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Overexpression of Anion Exchanger 2 in Human Hepatocellular Carcinoma

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Abstract

To compare gene expression patterns between the poorly-differentiated (HA22T/VGH) and well-differentiated (HepG2) hepatocellular carcinoma cells, messenger RNA was isolated form both kinds of cells and subjected to differential displays reversing transcriptase-polymerase chain reaction (DDRT-PCR) technology. Gene fragments showing difference in the expression were recovered, reamplified, cloned and sequenced. Anion exchanger 2 (AE2), an isoform of band 3 protein, was identified and chosen for further characterization. AE2 was strongly expressed in HA22T /VGH cells, while it was little expressed in the well-differentiated hepatocellular carcinoma cells (PLC/PRF5 and HepG2) or little in the normal liver cell (Chang liver). In the 28 pairs of HCC and adjacent non-tumor tissues, levels in the cancer tissue (32.7 $\pm$ 5.0) were significantly higher than that in the adjacent non-tumor tissue (9.1 $\pm$ 2.4) ($P < 0.01$). Moreover, 19 cases (68%) showed over-expression of AE2 in HCC tissues, 3 cases were similar in both tissues, and 6 cases exhibited little or undetectable signals. Twenty cases (71%) of adjacent normal tissue showed little or undetectable signals. The results indicated that overexpression of AE2 may be involved in the development of human HCC.

Key Words: DDRT-PCR, hepatocellular carcinoma, anion exchanger 2

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, particularly in Asia and Africa where HBV and HCV infections are endemic (3, 29). Since chemotherapy is unsuccessful in preventing metastasis and recurrence, hepatic resection remains the treatment for HCC (7, 28). However, the prognosis remains poor due to liver failure associated with cirrhosis and/or high recurrence rate, with a life expectancy of about 6 months from the time of diagnosis (9). The high liver recurrence rate is associated with the intrahepatic metastasis, which is a major target of recent research, involving

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prediction, treatment and prevention (28). Therefore, the identification of new gene targets from the metastatic sites or the malignant cell lines is needed for better HCC diagnosis and treatment.

Today, there are many ways to clone novel genes, such as subtractive hybridization (17, 31), cDNA microarray assay (14, 26) and mRNA differential display reverse transcription polymerase chain reaction (DDRT-PCR) (20). Comparatively, DDRT-PCR is the less labor-intensive method. In this study, DDRT-PCR analysis was used to identify and characterize differentially expressed genes in the human hepatocellular carcinoma cell line (HA22T) in comparison with the hepatoblastoma cell line (HepG2). The HA22T/VGH is poorly differentiated whereas HepG2 is well differentiated ones (1, 6). We report on a liver cancer related gene anion exchanger 2 (AE2). Expressions of AE2 in five liver cell lines and in human HCC were analyzed using RT-PCR.

Materials and Methods

Specimen Collection

Informed consent was given by patients and/or guardians for this study. Twenty-eight human hepatocellular carcinoma and paired adjacent normal liver tissue specimens (38-77 years old; 26 men and 2 women) were obtained from the Department of General Surgery, Taichung Veterans General Hospital. The tumors were graded according to the tumor-node-metastasis (TNM) stage as follows: 16 patients were grouped to be stage I or stage II, and 12 patients were grouped to be stage III or stage IV. Histopathological grading was then determined according to cell differentiation (well, moderately, or poorly). Among the HCC patients, 16 patients were found with hepatitis B and 12 were found with hepatitis C viral infections. All specimens were snap-frozen immediately in liquid nitrogen and stored at -70°C until use.

Cell Culture

The three cell lines (HA22T/VGH, PLC/PRF5 and HepG2) were cultivated with DMEM (Sigma Chemicals Co., St. Louis, MD, USA) supplemented with 100 μM non-essential amino acid, 2 mM glutamate, 10% FCS, 100 units/mL penicillin G, and 100 μg/ml streptomycin (Gibco BRL). Chang liver was cultivated with BME (Sigma) supplemented with 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Gibco BRL). PCL/PRF5 is developed from hepatoma and Chang liver is an immortalized non-tumor cell line.

RNA Preparation and Differential Display

Total RNA was extracted from the cell lines using guanidinium thiocyanate-phenol method (8). Targeted RNA DDRT-PCR was performed as per the manufacturer's instructions (RNAimage Kit 1, GenHunter Corporation, Nashville, TN, USA) with some modification. The primers used in the assay included 2 anchor primers (H-T11G and H-T11C) and 8 arbitrary oligonucleotide primers (H-AP-1 to H-AP-8). PCR products were resolved on 2% (w/v) denaturing polyacrylamide sequencing gels and visualized using Ethidium Bromide Staining under UV light. The selected bands were marked, recovered from the dried gel, and re-amplified according to the manufacturer's instructions using the same primer pairs.

Cloning and Sequencing

Re-amplified products were subcloned into pGEM-T-Easy vector (Promega Corporation, Madison, WI, USA) and confirmed using EcoR I (Life Technologies, Bethesda, MD, USA) digestion according to the manufacturer's instruction. Sequence analysis was performed with ABI PRISM 377 automated DNA sequencing system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems). The sequenced cDNA were analyzed via the BLAST program for homology matches in the GenBank database.

RNA Isolation and RT-PCR Analysis

The total RNA tissue and cell isolation was extracted using the guanidinium thiocyanate-phenol method (8). The RNA extract integrity was assessed on 1.5% agarose gel. The RNA was visualized using ethidium bromide staining. The total amount of RNA present was determined spectrophotometrically. The RT-PCR assay was performed as described (23) with some modification. Briefly, 0.5 μg of total RNA was reversely transcribed using 0.5 μM oligo d(T) primers in 50 μl of reaction solution containing 75 mM KCl, 50 mM Tris-HCL, pH 8.3, 3 mM $MgCl_2$, 10 mM DTT, 10 U RNase inhibitor (Promega), 0.8 mM total dNTPs (a 0.2 mM concentration of each) and 200 units of moloney murine leukemia virus reverse transcriptase (Promega, Madsion, WI, USA). After the addition of RNA and water, each sample was incubated at 42°C for 1 h and at 99°C for 5 min and then chilled on ice for 10 min. Four microliters of the RT reaction were diluted with the PCR buffer (50 mM KCl, 10 mM Tris-HCl, and 2 mM $MgCl_2$) to a final volume of 50 μl, which contained 0.5 μM for each primer and for each reaction, additional dNTPs (final concentration, 0.8 mM), and 0.5 unit of Super-Therm Taq DNA polymerase (Southern Cross Biotechnology, Cape

Table 1. Primer sequence.

	Sequence	Location[a]	Size	AT[b] (°C)	GenBank accession no.
AE2	5'-CAACATGGACTACCACGAGA -3' 5'-GCGGCAAAGTAGATGAAGAT -3'	1774-1793 2232-2213	459 bp	52	X62137
β2-MG	5'-ATCCAGCGTACTCCAAAGAT-3' 5'-TTACATGTCTCGATCCCACT-3'	74-93 373-354	300bp	52	AB021288

[a] Position of primer from the human mRNA sequence deposited in GenBank.
[b] AT, annealing temperature.

Town, South Africa). All specific primers were designed manually based on gene sequence (Table 1). The PCR was achieved on a GeneAmp PCR system 2400 with the indicated cycle profile. For each experiment, a limited number of PCR cycles were performed to avoid reaching the PCR plateau values for any of the mRNAs studies. The PCR products were analyzed on 1.2% agarose gel eletrophoresis. They were scanned directly from the agarose gel photographs using a Kodak Scientific IU-imaging System. The related level of AE2 mRNA (42 PCR cycles) was calculated by β_2-MG (30 PCR cycles). All of the values related to the expression of a certain gene were normalized against a reference sample, the HA22T/VGH cells extract, considered as 100%, which was included in each set of amplification reactions. The accuracy of the amplification reaction for each set of primers was determined by amplifying several dilutions of the same cDNA with the same cycling profiles and amplifying the same cDNA dilution with different cycling profiles. The specificity of the cDNA was also checked using DNA sequence analysis (data not shown).

Western Blot

To analyze the AE2 protein expression, the cultured cells were washed twice with PBS, then lysed with protein lysis buffer (50 mM Tris/HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 1 mM NaF, 1 mM sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) nonidet P40, 0.3% sodium deoxycholate). The cell lysates were centrifuged at 100000 g for 30 min at 4°C. The supernatant was then collected and the protein concentration was determined by the Bradford method (4). Samples (40 μg) were then separated using SDS-PAGE (8% (w/v) gel) and transferred electrophoretically onto a PVDF membrane (Millipore, Belford, MA, USA). The membrane was blocked with 5% (w/v) non-fat dried milk in TBST buffer (20 mM Tris, HCl (pH 7.4), 150 mM NaCl, 0.1% (v/v) Tween 20) and incubated with the specific anti-AE2 (Alpha Diagnostic International, San Antonio, TX, USA) or α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody at 4°C overnight. The membrane was then washed with TBST and incubated with the appropriate secondary antibody. The membrane was next washed with TBST and the reaction product was visualized using chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical Analyses

Data were expressed as mean ± SEM and statistical analyses were performed with the paired Student's *t* test and the Fisher exact test using the Analyze-it Software (UK). $P < 0.05$ represented significant difference.

Results

Differentially Amplified mRNAs

Purified total RNAs from human liver tumor cell lines (HA22T/VGH and Hepa G2), RT-PCR samples were processed for differential display. Since each primer combination displays an average of 15 bands per lane, 240 different mRNA species were screened in this study (Fig. 1.). At least 27 cDNAs appeared to be differentially expressed (Fig. 1.). The selective identification was confirmed after reproducibility and multiple display mRNA DDRT-PCR on DNA sequencing gel. Ten of these mRNAs are unique as analyzed by nucleotide sequencing (data not shown).

Expression of AE2 in Four Liver Cell Lines

After searching the GenBank database, one (anion exchanger 2; AE2; designed in Fig. 1.) of the differentially amplified mRNAs was chosen for further characterization. The result showed that AE2 was strongly expressed in the higher malignant HCC cell lines (HA22T /VGH). AE2 was low expressed in the low malignant HCC cell lines (PLC/PRF5) and the hepatoblastoma (HepG2) or little in the normal liver

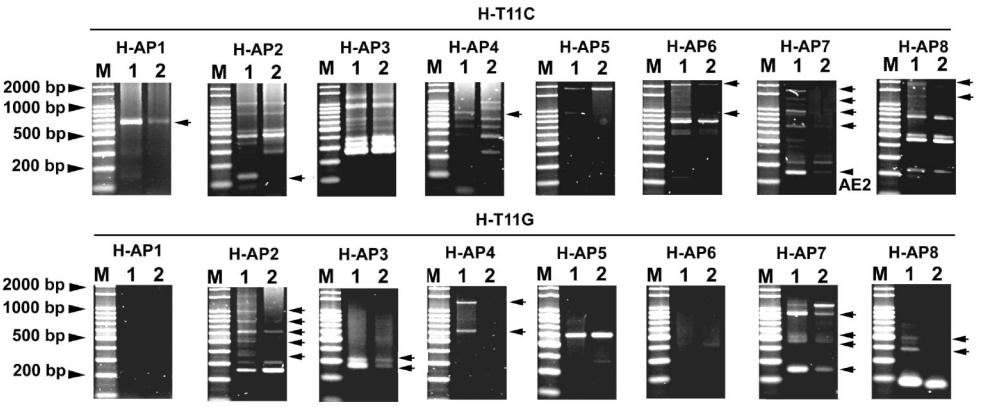

Fig. 1. The pattern of DDRT-PCR cDNA products differentially displayed from human liver tumor cell lines. The primers included 2 anchor primers (H-T11G and H-T11C) and 8 arbitrary oligonucleotide primers (H-AP-1 to H-AP-8) are described in Materials and Methods. The arrows were designed as the differentially expressed bands and the arrow head was the AE2 gene. M, refered to DNA molecular weight marker; 1, designed as the HA22T/VGH cell line; 2, designed as the HepG2 cell line.

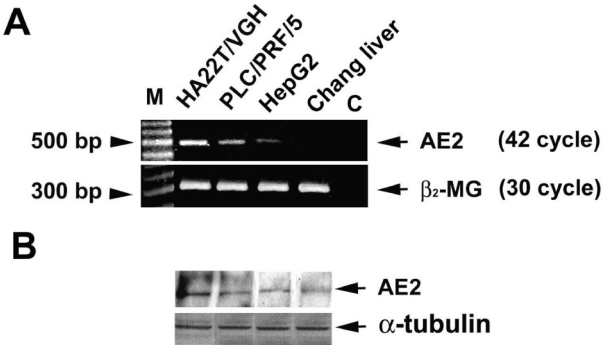

Fig. 2. The altered expression of AE2 in different cell lines. (A) Relative mRNA levels of AE2 detected using RT-PCR as described in Materials and Methods and the corresponding levels of β2-MG as an internal control. C, negative control for RT-PCR without RT; M, DNA size marker. (B) The AE2 protein in four cell lines measured using Western blot as described in Materials and Methods. α-tubulin is designed as an internal control. Data are presented as one of three experiments.

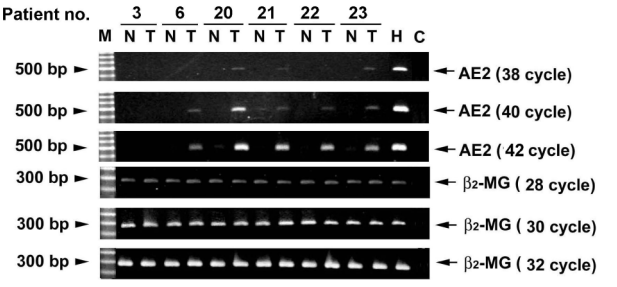

Fig. 3. Semiquantitative RT-PCR for AE2 and β2-MG in matched tumor (T) and normal (N) pairs. RT-PCR was performed for AE2 and β2-MG in matched tumor and normal pairs for the same cDNA dilution with different cycling profiles. H, corresponding to HA22T/VGH cells; C, negative control for RT-PCR without RT.

cell (Chang liver) (Fig. 2A.). The results were also confirmed by Western blot analysis (Fig. 2B.).

Semiquantitative RT-PCR for AE2 in Surgical Specimens

AE2 and β_2-MG were examined for first-strand cDNA from the matched tumor and normal liver tissue pairs using different numbers of PCR cycles (Fig. 3.). Using the paired Student's t test, levels in the cancer tissue (32.7 ± 5.0) were significantly higher than that in the adjacent non-tumor tissue (9.1 ± 2.4) ($P < 0.01$). Moreover, in 28 pairs of HCC and adjacent non-tumor tissues, 19 cases (68%) showed over-expression of AE2 in the HCC tissues, 3 cases (patient no. 9, 15 and 27) were similar and 6 cases (patient no. 1, 3, 5, 7, 10 and 18) showed little or undetectable signals. Furthermore, 20 cases (71%) of adjacent normal tissue (patient no. 1-8, 10, 12-13, 17-23, 25 and 28) were also showed little or undetectable signals. There was no significant difference in age, viral infection (HBV or/ and HCV), tumor size, histopathological grading and tumor stage (Table 3).

Discussion

AE2, a member of the Cl^-/HCO_3^- exchanger family, contributes to the regulation of intracellular pH, intracellular Cl^- concentration (16) and is regulated by acid-base balance (10). AE2 is expressed in various tissues (2, 5, 11, 22). In the human liver, AE2 mRNA signals are localized mainly in the terminal and interlobular bile-duct cells and in some hepatocytes, mostly periportal (12). Immuno-histochemical staining confirmed that AE2 is present at the canalicular

Table 2. The related ratio of AE2 expression (AE2/β2-MG) in HCC (T) and adjacent non-tumor tissue (NT) determined by RT-PCR.

Patient no.	Sex	Age (years)	HBs-Ag	Anti-HCV	Tumor size (cm)	TNM staging	AE2 (NT)	AE2 (T)
1	M	43	+[a]	–	4.0	I	3.8	3.2
2	M	67	–	+	4.0	I	3.6	27.8
3	M	52	+	–	7.0	I	UD[b]	UD
4	F	63	+	–	5.0	II	4.6	90.0
5	M	61	–	+	5.0	II	UD	8.0
6	M	60	+	–	5.0	II	UD	24.3
7	M	53	+	–	2.5	II	UD	UD
8	M	72	–	+	2.5	II	3.1	4.0
9	M	76	+	–	2.8	II	10.0	14.0
10	M	54	–	+	5.0	II	UD	UD
11	M	41	+	–	3.0	II	51.0	73.0
12	M	74	–	+	14.0	II	8.0	71.9
13	M	52	+	–	9.5	II	2.0	40.0
14	M	68	–	+	2.2	II	30.0	72.9
15	M	49	+	–	4.0	II	26.8	24.9
16	M	75	+	–	3.0	II	23.2	47.1
17	M	72	–	+	5.0	II	8.0	30.0
18	M	62	+	–	13.0	III	3.9	2.3
19	M	66	+	–	3.0	III	8.3	18.0
20	F	42	+	–	5.0	III	2.7	77.0
21	F	60	–	+	2.5	III	UD	24.0
22	M	77	–	+	9.0	III	UD	24.0
23	M	50	+	–	12.0	III	1.8	40.0
24	M	38	–	+	2.5	III	14.0	24.0
25	M	42	+	–	7.5	IV	UD	14.0
26	M	59	+	–	20.0	IV	14.0	30.2
27	M	48	–	+	13.0	IV	27.0	29.0
28	M	58	–	+	12.0	IV	10.0	51.0

[a] HBs-Ag and anti-HCV were measured by radioimmunoassay. +, Positive response; –, Negative response.
[b] UD, undetectable.

membrane of hepatocytes, and in the luminal side of bile duct epithelial cell membranes from the small and medium-sized bile ducts (21). This study is the first to demonstrate that AE2 is also expressed in human HCC, and found that sixty-eight percent of patients with HCC showed over-expression of AE2.

Several studies report that proliferation and transformation are associated with the cellular pH regulators. The Na^+/H^+ exchanger protein family, NHE-1, is a ubiquitously expressed transporter that appears to be a universal response of quiescent cells to growth promoting factors (30). Na^+/H^+ exchanger-dependent intracellular alkalinization is found in the activated ras p21- or HPV16 E7-transformated mouse fibroblasts (NIH3T3) (15, 24). The Na^+/H^+ exchanger-deficient cell lines either do not induce tumor formation or show severely retarded tumor growth when implanted in immuno-deprived mice (25). In the HepaG2 cell line, Na^+/H^+ exchanger activation plays a critical role in cell growth (27). These findings demonstrate a tumor formation association with the intracellular pH homeostasis in various cell lines. The Cl^-/HCO_3^- exchanger AE2 is also analogous to be an intracellular pH regulator, it is not clear yet whether the AE2 is associated with the tumor formation. In this study, we found that AE2 was strongly expressed in the higher malignant HCC cell lines (HA22T / VGH) and was weekly in the low malignant HCC cell lines (PLC/PRF5) and the hepatoblastoma (HepG2) (Fig. 2). Moreover, down-regulation of AE2 protein by antisense oligonucleotide treatment induced cell apoptosis in HA22T /VGH cells, but not in other cell lines (preparation for publication). These findings suggested that AE2 might have played an important role in the survival of human poorly-differentiated

Table 3. Association between the expression of AE2 and the clinical characteristics of patients with hepatocellular carcinoma.

Clinical characteristic	The value of AE2[a]		$P^{\dagger}$
	High	Low/UD	
Age			
≤ 60 years	5	9	0.5000
> 60 years	4	10	
HBs-Ag			
Positive	6	10	0.3884
Negative	3	9	
Anti-HCV			
Positive	3	9	0.3884
Negative	6	10	
Tumor size			
≤ 3 cm	6	6	0.6255
> 3 cm	3	13	
Histopathological grading			
well and moderately	9	12	0.4121
poorly	4	3	
TNM-staging			
T1 and T2	6	11	0.4928
T3 and T4	3	8	

[a] The value, which was higher than the average of the AE2 expression in HCC, was designed as the high expression, while the remaining ones were the low expression. UD, undetectable.

[†] Statistical analyses were performed by the Fisher exact test. $P < 0.05$ was considered significant.

HCC cells and may also provide a new approach in the development of human HCC, although the biological function of AE2 in HCC remains unclear.

Acknowledgments

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