

NNT-AS1 Impairs CD4+ T Cells Infiltration by Up-Regulating TGF- β Signaling in Hepatocellular Carcinoma

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Abstract

Background Nicotinamide Nucleotide Transhydrogenase-antisense RNA1 (NNT-AS1) is one of long non coding RNA, has been shown with high levels in several types of cancers. However, the molecular mechanism remains to be revealed for NNT-AS1 in the progression of hepatocellular carcinoma (HCC). TGF- β signaling pathway has been identified as one of negative factor for excessive immunological response. We sought to investigate the effects of NNT-AS1 mediating T cells infiltration by regulating TGF- β signaling pathway in patients with HCC.

Methods RNAscope *In Situ* Hybridization and Real Time Quantitative PCR assays were applied to detect NNT-AS1 levels in HCC tissues. Immunohistochemistry (IHC) assays were used to observe the co-expressions of TGF- β , TGFBR1, SMAD1/5/9, and CD4⁺ T cells. The mechanisms as to how NNT-AS1 orchestrates TGF- β signaling were further explored in HepG2 and Huh7 cells.

Results RNA-scope analyses revealed that the levels of NNT-AS1 were significant higher in cancerous tissues than in paired non-cancerous tissues ($P=0.0001$). Quantitative PCR assays validated the high expression of NNT-AS1 in HCC cancer tissues (n=64) when compared with normal tissues (n=26) ($P=0.0003$). The prognostic analysis indicated that the OS rates for HCC patients with high levels of NNT-AS1 were significantly lower than patients with low levels of NNT-AS1 ($P=0.0402$). Mechanistic analysis demonstrated that the downregulation of NNT-AS1 significantly reduced the expression of TGF- β , TGFBR1, and SMAD5. Moreover, the inhibition of NNT-AS1 decrease the expression of TGF- β , TGFBR1, and SMAD5. Importantly, IHC analyses indicated that the levels of NNT-AS1 were negatively correlated with CD4⁺ T lymphocyte cells infiltration in tissues from 16 HCC patients.

Conclusions Our study depicts a novel mechanism regarding NNT-AS1 activates TGF- β signaling pathway and thus impairs the CD4⁺ T lymphocyte cells infiltration in HCC.

Background

Hepatocellular carcinoma (HCC), as the dominant cause of death among liver cancer, is the 6th most common cancer in terms of incidence and has the 4th highest cancer-related mortality around the world[1]. In China, HCC morbidity and mortality rates rank as the 4th and 3rd in all malignant tumors respectively. Recently, immune checkpoint blockade (ICB), particularly antibody that targets PD-1, PD-L1, or CTLA-4, has been applied to treat HCC patients [2]. Nivolumab, pembrolizumab (PD-1 inhibitor) and tremelimumab (CTLA-4 inhibitor) have been demonstrated to be safe and effective in clinical trials [3–5]. Nivolumab has been approved to be as a second-line treatment for HCC[6]. In a phase I/II trial in advanced HCC (Checkmate-040 trial), nivolumab yielded a response rate of 20% [3]. Many factors contribute to prevent ICB therapy in HCC. Tumor infiltration lymphocytes (TIL) is one of key factors associated with ICB therapy effect. For example, high infiltration of Foxp3 + T-cells was associated with poor prognosis [7]. However, the molecular mechanisms governing the establishment of TIL still remained to be illustrated in HCC.

LncRNAs are a subgroup of non-coding RNAs that are composed of more than 200 nt, which are involved in almost all key aspects of the occurrence, development and process of different types of tumors [8]. Many

newly discovered lncRNAs have been reported to play as the oncogene or tumor suppressor role in a variety of tumorigenesis. lncRNA-ATB promotes metastasis of HCC [9]. HULC-mediated down-regulation of tumor suppressor p18 can promote cell proliferation in HCC [10]. lncRNA CUDR promotes the proliferation of HCC stem cells and affects the prognosis [11]. SNHG6-003 was found to function as a competing endogenous RNA to promote the progression and predict the poor prognosis of HCC [12]. NNT-AS1 is a novel identified lncRNA, which is located at 5p12 with 3 exons [13]. NNT-AS1 acts as one driver gene in cervical cancer [14], gastric cancer [15] and osteosarcoma [16]. NNT-AS1 was proved to induce Epithelial-to-Mesenchymal Transition (EMT) process in breast cancer by sponging miR-142-3p and up-regulating ZEB1 [17]. Downregulation of NNT-AS1 inhibits NSCLC progression via regulating miR-129-5p axis [18]. So far, the relationship between NNT-AS1 and TGF- β has not been studied. TGF- β signaling pathway plays key roles in regulating tumor immune reaction in Tumor Micro-Environment (TME) [19]. Tumor-derived TGF- β can induce tumorigenic and pro-metastatic responses in cancer cells and stroma, including the formation of an immune-suppressive TME [20]. Our previously data had proved that TGF- β signaling is hyper active in colon cancer. The loss of SMAD1/5/9 and TGFBR2 predicted a poor prognosis for colon cancer patients [21]. TGF- β consists of three isoforms (TGFB1, ~ 2, and ~ 3). TGFB1 is the most abundant and well-studied isoform [22]. There are two typical TGF- β receptors, TGF- β type I and type II receptors (TGFB1 and TGFB2), with active serine/threonine kinase [23]. Moreover, we demonstrated that the activation of TGF- β signaling could promote tumor angiogenesis, while the excessive activation of TGF- β signaling could prevent tumor angiogenesis [24]. TGF- β signaling mediates immune evasion by upregulating IDO in plasmacytoid DCs and CCL22 chemokine in myeloid DCs [25]. A recent study has provided that TGF- β induced CD8⁺CD103⁺ cells present in tumor beds exhibiting a tolerogenic phenotype that facilitates immune evasion [26]. An antibody against α v β 8 integrin that blocks the release of active TGF- β by cancer cells unleashed the immune system against tumors in pre-clinical models, and this therapeutic effect was largely potentiated by combining anti-PD1 antibodies [27].

Here, we studied the levels of NNT-AS1 in HCC tissues by RNAscope *in situ* hybridization. Further analysis focused on the correlation between NNT-AS1 and the prognosis of patients with HCC. We discussed the mechanism that NNT-AS1 promoted TGF- β signaling activation in HCC cell lines. We also explored the correlation among NNT-AS1, TGF- β signaling, and TIL cells in the tissue samples from HCC.

Methods

Hepatocellular carcinoma tissue samples

Informed consent was provided for the use of these clinical materials for our research, which was approved by the Beijing Chaoyang Hospital Ethics Committee of the Capital Medical University from 2016 to 2019. The tissue sources of HCC cancer are Beijing Chao-yang Hospital and Beijing Cancer Hospital. It consists of 16 pairs of liver cancer tissues and matched paracancerous tissues. Those tissues samples were gathered one Tissue Microarrays (TMA) of which included 32 sample dots. HCC cDNA microarrays (cDNA-HLivH090Su01) that contained 90 cDNA samples of HCC patients were provided by OUTDO (Shanghai OUTDO Biotech Co., LTD Shanghai, China).

NNT-AS1 RNAscope In Situ Hybridization Assay

The expression of NNT-AS1 was detected in HCC TMA slides by an RNAscope assay kit (RNAscope® 2.5 HD Assay-Brown, Advanced Cell Diagnostics, Hayward, CA, USA, Cat No. 322310). A RNAscope probe targeting NNT-AS1 was obtained from ACD (Advanced Cell Diagnostics, Cat No. 17268B). NNT-AS1 RNA molecules were detected with single-copy detection sensitivity in TMA tissues. To calculate the number of NNT-AS1 RNA molecules, the single-molecule signals were quantified on a cell-by-cell basis by manual counting. The signals per cell were divided into the following 5 levels: 0–1 dots/10 cells, 1–3 dots/cell, 4–10 dots/cell, > 10 dots/cell with dots in clusters and positive cells in clusters < 10%, and > 10 dots/cell with dots in clusters and positive cells in clusters > 10%. To explain more conveniently, these values were marked as -, +, ++, +++, and +++, respectively [28]. We used (Advanced Cell Diagnostics; catalog number 476701), a probe targeting human PPIB mRNA, as a positive control. A probe targeting *Bacillus subtilis* DapB mRNA (Advanced Cell Diagnostics; Cat No. 310043) was used as the negative control.

Analysis of the expression of NNT-AS1 in The Cancer Genome Atlas

The HCC (LIHC) transcription group expression profiles could be downloaded from TCGA data portal (<http://www.cbioportal.org/>). This could be used to validate differential expressions of NNT-AS1. The FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) values and prognostic information for each sample were downloaded from the TCGA database portal. Then, after standardizing the expression of RNA sequence, the difference level of NNT-AS1 between HCC tissues and adjacent noncancerous tissues were calculated. For OS analysis in the HCC transcription group dataset, the FPKM quartile value was used as the cutoff value that defined the high or low expressions of NNT-AS1.

Cell culture

HepG2, Huh7 and HL-7702 were obtained from Cell Center of the Institute of basic Medicine, Peking Union Medical College. HepG2 and Huh7 were maintained in DMEM Medium containing 10% FBS with 100 ug/ml penicillin and 100 µg ml/streptomycin and incubated at 37 °C under 5% CO₂. HL-7702, a normal cell line of human liver, was maintained in RPMI medium containing 20% fetal bovine serum, 37 °C, 5%CO₂.

siRNA transfection of HCC cells

Silencer select small interference RNAs (siRNAs) specific to NNT-AS1 (siRNA) and a control siRNA were obtained from SyngenTech (Beijing, China). Four siRNA targeting NNT-AS1(5'-GCCAGUCCUUGUCAUAUATT-3'; 5'-GCCUUUCUAGGCUGUACAATT-3';5'-GGAGACAGAUGGAUCAUUUTT-3'; 5'-GAAAAGAAAAAGAAGCUUAtt-3'),and one control siRNA (5'-UUCUCCGAACGUGUCACGUTT-3') were transfected into HepG2 and Huh7 cells using Lipofectamine 3000 (Life technologies) and Opti-MEM (Gibco, Carlsbad, CA, USA) according to the manufacturer's recommendations.

RNA extraction and reverse transcription

Total RNA was extracted from HCC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA quality was evaluated using a Nanodrop 2000 (Wilmington, DE, USA). Total RNA (1 µg) was reversely transcribed into complementary DNA (cDNA) using EasyScript® First-Strand cDNA Synthesis kit (Transgene, Beijing, China).

Real time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed by using the Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Gaithersburg, MD, USA) and the SYBR® Select Master Real-Time PCR assay (Transgene, Beijing, China). The PCR program was as follows: pre-denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min. The primers used for qRT-PCR detection of NNT-AS1 were as follows: forward primer, 5'-CAAAGGCGACCTCACGAAAT-3' and reverse primer, 5'-TTGATTGACAAGGACTGGCG – 3'. 18S was used as the internal reference gene: forward primer, 5'-AAACGGCTACCACATCCA-3' and reverse primer, 5'-CACCAGACTTGCCCCTCCA-3'.

Western blot analysis

Cells were washed twice with cold PBS, and 30 µL RIPA (Solarbio, Shanghai, China) containing a mixture of protease inhibitors (Invitrogen, Carlsbad, CA, USA) and a mixture of phosphatase inhibitors were added. The cells were then collected into a cold tube. The cell lysates were centrifuged at 4 °C at 12000 g for 20 minutes. Subsequently, the proteins in the lysates were separated on a 10% polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membrane. The membranes were blocked in 10% fat-free milk solution containing 0.1% Tween-20 for 2 hour at room temperature. Next, the PVDF membranes were incubated with primary antibodies of TGF-β (CST Danvers MA, 1:200 dilution), TGFBR1 (Abcam, 1:400 dilution), SMAD5 (Abcam, 1:200 dilution) and GAPDH (D16H11) (CST) at 4 °C. Then the second antibody (zhongshanjinqiao, China) was incubated at room temperature for 1 hour. Protein bands were visualized using Super Enhanced Chemiluminescence Detection reagents (Applygen Technologies, Beijing, China).

Immunohistochemical analysis

The TMA slices were baked in an oven at 65 °C for 2 hours. Xylene I and II dewaxing for 10 minutes respectively. The TMA slide was de-paraffinized and rehydrated. The slide was pre-treated with EDTA antigen retrieval solution (pH = 8.0) and rinsed in water. The sections were then blocked in 2% goat serum and were incubated with the primary antibody overnight at 4 °C. The primary antibodies included anti-TGF-β (dilution 1:100), TGFBR1 (dilution 1:100), SMAD1/5/9 (dilution 1:100, CST), and CD4⁺ (dilution 1:100, Abcam).

Statistical analysis

The chi-squared tests and Cox proportional hazards regression model were performed using SPSS 11.0 statistical software. Chi-squared tests were applied where appropriate to analyze the differential expression of NNT-AS1 between cancer and normal tissues and its associations with clinicopathological parameters. GraphPad Prism 5 software was used to plot the OS rates. The significance of the FPKM values from TCGA datasets was determined using the Mann-Whitney *U* test. *P* < 0.05 was considered to be significant.

Results

High levels of NNT-AS1 are observed by RNAscope In Situ Hybridization in HCC

16 HCC patients were enrolled to compare the levels of NNT-AS1 between cancerous tissues and corresponding adjacent noncancerous tissues. A total of 32 tissue samples were successfully stained with NNT-AS1 by RNAscope (Fig. 1A, B). The scores were classified as follows: “- , +” represented low levels, and “++ , +++ , ++++” represented high levels of NNT-AS1. NNT-AS1 levels were demonstrated to be significantly increased in 10 (62.5%) of 16 HCC tissues. However, the NNT-AS1 molecule was rarely detected in corresponding adjacent noncancerous tissues; no (0%) tissue was ranked as having a high expression of NNT-AS1 (*P* = **0.001**) (Table 1).

Table 1
Expression of NNT-AS1 in Hepatocellular Carcinoma

NNT-AS1 expression		
Score	Normal (n = 16)	Cancer (n = 16)
-	13 (81.25%)	2 (12.50%)
+	3 (18.75%)	4 (25.00%)
++	0	6 (37.50%)
+++	0	2 (12.50%)
++++	0	2 (12.50%)
<i>P value</i>	0.001	

Table 2
Correlation between NNT-AS1 and clinicopathological characteristics in HCC

	NNT-AS1 levels	<i>P</i> value
Sex		
Male (n = 60)	0.0001247 ± 0.0001082	0.3220
Female(n = 4)	0.0001078 ± 0.0000266	
Age		
≤ 60(n = 48)	0.0001180 ± 0.0001116	0.6756
> 60(n = 16)	0.0001135 ± 0.0000911	
Tumor size(cm)		
≤ 5(n = 31)	0.0001085 ± 0.0000776	0.6756
> 5(n = 33)	0.0001545 ± 0.0001253	
Number of lesions ^[a]		
A single lesion (n = 53)	0.0001163 ± 0.0001015	0.9183
Two lesions (n = 5)	0.0001589 ± 0.0001849	
Pathology classification ^[b]		
L, M(n = 27)	0.0001085 ± 0.0000988	0.8241
H(n = 9)	0.0001578 ± 0.0001264	
TNM staging ^[c]		
I(n = 43)	0.0001139 ± 0.0001006	0.2689
II-III(n = 16)	0.0001793 ± 0.0001274	
[a]. Six subjects lost.		
[b]. Twenty-eight subjects lost.		
[c]. Five subjects lost.		

High levels of NNT-AS1 are associated with shorter OS time in HCC tissues

NNT-AS1 expression was further examined in HCC tissues by qRT-PCR cDNA. The samples included both cancerous tissues and adjacent noncancerous tissues that were derived from 64 HCC patients. Significantly higher NNT-AS1 expression was found in the cancerous tissues than that in the noncancerous tissues ($P=0.0003$) (Fig. 2A). Paired *t* test analysis was applied to compare the differential levels of NNT-AS1 in 26 paired cDNA samples. it was further confirmed that the levels of NNT-AS1 in cancer tissues were

significantly higher than their levels that in adjacent non-cancerous tissues (Fig. 2B). Kaplan-Meier or Cox proportional hazards regression analyses were used to determine the prognostic relevant. In total, 64 subjects with HCCs were followed up for 2-113 months (mean \pm S.D., 44.25 ± 31.45 months). 39 (60.94%) patients had died at the endpoint of follow up. Kaplan-Meier analysis indicated that the higher levels of NNT-AS1 predicated a shorter overall survival (OS) time than HCC patients to whom shown low levels of NNT-AS1 ($P = 0.0402$) (Fig. 2C). By univariate analysis (Cox proportional hazards regression), NNT-AS1 expression level (Higher vs. Lower, $P = 0.044$), tumor size (≤ 5 cm vs. >5 cm, $P = 0.028$), TNM stage (I vs. II/III, $P = 0.036$) were identified as the prognostic factors (Table 3). To further validate our findings, we explored the difference in the expression levels of NNT-AS1 in the TCGA data. One of the HCC (LIHC) dataset that included 369 cancerous tissues and 50 normal tissues were used for the following investigations. We compared the normalized values (FPKM) that were determined by RNA sequencing of the cancerous tissues and normal tissues. In accordance with our results, the levels of NNT-AS1 were found to be significantly increased in HCC tissues when compared with NNT-AS1 levels in normal tissues ($P < 0.0001$) (Fig. 2D). Follow-up information was available for 146 liver cancer patients from the LIHC dataset. Kaplan-Meier analysis demonstrated that higher NNT-AS1 FPKM values were significantly associated with reduced OS time compared with the corresponding low expression groups ($P = 0.016$) (Fig. 2E).

Table 3
Independent influence on overall survival of HCC patients, assessed with Cox's proportional hazards model analysis

Variables	Univariate analysis		Multivariate analysis	
	HR(95%CI)	P value	HR(95%CI)	P value
NNT-AS1	1.918	0.044	1.701	0.245
(Low vs. High)	(1.017–3.619)		(0.694–4.167)	
Sex	1.335	0.691		
(Female vs. Male)	(0.321–5.546)			
Age	0.734	0.471		
(≤ 60 vs. >60)	(0.348–1.549)			
Tumor size	2.071	0.028	1.932	0.180
(≤ 5 cm vs. >5 cm)	(1.082–3.962)		(0.738–5.056)	
Tumor number	1.198	0.765		
(single vs. multiple)	(0.367–3.915)			
pathological grade	2.053	0.116	1.540	0.396
(H, M vs. L)	(0.838–5.029)		(0.568–4.178)	
TNM	2.113	0.036	2.806	0.039
(Ⅰvs.Ⅱ,Ⅲ)	(1.049–4.255)		(1.052–7.481)	
CI, confidence interval; HR, hazard ratio				

Altering TGF-β signaling is positively associated with the changes of NNT-AS1

Thorsson et al. had summarized six immune subtypes in cancer, that included C1 (wound healing), C2 (IFN-γ dominant), C3 (inflammation), C4 (lymphocyte depletion), C5 (immune silencing) and C6 (TGF-β dominance) based on TCGA datasets [29]. To investigate the roles for NNT-AS1 in HCC, we compared their levels in different immune types of HCC. NNT-AS1 were found to be significantly increased in C1 type (Fig. 3A). C1 patients showed less tumor TILs and had shorter OS time [29]. We thus speculated that NNT-AS1 may regulate immune response in HCC.

Next, We analyzed the correlation between the levels of NNT-AS1 and the genes involved in TGF-β signaling (included TGFB1, TGFB1, TGFB2, SMAD1, ~ 2, ~3,~4,~5,~6, ~ 7 ~ 9) and other genes as controls that were involved in interferon signaling, angiogenesis, immunoreaction (such as CD274, IFI27, CD33 and so on) (Fig. 3B). It demonstrated that NNT-AS1 was positively related to TGFB1, TGFB1, SMAD1, ~ 2, ~3, ~ 4, ~5, ~ 6, ~7). These results revealed that NNT-AS1 is positively associated with TGF-β signaling. Further, we tried to investigate the mechanism that mediated the interaction between NNT-AS1 and TGF-β signaling. At first,

we determined the levels of NNT-AS1 in cells lines including HL-7702, HepG2, and Huh7 (Fig. 3C). The levels of NNT-AS1 were significantly higher in HepG2 than in HL-7720, the latter is one of normal liver cell lines. To explore the effects of TGF- β signaling on NNT-AS1 expression, we used siRNA targeting TGF- β , TGFBR1, SMAD1, SMAD5, SMAD9 to transfect HepG2 cells. As a result, the downregulation of TGFBR1, SMAD1, SMAD5 by siRNA interference significantly decreased the levels of NNT-AS1 (Fig. 3D). On the other hand, human recombinant TGF- β significantly increased the expression of NNT-AS1 (Fig. 3E). SB431542, one of inhibitors that could block TGF- β receptor, significantly reduced the levels of NNT-AS1 in HepG2 cells (Fig. 3F). Moreover, qRT-PCR detection confirmed the positive correlation among NNT-AS1, SMAD5 and TGFBR1 in 15 samples from HCC patients (Fig. 3G).

Inhibition NNT-AS1 impairs TGF- β signaling in HCC cells

We used four siRNAs that targeted NNT-AS1 to disrupt the expression of NNT-AS1 in HepG2 cells. One of the most effective (siNNT3) siRNAs was used for further study (Fig. 4A). Transient transfections of siNNT significantly reduced mRNA levels of TGF- β , TGFBR1, and SMAD5 in HepG2 cells (Fig. 4B). Moreover, transient transfections of siNNT significantly reduced mRNA levels of TGFBR1 and SMAD5 in Huh7 cells (Fig. 4C). Besides, downregulation of NNT-AS1 by siNNT1 impaired protein levels of TGF- β , TGFBR1, and SMAD5 (Fig. 4D).

IHC analysis reveals that NNT-AS1 is positively related with TGF- β signal pathway and negatively related with immune cell infiltration

To determine the relationship among NNT-AS1, TGF- β signaling, and immune cells, IHC analyses were used to determine the levels of TGF- β , TGFBR1, SMAD1/5/9, CD4, and CD8 in continuous TMA slides. We successfully stained TGF- β (Fig. 5A), TGFBR1 (Fig. 5B) and SMAD1/5/9 (Fig. 5C) in TMAs. As expected, the levels of TGF- β , TGFBR1, and SMAD1/5/9 were significantly increased in HCC tissues (Table 4). On the contrary, the levels of CD4⁺ T cells were significantly reduced in cancer tissues (Fig. 5C) (Table 4). Pearson correlation analyses revealed that the levels of NNT-AS1 were positively related with the levels of TGF- β , TGFBR1, and SMAD1/5/9 (Fig. 5E). Interestingly, the levels of NNT-AS1 were negatively associated with the levels of CD4⁺ T cells (Fig. 5E). Collectively, those results indicated that NNT-AS1 activated TGF- β signaling pathway and then inhibited tumor infiltration of CD4⁺ T cells in HCC.

Table 4
Expression of TGF- β , TGFBR1, SMAD1/5/9 and CD4 in Hepatocellular Carcinoma.

	TGF- β		TGFBR1		SMAD1/5/9		CD4	
Score	Normal (n = 16)	Cancer (n = 16)	Normal (n = 16)	Cancer (n = 16)	Normal (n = 16)	Cancer (n = 16)	Normal (n = 16)	Cancer (n = 16)
-	5 (31.25%)	0	0	1 (6.25%)	9 (56.25%)	1 (6.25%)	1 (6.25%)	3 (18.75%)
+	9 (56.25%)	11 (68.75%)	13 (81.25%)	7 (43.75%)	7 (43.75%)	2 (12.50%)	2 (12.50%)	5 (31.25%)
++	2 (12.50%)	4 (12.50%)	2 (12.50%)	6 (37.50%)	0	11 (68.75%)	6 (37.50%)	8 (50.00%)
+++	0	1 (6.26%)	1 (6.26%)	2 (12.50%)	0	2 (12.50%)	7 (43.75%)	0
<i>P</i> <i>value</i>	0.046		0.005		0.008		0.002	

Discussion

Increasing evidence has indicated that lncRNAs participate in a wide range of cellular processes, including regulation of epigenetic signatures, gene expression and proliferation [30, 31].^[32] Since NGS detects the sequences in an unbiased way, NGS also enhances our knowledge about noncoding RNAs, such as long noncoding RNA (lncRNA), microRNA (miRNA) and circular RNA (circRNA), which were considered useless products of RNA splicing errors. Recently, Ye et al used NGS to find that non-coding RNA was found to be involved in the regulation of many important physiological and pathological processes[33]. Our present research demonstrated that NNT-AS1 is significantly increased and shows a robust correlation with HCC prognosis. In addition, we found that there was a positive correlation between the expression of NNT-AS1 and the activation of TGF- β signal. Importantly, the increased expression of NNT-AS1 inhibited CD4⁺ T cell tumor infiltration.

lncRNA was thought to be unstable and hard to preserve in paraffin-embedded tissues [32]. Here, we used the RNAscope method to evaluate the expression of NNT-AS1. This technique, which was first developed by Wang *et al.*, enabled the direct counting of mRNA molecules in single cells in routine formalin-fixed tissue specimens using bright-field microscopy[34]. More importantly, RNAscope has the advantage of avoiding false-negative results from the admixtures of many nonmalignant cells[35]. RNAscope analysis successfully captures RNA signals in paraffin files, thus accurate measurement of NNT-AS1 can be achieved. Through this advanced technology our study provided convincing evidence that HCC tissues showed significant increases in expression of NNT-AS1.

More and more lncRNAs have been reported to be involved in HCC immune reaction. lnc-EGFR suppressed tumor immune reaction by promoting Treg cell differentiation and promoted immune escape [36]. TP73-AS1 modulates HCC cell proliferation via miR-200a/ HMGB1/ RAGE pathway, being inversely correlated with miR-200a and positively correlated with HMGB1 and RAGE [37]. Previously, NNT-AS1 overexpression has been reported and predicted a poor prognosis of colon cancer [38]. Moreover, the significance of NNT-AS1 in orchestrating tumorigenesis had been addressed by many other reports. NNT-AS1 promoted cholangiocarcinoma cells proliferation and EMT through down-regulating miR-203 [39]. NNT-AS1 regulated the progression of lung cancer through the NNT-AS1/miR-3666/E2F2 axis [40]. NNT-AS1 promoted gastric cancer proliferation and invasion by regulating microRNA-363 expression [15]. Here, we conducted an analysis to address the novel mechanistic function of NNT-AS1 in HCC. Our analyses demonstrated that the expression of NNT-AS1 was positively associated with TGF- β signaling. TGF- β acts as immunosuppressive cytokine through effects on both immune cell differentiation and proliferation [41]. TGF- β inhibits proliferation of T-lymphocytes [42] and thymocytes [43], and TGF- β 2 was isolated as glioblastoma-derived T-cell suppressor factor, based on the observation that glioblastoma is frequently accompanied by immunosuppression [44]. TGF- β , in combination with IL-2, induces Treg cell differentiation through induction of Foxp3 expression in CD4⁺ T cells [45, 46]. Our study confirmed that the overexpression of NNT-AS1 would activate TGF- β signaling and inhibit the infiltration of CD4⁺ T cells. Future analysis needs to outline the molecular mechanism as to how NNT-AS1 and TGF- β signal transduction affect tumor immune response in HCC.

Collectively, we delineated NNT-AS1 as a prognostic factor with significantly increased levels in HCC. In addition, NNT-AS1 was proven to act as one of positive regulators of TGF- β , TGFBR1 and SMAD5. Further, NNT-AS1 was negatively associated with CD4⁺ cell TIL in HCC. Therefore, we demonstrated the novel mechanism that NNT-AS1 activated TGF- β signaling and further impaired CD4⁺ cell TIL. However, several limitations were present in this study. For instance, a retrospective approach was used to analyze the relationships between NNT-AS1 and clinical characteristics, and a relatively small cohort was enrolled. Further studies of the complete molecular mechanisms underlying NNT-AS1 knockdown would be useful to validate and expand our findings.

Conclusion

Our study depicts a novel mechanism that conducts the process of CD4⁺ T lymphocyte cells infiltration. Of which is regulated by NNT-AS1 that activates TGF- β signaling pathway and thus impairs the CD4⁺ T lymphocyte cells infiltration in HCC. Our investigations suggest that the upregulation of NNT-AS1 serves as a promising predictor and might be conducive for clinicians to estimate OS time.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Beijing Chao-yang Hospital(Capital Medical University).

Consent for publication

Authors confirmed that this work can be published. Also, all authors ensured that this manuscript is original and has not yet been accepted or published elsewhere.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Lei Yang and Yakun Wang designed the experiment. Yakun Wang performed the experiment, processed the data and wrote the manuscript. Lei Yang reviewed and approved the final submitted manuscript. Tao Wen supervised the study. Xichen Dong, Xin Yang, Xinxue Zhang, Zhe Liu and Xin Zhao collected samples. All authors read and approved the final manuscript.

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Not applicable.

Foundations

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Conflict of interest statement

The authors disclose no potential conflicts of interest.

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Figures

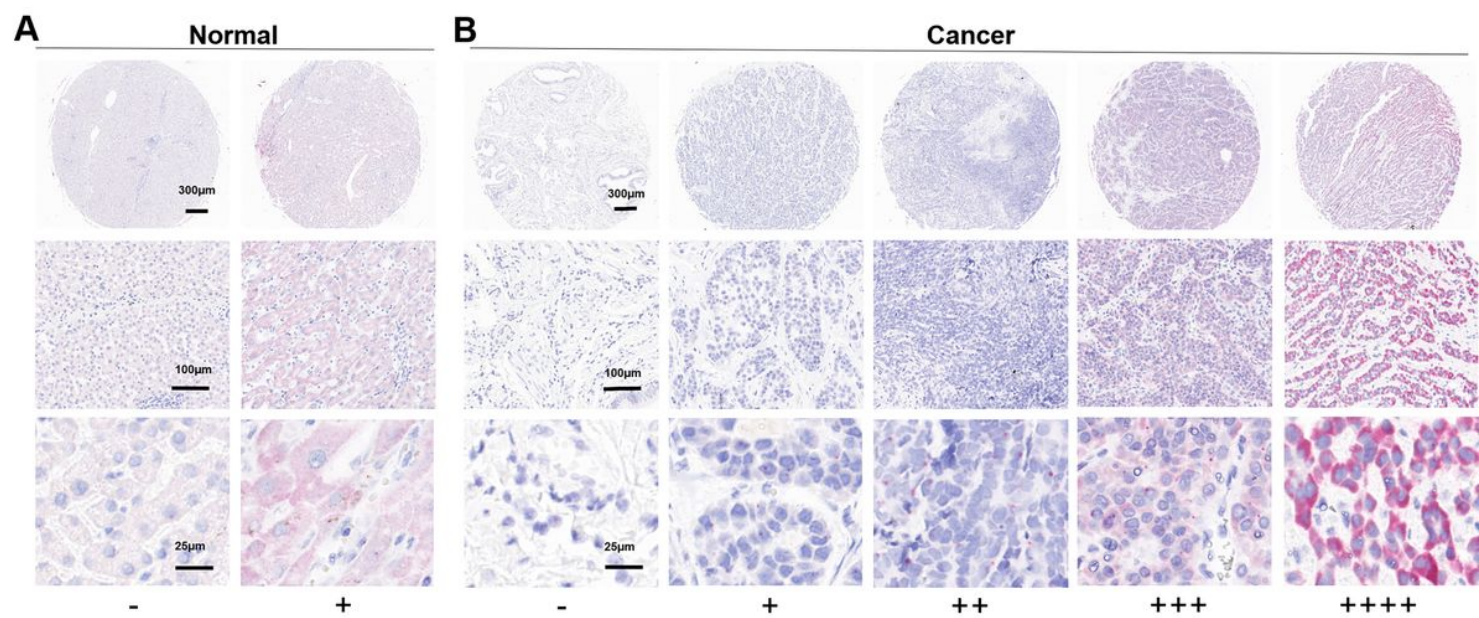


Figure 1

Representative staining of NNT-AS1 RNAscope in HCC adjacent normal tissues (A) and cancer tissues (B). ‘-’ (0-1 dots/10 cells), ‘+’ (1-3 dots/cell), ‘++’ (4-10 dots/cell), ‘+++’ (> 10 dots/cell with dots in clusters <10%), and ‘++++’ (> 10 dots/cell with dots in clusters >10%).

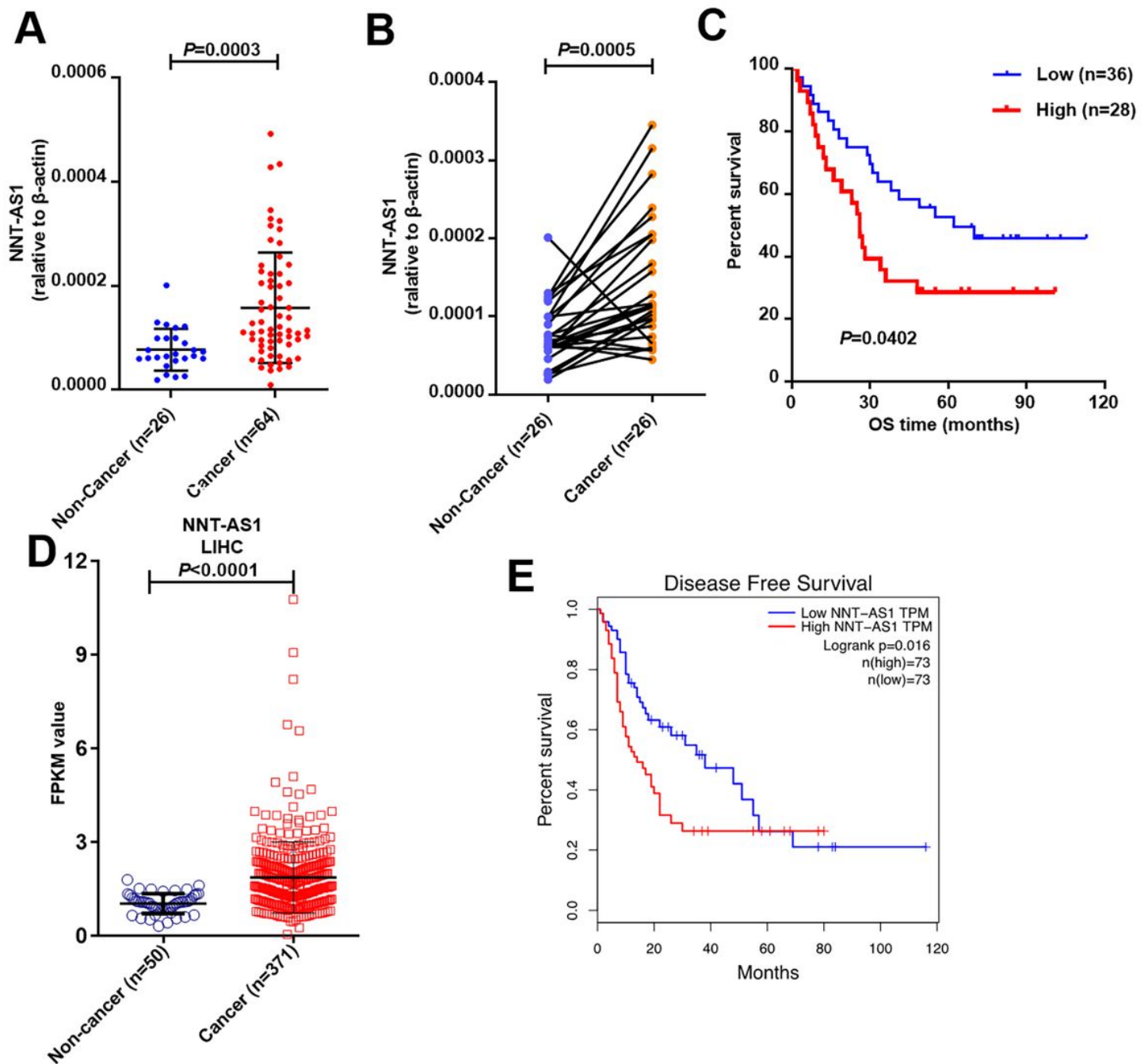


Figure 2

Quantitative real-time PCR was used to detect the expression of NNT-AS1 in cancer tissues and adjacent non-cancerous tissues from 64 patients with HCC (A). Detected the expression of NNT-AS1 in cancer tissues and adjacent normal tissues of 26 pairs of HCC patients by qRT-PCR (B). Kaplan-Meier was used to analyze the OS of 64 patients with HCC (C). The overexpression of NNT-AS1 is validated in TCGA LIHC dataset (D). Elevated expression of NNT-AS1 was found to be associated with a reduced overall survival time in LIHC (E).

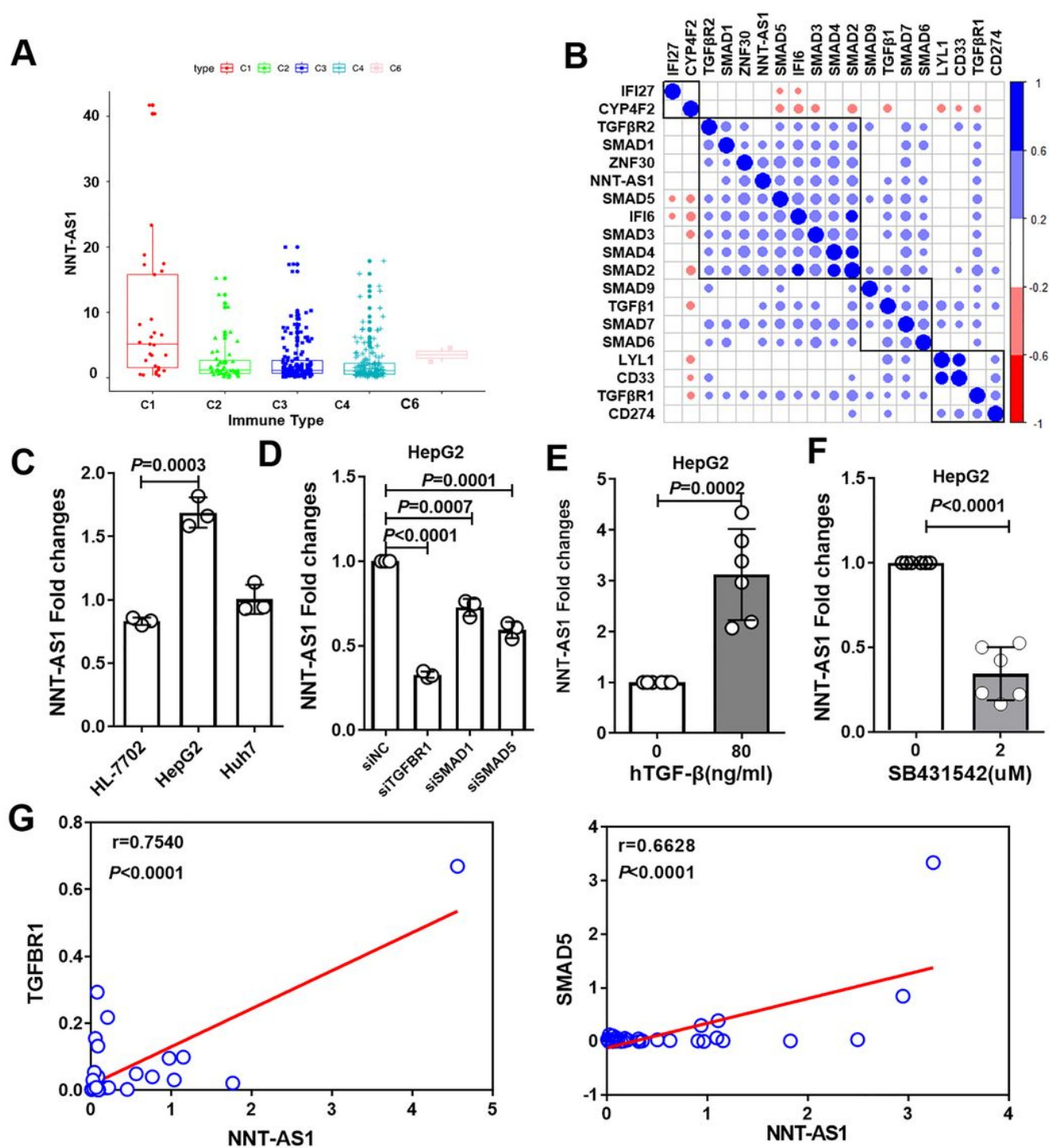


Figure 3

The levels of NNT-AS1 in different immune types of HCC(A). Pearson correlation analyzed the levels of NNT-AS1 and the genes that involved in TGF-β signaling and other genes as controls that involved in interferon signaling, angiogenesis, immunoreaction (B). qRT-PCR analysis of the relative mRNA levels of NNT-AS1 in three cell lines (HL-7702, HepG2, Huh7) (C). NNT-AS1 expression after transfecting siRNA that targeting TGF-β, TGFB1, and SMAD5 was confirmed by qRT-PCR in HepG2 (D). hTGF-β activated the TGF-β signaling pathway and increased the expression of NNT-AS1. SB431542 inhibited the TGF-β signaling pathway and

decreased expression of NNT-AS1 (E, F). The correlation between NNT-AS1 with SMAD5 and TGFBR1 was analyzed by qRT-PCR in 15 HCC patients (G).

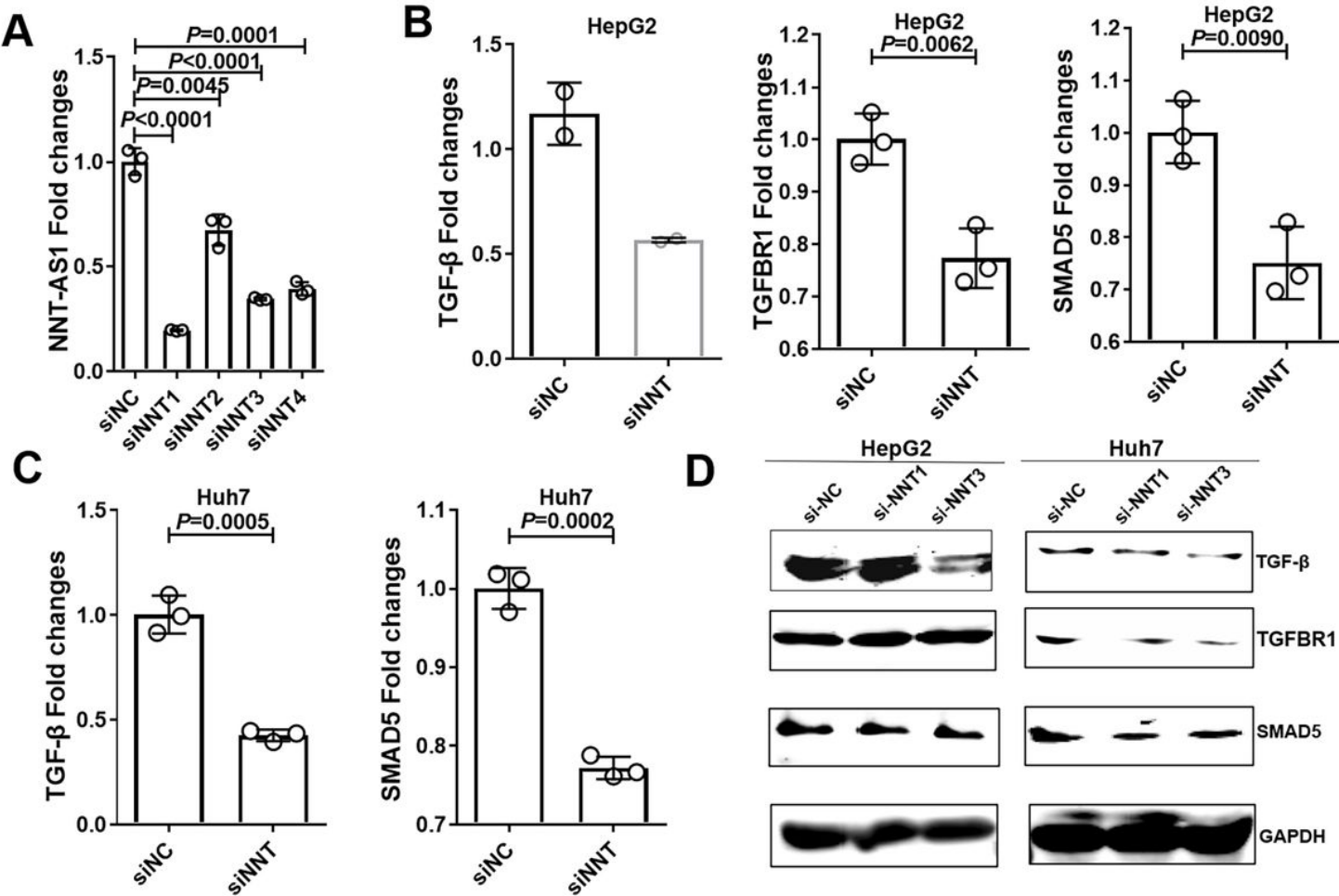


Figure 4

NNT-AS1 expression levels transiently transfected with NNT-AS1 specific siRNA (siNNT) or control siRNA (siNC). The NNT-AS1 expression was determined by qRT-PCR assay after siRNA transfection in HepG2 (A). qRT-PCR assays determined the mRNA levels of TGF-β, TGFBR1 and SMAD5 in HepG2 (B). qRT-PCR assays determined the mRNA levels of TGF-β and SMAD5 in Huh7 (C). Western-blotting determined the protein levels of TGF-β, TGFBR1 and SMAD5 in HepG2 and Huh7(D).

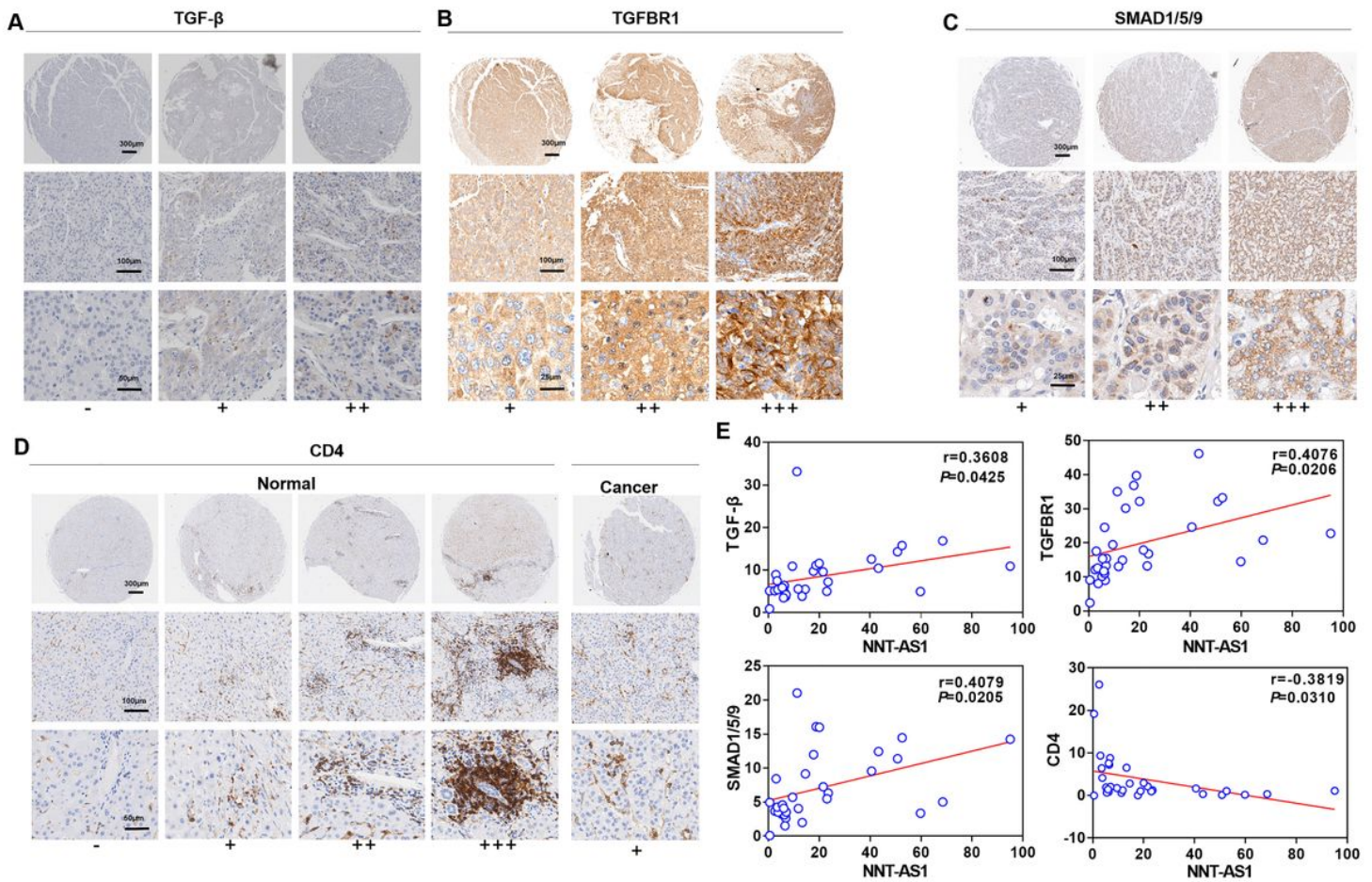


Figure 5

IHC analyses were used to determine the levels of TGF- β (A), TGFBR1 (B), SMAD1/5/9 (C), and CD4+ (D) in continuous TMA slides. Pearson correlation analyses showed that the correlation between NNT-AS1 levels and TGF- β , TGFBR1, SMAD1/5/9 and CD4+ cell levels (E).

Supplementary Files

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