DECREASED EXPRESSION OF THE HUMAN CARBONYL REDUCTASE 2 GENE HCR2 IN HEPATOCELLULAR CARCINOMA

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Abstract: Altered gene expression was associated with the induction and maintenance of hepatocellular carcinoma (HCC). To determine the significance of HCR2 in HCC, here we compare the expression levels of HCR2 in carcinoma and in paired non-carcinoma tissues using semiquantitative reverse-transcription polymerase chain reaction (RT-PCR), Western blot analysis, and immunohistochemical staining. The expression ratio (ER) of HCR2 between the tumor and paired tumor-free tissues was calculated for each case and the data was clinicopathologically analyzed. The expression of HCR2 mRNA was found to be significantly decreased in HCC tissues compared with paired normal tissues (𝑃 < 0.001). HCR2 was downregulated in 58% (𝑛 = 22) of 38 HCC patients. The ER of HCR2 was higher in Edmondson’s grade I/II carcinomas than that in Edmondson’s grade III/IV carcinomas (𝑃 < 0.05). Western blot analysis showed HCR2 to be notably depressed in carcinoma tissues in 3 out of 4 HCC patients. Immunohistochemical staining indicated most HCR2 protein accumulated in non-carcinoma cells. These results suggested that altered HCR2 expression might play roles in the carcinogenesis and progression of HCC, and it could be a clinical marker for prognosis, and a molecular target for screening potential anti-HCC drugs.

Key words: HCR2, Hepatocellular carcinoma, Gene expression

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Abbreviations used: AFP – alpha fetoprotein; β₂-MG gene – β₂-microglobulin gene; CAT – catalase; CBR1 – carbonyl reductase 1; ER – expression ratio; GPx – glutathione peroxidase; HCC – hepatocellular carcinoma; HCR2 – carbonyl reductase 2; IgG – immunoglobulin; ROS – reactive oxygen species; RT-PCR – reverse-transcription polymerase chain reaction; SDR – short chain dehydrogenases/reductases; SOD – superoxide dismutase
INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common human malignancies worldwide, and the second leading cause of cancer death in China [1]. Recently, altered gene expression was associated with the induction and maintenance of HCC [2-4]. The identification of novel genes that related to liver functions, especially those differentially expressed in the liver and HCC tissues, would be helpful in understanding the pathogenesis of HCC, and may facilitate the diagnosis and treatment of HCC.

Human carbonyl reductase 2 gene \( HCR2 \), also known as \( DCXR \), was isolated from tumor-free liver tissue of a Chinese HCC patient during the application of a differential display analysis between tumor tissue and its paired normal tissue in our laboratory. It was submitted to GenBank with the accession No. AF139841. As a new member of the short chain dehydrogenase/reductase (SDR) superfamily, \( HCR2 \) encodes a homotetrameric cytosolic enzyme that is highly expressed in the human liver and kidney, showing NADPH-dependent oxiddoreductase activity for alpha-dicarbonyl compounds and L-xylulose [5]. Reactive alpha-dicarbonyl compounds are generated by oxidative stress and prone to conversion into advanced glycation end products that accumulate in the plasma proteins and tissues, and interact with other long-lived proteins to cause renal failure, sclerotic disorders or retinopathy [6, 7]. In the course of these processes, HCR2 may play a role in detoxification by cleaning the reactive alpha-dicarbonyl compounds and excessive reactive oxygen species (ROS) generated in oxidative stress. This was demonstrated with two murine homologues of HCR2 [5].

It is believed that most HCC cases arise in the setting of chronic hepatitis virus infection; during persistent inflammation, sustained generation of ROS and the metabolic activations of dietary carcinogens such as aflatoxin B1 induce genetic mutations and chromosomal alterations that may eventually lead to the initiation of HCC [8, 9]. Therefore, disruption of the expression or activity of detoxification enzymes may impair the clearance of excessive ROS, thus contributing to the development of HCC. As stated above, HCR2 may function as a detoxification enzyme, and was found differentially expressed in carcinoma and matched normal liver tissues from one HCC patient, suggesting a possible linkage between HCR2 expression and clinical features. In this report, we use RT-PCR to detect the level of \( HCR2 \) mRNA in both hepatocellular carcinoma and matched normal tissue to explore the relationship of altered gene expression with clinico-pathological parameters. Western blot and immunohistochemical analysis were also used to examine the expression of HCR2 in the cells of carcinoma and non-carcinoma tissue.

MATERIALS AND METHODS

Tumor samples

Fresh surgical specimens of liver cancer tissues, diagnosed histologically as hepatocellular carcinoma, and of their adjacent normal tissues were obtained from
38 Chinese patients in the Liver Cancer Institute, Shanghai Zhongshan Hospital, China (Tab. 1). All the samples were fresh frozen in liquid nitrogen immediately after surgical removal and stored at -80ºC. The patients were 28 to 81 years old (49.39 ± 10.57), and the tumor volume ranged from 2 to 17 cm in diameter (5.94 ± 3.75 cm). Twenty-two (57.9%) patients had a serum alpha-fetoprotein (AFP) level above 20 ng/ml, and integument invasion was found in twenty-six patients (68.4%). The clinical degree of differentiation of lesions was defined based on Edmondson’s classification (grades I-IV) [10].

RNA isolation and primers for RT-PCR amplification
Using the manufacturer’s protocol, the total RNA was extracted from the carcinoma and paired non-carcinoma tissues using TRIzol® reagent (Life Technologies, Inc., Gaithersburg, MD). The RT-PCR primers were designed to flank a region that contains at least one intron. The unique primers for the HCR2 gene were HCR2-A (5’-GAC ATG CTG ACC AAG GTG ATG GC-3’ forward primer) and HCR2-B (5’-TGA GGT GTG TGG AGG GAG CTC AG-3’), which correspond to nucleotides 483-506 and 746-769 of the deposited sequence NM_016286 (GenBank) respectively, with an amplified DNA fragment of 286 bp. The primers for the β2-MG gene are β2-MG-C1 (5’-ATG AGT ATG CCT GCC GTG TGA AC-3’, sense primer) and β2-MG-C2 (5’-TGT GGA GCA ACC TGC TCA GAT AC-3’), with an amplified product of 284 bp [11].

Semiquantitative RT-PCR analysis
cDNA was synthesized using 2 μg total RNA, Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD) and oligo(dT)15 (Promega, Madison, WI), according to the manufacturer’s protocols. First-strand cDNA was synthesized by reverse transcription and followed by PCR amplification on an FS-918 DNA Amplifier (Shanghai Fusheng Institute of Biotechnology, Shanghai, China). The concentration of the templates was regulated to be about 1 μl per 25 μl PCR reaction volume by amplifying (60ºC, 30 s) for 30 cycles. To determine the best number of cycles, PCR was performed at 22-37 cycles. The PCR products from each cycle were run on 1.5% agarose gel electrophoresis, photographed, and then quantified using an image densitometer (GDS8000, UVP Inc). The growth curve of the PCR products was made according to the amount of PCR products from different cycles [12]. The best cycle was determined between the increased logarithmic phase and the plateau phase. They were 30 cycles for β2-MG C1C2 (60ºC, 30 s) and 31 cycles for HCR2 (62ºC, 30 s). Afterwards, the HCR2 genes were amplified using same amounts of the diluted templates with β2-MG as the control for HCR2. The RT-PCR products were separated on 1.5% agarose gels, then photographed and quantified. Finally, the HCR2/β2-MG ratio was evaluated for each sample.
Tab. 1. Clinical characteristics of the 38 Chinese HCC patients in this study.

| Case no | Sex | Age (yrs) | Tumour size | AFP (ng/ml) | HBV | Edmondson’s grade | Integument invasion | Cancer embolus |
|---------|-----|-----------|-------------|-------------|-----|-------------------|---------------------|-----------------|
| 1       | M   | 50        | 6.8         | 45          | +   | III               | +                   | -               |
| 2       | M   | 54        | 4.2*1.8     | 400         | +   | II                | -                   | -               |
| 3       | M   | 41        | 8*7         | >1000       | +   | III               | +                   | -               |
| 4       | M   | 61        | 3.5*2       | 0.4         | -   | III               | -                   | -               |
| 5       | M   | 28        | 4*4         | 7.7         | +   | III               | +                   | -               |
| 6       | M   | 42        | 8*6         | 400         | +   | III               | -                   | -               |
| 7       | M   | 64        | 16*14       | 5.9         | -   | III               | +                   | -               |
| 8       | M   | 48        | 3.2*1.6     | 0           | +   | I                 | -                   | -               |
| 9       | M   | 42        | 2*2         | 410.6       | +   | III               | -                   | -               |
| 10      | M   | 38        | 12*10       | 6.5         | +   | III               | +                   | -               |
| 11      | M   | 38        | 6*6         | 25.6        | +   | III               | +                   | -               |
| 12      | M   | 49        | 4*4         | 115         | +   | III               | +                   | -               |
| 13      | M   | 62        | 11*11       | >1000       | -   | II                | -                   | -               |
| 14      | M   | 32        | 2*2         | 76.9        | +   | III               | +                   | -               |
| 15      | M   | 44        | 5*4         | 52.3        | +   | III               | +                   | +               |
| 16      | M   | 66        | 4*3         | 8.9         | +   | I                 | +                   | -               |
| 17      | M   | 47        | 3.5*3.5     | 130         | +   | III               | +                   | -               |
| 18      | M   | 55        | 7.5*6       | 0           | +   | II                | -                   | -               |
| 19      | M   | 39        | 3*2.8       | 400         | +   | II                | +                   | -               |
| 20      | F   | 64        | 4*3         | 0           | +   | II                | +                   | -               |
| 21      | M   | 50        | 4*4         | 300         | +   | III               | +                   | -               |
| 22      | M   | 81        | 17*13       | >400        | +   | III               | +                   | -               |
| 23      | M   | 49        | 11*11       | >1000       | +   | IV                | +                   | +               |
| 24      | M   | 52        | 3*2.5       | 0           | +   | II                | +                   | -               |
| 25      | M   | 54        | 2.5*1.5     | 50          | +   | II                | -                   | -               |
| 26      | M   | 53        | 7*7         | 400         | +   | III               | +                   | -               |
| 27      | M   | 58        | 12*10       | 0           | +   | III               | -                   | -               |
| 28      | M   | 50        | 2.5*2       | 54          | +   | II                | -                   | -               |
| 29      | M   | 46        | 3*2         | 100         | +   | III               | +                   | -               |
| 30      | M   | 44        | 3*1.5       | 20          | +   | III               | -                   | -               |
| 31      | M   | 40        | 8*5         | 20          | +   | II                | +                   | -               |
| 32      | M   | 37        | 4*3         | 0           | +   | II                | +                   | -               |
| 33      | M   | 41        | 3.5*3       | 0           | +   | III               | +                   | -               |
| 34      | M   | 46        | 4.5*3       | 50          | +   | II                | +                   | -               |
| 35      | F   | 48        | 4*4         | 400         | +   | II                | +                   | -               |
| 36      | M   | 44        | 4.5*4.5     | 20          | +   | II                | +                   | -               |
| 37      | M   | 62        | 8*7         | 0           | +   | III               | +                   | -               |
| 38      | M   | 58        | 5*4         | 400         | +   | II                | -                   | +               |
Statistical analysis
All the statistical analyses were performed using statistical software (SPSS version 11.5, standard version, SPSS Inc., Chicago, IL). The paired t-test was used to compare the levels of HCR2 between the hepatocellular carcinoma and paired normal liver tissues. Student’s t-test was used to analyze the relationship between the gene expression level and clinical characteristics. A p value of less than 0.05 was considered to be significant.

Antibody production and Western blot analysis
To prepare a polyclonal antibody against human HCR2, a full length cDNA of HCR2 was amplified and digested with the restriction enzymes (Nde I/Hind III), then ligated into expression plasmids, pET28a (Invitrogen, Carlsbad, CA). The ORFs of the cDNAs in the expression plasmids were verified by nucleotide sequence analysis. The primers used to amplify the full length cDNA were HCR2-C (5′-CGC CGA CAT GGA GCT GTT CCT C-3′, forward primer) and HCR2-D (5′-GCA CGG CAT GGG GCT TGA GGT G-3′), which respectively correspond to nucleotides 9-30 and 763-784 of the deposited sequence NM_016286 (GenBank). Recombinant His-tag human DCXR protein was expressed in E. coli BL21(DE3) from an IPTG-inducible T7 promoter, and purified using a Ni-NTA agarose column (Qiagen, Chatsworth, CA). The antibodies in the antiserum were purified first on a protein-A column and subsequently with an affinity column cross-linked with the corresponding recombinant protein. Tumor and matched normal tissues from four HCC patients (Cases 13, 14, 15, 16) were homogenized with 4 volumes of 0.25 M sucrose at 4ºC and centrifuged at 105,000 × g for 1 h. The supernatant fractions were subjected to Western blot analysis using the above antibody and monoclonal mouse anti-human β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Immunohistochemical analysis
Immunohistochemical staining was used to evaluate the distribution of HCR2 in tumor and normal tissues (because of the limited amount of pathological tissues, we selected eight samples for analysis). The samples were fixed in neutralized 10% (v/v) formalin, embedded in paraffin, and sectioned at 4 µm. For immunohistochemistry, these sections were blocked in 3% normal goat serum and incubated with the primary antibody at 4ºC overnight, then with the secondary antibody for 1 h at room temperature, and with avidin-biotinylated peroxidase complex (BOSTER, China) for 1 h. Color was developed using a dianinobenzidine staining kit (BOSTER, China), followed by hematoxylin counterstaining. The respective primary and secondary biotinylated antibodies for HCR2 staining were the polyclonal rabbit anti-human antibody and a goat anti-rabbit immunoglobulin (IgG) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Negative controls were done for consecutive sections by replacing the primary antibody with a non-immune rabbit serum.
RESULTS

Changes in expression of HCR2 in the carcinoma and paired non-carcinoma tissues

Having determined the best number of cycles between the increased logarithmic phase and plateau phase (Fig. 1A) and confirmed the reproducibility of our semiquantitative RT-PCR assay for HCR2 mRNA expression, we next examined the relative expression level of HCR2 mRNA in 38 HCC patients (Fig. 1B). We did not find any difference in the expression of the control substance β2-MG in the HCC tissues, but found a significantly depressed expression of HCR2 (Tab. 2).

Fig. 1. Semi-quantitative RT–PCR analysis of HCR2. A - The best number of cycles of β2-MG and HCR2. The results showed 30 for β2-MG and 31 for HCR2 amplification. B - The expression of HCR2 mRNA in part of the HCC tissues and paired tumor-free tissues. β2-MG products as the control in RT-PCR analysis. T: carcinoma tissues, N: paired normal tissues.

Tab. 2  The density of RT-PCR products.

| Gene     | Normal (mean ± SD) | Tumor (mean ± SD) | Difference (95% CI) | P value |
|----------|--------------------|-------------------|---------------------|---------|
| β2-MG    | 106.64 ± 9.21      | 105.68 ± 9.11     | -0.96 (-3.54, 1.62) | 0.46    |
| HCR2     | 54.09 ± 14.10      | 37.03 ± 15.84     | -17.06 (-23.67, -10.44) | 7.07E-06 |
| HCR2/β2-MG | 0.51 ± 0.13      | 0.35 ± 0.16       | -0.16 (-0.22, -0.09) | 1.38E-05 |
The IPTG-induced recombinant protein was about 30 kDa (Fig. 2A). It specifically reacted with the polyclonal rabbit anti-human HCR2 antibody (ra-HCR2) (Fig. 2B). Using ra-HCR2 antibody to examine the expression levels of HCR2 in carcinoma and matched normal tissues from four HCC patients, we found HCR2 was remarkably depressed in the carcinoma tissues of three HCC patients (Cases 14, 15, 16). Immunohistochemical analysis showed that most of the carcinoma cells were stained weakly or negatively, and the HCR2 protein accumulated notably in the non-carcinoma cells (Fig. 3).

Fig. 2. Recombinant expression and western blot analysis of HCR2 protein. A - 12% SDS-PAGE of recombinant expression and purification of HCR2 protein. The IPTG-induced recombinant protein is about 30 kDa. B - Ra-HCR2 recognized HCR2 specifically. (1) Proteins (2-μg samples) were stained with Coomassie Brilliant Blue R-250 after SDS-PAGE. (2) Western blot analysis using the polyclonal rabbit anti-human antibody (ra-HCR2). Lanes: 1, recombinant human HCR2 protein; 2, human B3GALT7 protein (provided by Dr. Huang CQ). C - Western blot analysis of HCR2 expression in four HCC and paired tumor-free samples. (1) Using monoclonal mouse anti-human β-actin antibody. (2) Using polyclonal rabbit anti-human antibody (ra-HCR2). T: carcinoma tissues; N: paired normal tissues.
Fig. 3. Expression of HCR2 in paraffin-embedded sections of HCC and adjacent normal tissues. HCR2 was notably depressed in carcinoma tissue cells, while it accumulated in adjacent normal cells (arrows indicate the positively staining cells). The primary antibodies used were polyclonal rabbit anti-human HCR2 antibody (B, C) and preimmune serum (A, the control), with the dilution of 1:200. CT: carcinoma tissue, NT: non-carcinoma tissue; Original magnification: A, B 10x; C 40x.

Fig. 4. The expression level of HCR2 and clinical characteristics in 38 HCC cases. A - The expression ratio (ER) of HCR2 is shown in the histogram. In this study, we defined two critical ER values: ER = 1.5 and ER = 0.67 (ER = TR/NR, TR = RT-PCR product density of HCR2/RT-PCR product density of β2-MG in the tumor tissue, NR = RT-PCR product density of HCR2/RT-PCR product density of β2-MG in the paired tumor-free tissue). These values were used to determine whether the expression of HCR2 is downregulated or upregulated in a given case: if the ER value exceeded 1.5, the expression of HCR2 was judged as unregulated; if the ER value was under 0.67, the expression of HCR2 was judged as downregulated. The upper and lower lines in the histogram represent the two critical values, respectively. B - The association between the expression level of HCR2 and the tumor stage and integument invasion. Using ra-HCR2 antibody to examine the expression levels of HCR2 in...
carcinoma and matched normal tissues from four HCC patients, we found HCR2 was remarkably depressed in the carcinoma tissues of three HCC patients (Cases 14, 15, 16). Immunohistochemical analysis showed that most of the carcinoma cells were stained weakly or negatively, and the HCR2 protein accumulated notably in the non-carcinoma cells (Fig. 3).

The ER value was calculated for each case and used to evaluate the relative expression level of HCR2. As shown in Fig. 4A, HCR2 was down-regulated in 22 (58%) of the patients and up-regulated in 3 patients, while no obvious change was observed in the other 13 patients.

The relationship between gene expression level and clinical characteristics
Based on the ER of the tumor to matched tumor-free tissues, the relationship between the clinical characteristics and the HCR2 expression level was further analyzed by dividing patients into subgroups. The expression of HCR2 mRNA into grades I/II was higher than in grades III/IV (Fig. 4B) \((P = 0.014)\). No association was found between the expression level of HCR2 with other demographic and clinical variables, such as age, HbsAg, AFP level, integument invasion or cancer embolus.

DISCUSSION

Oxidative stress and the production of excessive ROS may induce toxicity and ultimately boost the development of HCC. During this progression, blocking of the detoxification system was thought to be a common pathway [13]. In previous studies, several oxidoreductases involved in the detoxification of ROS were reported to be coincidently depressed in most HCC cases, including catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and carbonyl reductase 1 (CBR1) [14-16]. Recent studies showed abnormal activity and expression alteration of quinone oxidoreductase 1 and glutathione S-transferase P1 in HBV-associated hepatocellular carcinomas and hepatoma cells [17, 18].

Our results suggested that disturbing the HCR2-related detoxification system may be another pathway to carcinogenesis and/or the progression of HCCs, and to the best of our knowledge, this was the first study reporting decreased levels of HCR2, a new oxidoreductase related to detoxification, in HCC tissues among Chinese patients; this was further proved by the Western blot and immunohistochemical analysis performed.

Furthermore, we found that the expression level of HCR2 was associated with the tumor stage. The lower level of HCR2 in Edmondson’s grade III or IV lesions may imply that the expression level of HCR2 was inversely related to the pathological grading of HCC, which concurred with the results of a previous study that showed depressed levels of CBR1 to be more frequently detected in high-grade HCCs and proportional to the dedifferentiation of the carcinoma [16].

CBR1 was the first carbonyl reductase identified in humans with important physiological functions in the detoxification of endogenous and xenobiotic carbonyl compounds and regulation of ROS [19]. HCR2 may contribute to the
multistage hepatocarcinogenesis the same way as CBR1 does in this malignant transformation process.

In organisms, carbonyl reduction and antioxidant enzymes have dual functions. On the one hand, they can detoxify chemical carcinogens to protect cell contents from genetic damage, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane, a potent carcinogen found in tobacco and playing a crucial role in lung cancer and hepatocellular carcinogenesis. On the other hand, they are supposed to change the pharmacological properties of some anticancer drugs like daunorubicin [19, 20]. So HCR2 with differential expression in carcinomas may not only be a potential predictor for people with a higher risk of lung cancer or HCCs, but also a molecular target for evaluating and screening potential anti-HCC drugs.

Two studies found that CBR1 participated in metastasis modulation, and that decreased expression of carbonyl reductase in epithelial ovarian cancer was associated with RLN metastasis and poor survival [21, 22], but our study did not find any association between integument invasion and HCR2 mRNA expression. We may need to do more research to investigate the relationship between HCR2 and tumor recurrence/metastasis. The significance of HCR2 gene expression in HCC needs to be further investigated with more samples.

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