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Association between the expression of carbonic anhydrase II and clinicopathological features of hepatocellular carcinoma

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Abstract. The present study aimed to examine the molecular marker associated with the therapy and prognosis of hepatocellular carcinoma (HCC), and further investigate the association between its expression and the clinicopathological features of HCC. To select the core genes closely associated with HCC, differentially expressed genes (DEGs) were analyzed and screened from Gene Expression Omnibus datasets (GSE 36376) using a bioinformatics approach. Tumor and adjacent tissues were collected form 112 patients of HCC who were treated by radical resection. The expression levels of carbonic anhydrase II (CA2) in the tumor and adjacent tissues were determined using reverse transcription-quantitative polymerase chain reaction analysis and immunohistochemistry. The χ^2 test was applied for observing the association between the expression of CA2 and clinicopathological features of patients with HCC. The effects of the expression of CA2 on the patients' overall survival (OS) and disease-free survival (DFS) were examined via Kaplan-Meier analysis. A total of 83 DEGs were screened and analyzed using gene network analysis, among which CA2 had direct interactions with more than one disease gene of HCC. The results of immunohistochemistry showed that CA2 was expressed at a lower level in the tumor tissues compared with the adjacent tissues (t=3.012, P=0.010). Single factor analysis revealed that the mRNA expression of CA2 was able to predict the recurrence of HCC, and was significantly associated with α-fetoprotein (AFP), microvascular invasion, tumor-node-metastasis (TNM) staging, and recurrence (P<0.05). The expression levels of AFP, CA2 and TNM staging were confirmed to be independent prognostic factors of HCC (P<0.05). Kaplan-Meier analysis demonstrated that the group with a high expression of CA2 showed increased DFS and OS, compared with the low expression group (P<0.05). These findings indicated that elevated CA2 increased DFS and OS of HCC, which suggested that CA2 may be a potential target for HCC therapy.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumor (1-5), with a mortality rate of >1,000,000 every year worldwide. HCC remains a global health problem with increasing morbidity rates and considerable mortality rates, having serious effects on human health. Although surgery is an effective therapy for HCC currently (6-8), there are malpractices in several aspects, including anesthesia, surgical risk and postoperative complications, and the majority of patients with HCC of occult onset have missed the optimal timing for surgical treatment. Therefore, biomarkers of HCC are an important and unmet requirement for the diagnosis, treatment and prognosis of HCC.

Gene chip technology provides a novel approach for the identification of biomarkers, and its high throughput has facilitated investigations of gene expression, disease diagnosis and treatment, targeted drugs and curative effects. In addition, using bioinformatics methods to analyze gene chip data can maximize the retrieval of hidden information in large quantities of data. In previous years, HCC and healthy control groups have been analyzed and compared through protein-protein interaction (PPI) analysis, which has identified markers of disease occurrence and development, providing a theoretical basis for the development of targeted drugs (9). Carbonic anhydrases (CAs), a crucial family of 16 isoenzymes, distribute in different tissues (10). Carbonic anhydrase II (CA2), first identified in red blood cells as monomers, have been suggested to be a prognostic factor of several tumor diseases, including pancreatic (11), colorectal (12) and gastric cancer (13), and

peritoneal myxoma (14). At present, the major biomarkers of HCC are ETS2 (15), Rac GTPase-activating protein 1 (16), matrix metalloproteinase, 3-phosphoinositide-dependent protein kinase-1 (PDK1) and FOS. The expression of PDK1 was found to be high in HCC cells following radical surgery, which was associated with the patient prognosis (17), however, the association between the expression of CA2 and HCC remains to be elucidated.

Through observing the change in the expression of CA2 in HCC, and assessing the clinical features and prognosis of patients, the present study investigated the association between the expression of CA2 and clinicopathological features of HCC, in order to provide an experimental and theoretical basis for the improvement of HCC treatment.

Materials and methods

Search and download of HCC gene expression data. Two gene expression data sets of HCC following radical surgery: GSE36376 and GSE22058, were obtained by searching 'Hepatocellular Carcinoma' and 'Curative Hepatectomy' in the Gene Expression Omnibus (GEO) datasets (http://www.ncbi.nlm.nih.gov/geo/). GSE22058 has three chip platforms: GPL6793, GPL9733 and GPL10457, with 397 samples in total. The GSE36376 dataset selected by Lim *et al* uses the chip platform GPL10558, with 433 samples in total. According to the standard of the 7th American Joint Committee on Cancer (AJCC) (18), the severity degrees of HCC can be ranked as I-III stages. The samples (193 tumor tissues and 240 adjacent tissues were sorted according to the Title, and the data of the tumor and adjacent tissues of the same patient were matched and downloaded.

Analysis of gene chip data and differentially expressed genes (DEGs). Gene chip data analysis in GEO was performed in R-software 3.4.0. software with its normalization perfected and innovated by Limma (http://www.bioconductor.org/packages/release/bioc/html/limma.html). The DEGs of different stages in the tumor and adjacent tissues were detected using a paired t-test. Multiple verification was executed using the NormalizeBetweenArrays method. The genes with adj $P<0.01$ and an absolute value of Log FC >1.8 were regarded as the DEGs.

HCC-associated genes and meta-analysis. The genes associated with HCC were searched in DigSee (http://210.107.182.61/digseeOld/) with 'Hepatocellular Carcinoma' as the key word (TP53, AFP, ADAM17, VEGFA, PTGS2, CTNNB1, CCND1, BCL2L1, AEN, TNF, MAPK1, IFNA5). Using the analysis tool of the Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/cgi/input.pl) database, the associations of these genes associated with the DEGs were identified. The molecular function, signaling pathways and biological ways of HCC, and the interaction of these DEGs were examined by functional enrichment analysis.

Patient and sample collection. A total of 112 patients undergoing radical surgery were selected from Fujian Provincial Cancer Hospital (Fujian, China) between January 2012 and January 2013. The inclusive criteria were as follows: Patients with pathological diagnosis confirmed; no radiotherapy and chemotherapy prior to surgery; no distant metastasis; provision of signed informed consent. The exclusion criteria were as follows: Patients with incomplete clinical data; presence of systemic disease; presence of other malignant tumors; pregnant or lactating. The tumor and adjacent tissue samples were collected from the patients with HCC. The degrees of severity of HCC were ranked as I-III stages in accordance with the standard of the 7th AJCC. Following the patient being admitted to hospital, 5 ml peripheral blood was collected into vascular tubes, and within 2 h serum was extracted at room temperature for 40 min, centrifuged for 1,000 x g for 10 min and preserved at -80˚C.

Follow-up survey. All 112 patients were followed-up by telephone and clinic visits 3, 6, 9, 12, 18 and 24 months following radical surgery, and during visits once each year from month 36 following radical surgery. The overall survival (OS) and disease-free survival (DFS) of the patients were observed until December 2016 with a median follow-up time of 34 months. The OS was determined as the time from randomization to mortality due to any cause. The DFS was defined as the time from randomization to the recurrence of primary disease or mortality.

Extraction of total RNA. The total RNA of the 122 patients' tumor and adjacent tissue samples were extracted using TRIzol (Beijing Biotechnology Co., Ltd., Beijing, China), according to the manufacturer's protocol. The tissue samples were added to 500 μl TRIzol and 200 μl trichloromethane, shaken and rested, and then placed in a centrifuge (Hunan Kaida Laboratory Co., Ltd., Hunan, China) at 12,000 x g for 10 min at 4˚C, followed by the addition of isopropanol and ethanol, centrifugation, and discarding the supernatant. The RNA was dissolved with 20-50 μl diethylpyrocarbonate (DEPC)-ddH_2O, dehydrated with ethanol and preserved in a refrigerator at -70˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The RNA was extracted by TRIzol (Beyotime Institute of Biotechnology, Beijing, China). The RT-qPCR analysis was performed according to the protocol of the reverse transcription and amplification kit (Roche Diagnostics, Basel, Switzerland) (2X SYBR-Green qPCR Mix; 10 μl; Forward primer (10 μmol/l), 1 μl; reverse primer (10 μmol/l), 1 μl; cDNA, 1 μl; DEPC-ddH_2O, 7.0 μl). The reaction conditions were as follows: 40 cycles of 5 min of pre-denaturation at 95˚C, 5 sec of denaturation at 95˚C, and 30 sec of annealing at 60˚C, replacing the cDNA template with DEPC-ddH_2O as a negative control. The primer sequence were as follows: CA2, forward 5'-AAACAAAGGGCAAGAGTGCT-3' and reverse 5'-GAGCACAATCCAGGTCACAC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CATGAGAAGTATGACAACAGCCT-3' and reverse 5'-AGTCCTFCCACGATACCAAAGT-3'. The primers were synthesized by Shanghai Biological Engineering Co., Ltd. (Shanghai, China). And the data was analysed by $2^{-\Delta\Delta Cq}$ method (19).

Immunohistochemistry. The tumor and adjacent tissue samples were paraffin-embedded and cut into 5-μm sections. Following dewaxing with dimethylbenzene and hydration, the sections were added to citrate, boiled, rinsed three

times (3 min each rinse) with PBS, blocked for 15 min with 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and incubated with CA2 antibody (dilution, 1:1,000; cat. no. ab191343) overnight in a wet box at 4°C. The tissue sections were then rinsed three times (3 min each rinse). The tissues were added to diaminobenzidine solution (Shanghai Fu Sheng Industrial Co., Ltd., Shanghai, China) for 10 min at room temperature, stained by hematoxylin, differentiated for 5 sec with HCI + ethanol, washed for 20 min, hydrated with an ethanol concentration gradient, and mounted. The median integral optical density (mIOD) was used as the cut-off point. All samples were divided into a high CA2 expression group (IOD > mIOD) and low CA2 expression group (IOD < mIOD).

Statistical analysis. R 3.4.0 software (https://cran.r-project.org/bin/windows/base/) was used for analyzing data. Quantitative data are presented as the mean ± standard deviation. A Kolmogorov-Smirnov test was applied to assess the normal distribution of data. If in a normal distribution, the mean of the measurement data among groups were compared using a Student t-test; if not, data were compared using a Mann-Whitney U test. The enumeration data are expressed as case number and percentage, and were examined using a χ^2 test. The expression of CA2, and patients OS and DFS were analyzed using Kaplan-Meier analysis. The patients survival rates were compared using a log-rank test. COX regression analysis was used to evaluate the prognostic value of CA2 and other parameters in HCC. P<0.05 was considered to indicate a statistically significant difference.

Results

DEGs in patients with and without relapse. There were 21,594 expressed genes (9,363 genes in stage I, 7,876 genes in stage II, and 4,355 genes in stage III) obtained through GEO analysis. Subsequently, the genes with an absolute value of Log FC >1.8 were selected and analyzed further. In total, 83 genes were differentially expressed in the three stages (Fig. 1).

Interaction network of the DEGs. According to the STRING database, there were 83 genes, which were differentially expressed in the three stages, among which 44 genes interacted with associated genes of HCC (Fig. 2) and the majority of these have been investigated in HCC. CA2 was expressed at a low level in HCC. However, there has been no report on the mechanism of CA2 in HCC or its association with the clinicopathological features of HCC.

Expression of CA2 in the tumor and adjacent tissues. The expression levels of CA2 in the tumor and adjacent tissues were observed via immunohistochemistry, which showed that the expression of CA2 in the tumor tissues was lower, compared with that in the adjacent tissues (Fig. 3). The patients were then divided into CA2 high and low expression groups to examine the association between the expression of CA2 and the clinicopathological features of HCC.

Greyscale maps of CA2 in the tumor and adjacent tissues. The grey ratios of CA2 in the tumor and adjacent tissues and their mean values were assessed. The grey ratio of CA2 in the adjacent tissues was (0.75±0.15), and in the tumor tissues was (0.57±0.12), which was a statistically significant difference (t=9.918, P<0.001; Fig. 4A). On comparing the expression of CA2 in the tumor and adjacent tissues, the expression of CA2 in the 112 tumor tissues was lower, compared with that in the adjacent tissues (t=3.012, P=0.010; Fig. 4B).

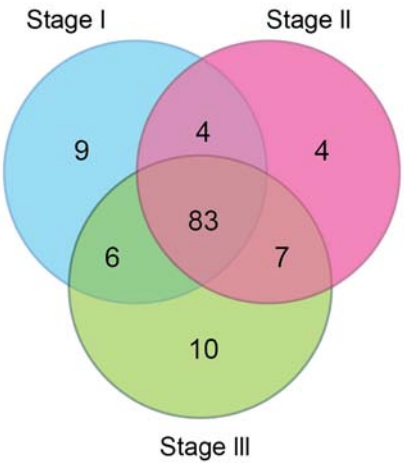

Figure 1. DEGs in tumor-node-metastasis stage. DEGs were integrated using online software (http://bioinformatics.psb.ugent.be/webtools/Venn/). The Venn diagram shows that there were 83 DEGs in the three stages of hepatocellular carcinoma. DEGs, differentially expressed genes.

Association between the expression of CA2 and clinicopathological features of HCC. There was a significant difference in α-fetoprotein (AFP), microvessel infiltration, tumor-node-metastasis (TNM) staging and recurrence under different expression levels of CA2 (P<0.05). There was no significant difference in age, sex, HBsAg, Child-Pugh score, chronic interstitial hepatitis, tumor diameter or tumor number between the two groups (P>0.05; Table I).

Association between the expression of CA2 and HCC prognosis. In the present study, 66 of the 112 patients suffered from relapse during the follow-up period. Of these, there were 54 cases of patients with intrahepatic metastasis, four with pulmonary metastasis, and eight with pulmonary and intrahepatic metastasis. Single factor analysis showed that the AFP level, TNM staging, microvascular invasion, and expression of CA2 may be risk factors in determining prognosis. Combined with the single factor analysis, the multivariate COX regression analysis showed that the AFP level (HR=1.378; 95% CI=0.209-0.897, P<0.05), expression of CA2 (HR=0.433; 95% CI=2.402-20.979, P<0.05), and TNM staging (HR=7.098; 95% CI=1.105-1.717, P<0.05) were independent prognostic factors for HCC (Table II). Kaplan-Meier analysis showed the high CA2 expression group presented with higher DFS (P<0.05) and OS (P<0.05), compared with the low expression group (Fig. 5A and B), which indicated that CA2 may be a potential target for the prognosis of HCC.

Discussion

HCC is the main cause of cancer-associated mortality (8,20). It is not easy to make an early diagnosis as there are no obvious

Table I. Association between the expression of CA2 and clinicopathologic features of hepatocellular carcinoma.

	Expression of CA2, n (%)			
Variable	Low (n=59)	High (n=53)	χ^2	P-value
Age (years)				
<50	41 (69.49)	37 (69.81)	0.001	0.971
≥50	18 (30.51)	16 (30.19)		
Sex				
Male	42 (71.19)	39 (73.58)	0.08	0.777
Female	17 (28.81)	14 (26.42)		
HbsAg				
Positive	10 (16.95)	9 (16.98)	<0.01	0.996
Negative	49 (83.05)	44 (83.02)		
Child-Pugh score				
A	55 (93.22)	50 (94.34)	0.06	0.807
B	4 (6.78)	3 (5.66)		
Liver cirrhosis				
Yes	50 (84.75)	46 (86.79)	0.096	0.757
No	9 (15.25)	7 (13.21)		
AFP (ng/ml)				
<100	19 (32.20)	29 (54.72)	14.48	0.006
100-400	7 (11.86)	11 (20.75)		
400-800	4 (6.78)	4 (6.78)		
800-1,210	4 (6.7842.37)	3 (5.66)		
≥1,210	25 (42.37)	6 (11.32)		
Tumor diameter (cm)				
≤5	41 (69.49)	37 (69.81)	0.001	0.971
>5	18 (30.51)	16 (30.19)		
Tumor number				
Single	12 (20.34)	12 (22.64)	0.088	0.767
Multiple	47 (79.66)	41 (77.36)		
TNM stage (30)				
I-II	11 (18.64)	28 (52.83)	14.377	<0.001
III-IV	48 (81.36)	25 (47.17)		
Microvascular invasion				
Yes	34 (57.63)	16 (30.19)	8.506	0.004
No	25 (42.37)	37 (69.81)		
Recurrence				
Yes	48 (81.36)	18 (33.96)	25.911	<0.001
No	11 (18.64)	35 (66.04)		

AFP, α-fetoprotein; TNM, tumor-node-metastasis; CA2, carbonic anhydrase II.

Table II. Multivariate COX regression analysis of factors associated with recurrence.

Variable	B	SE	Wald	P-value	Exp (B)	95% CI
AFP (>400, vs. ≤400 ng/ml)	0.320	0.112	8.128	0.004	1.378	0.209-0.897
TNM stage (I/II, vs. III/IV) (30)	1.960	0.553	12.566	<0.001	7.098	1.105-1.717
CA2 expression (low and high)	-0.836	0.371	5.079	0.024	0.433	2.402-20.979

AFP, α-fetoprotein; TNM, tumor-node-metastasis; CA2, carbonic anhydrase II.

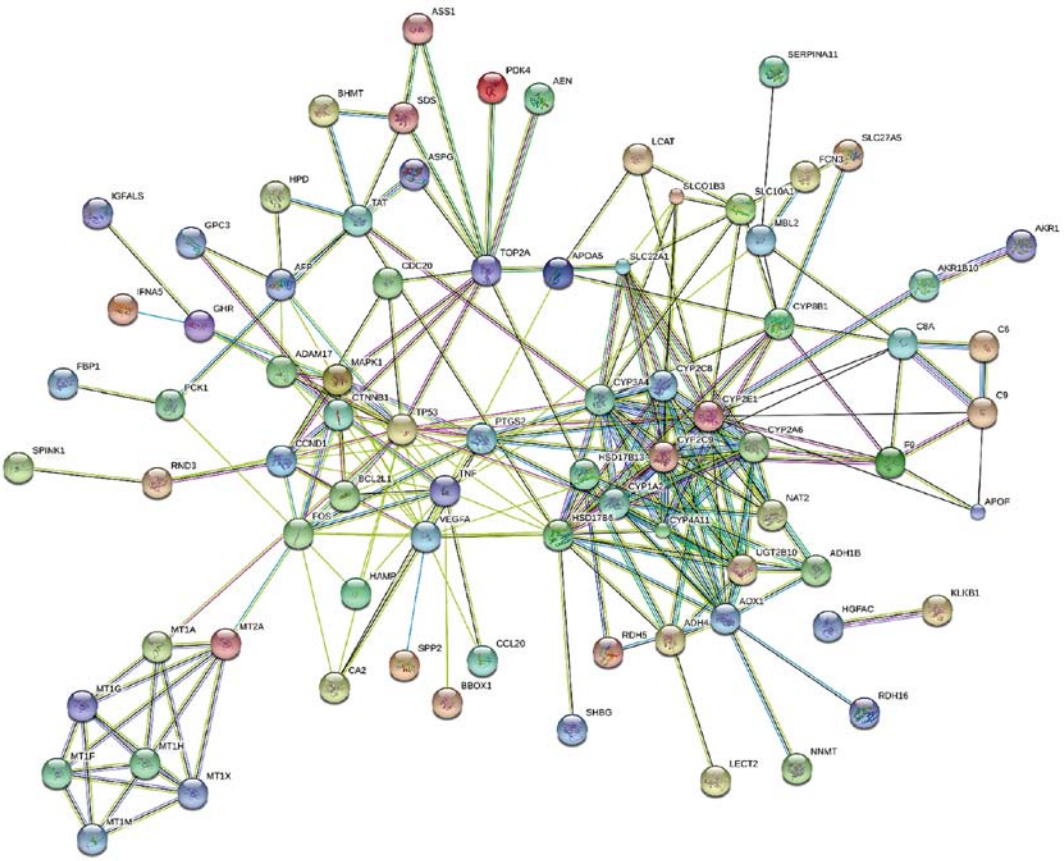

Figure 2. Network map of HCC genes. The protein-protein interaction data of HCC was obtained from the Search Tool for the Retrieval of Interacting Genes database, which indicated that carbonic anhydrase II was involved in the occurrence and development of HCC. Line: The protein-protein interaction. The thicker the line, the stronger the effect. Spheres: HCC genes. HCC, hepatocellular carcinoma.

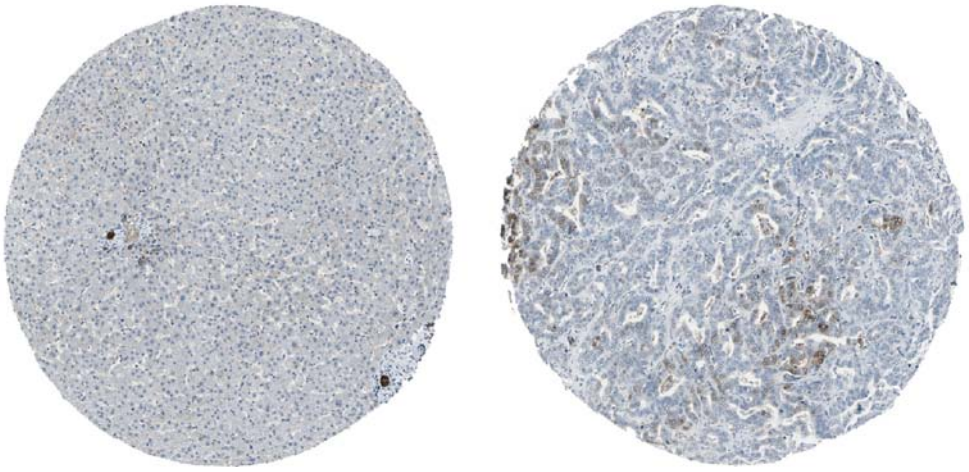

Figure 3. Expression of CA2 in serum samples. The expression of CA2 in serum samples of 112 patients with hepatocellular carcinoma was detected using immunohistochemistry (magnification, x200). Normal liver tissues showed a high level of expression whereas tumor tissues showed low expression. CA2, carbonic anhydrase II.

symptoms in the early stage. Therefore, the majority of patients lose the chance to undergo radical surgery, which seriously threatens patient health and quality of life (21). The prognosis of patients is poor (22). The traits of complex diseases are affected by the interaction of multiple genes. Gene analysis based on interaction between genes can effectively produce biological information, providing valid scientific reference for the diagnosis, treatment and improvement of prognosis. Following the rapid development of genomic science, a large quantity of genomic data and gene expression data have provided a novel basis for the investigation of gene-gene and gene-environment interactions (23-25). By analyzing the existing data through systems biology approaches, it is possible to investigate the association between gene function, protein interaction and the occurrence of diseases, and construct a detailed network of the occurrence and progression of diseases. Previously,

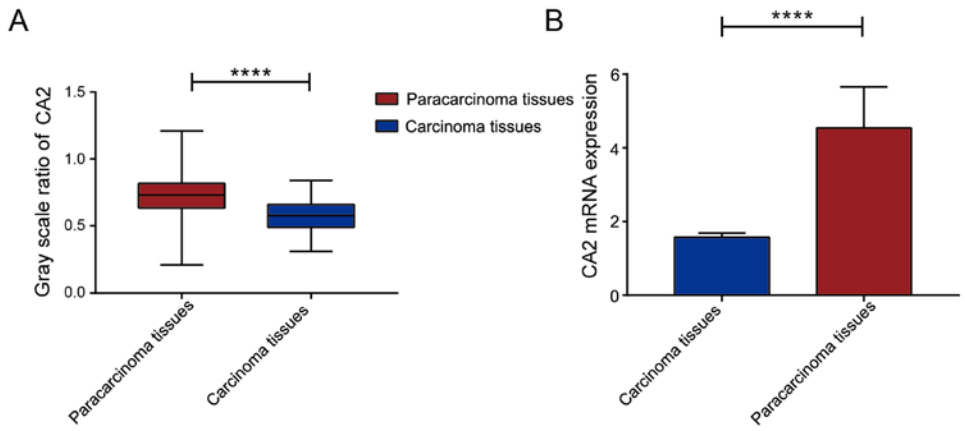

Figure 4. Expression of CA2. (A) Western blot analysis demonstrated that, in 112 HCC cases, the expression of CA2 was lower, compared with that in paired adjacent paracarcinoma tissues. (B) Expression of CA2 in tumor and adjacent tissues from 112 patients with HCC was determined using reverse transcription-quantitative polymerase chain reaction analysis and analyzed using a paired t-test. ****P<0.0001 vs. carcinoma. Blue, tumor tissues; red, adjacent tissues. HCC, hepatocellular carcinoma; CA2, carbonic anhydrase II.

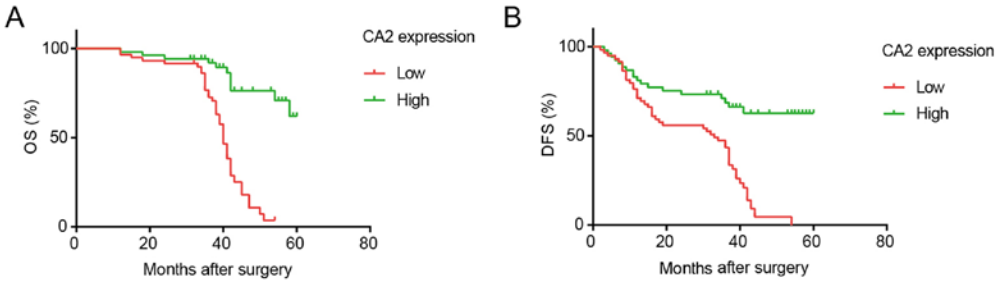

Figure 5. Association between CA2 and hepatocellular carcinoma patient survival rate. The association between the expression of CA2 and patient prognosis was assessed using Kaplan-Meier survival curves. Green, high expression of CA2; red, low expression of CA2. OS, overall survival; DFS, disease-free survival; CA2, carbonic anhydrase II.

the molecular mechanism of hepatitis C virus (HCV) protein in the progression of HCC was investigated by topological analysis of a PPI network (26). Through constructing the PPI network of DEGs in chronic HCV and HCC samples, interaction among DEGs and key microRNAs were confirmed using the STRING and GeneMANIA databases, which provided a foundation for subsequent investigations of mechanisms and clinical application. In the present study, it was found that 83 genes were differentially expressed in the three stages of HCC, based on the GEO of HCC. The gene network map of HCC was constructed according to the PPI data obtained using the STRING method, in which the majority of genes were found to be associated with the HCC genes. Among the DEGs, CA2 had a direct interaction with several HCC genes, but has received limited investigation. Therefore, the present study focused on the role of CA2 in HCC.

CAs have a variety of functions, including carbon dioxide (CO_2) transport, pH adjustment, ion transport, gastric acid formation, bone resorption, calcification and genesis, development, and tumor invasion (27,28). As one isoenzyme of CA, CA2 is widely distributed *in vivo* and has the highest activity. The main function of CA2 is to catalyze the hydration of CO_2, which can readily pass through the cell membrane. Therefore, CA2 being widely present in the cytoplasm can regulate HCO_3 balance by promoting CO_2 transmembrane transport. It has been suggested that the pH of cancer tissues is lower than that of adjacent normal tissues, possibly due to CA2 being involved in the acidification process of the extracellular microenvironment by the reversible catalysis of CO_2 and HCO_3-. The acidification of the microenvironment is beneficial to tumor invasion, which suggests that CA2 is an indicator of poor clinical prognosis. Therefore, differences in the expression of CA2 has a significant effect on the occurrence, progression and biological behavior of cancer. Previous studies have shown that CA2 was either absent or was expressed at low levels in the majority of tumors (12). In a study by Hu *et al* (13) CA2 in tumor and adjacent tissues were separately collected from patients with gastric cancer, and its content was analyzed following radical surgery via immunohistochemistry. The results showed that the content of CA2 in tumor tissues was significantly lower than that in normal gastric mucosa tissues, and that the downregulation of CA2 was correlated with tumor size and stage, and a low survival rate. AFP is a type of glycoprotein synthesized by endoplasmic reticulum in hepatocytes and is also a tumor marker of HCC, which is valuable for tumor prognosis. AFP content and the survival curve of patients with HCC were measured respectively using an electrochemiluminescence immunoassay and Kaplan-Meier analysis, which showed that the survival rate of patients with an AFP>400 μg/l was reduced, compared with that of patients with an AFP<400 μg/l (29). In the present study, Spearman's correlation analysis showed that CA2 was negatively correlated

with AFP content. The expression of CA2 in the HCC tissues was decreased and correlated with AFP, microvascular invasion, differentiation degree and tumor stage. Kaplan-Meier analysis suggested that patients with a low expression of CA2 had a poor prognosis, and COX regression analysis showed that the expression of CA2 was an independent prognostic factor for HCC, which was consistent with the study by Hu *et al* (13). Therefore, it was hypothesized that CA2 may be involved in the occurrence and progression of HCC, was correlated with the prognosis of patients, and may be used as an independent prognostic factor in patients with HCC.

The present study demonstrated firstly that CA2 was crucial in determining the clinical characteristics and prognosis of HCC by combining expression profiling and clinical data of cases of HCC. The results showed that the downregulation of CA2 in HCC was significantly correlated with the clinical characteristics and prognosis of HCC. These findings demonstrated the role of CA2 in the occurrence and progression of HCC, which is significant for predicting the postoperative survival rate of patients.

In conclusion, the present study examined the expression of CA2 in tumor and adjacent tissues using bioinformatics analysis, and analyzed the value of the expression of CA2 in the progression and prognosis of HCC. The findings suggested that the involvement of CA2 is pivotal in HCC, and is expected to be an independent prognostic factor and a potential target for the treatment of HCC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HZ designed the present study. CZ wrote the manuscript, contributed to the design of the present study and conducted the experiments. DZ, FZ, MC, SX and ZC assisted in the analysis and interpretation of the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Fujian Provincial Cancer Hospital (approval no. SQ2011-012-01) and signed informed consent was provided.

Patient consent for publication

Patients provided consent for the publication of the present study.

Competing interests

The authors declare that they have no competing interests.

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