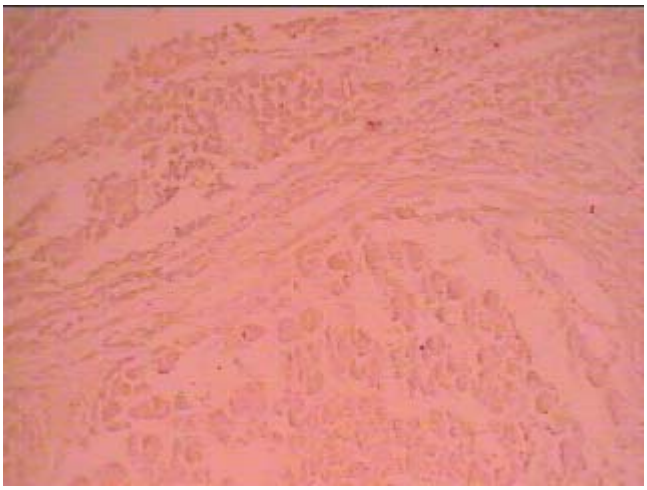


Figure 5: *Weak, almost negative immune reaction for SOD2 both in normal liver tissue and in cells of HCC (magnification - 100x)*

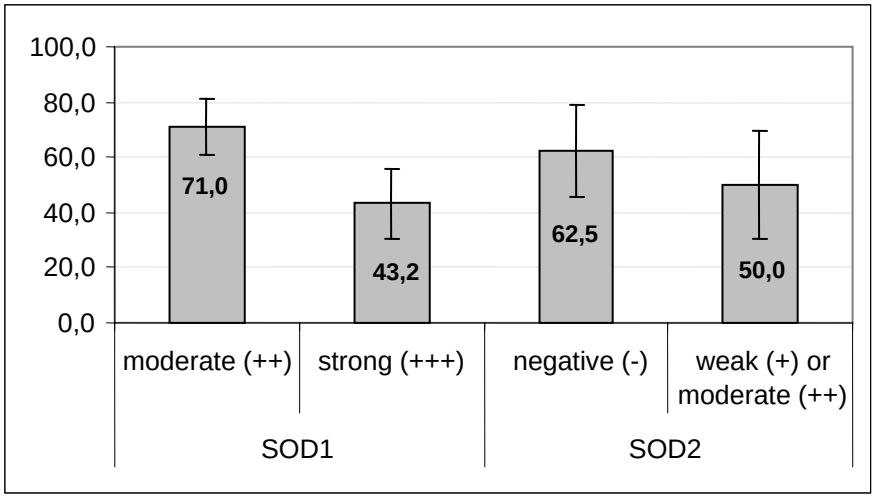


Figure 6: *Associations between the immunohistochemically assessed expression levels of SOD1 and SOD2 in normal non-affected liver tissue and age of the diagnosis of the HCC*

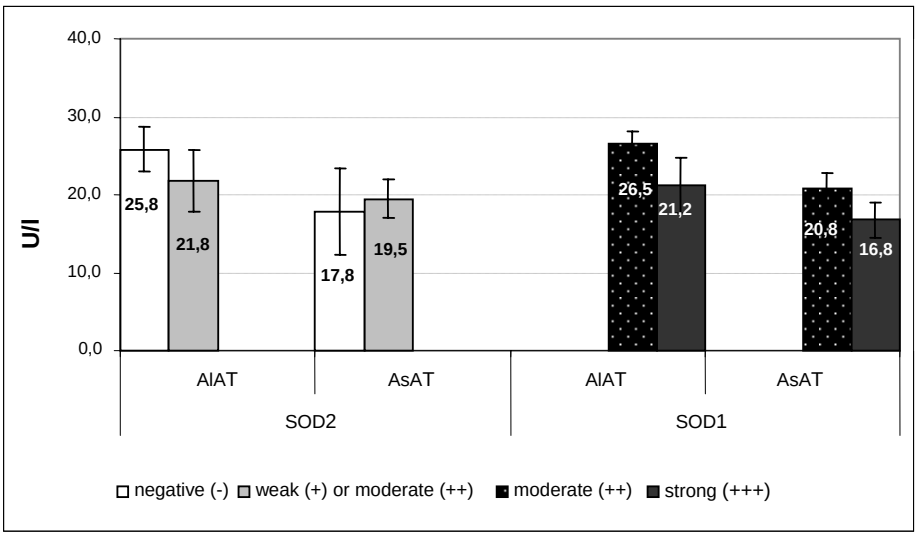


Figure 7. *Associations between the immunohistochemically assessed expression levels of SOD1 and SOD2 in normal non-affected liver tissue and serum concentrations of the transaminases, AlAT and AsAT*

DISCUSSION

This work focuses on a study using immunohistochemical staining to examine the expression of three antioxidant enzymes, GST-pi, SOD1 and SOD2 in normal non-affected liver tissue and in HCC. We observed a high expression of GST-pi in the cytoplasm of tumour cells of all HCC. This finding is in line with the results of several other research groups, suggesting that GST-pi may be a reliable marker enzyme for immunohistochemical detection and diagnosis of human HCC and its pre-neoplastic lesions (17, 18). These authors have found that GST-pi is a more sensitive tumour marker for HCC than alpha-fetoprotein (AFP) and could be used as an early marker for screening for HCC (17, 18). Because GST-pi conjugates the reactive oxygen intermediates that are generated by many anticancer agents, the high expression of this detoxification enzyme in HCC may be accountable for the inefficacy of systemic chemotherapy of this disease (1, 31).

Expression of SOD isoenzymes in normal liver tissues has been reported in several other studies (32-37). The findings of these studies concerning SOD1 expression were quite similar: the normal liver tissue has relatively strong expression of this antioxidant enzyme. On the other hand, the observed expression in the cells from different liver lesions, such as HCC, colorectal liver metastases, alcoholic fatty liver, viral liver diseases, hepatocytes with heavy iron burden, acute and chronic hepatitis, or liver cirrhosis was quite variable: in some liver diseases there was enhanced SOD1 expression compared to the normal non-injured liver tissue (37) or decreased level in other lesions (33, 34, 36, 38). Thus, our results that describe decreased level of SOD1 in tumour cells of HCC compared to the “normally” appearing liver cells adjacent to the tumour are in accord with those latter reports. They also confirm the results of research groups, presenting decreased activity of SOD1 in HCC compared to the surrounding “normal tissue (38).

Interesting observation of our current work is the association between the higher expression levels of SOD1 in adjacent non-affected liver tissue and the older age of diagnosis of the cancer. In addition, we found that higher level of SOD1 could be associated with a better liver function of the patients with HCC (lower serum transaminase concentrations). Those findings can be explained with the protective role of the studied antioxidant enzymes against the liver injury factors such as the reactive oxygen radicals, which are highly generated during the process of carcinogenesis and by the tumour cells. It is well documented that the malignant cells in general are more active than normal cells in the production of superoxide radicals and other ROS and are under intrinsic oxidative stress (39, 40). However the increased amount of these radicals leads to their diffusion in surrounding normal tissue and further damaging of it, which results in impairment of the functions in the liver in the case of HCC. Thus, the up-regulation of SOD and other antioxidant enzymes, such as GSTs, catalase, glutathione peroxidases, is most likely the mechanism for tolerating increased ROS stress protecting the normal hepatocytes and their functions.

In our work we also observed weak to negative expression of the mitochondrial isoenzyme of SOD, Mn-SOD (SOD-2) in tumour cells of HCC. This finding is in line with the reported lack of expression of Mn-SOD in hepatocellular carcinoma cells of all cases in the study of Inagaki et al (36). However, there is another report, which describes quite a high proportion of hepatocellular carcinomas expressing SOD2 in tumour cells and strong positive immunoreactivity in noncancerous liver tissues, especially in normal hepatocytes surrounding HCC (35). This discrepancy could be attributed to the different antibodies used for immunohistochemistry in the analyses.

Based on our finding for lower level of expression of SOD1 and SOD2 in hepatocellular carcinoma cells compared to the adjacent liver tissue, we suggest that the impaired expression of those antioxidant defence enzymes most probably contribute to generation of active oxygen species, organic peroxides and radicals in liver cells and cancer cells, resulting in further additional genetic damages and mutations, which probably lead to carcinogenesis and further increase of the aggressiveness of the tumour.

In conclusion, we suggest that the changes in the expression of xenobiotic- and ROS-detoxifying enzymes might be implicated in HCC development and progression via compromising the response to the injuring factors, their carcinogenic intermediators and free radical products.

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