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Long non-coding RNA NRON is downregulated in HCC and suppresses tumour cell proliferation and metastasis

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ABSTRACT

Dysregulation of long non-coding RNAs is a newly identified mechanism for tumour progression. Previous studies have suggested that the nuclear factor of activated T cells (NFAT) gene plays a very important role in cancer growth and metastasis. However, lncNRON is a newly identified repressor of NFAT, and its function is largely unknown, especially in hepatocellular carcinoma (HCC). Therefore, the expression levels of lncNRON in 215 pairs of HCC tissue were evaluated by qRT-PCR, and its relationship to clinicopathological parameters, recurrence, and survival was analysed. Furthermore, stably overexpressing lncNRON cell lines were constructed and evaluated for cell phenotype. Finally, we detected epithelial-to-mesenchymal transition (EMT) proteins to determine the underlying mechanism involved in lncNRON function. We observed that lncNRON was downregulated in HCC tumour tissues; low lncNRON expression was associated with poor tumour differentiation and the presence of vascular tumour thrombus, which tended to result in poor clinical outcomes, as demonstrated by the recurrence rate and survival curves. Functional analysis showed that lncNRON overexpression impaired colony formation and cell viability and inhibited cell migration and invasion. A study using tumour-bearing mice showed that lncNRON markedly limited tumour growth and lung metastasis in vivo. Importantly, western blot analysis revealed that the expression of the EMT-related epithelial marker, E-cadherin, increased, whereas the expression of mesenchymal markers *N*-cadherin, snail, and vimentin was attenuated by lncNRON overexpression in HCC cells. Therefore, lower lncNRON expression indicates a poorer clinical outcome in HCC. LncNRON overexpression can suppress HCC growth and metastasis via inhibiting the EMT, and lncNRON may function as a new HCC prognostic marker.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related death [1]. The number of HCC cases in 2020 is estimated to increase to 78,000 and 27,000 in Europe and the United States, respectively (IARC. http://www-dep.iarc.fr/; 2011). Therefore, studies focused on HCC biomarkers and new therapeutic targets have significant benefit for HCC prevention and the reduction in the physical and economic burden of the disease.

Long non-coding RNAs (lncRNA) are a class of RNA between 200 nucleotide (nt) and 100 kb in length and no protein-coding potential [2,3]. Research has revealed that they regulate biological events through chromatin modification, as well as via transcriptional and post-transcriptional processes [4]. LncRNAs have been suggested to participate in the regulation of gastric, lung, prostate, breast, cervical, and colorectal cancer development and progression and serve as prognostic markers and therapeutic targets in various forms of cancer [5–12]. Recently, several lncRNAs have been shown to demonstrate aberrant expression in HCC and are suggested to be involved in HCC tumourigenesis and metastasis. These include lncRNAs such as HOTAIR, H19, MALAT1, MEG3, and MVIH [13]. However, additional HCC-related lncRNAs need to be investigated to gain a better understanding of HCC pathogenesis and uncover additional potential prognostic markers.

Nuclear factor of activated T cells (NFAT) is a transcription factor that was first identified in nuclear extracts from activated T cells. The NFAT family comprises five members (NFAT-1, NFAT-2, NFAT-3, NFAT-4, and NFAT-5) [14,15] and is considered to play an important role in cancer invasion, migration, and angiogenesis [16–18]. NRON is reported to function as a NFAT repressor through the inhibition of NFAT activity by reducing NFAT nuclear translocation [19] and has

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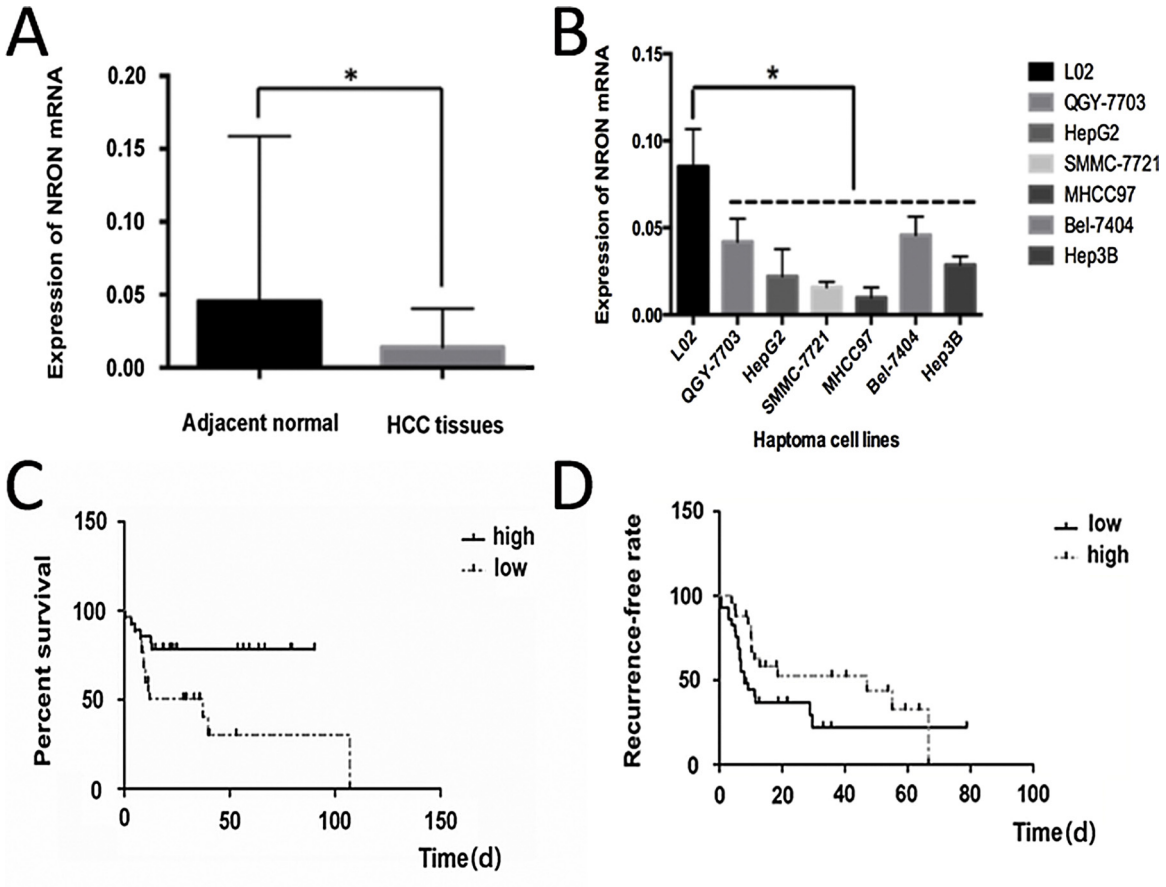

Fig. 1. NRON is down-expressed in hepatocellular carcinoma (HCC) tissues. (A) NRON mRNA expression levels in HCC tissue samples and paired adjacent normal tissue samples (215 cases) were determined by qRT-PCR. GAPDH was used as an internal quantitative control. (B) Lower expression levels of NRON were detected in HCC cell lines (QGY-7703, HepG2, BEL-7404, Hep3B, SMMC-7721 and MHCC97) than in normal L02 cells, according to the qRT-PCR assay. GAPDH was used as an internal quantitative control. The experiments were all repeated at least three times. *: $p < 0.05$ vs. the control. Kaplan-Meier survival curves for HCC patients according to the expression of lncRNA NRON. (C) Overall survival, (D) progression-free survival.

been suggested to suppress vein endothelial cell proliferation and invasion through its NFAT inhibition [20]. These findings demonstrate the inhibitory role of NRON in cell proliferation and the motile serotype. However, the understanding of NRON function remains limited. Because NFAT is involved in cancer pathology, we hypothesized that NRON may be involved in cancer development and prognosis.

In the present study, the expression of NRON in HCC clinical samples was analysed and correlated with clinical parameters to investigate whether NRON could function as a potential prognostic marker. Furthermore, its function in HCC tumour growth and metastasis, as well as its potential molecular mechanisms, were investigated in two HCC cell lines.

2. Material and methods

2.1. Patients and samples

Patients with HCC who underwent surgical resection were enrolled at The Third Affiliated Hospital of Sun Yet-sen University between May 2012 and August 2015. Only patients who did not survive the disease were included. All patient diagnoses were pathologically confirmed. HCC tumour tissues and adjacent normal tissues (2 cm from tumour tissue, confirmed by pathological diagnosis to be without tumour tissue) were fixed in formalin within 15 min after surgical section and stored at −80 °C.

2.2. Cell lines and culture

In this study, we detected long non-coding RNA NRON expression in 6 HCC cell lines (QGY-7703, HepG2, BEL-7404, Hep3B, SMMC-7721 and MHCC97) and 1 human hepatocyte cell line (L02) as a control; all cell lines were purchased from the Shanghai Cell Bank (Chinese Academy of Science). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (10% FBS DMEM) and grown at 37 °C in a 5% CO_2 atmosphere. The cells were maintained in an exponential growth phase for all experiments.

2.3. Quantitative reverse transcription-PCR (qRT-PCR)

The total RNA from 215 paired HCC patient samples was extracted using the Trizol reagent (Life Technologies, CA, USA). The qRT-PCR analyses were conducted on a Fast Real-time PCR 7500 System (Applied Biosystems) using SYBR-green PCR Master Mix, according to the manufacturer's instructions. The PCR program was set as 50 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The $2^{-\Delta Ct}$ value was used to determine the relative expression of NRON. The NRON primers used for qRT-PCR detection were Forward: 5′-ACGTTC CTTAATGTACGCCTTTGC-3′ and Reverse: 5′-TTGGCCGTGTCCTGAGT CCTT-3′.