Expression of c-erbB-2 and glutathione S-transferase-pi in hepatocellular carcinoma and its adjacent tissue

Zhao-Shan Niu, Mei Wang

Zhao-Shan Niu, Department of Pathology, Medical College of Qingdao University, Qingdao 266021, Shandong Province, China
Mei Wang, Institute of Education, the University of Reading, Britain RG6 1HY, Reading, United Kingdom

Supported by the Scientific Research Foundation of Shandong Provincial Education Committee (J94, K26)
Correspondence to: Zhao-Shan Niu, Department of Pathology, Medical College of Qingdao University, 38 Dengzhou Road, Qingdao 266021, Shandong Province, China. niumiao1993@hotmail.com
Telephone: +86-532-3812410
Received: 2004-09-22 Accepted: 2004-11-19

Abstract
AIM: To investigate the possible role of c-erbB-2 and glutathione S-transferase (GST-Pi) in primary hepatocellular carcinogenesis and the relationship between liver hyperplastic nodule (LHN), liver cirrhosis (LC), and hepatocellular carcinoma (HCC).

METHODS: The expression of c-erbB-2 and GST-Pi was detected immunohistochemically in 41 tissue specimens of HCC and 77 specimens of its adjacent tissue.

RESULTS: The positive expression of c-erbB-2 in LHN (28.6%) was significantly higher than that in LC (0%) ($P < 0.05$), but no significant difference was seen between HCC and LHN or LC ($P > 0.05$, $\chi^2 = 0.002$, 3.447). The positive expression of GST-Pi in HCC (89.6%) or LHN (71.1%) was significantly higher than that in LC (22.9%, $P < 0.001$, $\chi^2 = 49.91$, 16.96). There was a significant difference between HCC and LHN ($P < 0.05$, $\chi^2 = 6.353$).

CONCLUSION: The c-erbB-2 expression is an early event in the pathogenesis of HCC. GST-Pi may be a marker enzyme for immunohistochemical detection of human HCC and its preneoplastic lesions. LHN seems to be a preneoplastic lesion related to hepatocarcinogenesis.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Liver neoplasm; Hyperplastic nodule; C-erbB-2 gene; GST-pi gene; Immunohistochemistry

Niu ZS, Wang M. Expression of c-erbB-2 and glutathione S-transferase-pi in hepatocellular carcinoma and its adjacent tissue. World J Gastroenterol 2005; 11(28): 4404-4408
http://www.wjgnet.com/1007-9327/11/4404.asp

INTRODUCTION
Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors in Asia, and the incidence and mortality of HCC show a tendency to rise year by year. Therefore, the detection of preneoplastic lesions of HCC is crucial for the analysis of carcinogenic processes and developing strategies for prevention and treatment. For many years, liver hyperplastic nodule (LHN) has been considered as a preneoplastic lesion. Our previous studies have also confirmed that LHN is closely related to human HCC.

In the present study, the immunohistochemical LSAB method was used to detect the expression of c-erbB-2 oncogene and glutathione S-transferase-pi (GST-pi) in HCC and pericarcinomatous tissues, in order to investigate the possible roles of these genes in the HCC carcinogenesis, and to find out the relationship between LHN, liver cirrhosis (LC), and HCC.

MATERIALS AND METHODS

Materials
HCC specimens from 100 patients were selected during surgical resections or biopsies performed at the Affiliated Hospital of Medical College of Qingdao University, China. Of these patients, 76 were males and 24 females with an average age of 50.4 years. None of the patients received chemo- or radiotherapy before resection. We randomly selected 41 and 77 cases of HCC to detect the expression of c-erbB-2 and GST-pi, respectively. The same specimens from 16 cases were detected for both c-erbB-2 and GST-pi, which were too few to be analyzed in terms of the correlation between them in the present study. Forty-one specimens for detecting c-erbB-2 oncogene contained 18 pericarcinomatous tissues, including 14 LHN, 17 LC. Seventy-seven specimens for detecting GST-pi contained 40 pericarcinomatous tissues, including 38 LHN, and 35 LC.

Methods
All specimens were routinely processed, alcohol-fixed and paraffin-embedded. Serial paraffin sections of 4 µm thickness were cut and used for hematoxylin and eosin and immunohistochemical staining. Immunohistochemical LSAB method was used to detect c-erbB-2 and GST-pi. Anti-c-erbB-2 multiple clonal antibody, anti-GST-pi multiple clonal antibody and LSAB kits were purchased from Dako Co. Before staining, the sections were microwave heated in 0.05 mol citric acid solution for antigen retrieval. In each staining run, a known c-erbB-2 or GST-pi positive section was added as positive control, PBS was used as substitutes.
of the first antibodies for negative control.

**Analysis of immunohistochemical staining**

When brown granules were found on cell membrane of liver cells and cancer cells, c-erbB-2 was identified positive. When only cytoplasm was stained brown, c-erbB-2 was identified negative. The c-erbB-2 expression was graded semi-quantitatively according to the intensity and the percentage of positivity into negative (-): no positive cells or a weak staining of c-erbB-2 with positive cells<5%; positive (+): c-erbB-2 expression was relatively stronger with the positive cells>5%. For GST-pi, cells with brown granules in cytoplasm were regarded as positive. The criteria of the evaluation of GST-pi expression in this study were as follows.

The positive number was semiquantitatively evaluated by counting that in 5-10 randomly chosen medium power (×100 magnification), and the three grades for GST-pi were considered as negative (-): no positive cells; positive (+): the positive reaction being brown and the positive cells <30%; strong positive (++): the positive reaction being brown and the positive cells>30% according to the intensity and extent of GST-pi staining.

**Statistical analysis**

Results were analyzed by \( \chi^2 \)-test and direct probability calculation. \( P<0.05 \) was considered statistically significant.

**RESULTS**

**Expression of c-erbB-2 oncogene in HCC and its adjacent tissue**

The positive staining for c-erbB-2 in cancer cells was exclusively located in cell membrane stained brown, the expression of c-erbB-2 was observed in both cell membrane and cytoplasm in one of four cases of LHN expressing c-erbB-2 (Figure 1A). The c-erbB-2 positive rate was significantly higher in LHN (28.6%) than that in LC (0%, \( P \) value 0.032, direct probability calculation, \( P<0.05 \), but no significant difference was seen between HCC (Figure 1B) and LHN or LC (\( \chi^2 \) values 0.002, 3.447, \( P>0.05 \), Table 1).

| Table 1 | Expression of c-erbB-2 oncogene in HCC and its adjacent tissue |
|---------|---------------------------------------------------------------|
| Histological type | Case | Expression of c-erbB-2 oncogene | Positive rate (%) |
|---------|------|-------------------------------|------------------|
| HCC     | 41   | -                             | 24.4%            |
| LHN     | 14   | -                             | 28.6%            |
| LC      | 17   | -                             | 0%               |

\( ^*P<0.05 \) vs LC.

**Expression of GST-pi in HCC and its adjacent tissue**

The positive staining for GST-pi appeared as brown granules, which was predominantly located in the cytoplasm, and the staining of the nuclei was seen in part of the cancer cells. There was strong staining of GST-pi in bile duct epithelial cells. A weak staining of GST-pi was observed in LC with a positive rate of 22.9% (8/35). GST-pi expression was markedly stronger in HCC or LHN (Figure 1C). The rates of positivity were 89.6% (69/77) and 71.1% (27/38), respectively, and significantly higher than that in LC (\( \chi^2 \) values 49.91, 16.96, \( P<0.001 \)). There was also a significant difference between HCC (Figure 1D) and LHN (\( \chi^2 \) value 6.353, \( P<0.05 \), Table 2).

**DISCUSSION**

The c-erbB-2/neu is a transforming proto-oncogene encoding
c-erbB-2 overexpression was observed in 24.4% (n expression rate of c-erbB-2 in LHN was 28.6% (study, all four cases overexpressing c-erbB-2 oncogene in cancerous or have malignant phenotype. In the present those cells overexpressing c-erbB-2 oncogene are either role in transformation and tumorigenesis maintenance of malignant phenotype, but also plays a pivotal progression of HCC. c-erbB-2 expression can contribute to the initiation and maintains the transformed hepatocyte phenotype. Apparently, also occurs later in the process of hepatocarcinogeneis and into HCC in the present study, indicating that c-erbB-2 expression decrease in the transition from pericarcinomatous tissues erbB-2 oncogene plays an initial role in hepatocarcinogeneis, and might only have promoting effects of c-erbB-2 oncogene in carcinogenesis of HCC. Some studies reported that c-erbB-2 does not play a role in transformation activity. In human malignancies, the activation of c-erbB-2 is most frequently caused by gene amplification, there is extremely high concordance between copy number of gene amplification and protein overexpression[2,3]. The c-erbB-2 is frequently overexpressed in different tumors in humans, and the activation of c-erbB-2 appears to be an early event in tumorigenesis for some cancers[4,5]. However, there seems to be conflicting reports concerning the role of c-erbB-2 oncogene in carcinogenesis of HCC. Some studies reported that c-erbB-2 does not play a role in tumorigenesis of HCC[6], whereas others reported that the overexpression of c-erbB-2 is found in the middle stage of hepatocarcinogenesis, and might only have promoting effects during the development of this lesion[7]. In the present study, c-erbB-2 overexpression was observed in 24.4% (n = 41) of HCC tissue specimens and 22.2% (n = 18) of its adjacent tissue specimens, suggesting that the overexpression of c-erbB-2 oncogene plays an initial role in hepatocarcinogenesis, and is an early molecule change in the carcinogenesis of HCC. In addition, the expression of c-erbB-2 did not decrease in the transition from pericarcinomatous tissues into HCC in the present study, indicating that c-erbB-2 expression also occurs later in the process of hepatocarcinogenesis and maintains the transformed hepatocyte phenotype. Apparently, c-erbB-2 expression can contribute to the initiation and progression of HCC.

Many studies have demonstrated that c-erbB-2 gene product not only contributes to the development and maintenance of malignant phenotype, but also plays a pivotal role in transformation and tumorogenesis[8,9], because 3T3 cells or immortalized cells transfected with c-erbB-2 oncogene show a highly transformed and tumorigenic phenotype[10,11]. It can be inferred from these studies that those cells overexpressing c-erbB-2 oncogene are either cancerous or have malignant phenotype. In the present study, all four cases overexpressing c-erbB-2 oncogene in pericarcinomatous tissues were LHN, thus, the positive expression rate of c-erbB-2 in LHN was 28.6% (n = 14), and similar to that of HCC, suggesting that those cells overexpressing c-erbB-2 oncogene in LHN have at least acquired the malignant phenotype, which may reflect the alterations in different biologic state of LHN. Some studies reported that the increased oncogene expression brings cells into a state of active proliferation that results in an increased frequency of mutation[12]. Therefore, it is reasonable to postulate that the overexpression of c-erbB-2 oncogene may make LHN transform malignantly, and parts of LHN are actually in the preneoplastic state or might be cancerous. In contrast to LHN, the expression of c-erbB-2 oncogene is negative in LC, implying that there is no activation of c-erbB-2 in LC, i.e., it is impossible in this situation for malignant transformation. There is also no mutation of p53 in LC, consequently, LC does not necessarily link with hepatocarcinogenesis. Our findings further confirm the notion that it is not LC but LHN, is a preneoplastic lesion for the occurrence of HCC in humans.

GSTs play an important role in protecting cells against cytotoxic and carcinogenic agents. GST-pi is an acid GST, isolated and purified from human term placenta, which possesses catalytic and ligand-binding properties, and is regarded as a new marker enzyme for tumors. Some studies reported that GST-pi or GST-pi mRNA is hardly detectable in normal liver, but markedly increases in preneoplastic hepatic lesions such as hyperplastic nodules and in HCC by immunohistochemical staining or in situ hybridization[13,14], suggesting that GST-pi is a sensitive marker enzyme for preneoplastic lesions and neoplastic cells, not only at the protein level but also at the mRNA level, throughout the hepatocarcinogenesis in rat liver. In addition, other studies reported that single cells immunohistochemically positive for GST-pi induced in the rat liver carcinogenesis by chemical carcinogens are precursor-initiated cells of preneoplastic foci, GST-pi content in the single cells is higher than that in preneoplastic foci, and GST-pi is a more sensitive marker for detection of HCC than γ-GT or AFP[15-18]. Therefore, it has been generally considered that GST-pi is the most accurate marker enzyme for detection of the very early “initiated cells” in chemically induced hepatocarcinogenesis in rats. It is known that human GST-pi has highly immunological cross-reaction with rat GST-pi, and the alteration of GST-pi precedes that of cell morphology. Studies in human HCC are not as advanced as in rats but have revealed close similarities. To investigate the relationship between GST-pi and HCC and its preneoplastic lesions, accordingly, may offer an important enzyme for the early diagnosis of HCC.

The expression of GST-pi might increase abnormally in the course of the carcinogenesis of many tumors, and it has been associated with preneoplastic and neoplastic changes[21,22]. Recently, many researches demonstrated that loss of transcription activity of GST-pi gene promoter in human malignancies appears to be the result of CpG island DNA methylation, and this phenomenon is most frequent in breast and renal carcinomas, and might contribute to the carcinogenic process in these two carcinomas, while other tumor types show GST-pi promoter methylation only rarely or not at all[21,22]. However, some studies reported that GST-pi promoter hypermethylation changes occur frequently in human HCC, suggesting that somatic GST-pi inactivation via CpG island hypermethylation might contribute to the pathogenesis of HCC[24-27]. When cells display complete GST-pi hypermethylation in the CpG island, and they fail to express GST-pi mRNA and the corresponding protein.

### Table 2 Expression of GST-pi in HCC and its adjacent tissue

| Histological type | Case | Expression of GST-pi | Positive rate (%) |
|------------------|------|----------------------|-------------------|
| HCC              | 77   | 8 (10.4) 55 (71.4) 14 (18.2) | 89.6              |
| LHN              | 38   | 13 (28.9) 25 (65.8) 2 (5.3)  | 71.1              |
| LC               | 35   | 27 (77.1) 8 (22.9) 0 | 22.9              |

P<0.001, Χ² = 16.96, 49.91 vs LHN and HCC.

| Histological type | Case | Expression of GST-pi | Positive rate (%) |
|------------------|------|----------------------|-------------------|
| LHN              | 38   | 13 (28.9) 25 (65.8) 2 (5.3)  | 71.1              |
| LC               | 35   | 27 (77.1) 8 (22.9) 0 | 22.9              |

P<0.001, Χ² = 6.353 vs HCC, Χ² = 16.96, 49.91 vs LHN and HCC.
product, which is different from our findings. GST-pi expression was present in 89.6% of HCC and in 71.1% of LHN in the present study. There are several possibilities for this. (1) There is rare or no GST-pi promoter hypermethylation in pathogenesis of HCC. (2) There is GST-pi promoter hypermethylation, but loss of GST-pi promoter hypermethylation in HCC may be due to the emergence of tumor subclones unmethylated at the GST-pi promoter during HCC transformation. Such subclones may gain additional genetic lesions that rendered GST-pi inactivation or allowed a clonal expansion. Our findings suggest that cells overexpressing GST-pi in LHN may relate to the rapid emergence of GST-pi inactivation in HCC cells due to induction of resistance to anticancer drugs and might protect the tumor cells themselves against the cytotoxic effects of free radicals, as described in other tumors. It is well-known that GST-pi can detoxify not only electrophiles derived from xenobiotics, but also endogenous electrophiles usually with the consequence of free radical damage. These data indicate that GST-pi expression in LHN may be an effective marker enzyme for HCC and its precancerous lesions.

As an important enzyme of detoxification, GST-pi protects cells against the influence of carcinogenic materials. However, GST-pi is taken as a double-edged sword in tumorigenesis, namely, GST-pi protects all cells expressing GST-pi can detoxify not only electrophiles derived from xenobiotics, but also endogenous electrophiles usually with the consequence of free radical damage. These data indicate that GST-pi expression in LHN may be an effective marker enzyme for HCC and its precancerous lesions.

In contrast to LHN, the cells in LC appear to represent quiescent phenotypes unlikely to progress into HCC.

REFERENCES

1. Zhou BP, Hung MC. Dysregulation of cellular signaling by HER2/neu in breast cancer. Semin Oncol 2003; 30 (5 Suppl 16): 38-48
2. Bankfalvi A, HER-2 diagnostics. Magy Orv 2002; 46: 11-15
3. Nathanson DR, Culliford AT 4th, Shia J, Chen B, D Alessio M, Zeng ZS, Nash GM, Gerald W, Barany F, Pauly PB. HER 2/neu expression and gene amplification in colon cancer. Int J Cancer 2003; 105: 796-802
4. Lazar H, Bolltzer A, Gimmi C, Marti A, Jaggi R. Over-expression of erbB-2/neu is paralleled by inhibition of mouse-mammary-epithelial-cell differentiation and developmental apoptosis. Int J Cancer 2000; 85: 578-583
5. Kumar R. Yarmoud-Baegheri R. The role of HER2 in angiogenesis. Semin Oncol 2001; 28 (5 Suppl 16): 27-32
6. Zhang L, bewick M, Lafrenie RM. EGFR and ErbB2 differentially regulate Raf-1 translocation and activation. Lab Invest 2002; 82: 71-78
7. Vlasoff DM, Baschinsky DY, De Young BR, Morrison CD, Nuovo GJ, Frankel WL. C-erbB2 (Her2/neu) is neither overexpressed nor amplified in hepatic neoplasms. Appl Immunohistochem Mol Morphol 2002; 10: 237-241
8. Shi G, Sun C, Han X, Meng X, Wang M, Gu M. Expression of rasp21, C-myc, c-erbB-2, and AIP in 2-FUA induced experimental hepatocarcinogenesis. Zhonghwa Ganzangbing Zazhi 2001; 9: 98-99
9. Neve RM, Lane HA, Hynes NE. The role of overexpression HER2 in transformation. Ann Oncol 2001; 12(Suppl 1): S9-13
10. Yu D, Hamada J, Zhang H, Nicolson GL, Hung MC. Mechanisms of c-erbB-2/neu oncogene-induced metastasis and repression of metastatic properties by adenovirus 5 E1A gene products. Oncogene 1992; 7: 2263-2270
11. Kusakari T, Kariya M, Mandai M, Tsutuya Y, Hamid AA, Fukuhara K, Nanbu T, Takakura K, Fuji S. C-erbB-2 or mutant Ha-ras induced malignant transformation of immortalized human ovarian surface epithelial cells in vitro. Br J Cancer 2003; 89: 2293-2298
12. Lian ZR. HBV status and expression of ets-2, IGF-II, C-myc and N-ras in human hepatocellular carcinoma and adjacent nontumorous tissues--a comparative study. Zhonghua Zhongliu Zazhi 1991; 13: 5-8
13. Sawaki M, Hattori A, Tsuzuki N, Sugawara N, Enomoto K, Sawada N, Mori M. Chronic liver injury promotes hepatocarcinogenesis of the LEC rat. Carcinogenesis 1998; 19: 331-335
14. Nishikawa T, Wairibuchi H, Ogawa M, Kinoshita A, Morimura A, Hiroi T, Funae Y, Kishida H, Nakae D, Fukushima S. Promoting effect of monomethylarsonic acid, dimethylarsinic acid and trimethylarsine oxide on induction of rat liver preneoplastic glutathione S-transferase placental form positive foci: a possible reactive oxygen species mechanism. Int J Cancer 2002; 100: 136-139
15. Shukla Y, Arora A. Enhancing effects of mustard oil on preneoplastic hepatic foci development in Wistar rats. Hum Exp Toxicol 2003; 22: 51-55
16. Imai T, Masui T, Ichinose M, Nakanishi H, Yanai T, Masegi T, Zhong H, Yarmand-Baagheri R. The role of HER2 in angiogenesis. Carcinogenesis 2001; 22: 545-551
17. Satoh K, Itoh K, Yamamoto M, Tanaka M, Hayakari M, Okawa K, Yamazaki T, Sato T, Tsuchida S, Hatayama I. Nr1f2 transactivator-independent GSTP1-1 expression in “GSTP1-1 positive” single cells inducible in female mouse liver by DEN: a preneoplastic character of possible initiated cells. Carcinogenesis 2002; 23: 457-462
18. Satoh K, Hatayama I. Anomalous elevation of glutathione
S-transferase P-form (GST-P) in the elementary process of epigenetic initiation of chemical hepatocarcinogenesis in rats. *Carcinogenesis* 2002; 23: 1193-1198

19 Tatematsu M, Mera Y, Inoue T, Satoh K, Sato K, Ito N. Stable phenotypic expression of glutathione S-transferase placental type and unstable phenotypic expression of gamma-glutamyltransferase in rat liver preneoplastic and neoplastic lesions. *Carcinogenesis* 1988; 9: 215-220

20 Yusof YA, Yan KL, Hussain SN. Immunohistochemical expression of pi class glutathione S-transferase and alpha-fetoprotein in hepatocellular carcinoma and chronic liver disease. *Anal Quant Cytol Histol* 2003; 25: 332-328

21 Esteller M, Corn PG, Urena JM, Gabrielson E, Baylin SB, Herman JG. Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res* 1998; 58: 4515-4518

22 Miyanishi K, Takayama T, Ohi M, Hayashi T, Nobuoka A, Nakajima T, Takimoto R, Kogawa K, Kato J, Sakamaki S, Nishi Y. Glutathione S-transferase-pi overexpression is closely associated with K-ras mutation during human colon carcinogenesis. *Gastroenterology* 2001; 121: 865-874

23 Esteller M. CpG island hypermethylation and tumor suppressor gene: a booming present, a brighter future. *Oncogene* 2002; 21: 5427-5440

24 Zhong S, Tang MW, Yeo W, Liu C, Lo YM, Johnson PJ. Silencing of GSTP1 gene by CpG island DNA hypermethylation in HBV-associated hepatocellular carcinomas. *Clin Cancer Res* 2002; 8: 1087-1092

25 Bakker J, Lin X, Nelson WG. Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells. *J Biol Chem* 2002; 277: 22573-22580

26 Yang B, Guo M, Herman JG, Clark DP. Aberrant promoter methylation profiles of suppressor genes in hepatocellular carcinoma. *Am J Pathol* 2003; 163: 1101-1107

27 Lee S, Lee HJ, Kim JH, Lee HS, Jang JJ, Kang GH. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. *Am J Pathol* 2003; 163: 1371-1378

28 Ruscoe JE, Rosario LA, Wang T, Gate L, Arifoglu P, Wolf CR, Henderson CJ, Ronai Z, Tew KD. Pharmacologic or genetic manipulation of glutathione S-transferase P1-1(GSTpi) influences cell proliferation pathways. *J Pharmacol Exp Ther* 2001; 298: 339-345

29 Goto S, Kamada K, Soh Y, Ihara Y, Kondo T. Significance of nuclear glutathione S-transferase pi in resistance to anti-cancer drugs. *Jpn J Cancer Res* 2002; 93: 1047-1056

30 Hara T, Ishii T, Fujishiro M, Masuda M, Ito T, Nakajima J, Inoue T, Matsuse T. Glutathione S-transferase P1 has protective effects on cell viability against camptothecin. *Cancer Lett* 2004; 203: 199-207

31 Chandra RK, Bentz BG, Haines GK 3rd, Robinson AM, Radosevich JA. Expression of glutathione S-transferase pi in benign mucosa, Barrett’s metaplasia, and adenocarcinoma of the esophagus. *Head Neck* 2002; 24: 579-581