The Effect of AKR1C2 Gene on Hepatocarcinogenesis and Its Abnormal Expression in Hepatocellular Carcinoma From Qidong, China, a Liver Cancer High Risk Area

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Abstract Abnormal AKR1C2 expression has been observed in many malignant human tumors, but its relationship with hepatocellular carcinoma (HCC) is not well understood so far. In order to evaluate hepatocarcinogenic effect of AKR1C2 gene and the significance of its abnormal expression in hepatocellular carcinoma, AKR1C2 gene is analysed by preparing rabbit anti-human AKR1C2 polyclonal antibody, constructing of AKR1C2 frameshift mutant and exploring RT-PCR, *in situ* hybridization, immunohistochemistry, Western blot, Northern blot, cDNA expression microarray, co-immunoprecipitation and the tumorigenicity assay *in vivo* and *in vitro* etc. AKR1C2 gene expression and its effects was analyzed, including 68 pairs of HCC specimens and its adjacent para-cancerous tissues, 8 cases of normal liver tissues and QGY7703 cell line. Results showed AKR1C2 expression was up-regulated among these patients, when compared with those of para-cancerous and normal liver tissues. Over- expression of AKR1C2 is also found to be correlated with high metastasis potentiality of HCC. AKR1C2 overexpression stimulates DNA synthesis, apoptosis, growth in soft agar and promote tumor formation and lead to expression differences of tumor genes. AKR1C2 mediated the NF-κB-dependent resistance of QGY7703 cells to anti-fas killing. Intracellar binding of AKR1C2 and Cdk4 was found. Abnormal expression of AKR1C2 gene may contribute to the occurrence, advancement and invasiveness of HCC from Qidong, China, a liver cancer high risk area.

Key words liver tumor, AKR1C2 gene, metastasis, invasiveness gene expression, apoptosis, tumorigenicity assays

Hepatocellular carcinoma (HCC) is quite common in China, especially in Qidong, Jiangsu Province, where HCC mortality rate was 80/100 000. In recent years, great progresses have been made in the prevention and treatment of HCC, but the major problem is the high mortality rate and malignancy of HCC, that is, more than 50% of the patients receiving grossly radical treatment will suffer from recurrence within two years. So much effort has been put to investigate the molecular biological characteristics of HCC in order to lower the incidence. Abnormal AKR1C2 expression has been observed in many malignant human tumors[1~4]. But its relationship with HCC is not well understood so far. In order to evaluate hepatocarcinogenic effect of AKR1C2 gene and the significance of its abnormal expression in hepatocellular carcinoma, AKR1C2 gene is analysed by RT-PCR, *in situ* hybridization, immunohistochemistry, Western blot, Northern blot and the tumorigenicity *in vivo* and *in vitro* etc, using HCC samples from Qidong and QGY7703 line (Qidong liver cancer cell line).

1 Material and methods

1.1 Tissue

Sixty-eight samples (paired tumor and nontumor liver tissues) and eight cases of normal liver tissues used for analysis were obtained from Qidong patients who had undergone surgery in Qidong Liver Cancer Institute from January 2001 to December 2002. The tumor and nontumor tissues were fixed in formalin, and then embedded in paraffin. Paraffin sections were stained with HE for histological examination of HCC and were also used for immunohistochenistry, etc. All fresh tumor and nontumor tissues snap frozen in liquid nitrogen within 30 min of surgery were available for RT-PCR, Northern blot and Western blot, *in situ* hybridization analysis, etc.

1.2 Cell lines

The human HCC cell lines, QGY7703 (Qidong liver cancer cell line) were purchased from Shanghai Cell Institute. Cell lines were maintained in Dulbecco's modified Engle's medium (GIBCO BRL) in a humidified atmosphere containing 5% CO_2 at 37℃.

1.3 AKR1C2 mRNA extraction

Total RNA from human HCC cell lines and tissues were purified using the one-step method of Trizol reagents (Gibco) according to the manufacturer's protocol. In short, the cells were washed with cold PBS twice and broken with Trizol. Then total RNA was extracted with chloroform, sedimented with isopropyl

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alcohol one time and 75% alcohol for two times. PolyA$^+$ RNA was prepared using Oligotex mRNA midi isolation kit (Qiagen, Hilden, Germany).

1.4 RT-PCR

Complementary DNAs (cDNAs) were generated from total RNA using 200 U of SuperScript Ⅱ reverse transcriptase (GIBCOBR) and an antisense primer for AKR1C2. Primers for PCR were designed to amplify the consensus sequence for AKR1C2 gene. To verify the validity of amplification, the primers were designed within the region of AKR1C2 gene, and the amplification was performed by RT-PCR respectively. Primer A: 5′-ATTCGGATCCATGGATTCGAAATACC-AG-3′, Primer B: 5′-AGGGCTCGAGTTAATATTC-ATCAGAAAATGG-3′. PCR: PCR mixture, containing 100 pmol/L of primer A and B each, deoxyribonucleotide triphosphates at 200 μmol/L each, 1.5 mmol/L $MgCl_2$, 2 U Taq polymerase (Promega) and 2 μl DNA template was adjusted to 50 μl by adding double distilled water. Then the mixture was overlaid with 50 μl mineral oil and subjected to amplification for 35 cycles. Each cycle consisted of 94℃ for 30 s, 58℃ for 40 s, 72℃ for 50 s. β_2-MG mRNA was also amplified by RT-PCR as the internal control. The PCR products were identified first onto 12 g/L agarose gel and photographed. The photos of RT-PCR were scanned by optical density scanner (Shimadzu C-9000) and the gene expression index (*EI*) was arbitrarily defined as density Lum of AKR1C2/density Lum of β_2-MG.

1.5 Northern blot

Equivalent amounts (20 μg) of RNA from each cell line or tissue sample were analyzed by denaturing agarose gel electrophoresis and Northern blot hybridization using the AKR1C2 probe obtained from PCR select cDNA subtraction. This probe was radiolabled with α ^{32}P-dCTP by random priming (Prime-a-Gene System; Promega, Madison, WI), pT7Blue (R) was similarly prepared as a control probe. Hybridization and washing were performed under stringent conditions. After hybridization membranes were washed 4 times with 2 × sodium saline citrate containing 0.1% sodium dodecylsulfate at 65℃. Relative intensity of the signals was determined by laser densitometric analysis of the radiographic film.

1.6 *In situ* hybridization

The fresh frozen samples from tumor (HCC) and nontumor liver sections (5 μm) were obtained, dried for 2 h at room temperature, and placed in chloroform for 5 min. using the Oncor ISH and digoxigenin/biotin detection kits according to the enclosed instructions (Prime-a-Gene System; Promega, Madison, WI). Sections were fixed in 40 g/L paraformaldehyde for 7 min, rinsed in PBS for 3 min, rinsed twice in 2 × SSC for 5 min, and prehybridized at 42℃ for 60 min in hybridization solution (4 × SSC, 100 g/L dextran sulfate, 1 × Denhardt's solution, 2 mmol/L EDTA, 500 g/L deionized formamide, 500 mg/L salmon sperm DNA). Hybridization was carried out for 16 h in 100μl of prehybridization solution and 20μg/L digoxin labeled AKR1C2 oligonucleotides probe. Sections were counter-stained in nuclear methyl green, mounted with aqueous solution, and the final results of average density area and density lum of 500 signal positive cells were analyzed by a multifunctional true digital system (MTDS) using a computer. Albumin oligonucleotide probe and hybridization solution without probe were used as positive and negative control respectively.

1.7 Preparation of AKR1C2 polyclonal antibody

The full-length cDNA of AKR1C2 was amplified by PCR, then recombined into prokaryotic expression vector and transformed into BL21 for expression. The NEW Zealand rabbits were immunized with purified product by Ni-affinity chromatography as described. The product was identified to be a single component by SDS-PAGE and Western blot analysis showed higher titer rabbit anti-human AKR1C2 polyclonal antibody. The titer was initially be characterized in specific enzyme linked immunosorbent assays before the antisera were used in immunohistochemistry and Western blotting (>1:10 000). Each antiserum was used at a dilution of 1:500. Preimmune serum and preincubation of primary antibodies with an excess of the corresponding synthetic peptide(s) served as controls.

1.8 Immunohistochemical staining

Immunohistochemical analysis was carried out with the avidinbiotin complex immunoperoxidase technique according to the manufacturer's instruction. As the primary antibody, polyclonal human AKR1C2 antibody was used at ×500 dilution. As the secondary antibody, biotinylated anti-rabbit IgG (Dako) was used at ×100 dilution. Staining was performed using avidin-biotin reagents, 3,3-diaminobenzidine and hydrogen peroxide. Deparaffinized sections were heated for 5 min at 100℃ to reactivate the antigen, and treated with 0.3% H_2O_2 in methanol for 30 min to abolish endogenous peroxidase activity. Sections were blocked with 1% goat serum in PBS and incubated with 3 mg/L of anti-AKR1C2 antibody overnight at 4℃. The sections were incubated with biotinylated anti-rabbit IgG antibody followed by avidin-biotin-peroxidase complex. They were developed in a substrate solution of 0.01% diaminobenzidine-hydrogen peroxide and counterstained with hematoxylin. As a negative control, duplicate sections were immunostained without exposure to the primary antibodies. All cases were divided into two groups according to immunostaining pattern. Cases with a membranous staining pattern similar to that in normal hepatic cell were classified as membranous or normal and cases with marked cytoplasmic and nuclear staining

in addition to the membranous staining were defined as accumulated or abnormal. All immunohistochemical staining sections were reviewed independently by two pathologists. " - " group, tumor tissues were negative of AKR1C2 or the number of tumor cell positive of AKR1C2 was less than 30%; " + " group, tumor tissues were weakly positive of AKR1C2 compared with tissues closely adjacent to carcinoma, or the number of carcinoma cells positives of AKR1C2 was 30% ~60% of total tumor cells. " + + "group, tumor tissues were positive of AKR1C2 or the number of tumor cells positive of AKR1C2 was more than 60%. Cells from five randomized views were counted and the cell labeling index (*LI*) was arbitrarily defined as: *LI* = (positive cells counted/all cells counted) ×100.

1.9 Immunoblotting of AKR1C2

The fresh frozen samples from tumor (HCC) and nontumor liver or cell extracts of QGY7703 cell lines were homogenized in lysis buffer (50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% NP-40, 1 mmol/L phenylmethyl sulfonyl fluoride), and equal amounts of protein from each extract were separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to cellulose nitrate membranes (Advantec, Tokyo, Japan). After blocking with 5% milk in Tris-HCl pH 7.5 containing 0.1% Tween 20 at 4 ℃ overnight, the blots were incubated with 3 mg/L of anti- AKR1C2 antibody (dilution of 1:500) overnight at 4℃. The immunoblots were washed in Tris-HCl pH 7.5 with 0.1% Tween 20 and probed with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Tokyo, Japan, 1:2 000 diluted with 5% milk in Tris-HCl pH 7.5). After washing, the immunoblots were developed using the electrochemiluminescence system (Amersham).

1.10 Construction of AKR1C2 frameshift mutant

The simultaneous introduction of a translation "stop" codon and frameshift mutation was created by insertion of a thymidine at nucleotide position 128 of the full length AKR1C2 clone by a primer carrying the corresponding point mutation. The resulting plasmid encoded the first 7 amino acids of AKR1C2 prior to termination. This mutation was constructed using the QuikChange XL Site-Change XL Site-Directed Mutagenesis kit according to the instructions provided by the manufacturer (Stratagene, La Jolla, CA).

1.11 Transfection of AKR1C2 into QGY7703

AKR1C2 amplified using RT-PCR from total RNA or AKR1C2 frameshift mutant were digested with *Eco*R Ⅰ and *Xho* Ⅰ, and then ligated into pcDNA3.1 (Invitrogen). Plasmids encoding full length human AKR1C2 cDNA were confirmed by sequencing (ABI Prism310; Perkin-Elmer, Faster, CA). Transfections were performed using FuGENE™ 6 (Boehringer Mannheim, Mannheim, Germany) after serum starvation for 24 h and control cells received only pcDNA3.1-LacZ plasid (Invitrogen). Transfection efficiency was uniformly greater than 50%, as measured by LacZ staining of transfected control cells using a β-gal staining kit (Invitrogen) or as measured by anti-AKR1C2 staining of transfected or untransfected QGY7703 cell.

1.12 Growth of QGY7703-pcDNA3.1, and QGY7703-pcDNA3.1- AKR1C2, and QGY7703-pcDNA3.1- AKR1C2 frameshift mutant cells *in vitro* and *in vivo*

1.12.1 Growth curve: QGY7703-pcDNA3.1, QGY7703-pcDNA3.1- AKR1C2, QGY7703-pCDNA3.1-AKR1C2 mutant were grown in complete medium and the number of viable cells was determined at daily intervals after seeding for up to 5 days. Cell viability was determined by trypan blue staining. Cell viability was independently determined using the modified tetrazolium salt assay, as described by the manufacturer (Cell Titer 96 Non-radioactive Cell Proliferation Assay, Promega).

1.12.2 Cell cycle and apoptosis analysis: (1) Cell cycle distribution was determined by measuring the cellular DNA content using flow cytometry. To prepare samples for analysis, 1×10^6 cells were collected 24 h after the transfection and fixed with 70% cold ethanol. RNase A (10 mg/L) was added and the cells were incubated for 30 min at 37℃. The cells were resuspended in 0.5 ml Annexin-V (AV) and propidium iodide solution (each 50 mg/L in 0.1% sodium citrate with 0.1% NP-40). Propidium iodide-stained cells were analyzed with a FACScan cytometer using Cell Quest software (Becton Dickinson, Tokyo, Japan). All experiments were conducted in triplicate, and the results were evaluated blindly. (2) Apoptosis: 1×10^5 QGY7703 cell before and after the transfection were digested and then washed once with cold PBS, and then fluorescence dye Hoechst33258 and PI were added (terminal density was 10 ml/L and 20 ml/L, respectively), avoiding light for 30 min at 37℃. Dripping slides and then stimulating by ultraviolet ray, finally observed under fluorescence microscope.

1.12.3 Colony assay in agar: QGY7703 cells stably transfected with pcDNA3.1-Lac or with pcDNA3.1 encoding AKR1C2 or pCDNA3.1- AKR1C2 mutation were tested for anchorage-independent growth in soft agar. For growth in soft agar, 1×10^4 cells/well seeded in triplicate into 6-well plates and allowed to grow for 21 days. The colonies were then counted under code.

1.12.4 Tumorigenicity assays: 1×10^7 cells were injected subcutaneously at a single site in nude mice (Balb/C). The mice were observed over 6 weeks for tumor formation. The mice were then sacrificed and the tumors recovered. The number of tumors, as well as

the diameter and wet mass of each nodule, was determined for each mouse. The use of mice for this work was reviewed and approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of health guidelines (China).

1.12.5 cDNA expression microarray: The oncogene chip containing 200 genes (Chinese Shanghai Biostar Inc.) was used to analyze gene expression by hybridization of QGY7703 before and after transfection. PolyA$^+$ RNA was used to prepare probes hybridized with microarray, untransfection cells labeled with Cy3-dUTP and transfection cells labeled with Cy5-dUTP. Those probes were kept in 20 μl hybridization liquid (5 × SSC + 0.20% SDS). DNA chips and probes labeled with Cy3 or Cy5 were denatured for 5 min at 95℃. Then the probes were added to DNA chips and sealed with slide, hybridization process was placed at 60℃ for 15 ~ 17 h. After hybridizaton, the slide was taken off and chip was washed respetively with three kinds of liquid (2 × SSC + 0.2% SDS, 0.1% × SSC + 0.2% SDS, 0.1% × SSC) for 10 min. At last the chips were dried at room temprature. DNA chips were scanned by ScanArray 3000 (General Scanning) and analyzed with ImGene 3.0 to obtain the intensity of two kinds of fluorescence, Cy3 intensity of untransfection cells and Cy5 intensity of transfection cells. With correcting value of normalization coefficient, Cy5/Cy3 ratio of all genes was calculated. Ratio for more than 2.0 or less than 0.5 was considered to be significant. We performed a hierarchical clustering analysis using relative gene expression ratio (Cy5/Cy3) to examine the relatedness among expression patterns of 200 genes and those in HCCs such as etiology, histologic differentiation, and size. Cluster analysis was performed using Cluster software (version 1.45) written by Eisen *et al*.

1.13 Construction of dominant negative inhibitor of NF-κB cells

Two million QGY7703 cells were stably transfected with 15 μg of pSVzeo-dominant negative inhibitor of NF-κB (IκBα) using standard calcium phosphate precipitation. The cells were selected in 500 mg/L of zeocin for 3 weeks. All zeocin-resistant colonies were trypsinixed and used for further analysis.

1.14 Anti-Fas treatment

QGY7703-pcDNA3.1, QGY7703-pcDNA3.1-AKR1C2, QGY7703-pCDNA3.1- AKR1C2 mutant cultures were then treated with 0.5 mg/L of anti-Fas (Ab-2, monoclonal mouse IgG; Oncogene ResearchProducts) or 0.5 mg/L of mouse IgG (Sigma Chemical Co.) as control in the presence of actinomycin D (0.3 mg/L) or cycloheximide (2 mg/L). At 0, 24, 36, 48 and 72 h of treatment, all cells (adherent and floating) in each well were collected by trypsinization, stained by trypan blue, and counted in a hemocytometer. About 800 cells in each sample were counted. The percentage of live cells, as determined by trypan blue exclusion, was calculated as follows: percentage of live cells = (number of live cells in anti-Fas treated well/number of live cells in control well) × 100. All tests were done in duplicate, cell viability was independently determined using the modified tetrazolium salt assay.

1.15 Immunoprecipitation

QGY7703-pcDNA3.1-AKR1C2, QGY7703-pcDNA3.1 and QGY7703-pcDNA3.1-AKR1C2 mutant sample was pre-cleared with protein A Sepharose CL-4B (Pharmacia Biotech, Tokyo, Japan) and normal rabbit IgG for 1 h at room temperature. Precleared homogenates were incubated with 1 mg/L of anti- Cdk4 antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4℃. Immunoprecipitates were incubated with protein A Sepharose overnight at 4℃, then centrifuged. The precipitates were washed and resuspended in lysis buffer. Immunoblotting was performed using anti-AKR1C2 antibody.

1.16 Statistical analysis

The relationship between RT-PCR, Northern blot, Western blot, ISH and protein staining results for AKR1C2 have been evaluated in 2 × 2 tables in a 2-tailed analysis. The results were significant when $P < 0.05$. The mean difference in growth *in vivo* and *in vitro* among QGY7703 cells stably transfected with pcDNA3.1, pcDNA3.1- AKR1C2 or with the pcDNA3.1- AKR1C2 frameshift mutant were analyzed by the Student's t test. The same test was used to measure the mean difference in growth and survival of QGY7703 cells stably transfected with pSVzeo or pSVzeoIκBα and treated with anti-Fas. A signficant difference was recognized when $P < 0.05$. The other data were statistically analyzed using Student's t test, U test and χ^2 test.

2 Results

2.1 Northern blot of AKR1C2 in normal human tissue

Northern blot analysis showed that full-length AKR1C2 mRNA was approximately 1 900 bp. We found that AKR1C2 expression is in brain, colon, lung, liver, kidney, spleen and prostate. There is higher expression in liver than the other tissue. There is no expression in heart, placenta, sekletal muscle, ovary, pancreas (Figure 1). It was shown that the AKR1C2 expression in normal human tissue were specificity.

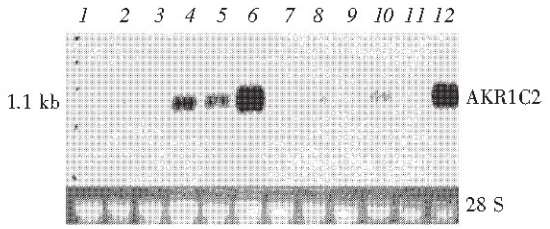

Fig. 1　Northern blot of AKR1C2 in human normal tissue
1: heart, *2*: brain, *3*: placenta, *4*: colon, *5*: lung, *6*: liver, *7*: sekletalmuscle, *8*: kidney, *9*: ovary, *10*: spleen, *11*: pancreas, *12*: prostate.

2.2　Differential expression of AKR1C2 in tumor and nontumor tissue

Experiments were then designed to determine whether AKR1C2 mRNA was differentially expressed in HCC compared with surrounding nontumor liver and/or whether there was a difference in expression.

Using *in situ* hybridization, we also found the signal corresponding to AKR1C2 mRNA was particularly strong in cytoplasm of HCC（*EI*: 0.95 ± 0.16）when compared with those of para-cancerous tissues（*EI*: 0.15 ± 0.01）and normal liver tissues（*EI*: 0.12 ± 0.2）（Figure 2a, b, c）and stronger signal of AKR1C2 mRNA was also closely related to incomplete capsule, intrahepatic metastasis and portal vein thrombus, Edmondson's grade（Table 1）. When ISH was conducted on liver tissue from the patient with the pT7Blue（R）control vector as probe, no hybridization was observed, suggesting that the ISH signals were specific.

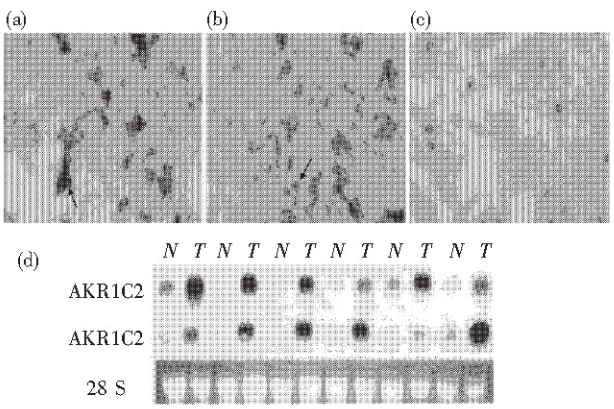

Fig. 2　ISH and Northern blot analysis of AKR1C2 in liver cancer tissue and its surrounding liver tissue
ISH was also performed with the isolated AKR1C2 fragment as probe in tumor tissue（a）, surrounding nontumor liver（b）, and in uninfected liver（c）. Original magnification of tissue sections was × 200.（d）Northern blot analysis of AKR1C2 mRNA from the liver of tumor tissue（*T*）and surrounding nontumor liver（*N*）. The corresponding 28 S RNA controls for each lane.

Table 1　Relationship between labeling index of AKR1C2 accumulated type, expression index of AKR1C2 mRNA and clinicopathological characteristics of HCC

	n	*LI* of AKR1C2 accumulated type	*EI* of AKR1C2 mRNA
Male	62	61.7 ± 13.1	0.8 ± 0.2
Female	6	62.3 ± 14.1	0.8 ± 0.1
AFP ≤ 20 μg/L	18	60.3 ± 15.1	0.9 ± 0.1
AFP > 20 μg/L	50	61.4 ± 13.1	0.8 ± 0.1
Tumor size			
≤5 cm	30	67.1 ± 19.1	0.8 ± 0.2
5 ~ 10 cm	14	60.7 ± 20.3	0.8 ± 0.2
> 10	24	60.8 ± 16.8	0.8 ± 0.1
Capsule			
Complete	30	74.3 ± 22.4	0.6 ± 0.1
Incomplete	38	41.2 ± 11.1[2)]	0.9 ± 0.1[1)]
Intrahepatic metastasis Yes	28	81.3 ± 20.7	0.9 ± 0.2
Intrahepatic metastasis No	40	41.7 ± 17.7[2)]	0.6 ± 0.1[1)]
Portal vein thrombus Yes	38	80.4 ± 13.1	0.9 ± 0.2
Portal vein thrombus No	30	42.3 ± 20.8[1)]	0.5 ± 0.2[2)]
Edmondson's Grade Ⅱ	38	39.6 ± 18.1	0.5 ± 0.4
Edmondson's Grade Ⅲ	30	80.2 ± 14.3[2)]	0.9 ± 0.4[1)]
Cirrhotic nodule ≤ 0.5 cm	46	61.1 ± 11.3	0.8 ± 0.2
Cirrhotic nodule > 0.5 cm	22	62.3 ± 17.8	0.7 ± 0.1

[1)] $P < 0.05$, [2)] $P < 0.01$.

Confirmation that AKR1C2 was differentially expressed in tumor tissue compared with para-canerous tissue was provided by Northern blot analysis with RNA isolated from snap frozen tissue samples. Our results showed that 91.2%（62/68）was up-regulation expression, 8.8%（8/68）was down-regulation（$P < 0.01$）（Figure 2d）. AKR1C2 mRNA is up-regulated in cancer tissue compared with para-canerous tissue. The hybridization signals of RNA isolated from nontumor tissue were weak or absent, in accordance with the ISH results. Hence, AKR1C2 mRNA is overexpressed in HCC tissue compared with para-canerous tissue.

The development of antibodies to AKR1C2. Accordingly, consecutive sections of paired, formalin-fixed, paraffinembedded liver and tumor samples were stained for AKR1C2. It was found from immunohistochemistry that all normal liver tissues and para-cancerous tissues examined showed membranous type, occasionally with weak expression of AKR1C2 in the cytoplasm, but no AKR1C2 accumulation in nuclei was observed, while for HCC, 65 cases（95.3%）showed accumulated type（Figure 3a, b, c）. The *LI* of accumulated type for tumor tissue and paracancerous tissue were（61.4 ± 27.8）and（10.2 ± 8.7）respectively（$P < 0.01$）, while the *LI* of membraneous

type for tumor tissue and paracancerous tissue were (12.6 ± 6.5) and (95.7 ± 11.7) respectively ($P < 0.01$), suggesting up-regulated gene expression at both the mRNA and protein levels in clinical samples from the same patients. Consecutive tissue sections were negative using preimmune rabbit sera. Immunohistochemical staining was also completely blocked by preincubation of primary antibodies with an excess of the corresponding synthetic peptides prior to staining, confirming the specificity of staining. When *LI* if accumulated type was analyzed according to the clinicopathological characteristics of HCC, close relationship could be seen with capsule, portal vein tumor thrombus, pathological grade, intrahepatic metastasis and postoperative recurrence (Table 1). Labeling index (*LI*) of AKR1C2 in recurrent patients ($n = 30$) was much higher than that in non-recurrent patients ($n = 38$), (89.1 ± 16.3) vs (25.3 ± 11.7) ($P < 0.01$).

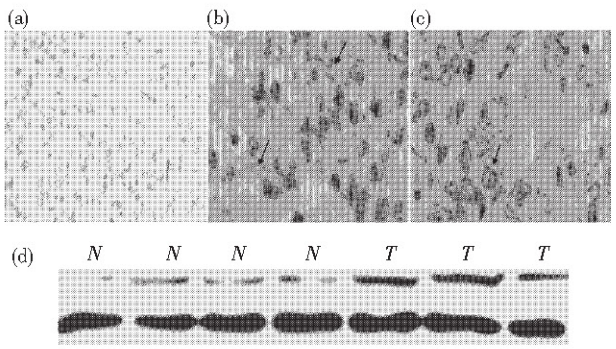

Fig. 3 Immunohistochemical staining and Western blot analysis for AKR1C2

(a) In normal liver tissue, the staining was mainly positive on the cellular membrane, with very weak cytoplasmic staining (DAB, × 200); (b) Para-cancerous liver tissue showed membrane staining like normal liver tissue (DAB, × 400); (c) For HCC, cytoplasmic and nuclear staining was dominant whereas membrane staining was rare (DAB, × 400); (d) Western blot analysis for AKR1C2 in liver cancer tissue (*T*) and surrounding nontumor liver tissue (*N*), the β-actin loading controls for each lane.

Elevated expression of AKR1C2 was also shown by Western blotting, which revealed a strong band at the expected molecular mass of approximately 31 ku from liver cancer, compared with normal liver. Based on gel scanning, the ratio of the bands is 12.3 (Figure 3d). When extrahepatic tissues from uninfected individuals were stained for AKR1C2, faint staining was consistently observed in breast, and in some samples from stomach, colon, prostate, lung, kidney and uterus (data not shown). No staining was observed in thyroid (data not shown). These results show that AKR1C2 is expressed in selected tissue types other than the liver.

RT-PCR amplification products were 972 bp in para-cancerous tissues and HCC tissues, but there are some sequence difference between HCC tissue and para-cancerous tissues, resulting in transform or tranversion at 19, 48, 77, 157, 158, 159, 268, 338, 454, 622, 733, 821, 865, 866, 923, 924 site and leading to change the sequence of amino acids and these are all mis-sense mutation. (data not showed). RT-PCR results showed the AKR1C2 mRNA *EI* were (0.81 ± 0.15) and (0.43 ± 0.10) ($n = 68$) for HCC tissues and para-cancerous tissues respectively ($P < 0.05$). In HCC, higher *EI* of AKR1C2 mRNA attempted to be seen in cancer with incomplete capsule, intrahepatic metastasis and portal vein thrombus, Edmondson's grade (Table 1).

2.3 Test and verify of transfection of AKR1C2 into QGY7703

ICC experiments were then performed to detemine whether the AKR1C2 PCR fragment had been integrated into chromosome of QGY7703-pcDNA3 or QGY7703-pcDNA3- AKR1C2. The results show a strong signal for AKR1C2 protein in the cytoplasm of QGY7703-pcDNA3- AKR1C2, whereas weak or no signals were observed in QGY7703-pcDNA3.1 (Figure 4).

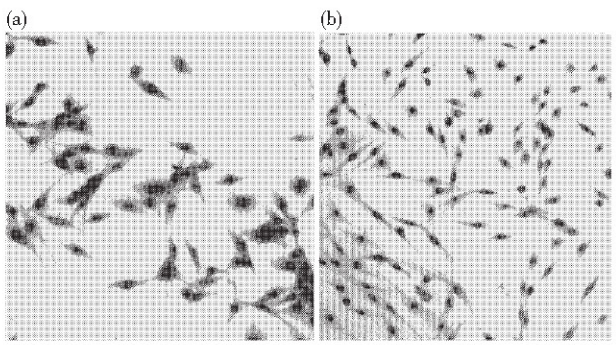

Fig. 4 ICC of QGY7703-pcDNA3.1-AKR1C2 (a) or QGY7703-pcDNA3.1 (b) using the anti-AKR1C2

2.4 Overexpression of AKR1C2 promotes hepatocellular carcinoma growth *in vitro* and tumorigenesis *in vivo*

To test whether AKR1C2 mediates the growth stimulatory properties, the growth curves of QGY7703 cells stably transfected with vector, with pcDNA3.1, pcDNA3.1- AKR1C2, or with the pcDNA3.1-AKR1C2 frameshift mutant were compared in medium containing 10% fetal calf serum. The results show that AKR1C2 stimulates the growth of QGY7703 cells to a significantly greater extent when compared with QGY7703-pcDNA3 cells ($P < 0.008$ after 96 h) or frameshift mutant transfected QGY7703 cells ($P < 0.007$) (Figure 5). In comparison, when the experiment was repeated in serum-free medium, the cells overexpressing AKR1C2 were significantly

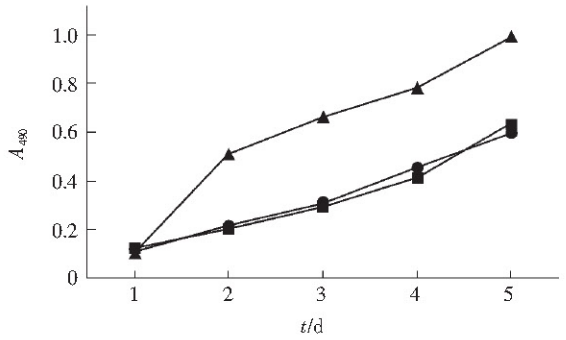

Fig. 5 Growth curve of QGY7703 transfected with pcDNA3. 1, pcDNA3. 1AKR1C2, pcDNA3. 1AKR1C2 mutant in medium containing 10% fetal calf serum

●—●: pcDNA3. 1; ■—■: pcDNA3. 1AKR1C2 mutant; ▲—▲: pcDNA3. 1AKR1C2.

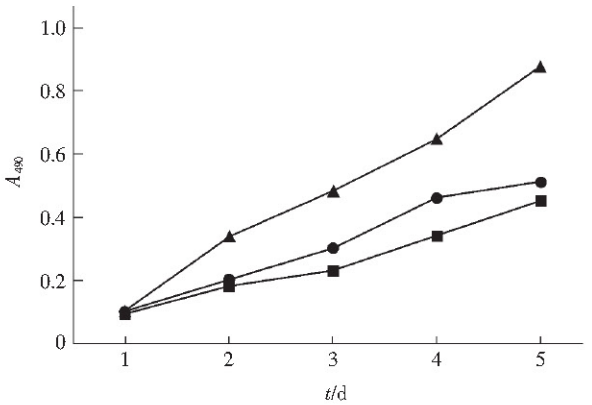

Fig. 6 Growth curve of QGY7703 transfected with pcDNA3. 1, pcDNA3. 1AKR1C2, pcDNA3. 1AKR1C2 mutant in serum-free medium

●—●: pcDNA3. 1; ■—■: pcDNA3. 1AKR1C2 mutant; ▲—▲: pcDNA3. 1AKR1C2.

stimulated to obtain more survival rate than that of control cells ($P < 0.01$ after 96 h) or AKR1C2 frameshift mutant transfected cells (Figure 6). These results were confirmed by FACS analysis in serum starved synchronized cells. In these experiments, 34% of QGY7703-pcDNA3. 1 control cells were in S or G2 phase at 24 h after the addition of serum. Whereas this number was 67% for QGY7703-pcDNA3. 1- AKR1C2 cells analyzed at the same time ($P < 0.01$). Apoptosis in QGY7703-pcDNA3. 1- AKR1C2 is less than QGY7703-pcDNA3. 1 (Figure 7). Together these results suggest that AKR1C2 overexpression stimulates DNA synthesis in QGY7703 cells.

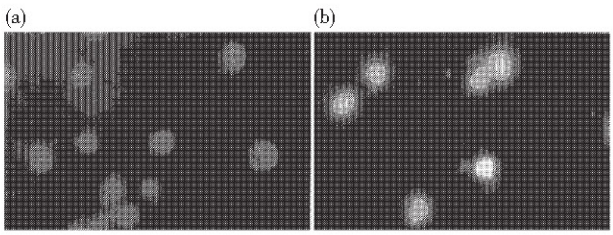

Fig. 7 Fluorescence staining analysis of apoptosis

(a) QGY7703-pcDNA3. 1- AKR1C2; (b) QGY7703-pcDNA3. 1.

Additional experiments were conducted to test whether AKR1C2 overexpression correlated with increased anchorageindependent growth in soft agar. Accordingly, 1×10^4 cells were seeded into individual wells and allowed to grow for 21 days.

The results (Figure 8) show that AKR1C2 overexpressing cells are stimulated to grow in soft agar (104 clony per field of vision) compared with vector transfected control cells (44 clony per field of vision) ($P < 0.01$) or QGY7703 cells stably transfected with the AKR1C2 frameshift mutant (47 clony per field of vision) ($P < 0.01$).

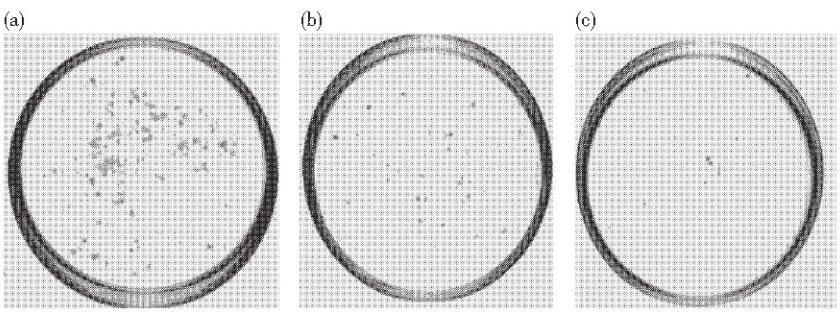

Fig. 8 Experiments were conducted to test whether AKR1C2 overexpression correlated with increased anchorage independent growth in soft agar, accordingly, 1×10^4 cells were seeded into individual wells and allowed to grow for 21 days

The results show that AKR1C2 overexpressing cells are stimulated to grow in soft agar (a) compared with vector transfected control cells (b), QGY7703 cells stably transfected with the AKR1C2 frameshift mutant (c).

Moreover, in a tumorigenesis assay, QGY7703-pcDNA3. 1- AKR1C2 cells form larger tumor formation than that of QGY7703-pcDNA3. 1, QGY7703-pcDNA3- AKR1C2 frameshift mutant. In all cases, there was statistically significant difference between the number of tumors obtained, their average size (i. e., wet tumor mass) and diameter, or their onset of appearance (Figure 9 ~ 11).

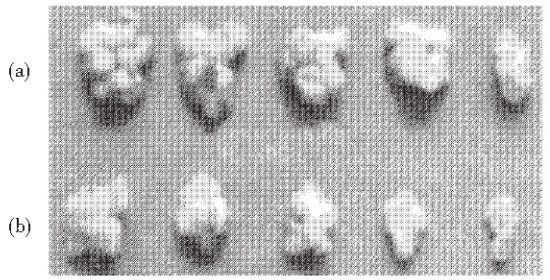

Fig. 9 Tumorigenesis assay
(a) QGY7703-pcDNA3- AKR1C2 cells; (b) QGY7703-pcDNA3. 1.

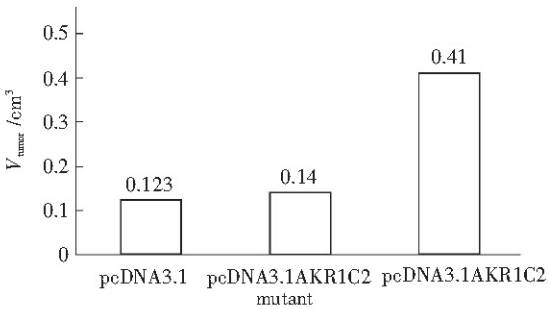

Fig. 10 Tumor average size in nude mice transfected with pcDNA3. 1, pcDNA3. 1AKR1C2, pcDNA3. 1AKR1C2 mutant
□: QGY7710.

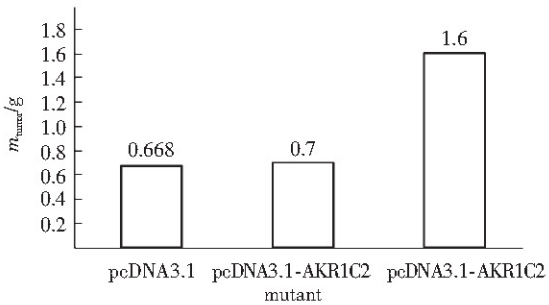

Fig. 11 Tumor mass of nude mice transfected with pcDNA3. 1, pcDNA3. 1AKR1C2, pcDNA3. 1AKR1C2 mutant
□: QGY7710.

2. 5 Difference on cDNA expression microarray between QGY7703-pcDNA3. 1- AKR1C2 and QGY7703-pcDNA3. 1

The hybridization results of gene expression chip were shown in the Figure 12a, b (The probes were labled by Cy3 and Cy5 fluorescence). The results that intensity of Cy3 and Cy5 fluorescence at the correspondence position on the chip was added together, were shown in Figure 12c.

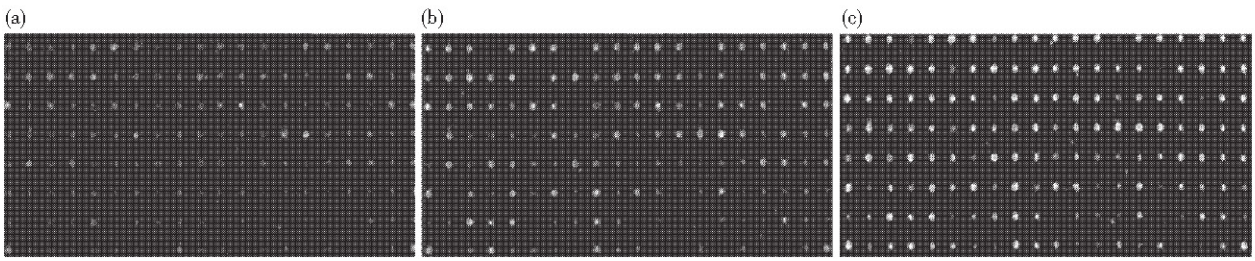

Fig. 12 The hybridization results of gene expression chip
(a) QGY7703-pcDNA3. 1; (b) QGY7703-pcDNA3. 1-AKR1C2; (c) addition Cy3 up Cy5 for same position point

According to results of two repeated experiments, expression differences of 10% (20/200) tumor genes were considered significant. Those genes are shown in Figure 13 and listed in Table 2. In the Figure 13, that Cy5/Cy3 value is between 0. 5 and 2. 0 shows no different gene expression; that Cy5/Cy3 value is less than 0. 5 or more than 2. 0, shows significant difference of gene expression. In table 2, there are twenty tumor genes for significant difference on gene expression.

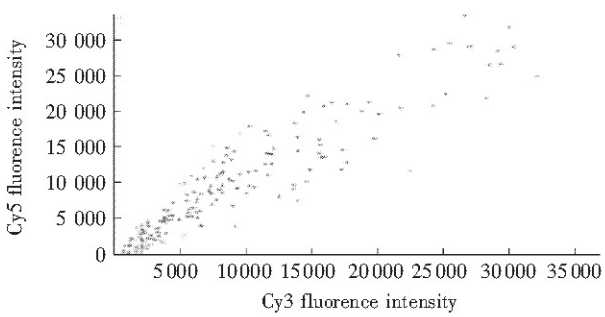

Fig. 13 The scatter plots of hybridizing signal intensity on gene

Table 2 Gene differential expression between QGY7703-pcDNA3.1 and QGY7703-pcDNA3.1-AKR1C2

Gene name	Average ratio (Cy5/Cy3)
C-myc transcription factor (puf)	2.778
GRO2 oncogene	4.675
P16	0.114
APC	0.213
GADD34	0.132
Gadd153	0.206
ING1	0.156
Kip2p57	0.186
MDM2	0.236
P15	0.122
P63	0.325
RAD51	0.321
XRCC1	0.224
TSG101	0.213
MLH1	0.321
PTEN	0.334
CALLA	0.111
NF-IL16	0.164
Growth arrest-specific 1	0.170
P53-induced protein (P I G11) mRNA	0.387

2.6 AKR1C2 mediates the NF-κB-dependent resistance of QGY7703 cells to anti-fas killing

QGY7703 cells transfected with pcDNA3.1, PcDNA3.1-AKR1C2 or pcDNA3- AKR1C2 frameshift mutant were treated with anti-Fas or an equivalent amount of normal mouse IgG in the presence or absence of either cycloheximide or actinomycin D for up to 72 h. Approximately 90% of QGY7703-pcDNA3.1 cells were killed by anti-Fas by 48 h of treatment, whereas only about 35% of the QGY7703-pcDNA3- AKR1C2 cells took up trypan blue ($P < 0.001$). The survival curves for the AKR1C2 frameshift mutant were the same as those for pcDNA3-transfected cells. Independent date collected from the modified tetrazolium salt assay confirmed these findings (data not shown). Controls using an equivalent amount of normal IgG in place of anti-fas showed less than 6% trypan blue, suggesting that the killing was associated with anti-Fas treatment. Hence, the up-regulation of AKR1C2 appears to mediate the resistance of cells to killing by anti-Fas.

The finding that AKR1C2 appears to mediate resistance to anti-Fas treatment and that activated NF-κB contributes importantly to mediating this resistance, suggest that AKR1C2 may be an effector of activated NF-κB. To test this hypothesis, QGY7703 cells were stably transfected with exogenous IκBα. Introduction of exogenous IκBα resulted in an approximate 2-fold reduction in the steady state levels of AKR1C2 mRNA in QGY7703 cells. However, the levels of endogenous IκB mRNA were depressed more than 30-fold in AKR1C2-positive compared with AKR1C2-negitive cells ($P < 0.001$, Figure 14a). An IκB expression plasmid restored most of the steady state levels of IκB mRNA to that observed in QGY7703-pcDNA3.1 cells. Importantly, the levels of AKR1C2 polypeptide observed was depressed about 2.7-fold (Figure 14b). This fold difference in endogenous AKR1C2 protein levels were approximately the same as that observed in QGY7703-pcDNA3.1 compared with QGY7703-pcDNA3.1- AKR1C2 cells. This magnitude of difference is associated with significant differences in the growth and survival of QGY7703 cells as well as the resistance of the cell line to anti-Fas treatment. Hence, it is likely that AKR1C2 is an effector of NF-κB, and that the activation of NF-κB may up-regulate AKR1C2.

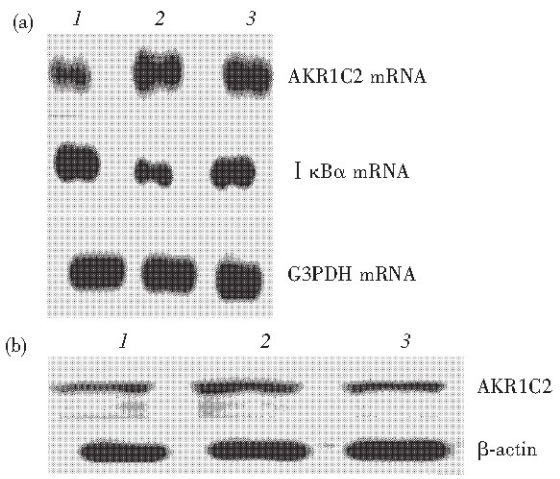

Fig. 14 AKR1C2 mRNA and protein in QGY7703 cells expressing exogenous IκBα

(a) The upper portion of the panel are the results of Northern blot analysis for AKR1C2 mRNA in QGY7703-pcDNA cells (*1*), QGY7703-pcDNA-AKR1C2 cells (*2*), and in QGY7703-pcDNA-AKR1C2 stably transfected with IκBα (*3*). The ratio of AKR1C2 mRNA in lane *1*, *2*, *3* is 1:4.3:2.2 based on densitometry measurements. The middle portion of the panel shows Northern blot analysis of IκBα mRNA from the same cells. The ratio of mRNA in lane *1*, *2*, *3* is 1:0.43:0.80. The lower portion of the panel is a Northern blot analysis of G3PDH from the same membrane as a control for loading. (b) Western blot analysis of AKR1C2 protein (using AKR1C2 antisera) in QGY7703-pcDNA cells (*1*), QGY7703-pcDNA-AKR1C2 cells (*2*), and QGY7703-pcDNA-AKR1C2 cells stably transfected with IκBα (*3*). The ratio of protein in lane *1*, *2*, *3* is 1.0:2.0:1.6. The Western blot for the β-actin controls are shown directly below each respective lane. Both Northern blot and Western blot data shown here are from one of three independently conducted experiments.

2.7 Intracellar binding of AKR1C2 and Cdk4

Using a co-immunoprecipitation approach, cell cycle-associated factors were examined for potential interactions with AKR1C2. Among the cell cycle-associated factors tested (Cdk1/Cdc2, Cdk2, cdk4, cyclinB, CyclinD1, PCNA and Rb2), only Cdk4 interacted with AKR1C2 by co-immunoprecipitation experiments using anti-AKR1C2 antibody. Therefore, we investigated the association of Cdk4 with AKR1C2 in QGY7703 cell line (Figure 15). Immunoprecipitations of cell extracts were done with anti-Cdk4 antibody. The precipitated protein were separated by SDS-PAGE, and associated proteins were examined by immunoblot analysis using anti-AKR1C2 antibody. A single 32 ku band was observed from each sample blotted with the anti-AKR1C2 antibody, which is consistent with the known molecular size of AKR1C2 in QGY7703 cell line.

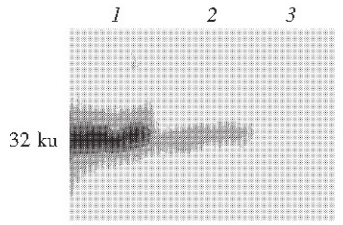

Fig. 15 Cdk4/AKR1C2 complex in QGY7703 cell line

Cell extracts were immunoprecipitated with anti-Cdk4 antibody. Anti-Cdk4 antibody immunoprecipitates were separated by 14% SDS-PAGE under reducing condition, and immunoblotting was performed using anti-AKR1C2 antibody. *1*: QGY7703-pcDNA3.1-AKR1C; *2*: QGY7703-pcDNA3.1-AKR1C2 mutation; *3*: QGY7703-pcDNA3.1.

3 Discussion

AKR1C2 is a new gene cloned from mouse liver cDNA library[1]. Its cDNA full-length is 1 900 bp and its open reading frame is 972 bp encoding 322 amino acids. AKR1C2 is one member of the Aldo-Deto Reductase (AKR) gene family (Gene Bank AB02165)[1~3]. The gene is mainly expressed in the liver tissue and secreting hormone tissue, such as breast, and it related with sex hormone, especially steroid hormone such as DHT, androstenedione. AKR1C2 called 3-HSD Ⅲ type was originally identified and purified by its high affinity binding for bile salts[4~6]. Some research showed that it is also a signaling molecule and can activate gene transcription by forming a heterodimer with the T-cell factor/lymphoid enhancer-binding factor family of DNA binding protein[7,8]. Some researches showed that AKR1C2 plays an important role in the interactions between cadherins and other transmembrane receptor proteins and it maybe lead to change of gene message pathway resulting in the biology nature alter of hepatocellular carcinoma, such as apoptosis and metastas[9]. Previous studies have shown that AKR1C2 is involved in pathways that regulate cellular differentiation and proliferation. In the absence of differentiation signals, cytoplasm AKR1C2 is rapidly turned over under the control of the APC protein and the GSK-2β resulting in low level of cytoplasm AKR1C2 level in normal cells[10].

Our results showed that there is expression difference of AKR1C2 between liver cancer and paracancerous tissue and suggested it is an up-regulation gene. Interestingly, there is some sequence difference between HCC and paracancerous tissue, resulting in transform or tranversion at 19, 48, 77, 157, 158, 159, 268, 338, 454, 622, 733, 821, 865, 866, 923, 924 site, and leading to change the sequence of amino acids and these are all mis-sense mutation, whether it is an SNP or point mutation will be studied (data not shown). We examined the sub-cellular localization of AKR1C2 in 68 pairs of HCC samples with the result that 95.3% of HCC specimen showed that to be accumulated type, suggesting cytoplasmic stabilization of the protein. This showed that activation of the AKR1C2 maybe of importance in the carcinogenesis of HCC from Qidong. Mutation of AKR1C2 gene is probably one of the most important factor causing accumulation of AKR1C2 protein in the cytoplasms in HCC. Using *in situ* hybridization, we also found the signal corresponding to AKR1C2 mRNA was particularly strong in cytoplasm of HCC (*EI*: 0.95 ± 0.16) when compared with those of paracancerous tissues (*EI*: 0.15 ± 0.01) and normal liver tissues (*EI*: 0.12 ± 0.2) and stronger signal of AKR1C2 mRNA was also closely related to incomplete capsule, intrahepatic metastasis and portal vein thrombus. Compared with normal liver, elevated expression of AKR1C2 was also shown from liver cancer by Western blotting, which revealed a strong band at the expected molecular mass of approximately 31 ku. Based on gel scanning, the ratio of the bands is 12.3. These results also show that AKR1C2 is expressed in selected tissue types other than the liver.

Although some have been made to investigate AKR1C2 mutation and abnormal in tumor. No previous results have been reported concerning about the relationship between expression abnormality of AKR1C2 and clinico-pathological features of HCC. Furthermore, research reports about the relationship between AKR1C2 abnormal expression and clinico-pathological features of tumors such as colon cancer, melanoma, breast carcinoma, gastric carcinoma, and lung carcinoma are rather various and some of the results were even totally contradictory[11,12]. Most tumors showed reduced AKR1C2 in the cytosolic

fraction and truncated AKR1C2 protein which was encoded by mutational AKR1C2 gene was found bound weakly to AKR1C2 monclonal antibody when compared with non-truncated AKR1C2[13]. This is the reason why we chose polyclonal antibody instead of monoclonal antibody in our study. In this study we aimed to determine which type of expression abnormalities for AKR1C2 correlate with clinicopathological feature and postoperative recurrence in HCC. Our results demonstrated that although great difference existed between cancer tissue and non-cancer tissue, we failed to show the *LI* of membraneous type to be correlated with the invasiveness of HCC. But the *LI* of accumulated type was discovered closely related with the invasive characteristics of HCC, higher *EI* would predict high ability of invasiveness of HCC and thus a worse prognosis. This was different from another article about colon cancer, which showed that membraneous type, instead of accumulated type, was related to the invasiveness and prognosis of the tumor.

Since abnormal expression of AKR1C2 protein can be caused by both AKR1C2 gene mutation and over expression and in some HCCs, accumulated type of staining could be observed, it is our logical thoughts to figure out whether over expression on AKR1C2 gene existed and what its relationship with the invasiveness of HCC was. In this article, we studied the AKR1C2 gene expression in HCC at mRNA level. We used RT-PCR and Northern blot to examine the expression of AKR1C2 gene. Since RT-PCR and Northern blot was not very accurate in semi-quantitive analysis of gene expression, we chose *in situ* hybridization to reconfirm the results of RT-PCR. The results of them are the same, that is overexpression did exist in HCC and it showed relationship with the invasiveness of HCC. This could give some explanation why strong membranous and cytoplasmic distribution of AKR1C2 was observed on immunohistochemistry in some HCC. It was the accumulation of AKR1C2 though apparently normal, that exceeded the capacity of E-cadherin combination and GSK-3β degradation, resulting in increase and stabilization of this protein in the cytoplasm[14,15].

Although we found that *LI* of AKR1C2 accumulated type was related with HCC recurrence, we were unable to find there was such relationship between AKR1C2 gene *EI* and HCC recurrence, either by RT-PCR or *in situ* hybridization. This implies that the *LI* of AKR1C2 accumulated type would be of greater value in predicting recurrence of HCC. From above we can see that abnormal expression of AKR1C2 protein, especially the accumulated type, is closely related to the invasiveness of HCC from Qidong HCC patients. Further study should be carried out to confirm this and to investigate what the other mechanism causing abnormal expression of AKR1C2 gene is.

When AKR1C2 was stably overexpressed in QGY7703 cells, it promoted cell growth and survival in medium containing 10% serum or free-serum compared with vector-transfected cells. This suggests that AKR1C2 mediates some of the growth stimulatory properties. This stimulatory activity was also observed by FACS analysis, where AKR1C2 significantly increased the fraction of cells in S and G2 phases at the expense of cells in G1 and decreased apoptosis. Together these results suggest that AKR1C2 overexpression stimulates DNA synthesis in QGY7703 cells.

Moreover, in a tumorigenesis assay, QGY7703-pcDNA3.1- AKR1C2 cells promote tumor formation any more than QGY7703-pcDNA3.1, QGY7703-pcDNA3- AKR1C2 frameshift mutant. In all cases, there was statistically significant difference between the number of tumors obtained, their average size (i.e., wet tumor mass) and diameter, or their onset of appearance. The results show that AKR1C2 overexpressing cells are stimulated to grow in soft agar compared with vector transfected control cells ($P < 0.01$) or QGY7703 cells stably transfected with the AKR1C2 frameshift mutant. AKR1C2 promoted QGY7703 growth in soft agar. Moreover, AKR1C2 promoted tumor formation in immunodeficient mice. These results suggest that AKR1C2 is involved in the stimulation of hepatocellular carclnoma growth, but bring about transformation.

Our experiment showed differential cDNA expression microarray between QGY7703-pcDNA3.1-AKR1C2 and QGY7703-pcDNA3.1. Expression differences of 10% (20/200) tumor genes were considered significant, that is, here are twenty tumor genes for significant diffrence of gene expression. Of these oncogene, eighteen genes showed down-regulation and two genes showed up-regulation. Our study showed that QGY7703 cell showed distinct expression profiles of genes, including 20 genes, the connection of which to hepatocarcinogenesis are yet to be elucidated. Clearly, the interpretation of results using cell lines is limited in the prediction of the behavior of tumors. Use of cDNA microarrays to define gene-expression profiles in HCC may be an important way to uncover the specific molecular derangements that contribute to HCC pathogenesis, and thus to identify potential targets for therapeutic intervention.

Although the mechanism(s) whereby AKR1C2 prevents cell death and/or promotes cell growth remain(s) to be clarified, NF-κB, an important mediator of hepatocellular carcinoma growth and survival may be mediated by AKR1C2. Accordingly, the stable introduction of the dominant negative NF-κB inhibitor, IκBα, into QGY7703 cells resulted in decreased AKR1C2 protein expression. Interestingly,

the 2.7-fold decrease in AKR1C2 protein expression in QGY7703-pcDNA3.1-AKR1C2-I κBα cells compared with QGY7703-pcDNA3.1-AKR1C2 cells is the same that distinguishes the AKR1C2 protein levels in QGY7703-pcDNA3.1 from HepG2-pcDNA3.1-AKR1C2 overexpressing cells. Because the latter 2 cell lines have different growth and survival characteristics in culture, it is likely that the 2.7-fold difference in AKR1C2 expression is biologically significant. Together, these results suggest that AKR1C2 is an effector of NF-κB that promotes hepatocellular carcinoma growth and survival and helps to prevent cell death[16]. This may occur in part by conferring resistance to fas-mediated hepatocytolysis.

Using a coimmunoprecipitation approach, we investigated the association of Cdk4 with AKR1C2 in QGY7703 cell line. Our research tested intracellar binding of AKR1C2 and Cdk4. We found AKR1C2 interacting with cell cycle regulators, especially those of the G1 phase, and found AKR1C2 directly interacted with Cdk4. AKR1C2 may counteract a default induction of apoptosis in G2/M phase. At the beginning of mitosis, AKR1C2 associates with the mitotic spindle, and disruption of this interaction results in losing its anti-apoptosis function[16]. Overexpression of AKR1C2 in cancer cells may overcome this apoptosis-related cell cycle checkpoint. We propose that this function of AKR1C2 plays an important role in the development and progression of maligant transformation in HCC. Further evaluation of these possible roles of AKR1C2 and regulation of AKR1C2 expression in malignancy cell is critically important for the development of new strategies for controlling the growth of malignant cells.

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中国启东肝癌高发区肝癌中 AKR1C2 基因异常表达及其在肝癌发生中作用

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摘要 已在许多肿瘤中发现 AKR1C2 基因的异常表达. 为研究启东肝癌中 AKR1C2 基因异常表达的意义及其在肝癌发生中作用. 通过制备兔抗人 AKR1C2 多克隆抗体、免疫组化、蛋白质印迹、RT-PCR、RNA 印迹、原位杂交、cDNA 表达芯片、免疫共沉淀、体内外致瘤试验等方法，对 68 例启东肝癌标本、8 例正常肝组织、QGY7703 启东肝癌细胞株中 AKR1C2 表达及作用进行分析. 并研究了 AKR1C2 蛋白、mRNA 表达与肝癌临床病理特征，侵袭性间关系. 研究表明正常及癌旁肝组织

中 AKR1C2 蛋白为膜染色，偶见弱的细胞浆染色. 95.3% 肝癌显示胞浆或核染的累积型. 癌及癌旁肝组织中标记指数（*LI*）分别为 61.4 ± 27.8，10.2 ± 8.7（$P < 0.01$）. 较高的 *LI* 与 HCC 侵袭性密切相关. 蛋白质印迹显示癌组织中 AKR1C2 表达升高. RT-PCR 显示，肝癌中 AKR1C2 表达指数（*EI*）高于癌旁及正常组织，而且存在序列差异. RNA 印迹显示 91.2% 为上调表达. 原位杂交显示肝癌细胞胞浆中染色强于癌旁及正常肝. AKR1C2 过表达与肝癌转移潜能有关. AKR1C2 过表达刺激 QGY7703 细胞中 DNA 合成与阻止细胞凋亡. 转染 AKR1C2 基因的 QGY7703 细胞在软琼脂上集落形成能力增强，并能促进 QGY7703 在裸鼠体内肿瘤形成能力. cDNA 表达芯片显示转染 AKR1C2 后导致 QGY7703 细胞中一些基因表达改变. AKR1C2 介导 NF-κB 阻止抗-Fas 对 QGY7703 的抑制作用，并且在细胞内 AKR1C2 能与 Cdk4 结合，产生免疫共沉淀. AKR1C2 的异常表达在启东肝癌的发生、发展及转移中可能起重要作用.

关键词 肝肿瘤，AKR1C2 基因，转移，侵袭性，基因表达，细胞凋亡
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知识与动态

角质细胞生长因子-2 研究进展

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角质细胞生长因子-2（KGF-2）也叫成纤维细胞生长因子-10（FGF-10），是成纤维细胞生长因子家族的一员. 能特异性促进上皮细胞的增殖、分化和迁移，对脊椎动物多种组织和器官的发育起重要调控作用，对临床上多种疾病的治疗也有很好的应用前景.

1 KGF-2 与受体

KGF-2 有两种细胞膜表面受体：FGFR1Ⅲb 和 FGFR2Ⅲb. KGF-2 与 FGFR2Ⅲb 的亲和力很高，而与 FGFR1Ⅲb 的亲和力很低，只有在高浓度 KGF-2 存在时才与 FGFR1Ⅲb 结合. KGF-2 与受体结合后，促使受体胞内的 C 端酪氨酸残基磷酸化，磷酸化的受体具有了酪氨酸蛋白激酶活性，并与一系列靶蛋白发生作用，引发信号级联反应，发挥生物学功能. FGFR2Ⅲb 仅在上皮细胞中表达，KGF-2 特异的促上皮细胞增殖作用主要是通过 FGFR2Ⅲb 介导的. FGFR1Ⅲb 在许多类型的细胞中都表达，但对其介导的 KGF-2 的生物学作用仍不清楚.

2 KGF-2 与发育

KGF-2 参与并调控脊椎动物多种组织和器官的形成. 肢发育过程中，肢间充质细胞表达的 KGF-2 通过 Wnt/β-catenin 介导的信号通路诱导尖外胚层嵴表达 FGF-8，促进尖外胚层嵴的发育，FGF-8 又可反作用于中胚叶，促进间充质细胞的生长并维持 KGF-2 的稳定表达. 在肺分支形态建成过程中，KGF-2 由肺节周围的中胚层间充质细胞表达，促进分支形成. 同时，中胚层间充质细胞表达的 KGF-2 能上调内胚层 *Bmp4* 基因的表达水平，而 Bmp4 则抑制肺分支和形态建成. 通过二者促进与抑制作用的平衡来调节肺的发育.

3 KGF-2 与疾病

动物模型的研究表明，KGF-2 对移植皮肤的愈合具有极显著的促进作用. 它能促进表皮细胞的生长，起始和加速创口愈合，加快上皮和肉芽组织的形成. 在预防辐射对正常机体造成的损伤方面，KGF-2 也有非常显著的作用. 它能提高骨髓对全身辐射的耐受性，保护小肠隐窝免受辐射损伤，降低辐射引起的胃肠道损伤，预防粘膜炎的发生，并能调节和维持干细胞的分裂及存活. 此外，KGF-2 对溃疡和肠炎也有很好的疗效，静脉注射 KGF-2 几乎能完全恢复小鼠肠道表面的正常细胞构造，显著降低急性和慢性肠损伤. 持续的 KGF-2 给药，能维持小鼠正常体重，改善宏观和微观肠炎症和溃疡，并且没有明显的毒副作用.

目前，美国人类基因组科学公司正在进行三项 KGF-2 的Ⅱ期临床实验：一是静脉溃疡；二是骨髓移植前高剂量化疗引起的粘膜炎；三是溃疡性大肠炎。其中静脉溃疡的实验已基本完成，实验结果显示了 KGF-2 良好的疗效和安全性. KGF-2 作为一种新型多肽类药物有着广阔的开发和应用前景.