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ORIGINAL ARTICLE

NOR1 expression and its relationship with prognosis in patients with hepatocellular carcinoma

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Summary

Purpose: *This study aimed to investigate the expression of NOR1 in hepatocellular carcinoma (HCC) tissue and its relationship with prognosis.*

Methods: *A sample of 60 specimens including HCC and adjacent normal tissues were collected from postoperative HCC patients from February to September 2011. Immunohistochemistry was used to detect the positive expression of the oxidored-nitro domain containing protein 1 (NOR1), the postoperative disease-free survival (DFS) rate and then the overall survival (OS) was analyzed by the Kaplan-Meier method.*

Results: *The results showed significantly lower expression of NOR1 in HCC tissue than that in adjacent normal tissue. Moreover, the OS and DFS rate of NOR1 (++) patients were significantly higher than those of other groups.*

Conclusion: *Consequently, NOR1 is a protective protein in HCC, and the increase of its expression is favorable for the prognosis of patients.*

Key words: *hepatocellular carcinoma, oxidored-nitro domain containing protein 1, prognosis*

Introduction

Primary HCC is one of the common malignant tumors in China. The standardized mortality ratio ranks third among a variety of malignant tumors after gastric cancer and esophageal cancer. The incidence of HCC is particularly high in the southeast coastal area of China, which seriously threatens the quality of life and life expectancy of urban residents in China. The occurrence of HCC is a gradual, dynamic and multi-gene regulatory pathological process. Therefore, understanding the molecular mechanism of the pathogenesis of HCC and screening based on relevant factors in high-risk groups can prevent and treat HCC [1,2]. In recent years, an association has been confirmed of NOR1 gene with the occurrence of HCC. NOR1, being an anti-cancer protein, plays a biological role by regulating autophagy in the tumor microenvironment or apoptotic molecular pathway. It was found that NOR1 gene is significantly reduced in nasopharyngeal carcinoma, but its mechanism of down-regulation and transcription regulation has not been elucidated yet. NOR1 protein is located in the mitochondria and cytoplasm, and can interact with oligomycin sensitivity confering protein (OSCP). Moreover, NOR1 expression inhibits the growth and proliferation of nasopharyngeal carcinoma cells and promotes hypoxia-induced apoptosis. However, the mechanism of NOR1 in the occurrence and development of HCC and its biological significance are still not clear. Currently, *in vitro* experiments have confirmed that it played an important role in the occurrence and development of HCC [3].

In the present study, by detecting the expression of NOR1 in HCC and adjacent normal tissues, we further explored its role in the development

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and progression of HCC, its pathological significance and its correlation with clinical indicators. This may provide a considerable help for the clinical diagnosis and treatment of HCC.

Methods

Experimental reagents

The following reagents were used: sodium oxalate anticoagulant, triethanolamine (Amresco,CA,USA), chloroform, dithiothreitol, citric acid, EDTA (Sigma,CA,USA), ferric chloride (Sigma,USA), diethyl oxydiformate (Haloccarbon,CA,USA), NH2SO4 (BDH Laboratory Supplies,UK), rabbit anti-human NOR1 antibody, HRP conjugated goat anti-rabbit IgG secondary antibody (Abcam,USA), and PBS (Glibco, CA,USA).

Experimental equipments

The experimental equipments used in this study were the following: 4 °C refrigerator (Thermo, USA); -80°C low temperature refrigerator (Thermo,USA); microtome (Leica, Germany); electronic balance (OHAUS,USA); thermostatic water bath (Thermo Fisher, USA); pipettes (Eppendorf, Germany); high pressure sterilization pot (Market Forge, USA); ultra-clean bench (Labconco, USA); inverted fluorescence microscope (Olympus, Japan); microscope imaging system (Lumenera, Canada); mortar (Dingguo Biotechnology Co. Ltd., Chongqing); PVDF membrane (0.45 microns) (Biyuntian Biotechnology Co. Ltd., Shanghai); stabilized voltage supply (Bio-Rad, USA); 37 °C thermostat incubator (Liuyi Instrument Factory, Beijing); Imaging analysis system software (Imaging Corporation, USA); glass homogenizer (Changhong Glass Instrument Factory, Zhuozhou); multifunctional microplate reader (Bio-Rad, model 680, Austria); and vortex mixer (Liuyi Instrument Factory, Beijing).

Clinical data collection

From January to September 2014, 60 HCC patients in the Digestive Department of our hospital were randomly selected on 1:1 matching method in accordance with the inclusion and exclusion criteria. The baseline, preoperative, intraoperative, postoperative clinical and pathological patient data were retrieved from the hospital's electronic medical records database. The inclusion criteria were: a) patients older than 18 years, with pathologically diagnosed HCC and b) patients who agreed to participate in the study and signed the informed consent. Exclusion criteria included patients who had a) liver metastases from other organs/systems; b) were smokers, drinkers, or taking one of the following drugs such as non-steroidal anti-inflammatory drugs, antipsychotics, anxiolytics, sedatives, tryptophan or beta-receptor blockers; c) participated in clinical trials of any drug or medical device 12 weeks prior to the commencement of this study, or planning to participate in other clinical trials during the study; and d) had moderate or severe liver, heart, lung, and kidney dysfunction, hematopoietic system diseases or psychiatric diseases.

Ethics of the study

This study followed the Helsinki Declaration. It was a retrospective study and was approved by the Medical Ethics Committee of the Third Hospital of Xiangya Medical College, Central South University. Informed consent was signed by all subjects in this study.

Exiting criteria

Exiting subjects were in fact those who had filled out the informed consent but could not continue the trial. For instance, subjects who experienced severe adverse reactions, complications, cardiac arrest, and rapid ventricular arrhythmia during the trial; subjects who asked to withdraw from the study and withdrew their informed consents and subjects for whom researchers discontinued the trial under the premise of safety.

Immunohistochemical staining and interpretation

Cancer and adjacent normal tissues were fixed in formalin solution, paraffin-embedded and sectioned. After heat-treated antigen retrieval, sections were washed with PBS for 3 times and incubated in 3% hydrogen peroxide (H_2O_2) at room temperature for 20 min. After washing, sections were incubated with primary antibody overnight at 4 °C, followed by triple washing in PBS. The sections were then incubated with enzyme-conjugated secondary antibody at room temperature for 20 min. Next, after washing with PBS the sections were incubated with diaminobenzidine (DAB) for 3-5 min under the microscope to monitor the color development. Then the sections were washed with distilled water for 3 times, and differentiated in Mayer hematoxylin for 10-20s until turning blue. After gradient ethanol dehydration, transparent with xylene images were observed and photographed. In positive cells which showed yellow-brown granules in the nucleus, semi-quantitative analysis was performed according to the number of positive cells and staining intensity and the results were categorized as follows: 0 points for no positive cell, 1 point for less than 25% positive cells, 2 points when the number of positive cells was between 26 and 50%, 3 points when the number of positive cells was greater than 50%. Based on the color staining the results were categorized as follows: 0 point for no color, 1 point for light brown color, 2 points for brown color, 3 points for strong brown color. The multiplied two scores of each section were categorized as follows: 0 points when the result was negative, 1-2 points for weak positive (±), 3-4 points for moderate positive (+) and 4 or more points for strong positive (++). Finally, 3 points or more were considered as NOR1 positive expression.

Statistics

The data was analyzed by SPSS 21.0 software (IBM, NY, USA). Quantitative data was analyzed by using one way analysis of variance (ANOVA). However, qualitative data were compared by *t*- test or chi-square test. If the data did not meet the assumptions, then the Fisher method was used to calculate the exact probability. For all test, $p<0.05$ was considered statistically significant.

The survival of patients with HCC was analyzed by Kaplan-Meier method and differences were assessed by log-rank test.

Results

Results of immunohistochemical staining

HCC tissues were stained by immunohistochemical streptavidin-peroxidase method and results were scored semi-quantitatively. There were 18 cases of NOR1 (-), 10 cases (±), 12 cases (+) and 20 cases (++). Furthermore, there was no significant difference in terms of age, time of onset, and the tumor volume among the 4 groups (Table 1).

Comparison of the positive expression rates of NOR1 in cancer and adjacent normal tissues of patients with HCC

The results in Table 2 show that the positive expression rate of NOR1 in cancer tissues of patients with HCC was significantly lower than that in adjacent normal tissue ($p<0.05$). However, the positive expression rate of NOR1 in the NOR1 (++) group was significantly higher than that in other groups ($p<0.05$).

Comparison of the prognosis of HCC patients with different NOR1 expression levels

In order to compare the postoperative DFS and OS of patients with different NOR1 expression levels, the Kaplan-Meier method was used. The results showed that DFS rates gradually decreased from NOR1 (++) to NOR1 (-) ($p<0.05$) and the same was observed for OS ($p<0.05$; Figure 1 A,B).

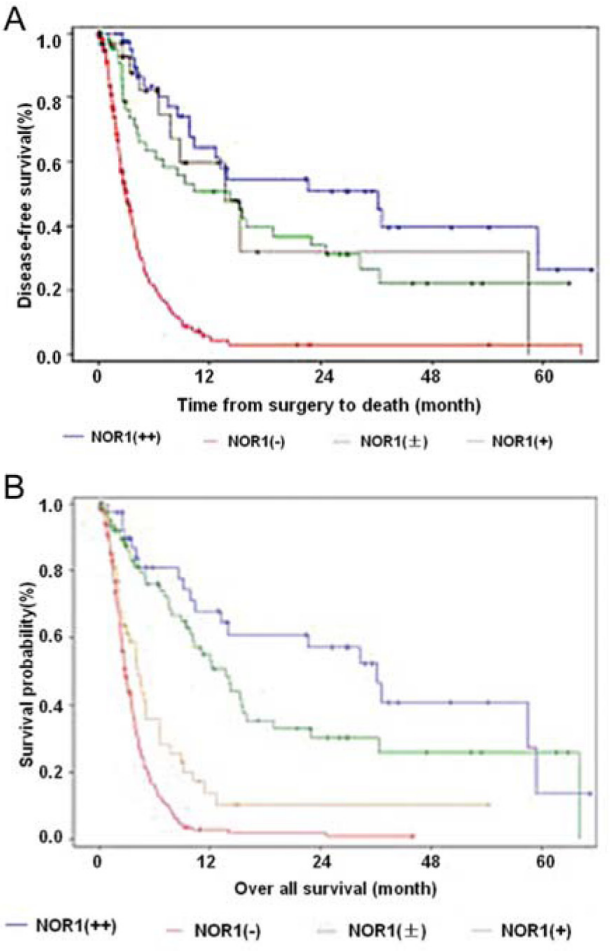

Figure 1. (A) Disease-free survival rates of patients with HCC. From NOR1 (++) to (-), disease-free survival rates of HCC patients gradually decreased (log rank test, Z=18.23, $p<0.05$). **(B)** Overall survival. The results suggested that the overall survival of patients with HCC was shortened gradually (log rank test, Z=8.39, $p<0.05$).

Table 1. Correlation of age and disease characteristics with different NOR1 expression levels

Group	*Cases, n*	*Age (years)* *Mean±SD*	*Duration of HCC (months)* *Mean±SD*	*Tumor volume (cm^3)* *Mean±SD*
(-)	18	51.3±10.9	19.2±8.1	63.3±12.3
(±)	10	52.4±11.3	20.8±9.3	61.5±10.8
(+)	12	58.3±12.8	18.2±8.1	59.4±9.7
(++)	20	54.6±10.9	20.4±9.4	65.3±8.8
t/x^2 value	-	0.43	0.79	0.44
p value	-	0.59	0.22	0.57

Table 2. Comparison of the different expression levels of NOR1 in cancer and adjacent normal tissues

Group	*Cases, n*	*Cancer tissue (%)* *Mean±SD*	*Adjacent normal tissue (%)* *Mean±SD*	*t value*	*p value*
(-)	18	0.17±0.2	74.3±9.8	12.8	0.014
(±)	10	18.7±4.8	72.1±8.4	10.8	0.016
(+)	12	41.4±3.3	70.5±7.3	9.6	0.033
(++)	20	57.4±12.5	75.3±10.2	7.2	0.044
t value	-	22.5	0.39	-	-
p value	-	0.007	0.67	-	-

Discussion

The occurrence of HCC is a gradual and dynamic process regulated by multiple genes [4]. The overall investigation and exploration for the molecular mechanism of the occurrence and development of HCC are conducive to the clinical screening, prevention, and treatment of HCC patients [5]. NOR1 is a novel nitro reductase gene found in 2003 by gene chip and bioinformatics prediction techniques [4-7]. In the last ten years since its discovery, the understanding of NOR1 gene has been gradually enriched [8], but now what we know about NOR1 gene is still little. Further study of NOR1 gene function and the mechanism of its abnormal expression is still a main task [9-13]. To develop genetically engineered anticancer drugs by using NOR1 related knowledge is the ultimate goal of our team commitment to NOR1 research. With more than ten years of research and development, our team has known the role and mechanism of NOR1 gene in human tumors such as nasopharyngeal carcinoma, testicular cancer, multiple myeloma etc. It has been initially believed that NOR1 is an anticancer protein and can play a biological role by regulating tumor microenvironment autophagy/apoptotic molecular pathways [14]. However, in recent years, several authors suggested that NOR1 gene overexpression significantly increased cancer cell apoptosis in *in vitro* studies of HCC cells [15]. In this study, liver tissue specimens obtained from 60 HCC patients were stained immunohistochemically to reveal the pathophysiological and clinical significance of NOR1 gene in the development and progression of HCC. Our study found that in all cases the expression of NOR1 in HCC tissue was significantly reduced. In addition, DFS and OS of patients with high expression of NOR1 protein were significantly higher than in patients with low expression of NOR1 ($p<0.05$). The expression level of NOR1 protein in cancer tissue was significantly lower than that in normal liver tissue. This result implies that NOR1 may play a protective/preventive role in HCC. However, compared with normal liver tissue, NOR1 in cancer tissue was significantly reduced, and therefore, increasing the expression level of NOR1 in cancer tissue by using specific targeting drugs may have some biological significance for improving the prognosis of HCC patients.

In summary, we believe that in the occurrence and development of HCC, NOR1 gene is a newly discovered tumor suppressor gene [4-7]. NOR1 gene affects the biological behavior of cells through a number of pathways, it can inhibit tumor cell growth and proliferation [16-18] and has important clinical significance for the survival and prognosis of HCC patients.

Conflict of interests

The authors declare no confict of interests.

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