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# Impaired autophagy response in human hepatocellular carcinoma

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ABSTRACT

*Background:* Autophagy is a cellular lysosomal degradation mechanism that has been implicated in chronic liver diseases and hepatocellular carcinoma (HCC). Association of autophagy defect with the development of human HCC has been shown in transgenic mouse model.
*Aim:* We performed this study to verify whether a defect in autophagy would play a role in human hepatocellular carcinoma (HCC).
*Methods:* Archival tissue sections of 20 patients with HCC with or without hepatitis C virus (HCV) infection were studied. All slides were immunostained using monoclonal antibodies to p62 and glypican-3 with appropriate positive and negative controls. The expression of p62 and glycican-3 in the HCC and the surrounding non-tumor was semiquantitated. The cytoplasmic staining was graded as negative, weak or strong.
*Results:* Positive p62 staining was found in 20 out of 20 (100%) HCCs and negative staining was observed in 20 out of 20 non-tumor areas and cirrhotic nodules. Positive glypican-3 staining was found in 70% of HCCs and negative staining was seen in all non-tumor areas. An autophagy defect leading to increased expression of p62 and glypican-3 was also seen in the HCC cell line (Huh-7.5), but not in the primary human hepatocytes. Activation of cellular autophagy in Huh-7.5 cells efficiently cleared p62 and glypican-3 expression and inhibition of autophagy induced the expression of p62 and glypican-3.
*Conclusions:* This study shows that p62 is increased in HCC compared to the surrounding non-tumorous liver tissue suggesting that human HCCs are autophagy defective. We provide further evidence that glypican-3 expression in HCC may also be related to defective autophagy. Our study indicates that p62 immunostain may represent a novel marker for HCC.



## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the incidence of HCC is increasing in the Western world (Befeler and Di Bisceglie, 2002; El-Serag and Rudolph, 2007; Thorgeirsson and Grisham, 2002). In the majority of cases, HCC develops as a result of chronic inflammation and cirrhosis secondary to hepatitis B and hepatitis C viral infection (HBV, HCV) and non-viral etiologies including non-alcoholic and alcoholic fatty liver diseases (Rustgi, 1987). HCCs detected at a very early stage are treatable, but HCCs diagnosed at later stages are difficult to treat and have worse prognosis. Therefore early diagnosis and development of newer targeted therapy are urgently needed to improve HCC patient survival.

The serum alpha-fetoprotein (AFP) level has been used as a marker for diagnosis and early detection of HCC (Johnson, 2001). However, it is not specific for HCC since elevated AFP levels have also been detected in a considerable number of patients with chronic liver disease and liver cirrhosis (Collier and Sherman, 1998; Sherman, 2001). Recently, a number of studies have demonstrated that glypican-3 is another reliable tumor marker for hepatocellular carcinomas (Jakubovic and Jothy, 2007; Kandil et al., 2007; Mounajjed et al., 2013; Shirakawa et al., 2009). Glypican-3 immunostaining shows strong membranous and cytoplasmic staining of HCC and the expression was undetectable in normal and cirrhotic livers. Glypican-3 is a membrane-bound proteoglycan localized on the cell membrane of hepatocellular carcinoma (Filmus et al., 2008). Glypican-3 has been considered a better marker compared to AFP in the diagnosis of early HCC (Capurro et al., 2003; Hippo et al., 2004; Man et al., 2005; Wang et al., 2006; Yamauchi et al., 2005). The mechanism as to why glypican-3 is expressed at a high level only in the tumor and not in the surrounding non-tumor liver is unknown. This could be due to the incomplete understanding of the complex molecular mechanisms linking to the multifactorial etiology involved in hepatocarcinogenesis. Recent studies have suggested that glypican-3 expression in HCC is regulated by the expression of Sulfatase-2, c-myc and microRNAs (Lai et al., 2008; Li et al., 2012; Maurel et al., 2013). Since these reports have not been consistent, additional mechanisms of glypican-3 regulation in HCC need to be explored. A search for a highly reliable tissue marker for the detection of early HCC is needed.

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Autophagy is an evolutionary conserved lysosomal degradation process occurring in chronic liver diseases including viral hepatitis, alcoholic liver disease and fatty liver disease (Eskelinen and Saftig, 2009). Cellular autophagy, which is maintained during chronic liver injury, may play an important role in the sustainment of chronic liver disease and cancer development (Kotsafti et al., 2012; Rautou et al., 2010; Rautou et al., 2010). In this regard, there is evidence to suggest that mice with deletion of autophagy related genes, i.e. ATG 5 and ATG 7, develop liver adenoma (Chen and Karantza-Wadsworth, 2009; Cui et al., 2013; Takamura et al., 2011). The involvement of a highly conserved cellular autophagy process in human hepatocarcinogenesis is unknown and needs to be explored.

This study was performed to verify our hypothesis that defective autophagy may play a role in the development of hepatocellular carcinoma in humans. Cellular autophagy is a multistep process that begins with an initiation step, followed by a nucleation step, an elongation step and finally a maturation step Wang et al., 2008. It is thought that several cellular compartments including the endoplasmic reticulum (ER), the Golgi apparatus and plasma membrane participate in the autophagy process. Cellular autophagy process is controlled by complex interactions of many cellular proteins, but how these protein–protein interactions when altered lead to autophagy defect is not well understood. To confirm that HCC cells are autophagy defective, we examined the accumulation of p62, which has been used as a marker for autophagy deficiency (Chen and Karantza-Wadsworth, 2009). This protein is used as an index for autophagy flux measurement since p62 is degraded during the autophagy process (Kirkin et al., 2009; Puissant et al., 2012). A monoclonal antibody to p62 was used to determine autophagy defect in HCC samples. The expression of p62, an autophagy defect marker, was compared with glypican-3, another reliable marker for human hepatocellular carcinoma. Our results indicate that 100% of viral and non-viral related HCC samples have positive expression of p62, and the adjacent non-tumor areas are negative for p62. We also found that an impaired expression of glypican-3 in HCC cells is related to autophagy defect, since activation of cellular autophagy by mTOR inhibitor clears the expression of p62 and glypican-3.

## Materials and methods

### *Tissue specimens*

Paraffin blocks of 20 HCCs from patients with and without hepatitis C virus infection were obtained from the Department of Pathology, The Mount Sinai Medical Center, New York. Hematoxylin and eosin (H&E)-stained sections of all specimens including cancer and non-cancer areas of the liver tissue were examined by three pathologists (SNT, TW and KM).

### *Antigen retrieval and immunohistological staining*

Five-micron tissue sections were prepared and the slides were deparaffinized for 15 min at 50–60 °C followed by treatment with xylene twice for 5 min. The tissue sections were rehydrated by sequential treatment with 100%, 95% and 80% alcohol. Peroxidase quenching was carried out by incubation with 3% hydrogen peroxide and 100% methanol for 5 min. The slides were placed in a plastic Coplin jar with Reveal Decloaker RTU (Biocare Medical) for 25 min at 95 °C in a steamer for heated antigen retrieval. Following this step, the slides were allowed to cool down at room temperature for 20 min. The tissue sections were rinsed in deionized distilled water and marked using a PAP pen. The slides were incubated with a blocking sniper (Biocare Medical) for 10 min and incubated with a primary antibody for 1 h at room temperature. The primary antibodies we used were p62 mouse monoclonal antibody (Cell Signaling) (1:200 dilution) and pre-diluted antibody to glypican-3 (Biocare Medical). After the primary antibody incubation, slides were washed 3 times in Tris Buffered Saline (TBS) (pH 8.0), and incubated with a MACH 4 mouse probe (Biocare Medical, UP534) for 20 min and MACH 4 HRP Polymer (Biocare Medical, MRH534) for 30 min each, then washed 3 times using TBS. Finally, tissue sections were treated with diaminobenzidine (DAB) chromogen (Dako Cytomation, Carpinteria, CA) for 1–5 min. The slides were then counterstained with hematoxylin for 30 s and Tacha's bluing Solution (Biocare Medical, HTBLU) for 30 s, dehydrated with 95% and 100% alcohol, mounted and observed by light microscopy.

### *Evaluation of immunohistochemical staining*

Immunohistochemical staining of HCC tissue sections was examined by two pathologists (TW and KM). Scores were assigned to the intensity and percentage of positive staining of all the slides used in this study. Score 0 means negative staining, score (+) when 1–10% of cells were positive, score (++) when 10–50% of cells were positive and score (+++) when 50–100% cells were positive. Discrepancies were resolved by a consensus between the two pathologists using a multiheaded microscope in the Pathology Department, Tulane University Health Sciences Center. H&E-stained sections of all specimens including cancer and non-cancer cases were examined by the same two pathologists following the immunohistochemical evaluation.

### *Immunostaining of cultured hepatoma cells and primary human hepatocytes*

Cultured Huh-7 cells and primary human hepatocytes (Xenotech) were mounted onto glass slides via Cytospin. The cells were washed twice with 10 mM PBS pH 7.4 (Sigma-Aldrich, St Louis, MO) for 5 min, fixed in chilled acetone for 15 min and then permeabilized by treatment with Reveal Decloaker RTU (Biocare Medical, RV 100) for 25 min at boiling point. Slides were then cooled down to room temperature for 20 min. Blocking was performed utilizing Background Sniper (Biocare Medical, BS966) for 10 min at room temperature. The cells were incubated with monoclonal anti-p62 antibody (Cell Signaling) at 1:200 diluted with Da Vinci Green Diluent (Biocare Medical, PD900) for 1 h at room temperature. Following the primary antibody incubation, the cells were washed 3 times in Tris Buffered Saline (pH 8.0), and incubated with MACH 4 mouse probe (Biocare Medical, UP534) for 20 min. After the mouse probe treatment, the cells were incubated with MACH 4 HRP Polymer (Biocare Medical, MRH534) for 30 min, and cells were washed with TBS 3 times. Next, the cells were treated with diaminobenzidine (DAB) chromogen (Dako Cytomation, Carpinteria, CA) for 5 min. The slides were counterstained with hematoxylin for 30 s and Tacha's bluing Solution (Biocare Medical, HTBLU) for 30 s, dehydrated with 95% and 100% alcohol, mounted and observed by light microscopy.

## Results

### *Expression of p62 in human hepatocellular carcinomas*

The expression of p62 was examined following immunocytochemical staining of 20 archival formalin-fixed, paraffin-embedded HCC samples and their surrounding non-tumorous liver tissue. Most of the specimens used in our study have liver cirrhosis and all have HCC with or without HCV infection (Table 1). The expression of p62 was absent in the control livers and cirrhotic nodules. Most of the HCCs showed positive expression of p62 with a variation in the staining intensity. The expression of p62 in HCC was localized mostly in the cytoplasmic vacuoles and also in perinuclear in some samples (Fig. 1). The specimens with high expression of p62 and low expression of p62 are shown in Table 1. p62 expression was not detectable in non-tumor areas and cirrhotic nodules. The expression of p62 was detected in 20 out of 20 tumor specimens. There was no correlation between the amount of p62 expression and the degree of differentiation, i.e. well