Research Article

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SUOX is a promising diagnostic and prognostic biomarker for hepatocellular carcinoma

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Background & Aims: To investigate diagnostic and prognostic values of sulfite oxidase (SUOX) in patients with hepatocellular carcinoma (HCC) who underwent curative resection.

Methods: We investigated immunohistochemically the expression dynamics of SUOX, aldo-ketoreductase family 1 member B10 (AKR1B10) and CD34 at different stages of HCC. The differential diagnostic performance of three markers or their combinations in high-grade dysplastic nodules (HGDNs) and well-differentiated small HCC (WD-sHCC) were investigated by logistic regression models and validated in an independent testing set. Overall survival (OS) and time to recurrence (TTR) were evaluated in 300 patients with HCC as the testing cohort, and validated in 198 patients with HCC.

Results: SUOX was decreased and AKR1B10 and CD34 were increased with the stepwise progression of hepatocarcinogenesis. For differential diagnosis of WD-sHCC from HGDNs, the sensitivity and specificity of the SUOX + AKR1B10 + CD34 combination for WD-sHCC detection were 93.8% and 95.2%, respectively, and overall accuracy was much higher than any of the three individual markers and two marker combinations. In addition, SUOX, but not AKR1B10 and CD34, was an independent prognostic factor for OS and TTR, and showed better correlation with OS and TTR if combined with serum α-fetoprotein (AFP) for both the testing and validation cohorts.

Conclusions: SUOX + AKR1B10 + CD34 combination could make a substantial contribution to hepatic immunopathological diagnosis to distinguish WD-sHCC from HGDNs. Meanwhile, SUOX combined with serum AFP may predict postoperative outcome and tumor recurrence risk.

Keywords: High grade dysplastic nodules; Well-differentiated small hepatocellular carcinoma; Sulfite oxidase; Aldo-keto reductase family 1 member B10; CD34; Diagnosis; Prognosis.

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; DN, dysplastic nodules; HGDN, high-grade dysplastic nodules; WD-sHCC, well-differentiated small hepatocellular carcinoma; SUOX, sulfite oxidase; AKR1B10, aldo-ketoreductase family 1 member B10; MD-sHCC, moderately differentiated HCC; AFP, α-fetoprotein; OS, overall survival; TTR, time to recurrence; ROC, receiver operating characteristic.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors in the world and 82% of cases occur in developing countries (55% in China) [1]. HCC occurs mainly in patients with chronic liver diseases such as hepatitis B virus (HBV) or hepatitis C virus infection-based liver cirrhosis. Dysplastic nodules (DNs) are precancerous lesions of HCC and high-grade DNs (HGDNs) have a high risk of malignant transformation [2–5]. However, detection of DNs, especially HGDNs, and correct differentiation from well-differentiated small HCC (WD-sHCC), are sometimes difficult on the basis of clinical, imaging, and even morphological examination. Although current progress in imaging techniques has increased the frequency of detection of small liver lesions, there are still issues to be explored such as low specificity for identifying their nature [6,7].

Although it has been reported that CD31 and CD34 could serve as distinguishing biomarkers for HCC or sHCC from DNs or HGDNs, the sensitivity or specificity of the above-mentioned immunohistochemical markers is still limited. For instance, CD31 or CD34 staining positive capillaries usually shows a smaller difference between HGDNs and HCC [8,9]. Therefore, it has been questioned whether CD31 or CD34 alone can be used to distinguish HCC from HGDNs because of their low specificity. Thus, immunohistochemical markers that can assist in the differential diagnosis between WD-sHCC and HGDNs are still needed.

Recently, we have reported that sulfite oxidase (SUOX) could be a candidate immunohistochemical marker for distinction of sHCC from DNs [10], and Satow *et al.* have reported that aldo-keto

reductase family 1 member B10 (AKR1B10) is increased in HCC [11].

To the best of our knowledge, differential diagnosis between WD-sHCC and HGDNs using SUOX and the prognostic value of SUOX have not yet been studied. Therefore, in the present study, we analyzed the expression pattern of SUOX, AKR1B10, and CD34 in liver cirrhosis (LC), low-grade DNs (LGDNs), HGDNs, WD-sHCC, and moderately differentiated HCC (MD-sHCC), and the diagnostic accuracy of panels of markers using SUOX, AKR1B10, and CD34. In addition, four differential diagnostic models were established by logistic regression analyses to evaluate their diagnostic value to distinguish WD-sHCC from HGDNs, and were externally validated in an independent testing set. In addition, we also evaluated the prognostic value of SUOX, and demonstrated that SUOX in combination with serum α-fetoprotein (AFP) was an independent prognostic factor for overall survival (OS) and time to recurrence (TTR).

Materials and methods

Patients and specimens

The current study was designed according to the REMARK guidelines for reporting prognostic biomarkers in oncology [12]. The inclusion and exclusion criteria used in the current study were: (i) pathological diagnosis of hepatocellular lesions; (ii) consistent with histological diagnostic criteria of WHO; (iii) without pre-operative anti-cancer treatment and no evidence of extrahepatic metastases; and (iv) complete follow-up data. Institutional review board approval and written informed consent from each patient were obtained.

As the diagnostic group, 202 formalin-fixed paraffin-embedded (FFPE) tissues of liver nodules (LC = 60, DNs = 67, and sHCC = 75) were randomly collected retrospectively from patients who underwent curative resection between 2005 and 2011 at the Eastern Hepatobiliary Surgery Hospital (EHBH), Second Military Medical University, Shanghai, China (diagnostic group in Supplementary Table 1).

According to inclusion and exclusion criteria, a total of 1568 eligible cases were identified from the two hepatic surgery departments of EHBH between May 2003 and September 2006. Using computer–generated random numbers via SPSS software, 300 patients were selected for follow-up and used as testing group of prognosis. In parallel, we assessed another randomly collected, validation cohort collected as follows: according to inclusion and exclusion criteria, a total of 1011 eligible cases were identified from the same two hepatic surgery departments of EHBH between January 1996 and September 2001. Using computer–generated random numbers via SPSS software, 198 patients were selected for follow-up and used as validation group of prognosis (prognostic group in Supplementary Table 1 and Supplementary Fig. 1).

Complete follow-up data for patients in the prognostic group were available. Patients were followed until October 2010. The overall survival (OS) was defined as the length of time between surgery and death or the last follow-up examination. The time to recurrence (TTR) was calculated from the date of tumor resection until the detection of tumor recurrence, death or last observation. Detailed follow-up procedures are described in Supplementary data.

The mean follow-up of the testing cohort was 57.8 months (range, 1–90 months) and the postoperative cumulative survival and recurrence rates (in brackets) at 1, 3, and 5 years were 71% (57%), 57% (44%), and 55% (38%), respectively. In the validation cohort, the mean follow-up was 39.3 months (range, 1–141 months) and the postoperative cumulative survival and recurrence rates (in brackets) at 1, 3, and 5 years were 61% (43%), 42% (28%), and 34% (20%), respectively. Computed tomography (CT) and/or magnetic resonance imaging (MRI) and an elevated serum AFP level (>20 ng/ml as positive) were used to verify tumor recurrence in suspected cases.

Hematoxylin and eosin (HE)-stained slides were made from each FFPE tissue and were reviewed by two experienced hepatopathologists (WM Cong and H Dong). Diagnosis of LC, LGDNs, and HGDNs was based on the criteria proposed previously [13,14]. The hepatocytes in LGDNs appeared normal or showed minimal nuclear atypia and slightly increased nucleus to cytoplasm (N:C) ratio, but mitotic figures were absent. HGDNs were identified if there was cytological and/or structural atypia, but insufficient for diagnosis of WD-sHCC. The cytological atypia may have been diffuse or focal and was characterized by nuclear hyperchromasia, nuclear contour irregularities, cytoplasmic basophilia or clear cell change, high N:C ratio, and occasional mitotic figures. Architecturally, the cell plates were thickened by up to three cells, with occasional foci of pseudoglandular formation. All WD-sHCC and MD-sHCC in the diagnostic group were <3 cm in diameter. WD-sHCC (early HCC) was mainly diagnosed based on the following major histological features proposed by the World Health Organization: (i) increased cell density, more than twice that of the surrounding liver, with increased N:C ratio; (ii) irregular, thin trabecular pattern or growth; (iii) pseudoglandular structures; (iv) fatty changes; (v) unpaired arteries; (vi) intratumoral portal tracts; and (vii) stromal invasion.

Tumor stage was defined according to the 2002 American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis (TNM) classification system [15].

Tissue microarrays and immunohistochemistry and scoring

Four hundred and forty-seven specimens were selected randomly and tissue microarrays were constructed from two representative cores from each specimen. Immunohistochemistry was performed and integrated optical density (IOD) was measured as reported previously [10]. The imaging system comprised a Leica CCD camera, DFC420, connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions, Cambridge, UK). Photographs of two representative fields were captured from each core under high-power magnification (200×) using Leica QW in Plus v3 software. Therefore, the IOD of a total of four photographs was counted and measured using Image-Pro Plus v6.0 software (Media Cybernetics, Bethesda, MD, USA) with the three parameters: area sum, mean density, and IOD. Finally, mean IOD was calculated from four photographs and used as IOD of specimen. Primary antibodies were diluted as follows: a mouse monoclonal antibody against SUOX (ab88346; Abcam, Hong Kong; 1:200 dilution, cytoplasmic staining), a mouse polyclonal antibody against AKR1B10 (H00057016; Abnova, Walnut, CA, USA; 1:500 dilution, cytoplasmic staining), a mouse monoclonal antibody against CD34 (ab6330, clone BI-3C5; Abcam, Hong Kong; 1:50 dilution, vascular endothelial cell staining). Immunostaining scores were independently evaluated by two pathologists (WM Cong and H Dong), who were blinded to the clinicopathological data. The mean percentage value of two cores was considered representative of one tumor, and discrepancies were resolved by consensus.

The intensity of immunostaining was scored on the basis of the percentage of positive cells: 0 (0–5%), 1 (6–25%), 2 (26–50%), and 3 (>51%) for SUOX and AKR1B10. For CD34, negative and positive were defined as reported previously [16,17], with minor modification: cases showing staining of no or only a few sinusoids were defined as negative (0), and those showing diffuse staining of sinusoidal endothelium throughout the lesion area were regarded as positive, and intensity of immunostaining was scored as weak (1), moderate (2), and strong (3). The immunostaining was considered negative if the final score was 0 (–), and positive if the final score was 1 (+), 2 (++), or 3 (+++). The final score in each case was calculated from both cores.

Diagnostic model construction and validation of diagnostic efficiency

HGDN (n = 21) and WD-sHCC (n = 32) scores from immunohistochemistry were used in diagnostic model construction (training set). The scores (0–3) for SUOX, AKR1B10, and CD34 were subjected to binary logistic regression using the method of "enter" to generate differential diagnostic models for detection of WD-sHCC. The output was the diagnostic score in the range of 0–1. During model construction, the diagnostic score of an HGDN lesion was defined as 0, whereas that of a WD-sHCC lesion was defined as 1. The predictive probability of this model was applied to the same data set (HGDN = 21, WD-sHCC = 32), and receiver operating characteristic (ROC) analysis was performed. The ROC curve showed sensitivity plotted against 1 – specificity for each cut-off value and it was used to determine the best cut-off of each combination for regression analyses. The sensitivity and specificity for each cut-off value were plotted, thus generating ROC curves. The score was selected as the cut-off value, which was closest to the point with both maximum sensitivity and specificity.

The differential diagnostic models were used to classify HGDN and WD-sHCC cases in the independent validation set (HGDN = 21, WD-sHCC = 24). The diagnostic scores, which were calculated from the model using the immunostaining scores of SUOX, AKR1B10, and CD34 of individual cases, were used as an index for classifying WD-sHCC and HGDNs.

Statistical analysis

Statistical analyses were carried out with SPSS 13.0 software (SPSS, Chicago, IL, USA). The relationship between the expression of biomarkers and hepatocellular tumors was analyzed by calculating Spearman's correlation coefficient (r).