RESEARCH ARTICLE

# MACC1 Expression Correlates with PFKFB2 and Survival in Hepatocellular Carcinoma

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## Abstract

**Objective: To validate the relationship between MACC1 and 6-phosphofructo-2-kinase/fructose 2, 6 bisphosphatase (PFKFB2) expression as well as its clinicopathological features and prognostic significance in hepatocellular carcinoma. Methods: By using immunohistochemistry, we investigated the MACC1 and PFKFB2 protein expression in 60 pairs of hepatocellular carcinoma and corresponding non-tumor tissues. Using the Mann-Whitney U test, the Chi-square test, Kaplan-Meier survival analysis, Cox proportional hazard regression analysis and Spearman analysis, we studied the relationship between MACC1 and PFKFB2 protein expression and postoperative overall survival (OS) of the HCC patients. Results: MACC1 and PFKFB2 positive staining rates were significantly higher in hepatocellular carcinoma than in the corresponding nontumor tissues ($P$=0.012 and 0.04, respectively). The clinicopathological features evaluation revealed that positive expression of MACC1 was associated with a high Edmondson classification ($P$=0.007) and advanced TNM stage ($P$=0.027). Similar findings were evident for PFKFB2 expression ($P$=0.002 and $P$=0.027). MACC1 and PFKFB2 positive expression was associated with a lower OS rate ($P$=0.004 and 0.03, respectively). Kaplan-Meier survival and Cox proportional hazard regression analyses revealed MACC1 positive expression to be a prognostic factor for postoperative OS, but PFKFB was not. Conclusion: Highly expressed MACC1 and PFKFB2 protein were associated with TNM stage, Edmondson –Steier classification and overall survival. MACC1 may affect tumor metabolism partly through expression and phophorylation of PFKFB2.**

**Keywords:** MACC1 - PFKFB2 - glycolysis - hepatocellular carcinoma - immunohistochemistry

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## Introduction

Hepatocellular carcinoma is one of most common malignant tumor in the world. Its incidence has increased in recent years, and it is the third most common cause of cancer mortality (Ferlay et al., 2010). At present, the change of the key enzyme in tumor glycolysis was drawing more and more attention, and now it has became a hot spot in cancer research (Minchenko et al., 2005; Bobarykina et al., 2006; Scatena, et al., 2008; Yalcin et al., 2009). At the same time, the liver as the center biochemical metabolism of human body, the changes of glycolysis due to tumor growth and progression in the liver may be very significnant. The changes of the tumor glycolysis were often caused by the changes of the key enzymes in the metabolic pathway. Previous study showed that the change of ras , c-myc , src, p53 were associated with change of tumor glycolysis (Bosca, et al., 1986; Blum, et al., 2005; Zeller et al., 2006; Kawauchi, et al., 2008). In recent years , more and more study found that the activation of the PI3K/Akt signaling pathway play an important role in promoting glycolysis of tumor (Moon et al., 2011; Novellasdemunt et al., 2013), especially on the regulation of the key enzyme in glycolysis, 6-phosphoric acid fructose kinase-1 (PFK-1) is the most important enzyme on regulation sugar flow and energy supply in glycolysis pathway, and its active height was often affect by a potent allosteric agent-fructose 2, 6-bisphosphate, which was produced by a bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2, 6 bisphosphatase (PFK-2/FBPase-2) (Van Schaftingen, 1987; Okar et al., 2001; Rider et al., 2004). PFKFB2 is one of isoenzymes of PFK-2/FBase-2.And recent study found that the activation of Akt contribute to enhance the PPFKFB2's function (Moon et al., 2011; Novellasdemunt et al., 2013).

MACC1 was a new gene that was found by Stein, etc. in 2009. More and more studies showed that MACC1 was an important key factor in regulation of HGF/c-MET signaling pathway (Stein, et al., 2009), and it can also activate PI3K/Akt signaling pathway, which was drawing more and more attention on the role of tumor glycolysis. MACC1 as newly discovered gene, there was more and more reports about it. But it is not reported in field of tumor glycolysis.

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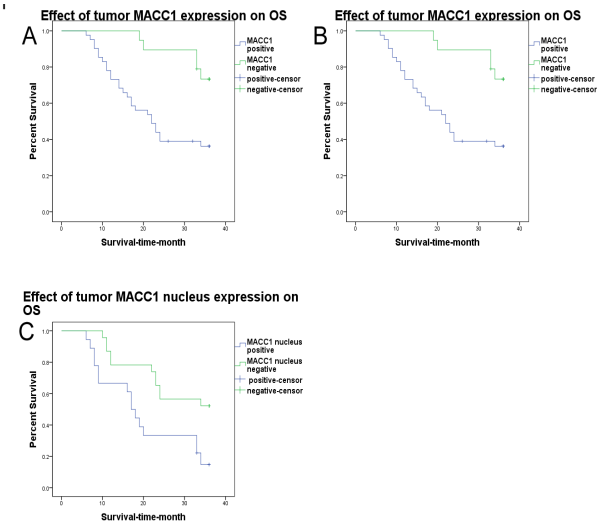


**Figure 1. Green Lines Represent Immunohistochemistry Positive Samples while Blue Lines Represent Immunohistochemistry Negative Samples in Picture A and B, but in Picture C the Color was Opposite.** A) OS was significantly worse for patients with MACC1 positive tumors compared to patients with MACC negative tumors (Log Rank test *P*=0.004). B) OS was significantly worse for patients with PFKFB2 positive tumors compared to patients with PFKFB2 negative tumors (Log Rank test *P*=0.013). C) OS was significantly worse for patients with MACC1 positive tumors compared to patients with MACC1 negative tumors (Log Rank test *P*=0.007)

The purpose of this study was to preliminary evaluate the association between MACC1protein expression and PFKFB2 protein expression in HCC and provide valuable reference for further research.

## Materials and Methods

*Patients and specimens*

All the samples were collected from July 2009 to June 2010 in the first affiliated hospital of the medical college of Xi an Jiao tong University and the pathologiacal comfirmed for hepatocellular carcinoma. There were 40 men and 20 women (median age 52 years. range 24-76). All patient had not received pre-operative chemotherapy or ablation and provided written informed consent for their tissues to be used in in this study.Tumor tissues and the corresponding nontumor tissues (>2 cm distance from the resection margin) were collected and immediately stored in paraformaldehyde for immunohistochemistry.

*The Reagents*

The primary rabbit antibody MACC1 was purchased from Abcam (Hong Kong). PFKFB2 was the product of ABGENT.

*Methods*

Immunohistochemistry was used to detect the expression of MACC1 and PFKFB2.Tissues was fixed, paraffin embedding, serial section, etc. Incubated with the primary antibodies directed against MACC1 (1:200) and PFKFB2 (1:100) at 4°C overnight. Negetive control using PBS instead of the primary antibody. And then using biotinylated secondary antibody (Zhongshan Goldenbridge Biotechnology Itd.co.) according to the manufacturer's recommendations .after that, adding HRP3 streptavidin conjugates for MACC1 and PFKFB.DAB visualized the section, and then dehydrated in alcohol and xylene and mounted onto glass slides.

All section were assessed independently by two experienced pathologists. Specific immunoreactivity was judged according to the following two aspects. 1) Staining intensity:0, none; 1, weak; 2, moderate; and 3, strong. 2) The percentage of positive cell:0, <10%; 1, 10-25%; 2, 26-50%; 3, 50-75%; and 4, >75%; The total score was calculated by summating the stain-ing intensity and the percentage of positive cells. Sections with a total score of ≥ 3 was defined as positive staining

*Statistical analysis*

All laboratory data was analyzed with the software package SPSS ver.13a.0. Differences in MACC1 and PKFB2 proteins expression in the HCC tissues compared to non-tumor-adjacent was compared by the Chi-square test. Associations between protein expression and clinical pathologic features was analyzed by the Chi-square test or Fisher's exact test. The Correlation between MACC1, PFKFB2 protein expression in the HCC were analyzed by the Spearman's rank correlation coefficient. Kaplan-Meier method was used to estimate the survival curves. Long-rank test was used to analyze the differences between the survival curves. Hazard ratios (HR) and 95% confidence Interal (CI) was calculated using Cox proportional hazards regressions modeling. Value of $P<0.05$ was considered to be statistically significant.

## Results

*MACC1 and PFKFB2 expression in HCC and adjacent liver tissues*

IHC staining showed that PFKFB2 was mainly located in cytoplasm. It is positive in 38/60 (63.30%) of HCC tissues and lower than adjacent liver tissues in 26/60, 43, 3% (*P*=0.04). In addition, immunohistochemical scores showed that the expression of MACC1 and PFKFB2 protein was higher in the HCC tissues than in the adjacent liver tissues (3.27±1.582 vs 2.43±1.640, *P*=0.007; 3.05±1.682 vs 2.23±1.466, *P*=0.005, respectively ). MACC1 also showed a higher expression in tumor tissues than the adjacent liver tissues (68.3%vs43.3%, $P<0.05$) (Figure 2). IHC assay showed that MACC1 protein mainly located in cytoplasm and nucleus. In addition, there were 43.9% (18/41) MACC1 nucleus positive expression among MACC1 positive expression (Figure 3).

*Correlation between MACC1 and PFKFB2 protein expression with clinical features*

By comparing the relationship between the two protein expression and clinical characteristics, we found that PFKFB2 was significantly correlation with serm α-fetoprotein level (*P*=0.020), TNM stage (*P*=0.027), portal vein invasion (*P*= 0.037)and Edmonson-Steiner classification (*P*=0.002) , and there was no correlation between PFKFB2 expression and age , gender, HBV

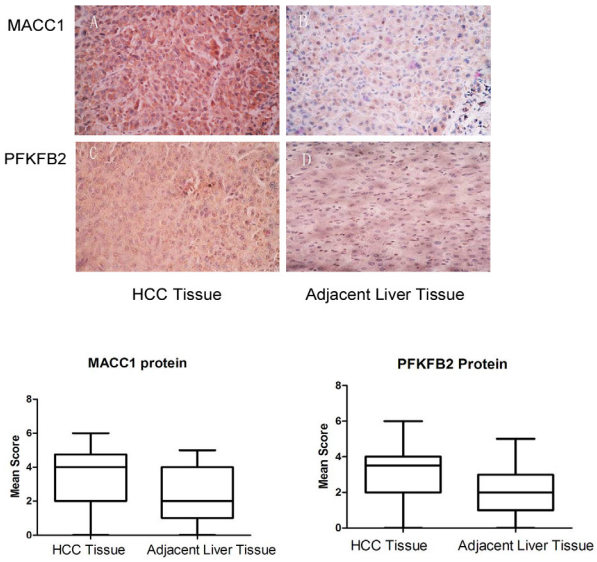


**Figure 2. Immunohistochemical Staining of MACC1 and PFKFB2 in HCC Tissues and Adjacent Liver Tissues.** A, B) The expression of MACC1 protein in HCC tissues and matched adjacent liver tissues (60). C, D) The expression of PFKFB2 protein in HCC tissues and matched adjacent liver tissues (n=60).Values are mean ± standard error. $P < 0.05$

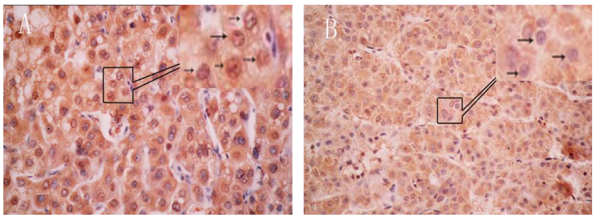

**Figure 3. A) MACC1 Nucleus Positive, B) MACC1 Nucleus Negative**

infection, live cirrhosis and (Table 1). MACC1 protein expression were significantly correlation between the TNM stage (*P*=0.027) and Edmonson-Steiner classification (*P*=0.007) , there was no correlation with other characteristics (Table 1). In addition, MACC1 nucleus positive was just significantly associated with Edmondson classification (*P*=0.01) (Table 2). Spearman correlation analysis found that the expression of MACC1 has a positive correlation with the expression of PFKFB2 (0R=3.375 95%CI=1.196-11.762, Spearman correlation=0.300, *P*=0.02).

*Survival associated with MACC1 expression and PFKFB2 expression in HCC patients*

There were 60 patients followed up by us, 55 patients was retrospective analyzed in 36 months and the median survival time of them was 34 month. The median survival time of MACC1 positive expression were 22 months, which were lower than the negative expression (>36 months). The median survival time of PFKFB2 positive expression was lower than the negative expression (24month vs 36month). The overall survival rate of MACC1 positive expression and PFKFB2 positive expression was higher than the negative expression in respectively 1 year ( 73.2% vs 100%, 75.6%vs100%) and 3 year (36.2% vs 75.0%, 39.3% vs 73.3%). Long rank test showed that the overall survival rate between the positive expression of the above two proteins and the negative expression have a significant difference (*P* <0.05). In addition, MACC1 nucleus positive expression has a poor overall survival compared to MACC1 nucleus negative expression (*P*=O.OO7) (Figure 1).

**Table 1. Distribution of MACC1 Positive Tumors in Groups Defined by Clinicopathological Variables**

| Variables | MACC1 | | | PFKFB2 | | |
|---|---|---|---|---|---|---|
| Groups | positive | negative | *p* value | positive | negative | *p* value |
| Age | | | | | | |
| ≥45 | 24 | 12 | 0.734 | 21 | 15 | 0.325 |
| <45 | 17 | 7 | | 17 | 7 | |
| Gender | | | | | | |
| Male | 27 | 14 | 0.544 | 29 | 12 | 0.081 |
| Female | 14 | 5 | | 9 | 10 | |
| HBV Infection | | | | | | |
| Yes | 28 | 14 | 0.672 | 28 | 14 | 0.413 |
| No | 13 | 5 | | 10 | 8 | |
| Liver Cirrhosis | | | | | | |
| Yes | 28 | 15 | 0.394 | 29 | 14 | 0.294 |
| No | 13 | 4 | | 9 | 8 | |
| AFP(ng/ml) | | | | | | |
| ≤400 | 27 | 14 | 0.554 | 8 | 11 | 0.02 |
| >400 | 14 | 5 | | 30 | 11 | |
| Tumor Size(cm) | | | | | | |
| ≤5 | 27 | 16 | 0.142 | 24 | 19 | 0.055 |
| >5 | 14 | 3 | | 14 | 3 | |
| Edmondson | | | | | | |
| I+II | 25 | 18 | 0.007 | 22 | 21 | 0.002 |
| III+IV | 16 | 1 | | 16 | 1 | |
| TNM Stage | | | | | | |
| I+II | 20 | 15 | 0.027 | 20 | 15 | 0.027 |
| III+IV | 21 | 4 | | 21 | 4 | |
| Intrahepatic Metastases | | | | | | |
| Yes | 16 | 5 | 0.337 | 14 | 7 | 0.435 |
| No | 25 | 14 | | 24 | 15 | |
| Portal Vein Invasion | | | | | | |
| Yes | 13 | 7 | 0.695 | 9 | 11 | 0.037 |
| No | 28 | 12 | | 29 | 11 | |
| Capsular Invasion | | | | | | |
| Yes | 21 | 8 | 0.511 | 19 | 10 | 0.734 |
| No | 20 | 11 | | 19 | 12 | |

**Table 2. Distribution of MACC1 Nucleus Positive Tumors in Groups Defined by Clinicopathological Variables**

| Variables groups | | MACC1 nucleus | | |
|---|---|---|---|---|
| | | positive | negative | *p* value |
| Edmondson | I+II | 7 | 18 | 0.01 |
| | III+IV | 11 | 5 | |
| TNM Stage | I+II | 7 | 13 | 0.262 |
| | III+IV | 11 | 10 | |

*Cox proportional hazard regression analysis*

Analysis the affect of age, sex , TNM stage, Edmonson-Steiner classification , intrahepatic metastases, MACC1, PFKFB2 for the prognosis of patients with HCC in Cox regression model. Univariate analysis showed that TNM stage, Edmonson-Steiner classification ,

**Table 3. Cox Proportionalhazard Regression Analysis of MACC1 and PFKFB2 Expression and the Relationship Between Clinicopathologic Features and Overall Survival Rate of HCC Patients**

| Clinicopathologic features | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | RR (95%CI) | *p* value | RR (95%CI) | *p* value |
| MACC1 + vs - | 2.410 (1.096-5.296) | 0.007 | 2.827 (1.030-7.759) | 0.044 |
| PFKFB2 + vs - | 1.985 (1.028-3.833) | 0.019 | 2.140 (0.691-6.626) | 0.187 |
| TNM I+IIvs III+IV | 0.451 (0.271-0.750) | 0.001 | 1.890 (0.081-4.457) | 0.146 |
| Edmonson I+IIvsIII+IV | 0.371 (0.242-0.567) | <0.001 | 0.354 (0.145-0.865) | 0.023 |

MACC1 and PFKFB2 were the prognosis factors of HCC patients. Multvariate analysis showed that MACC1 and Edmongson-Steiner classification were the independent prognosis factors of the HCC patients (Table 3).

## Discussion

Warburg Effect is reported by Otto Warburg, ect in the 1920s.He found that tumor cells have a high glycolytic rate compared to normal cells, even in the presence of adequate oxygen levels (WARBURG, 1956) .And now the glycolysis of the tumor has became a hot spot in the field of cancer research. At the same time , it is also provided a new train of thought for the treatment of athe cancer (Pelicano et al., 2006; Scatena et al., 2008).

Previous study found that several cancer cell lines express elevated levels of pfkfb enzymes, which catalyses both the biosynthesis and degradation of Fru-2, 6-P2 , and the Fru-2, 6-P2 is an signal metabolite that controls glycolysis (Minchenko et al., 2005; Bobarykina et al., 2006; ). Recent study found that the activation PI3K/Akt signaling pathway contribute to enhance the function of PFKFB2 (Moon et al., 2011; Novellasdemunt et al., 2013), which is essential in the regulation of glycolysis in heart. In our study, we found that the rate of PFKFB2 protein positive expression in HCC was increased compared to the corresponding non-tumor liver tissues, and the increased PFKFB2 protein expression was significantly associated with TNM stage, Edmondson-Steiner classification and also implied an poor overall survival.

MACC1 was a newly found oncogene in 2009.Some study found that MACC1 can contribute to the neoplasm growth, invasion and metastasis by the activation of HGF/c-MET signaling in several tumor (Boardman, 2009; Kokoszynska, et al., 2009; Stein et al., 2009). In our study, we found that MACC1 protein expression was significantly associated with high Edmonson-Steiner classification and advanced TNM stage,which was in consistent with previous study in several tumor. In addition, previous study found that MACC1 protein would translocate from the cytoplasm into the nucleus gradually with the progression of tumor (Gao, et al., 2013). In our study, we also found this phenomenon , there was higher MACC1 nucleus stain positive rate in high Edmondson stage when compared to in lower stage ($P$<0.05). Moreover , the MACC1 nucleus stain positive HCC patients had a lower survival. In a word, in our data, we found that this phenomenon of MACC1 protein translocate from the cytoplasm into the nucleus meant a poor prognosis for HCC patients and this strengthen the role of MACC1 protein as a prognosis for HCC patients.

As we known, previous study had found that MACC1 contributed to the activation of HGF/c-MET signaling pathway, and PI3K/Akt signaling pathway, as a part of the downstream of HGF/c-MET, was well-known to be one of the basic elements in the development of many tumor, involved in tumor cell growth , differentiation and apoptosis (Gottlob K FAU Majewski et al., 2001; Bijur GN FAU Jope et al., 2003; ). While recent study found that the activation of PI3K/Akt signaling pathway increased PFKFB2 phophorylation and Fru-2, 6-P2 production (Moon et al., 2011; Novellasdemunt et al., 2013), but did not increased the expression of PFKFB2 . But our present data show that highly MACC1 expression was associated with PFKFB2 expression .Moreover, some study had found that PI3K/Akt signaling pathway involved in the glucose metabolism of tumor (Gottlob K FAU Majewski et al., 2001; Neary CL FAU Pastorino et al., 2013; Roberts DJ FAU Tan-Sah et al., 2013). In a word, the previous study and our present data all indicated that MACC1 might affect the tumor glucose metabolism by controlling expression and phophorylation of PFKFB2 protein through PI3K/Akt signaling pathway, and our data also found that when we knockdown MACC1 expression , the concentration of glucose and lactic acid was lower than control group (the data are still unpublished) , but we are still unclear what mechanism implied in them.

In this study, there was still some shortcoming, the number of patient was not enough, and the following was short. But this study had made some beneficial attempts in the field of MACC1 in metabolism of liver cancer.MACC1 as a new founded gene, there was still no report about its function in the tumor metabolism. The study provide us valuable reference for next depth study.

In conclusions, our study show that highly expressed MACC1 and PFKFB2 protein were associated with TNM stage, Edmondson –Steier classification and overall survival.MACC1 was closely related with PFKFB2 expression. and we thought MACC1 may affect the tumor metabolism in part through control the expression and phophorylation of PFKFB2.

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