Oncogenic Wnt3a expression as an estimable prognostic marker for hepatocellular carcinoma

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AIM: To investigate member 3a of Wingless-type MMTV integration site family (Wnt3a) expression in cancerous and surrounding tissues and the relationship between clinicopathologic features of hepatocellular carcinoma (HCC) and Wnt3a expression.

METHODS: Wnt3a expression and cellular distribution and clinicopathologic characteristics in cancerous tissue and matched surrounding tissues were analyzed in 80 HCC patients from January 2006 to August 2008 by tissue microarrays and immunohistochemistry. The overall and disease-free survival rates were estimated using the Kaplan-Meier method and compared with the log-rank test. The prognostic analysis was carried out with univariate and multivariate Cox regressions models.

RESULTS: The incidence of oncogenic Wnt3a expression in the cancerous group was up to 96.25% (77 of 80), which was significantly higher ($\chi^2 = 48.818, P < 0.001$) than that in the surrounding group (46.25%, 37 of 80). Brown Wnt3a staining gradually increased with clinical staging that showed very strong staining in advanced HCC. The clinicopathologic features of high Wnt3a expression in HCC were related to poorly-differentiated grade ($\chi^2 = 20.211, P < 0.001$), liver cirrhosis ($\chi^2 = 8.467, P < 0.004$), hepatitis B virus (HBV) infection ($\chi^2 = 12.957, P < 0.001$), higher tumor-node-metastasis stage ($\chi^2 = 22.960, P < 0.001$), and 5-year survival rate ($\chi^2 = 15.469, P < 0.001$).

CONCLUSION: Oncogenic Wnt3a expression asso-
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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and rapidly fatal malignancies worldwide[1,2]. It develops mostly in cirrhotic livers, and risk factors include chronic persistent infection by the hepatitis B viruses (HBV) and C viruses (HCV) as well as non-viral liver diseases[3,4]. HCC is a highly chemo-resistant cancer with no effective systemic or well-established adjuvant therapies, and prognosis remains poor because of its high recurrence[5,6]. Hepatic resection or transplantation is the only potential curative treatment for HCC patients[7,8]. Unfortunately, the molecular mechanisms of hepatocarcinogenesis remain poorly understood. Recently, abnormal expression of some key molecules in the Wingless (Wnt)/β-catenin pathway was associated with the development and progression of HCC[9,10]. The Wnt proteins are secreted glycoproteins that regulate multiple cellular events, such as cell proliferation, differentiation, and apoptosis through β-catenin- dependent canonical and β-catenin-independent non-canonical pathways, and play an important role in HCC prognosis[11,12].

The member 3a of Wnt-type MMTV integration site family (Wnt3a) is a canonical Wnt ligand[13,14]. Its gene is located on human 17 chromosome (17q21) and has been regarded as an activator of the canonical Wnt signaling pathway, inducing β-catenin accumulation and activating the canonical Wnt signaling pathway[15]. Studies on human Wnt3a have focused primarily on its key role in human malignancy, and its high expression in many kinds of tumors was correlated with a worse outcome[15-17]. However, limited data are available on the relationship between Wnt3a and HCC, and its prognostic value remains to be explored. The objective of the current study, therefore, was to investigate alterations in the expression of oncogenic Wnt3a in cancerous tissues and to explore its clinical utility as a novel molecular marker for HCC prognosis.

MATERIALS AND METHODS

Liver specimen

Human liver cancer and surrounding tissues in this study were obtained from 80 HCC patients who underwent operations between January 2006 and August 2008 at the Affiliated Hospital of Nantong University, Jiangsu Province, China. The tissues were immediately frozen in liquid nitrogen and kept at -80 ℃ until required. The patient cohort inclusion and exclusion criteria included: (1) accurate pathologic diagnosis of primary HCC; (2) complete clinicopathologic and follow-up data; and (3) previously untreated, with surgery as the first treatment. Thus, analysis of the data would reflect the actual impact of tumor biology on the clinical outcome. This study was approved by the Ethics Committee permission (No. TDFY2013008) of the Affiliated Hospital of Nantong University, and all patients signed informed consent. The related important clinical information of each patient was collected from their medical records.

Clinical data of HCC patients

The patient characteristics are listed in Table 1. The patient population consisted of 62 males and 18 females, with a median age of 51 years (range: 35-74). The sizes of tumors were 44 cases with > 5 cm and 36 with < 5 cm. α-fetoprotein (AFP) levels were 28 cases with ≥ 400 ng/mL and 52 with < 400 ng/mL. The tumor number was 64 cases with single and 16 with multiple. Tumor differentiation was graded using the Edmondson grading system[18] with 5 well-, 41 middle-, and 34 poor- differentiation. Tumor staging was 36 cases (45%, 36 of 80) at I and II and 44 cases (55%, 44 of 80) at III and IV based on the 6th edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer (IUAC). The incidence of hepatitis B surface antigen (HBsAg) was 81.25% (65 of 80) in these HCC tissues, and 63.75% (51 of 80) had a history of liver cirrhosis. All patients had regular follow-up. The follow-up period was defined as the duration from the date of the operation to the date of either death or the last follow-up. The last follow-up was October 2013. Overall survival (OS) was evaluated by the duration between the dates of surgery and death. During the follow-up period, a total of 48 patients died.

Pathologic examination and HCC diagnosis

Histopathologic examination of the livers with...
hematoxylin and eosin (HE) staining showed that all cancerous tissues were accurate pathologic diagnosis of HCC according to the World Health Organization criteria. HCC diagnosis was confirmed according to the criteria set by the Chinese National Collaborative Cancer Research Group. Tissue microarrays

All HCC tissues were stained by HE staining and reviewed by two histopathologists. Representative areas free from necrotic and hemorrhagic materials were marked in paraffin blocks. Tissue cores (2.0 mm) were taken from each representative tumor tissue and peritumoral tissue to construct the tissue microarray (TMA) slides.

Immunohistochemistry

The TMA slides used for immunohistochemistry (IHC) were deparaffinized, and peroxidase was quenched with methanol and 3% H2O2 for 15 min. For antigen retrieval, the sections were boiled under pressure in citrate buffer (pH 6.0) for 3 min. After that, the tissues were incubated for 120 min with primary mouse anti-Wnt3a (ab81614; Abcam, Cambridge, United Kingdom) at 1:50 dilution. Following washing with phosphate buffered saline (PBS), the sections were incubated with horseradish peroxidase conjugated goat anti-mouse IgG (A21010; Abcam, Cambridge, United Kingdom) for 15 min at 1:5000 dilution and washed again with PBS. The sections were incubated in diaminobenzidine solution, counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and cover-slipped. For the negative control reactions, PBS was used instead of the primary antibody.

Evaluation of the IHC findings

The results of IHC staining were assessed by two independent pathologists without knowledge of the clinicopathologic features. The percentages for Wnt3a-positive cells were scored as follows: 0 (0%), 1 (1%-33%), 2 (34%-66%), and 3 (67%-100%). Staining intensity was stratified into four categories: 0 (negative), 1 (weakly), 2 (moderate), and 3 (strongly). The sum of the percentage and intensity score was defined as the IHC staining score. According to above criterion, the HCC tissues with Wnt3a expression were divided into two groups: low with 0-2 scores and high 3-6 scores. The higher score for positive percentages and staining intensity was taken as the final score when there was a difference between duplicate tissue scores.

Statistical analysis

Statistical analysis was carried out by using SPSS software (version 20.0, Armonk, NY, United States). The difference between HCC and para-cancerous groups was analyzed by χ2 test. Quantitative variables were analyzed by Mann-Whitney U test (Z). The relationship between Wnt3a expression and clinicopathological factors was analyzed by χ2 test. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. Factors shown to be of prognostic significance in the univariate models were evaluated in a multivariable Cox regression model. For all tests, the significance level for statistical analysis was set at P < 0.05.

RESULTS

Hepatic Wnt3a expression in cancerous and surrounding tissues

The over-expression of hepatic Wnt3a and its cellular distribution in cancerous or surrounding tissues analyzed by immunohistochemistry with human anti-Wnt3a antibodies are shown in Figure 1. Positive Wnt3a staining, identified as brown particles, was distributed in the cytosol and the membrane of hepatocytes or only a few cell nuclei. The incidence of hepatic Wnt3a expression in cancerous tissues (96.25%, 77 of 80) was significantly higher (χ2 = 48.818, P < 0.001) than that in surrounding tissues (46.25%, 37 of 80). Liver Wnt3a staining and its expression frequency in cancerous or surrounding tissues are shown in Table 2. The liver Wnt3a staining scores were significantly lower than those in cancerous tissues.

Table 1  Clinical characteristics of hepatocellular carcinoma patients

| Parameter                  | n    |
|---------------------------|------|
| Age (yr)                  | 51 (35-74) |
| Gender                    |      |
| Male                      | 62   |
| Female                    | 18   |
| Differentiation           |      |
| Poor                      | 34   |
| Moderate                  | 41   |
| Well                      | 5    |
| Tumor number              |      |
| Single                    | 64   |
| Multiple                  | 16   |
| Liver cirrhosis           |      |
| Absent                    | 29   |
| Present                   | 51   |
| HBV infection             |      |
| Absent                    | 15   |
| Present                   | 65   |
| Periportal embolus        |      |
| Absent                    | 9    |
| Present                   | 71   |
| AFP (ng/mL)               |      |
| < 400                     | 52   |
| ≥ 400                     | 28   |
| TNM staging               |      |
| I - II                    | 36   |
| III-IV                    | 44   |
| Five-year survival        |      |
| Yes                       | 32   |
| No                        | 48   |

HBV: Hepatitis B virus; AFP: α-fetoprotein; TNM: Tumor-node-metastasis.
different ($Z = 8.144, P < 0.001$) between cancerous tissues and surrounding tissues, with strong expression (3-6 scores) in 71.25% (57 of 80) of cancerous tissues and low or no expression (0-2 scores) in 86.25% (69 of 80) of surrounding tissues.

**Relationship between Wnt3a expression and HCC staging**

The relationship between hepatic Wnt3a expression and clinical staging of HCC is shown in Figure 2. According to the IUAC clinical staging criteria of HCC patients, there were nine cases at staging I (11.25%, 9 of 80), 27 at II (33.75%, 27 of 80), 38 at III (47.5%, 38 of 80), and six at IV (7.5%, 6 of 80) among 80 cancerous tissues. The incidences of high or low Wnt3a expression in HCC tissues were 22.2% (two of nine) or 77.8% (seven of nine) at I staging, 51.9% (14 of 27) or 48.1% (13 of 27) at II staging, 94.7% (36 of 38) or 5.3% (two of 38) at III staging, and 83.3% (five of six) or 16.7% (one of six) at IV staging, respectively. Brown Wnt3a expression was gradually increased in different clinical stages, with very strong staining at the advanced stage.

**Clinicopathological characteristic of Wnt3a expression**

The correlation between Wnt3a levels and the clinicopathological parameters of 80 HCC patients is shown in Table 3. High Wnt3a expression was associated with poorly-differentiated grade ($P < 0.001$), liver cirrhosis ($P = 0.004$), HBV infection ($P < 0.001$), and higher TNM stage ($P < 0.001$). By contrast, there were no significant associations between hepatic Wnt3a expression and other clinicopathological features, such as HCC patients’ gender or age, tumor number, circulating AFP level, and periportal cancer embolus.

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**Table 2 Incidence of liver Wnt3a expression and its scores with staining strength in cancerous and surrounding tissues**

| Group      | $n$ | Wnt3a | $\chi^2$ | P value | Wnt3a strength | $Z$ value | P value |
|------------|-----|--------|----------|---------|----------------|-----------|---------|
|            |     | Neg.   | Pos.     |         | 0              | 1-2       | 3-4     | 5-6     |         |         |
| HCC        | 80  | 3      | 77       | 48.818  | < 0.001        | 3         | 20      | 34      | 23      | 8.274   | < 0.001 |
| Para-can   | 80  | 43     | 37       |         | 41             | 28        | 11      | 0       |         |         |         |

1Compared with the para-cancerous group. HCC: Hepatocellular carcinoma; Para-can: Para-cancerous tissues; Neg.: Negative Wnt3a expression; Pos.: Positive Wnt3a expression.
As expected, the overexpression of hepatic Wnt3a showed a significant relationship with 5-year survival of the 80 HCC patients ($P < 0.001$). Meanwhile, certain HCC clinical prognostic factors, such as liver cirrhosis ($P = 0.035$) and HBV infection ($P = 0.029$), also showed a statistically significant correlation with 5-year survival rate based on Cox regression univariate analysis (Table 4). All these factors were enrolled in a multivariable analysis. High Wnt3a expression ($P < 0.001$), liver cirrhosis, and HBV infection were independent risk factors for decreased 5-year survival in HCC patients.

![Figure 2](image_url)  
**Figure 2** Expression of hepatic Wnt3a at different hepatocellular carcinoma stages. A1 and B1: Low or no Wnt3a expression in the para-cancerous tissues. A2-A5 and B2-B5: The brown staining of Wnt3a gradually increases in cancerous tissues from stage Ⅰ to Ⅳ. A1-A5: Original magnification × 200; B1-B5: Original magnification × 400.

| Groups                          | Patients, $n$ | % of total | Wnt3a high expression, $n$ | % of total | $\chi^2$ | $P$ value |
|---------------------------------|--------------|------------|----------------------------|------------|----------|-----------|
| Differentiation                 |              |            |                            |            |          |           |
| Poor                            | 34           | 42.50      | 31                         | 91.18      | 20.211   | $< 0.001$ |
| Moderate                        | 41           | 51.25      | 26                         | 63.41      |          |           |
| Well                            | 5            | 6.25       | 0                          | 0.00       |          |           |
| Tumor number                    |              |            |                            |            | 0.976    | 0.323     |
| Single                          | 64           | 80.00      | 44                         | 68.75      |          |           |
| Multiple                        | 16           | 20.00      | 13                         | 81.25      |          |           |
| Liver cirrhosis                 |              |            |                            |            | 8.467    | 0.004     |
| Absent                          | 29           | 36.25      | 15                         | 51.72      |          |           |
| Present                         | 51           | 63.75      | 42                         | 82.35      |          |           |
| HBV infection                   |              |            |                            |            | 12.957   | $< 0.001$ |
| Absent                          | 15           | 18.75      | 5                          | 33.33      |          |           |
| Present                         | 65           | 81.25      | 52                         | 68.00      |          |           |
| Periportal embolus              |              |            |                            |            | 3.557    | 0.059     |
| Absent                          | 9            | 11.25      | 4                          | 44.44      |          |           |
| Present                         | 71           | 88.75      | 53                         | 55.56      |          |           |
| AFP (ng/mL)                     |              |            |                            |            | 1.020    | 0.313     |
| $< 400$                         | 52           | 65.00      | 39                         | 75.00      |          |           |
| $\geq 400$                      | 28           | 35.00      | 18                         | 25.00      |          |           |
| TNM staging                     |              |            |                            |            | 22.960   | $< 0.001$ |
| Ⅰ - Ⅱ                          | 36           | 45.00      | 16                         | 44.44      |          |           |
| Ⅲ - Ⅳ                          | 44           | 55.00      | 41                         | 55.56      |          |           |
| Five-year survival              |              |            |                            |            | 15.469   | $< 0.001$ |
| Yes                             | 32           | 40.00      | 15                         | 46.88      |          |           |
| No                              | 48           | 60.00      | 42                         | 53.12      |          |           |

HBV: Hepatitis B virus; AFP: α-fetoprotein; TNM: Tumor-node-metastasis.

**High Wnt3a expression with shorter survival in HCC**

As expected, the overexpression of hepatic Wnt3a showed a significant relationship with 5-year survival of the 80 HCC patients ($P < 0.001$). Meanwhile, certain HCC clinical prognostic factors, such as liver cirrhosis ($P = 0.035$) and HBV infection ($P = 0.029$), also showed a statistically significant correlation with 5-year survival rate based on Cox regression univariate analysis (Table 4). All these factors were enrolled in a multivariable analysis. High Wnt3a expression ($P < 0.001$), liver cirrhosis, and HBV infection were independent risk factors for decreased 5-year survival in HCC patients.

Table 3  Clinicopathological characteristics of high Wnt3a expression in 80 hepatocellular carcinoma patients

| Groups                          | Patients, $n$ | % of total | Wnt3a high expression, $n$ | % of total | $\chi^2$ | $P$ value |
|---------------------------------|--------------|------------|----------------------------|------------|----------|-----------|
| Differentiation                 |              |            |                            |            |          |           |
| Poor                            | 34           | 42.50      | 31                         | 91.18      | 20.211   | $< 0.001$ |
| Moderate                        | 41           | 51.25      | 26                         | 63.41      |          |           |
| Well                            | 5            | 6.25       | 0                          | 0.00       |          |           |
| Tumor number                    |              |            |                            |            | 0.976    | 0.323     |
| Single                          | 64           | 80.00      | 44                         | 68.75      |          |           |
| Multiple                        | 16           | 20.00      | 13                         | 81.25      |          |           |
| Liver cirrhosis                 |              |            |                            |            | 8.467    | 0.004     |
| Absent                          | 29           | 36.25      | 15                         | 51.72      |          |           |
| Present                         | 51           | 63.75      | 42                         | 82.35      |          |           |
| HBV infection                   |              |            |                            |            | 12.957   | $< 0.001$ |
| Absent                          | 15           | 18.75      | 5                          | 33.33      |          |           |
| Present                         | 65           | 81.25      | 52                         | 68.00      |          |           |
| Periportal embolus              |              |            |                            |            | 3.557    | 0.059     |
| Absent                          | 9            | 11.25      | 4                          | 44.44      |          |           |
| Present                         | 71           | 88.75      | 53                         | 55.56      |          |           |
| AFP (ng/mL)                     |              |            |                            |            | 1.020    | 0.313     |
| $< 400$                         | 52           | 65.00      | 39                         | 75.00      |          |           |
| $\geq 400$                      | 28           | 35.00      | 18                         | 25.00      |          |           |
| TNM staging                     |              |            |                            |            | 22.960   | $< 0.001$ |
| Ⅰ - Ⅱ                          | 36           | 45.00      | 16                         | 44.44      |          |           |
| Ⅲ - Ⅳ                          | 44           | 55.00      | 41                         | 55.56      |          |           |
| Five-year survival              |              |            |                            |            | 15.469   | $< 0.001$ |
| Yes                             | 32           | 40.00      | 15                         | 46.88      |          |           |
| No                              | 48           | 60.00      | 42                         | 53.12      |          |           |

HBV: Hepatitis B virus; AFP: α-fetoprotein; TNM: Tumor-node-metastasis.
Table 4 Univariate and multivariable analysis of prognostic factors in hepatocellular carcinoma for 5-year survival

| Variable                        | Univariate analysis          | Multivariable analysis          |
|---------------------------------|------------------------------|---------------------------------|
|                                 | HR  | P > |z|  | 95%CI  | HR  | P > |z|  | 95%CI  |
| Age (yr) < 60 vs > 60           | 0.828 | 0.662 | 0.355-1.930 |
| Gender Male vs female           | 1.005 | 0.990 | 0.440-2.296 |
| Differentiation Low vs high     | 1.037 | 0.914 | 0.539-1.993 |
| Tumor number Single vs multiple | 0.867 | 0.720 | 0.397-1.893 |
| Liver cirrhosis Absent vs present | 2.101 | 0.035 | 1.053-4.195 | 2.021 | 0.033 | 1.057-3.864 |
| HBV infection Absent vs present | 0.327 | 0.029 | 0.120-0.892 | 0.325 | 0.010 | 0.138-0.765 |
| Periportal embolus Absent vs present | 0.499 | 0.360 | 0.113-2.210 |
| AFP (ng/mL) < 400 vs ≥ 400      | 0.938 | 0.725 | 0.655-1.342 |
| TNM staging I, II vs III, IV     | 0.686 | 0.318 | 0.328-1.436 |
| Wnt3a expression Low vs high    | 5.656 | < 0.001 | 2.682-11.926 | 3.651 | < 0.001 | 1.973-6.757 |

HBV: Hepatitis B virus; AFP: α-fetoprotein; TNM: Tumor-node-metastasis.

DISCUSSION

HCC is one of the most frequently occurring tumors worldwide. Accumulating data have indicated that the Wnt/β-catenin pathway is associated with tumor progression by interacting with microenvironment and oncogenesis, and high expression of Wnt3a in different kinds of tumors was related to worse outcome. Wnt3a is an important regulator of HCC cell growth in vitro, and induction of Wnt3a activates the canonical Wnt pathway after binding with sulfatase 2 and glypican-3 and increases cell proliferation in nude mouse xenografts in vivo. However, limited data was available until now on the relationship between Wnt3a expression and HCC prognosis. In this present study, therefore, the alterations of oncogenic Wnt3a expression in cancerous tissues were investigated, and it was confirmed that Wnt3a may be a useful biomarker for HCC prognosis.

Recent advances have shown that, apart from autocrine stimulation by growth factors and the dysregulation of different growth regulatory pathways, the Wnt/β-catenin pathway is frequently involved in hepatocarcinogenesis. Growing evidence has highlighted the key molecules of the Wnt/β-catenin pathway in the control of HCC suppressors, growth, invasiveness, and curability. Wnt3a expression in a large array of HCC tissues demonstrated that HCC tissues exhibited unique immunostaining patterns and that the staining was positively correlated with the clinical stage. The incidence of oncogenic Wnt3a expression in the cancerous group was significantly higher than that in the surrounding tissue group.

Kaplan-Meier survival estimates, by WNT3a

Figure 3 Overall survival curves of 80 hepatocellular carcinoma patients. The Wnt3a expression curves were calculated by the Kaplan-Meier method. Survival curves of 80 hepatocellular carcinoma patients were made according to cancerous tissues expressing a low or high level of Wnt3a (log-rank test, P < 0.001). The green line is the high Wnt3a group; and the blue line is the low or without Wnt3a group.
Wnt3a staining was gradually increased with clinical staging and showed very strong staining in advanced HCC, indicating that hepatic Wnt3a expression might be related to HCC progression.

The management of advanced HCC is entering a new era of molecular targeting therapy, which is of particular significance for HCC because of the lack of effective systemic therapies for HCC. Although the clinical significance of Wnt3a expression in tissue has been demonstrated in different human malignancies[13,14,16], little data until now was available on the relationship between Wnt3a expression and HCC. In this study, the clinicopathologic features of high Wnt3a expression in HCC were reported and associated with the poorly-differentiated grade, liver cirrhosis, HBV infection, higher TNM stage, and a relatively shorter survival time. Furthermore, univariate and multivariate analyses suggested that high Wnt3a expression in HCC patients might be an independent prognostic indicator, like the factors liver cirrhosis and HBV infection[29,30].

There are some possible limitations associated with this study. We evaluated a relatively small number of patients, and the study design was retrospective. In addition, this study is limited by the inclusion of only patients with resectable tumors. It is possible that these findings pertain only to those patients who have undergone resection. A larger prospective study examining Wnt3a expression is necessary to validate further the usefulness of this system. Inhibiting Wnt3a overexpression may prevent invasive progression and metastatic relapse, which would improve the prognosis and quality of life for HCC patients after hepatic resection. Targeting the Wnt3a pathway may be a therapeutic strategy for HCC that could be used in combination with conventional chemotherapy or radiotherapy. Wnt3a may play an important role in promoting HCC progression and metastasis.

In conclusion, to the best of our knowledge, this is the first report to investigate cancerous Wnt3a expression and to indicate that it may be a novel prognostic marker for HCC. Multiple key factors along the Wnt/β-catenin signaling cascade likely represent potential diagnosis, prognosis, or therapeutic targets. The present findings are promising, and the initial evidence confirmed that Wnt3a is a key molecule in the Wnt/β-catenin pathway and further studies should clarify the molecular mechanisms of the upregulation of oncogenic Wnt3a expression and its important role in hepatocarcinogenesis[33,34].

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Pan LH et al. Wnt3a in HCC

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