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Association of NET-1 Gene Expression With Human Hepatocellular Carcinoma

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NET-1 is a member of the NET-*x* family. To explore the potential role of NET-1 in hepatocellular carcinoma (HCC), the expression of NET-1 and the relationship with HCC were examined for the first time. We found that NET-1 was frequently expressed in HCC and the peritumor tissue. The relative amounts of NET-1 mRNA in HCC and peritumor tissue were 0.645 ± 0.37 and 0.466 ± 0.30, respectively, indicating a higher expression level in HCC than in the peritumor ($P < .05$). NET-1 protein is usually located on the cell membrane and in the cytoplasm of HCC cells. NET-1 immunoreactivity was found in 126 out of 130 samples of HCC tissue (96.92%). An association of NET-1 expression with cytological variants, histopathological grading, and clinical stages of HCC was also found ($P < .05$). Detection of NET-1 gene expression in liver biopsy may provide useful information about the biological behavior of HCC.

Keywords: NET-1; liver; carcinoma; gene expression; protein expression

Introduction

Hepatocellular carcinoma (HCC) is a common type of malignance worldwide, especially in Africa and Asia. Although significant progress has been made in both diagnosis and treatment in the past decades, the prognosis for HCC remains poor, resulting in nearly 1 million deaths annually.[1] One of the reasons for poor prognosis is that most patients are already in the late stage of the disease when they are clinically diagnosed. In China, the overall resection rate of HCC is only around 20%, and many cancer patients are left even without surgical therapy.[2] Current early detection of liver carcinoma involves several serum markers, among which A-fetoprotein (AFP) has been the best for liver carcinoma diagnosis. However, increase in the AFP level in sera may not always indicate an early stage of the cancer.[3] There have been reports of several other serum markers for HCC, including matrix metalloproteinases (MMP),[4] E-selectin,[5] vascular endothelial growth factor,[6] tissue polypeptide specific antigen (TPA), and des-gamma-carboxy prothrombin (DCP),[7,8] although none of them have provided a more reliable diagnosis for HCC than serum AFP.

NET-1 is a member of the NET-*x* family, currently including NET-2, NET-3, NET-4, NET-5, NET-6, and NET-7. All NET genes were originally identified as EST clones with sequences homologous to tetraspan, a superfamily that is distinguished by the presence of 4 transmembrane domains defining 2 extracellular regions with conserved amino acid residues,[9-11] and all have been implicated in signal transduction, cell adhesion, migration, proliferation, and differentiation.[12-13] Deregulation of the expression of NET-*x* and several other members of the tetraspan family, including CD9, CD63, CD82, and CO-029, have been found in certain types of human tumors.[9-10] So far, there have been no reports about the involvement and significance of NET-*x* in HCC.

In this study, we examined the expression of NET-1[14] in human liver and HCC tissue for the first time by reverse transcription-polymerase chain reaction (RT-PCR), fluorescent immunohistochemisty,

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and Western blot. The relationship between NET-1 expression and the clinicopathological factors of human HCC was also examined. We found that NET-1 was frequently expressed in HCC tissue at a higher level than in peritumor tissue, showing an association with cytological variants, histopathological grading, and clinical stages of HCC.

Materials and Methods

Materials

GetRare cDNA Panels[15] (Genemed, South San Francisco, CA), including a total of 53 cases of various normal and cancerous tissue samples, were used in this study for screening for the expression of NET-1. This panel includes 3 groups: Group 1 contains 29 items of human normal tissue and cells in adults—namely, adrenal gland, bone marrow, brain, cervix, colon, heart (2 cases), kidney, leukocyte, liver (2 cases), lung, lymph node, mammary gland, pancreas, pituitary gland, placenta, prostate, rectum, retina, salivary gland, skeletal muscle, small intestine, spinal cord, spleen, stomach, testis, thymus, thyroid, and uterus. Group 2 contains 7 items of human normal fetal tissue: fetal brain, fetal heart, fetal kidney, fetal liver, fetal lung, fetal muscle, and fetal skin. Group 3 contains 11 items of human cancerous tissue and cells: liver cancer, lung cancer (2 cases), leukemia (3 cases), lymphoma (2 cases), melanoma, pancreatic cancer, kidney cancer (2 cases), rectal cancer, stomach cancer, uterus cancer, and Hela cells.

Twenty-eight cases of HCC and corresponding peritumor tissue (2 cm beyond tumor foci) and 130 cases of paraffin-embedded sections were obtained from Nantong Tumor Hospital and Affiliated Hospital, Nantong University (Jiangsu, China). Freshly isolated tissue samples were stored at −70°C for future use.

1.3 SMMC-7721 HCC cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China).

Methods

Screening of NET-1 gene expression in a series of human normal or cancerous tissue samples by RT-PCR. RT-PCR was performed in screening of 53 cases of normal and cancerous tissue for NET-1 RNA expression. PCR amplification was carried out in a 50 μL reaction buffer containing 5 μL (50 ng) of each type of GetRare cDNA; 40 mM Tricine-KOH, pH 9.2; 15 mM KOAc; 3.5 mM $Mg(OAc)_2$; 0.2 μM 5′ NET-1 primer (5′-CAG-TTC-CCT-CTT-TCA-GAA-CTC-ACT-G-3′); 0.2 μM 3′ NET-1 primer (5′-ATC-CAC-CCA-GAG-GCT-CTG-CTG-ATT-TCA-CCT-3′); 0.2 mM dNTP; and 1 μL of Advantage™ cDNA polymerase mix (50×, containing KlenTaq-1 and Deep Vent polymerases, Clontech, CA). The PCR cycling was set at 1 cycle of 94°C for 20 seconds, followed by 30 cycles of 96°C for 6 seconds (denaturation), 60°C for 20 seconds (annealing), and 72°C for 1 minute (extension). After PCR, 5 μL of the DNA product was analyzed in 1% agarose gel in the presence of ethidium bromide. Appearance of the expected amplicon of 1159 base pairs (bp) was defined as the sign for a positive expression (+). Otherwise, negative expression (−) was assigned. DNA sequencing was used to confirm the band of 1159 bp as the amplicon.

Semiquantitative RT-PCR analysis of normal liver and tumor tissue samples. Total RNA was prepared using RISO reagent (Genemed, South San Francisco, CA). Poly(A)+ RNA was purified using Oligotex mRNA Mini Kit (QIAGEN, Valencia, CA). Semiquantitative PCR was performed by coamplification of the NET-1 gene and β-actin. A 50 μL PCR reaction contained approximately 50 ng of double-stranded cDNA; 40 mM Tricine-KOH, pH 9.2; 15 mM KOAc; 3.5 mM $Mg(OAc)_2$; 0.2 μM 5′ NET-1 primer; 0.2 μM 3′ NET-1 primer; 0.1 μM 5′ β-actin primer (5′-TTA-CAC-CCT-TTC-TTG-ACA-AAA-CCT-A-3′); 0.1 μM 3′ β-actin primer (5′-CAA-AAG-CCT-TCA-TAC-ATC-TCA-AGT-3′); 0.2 mM of dNTP; and 1 μL of Advantage™ cDNA Polymerase Mix. PCR was carried out as described above. The predicted amplicon was 1159 bp for NET-1 and 800 bp for β-actin. The density of bands was measured by Four-Star image analysis software, and the relative quantity of NET-1 expression was calculated based on the ratio NET-1/β-actin.

Preparation of NET-1 polyclone antibody. A peptide of amino acid sequence NYTDFEDSPYFKENS linked to cysteine at the N-terminus was used to generate polyclonal antibody in rabbits with the help of American San Francisco Gene Biotechnology Inc. Polyclonal antibody against a peptide of ISEISRAS-GWMCRRHFKMHKPVTN was used as a negative control.

Western blot analysis. Cancerous and peritumor tissue samples were lysed and centrifuged at 1200 rpm

at 4°C for 15 minutes. The supernatants were stored at −70°C. Proteins were fractionated on a gradient SDS polyacrylamide gel (4-12%) and electro-transferred onto a PDVF (polyvinylidene diflouride) membrane (Bio-Rad). Sample loading and transfer efficiency were confirmed by staining with 0.5% Ponceau S in 1% acetic acid; this was followed by 3 washes with TBST buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, and 0.1% Tween-20). The membrane was blocked with 5% nonfat milk in TBST buffer for 1 hour at room temperature followed by incubation with NEI-1 antibody for 1 hour and secondary antibody conjugated to horseradish peroxidase (1:10 000 dilution; Amersham Pharmacia Biotech, UK) for 2 hours. NET-1 protein was visualized by enhanced chemiluminescence detection (ECL, Amersham Corporation, Arlington Heights, IL).

Cell culture and confocal microscopy. SMMC-7721 human liver carcinoma cell line was obtained from Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China). Cells were grown at 37°C, 5% CO_2 in RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 15% heat-inactivated newborn calf serum. For microscopic analysis, cells were cultured on glass coverslips in a 24-well plate to 70% confluence and fixed in 4% paraformaldehyde for 1 hour at room temperature followed by 3 washes with phosphate-buffered saline (PBS). Cells were then incubated with NET-1 antibody at a concentration of 0.5 μg/mL for 2 hours at room temperature, washed and incubated with a secondary FITC-conjugated anti-rabbit antibody at 1:100. After antibody incubation, the coverslips were mounted on glass slides using VECTORSHIELD agent (Vector). Confocal immunofluorescence microscopy was performed on a BioRad MRC1024 system, and the images were captured at 630× magnification with the aid of Laser Sharp software.

Detection of NET-1 protein expression in HCC by immunohistochemistry S-P method. Liver samples were immediately fixed in 40 g/L formaldehyde solution after isolation and embedded subsequently in paraffin. The paraffin-fixed samples were sliced into sections of 4 μm and subjected to immunohistochemical staining using Dako Elivision™ Plus Two-step System. Briefly, paraffin sections were de-waxed in xylene, rinsed in alcohol, and further dehydrated in graded alcohol. The sections were then subjected to antigen retrieval treatment by boiling in 0.01 M citric acid, pH 6.0, for 5 minutes in a pressure cooker and then to treatment with 0.3% hydrogen peroxide for 15 minutes in absolute methanol to inhibit endogenous peroxidase. After treatment, the sections were blocked with diluted normal calf serum followed by incubation overnight at 4°C with polyclonal anti-NET-1. The color reaction was carried out using PV-6000 kit (Zymed, CA). The stained samples were counterstained with Mayer's hematoxylin and then mounted. Negative control reactions were performed in parallel without primary antibodies. Cervical adenocarcinoma, which expresses abundant NET-1, was used as the positive control.

Cells with distinct brown cytoplasmic or membrane staining were judged to be NET-1 positive samples. For each sample, 10 random microscopic fields (×400), which correspond to approximately 2000 cells, were inspected. The samples were initially graded based on the percentage of positively stained cells: 0, positively stained cells <5%; 1, 6% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, >75%. The samples were further graded based on intensity: 1, weak yellow; 2, yellow; 3, dark yellow, and 4, brown. Overall, the samples were scored based on the grades of both percentage and intensity: negative (–) (score 0), positive (+) (score 1—3), middle positive (++) (Score 4-6), and strong positive (+++) (score ≥7).

Observation of paraffin-embedded sections by microscopy. According to the World Health Organization, HCC architecture patterns were classified into trabecular, pseudoglandular, and acinar. The cytological variants of HCC were divided into 4 groups: (1) hepatocellular type, the carcinoma cells similar to normal cells with abundant cytoplasm; (2) clear cell type, tumors consisting of cells with abundant glycogen; (3) pleomorphic cell type, tumors with bizarre multinucleated or mononuclear giant cells; (4) sarcomatous change, tumors containing many proliferating spindle-like or bizarre giant cells.[16]

Edmondson grading of HCC differentiation: *grades I to IV*.[17] HCC backgrounds: (–)—no hepatitis or cirrhosis; (+)—hepatitis or/and cirrhosis. HCC Clinical stage (according to UICC (1987) and Japanese HCC research team): I stage $T_1N_0M_0$; II stage $T_2M_0N_0$; III stage $T_3N_0M_0$ or $T_{1\sim3}N_1M_0$; IVa stage $T_4N_{0\sim1}M_0$; IVb stage $T_{1\sim4}N_{0\sim1}M_1$. Presence of portal vein tumor thrombus is denoted by (+) and absence by (–).

Statistical analysis. Paired data were analyzed using Student's *t* test; rank correlation data were analyzed using the Spearman test; relationships between

Table 1. NET-1 Gene RT-PCR Systemic Screening Results

System	Tissue	Adult (Group 1)	Fetal (Group 2)	Malignant (Group 3)
Digestive	Stomach	+	NA[a]	+
	Salivary gland	+	NA	NA
	Small intestine	+	NA	NA
	Colon	+	NA	NA
	Rectum	+	NA	+
	Pancreas	–	NA	+
	Liver	–	–	+
Urological and reproductive	Kidney	+	+	+
	Testis	+	NA	NA
	Cervix	+	NA	Hela cell +
	Uterus	+	NA	NA
	Prostate	+	NA	NA
	Mammary gland	+	NA	NA
Endocrine	Pituitary gland	+	NA	NA
	Thyroid	+	NA	NA
	Adrenal gland	+	NA	NA
Lymph Hemopoietic	Hematopoietic cells	–	NA	–[b]
	Lymph node	+	NA	NA
	Bone marrow	–	NA	NA
	Spleen	+	NA	NA
	Thymus	+	NA	NA
Neural	Brain	+	+	NA
	Spinal cord	+	NA	NA
	Retina	+	NA	NA
Others	Heart	–	+	NA
	Lung	+	+	+
	Muscle	+	+	NA
	Skin	NA	+	NA
	Placenta	+	NA	NA
Total		28	7	Melanoma + 9

a. NA, not available.
b. Including lymphoma and leukemia.

NET-1 expression and clinicopathological parameters were analyzed using the χ^2 test. For all statistical analyses, a difference with $P < .05$ was considered significant.

Results

RT-PCR Analysis of NET-1 Gene Expression in Human Tissue Samples

The RT-PCR analysis result is summarized in Table 1. In group 1, NET-1 expression was found in most types of normal adult tissue except the pancreas, liver, heart, bone marrow, and hematopoietic cells. In group 2—fetal tissue—NET-1 was negative only in the fetal liver. In group 3, NET-1 was positive in cancerous tissue samples derived from 9 origins, including the liver and pancreas, as well as in Hela cells, which are a cervix-derived tumor cell line. However, NET-1 remained negative in hematopoietic malignant diseases including lymphoma and leukemia.

The Pattern of NET-1 Protein Distribution in Cultured Human Liver Cancer Cells

NET-1 protein was examined by immunofluorescent microscopy in SMMC-7721 human liver tumor cells. As shown in Figure 1, NET-1 protein stained with FITC was mainly found around the nucleus, which was stained with Hoechst, the area where the Golgi apparatus is frequently located, indicating that NET-1 may be associated with the membrane of secretion organelles.

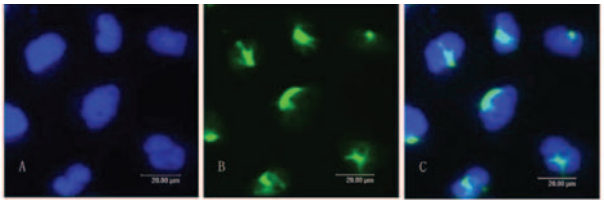

Figure 1. NET-1 distribution in the cytoplasm of SMMC-7721 cells (A). The nuclei of SMMC-7721 cell lines were homogeneously stained by Hoechst. (B) NET-1 stained by FITC in green was located in the cytoplasm of SMMC-7721 cell lines. (C) Merged figure, NET-1 was distributed in the cytoplasm.

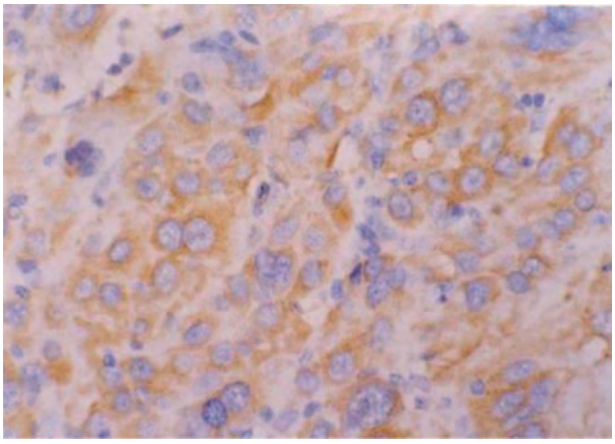

Figure 2. NET-1 expression in hepatocellular carcinoma (HCC) tissue. HCC were recognized as orbicular cells with large nuclei. NET-1 immunoactivity was indicated by numerous yellowish granules in the cytoplasm; magnification, ×200.

The Pattern of NET-1 Protein Distribution in Human Liver Cancer Tissue

Immunohistochemisty was performed to evaluate NET-1 protein expression in the liver tumor tissue. NET-1 immunoreactivity was found in granules of the cytoplasm (Figure 2) or in the membrane of tumor cells (Figure 3). No staining was seen when a negative control antibody was used (Figure 4).

Comparison of NET-1 Protein Expression in HCC Tissue and Adjacent Noncancerous Tissue

NET-1 mRNA was detected in 85.71% (24/28) of HCC samples and in 50.00% (14/28) of peritumor tissue samples. Based on the ratio of amplified NET-1 and actin cDNA after PCR, the relative expression level of NET-1 was 0.645 ± 0.37 and 0.466 ± 0.30 in HCC and peritumor samples, respectively, with a statistical significance of $P < .05$ (Figures 5 and 6). In addition, NET-1 protein was detected immunohistochemically in HCC and peritumor tissue samples. Similarly, expression was elevated in cancerous tissue samples as compared with peritumor tissue samples (Table 2).

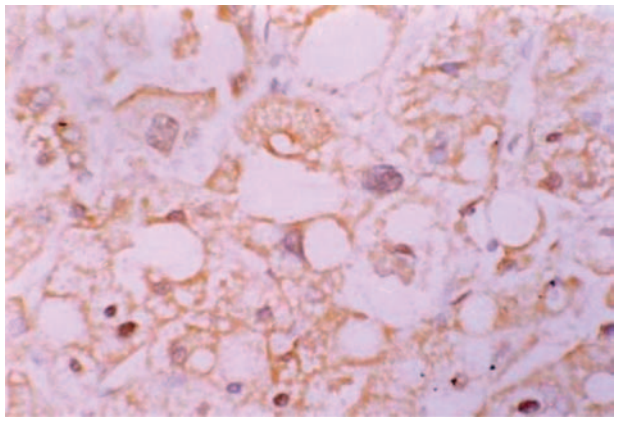

Figure 3. NET-1 expression in hepatocellular carcinoma (HCC) tissue. In spatially spread HCC cells with large and transparent cytoplasm, NET-1 staining was apparently associated with both the cytoplasm and the plasma membrane; magnification, ×400.

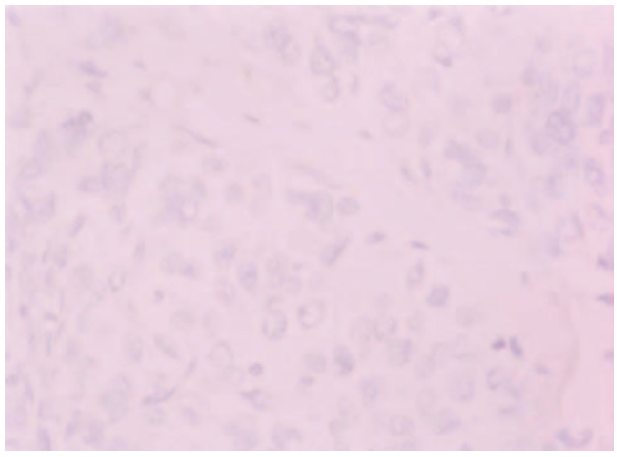

Figure 4. NET-1 expression in hepatocellular carcinoma (HCC) tissue. Control was used to stain the same liver tissue; magnification, ×400.

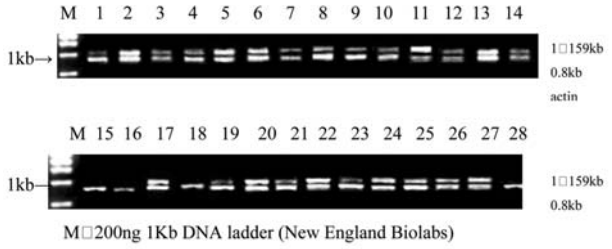

Figure 5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of NET-1 and β-actin mRNA expression in 28 cases of hepatocellular carcinoma (HCC).

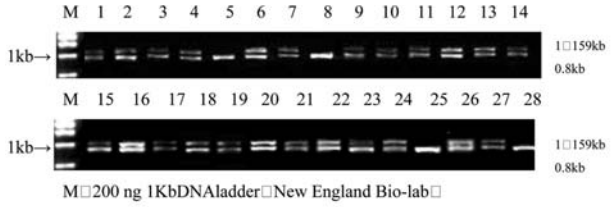

Figure 6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of NET-1 and β-actin mRNA expression in 28 cases of peritumor tissue.

Table 2. Comparison of NET-1 Immunoexpression in HCC Tissue and Adjacent Noncancerous Tissue

		NET-1 Expression				
Group	N	− (%)	+ (%)	++ (%)	+++ (%)	Statistics
Adjacent tissue	28	8 (28.57)	12 (42.86)	8 (28.57)	0 (0.00)	$P < .005$
HCC	28	1 (3.57)	3 (10.70)	8 (28.57)	16 (57.14)	$\chi^2 = 26.8454$

The Relationship Between NET-1 Gene Expression and Clinical Pathological Factors

The clinical and pathological data were as follows: male 87.69% (114/130), female 12.31% (16/130); average age was 50 years (25-70); early HCC (<2 cm), 5.16% (7/130); advanced HCC, 94.84% (123/130). Architectural patterns of cell growth: trabecular, 30.77%; pseudoglandular and acinar, 23.85%; and compact, 45.38%. Cytological variants: hepatocellular type, 50.77%; clear cells, 25.38%; pleomorphic, 12.31%; and sarcomatous change, 11.54%. Edmondson grading: I, 3.08%; II, 35.38%; III, 44.62%; and IV, 16.92%. Portal vein thrombosis accounts for 47.69%; 82.31% of HCC cases had either hepatitis or cirrhosis.

Table 3 shows the relationship between NET-1 protein expression and clinicopathological factors. The level of NET-1 protein expression was closely associated with HCC cytological variants with stronger staining found in clear cells, pleomorphic cells, and sarcomatous-like cells than in the hepatocellular type ($P < .05$). Moreover, the intensity of NET-1 staining was apparently correlated with tumor grading and clinical stages as well ($P < .01$). Whereas HCC I to II graded tumors were poorly stained with NET-1 antibody, tumors with III to IV grades showed stronger NET-1 staining ($P < .05$). Furthermore, the level of NET-1 expression was notably increased in HCC accompanied by hepatitis or cirrhosis than in HCC alone. However, we found no clear relationship between NET-1 protein expression and tumor size, vein thrombus, and serum AFP levels ($P > .05$). Furthermore, we performed a multivariate analysis to determine which of the above factors was independently associated with NET-1 expression, and found that the clinical stage was the specific marker.

RT-PCR Analysis of NET-1 mRNA Expression in HCC Tissue

Total RNA was extracted from 28 cases of HCC tissue and their peritumor tissue. RT-PCR analysis of NET-1 expression was performed, and the relative amount of NET-1 RNA levels in the two types of tissue samples were assessed based on the actin control. The relative amounts of NET-1 mRNA in HCC and peritumor tissue were 0.645 ± 0.37 and 0.466 ± 0.30, respectively, indicating a higher expression level in HCC than in the peritumor tissue ($P < .05$) (Figures 5 and 6).

Discussion

In this study, we examined a cDNA panel derived from 53 cases of human normal tissue and found

Table 3. The Relationship Between NET-1 Protein Expression and Clinicopathological Factors

Clinicopathological Factors	N	NET-1 Expression				Statistics
		– (%)	+ (%)	++ (%)	+++ (%)	
Cytological variants						
Hepatocellular type	66	4 (6.06)	17 (25.76)	21 (31.82)	24 (36.36)	$P = .0181$
Clear cells	33	0 (0.00)	0 (0.00)	10 (30.30)	23 (69.70)	$\chi^2 = 10.060$
Pleomorphic cells	16	0 (0.00)	0 (0.00)	7 (43.75)	9 (56.25)	
Sarcomatous-like	15	0 (0.00)	0 (0.00)	5 (33.33)	10 (66.67)	
Tumor grading						
I	4	2 (50.0)	1 (0.25)	1 (0.25)	0 (0.00)	$P = .0021$
II	46	1 (2.17)	13 (28.26)	13 (28.26)	19 (41.30)	$v = 0.2650$
III	58	0 (0.00)	2 (3.49)	24 (41.38)	32 (55.17)	
IV	22	1 (4.54)	1 (4.54)	5 (22.73)	15 (68.18)	
Background						
–	23	0 (0.00)	6 (26.09)	9 (39.13)	8 (34.78)	$P = .0415$
Hepatitis/cirrhosis	107	4 (3.74)	11 (10.28)	34 (31.78)	58 (54.21)	$v = 0.1788$
Clinical stage						
I	4	0 (0.00)	1 (25.0)	2 (50.00)	1 (25.00)	$P = .0198$
II	81	3 (3.70)	14 (17.28)	28 (34.57)	36 (44.44)	$v = 0.2019$
III	36	1 (2.78)	2 (5.56)	11 (30.56)	22 (61.11)	
IV	9	0 (0.00)	0 (0.00)	2 (22.22)	7 (77.78)	

v, Variable.

that NET-1 RNA is present in most adult tissue except the liver and hematopoietic cells. NET-1 expression is also negative in fetal livers. However, high levels of NET-1 expression were found in liver cancer tissue samples as analyzed by either RT-PCR or immunohistochemical analysis. Furthermore, there is a correlation between NET-1 protein expression and the multiple clinicopathological factors of HCC. Consistent with our finding, deregulation of NET-1 expression at the transcriptional level has also been found in multiple human cell lines, including cervical cancer, lung cancer, colon carcinoma, and breast cancer cells.[18-23] High levels of NET-1 protein expression in CIN III, cervical squamous carcinoma, undifferentiated carcinoma cells, and adenocarcinomas were also recently reported.[24] The mechanism of deregulation of the NET-1 gene is currently unknown. These findings imply a possibility that overexpression of NET-1 protein may be associated with the biological behavior indicating a high degree of malignancy.

We also detected NET-1 gene expression in the fetal lung—a result that is apparently inconsistent with another report in which no NET-1 expression was detected in a fetal lung cell line IGIG7.[9] The reason for this variation might be a result of the differences in tissue samples and cell lines used in these studies.

In the RT-PCR analysis of 28 cases of HCC tissue and normal liver tissue samples, we attempted to minimize the variations caused by RNA sample preparation and PCR amplification: we co-amplified the NET-1 gene and β-actin in the same reaction.[11] Our results indicated that the level of β-actin expression was similar in both HCC and non-HCC tissue, whereas the level of NET-1 expression was higher in HCC tissue than in tissue adjacent to HCC. No NET-1 RNA was detected in normal or fetal liver tissue under the same conditions.

The immunohistochemical analysis of paraffin-embedded sections derived from 130 cases of HCC revealed that NET-1 protein expression was associated with cytological variants of HCC. Positive NET-1 staining was significantly higher in cells with a clear shape and cells with pleomorphic and sarcomatous changes than in those with normal hepatocellular morphology. Pleomorphic and sarcomatous changes are specific phenomenon in HCC at lower differentiation stages, which are often followed by tumor invasion and metastasis. There is also a strong correlation between the level of NET-1 expression and HCC pathological grading and clinical stages. A multivariate analysis was performed and indicated that the clinical stage was an independent factor associated with the NET-1 expression. These results suggest that NET-1 overexpression in HCC cells may facilitate the development of tumors. In addition, we noticed that NET-1 expression was elevated in HCC accompanied with hepatitis and/or cirrhosis

background. Patients with severe hepatitis and/or cirrhosis should have regular follow-ups to monitor the clinical changes. Detection of NET-1 gene expression in liver biopsy may provide useful information for the diagnosis of HCC.

We still don't know the detailed function of NET-1 in HCC, though the present result indicates that NET-1 is related to the degree of malignancy of HCC and implies the possibility that NET-1 may promote the development of the cancer. We are carrying out cell experiments using NET-1 siRNA to inhibit the expression of NET-1 in HCC cells, after which we can observe the changes in cell growth and motility. Because confocal microscopy showed that NET-1 protein was mainly distributed near the nuclear region, we will also examine the possible association of NET-1 function with protein secretion–related cell processes.

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