CA II, a potential biomarker by proteomic analysis, exerts significant inhibitory effect on the growth of colorectal cancer cells

RUI ZHOU[1,3*], WENJUN HUANG[3*], YUQIN YAO[1], YUXI WANG[1], ZIQIANG LI[1], BIN SHAO[1], JIAN ZHONG[1], MINGHAI TANG[1], SHUFANG LIANG[1], XIA ZHAO[2], AIPING TONG[1] and JINLIANG YANG[1]

[1]State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, [2]Department of Gynecology and Obstetrics, West China Second Hospital, Sichuan University, Chengdu 610041; [3]Department of Electrophysiology, Institute of Cardiovasology, Luzhou Medical College, Luzhou, Sichuan 646000, P.R. China

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Abstract. In the Western world, colorectal cancer (CRC) is the third most common cancer with poor prognosis. To identify the proteins and to elucidate the possible mechanisms involved in colorectal carcinogenesis, 2-DE coupled with MS/MS analysis were employed to compare the global protein profile between CRC and individual matched normal tissues from 8 CRC patients. Of 36 proteins identified, carbonic anhydrase II (CA II) was one of most significantly altered and its downregulation in CRC tissues was verified by RT-PCR, western blotting and immunohistochemistry methods, suggesting that CA II may serve as a potential biomarker for CRC diagnosis. To investigate the function and mechanisms of CA II in CRC, a stable SW480 colorectal cancer cell line overexpressing CA II was established. It was shown that overexpression of CA II remarkably suppressed tumor cell growth both *in vitro* and *in vivo*, which was in part interpreted by cell cycle arrest at G0/G1 and G2 phase. Further mechanism analysis revealed that the sensitivity of colorectal cancer cells to chemotherapy drugs could be increased by CA II overexpression. Taken together, these data suggest that CA II may be a potential biomarker for early diagnosis of CRC and the results may contribute to a better understanding of the molecular mechanism of CRC and colorectal cancer treatment.

Correspondence to: Professor Jinliang Yang or Dr Aiping Tong, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, 1 Keyuan Rd 4, Gaopeng St, Chengdu 610041, P.R. China
E-mail: jlyang01@163.com
E-mail: aipingtong@gemail.com

*Contributed equally

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Introduction

Colorectal cancer (CRC) is a major international health problem that is the third most frequent type of cancer and the second most common cause of cancer related death in the Western world (1). It has been reported that when CRC is diagnosed at early stage, nearly 90% of the patients can be cured by surgery. However, this disease is very often diagnosed at an advanced stage, resulting in poor prognosis subsequently (2-4). In addition, the mechanisms of CRC development and progression are not quite clear and have yet to be further explored. Therefore, more insight and new methods to investigate the underlying mechanisms of CRC are needed to identify effective biomarkers and this is critical for proper control of CRC.

In recent years, proteomics have burst onto the scientific scene rapidly (5). Based on 2-DE and mass spectrometry, hundreds of proteins can be identified simultaneously and precisely through high-throughput identification. Therefore, proteomics have been widely applied to search for diagnostic biomarkers in early disease detection, as well as mechanism analysis of disease, especially in the field of cancer research (6-8). In the present study, differentially expressed proteins between individually matched CRC and normal tissues were profiled from 8 CRC patients. Of 36 proteins identified, carbonic anhydrase II (CA II) was chosen for verification and function and mechanism analysis. It was expected that the results from the study may contribute to a better understanding of the molecular mechanism of CRC and provide insight into colorectal cancer treatment.

Materials and methods

Patients and tissue preparation. For proteomic analysis, 8 cases of CRC and pared adjacent normal tissues were obtained from West China Hospital, Sichuan University. The clinical characteristics of the patients are summarized in Table I. Fresh tissues samples were obtained immediately after the surgery, snap-frozen immediately in liquid nitrogen and then stored at -80˚C before analysis. For the validation studies, 25 cases of

Table I. Clinical features of all human tissue samples.

Sample	Age	Gender	Location[a]	UICC staging
1	84	Male	A	I
2	53	Male	R	III
3	66	Male	A	II
4	72	Male	D	III
5	59	Female	T	III
6	79	Male	S	II
7	60	Female	R	III
8	57	Male	R	II

[a]A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum.

paraffin-embedded primary CRC tissues and pared adjacent normal tissues were collected consecutively from patients at West China Hospital in 2009. Written informed consent was obtained from all patients and the study procedures were approved by the Scientific and Ethics Committee of Sichuan University (Chengdu, China).

Proteomic analysis and protein identification. 2-DE was carried out as previously described (9) with minor modifications. Briefly, tissue sample was ground into powder in liquid nitrogen and sonicated in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% ampholyte pH 3.0-10.0; Bio-Rad, Hercules, CA, USA) containing protease inhibitor cocktail. IPG strips loaded with 1 mg protein (17 cm, pH 3.0-10.0, non-linear; Bio-Rad) were passively rehydrated for 12-16 h. Having been separated according to their pI for the first dimension, the strips were transferred to the second dimension 12% SDS-PAGE for the separation according to the molecular weight. Spots that showed consistent and significant differences (>2-fold) were selected for mass spectrometry (MS) analysis.

In-gel digestion of protein was conducted using MS-grade Trypsin Gold (Promega, Madison, WI, USA) by following the manufacturer's instructions. ESI-Q-TOF MS/MS analysis and protein identification were performed as described in our previous proteomic studies (9). Briefly, peptide mass maps were acquired using a Q-TOF mass spectrometer (Micromass, Manchester, UK) fitted with an ESI source. For MASCOT analysis, peptide and fragment mass tolerance were set at 0.1 and 0.05 Da, respectively.

Semiquantitative RT-PCR. Total RNA extraction was performed using TRIzol reagent (Invitrogen). cDNA was then synthesized using the ExScript™ reagent kit (Takara, Shiga, Japan) following the manufacturer's instructions. The primer sequences and the expected sizes for PCR products were as follows: CA II, 5'-GTCCCATAGTCTGTATCCAA-3' (sense) and 5'-GAGTGCTCATCACCCTACAT-3' (antisense) (301 bp); GAPDH, 5'-TGGAAGGACTCATGACCACA-3' (sense) and 5'-GCTTCCCACCTTCTTGATG-3' (antisense) (280 bp). The amplification parameters consisted of 25 (CA II) or 20 cycles (GAPDH) at 94˚C for 30 sec, 60˚C (CA II) or 57˚C (GAPDH) for 30 sec and 72˚C for 30 sec. The PCR products were analyzed by electrophoresis in 1.2% agarose gels and visualized by Gold View (Takara) staining.

Western blotting. CRC tissues and cells were lysed with cold RIPA lysis buffer containing protease inhibitors. Thirty micrograms of protein extraction were applied to 12% SDS-PAGE gels and then transferred to polyvinylidene fluoride membrane. The membrane was probed with primary antibodies against CA II (1:1,000, GeneTex), E-cadherin (1:1,000, Cell Signaling Technology, MA, USA), vimentin (1:1,000, Cell Signaling Technology), PKM2 (1:1,000, Cell Signaling Technology) and GAPDH (1:1,000, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), respectively. Blots were developed with HRP-conjugated secondary antibodies (1:5,000, Santa Cruz) and chemiluminescent substrate (Millipore, MA, USA) on Kodak X-ray film.

Immunohistochemistry and immunocytochemistry. Tissue slides or SW480 cells fixed in polystyrene culture were stained with the rabbit anti-human CA II antibody (diluted 1:200, GeneTex) using the DAB substrate solution according to the manufacturer's instructions.

Cell culture and establishment of a stable cell line. Four human colorectal cancer cell lines, SW480, SW620, HCT116 and LoVo cell were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in DMEM medium (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a humidified environment containing 5% CO_2 at 37˚C.

For establishment of the stable cell line SW480, cells were transfected with DNA constructs (OriGene) encoding EGFP-CA II (SW480-CA II-over) or EGFP (SW480-control). Forty-eight hours after transfection, cells are harvested, diluted (1:10) and plated in fresh medium containing G418 (800 μg/ml, Invitrogen, Carlsbad, CA, USA). Colonies with green fluorescent signal were then picked and expanded.

Drug treatments and MTT assay. Tumor cells were seeded in 96-well plates at $5x10^3$ cells per well. After 16 h, cells were incubated with various concentrations of drugs. SW480 cells were treated with different concentration of oxaliplatin (10, 20, 30, 40 and 50 μM respectively, Sigma, St. Louis, MO, USA); and, after pretreatment with 100 μM acetazolamide (Sigma) for 8 h, HCT116 cells were treated with oxaliplatin as in SW480 cells. Forty-eight hours later, the effects of drugs on cells were assessed using MTT methods. Briefly, cells were incubated with 20 μl of MTT reagent (20 mg/ml) for 4 h, followed by addition of 100 μl of solubilization solution into each well. The plates were left in the dark room overnight and optical density (OD) was measured at 590 nm wavelength. Results are expressed as percentage of viable cells compared with untreated cells (with 100% viability). The results are based on three independent experiments. Drug concentrations that inhibit 50% of cell viability (IC_{50}) for oxaliplatin were determined using the method described previously (10).

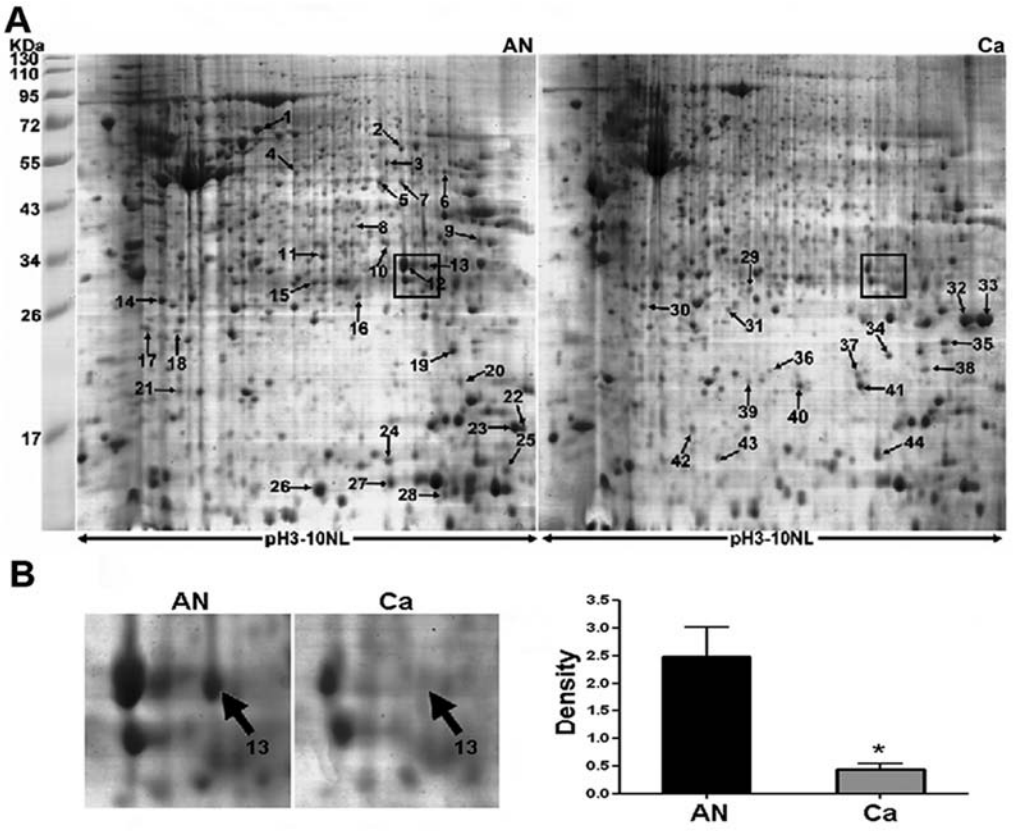

Figure 1. Representative 2-DE gel images of CRC and adjacent normal tissues. (A) Forty-four differentially expressed spots were identified by MS/MS analysis (marked with arrow and number). Information on each numbered spot is reported in Table II. (B) A representative protein spot no. 13 shows significant downregulation in CRC compared with normal tissues. The asterisk represents P<0.05 between two groups. AN, adjacent normal tissue; Ca, cancer tissue.

Colony formation assay. SW480 cells overexpressing CA II and control cells were seeded at 300 cells/well in a 6-well plate with triplicate wells for each group. After 14 days of culture, cells were fixed in methanol for 30 min and stained with Giemsa (Beyotime). The number of clones consisting of >50 cells was counted. The colony forming efficiency was calculated according to the formula: (the clone number/the plated cell number) x 100.

Flow cytometry. The cells were harvested, washed twice with PBS and fixed in 70% ethanol overnight. After incubation with RNAse A and propidium iodide (Beyotime) for 30 min at 4˚C in the dark, cell cycle data were collected on a flow cytometer with a 488-nm laser and analyzed with the manufacturer's software.

Statistical analyses. All quantitative data were expressed as mean ± SD. Comparisons between two groups were performed by Student's t-test. Statistical calculations were performed with SPSS 11.0.0 statistical software. Data were considered as statistically significant at P<0.05.

Results

Identification of differentially expressed proteins between CRC and the corresponding normal tissues. The proteome of individual-matched CRC and normal colorectal tissues from 8 patients (mean age 66.25±10.39 years; range 53-84 years) were compared by 2-dimensional gel electrophoresis (2-DE) using a broad pH gradient (pH 3.0-10.0 non-linear). Coomassie staining of 2-D gels visualized 852±46 and 871±34 protein spots within normal colorectal tissues and CRC, respectively. Representative 2-DE maps are showed in Fig. 1A and spot no. 13 (boxed in Fig. 1A) as a selected example, was significantly downregulated in CRC as shown in enlarged form in Fig. 1B. As a result, 52 spots showed >2.0-fold change (P<0.05). Differentially expressed protein spots were subsequently subjected to MS/MS analysis. Of 52 spots, 44 spots corresponding to 36 unique proteins were identified probably due to post-translational modification such as protein phosphorylation (Table II). Notably, carbonic anhydrase II (CA II) corresponding to spot no. 13 (Fig. 1B) was found to be one of the most significantly differential expression between cancer and normal tissues. It was downregulated >5-fold in CRC compared with the normal tissues. The mass spectra of CA II is shown in Fig. 2. MS/MS analysis of CA II revealed 8 matched-peptides, 50% sequence coverage and a MOWSE score of 226. Due to the confident identification, CA II was chosen as the subsequent focus of this study.

Validation of CA II by semiquantitative RT-PCR and western blot analysis. To confirm the differential expression of CA II between CRC and corresponding normal tissues, validation experiments were performed by RT-PCR and western blot

Table II. Identified proteins by MS/MS analysis.

	Protein description	Gene name	Function	Accession no.	Theoretical Mr/pI[a]	Score[b]	No. of pep[c] (%)	Fold-change[d] (mean ± SD)
1	Protein disulfide-isomerase A3	PDIA3	Protein folding	P30101	57146/5.98	388	12/28	↓2.1±0.7
2	Hydroxymethylglutaryl-CoA synthase, mitochondrial	HMCS2	Energy metabolism	P54868	57113/8.40	228	7/29	↓2.4±0.6
3	Isocitrate dehydrogenase [NADP] cytoplasmic	IDHC	Energy metabolism	O75874	46915/6.53	263	10/41	↓2.2±0.7
4	Leukocyte elastase inhibitor	ILEU	Proteolysis	P30740	42829/5.90	205	12/41	↓3.2±0.9
5	Sialic acid synthase	SIAS	Glucose metabolism	Q9NR45	40738/6.29	196	7/37	↓2.2±0.4
6	Creatine kinase U-type, mitochondrial	KCRU	Metabolism	P12532	47406/8.60	519	9/41	↓2.0±0.6
7	Poly(rC)-binding protein 1	PCBP1	RNA binding	Q15365	37987/ 6.66	251	10/54	↓2.3±0.8
8	Ribose-phosphate pyrophosphokinase 2	PRPS2	Nucleic acid metabolism	P11908	35146/6.15	191	6/30	↓2.2±0.8
9	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	HCDH	Energy metabolism	Q16836	34313/8.88	218	6/39	↓3.1±0.7
10	Sulfotransferase family cytosolic 1B member 1	ST1B1	Protein modification	O43704	35048/6.57	64	1/4	↓4.2±0.9
11	Sulfotransferase 1A1	ST1A1	Protein modification	P50225	34289/6.16	304	11/51	↓3.6±1.1
12	Carbonic anhydrase 1	CAH1	Carbonate dehydratase	P00915	28909/6.59	1130	10/61	↓2.5±0.8
13	Carbonic anhydrase 2	CAH2	Carbonate dehydratase	P00918	29285/6.87	226	8/50	↓5.6±1.5
14	Rho GDP-dissociation inhibitor 1	GDIR1	GTPase activator	P52565	23250/5.02	793	9/57	↓2.4±0.6
15	Protein ETHE1, mitochondrial	ETHE1	Energy metabolism	O95571	28368/6.35	875	11/74	↓1.6±0.4
16	Cytochrome b-c1 complex subunit Rieske, mitochondrial	UCRI	Electron transport	P47985	29934/8.55	272	6/29	↓2.3±0.9
17	Translationally-controlled tumor protein	TCTP	Calcium ion binding	P13693	19697/4.84	129	5/36	↓2.9±0.8
18	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	NDUS8	Energy metabolism	O00217	24203/6.00	287	6/32	↓2.2±0.6
19	Superoxide dismutase [Mn], mitochondrial	SODM	Redox regulation	P04179	24878/8.35	703	9/71	↓2.4±0.8
20	Phosphatidylethanolamine-binding protein 1	PEBP1	ATP binding	P30086	21158/7.01	1276	11/76	↓N/A[e]
21	Plasma cell-induced resident endoplasmic reticulum protein	PERP1	Protein binding	Q8WU39	21023/5.37	397	5/55	↓2.1±0.6
22	Anterior gradient protein 2 homolog	AGR2	Protein binding	O95994	20024/9.03	462	9/42	↓2.3±0.7
23	Anterior gradient protein 2 homolog	AGR2	Protein binding	O95994	20024/9.03	279	9/42	↓3.4±0.6

Table II. Contimued.

	Protein description	Gene name	Function	Accession no.	Theoretical Mr/pI[a]	Score[b]	No. of pep[c] (%)	Fold-change[d] (mean ± SD)
24	Cytochrome *c* oxidase subunit 5B, mitochondrial	COX5B	Electron transport	P10606	13915/ 9.07	47	2/19	↓2.4±0.8
25	Cytochrome b-c1 complex subunit 7	QCR7	Electron transport	P14927	13522/8.73	112	3/34	↓2.1±0.8
26	Fatty acid-binding protein, liver	FABPL	Lipid metabolism	P07148	14256/6.60	367	9/61	↓3.0±0.9
27	Fatty acid-binding protein, liver	FABPL	Lipid metabolism	P07148	14256/ 6.60	67	2/33	↓3.6±1.3
28	D-dopachrome decarboxylase	DOPD	Protein modification	P30046	12818/6.71	145	5/44	↓2.2±0.7
29	Myosin-11	MYH11	Muscle contraction	P35749	228054/5.42	68	2/2	↑N/A
30	ATP synthase subunit d, mitochondrial	ATP5H	Metabolism	O75947	18537/5.21	116	4/43	↑2.6±0.5
31	Triosephosphate isomerase	TPIS	Glucose metabolism	P60174	26938/6.45	160	7/41	↑2.3±0.6
32	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	858	13/59	↑N/A
33	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	1428	15/55	↑N/A
34	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	487	11/64	↑2.2±0.7
35	Transgelin-2	TAGL2	Not determined	P37802	22548/8.41	640	16/67	↑2.3±0.7
36	Actin-related protein 2/3 complex subunit 5-like protein	ARP5L	Structural component	Q9BPX5	16931/6.15	84	1/16	↑2.2±0.7
37	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	184	7/43	↑3.3±0.7
38	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	487	11/64	↑N/A
39	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	326	10/52	↑N/A
40	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	102	10/52	↑3.3±0.9
41	Transgelin	TAGL	Actin binding	Q01995	22653/8.87	326	6/55	↑4.3±1.3
42	Transthyretin	TTHY	Thyroid hormone-binding	P02766	15991/5.52	423	6/55	↑N/A
43	Protein S100-A9	S10A9	Calcium ion binding	P06702	13291/5.71	100	4/49	↑2.3±0.7
44	Eosinophil lysophospholipase	LPPL	Lipid metabolism	Q05315	16584/6.82	42	3/25	↑2.0±0.9

[a]Theoretical molecular weight (kDa) and pI from the ExPASy database. [b]Probability-based MASCOT scores. [c]The number of unique peptides identified by MS/MS sequencing (multiple matches to peptide with the same primary sequence count as one). [d]Average expression level (fold-change) in CRC group compared with ad from all analyses (↑, increase; ↓, decrease). [e]N/A, not applicable because the spots on one of the paired gels were too weak or non-detectable.

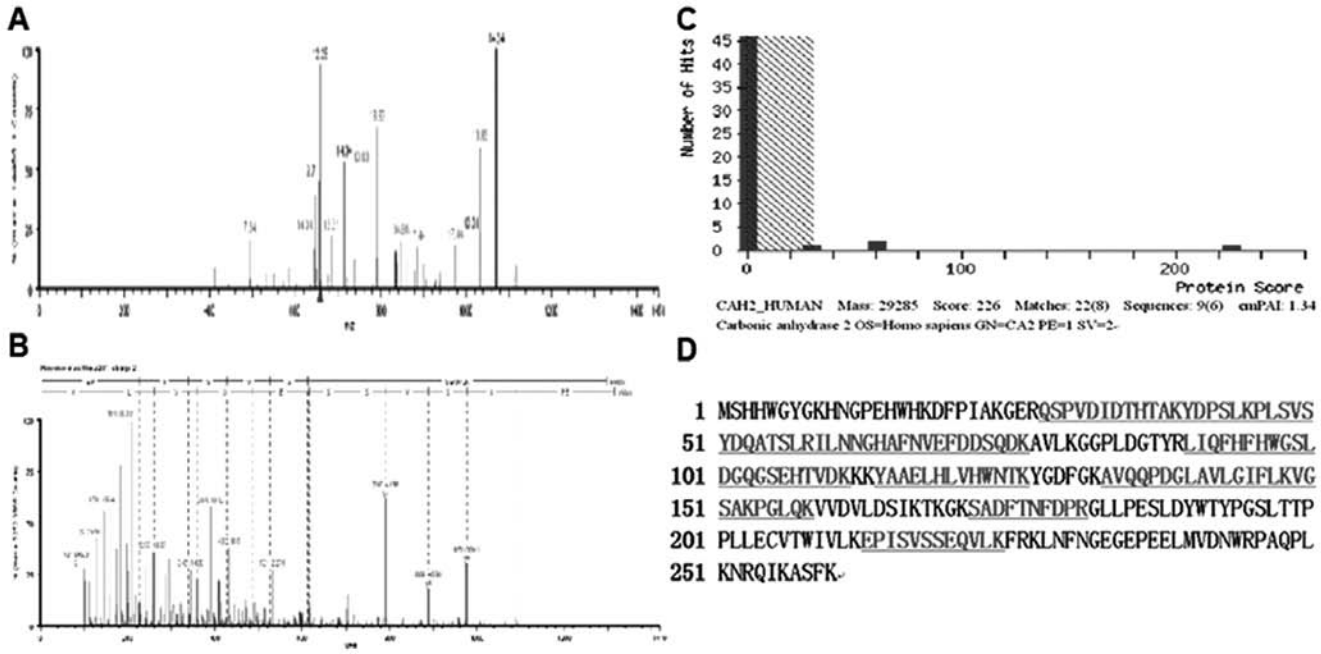

Figure 2. Results of CA II (spot no. 13) as the representative of protein using ESI-TOF/TOF. (A) Mass spectrogram of tryptic peptides from spot no. 13. (B) An example of an MS/MS spectrum of parent ion 858.3201. (C) Output of the database searching by the MASCOT program using MS/MS data resulted in the identification of CA II. (D) Protein sequence of CA II is shown and the matched peptides are underlined.

Table III. The expression of carbonic anhydrase II in colorectal cancer tissues.

	No.	-	+	++	+++[a]	Total score	Average score[b]
AN	25	0	20% (5/25)	32% (8/25)	48% (12/25)	161	6.45±.84
Ca	25	52% (13/25)	28% (7/25)	20% (5/25)	0	39	1.57±0.86

[a]Rank-sum test, P<0.05; [b]Student's t-test, P<0.01. AN, adjacent normal tissue; Ca, cancer tissue.

analysis at mRNA and protein level, respectively. The result of RT-PCR analysis showed significantly different mRNA level of CA II between CRC and normal tissues (cancer tissues, 0.31±0.07; normal tissues, 0.98±0.25; Student's t-test, P<0.01) (Fig. 3A). Western blot analysis was performed using anti-CA II antibody and remarkable CA II downregulation was observed in CRC tissues (cancer tissues, 0.22±0.05; normal tissues, 0.85±0.28; Student's t-test, P<0.01) (Fig. 3B). Taken together, our data demonstrated that CA II expression notably decreased in CRC compared with normal tissues, which was consistent with the results of 2-DE.

Further verification of CA II expression by immunohistochemistry. To further confirm the reduction of CA II expression in CRC, 25 paraffin-embedded individual-matched CRC and normal colorectal tissues were stained using anti-human CA II antibody. Strong positive staining for CA II mainly located in the cytoplasm and nucleus of epithelium and gland cells in normal colorectal tissues. In contrast, there were weakly or negative staining signal in CRC tissues (Fig. 3C). As shown in Table III significant differences in staining intensity and positive cells were observed between CRC and normal colorectal specimens (rank-sum test, P<0.05). The semiquantitative scoring of immunoreactivity for normal tissues and CRC was 6.45±2.84, 1.57±0.86, respectively (Student's t-test, P<0.01), suggesting the expression of CA II had a decreased tendency in both frequency and intensity from normal tissues to CRC.

CA II overexpression exerts inhibitory effect on CRC cell growth both in vitro and in vivo. In order to investigate the function of CA II in colorectal carcinoma, CRC cancer cell line SW480 was used to establish a stable cell line overexpressing CA II (SW480-CA II-over), since CA II was not detected in SW480 cells (Fig. 4A). Western blot and immunocytochemistry analysis showed that in contrast with no expression in control stable cell line (SW480-control), remarkable expression of CA II was observed in SW480-CA II-over stable cell line (Fig. 4A and B), suggesting successful establishment of stable SW480-CA II-over cell line.

To examine the effect of on CRC cells, MTT assay was carried out. As shown in Fig. 5A, CA II overexpression notably suppressed SW480 cell viability in a time-dependent manner. Colony formation assay showed that overexpression of CA II in SW480 cell significantly suppressed colony formation efficiency compared with control cells (Fig. 5B). Further flow cytometry analysis demonstrated that SW480 cells stably and

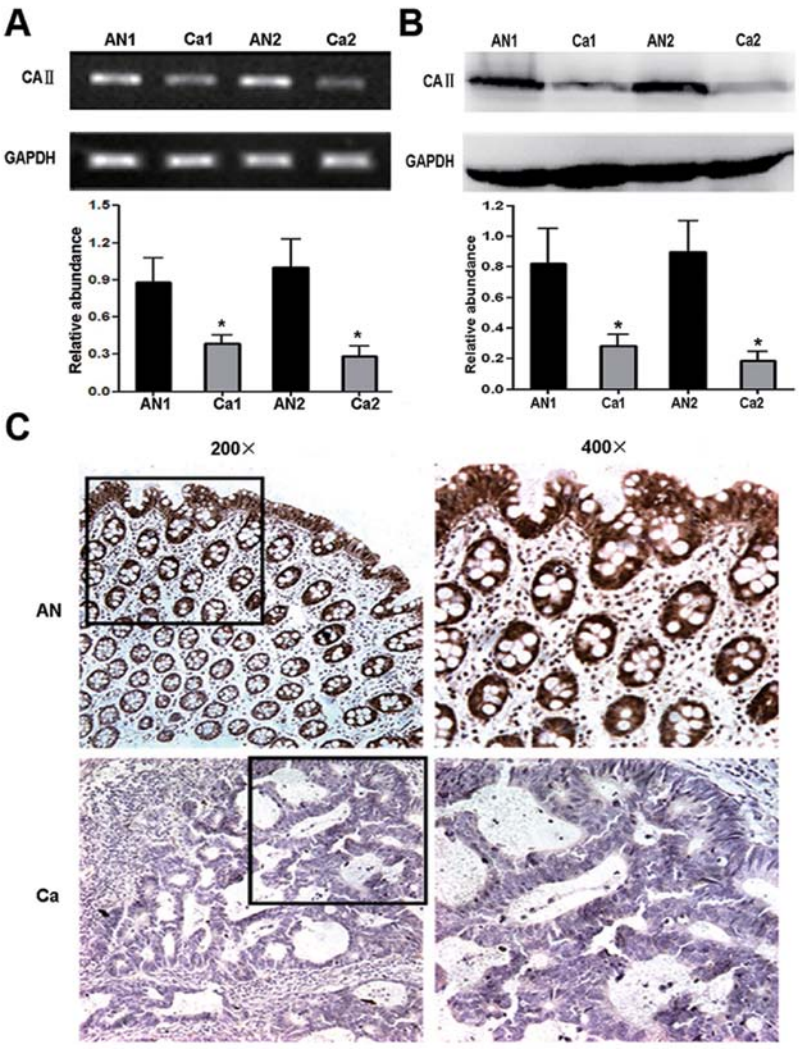

Figure 3. Validation of CA II downregulation in CRC tissues. (A and B) Results of semiquantitative RT-PCR (A) and western blotting (B) showed that the expression of CA II at mRNA, and protein levels were remarkably decreased in CRC tissues. (C) Representative immunohistochemical staining for CA II in 25 cases of CRC patients showing significant downregulation of CA II in CRC tissues.

highly expressing CA II were stalled at G0/G1 and G2 phase with subsequent decrease in S phase compared with control stable cell line (Fig. 5C).

Moreover, the inhibitory effects on CRC cancer cell growth were examined in an animal model. Tumor growth curve drawn based on the data from *in vivo* tumor model showed that SW480 overexpressing CA II had a slowed growth rate (Fig. 5D). Representative images of tumor dissection showed that high expression of CA II in tumor cell resulted in suppressed tumor volume (Fig. 5E). As shown in Fig. 5F, there was a remarkable difference in average tumor weight between CA II-overexpression and control group (CA II-over, 301.3±120.7 mg; control, 730±240.5 mg; Student's t-test, $P<0.01$). Our results *in vitro* and *in vivo* suggested that CA II could serve as a tumor suppressor gene and suppress colorectal carcinoma growth and development.

Cytotoxicity assay suggests CA II increases the sensitivity of CRC cells to oxaliplatin. Through catalyzing the reversible reactions of CO_2 and water: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$, CA II exerts an important role in acid-base balance in living organism. Thus, it was hypothesized that some signal pathways underling tumor development might be regulated by CA II. As a rate-limiting enzyme of aerobic glycolysis in tumor, embryonic M2 isoform of pyruvate kinase (PKM2) was first determined by western blotting. Similar protein level of PKM2 was observed in control and SW480-CA II-over cells (Fig. 6A); there was no significant difference of PKM2 expression in CRC and corresponding normal tissues (Fig. 6B). E-cadherin and vimentin, two markers corresponding to epithelial and mesenchymal cells, respectively, in epithelial-mesenchymal transition (EMT) were then examined. As shown in Fig. 6A and B, both *in vitro* cells and CRC tissues, no differential expression of CA II was observed.

Since extracellular acidification of cancer cells in tumor tissues leading to decreased anticancer drug uptake is one of drug-resistance mechanism, it was hypothesized that through pH regulation, CA II may affect sensitivity of CRC cancer cells to anticancer drug. Chemosensitivity tests by MTT showed that CA II overexpression increased the sensitivity of

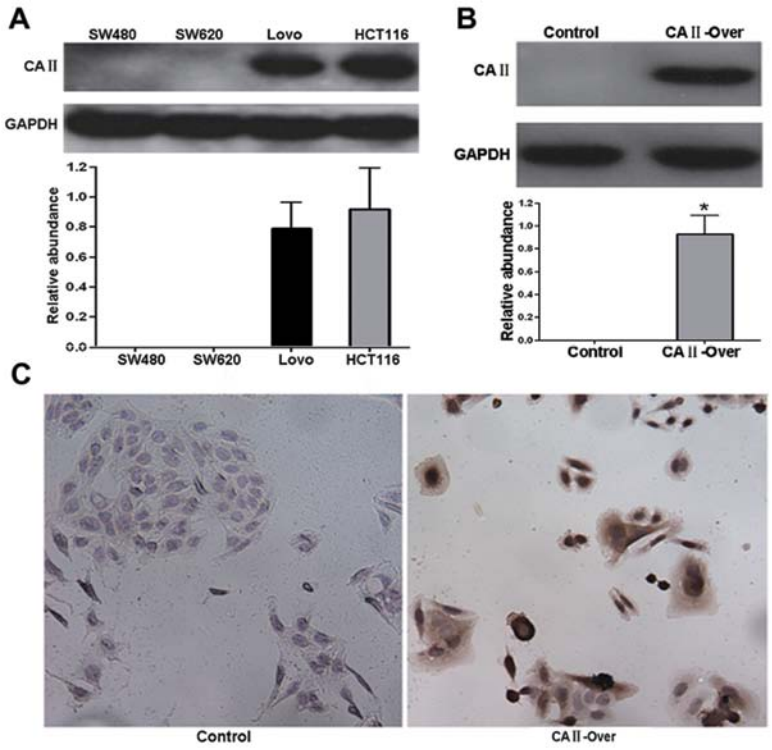

Figure 4. Establishment and identification of the stable SW480 cell line expressing CA II (SW480-CA II). (A) CA II was showed to be highly expressed in LoVo and HCT116, while could not be determined in SW480 and SW620 cells. (B and C) Western blotting (B) and immunocytochemistry (C) demonstrated high expression of CA II in CA II gene transfected SW480 cells (SW480-CA II) while not in control.

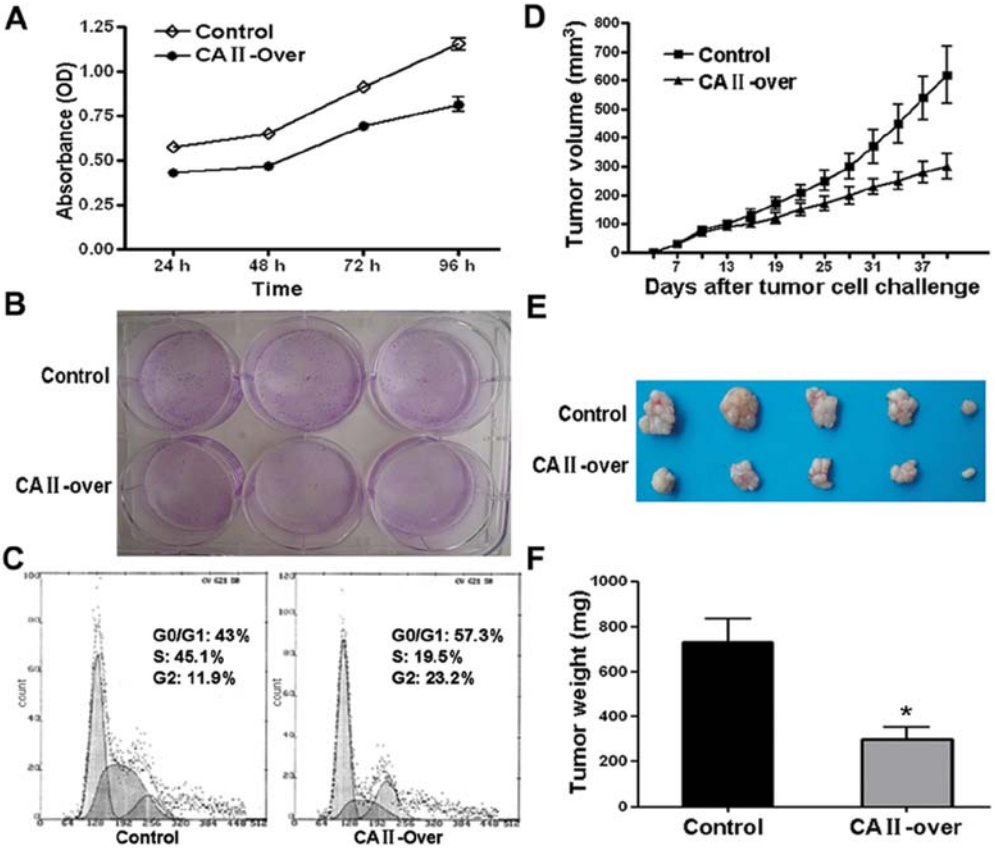

Figure 5. CA II exerts antitumor activity against colorectal cancer both *in vitro* and *in vivo*. (A) MTT assay showed overexpression of CA II inhibited proliferation potential of SW480 cells time-dependently. (B) Colony formation assay showed that in contrast to control, SW480 cells with overexpressed CA II displayed low colony-forming efficiency. (C) Flow cytometry analysis demonstrated that the cell cycle of SW480 with CA II stalled at G0/G1 and G2 phase compared with control group, resulting in significant decrease in S phase. (D, E and F) Inhibitory effects of CA II on the growth of SW480 xenograft tumor in nude mice. (D) The tumor volume was monitored regularly and growth curve was drawn. (E) Representative images of dissected tumors are shown. (F) Average tumor weight was 301.3±120.7 in CA II-over vs 730±240.5 mg in control group.

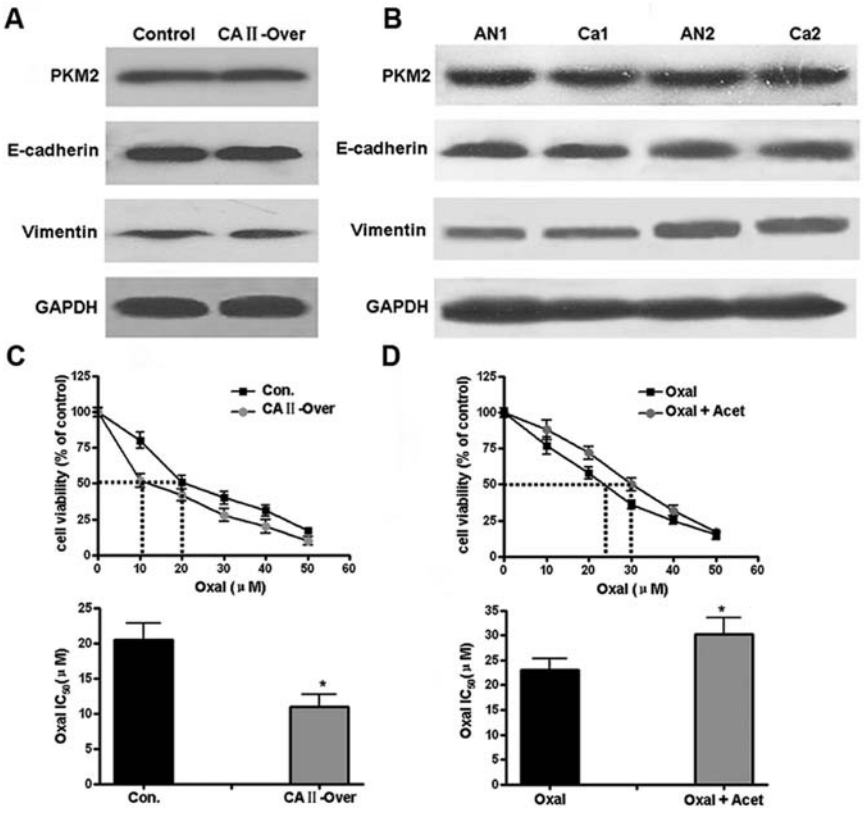

Figure 6. Mechanism analysis of antitumor activity against CRC by CA II. (A) Western blotting showed that overexpression of CA II had no obvious effect on regulation on ether PKM2 or E-cadherin and vimentin expression. (B) There was no obvious difference in CRC versus adjacent normal tissues by western blotting. (C and D) CA II increased the sensitivity of colorectal cancer cells to oxaliplatin. (C) Overexpression of CA II enhanced cytotoxic potency to oxaliplatin and accordingly decreased IC_{50} values for oxaliplatin in SW480 cells in contrast with control group. (D) In HCT116 cells, inhibition of endogenous CA II by acetazolamide weakened cytotoxic potency to oxaliplatin and increased IC_{50} values for oxaliplatin. Data are shown as representative of three independent experiments (Student's t-test, $^{*}P<0.05$).

SW480 cells to oxaliplatin (IC_{50}), from 20.5±4.3 μM in control group to 11±5.5 μM (Student's t-test, P<0.05). In contrast, for HCT116 cells with high expression of CA II, with or without pretreatment with acetazolamide, a non-specific antagonist against CA II, the corresponding IC_{50} for oxaliplatin was 30.3±3.2 and 23±4.1 μM, respectively (P<0.05), suggesting CA II could induce the chemotherapeutic sensitivity of colon cancer cells.

Discussion

In the Western world colorectal cancer (CRC) is the third most frequent type of cancer and the second most common cause of cancer related death (1). Clearly, early diagnosis and prognosis are urgent for efficient control of CRC and this largely dependents on more advances in the knowledge of mechanisms associated with CRC. In the present study, we compared the proteome between CRC and corresponding normal tissues with 2-DE and MS/MS-based approach. Thirty-six differentially expressed proteins were identified between two groups and most of these proteins were involved in fundamental biological processes. Among these 36 proteins, CA II was further studied according to the following selection criteria: i) it is one of the most significantly and differentially expressed proteins between CRC and matched normal tissues; ii) good reliability of the MS identification of protein; iii) evolutionarily conserved sequence and physiological function is crucial; iv) no or few studies reported the function and mechanism in tumor.

Carbonic anhydrase II (CA II), one of CA family isozymes, catalyzes the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$, which is involved in many critical physiological or biochemical processes based on ion transport and pH balance such as respiration, digestion, bone resorption and renal acidification (11). In addition to physiological function, it has recently been found that CA II is abnormally expressed in many types of human cancer. It was noteworthy that there was no consistent expression profile of CA II in different types of cancer tissues (12-15). Moreover, there is a contradictory correlation between CA II and the prognosis, progression of cancer patient among different types of cancer (12-15). It was shown that CA II was overexpressed in most gastrointestinal stromal tumors and strong staining of CA II indicated significantly better survival rates, suggesting CA II may serve as a diagnostic and prognostic biomarker for gastrointestinal stromal tumors (12). In contrast, immunostaining of the tumors and normal tissues from melanoma, esophageal, renal and lung cancers revealed that CA II was expressed in the tumor vessel while not in normal vessel endothelium (13). Furthermore, compared with negative staining, positive staining of CA II in vessel endothelial cells from meningiomas and glial tumors predicted worse survival rates (14,15). In our study, compara-

tive proteomic analysis showed expression of CA II notably decreased in CRC compared to normal colorectal tissues. RT-PCR, western blot analyses and immunohistochemistry were further performed to validate downregulation of CA II in CRC tissues and these results were also consistent with previous studies that paralleled with increasing severity of colorectal tissue lesions and progression of CRC, the staining intensity of CA II among normal tissues, benign lesions and malignant lesions revealed clearly decreased tendency (16-18). However, function and mechanism of CA II in the CRC development and progression have not been investigated.

Considering that CA II expressed is low in CRC tissues, gain of function strategy was utilized to study function of CA II in CRC. Stable cell line overexpressing CA II, SW480-CA II-over was then established given that CA II could not be examined in colorectal cancer cell line SW480. Serial *in vitro* as well as *in vivo* experiment results demonstrated that overexpressing CA II significantly suppressed colorectal cancer cell SW480 proliferation both *in vitro* and *in vivo*, which could be partially explained by remarkable cell cycle arrest at G0/G1 and G2 phase. To our knowledge, there is no report on how CA II functions in cancer development and progression, in spite of its abnormal expression in many types of cancer. We report that at least in colorectal cancer, CA II may play a role as tumor suppressor gene in cancer development and progression.

A distinguishing phenotype of acidic extracellular pH (pHe) and alkaline intracellar pH (pHi) in solid tumors appears to give selective advantage for tumor growth and development (19). Since carbonic anhydrase isoenzymes were involved in generating acidic tumor microenvironment (20,21), we hypothesized that CA II may also influence the processes associated with tumor microenvironment. Embryonic M2 isoform of pyruvate kinase (PKM2) was first determined by western blotting since it is a rate-limiting enzyme of aerobic glycolysis in tumors and this is specific to metabolism of solid tumors originally described by Otto Warburg (22,23). Overexpressing CA II failed to alter PKM2 protein level and there was no significant change of CA II between matched CRC and normal tissues. Epithelial-to-mesenchymal transition (EMT) is a transdifferentiation shift in which epithelial cells lose adhesiveness and polarity and acquire spindle morphology and migratory capacity characteristic of fibroblasts (24,25). It has been shown that EMT plays crucial roles in acquisition of tumoral invasiveness, the initial step of the metastatic cascade in cancer (24,25). Differently expressed E-cadherin and vimentin, two markers corresponding to epithelial and mesenchymal cells, respectively, were not observed *in vitro* or in tissues. Changed tumor microenvironment can influence the uptake of anticancer drugs and modulate the response of tumor cells to anticancer drugs (19). In the present study, overexpression of CA II decreased the oxaliplatin IC_{50} compared with that in control SW480 cells. In contrast, in HCT16 cells with high CA II expression, oxaliplatin IC_{50} increased after pretreatment with CA II antagonist, which suggested that CA II could increase the sensitivity of colorectal cancer cells to chemotherapy drugs. Inconsistent with our results, Mallory *et al* (26) found that in highly tumorigenic MDA-MB-231 breast cancer cells, knockdown of CA II expression using RNAi strategy resulted in less IC_{50} than in control cells for doxorubicin, an antineoplastic drug, implicating that CA II may negatively regulate sensitivity of breast cancer cells to chemotherapy drugs. In order to explain this contradiction and get more precise results, it is obviously necessary to increase the number of cell types and chemotherapy drugs for IC_{50} determination.

In the present study, we only utilized gain of function strategy to study the functions of CA II. Through establishing the colorectal cancer cell line stably overexpressing CA II, we concluded that CA II might serve as a tumor suppressor gene in at least CRC development and progression. In future research, it is necessary to further strengthen our conclusion by using loss of function strategies such as knockout or knockdown of CA II gene expression. Another limitation is the mechanism by which CA II suppressed the development and progression of CRC has yet to be thoroughly revealed, although it was shown that CA II could increase the sensitivity of CRC cells to anticancer drugs. Further study to explore the mechanism of CA II tumor inhibitory effects will be conducted in our research group.

In this study, CA II was identified by proteomic analysis as a potential biomarker for diagnosis of CRC followed by further verification by molecular biology methods. Moreover, it was shown that CA II might play a role as tumor suppressor gene in cancer development and progression. In conclusion, our data may contribute to a better understanding of the molecular mechanism of CRC and provide insight into colorectal cancer treatment.

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