ORIGINAL PAPER

Expression and clinical significance of NET-1 and PCNA in hepatocellular carcinoma

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Abstract *Objective* Objective of this study was to detect the expression of neuroepithelial transforming gene-1 (NET-1) and proliferating cell nuclear antigen (PCNA) in hepatocellular carcinoma (HCC) and adjacent tissues, and to investigate the relation of the expression of NET-1 in HCC tissue with cancer proliferation, metastasis and clinic stages. *Methods* The expression of NET-1 mRNA was detected by reverse transcription-polymerase chain reaction method in 34 human HCC tissues, and it was matched with 34 paracarcinoma tissues. The expression of PCNA in HCC was analyzed by immunohistochemistry. Meanwhile, the relation of the expression with clinic pathological features of HCC was evaluated, and the correlation between the expression of NET-1 and PCNA in HCC was investigated. *Results* Expression of NET-1 was significantly higher in HCC than that in matched paracarcinoma tissues. The expression of NET-1 was significantly higher in TMN III–IV HCC tissues when compared with TMN I–II HCC tissues ($P < 0.05$). The expression level of NET-1 in HCC tissues was related to intrahepatic metastasis and portal vein infiltration. The expression of NET-1 in HCC tissues was positively correlated with PCNA. *Conclusions* The expression of NET-1 may relate to proliferation, metastasis and clinic stages of HCC. The expression of NET-1 in HCC tissues may positively correlate to the TMN stages.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer in men and the eighth in women worldwide [1]. A detailed understanding of epidemiologic factors and molecular mechanisms associated with HCC ultimately may improve our current concepts for screening and treatment of this disease.

Cell homeostasis is regulated by the balance between proliferation and programmed cell death. That proliferation exceeds and disbalance between proliferative and apoptotic processes are fundamental features of neoplasms. Proliferating cell nuclear antigen (PCNA) and neuroepithelial transforming gene-1 (NET-1) have been the most widely used cell proliferative markers.

In this study, the expression of NET-1 mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) method in 34 human HCC tissues, and it was matched with 34 paracarcinoma tissues. The expression of PCNA in HCC was analyzed by immunohistochemistry. Meanwhile, the relation of the expression to clinic pathological features of HCC was evaluated, and the correlation between the expression of NET-1 and PCNA in HCC was investigated.

Materials and methods

Patients

Primary HCC tissues and corresponding paracarcinoma tissues were obtained from 34 HCC patients who underwent resection at the Renmin Hospital of Wuhan University. Two matched tumor samples were collected from each patient.

After surgical removal, all samples were immediately snap-frozen and stored in liquid nitrogen until further use. Some parts of tumor tissues were fixed in 4% paraformaldehyde overnight and embedded in paraffin. The diagnosis of HCC was confirmed by the pathologic separate criteria of WHO in 2000. The study group comprised 22 men and 12 women ranging in age from 38 to 66 years.

RNA extraction

Total RNA was extracted from 10 mg of each tissue sample using Trizol (Invitrogen Ltd, UK), according to the manufacturer's instructions. The concentration of total RNA was quantified by the absorbance at A_{260} and A_{280} through spectrophotometer.

Semi-nested reverse transcription-polymerase chain reaction

cDNA synthesis was carried out with the M-MULV reverse transcriptase (Invitrogen Ltd, UK) on 5 μg of total RNA mixed with random primers. Two milligrams of total RNA was treated with DNAse I and reverse-transcribed using random hexamers and SuperScript II reverse transcriptase (Invitrogen Ltd, UK). The PCR amplifications were performed as following. The 50 μl reaction mixture for PCR contained cDNA, specific primers, dNTPs, 10× Taq Buffer, Taq DNA Polymerase and $MgCl_2$. Each thermal cycle for amplification included denaturation at 94°C for 15 s, annealing temperature range from 45 to 65°C for 45 s and extension at 72°C for 1 min. This cycle was repeated 35–40 times with final extension for 5 min at 72°C. The following primers were used for PCR; primers specific for NET-1: forward 5′-GTGGGCATCTGGGTGTCA-3′ and reverse 5′-GCTCAGCCATTGTGGTGTA-3′. Amplifications yielded products of 186 bp. β-action was used as internal control (475 bp): forward 5′-TGACGGGGGTCACCCACACTGTG-CC-3′and reverse 5′-CTGCATCCTGTCGGCAATGCCA G-3′. Amplified PCR products were electrophoresed through 1.5% agarose gels (voltage: 100 V) stained with ethidium bromide. A 100 bp molecular DNA marker was run in each gel. Gels were illuminated with UV light and gel scanner (Kodak1D3.5, USA), then analyzed with an image analysis system and related software. Finally, we got the density indexes of production and used the ratio between density indexes of NET-1 lane and that of internal control as NET-1 expression level parameter.

Immunohistochemical staining

Immunohistochemistry S-P method was used to detect PCNA. The tissues were treated with endogenous peroxidase blocking solution at room temperature for 10 min and then incubated in normal nonimmune serum at room temperature for 10 min. The mouse anti-PCNA antibodies (DAKO Company, Denmark) were added to adjacent tissue sections respectively and incubated overnight at 4°C. Biotin-conjugated second antibody was added to the sections and incubated at room temperature for 10 min. S-P complex was added at room temperature for 10 min and then DAB was used for the color reaction. The tissue sections were washed with PBS between each step. Positive and negative controls were simultaneously used to ensure specificity and reliability of the staining process. A positive section was taken as positive control. In negative control, PBS was used to replace the first antibody. All the nuclei that stained brown (irrespective of intensity) were regarded as positive for PCNA. The PCNA labeling indexes were determined by counting at least 500 hepatocytes systematically in each high power field, and the percentages of PCNA-labeled nuclei were used as the cell proliferation indexes.

Statistical analysis

A statistical evaluation was performed using the Statistical Program for Social Sciences for Windows (SPSS, version 13.0). All experimental results and measurements were expressed as means ± standard deviation (SD). Differences between groups were examined for statistical significance using the Bivariate method and Student's t test. Values of $P < 0.05$ were considered statistically significant.

Results

Differential expression of Net1 in HCC tissues and paracarcinoma tissues

PCR confirmed elevated levels of Net1 expression in all cancer tissue specimens studied, in comparison with paracarcinoma tissues (Fig. 1). The positive rate of Net1 expression was 88.2% (30/34) and 75.5% (25/34), respectively.

The relation of the expression to clinic pathological features of HCC

Expression of NET-1mRNA was obviously different in each HCC tissues by TNM stages ($P < 0.01$), and the expression was higher if there was intrahepatic metastasis and portal vein infiltration. The density indexes of NET-1mRNA expression were not significantly different in HCC
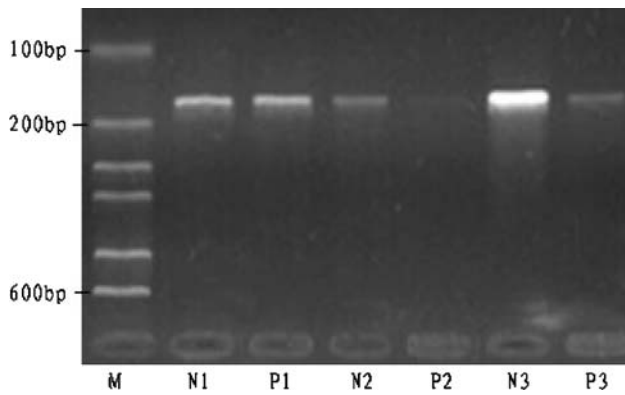
Fig. 1 RT-PCR of NET-1. *Lane M β*-action as internal control, *lanes N* the expression of NET-1 in HCC tissues, *lanes P* the expression of NET-1 in paracarcinoma tissues

tissues of Edmondson I–II and III–IV stages. There was no obvious correlation of expression of NET-1mRNA with the level of AFP (Table 1).

Expression of Net1 and PCNA in HCC tissues

The expression of PCNA can be seen from all HCC samples of NET-1mRNA positive expression. A positive linear correlation ($r = 0.490$) was observed between the expression of NET-1 and PCNA in HCC tissues.

Table 1 Relation of the expression to clinic pathological features of HCC

Clinic pathological features	Number	Density indexes of NET-1 mRNA expression	P
Tumor membrane			
Yes	12	0.47 ± 0.11	0.238
No	22	0.52 ± 0.12	
Intrahepatic metastasis			
Yes	7	0.62 ± 0.10	0.003
No	27	0.47 ± 0.00	
Portal vein infiltration			
Yes	8	0.63 ± 0.09	0.000
No	26	0.46 ± 0.09	
Edmondson classification			
I, II	21	0.50 ± 0.11	0.889
III, IV	13	0.51 ± 0.13	
TNM classification			
I, II	21	0.45 ± 0.08	0.000
III, IV	13	0.59 ± 0.10	
AFP			
Positive	29	0.51 ± 0.12	0.563
Negative	5	0.47 ± 0.11	

Discussion

Net1 is a member of the guanine nucleotide exchange factor (GEF) family, which is involved, through their regulation of RhoA activity, in a range of biological processes including cell proliferation, apoptosis, differentiation and cytoskeletal reorganization [2]. At the amino acid level, Net1 is most closely related to CD82, CO-029 and A15. A database search with the DNA sequence of cDNA clone NET-1 revealed absolute identity with a recently described gene NET-1 (accession number AF065388), which is a new member of the Tetraspanin/TM4SF family [3]. NET-1 locates at chromosome 1p34.1. Its mRNA span is 1,297 bp; the code sequence is 128–853 bp; it has an opening reading frame with 241 amino acids [4]. Some studies provided evidences that upregulation of NET-1 proteins is clearly associated with carcinogenesis [5].

There are few reports about the relation of the expression of NET-1 in HCC tissue to cancer proliferation, metastasis and clinic stages. In our study, we detected the expression of NET-1 mRNA by RT-PCR method in 34 human HCC tissues and matched it with 34 paracarcinoma tissues. We found that the cDNA expression of NET-1 is amplified. The positive rates in HCCs and paracarcinoma tissues were 88.2% (30/34) and 75.5% (25/34), respectively. Expression of NET-1mRNA was obviously different in each HCC tissues by TNM stages ($P < 0.01$), and the expression was higher if there was intrahepatic metastasis and portal vein infiltration. The density indexes of NET-1mRNA expression were not significantly different in HCC tissues of Edmondson I–II and III–IV stages. There was no obvious correlation of expression of NET-1mRNA with the level of AFP ($P > 0.05$). Whether tumor had the membrane or not, the expression did not change much ($P > 0.05$). It was thus evident that expression of NET-1 may relate to proliferation, metastasis and clinic stages of HCC. In this study, all paracarcinoma tissues had hyperplasy in pathological features. Moreover, most patients accompanied with a background of hepatitis or cirrhosis. The expression of NET-1 can be discovered in some parts of samples, but the positive rates were lower than that of HCC tissues.

Primary liver cancer, which consists predominantly of hepatocellular carcinoma (HCC), is the fifth most common cancer worldwide and the third most common cause of cancer mortality. HCC has several interesting epidemiologic features including dynamic temporal trends, marked variations among geographic regions, variation between racial and ethnic groups, variation between men and women and the presence of several well-documented environmental potentially preventable risk factors. Moreover, there is a growing understanding on the molecular mechanisms inducing hepatocarcinogenesis, which almost never occurs in healthy liver, but the cancer risk increases

sharply in response to chronic liver injury at the cirrhosis stage [6–8].

Dysplastic nodules (DNs) have been identified as premalignant lesions in the multistep process of hepatocarcinogenesis in humans. A clonal expansion of hepatocytes after the early carcinogenic events, in response to diffuse injury of the liver, lead to a clonal expansion of hepatocytes that spreads around adjacent portal structures. As the rest of the liver becomes scarred, developing to disease and cirrhosis, the island of clonal hepatocytes maybe remain intact and it is mainly composed of hepatic parenchyma. Then, the clonal expansion presents the appearance of a large cirrhotic nodule. Having already experienced the earliest transformation of hepatocarcinogenesis, the clonal hepatocyte expansion remains at augmented risk for later developments; thus, the lesion becomes the likeliest site for full malignant transformation [9].

It has been reported that balance between proliferation and apoptosis is important in the progression of hepatocarcinogenesis. Excessive cell accumulation during carcinogenesis can result not only from increased cellular proliferation, but also from diminished cell death [10].

PCNA is an auxiliary protein of DNA polymerase and is thought to play an important role in the elongation or replication of the DNA chain. The percentage of PCNA-positive cell is correlated with the proliferative activity and the prognosis of various malignant tumors [11–13].

Net1 is a guanine nucleotide exchange factor (GEF) that activates Rho family proteins [14]. The Net1 gene was originally isolated in a tissue culture screen for novel oncogenes in NIH 3T3 fibroblasts. Net1 regulates Rho-GTPases, a main branch of the Ras superfamily of small GTPases. Rho proteins, once activated, stimulate signaling in multiple pathways by binding to downstream effector proteins, modulating their activities and thereby regulating a range of cellular processes including cell proliferation, apoptosis, differentiation and cytoskeletal reorganisation. They are also thought to play a role in transformation and metastasis [15–17].

Our results demonstrated that the positive rates of PCNA and NET-1 increased significantly in the paratumorous tissue and HCC. The expression of NET-1 in HCC tissues was positively correlated with PCNA. The increase of cell proliferation and the integration of viral genome caused disturbed proteins synthesis abnormality in metabolism enzymes. So repeated degeneration, necrosis and hyperplasia occurred in the hepatitis. It might be a gradual developmental process from quantitative to qualitative change. So the expression of NET-1 also may relate to the proliferation, metastasis and clinic stages of HCC. The expression of NET-1 in HCC tissues may positively correlate with the TMN stages.

Net1 is a new member of the tetraspanin superfamily. Members of this family are molecular facilitators with many different functions. They play a role in signal transduction and regulate adhesion, migration, proliferation and differentiation of cells [18].

The molecules of the tetraspan superfamily (TM4SF) are characterized by the existence of four predicted transmembrane domains delimiting two extracellular regions of unequal size. These molecules have a significant sequence similarity to each other, and for some of them, a signature sequence is present between transmembrane domains 2 and 3. The tetraspans could play a role in interconnecting several cell surface molecules and coupling various cellular functions. This hypothesis is strengthened by the fact that mAbs directed to different tetraspans induce similar effects in relation to adhesion, cell migration and costimulation [19].

Activation of tetraspanins results in changes in cell morphology, cell–cell and cell–matrix adhesion, and motility. Crosslinking tetraspanin molecules on the cell surface can provide costimulatory signals, possibly by virtue of their association with lineage-specific signaling molecules. Most of the observed functions of tetraspanins relate to their ability to facilitate interactions between other proteins, generating functional complexes. The term "molecular facilitators" can be used to describe this general role [20].

As one of the new members of TM4SF, NET-1 may take part in the process of proliferation, canceration and metastasis during carcinomatous development. Among the tetraspans, CD81 is associated preferentially with the $\alpha 4\beta 1$ integrin, and CD151 with both $\alpha 3\beta 1$ and $\alpha 6\beta 1$. These two tetraspans are likely to be responsible for the connection of these integrins to other tetraspans, through tetraspan–tetraspan interactions. Other tetraspans may similarly link other molecules to the whole set of tetraspans. The organization of the tetraspans in a tetraspan web may allow the crosstalk of different kinds of associated molecules on the cell surface [21]. Whether NET-1 produces a marked effect by this pathway or not still needs to be clarified.

In conclusion, the expression of NET-1 may relate to the proliferation, metastasis and clinic stages of HCC. The expression of NET-1 in HCC tissues may positively correlate with the TMN stages. In addition, there was no expression of NET-1 mRNA in normal liver tissues, but there was more expression in HCC tissues (32/34), which showed that the NET-1 protein possibly has a certain value in the early diagnosis of liver cancer.

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