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NRSF/REST levels are decreased in cholangiocellular carcinoma but not hepatocellular carcinoma compared with normal liver tissues: A tissue microarray study

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Abstract. The transcription factor neuron-restrictive silencer factor (NRSF), also termed repressor element 1-silencing transcription factor (REST), has been previously demonstrated to repress the expression of neuronal genes in non-neuronal cells, facilitating the controlled development and organization of nerve tissue. However, previous studies have reported NRSF/REST to be upregulated or downregulated in multiple types of carcinoma. Liver diseases are a major global health concern, with cirrhosis and liver carcinoma among the most common causes of mortality worldwide. A previous study demonstrated that there were >400 NRSF/REST target genes in mouse liver cells; however, the expression profile of NRSF/REST in human liver disease remains unclear. The present study examined NRSF/REST expression in human normal and liver carcinoma samples using tissue microarray immunohistochemistry. The results demonstrated that in normal liver tissues, NRSF/REST can be detected in the cytoplasm and nuclei of the cell; whereas in the liver carcinoma tissue, NRSF/REST is only detected in the cytoplasm. Furthermore, the number of samples with high levels of NRSF/REST was significantly lower in cholangiocellular carcinoma samples compared with normal tissues. Additionally, no detectable sex- or age-associated differences were identified in NRSF/REST expression among all the tissues examined. In conclusion, the results of the present study revealed nuclear loss of NRSF/REST in hepatic carcinomas and decreased expression of NRSF/REST in cholangiocellular carcinoma, indicating that the cytoplasmic translocation of NRSF/REST may be involved in liver tumorigenesis. A low expression level of NRSF/REST may be a novel biomarker for cholangiocellular carcinoma.

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Introduction

Neuron-restrictive silencer factor (NRSF), also termed repressor element 1-silencing transcription factor (REST), is a zinc-finger transcription factor and an important regulator of neural genes (1). Chong *et al* (2) first reported that NRSF/REST is a silencer protein that reduces the expression of sodium channel genes in neurons. A previous study demonstrated that NRSF/REST is an important regulator of neurogenesis *in vitro* and *in vivo*. For example, downregulation of NRSF/REST in embryonic stem cells induces neuronal lineage differentiation (3), and knockdown of NRSF/REST in cultured neural stem cells induces the expression of pro-neuronal genes, including neuronal differentiation 1, neuron-specific class III β-tubulin and doublecortin (4). In *Xenopus* and chicken embryos, NRSF/REST inactivation induces abnormal neurogenesis and inhibits the repression of neuronal tubulin and several other neuronal target genes (5,6). Additionally, NRSF/REST overexpression represses the expression of neuronal genes, including N-tubulin and neuronal cell adhesion molecule (7).

Numerous studies have investigated the expression and, to a lesser extent, the function of NRSF/REST in tumors of the nervous system (8-15). Certain tumors, including neuroblastoma, share a number of biological properties with neuronal progenitor cells and, thus, can acquire neuronal phenotypes in response to a variety of agents. A study by Nishimura *et al* (8) examined the levels of NRSF/REST mRNA in a human neuroblastoma cell line following induced differentiation. The study demonstrated that the NRSF/REST mRNA level was evidently decreased following induction, indicating that NRSF/REST expression is a biochemical marker of neuronal differentiation in neuroblastoma cells (8). A similar downregulation of NRSF/REST was also demonstrated in other studies using different neuroblastoma cell lines (9,10). In addition,

high NRSF/REST levels have been detected in human medulloblastoma cell lines and tumors (11-13). Similarly, in human glioblastoma multiforme (GBM), NRSF/REST is highly expressed (14) and its inhibition suppresses the proliferation and migration of GBM cells (15).

Certain studies have also investigated the expression and/or function of NRSF/REST in non-neuroepithelial tumors *in vitro* and *in vivo*. Gurrola-Diaz *et al* (16) reported that exogenous overexpression of NRSF/REST in NRSF/REST-deficient small-cell lung cancer (SCLC) cell lines induced apoptosis of SCLC cells, indicating that the inhibition of NRSF/REST activity is a crucial step in the carcinogenesis of a subgroup of SCLCs (16). Similar results were also observed in human non-SCLC cell lines (17). Kreisler *et al* (18) reported that loss of NRSF/REST expression was associated with the malignant progression of SCLC. In human breast cancer cells, NRSF/REST activity was required for estradiol stimulation of the cell cycle (19). Immunohistochemistry previously demonstrated that NRSF/REST expression is significantly lower in breast cancer samples compared with normal and benign breast samples, and that knockdown of NRSF/REST expression by short hairpin RNA in MCF-7 human breast cancer cells resulted in an increase in cell proliferation, suppression of apoptosis and reduced sensitivity to anticancer drugs (20). Previous studies have also demonstrated the important function of NRSF/REST in the pathogenesis of uterine fibroids (21). However, the expression of NRSF/REST in liver tumors remains unclear. Thus, the present study determined the expression profile of NRSF/REST in liver tumors using tissue microarray (TMA) immunohistochemistry.

Materials and methods

TMA and pathology. All paraffin-embedded TMAs used in the present study were purchased from US Biomax, Inc. (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The hepatic carcinoma and normal hepatic tissue TMAs (cat. no. BC03118) contained 90 carcinoma samples, including 15 cholangiocellular carcinoma (CCC), 75 hepatocellular carcinoma (HCC), and 10 normal hepatic tissue samples. On the basis of morphology, the liver carcinoma samples were graded 1-3 (or I-III), according to the Tumor-Node-Metastasis grading system (22) by the supplier, indicating well-, moderately- or poorly-differentiated tissue, respectively. In total, there were 200 tissue samples on the microarray, with two samples from each patient.

Immunohistochemistry. TMAs were deparaffinized with xylene, rehydrated with a graded alcohol series and subjected to heat-mediated antigen retrieval [0.01 M sodium citrate buffer, (pH 6.0)], according to a previously described protocol (23,24). TMAs were then rinsed with phosphate-buffered saline [PBS; 0.01 mol/l, (pH 7.4)] and blocked with 3% H_2O_2 (v/v in PBS) for 15 min at room temperature. The sections were then incubated for 20 min at room temperature with 2% normal goat serum (v/v; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at room temperature to block the non-specific binding. Subsequently, the TMAs were incubated overnight at 4˚C with a polyclonal rabbit antibody against NRSF/REST (cat. no. ab21635; Abcam, Cambridge, UK) that was diluted 1:100 with antibody diluent (cat. no. S3022; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Following several washes with PBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (cat. no. ZB2010; 1:200; Beijing Zhongshan, China) for 1 h at room temperature. The sections were then washed with PBS and incubated with horseradish peroxidase-labeled streptavidin (cat. no. ZB2404; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 1 h at room temperature. Finally, the sections were incubated with a diaminobenzidine-peroxidase substrate kit (cat. no. ZLI-9018; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 5 min at room temperature. The equivalent procedure was conducted for the blank controls, with the primary antibody replaced by antibody diluent.

Imaging and data analysis. Images of the immunohistochemical staining were captured with a DP70 digital camera (Leica Microsystems GmbH, Wetzlar, Germany) mounted to a BX60 Olympus microscope (Olympus Corporation, Tokyo, Japan). The staining was scored according to the previously described four-point system (score 0-3) (24) by a pathologist (double-blinded) as follows: Score 3, dark staining that is easily visible and present in >50% of cells; score 2, focal areas of dark staining (<50% of cells) or moderate staining of >50% of cells; score 1, focal moderate staining in <50% of cells or pale staining in any proportion of cells not easily observable at low power; and score 0, none of the above. A high level of expression was defined as a score of 2-3 and low level of expression was defined as a score of 0-1, as described previously (24). Considering the comparatively small sample size, an early tumor stage was defined as stages I and II, and the advanced stage was defined as stages III and IIIb. Well-differentiated carcinoma (WDC) was defined as grade 1, moderately-differentiated carcinoma (MDC) as grade 2 and poorly-differentiated carcinoma (PDC) was defined as grade 3 (24).

Statistical analysis. All data are expressed as n (%) and were compared using a χ^2 test. A Fisher's exact test was used for correction when necessary. Statistical analysis was performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). All P-values were 2-tailed and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Subcellular localization of NRSF/REST immunohistochemical staining. The immunohistochemical analysis demonstrated that, in normal hepatic tissue, NRSF/REST was present in the nuclei and cytoplasm of hepatocytes and cholangiocytes (Fig. 1A and B). In CCC and HCC tissues, NRSF/REST was predominantly detected in the cytoplasm, with the nuclei clearly unstained. Additionally, the NRSF/REST immunohistochemical staining of HCC tissues seemed stronger than that of CCC tissues (Fig. 1C-F).

Expression of NRSF/REST in liver carcinoma. The levels of NRSF/REST immunoreactivity were compared between normal and liver carcinomas. As presented in Table I, among the 10 cases of normal liver tissue on the TMA, 90% exhibited

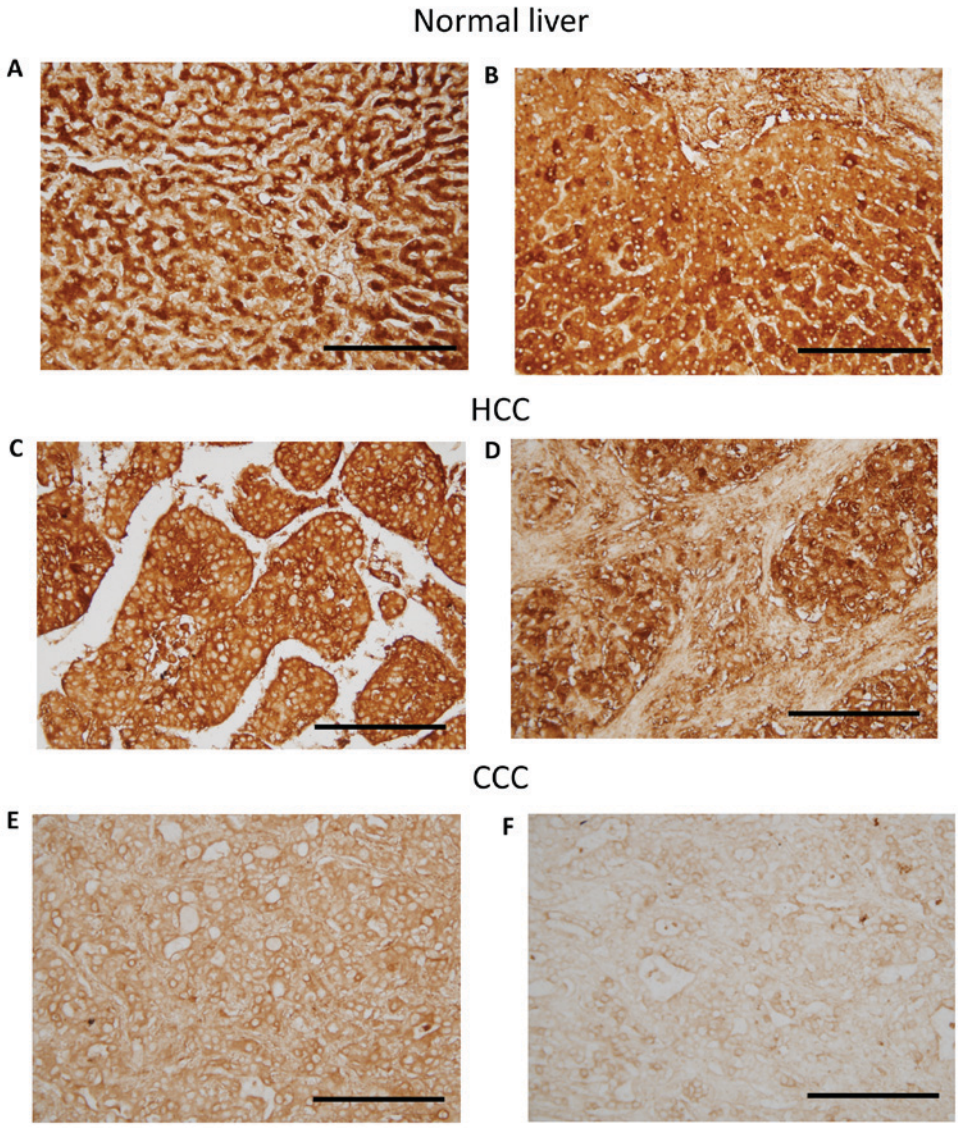

Figure 1. Representative immunohistochemical staining for NRSF/REST in normal and cancerous liver tissues. Normal liver tissue from (A) a 40-year-old female and (B) a 3-year-old male. HCC tissue from (C) a 45-year-old female (grade 2; stage III) and (D) a 51 year-old male (grade 2; stage III). CCC tissue from (E) a 24-year-old male (grade 2; stage III) and (F) a 40-year-old female (grade 1; stage III). Scale bar, 200 μm. The number of samples with high levels of NRSF/REST was increased in normal liver tissue and HCC as compared with CCC. Additionally, in normal liver tissue, nuclear and cytoplasmic NRSF/REST expression was detected; whereas in liver carcinomas, NRSF/REST was predominantly detected in the cytoplasm. NRSF/REST, neuron-restrictive silencer factor/repressor element 1-silencing transcription factor; HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma.

high levels of NRSF/REST (score 2-3; Fig. 1A and B). Among the 90 cases, 66% (59/90) of liver carcinoma samples and 77% (58/75) of HCC samples demonstrated high levels of NRSF/REST (Table I; Fig. 2). There was no significant difference in the percentage of samples with high NRSF/REST levels between the normal liver tissue and liver carcinoma samples (P>0.05; Fig. 2A; Table I) or between normal liver tissue and HCC (P>0.05; Fig. 2B; Table I). However, the percentage of samples with high NRSF/REST expression was significantly reduced in CCC samples (7%; 1/15) compared with normal liver tissues (P<0.001; Fig. 2C; Table I) and with HCC samples (P<0.001; Fig. 2D; Table I).

Among the 90 cases of liver carcinomas, 11 cases were graded as WDC, 64 as MDC and 11 as PDC (including grade 2-3). The grading of the remaining 4 cases was undetermined. The percentage of high NRSF/REST immunoreactivity was 73% (8/11) in WDCs, 69% (44/64) in MDCs and 36% (4/11) in PDCs. There was no statistical difference between the percentages of samples with high NRSF/REST staining among the three groups (P>0.05; Fig. 2E; Table I).

Additionally, the association between NRSF/REST expression and tumor stage was analyzed. Stages I and II were defined as early stage, and stages III and IIIb as advanced stage. Thus, 43 cases were early stage and 47 cases were advanced stage. High levels of NRSF/REST were observed in 67% (29/43) of early stage and 64% (30/47) of advanced stage samples, with no statistical difference between the two stages (P>0.05; Fig. 2F; Table I).

Sex-associated differences in NRSF/REST in normal and cancerous liver tissue. The occurrence of liver diseases exhibits a certain degree of sex bias, with a higher percentage

Table I. Expression of NRSF/REST in normal and abnormal liver tissues.

Tissue	High	Low	χ^2	P-value
Carcinoma vs. normal			1.476	0.224
Carcinoma	59	31		
Normal	9	1		
CCC vs. normal			14.063	<0.001
CCC	1	14		
Normal	9	1		
HCC vs. normal			0.259	0.611
HCC	58	17		
Normal	9	1		
CCC vs. HCC			27.645	<0.001
CCC	1	14		
HCC	58	17		
Carcinoma early/ advanced stage			0.130	0.719
Early	29	14		
Advanced	30	17		
Carcinoma differentiation stage			4.656	0.097
WDC	8	3		
MDC	44	20		
PDC	4	7		

High NRSF/REST expression was defined as score 2-3 and low expression as score 0-1. WDC was defined as grade 1, MDC was defined as grade 2 and PDC was defined as grade 3 and grade 2-3. Early stage was defined as stage I-II and advanced stage was defined as stage III (including IIIb). NRSF/REST, neuron-restrictive silencer factor/repressor element 1-silencing transcription factor; HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma; WDC, well-differentiated carcinoma; MDC, moderately-differentiated carcinoma; PDC, poorly-differentiated carcinoma.

Table II. Sex and age-associated differences of NRSF/REST in normal and cancerous liver tissues.

A, Sex-associated differences

Tissue	High	Low	χ^2	P-value
Normal			0.000	1.000
Male	5	1		
Female	4	0		
Carcinoma			3.176	0.075
Male	51	22		
Female	8	9		
CCC			0.000	1.000
Male	1	7		
Female	0	7		
HCC			0.000	1.000
Male	50	15		
Female	8	2		

B, Age-associated differences

Tissue	High	Low	χ^2	P-value
Normal			0.046	0.830
≥26.8	3	1		
<26.8	6	0		
Carcinoma			0.945	0.331
≥50.4	26	17		
<50.4	33	14		
CCC			0.005	0.945
≥48.5	0	8		
<48.5	1	6		
HCC			0.792	0.373
≥50.8	27	10		
<50.8	31	7		

High levels of NRSF/REST expression were defined as score 2-3 and low levels of expression were defined as score 0-1. Ages are presented in years. No sex- or age-associated differences were detected. NRSF/REST, neuron-restrictive silencer factor/repressor element 1-silencing transcription factor; HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma.

detected in males. A total of 74.11% of new liver cancer cases in 2014 in the USA were in males (25). Thus, the present study compared the expression of NRSF/REST in normal and cancerous liver tissues from men and women. Among the 10 cases of normal liver tissue, the percentage of samples with high NRSF/REST levels was 83% (5/6) in males and 100% (4/4) in females, with no significant difference between the sexes (P>0.05; Fig. 3A; Table II). Among the 90 cases of liver carcinoma, the percentage of samples with high levels of NRSF/REST was 70% (51/73) in males and 47% (8/17) in females, demonstrating no significant difference between the sexes (P>0.05; Fig. 3B; Table II).

Among the 15 cases of CCC, only 1 male demonstrated high levels of NRSF/REST and the other 14 cases (7 male and 7 female) all demonstrated low levels of NRSF; thus, no significant sex-associated difference was identified (P>0.05; Table II). Among the 75 cases of HCC, the percentage of samples with high levels of NRSF/REST was 77% (50/65) in males and 80% (8/10) in females; thus, there was no significant sex-associated difference detected (P>0.05; Table II).

Age-associated differences in NRSF/REST in normal and cancerous liver tissue. The present study also investigated the effect of age on NRSF/REST immunoreactivity in normal and cancerous liver tissues. Among the 10 normal liver tissues, the mean age of the patients was 26.8 years. The percentage of samples with high expression of NRSF/REST was 75% (3/4) in samples from patients aged ≥26.8 years, and 100% (6/6) in those aged <26.8 years. The statistical analysis demonstrated no significant difference between the two age groups (P>0.05; Fig. 3C; Table II). Among the 90 cases of liver carcinoma, the mean age was 50.4 years. The percentage of samples with high NRSF/REST expression was 60% (26/43) in patients

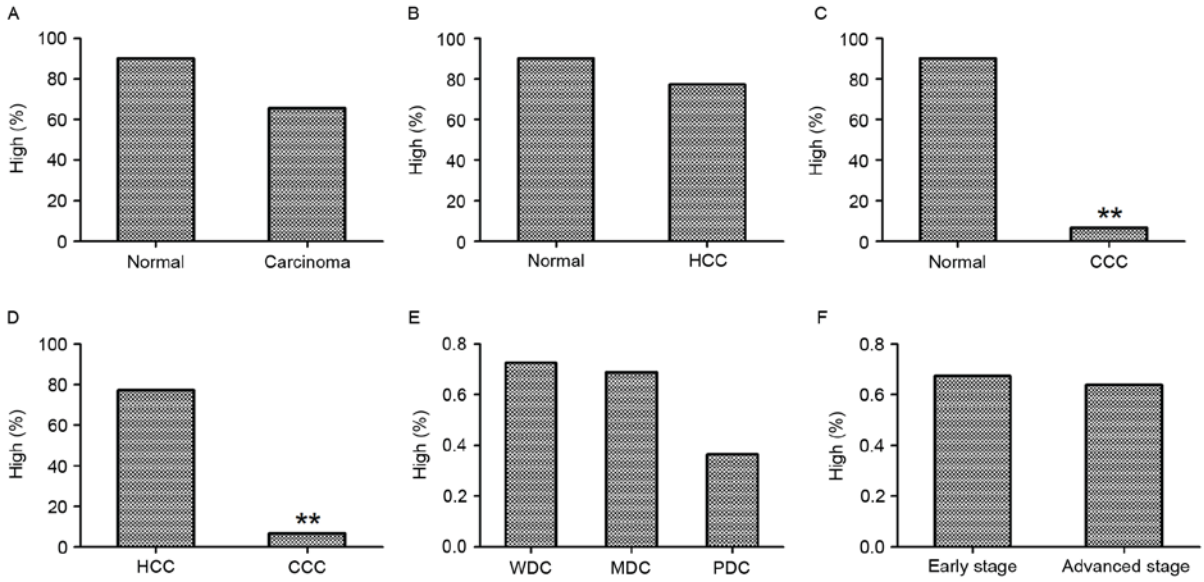

Figure 2. Statistical analysis of NRSF/REST immunoreactivity in normal and cancerous liver tissues and the association with tumor differentiation and stage. (A) Percentage of samples with high NRSF/REST levels between normal and liver carcinoma tissues. (B) Percentage of samples with high NRSF/REST levels between normal and HCC liver tissues. (C) Percentage of samples with high NRSF/REST levels between CCC and normal liver tissue. (D) Percentage of samples with high NRSF/REST levels between CCC and HCC. (E) Percentage of samples with high NRSF/REST levels among the differentiation status. (F) Percentage of samples with high NRSF/REST levels between tumor stages. **P<0.001. NRSF/REST, neuron-restrictive silencer factor/repressor element 1-silencing transcription factor; HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma; WDC, well-differentiated carcinoma; MDC, moderately-differentiated carcinoma; PDC, poorly-differentiated carcinoma.

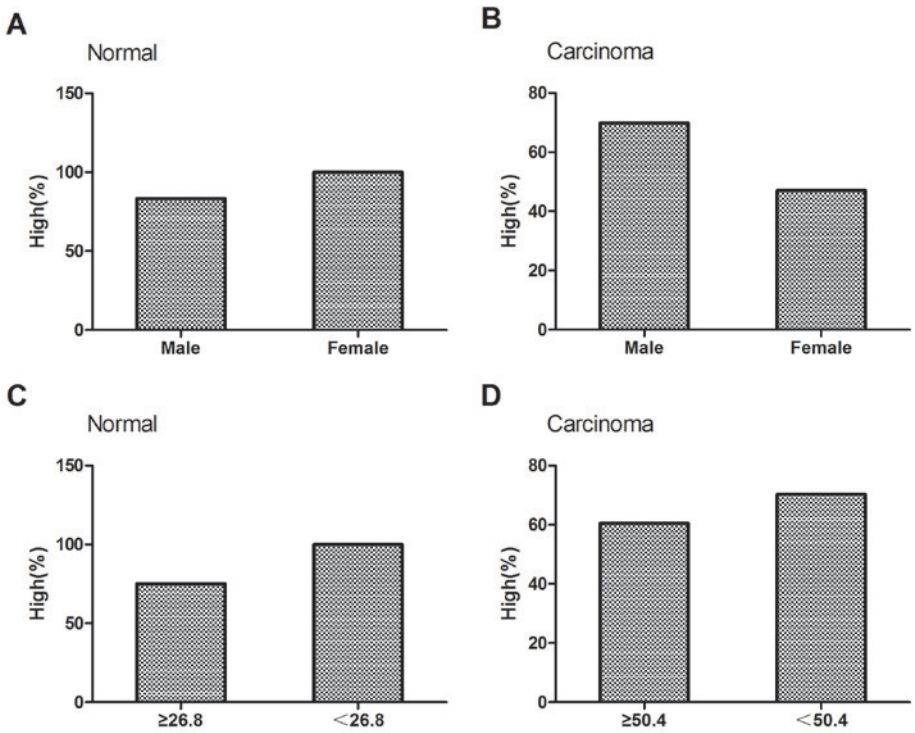

Figure 3. Sex and age analysis of NRSF/REST immunoreactivity in normal and cancerous liver tissues. Sex-associated differences of NRSF/REST levels in (A) normal liver tissue and (B) hepatic carcinoma. Age-associated differences of NRSF/REST levels in (C) normal liver tissue and (D) hepatic carcinoma. No sex- or age-associated differences were detected among the liver tissues. NRSF/REST, neuron-restrictive silencer factor/repressor element 1-silencing transcription factor.

aged ≥50.4 years and 70% (33/47) in those aged <50.4 years. Statistical analysis demonstrated no significant difference between the two age groups (P>0.05; Fig. 3D; Table II).

The age-associated difference of NRSF/REST immunoreactivity in individual liver carcinoma tissues was also analyzed. Among the 15 cases of CCC, the mean age of the patients was

48.5 years. Only 1 patient aged <48.5 years demonstrated high levels of NRSF/REST and statistical analysis revealed no significant difference between the two age groups (P>0.05; Table II). Among the 75 cases of HCC, the mean age of the patients was 50.8 years. The percentage with high expression of NRSF/REST was 73% (27/37) in patients ≥50.8 years old and 82% (31/38) in those <50.8 years old. Statistical analysis demonstrated no significant difference between the two age groups (P>0.05; Table II).

Discussion

The present study examined the expression of NRSF/REST in normal liver tissue and liver carcinomas using TMA immunohistochemistry. The results demonstrated that, in the normal liver tissue, NRSF/REST expression was present in the nuclei and cytoplasm, whereas, in liver tumor tissue, nuclear NRSF/REST staining was clearly reduced and expression was detected in the cytoplasm. Furthermore, the highest levels of NRSF/REST were detected in normal liver tissue (90% of cases); however, the expression of NRSF/REST among all liver tumors, including HCC and CCC, did not demonstrate any significant difference compared with the normal liver tissue. However, in CCC tissues, the number of samples with high NRSF/REST expression was significantly decreased compared with normal or HCC tissues, with only 7% of CCCs exhibiting high levels of NRSF/REST. The expression of NRSF/REST was not statistically different among the grades of tumor differentiation (WDC, MDC and PDC) or between pathological stages. Finally, no age- or sex-associated differences were identified in the number of samples with high NRSF/REST immunoreactivity among all the tissues examined, including normal and cancerous liver tissues.

As a transcription factor, NRSF/REST expression has typically been detected in cell nuclei in previous studies. For example, in human medulloblastoma tumors (13) and normal human brain tissue (26), NRSF/REST staining was observed to be localized to the nuclei. Conti *et al* (14) also reported that in gliomas, NRSF/REST exhibited a nuclear staining pattern (14). In the present study, positive NRSF/REST staining was detected in the nuclei and cytoplasm in normal liver tissue; whereas, in liver carcinoma, NRSF/REST was predominantly detected in the cytoplasm. Similar results have been previously observed in other tissues. For example, Conti *et al* (14) also reported that in normal human tissue, NRSF/REST immunoreactivity was detected in the cytoplasm of selected neurons. Furthermore, Orta-Salazar *et al* (27) reported that in the hippocampus of a 3xTg-AD mouse (a mouse model of Alzheimer's disease), the nuclei and cytoplasm were NRSF/REST-positive. Additionally, these mice exhibited decreased cytoplasmic and increased nuclear NRSF/REST staining compared with control mice, which was indicated to be associated with the degeneration observed in Alzheimer's disease. Lu *et al* (26) reported that in normal human brain tissue, NRSF/REST was predominantly localized in the cell nuclei; however, in several brain tissue samples from patients with dementia, NRSF/REST was predominantly absent from the cell nuclei, but present in the cytoplasm. This nuclear loss and cytoplasmic translocation was indicated to be one of the causes of the reduced repression of certain dementia/stress-associated genes that are highly expressed in dementia (26). In the present study, nuclear/cytoplasmic translocation of NRSF/REST was clearly observed; this shift indicated that loss of nuclear NRSF/REST may contribute to hepatic carcinogenesis.

It has been previously reported that NRSF/REST may be tumor-suppressive or exert an oncogenic effect (28). Using array-comparative genomic hybridization analysis, Westbrook *et al* (29) identified that NRSF/REST is frequently deleted in colorectal cancer, and proposed that it functions as a tumor suppressor. Additionally, Blom *et al* (30) used gene copy number analyses to demonstrate that the majority of brain tumors exhibited low-level amplification of NRSF/REST. Wagoner *et al* (31) reported that expression of NRSF/REST was lost in aggressive breast cancer, and that this loss was positively correlated with poor prognosis and higher recurrence rates. Similarly, Lv *et al* (20) reported that NRSF/REST expression was decreased in breast cancer samples. Furthermore, Varghese *et al* (21) demonstrated that expression of NRSF/REST was reduced in uterine fibroids (leiomyomas). A decreased NRSF/REST expression profile was also detected in human brain tissue from patients with dementia and in the brain tissue from an Alzheimer's disease mouse model (26,27).

However, certain studies have also reported that NRSF/REST expression is increased in individual tumors or other tissues. For example, Lawinger *et al* (11) reported that NRSF/REST levels were high in three types of human medulloblastoma cells. Furthermore, Fuller *et al* (13) reported that expression of NRSF/REST was increased in human medulloblastoma tumors compared with normal brain tissue samples (13) and Conti *et al* (14) observed that NRSF/REST expression was increased in human GBM. The present study demonstrated that the number of samples with high NRSF/REST expression was reduced in CCC compared with normal tissues, indicating that decreased NRSF/REST levels may be associated with the occurrence and/or poor prognosis of CCC.

Liver disease is a major global health concern (32), with liver and intrahepatic bile duct cancer among the top 10 causes of cancer-associated mortality in the United States (25). In China, liver carcinomas (including HCC and CCC) are also commonly diagnosed and have been identified as one of the leading causes of cancer-associated mortality (33). Therefore, it is important to investigate the mechanisms of liver disease and to identify potential novel therapeutic targets. A study by Sedaghat *et al* (34) reported that in mouse liver, NRSF/REST regulates the expression of neuronal markers, including brain-derived neurotrophic factor and various other genes (a total of 433 genes, of which 25% were downregulated and 75% upregulated), particularly those associated with the cell cycle, cell growth, proliferation and cancer.

The present study observed cytoplasmic translocation of NRSF/REST in liver carcinomas compared with NRSF/REST detected in normal liver tissues and the levels of NRSF/REST expression were reduced in CCC. In conclusion, the results of the current and previous studies indicate that NRSF/REST has an important function in liver carcinomas. Furthermore, the observed nuclear/cytoplasmic translocation may contribute to tumor formation and the reduced levels of NRSF/REST

may potentially be used as a biomarker of CCC. Notably, as NRSF/REST may paradoxically exert tumor suppressive or oncogenic effects (28), the expression and importance of NRSF/REST in normal and abnormal liver tissues requires further investigation.

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