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The differential expression of cytosolic carbonic anhydrase in human hepatocellular carcinoma

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Abstract

Cytosolic carbonic anhydrases (CAs), including CAI, CAII and CAIII are present in normal hepatocytes. This study was aimed to investigate the expression status of CAs in hepatocellular carcinomas (HCC) and cholangiocellular carcinoma (CCC) and the role of tumor progression. The activity, protein expression pattern and messenger RNA of cytosolic CA were analyzed by CA activity analysis, immunoblot and RT-PCR in 60 human hepatocellular carcinomas and 10 human cholangiocellular carcinoma surgical specimens. The in situ distribution of CAI, CAII and CAIII in hepatocellular carcinomas tissues were analyzed by immunohistochemistry. The result showed that in each of 60 human hepatocellular carcinomas and 10 cholangiocellular carcinoma, CA activity and protein expression in tumor area was significantly lower than that of paired adjacent normal tissues ($P < 0.01$), and mRNA expressions in tumor areas were also reduced ($P < 0.001$). Furthermore, the immunohistochemical studies have further confirmed this reduction of CAI, CAII and CAIII protein expression in tumor areas. There was a statistically significant reduction in the expression of cytosolic CAII in poorly differentiated cancer ($P < 0.001$). Furthermore, the reduction of CAI, CAII and CAIII in HCC tumor areas was also revealed in this study and this reduction might promote tumor cell motility and contribute to tumor growth and metastasis.
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Keywords: Cytosolic carbonic anhydrase; Human hepatocellular carcinomas; Cholangiocellular carcinoma

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Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are wide-spread zinc enzymes, present in mammals in at least 14 different isoforms. Some of these isozymes are cytosolic (including CA I, CA II, CA III, and CA VII), and others are membrane-bound (including CA IV, CA IX, CA XII and CA XIV). Among these isozymes, CA V is mitochondrial and CA VI is secreted in the saliva. Three acatalytic forms are also known (CARP VIII, CARP X and CARP XI). In the aspect of CA function, respiration (CO_2 transport and excretion) and ion transport are the two most extensively investigated areas and have been studied primarily on the systemic level. Several CA isozymes are responsible for important physiological and pathological functions. Three recently discovered isoenzymes (CA IX, XII, and XIV) are transmembrane proteins whose active sites are located on the cell exterior (Fujikawa-Adachi et al., 1999; Mori et al., 1999; Opavsky et al., 1996). Two of them, i.e. CA IX and XII, are highly expressed in some in some renal cell cancers and may be functionally related to oncogenesis (Tureci et al., 1998; Ivanov et al., 1998; Zavada et al., 1993; Pastorek et al., 1994). Although the exact role of CA activity in carcinogenesis has not been established, it has been recently hypothesized that tumor-associated-transmembrane isoenzymes CA IX and XII may be implicated in acidification of extracellular milieu surrounding the cancer cells and thus create a microenvironment conducive to tumor growth and spread (Ivanov et al., 1998). Supuran et al. proposed that sulfonamide CA inhibitors have exhibited a high potency as tumor cell growth inhibitors in a variety of very tumor cell lines (Supuran et al., 2001, 2003). Previous immunohistochemical studies have also indicated that CA II was highly expressed in several types of tumors, including malignant brain tumors (Parkkila et al., 1995a,b), gastric and pancreatic carcinomas (Parkkila et al., 1995a,b; Pastorekova et al., 1997). On the contrary, the cytosolic CAs activity and protein expression were significantly decreased in lung cancer (Chiang et al., 2002) and colon cancer (Bekku et al., 2000).

Primary hepatocellular carcinomas (HCCs) is an epithelial cancer that originates from hepatocytes and the most common primary malignant tumor of the liver, which is frequently (in up to 80% of cases) associated with cirrhosis (Colombo et al., 1989). Furthermore, the incidence of HCC closely parallels with that of chronic liver disease. Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most frequent causes of cirrhosis worldwide. HCC also occurs in chronic liver disease of nonviral etiology, such as aflatoxin exposure and alcohol rated disease (Hashem, 2002; Yu et al., 2002).

Carbon dioxide and ammonia are derived from the catabolisms of carbohydrate and nitrogen, respectively. They both are primarily waste products eliminating from the animal by various routes of excretion. CA can potentially play a role in metabolism in the specific steps involving carbon fixation. Most of the catabolic reactions that produce carbon dioxide liberate CO_2 gas, and conversely, many of the carbon fixing reaction utilize HCO_3^-. Intracellular CA, by maintaining a virtually instantaneous chemical equilibrium between CO_2 and HCO_3^-, could allow for some of the metabolically produced CO_2 to be transported into synthetic pathways. At least three forms of CA have been found in the livers of a variety of species. Both the high-activity isoenzyme (CAII) and the low-activity isoenzyme (CAIII) have been detected in hepatocytes (Jeffery et al., 1986) and the third isoenzyme (CAV) has been associated with the mitochondria of liver. CA is traditionally considered to be a transport enzyme, it is also an integral part of many biosynthetic pathways. Their critical contribution in systemic acid-base regulation is based upon the fact of ureagenesis and gluconeogenesis as described above (Dodgson et al., 1984).

This study was designed to elucidate the possible relationship between the expression of cytosolic CAs and HCC. Collectively, these observations suggest that cytosolic CAs may be critically involved in the acidity condition formation and tumor progression.

Patients and methods

The study group consisted of 60 HCC patients, including 46 males and 14 females, aged between 25 to 73 year-old and the mean age is 51.8 year-old. Furthermore, 21 study subjects were positive with HBsAg. HCC was diagnosed histologically in surgically resected tissues. A total of 60 resected tumors were obtained from the Changhua Christian Hospital, Changhua and the Armed-Force Taichung General Hospital, Taichung, Taiwan. Classification on histological type, pathological stage, and tumor-node-metastasis (TNM) of these tumor samples were determined according to the criteria of the American Joint Committee on Cancer (Fleming et al., 1997). There are 3 cases of grade I HCC, 33 cases of grade II, 16 cases of grade III and 9 cases of grade IV. Surgical specimens of human HCC and the corresponding paired adjacent normal liver tissue were obtained right after the surgery, fresh tissue was snap-frozen in liquid nitrogen and stored at -70 °C until processing.

Measurement of carbonic anhydrase activity

This method has been carried out for the direct staining of site containing CA when its presumptive native substrate, CO_2 and water were used (Edwards and Patton, 1966). Purified human erythrocyte CA isoenzymes (CAI, C4396 and CAII, C6165) were purchased from (St. Louis, MO) and electrophoresis as a control. The CA activity of lung tissues homogenate was directly determined by activity stain on a 15% SDS-PAGE gel modified as followed (Hou and Lin, 1998). After electrophoresis, the gel was immersed and shaken twice in 2.5% Trion X-100 for 30 min, followed by a immersion in 25% isopropanol at 4 °C overnight, and finally washed in 20 mM Tris buffer (pH 8.1) for 10 min. Six to eight drops of bromthymol blue (0.2% in 50% ethanol) were added to gel. After standing for about 30 min at 4 °C, the gel was then placed in CO_2 saturated water at 4 °C. After 15–30 sec, a distinct yellow band appeared at the position of the CA in the gel. Relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation).

Table 1
Summary of cytosolic CA expression in HCC by CA activity, immunoblotting analysis and RT-PCR

	Normal tissues (TN) (Mean ± S.E.[a])	Tumor tissues (TC) (Mean ± S.E.[a])
CA activity stain	396.71 ± 62.45	56.14 ± 7.62***
Immunoblot analysis		
CAI	2158.35 ± 768.22	386.58 ± 38.42***
CAII	1852.2 ± 131.97	250.51 ± 13.45***
CAIII	666.80 ± 73.59	126.43 ± 21.87***
RT-PCR		
CAI	356.81 ± 32.18	38.79 ± 10.48***
CAII	297.37 ± 20.46	64.85 ± 11.75***
CAIII	316.28 ± 28.89	72.44 ± 18.68***

Student's t test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
[a] Relative density × 10^2.

Immunoblot analysis

Procedures for immunoblotting and antibody production have been described previously (Chiang et al., 2001). Briefly, protein samples were separated in a 12.5% polyacrylamide gel, transferred onto a nitrocellulose membrane and then incubated separately with CAI, CAII and CAIII specific antibodies (Chiang et al., 2001). These utilized isoform-specific antibodies against CAIII were generously provided by Dr. Chong-Kuei Lii in Department of Nutrition, Chung-Shan Medical University, Taichung, Taiwan. After incubation with horseradish peroxidase goat anti-rabbit IgG, the color signal was developed by 4-

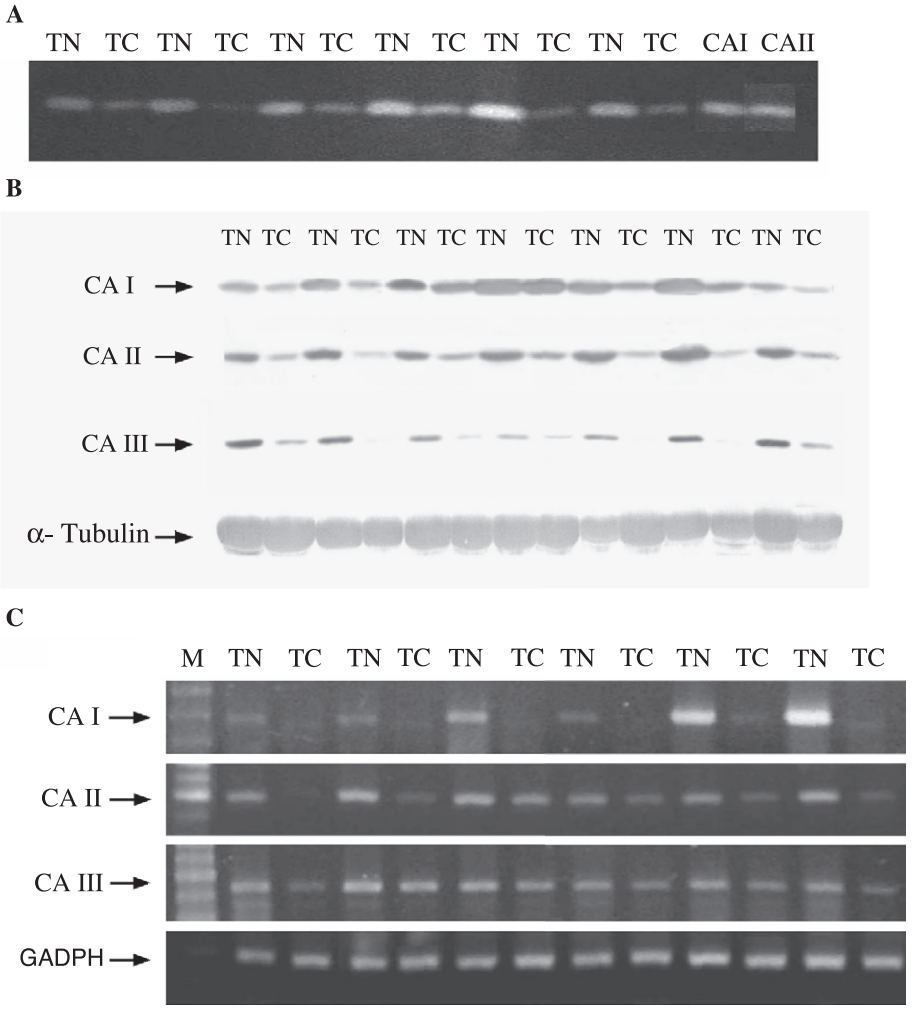

Fig. 1. Analysis of the carbonic anhydrase in tumor tissue and adjacent normal tissue of HCC. (A). Activity stain on polyacrylamide gel electrophoresis. (B). Immunoblotting analysis of CAI, CAII and CAIII, α-tubulin is used as a loading control. (C). Analysis of CAI, CAII and CAIII expression in tumor tissue and adjacent normal tissue of HCC by RT-PCR. (TC, tumor tissue; TN, adjacent normal tissue).

chloro-1-napthol/ 3,3′-diaminobenzidine, 0.9% (w/v) NaCl in Tris-HCl. Relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation).

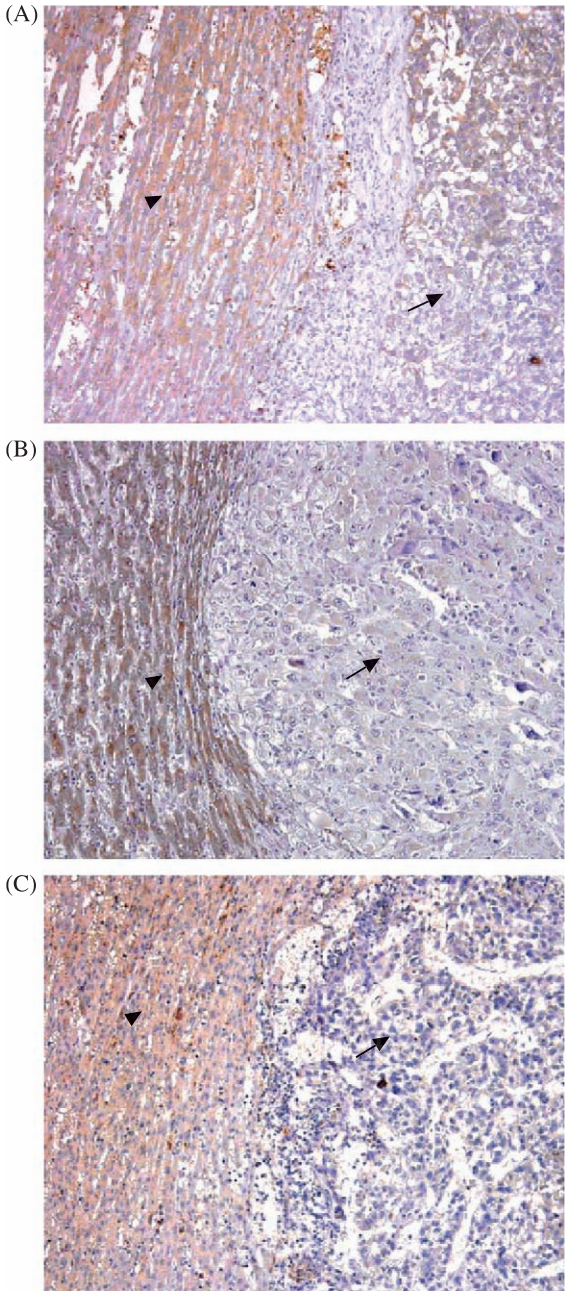

Fig. 2. Analyses of CAI, CAII and CAIII by immunohistochemical stain in HCC. (Arrow, normal tissue; Arrowhead, the tumor tissue. Original magnifications, × 100).

Immunohistochemical analyses

CAI, CAII and CAIII isoenzyme was localized in 5 ìm tissue sections using the avidin-biotin-peroxidase complex technique (Hsu et al., 1981a,b). Deparaffinized and rehydrated sections were treated with 1% hydrogen peroxide for 5 minutes at 25 °C to inactivate endogenous peroxidase activity. The primary antiserum as a polyclonal CAI, CAII and CAIII antibody was diluted with 0.1 mol/L phosphate-buffered saline at 1:600 for 1 hour at room temperature. After rinsing twice with Tris buffered saline for a total of 20 min, the bound primary antibody was detected by sequential incubations with a biotinylated secondary antibody (LSAB kit from DAKO or ABC kit from Invitrogen) for 1 hour and DAB for 5–10 min, at room temperature with two rinses of TBS in between. After hematoxylin counterstain, sections were dehydrated through graded alcohols and coverglasses were applied using a histologic mounting medium.

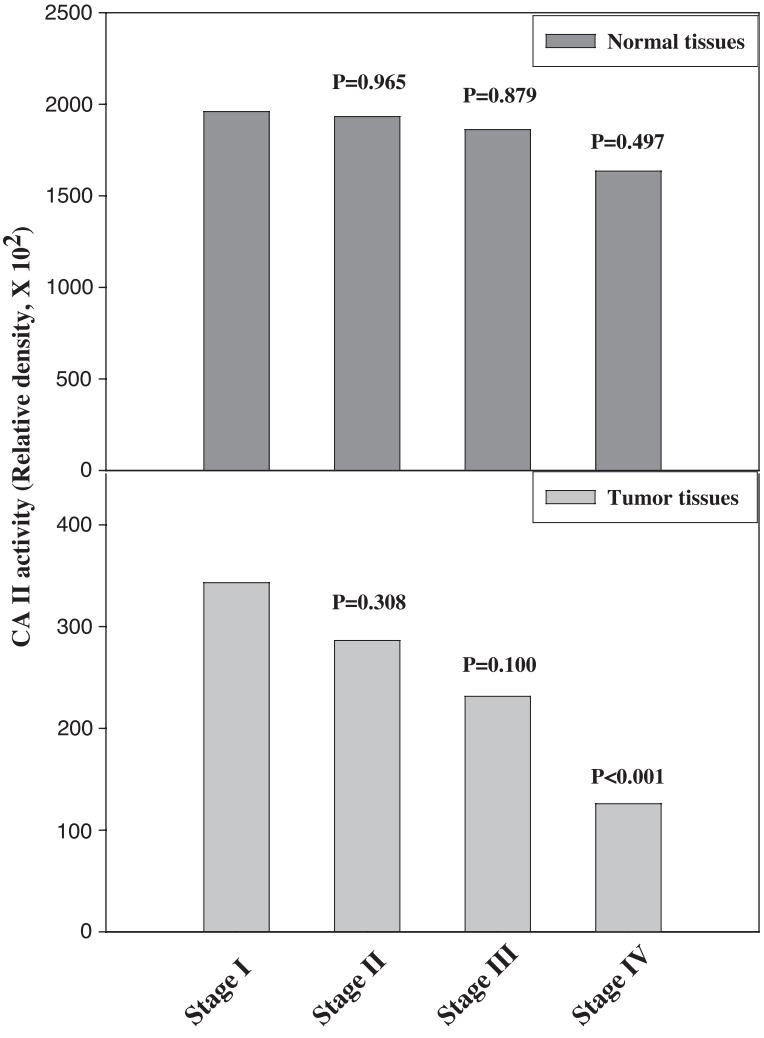

Fig. 3. Relative CA II activity various stages of HCC.

Reverse transcripition-polymerase chain reaction (RT-PCR)

Total RNA was isolated from fresh tissues using a guanidinium chloride procedure (Durnam et al., 1980). For RT-PCR, 15 *u*g of RNA were used as templates for Mooney murine leukemia virus RT (300 units) in a 20 *u*l reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 20 units Rnasin (Promega), that amplify with two oligonucleotide primers based on the published cDNA sequence of cytosolic CA (Okuyama et al., 1992; Bennett et al., 1998). RT-PCR products were electrophoresis in 1.8% agarose native gel and detected by ethidium bromide staining.

Statistical analysis

Values were expressed as means ± SE. The statistic analysis was performed by Mann-Whitney Rank sum test between groups. SigmaStat software (Jandel Scientific Software, USA) was used for all statistical analyses. A P value of less than 0.05 was considered statistically significant.

Results

Tumors from 60 HCC patients were subjected to CA activity assay; immunoblot analysis and RT-PCR (Table 1). In this study, 46 men and 14 women were enrolled, meaning a significantly higher incidence in male than in female.

First of all, to examine alterations in activity and quantity of cytosolic CA isoenzymes in HCC, CA activity was determined by activity stain on a SDS-PAGE (Fig. 1A) and the expressed CAI, CAII and CAIII were determined by immunoblot analysis (Fig. 1B). The results showed that the mean total CA activity of HCC is significantly lower than that of paired adjacent normal tissues ($P < 0.01$), indicating the weaker expression of CAI, CAII and CAIII in tumor tissues. In the statistical analysis, the expression of CAII and CAIII was significantly decreased in HCC ($P < 0.001$).

Table 2
Clinical profiles of patients with HCC and CCC: comparison with CA II protein expression

Variables	N	Normal tissues *(Mean ± S.E.[a])*	Tumor tissues *(Mean ± S.E.[a])*
HCC[b]	60(M/F)	1852.2 ± 131.97	250.51 ± 13.45
Age (years)			
< 40	6(4/2)	1629.53 ± 339.26	207.75 ± 43.04
40–60	21(14/7)	1854.01 ± 234.89	268.61 ± 21.91
> 60	33(28/5)	1895.5 ± 180.38	246.75 ± 18.6
Gender			
Male	46	1871.55 ± 155.87	262.58 ± 15.15
Female	14	1771.15 ± 242.95	259.30 ± 32.73
CCC[b]	10	2454.21 ± 308.08	375.07 ± 75.39

[a] Relative density × 10^2.
[b] HCC, hepaocellular carcinoma; CCC, cholangiocellular carcinoma.

Table 3
Relationship between HCC subjects and histopathological features of tumor: comparison with CA II protein expression

Causes of HCC	N	Normal tissues *(Mean ± S.E.[a])*	Tumor tissues *(Mean ± S.E.[a])*
HBV	18	2019.81 ± 256.83	252.17 ± 24.19
HCV	26	1911.1 ± 204.8	259.11 ± 21.21
HBV/HCV	3	1727.14 ± 633.52	325.68 ± 80.34
Others	13	1848.97 ± 202.71	263.98 ± 28.03
TNM stage			
I	3	1959.37 ± 762.11	343.24 ± 64.71***
II	32	1932.17 ± 173.99	286.38 ± 15.81***
III	16	1861.09 ± 242.96	231.44 ± 25.21***
IV	9	1635.37 ± 133.23	125.9 ± 6.14***
Tumor Size (mm)			
≦ 50	38	1862.64 ± 175.24	246.31 ± 16.88
> 50	22	1743.25 ± 169.36	209.57 ± 15.14

Student's t test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
[a] Relative density × 10^2.

To further investigate the mRNA level of cytosolic CA isoenzymes in HCC, RT-PCR amplification of mRNAs from resected tumors and the corresponding paired adjacent normal liver tissue was performed. The results demonstrated that the mRNA expressions of cytosolic CA in HCC tissues were reduced to CAI 10.9%, CAII 21.8% and CAIII 22.9% of paired adjacent normal tissues ($P < 0.001$) (Fig. 1C).

Finally, to understand the distribution of the significantly reduced expression of CAI, CAII and CAIII in situ, we have performed the immuniohistochemical staining on tissue sections from HCC. As the results shown, strong signal of CAII and CAIII were detected in the paired adjacent normal region of HCC, but weak in the tumor area (Fig. 2). The strength of CAI signal in normal tissue, although not as strong as that of CAII and CAIII, was still distinguishable from that of CAI in tumor area. This weaker signal of CAI could be due to the lower expression of CAI in hepatocytes compared to that of CAII and CAIII. There was a significant tendency of reduction in the expression of cytosolic CAII in poorly differentiated cancer, based on the significant decrease in the comparison among cancer tissues with different stages (Fig. 3, $P < 0.001$). In the other hand, the mean age, gender and HBV infection status of HCC patients with various grades were statistically different and therefore these parameters should have no impact on the expression of CAII (Tables 2 and 3). A larger scale of study shall be conducted to provide more definite conclusion on this issue.

Discussion

Hepatoma is the number one cause of cancer death with 24.05/100,000 populations in Taiwan with an annually increasing rate of 11.23%. About 5000 people die from hepatoma each year in Taiwan (Beasley et al., 1981). Taiwan is a hyperendemic area for hepatitis B virus (HBV) infection with HBsAg being

detected in 75–80% of chronic hepatitis, 34% of cirrhosis and 72% of HCC (Merican et al., 2000). However, the mean age, gender and HBV infection status of HCC patients were not significantly related to cytosolic CA expression in this study.

Carbonic anhydrases are evolutionarily ancient enzymes, which are expressed in most normal mammalian tissue. The cytosolic high-activity isoenzyme, CAII, appears to be the most widely distributed isoenzyme of the CA gene family. A deficiency of carbonic anhydrase II is the primary defect in a human recessive inherited syndrome known as osteopetrosis, involving renal tubular acidosis and cerebral calcification (Sly et al., 1983; Sly and Hu, 1995a,b) together with growth failure, dental malocclusions and mental retardation (Strisciuglio et al. 1990). Recently, many studies support that the expression of CAIX and CAXII is elevated in some malignant tumor tissues including breast cancer (Chia et al., 2001), colon cancer (Kivela et al., 2000; Saarnio et al., 1998), lung cancer (Vermylen et al., 1999), and other malignancies (McKiernan et al., 1997). These investigations have also shown that the hypothetical pH sensing functions of CA isoenzymes may be associated with histidine residues in their catalytic center outside the cell as well as with internal histidines in the cytoplasmic domains (Ivanov et al., 2001). Over the past decades, serious efforts have been made to find the role of CA in tumor progression, either as a biomaker or a tumor-associated protein. The expression of CAI and CAII has also been frequently investigated in a variety of tumor cells and cell lines (Chegwidden et al., 2000), but a clear-cut relationship between the expression of CA in normal and malignant cells has still not been established. Role of CAI and CAII is ambiguous although several studies have been conducted on pancreatic cancer (Pastorekova et al., 1997), lung cancer (Chiang et al., 2002) and colon cancer (Bekku et al., 2000).

Several CA isoenzymes have been reported in the hepatobiliary tract. The biliary epithelial cells express cytoplasmic CAII, an apical membrane-associated CAIV and a basolateral transmembrane MN/CA IX, but are absent from hepatocytes (Parkkila et al., 1994, 1996). In hepatocytes, the most abundant isoenzyme is the cytosolic CAII and mitochondrial CAV (Shah et al., 2000). The significance of hepatic urea synthesis resides not only in the removal of potentially toxic ammonium ions, but also in the removal of bicarbonate. The impairment of hepatic urea synthesis may lead to hyperammonemia, and to a decreased HCO_3^- removal by the liver, consistent with the proposed role of the liver in homeostasis and systemic pH regulation. Cytosolic carbonic anhydrases plays a role in systemic acid-base regulation based upon the fact of ureagenesis and gluconeogenesis (Haussinger and Gerok, 1985).

Microelectrode-measured pH values have indicated that the extracellular pH in solid tumors is more acidic than in adjacent normal tissue (Webb et al., 1999). In contrast, the intracellular pH estimated by ^{31}P-magnetic resonance spectroscopy is identical or slightly more basic in tumor compared with normal tissue (Gerweck, 1998). To establish the pH gradient between the extracellular and intracellular compartments, tumor cells express ion transport proteins, including vacuolar H^+-ATPase, Cl^-/HCO_3^- exchanger, and Na^+/H^+ exchanger (Montcourrier et al., 1997; Lee and Tannock, 1998). Many tumors also express carbonic anhydrases (CAs), which catalyze the production of H^+ and HCO_3^- ions in the reversible reaction $H_2O + CO_2 \leftrightarrows H^+ + HCO_3^-$ (Parkkila et al., 1995a,b; Kivela et al., 2000). Recently, it was hypothesized that tumor-associated transmembrane isoenzymes CA IX and XII may be implicated in acidification of extracellular milieu surrounding the cancer cells and thus create a microenvironment conducive to tumor growth and spread (Ivanov et al., 1998). CAIV, CAIX, or CAXII may produce bicarbonate ions outside the cell that are transported inside by bicarbonate/chloride exchangers and used by the cytosolic CAII to titrate protons. On the other hand,

CAII binds to the carboxyl terminus of human bans 3 protein, which catalyses the electroneutral exchange of bicarbonate for chloride (Vince and Reithmeier, 1998). Buffering protons facilitate proton secretion and protect the cell from intracellular acidification. Therefore, on the basis of above considerations, we have observed in this study that there was a significant reduction in the expression of cytosolic CAII in poorly differentiated cancer based on the significant decrease in stage IV of HCC ($P < 0.001$). Furthermore, a similar tendency of reduction, although not statistically significant, was also observed in the CAII expression in the adjacent normal tissue. The difference in expression of CAs did not reach statistical significance which might be due to insufficient number of cases being analyzed. Kivela et al. has proposed a hypothesis to indicate that loss of expression of the closely linked CAI and II genes could be resulted from loss of alleles specifying these genes. However, although they play a role in regulation of pH homeostasis and water and ion transport, loss of expression of these cytoplasmic isozymes consistently accompanies progression to malignant transformation (Kivela et al., 2001). We used a transient expression system to transfect a hepatoblastoma cell line (Hep 3B) with cDNA of CAII that was amplified by RT-PCR and cloned into pGFP vector. The preliminary result showed that the viable number of CAII-transfected cancer cells gradually decreased as the culture time increased with morphology different from that of control cells. Furthermore, these cells were unable to continue to grow. The results of TUNEL assay further indicated that these cells might be apoptotic cells (data not shown). Concluded from the above observations, it was suggested that overexpression of CAII might be toxic to cancer cells. However, further study will be needed to clarify the associations of CAI, CAII and CAIII expressions with the occurrence of metastasis and with survival.

Recently, Supuran's group has showed that by modulating the CA activity the pH of the tumor environment can be changed, which may favorably influence the anticancer effect of the drug per se (i.e., the sulfonamide CA inhibitor) or that of another anticancer agent used concomitantly with the CA inhibitor/activator. Therefore, the potential of CA inhibitor/activator in treating cancers and inhibiting tumor progression would be especially significant and warrants more relevant studies (Supuran and Scozzafava, 2001).

Additionally, CAIII levels of HCC patients are lower than those of paired adjacent normal tissues. CAIII has two sulfhydryl groups; on this basis it has been suggested that it may have a role in scavenging oxygen radical in cells and oxidative stress has been suggested to play a role as a common mediator of apoptosis or programmed cell death. These findings suggested that CAIII might be a novel genes involved in human HCC cancer biology (Calvo et al., 2002).

Conclusion

The reduction of CAI, CAII and CAIII in HCC tumor areas was firstly revealed in this study and such reduction might promote tumor cell motility and contribute to tumor growth and metastasis.

Acknowledgements

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