

Aim of the study: Hepatocellular carcinoma suppressor 1 (HCCS1) has been identified as a tumor suppressor gene in the high-frequency loss of heterozygosity (LOH) region on chromosome 17p13.3 in hepatocellular carcinoma (HCC). There was also a high frequency of LOH on chromosome 17p13.3 in non-small cell lung cancer (NSCLC). Therefore, the aim of this study was to explore the expression of HCCS1 in NSCLC as well as its clinical significance.

Material and methods: Real-time PCR and immunohistochemistry were performed to detect the expression level of HCCS1 mRNA and protein in NSCLC and noncancerous tissues, respectively. Further, we explored the relationship between HCCS1 expression and various clinical features in NSCLC.

Results: The mRNA and protein expression of HCCS1 were both significantly lower in NSCLC samples than those in noncancerous tissues. That is, the mRNA level of HCCS1 was 0.0044 ± 0.0036 and 0.0067 ± 0.0054 in NSCLC samples and noncancerous tissues, respectively. The protein level of HCCS1 was 4.67 ± 1.15 and 6.13 ± 1.24 in NSCLC samples and noncancerous tissues, respectively. Importantly, this difference in expression was significantly correlated with tumor lymph node metastasis (TNM) in NSCLC ($p < 0.05$), but not with gender and age of the patients, pathological types, TNM stages, or grades of cancers ($p > 0.05$).

Conclusion: Our results suggest that HCCS1 may be involved in NSCLC carcinogenesis.

Key words: HCCS1, non-small cell lung cancer, loss of heterozygosity.

Expression and clinical significance of HCCS1 in non-small cell lung cancer

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Introduction

Lung cancer is one of the most common malignancies and has been the leading cause of cancer-related deaths in the world [1]. Fine deleted mapping studies found that loss of heterozygosity (LOH) was frequently present on the chromosome 17p13.3 region in lung cancer, which is independent of and distal to the p53 gene at 17p13.1. This indicates that some unidentified genes may reside on these deleted regions and play an important role in progression and differentiation of lung carcinogenesis [2–4]. Therefore, recent studies pay more attention to identify the lung cancer-related gene in the 17p13.3 region.

Of them, HIC-1 (hypermethylated in cancer-1), a candidate tumor suppressor gene, has been identified in this region, which is telomeric from TP53 and often deleted in surgically resected lung cancers. The reduced expression of the HIC-1 gene in the tumor had a direct link with the clinical outcome, such as short survival [5]. The 14-3-3 gene, which also resides within the commonly deleted region at 17p13.3 in lung cancers, was found homozygously deleted in two SCLC cell lines. The introduction of 14-3-3 induced significantly restored G2 checkpoint responses, which resulted in the reduction of mitotic cells as well as of aberrant mitotic figures in the X-ray-irradiated 14-3-3-null SCLC cell line [6]. A novel gene, LOST1, was recently shown to be disrupted by the homozygous deletion at 17p13.3. Significantly reduced expression of the LOST1 gene was detected in 69% of lung cancer specimens [7].

Hepatocellular carcinoma suppressor 1 (HCCS1) is also a novel tumor suppressor gene that has been identified in high-frequency loss of heterozygosity on chromosome 17p13.3 in HCC [8]. However, there has been no report of HCCS1 in lung cancer. Thus, in this study, we aim to explore the HCCS1 expression in NSCLC and further evaluate its clinical significance.

Material and methods

Tissue sample

Thirty-one resected NSCLC specimens and matched noncancerous tissues were obtained from the First People's Hospital (Shanghai, China) and the Huashan Hospital (Shanghai, China) from April 2010 to December 2010. The specimens were divided into two equal parts; one was immediately immersed in liquid N₂ and the other fixed in 10% formalin. The clinical features of these patients are listed in Table 1. All of the patients have no history of chemotherapy or radiotherapy before the operation.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from NSCLC specimens and noncancerous tissues according to the protocols of the Triblue system (Shenergy Biocolor, Shanghai, China). A 50 mg frozen tissue sample was ground into a fine powder with a pre-cooled pestle and mortar under liquid N₂. The powder was transferred

Table 1. The relationship between HCCS1 mRNA expression and various clinical parameters in NSCLC

Clinical parameter		HCCS1 mRNA expression			p
		N	T ≥ N	T < N	
gender	male	14 (54%)	3	11	> 0.05
	female	12 (46%)	3	9	
age/year	≤ 60	15 (58%)	4	11	> 0.05
	>60	11 (42%)	2	9	
pathological type	squamous cell carcinoma	3 (12%)	1	2	> 0.05
	adenocarcinoma	23 (88%)	5	18	
lymph nodes metastasis	N0	12 (46%)	6	6	< 0.05
	N1–N3	14 (54%)	0	14	
TNM stage	I–II	16 (62%)	5	11	> 0.05
	III–IV	10 (38%)	1	9	
tumor grade	high-middle	23 (88%)	4	19	> 0.05
	low	3 (12%)	2	1	

into a 15 ml centrifuge tube and mixed with 1 ml Trizol buffer and 200 μ l chloroform. After centrifuging at 12 000 rpm for 10 min, RNA was precipitated from the aqueous phase by adding 3-fold volume of isopropanol. The RNA pellet was washed with 200 μ l 75% (v/v) ethanol and dissolved in RNase-free water. The RNA purity and concentration were determined by spectrophotometric absorbance at 260 and 280 nm. 1 μ g RNA was used to synthesize cDNA with Random Primers primers and M-MLV Reverse Transcriptase (Promega, Madison, USA). The 25 μ l PCR reaction mixture contained 5 μ l 5 × RT Buffer, 2 μ l dNTPs (10 mmol/l, 0.6 μ l RNase inhibitor, 1 μ l Random Primers (25 μ M), 1 μ l Reverse Transcriptase, and 15.4 μ l RNase-free H₂O. The PCR condition was 37°C for 60 min, 85°C for 10 min, and 4°C for 5 min.

Real time quantitative PCR

The quantitative PCR was performed in an IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) and the HCCS1 gene expression levels were determined by comparison with the GAPDH gene. The amplification product was 240 bp for GAPDH (forward primer: 5'-GGTGAAGGTCGGAGTCAACGGA-3', backward primer: 5'-GAGGGATCTCGCTCCTGGAAGA-3'); for HCCS1 (hVPS53), 103 bp (forward primer: 5'-CTGCACAGACTGAGTTA-GGACA-3', backward primer: 5'-TCTCGTAG AACATTGCTGGGT-3'). The PCR reaction mixture consisted of 10 μ l 2 × ExTaq buffer, 1 μ l cDNA, each 0.2 μ l sense and anti-sense primers (25 μ mol/l), and 8.6 μ l ddH₂O. The PCR cycling parameters were initial denaturation at 94°C for 30 s; 40 cycles of amplification (94°C for 10 s, 60°C for 60 s, 76°C for 10 s with a single fluorescence measurement). Melting curve analysis was conducted 60–95°C at a 0.2°C/s melt rate, and finally a cooling step to 60°C. The relative mRNA expression was determined by the expression $2^{-\Delta\Delta Ct}$ [$\Delta Ct = Ct (HCCS1) - Ct (GAPDH)$, $\Delta\Delta Ct = \Delta Ct (NSCLC) - \Delta Ct (noncancerous)$].

Immunohistochemistry (IHC) assays

Formalin-fixed samples were dehydrated with gradient ethanol and embedded in paraffin. The paraffin sections (4 μ m) were mounted onto poly-lysine-coated glass slides and dried for 1 h at 60°C followed by deparaffinizing and rehydration according to a standard protocol. For antigen

retrieval, slides were immersed in sodium citrate buffer (pH 6.0) and boiled twice for 5 min in a microwave oven. The slides were then washed with PBS (3 min) twice and treated with 3% H₂O₂ for 10 min to inhibit the endogenous peroxidase. This was followed by incubation with rabbit anti-human HCCS1 primary antibody (Sigma, USA) overnight at 4°C. After being rinsed in PBS three times for 3 min each, the slides were covered with DAKO EnVision, horseradish peroxidase/DAB (3,3'-diaminobenzidine), Rabbit/Mouse (DAKO, Carpinteria, CA) for 30 min at room temperature. The slides were developed in substrate-chromogen solution (DAB), counterstained with hematoxylin, and mounted. Faint yellow, tan, and brown color could be observed in cytoplasm under the optical microscope as positive results. The positive cell counting was performed according to the percentage of positive cells in 5 visual fields, that is: 5–25%, 1 point; 25–50%, 2 points; 50–75%, 3 points; > 75%, 4 points. The dyeing degree classification was as follows: faint yellow, 1 point; yellow or deep yellow, 2 points; tan or brown, 3 points. The average protein expression level of HCCS1 was obtained by multiplying positive cell scores and dyeing degree scores. If the difference was more than 1 in the NSCLC and noncancerous tissue, we considered it significant.

Statistical analysis

All the statistical analysis was performed using SPSS13.0 software. Normal distribution and paired t test were used for comparing HCCS1 expression between NSCLC and noncancerous tissue. The relationship between HCCS1 expression and various clinical parameters in NSCLC were evaluated with chi-square statistics; $p < 0.05$ was considered as statistically significant.

Results

HCCS1 mRNA expression in NSCLC and noncancerous tissue

Quantitative PCR was used to analyze HCCS1 mRNA expression in 26 NSCLC and their matched noncancerous tissues. The results showed that the mRNA level of HCCS1 was 0.0044 ± 0.0036 in NSCLC samples, but 0.0067 ± 0.0054 in matched noncancerous tissues. This indicated that the expres-

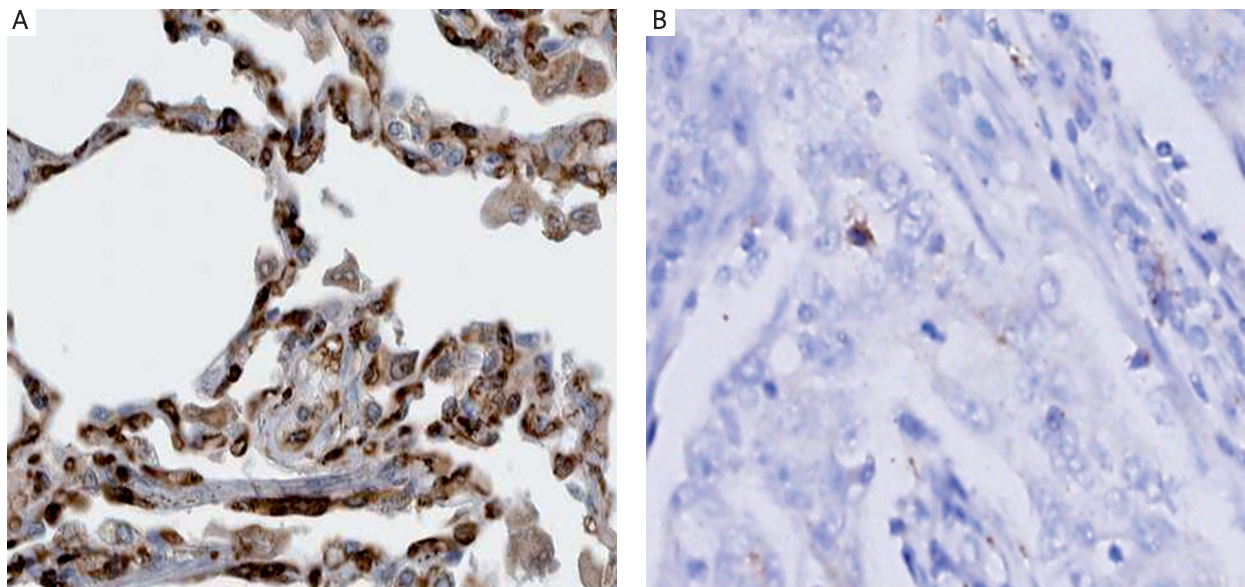


Fig. 1. The protein expression of HCCS1 in NSCLC and noncancerous tissues (400×). A – higher expression in noncancerous tissue; B – lower expression in NSCLC tissue

sion of HCCS1 mRNA was significantly lower in NSCLC than that in noncancerous tissues ($p = 0.023$).

HCCS1 protein expression in NSCLC and noncancerous tissue

IHC was used to analyze HCCS1 protein expression in 31 NSCLC and their matched noncancerous tissues. The results revealed that the protein level of HCCS1 was 4.67 ± 1.15 in NSCLC samples, but 6.13 ± 1.24 in matched noncancerous tissues. This suggested that the expression of HCCS1 protein was significantly lower in NSCLC than that in noncancerous tissues ($p = 0.038$) (Fig. 1).

The relationship between HCCS1 mRNA expression and various clinical parameters in NSCLC

From table 1, we can observe that the HCCS1 mRNA expression was significantly correlated with tumor lymph node metastasis (TNM) in NSCLC ($p = 0.003$), but not with gender and age of the patients, pathological types, TNM stages, and grades of cancers ($p > 0.05$) (Table 1).

The relationship between HCCS1 protein expression and various clinical parameters in NSCLC

Our results also showed that the HCCS1 protein expression was significantly correlated with tumor lymph node metastasis (TNM) in NSCLC ($p = 0.01$), but not with gender and age of the patients, pathological types, TNM stages, or grades of cancers ($p > 0.05$) (Table 2).

Discussion

As a novel tumor suppressor gene, HCCS1 was identified through screening the minimum region of high-frequency loss of heterozygosity on chromosome 17p13.3 and positional cloning in HCC [8–10]. The expression level of HCCS1 in HCC samples was significantly lower than that

in noncancerous liver cells. Moreover, expression of the exogenous HCCS1 gene in human hepatocarcinoma cells could remarkably suppress their abilities to develop tumors in nude mice and to form colonies in soft agar [8]. Recently, many studies have also demonstrated that HCCS1 showed reduced expression in other cancer cell lines, such as breast cancer cell lines, HeLa-a cervical cancer cell line [11], and colorectal cancer cells [12].

However, there has been no report on the HCCS1 gene in lung cancer. In this study, we first investigated HCCS1 gene expression in the NSCLC sample by quantitative real time PCR and immunohistochemistry assays. As we expected, the results showed that the mRNA and protein expression of the HCCS1 gene were both significantly lower in the NSCLC sample than those in noncancerous tissues, indicating that HCCS1 may be involved in NSCLC development and progression. Further, we explored the relationship between HCCS1 expression and various clinical parameters in NSCLC. The results indicated that the mRNA and protein expression of HCCS1 were significantly correlated with tumor lymph node metastasis (TNM) in NSCLC ($p < 0.05$), but not with gender and age of the patients, pathological types, TNM stages, or grades of cancers ($p > 0.05$). Taken together, our data suggest that HCCS1 is a promising therapeutic gene for the treatment of lung cancers.

The exact molecular mechanisms of the HCCS1 gene in human cancer remain unknown. It is believed that HCCS1 may be involved in human cancer development through a pro-apoptosis pathway. The amphipathic tail-anchoring peptide (ATAP) domain of HCCS1 has been demonstrated to induce mitochondrial permeability transition via its amphipathic property that perturbs the integrity of the mitochondrial membrane and leads to caspase-dependent apoptosis that does not require Bax or Bak [13]. It was also found that HCCS1 overexpression may induce lysosomal cathepsin D release into the cytosol and consequently triggers Bax insertion into the mitochondrial membrane, leading to the release of

Table 2. The relationship between HCCS1 protein expression and various clinical parameters in NSCLC

Clinical parameter		HCCS1 protein expression			p
		N	T ≥ N	T < N	
gender	male	16 (52%)	4	12	> 0.05
	female	15 (48%)	4	11	
age (year)	≤ 60	19 (61%)	5	14	> 0.05
	> 60	12 (39%)	3	9	
pathological type	squamous cell carcinoma	5 (16%)	1	4	> 0.05
	adenocarcinoma	26 (84%)	7	19	
lymph node metastasis	N0	15 (48%)	7	8	< 0.05
	N1–N3	16 (52%)	1	15	
TNM stage	I–II	19 (61%)	7	12	> 0.05
	III–IV	12 (39%)	1	11	
tumor grade	high-middle	27 (87%)	6	21	> 0.05
	low	4 (13%)	2	2	

cytochrome c. The released cytochrome c activates downstream caspase, resulting in the occurrence of the late stages of apoptosis [11]. Importantly, the disruption of HCCS1 in mice leads to embryonic lethality, accompanied by abnormal labyrinth architecture resulting from the excessive proliferation of trophoblast cells in the placenta [14]. In conclusion, these reports suggest that HCCS1 plays a role in apoptosis regulation and development.

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