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Original Article

Nicotinamide nucleotide transhydrogenase acts as a new prognosis biomarker in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Lipid metabolism is essential for cancer development. Nicotinamide nucleotide transhydrogenase (NNT) is abnormally expressed in multiple cancers; however, its role in HCC is unclear. We assessed the NNT expression level in The Cancer Genome Atlas (TCGA) cohort and Gene Expression Omnibus (GEO) datasets and found that the expression level of NNT was lower in HCC patients than non-cancer control subjects in the public databases. Survival analysis was conducted according to high and low NNT expression. Low NNT expression was significantly associated with a poor prognosis. For confirmation, the gene and protein expression of NNT in cancer and adjacent non-cancer tissues from HCC patients at our institute cohort indicated the lower expression level of NNT in cancer compared to adjacent non-cancer tissues using quantitative polymerase chain reaction and western blot, respectively. Bioinformatics was used to analyze the underlying mechanisms and establish the protein-protein interaction network of NNT. It showed that NNT is associated with functions of bile acid and fatty acid metabolism and their related genes. To conclude, our results supported that NNT expression is downregulated in HCC, and can serve as a novel prognostic biomarker.

Keywords: Hepatocellular carcinoma, nicotinamide nucleotide transhydrogenase, prognosis, lipid metabolism

Introduction

Hepatocellular carcinoma (HCC) ranks high with respect to incidence and mortality worldwide [1]. HCC is one of the leading causes of cancer-related mortality with more advanced disease cases and poor 5-year overall survival [2]. Despite common treatments of surgical resection, interventional therapy, and systemic pharmacological treatments such as targeted therapy, the survival time of patients with HCC remains poor [3-6]. Hepatitis, cirrhosis, and fatty liver are the main risk factors for HCC development [7]. Patients with chronic hepatitis B and C infection have a higher probability of developing HCC [8]. Fatty liver disease and diabetes are also major risk factor for HCC [9, 10]. Alpha-fetoprotein is a recognized marker of HCC; however, its prediction performance is not satisfactory [11]. Therefore, new markers, including prognostic predictors, and drug targets of HCC need to be discovered.

Deregulated cellular metabolism is one of the hallmarks of cancer [12]. Indeed, the majority of HCC patients show metabolic dysfunction. Lipid metabolism is an essential component of cancer metabolism [13]; lipids promote malignant biological behaviors such as tumor cell proliferation, invasion, and metastasis by regulating the fluidity of cell membranes and driving signal transduction cascades [14].

Recently, many studies have focused on understanding the mechanisms contributing to aberrant metabolic processes in cancer. Nicotinamide nucleotide transhydrogenase (NNT) is an antioxidative enzyme that converts nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide phosphate (NADPH) [15, 16]. NADH and NADPH can be reduced in oxidative reactions in the cytosol and mitochondria during energy metabolism [17]. Accordingly, NNT plays a key role in redox metabolism [18], and is found to be dysfunc-

tional in several cancer types. For example, in gastric cancer, NNT can regulate redox homeostasis to promote cancer growth and metastasis [19]. Moreover, NNT expression is upregulated in adrenocortical carcinoma, and triggered anti-apoptosis pathways in cancer cells [20]. NNT is also used as a sensor in mitochondrial biology [21]. The lack of NNT in macrophages was shown to exacerbate atherosclerosis in hypercholesterolemic mice [22]. The absence of NNT was also shown to inhibit cell proliferation and aggressive behavior via alteration of hypoxia inducible factor-1 alpha- and histone deacetylase 1-dependent pathways [23]. However, its role in HCC remains unclear.

Therefore, in the present study, we explored the expression profiles of NNT in HCC based on data from public databases and samples collected from patients at our institute, and determined their associations with survival. We further conducted bioinformatics analysis to identify the functions, and related genes and pathways of NNT. These results can provide new insight into the potential mechanisms contributing to HCC development, particularly with respect to the role of lipid metabolism, and highlight new targets for treatment.

Materials and methods

Clinical sample

Fresh HCC and paired adjusted tissues were collected at Nanfang Hospital, Southern Medical University (Guangzhou, China). Tissues were immediately frozen with liquid nitrogen after liver resection and stored in a refrigerator at -80°C for protein and RNA extraction. The study protocol was accepted by the Nanfang Hospital Institutional Review Board.

Expression data sets

HCC patients and health donors RNA-seq data in The Cancer Genome atlas (TCGA) cohort (https://tcga-data.nci.nih.gov/tcga/) were included in our study to explore the expression of NNT. A set of array data (GSE20140, GSE14520 and GSE74656) were downloaded from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/).

Quantitative polymerase chain reaction

Total RNA was extracted from tissues using TRIzol reagent (TaKaRa Biotechnology, Dalian, China). Complementary DNA (cDNA) was synthesized by reverse transcription kit (TaKaRa Biotechnology, Dalian, China). Primer sequences were synthesized as follows: NNT primer: Forward: CAGGTACCCGGAGTGGAGTT, Reverse: TCCTTGAAGGCAAATCACTGGT; β-actin primer: Forward: GATTCCTATGTGGGCGACGA, Reverse: AGGTCTCAAACATGATCTGGGT. Q-PCR was performed using the SYBR Green PCR kit (TaKaRa Biotechnology, Dalian, China). β-actin was chosen as an internal quantitative reference. Each experiment was conducted in triplicate.

Western blot

Tissues were lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Total Protein concentrations were measured by the BCA protein assay kit (Thermo Fisher, Shanghai, China). Protein lysates were separated on a 10%-12% sodium dodecyl sulfate-polyacrylamide gel and electro-transferred to a polyvinylidene difluoride membrane (Bio-Rad) that was blocked with 5% bovine serum albumin for 1 h at room temperature and probed overnight at 4°C with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA). Immunodetection was performed using enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). Antibodies against, NNT (1:1000, from Proteintech, Chicago, IL, USA); β-actin (1:3000, from Abcam, Cambridge, MA, USA) were used.

GSEA

The GSEA program provided in broad institute was used (http://www.broadinstitute.org/gsea/index.jsp). RNA-seq data from TCGA HCC cohort were analyzed. The data were split into NNT low or high group and were entered for GSEA program. The program was exploited to test whether numbers of a gene set or signatures were randomly distributed at the top or bottom of the ranking.

Statistical analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Prism Software Inc., San Diego, California) and expressed as mean ± SEM of at least three independent experiments. The

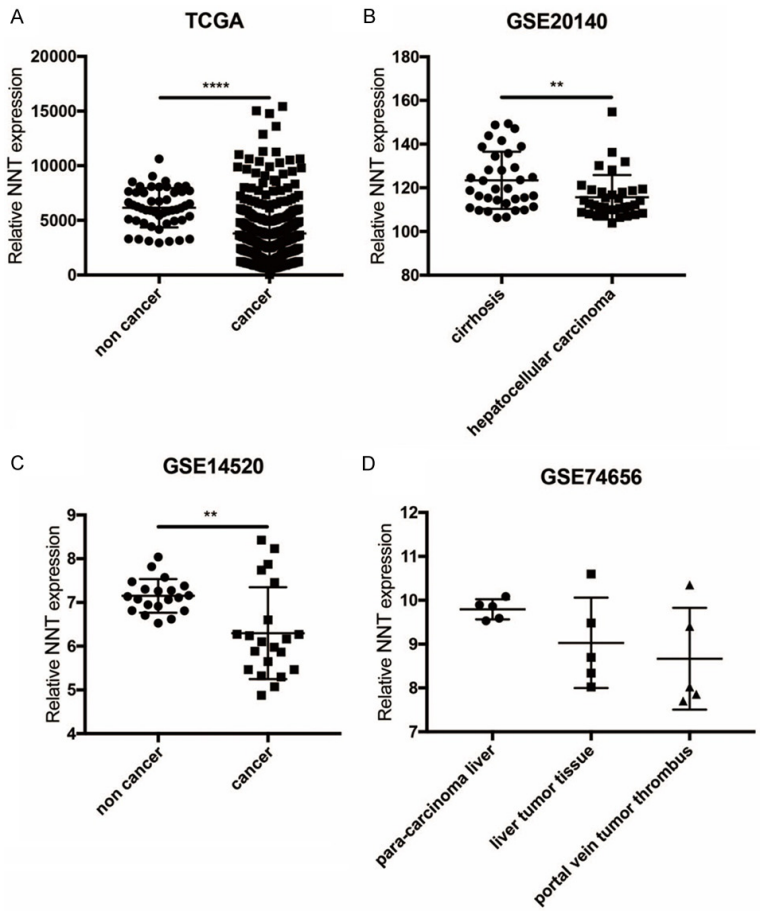

Figure 1. NNT expression was downregulated in patients with hepatocellular carcinoma (HCC). A. The expression of NNT in the TCGA cohort was analyzed in a group of non-cancer donors and HCC patients ($P < 0.0001$). B. NNT expression level in patients with cirrhosis or HCC from the GSE20140 dataset ($P < 0.01$). C. Expression of NNT in liver non-cancer tissues and liver cancer tissues in the GSE14520 cohort ($P < 0.01$). D. NNT expression in para-carcinoma liver, liver cancer, and portal vein cancer thrombus tissue samples in the GSE74656 cohort.

Student's t-test was used to assess the significance of differences between two groups.

All statistical tests were two-sided. $P < 0.05$ was considered statistically significant. Single, double and triple asterisks indicate statistical significanced $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Results

NNT expression was downregulated in HCC

Analysis of RNA-seq data from the TCGA cohort showed lower expression of NNT in the cancer group than in the non-cancer group (**Figure 1A**, $P < 0.0001$). Similarly, in the GSE-20140 dataset, patients with HCC had lower NNT expression levels than cirrhosis patients as the control group (**Figure 1B**, $P < 0.01$), and NNT levels were lower in the cancer group than those in the non-cancer group in the GSE14520 dataset (**Figure 1C**, $P < 0.01$). In the GSE74656 dataset, the expression level of NNT showed a gradual decline from the para-carcinoma liver tissues, liver tumor tissues, and portal vein tumor thrombus tissues (**Figure 1D**).

Low NNT expression was associated with a poor prognosis

The patients in the TCGA cohort were split into a high and low NNT expression group for survival analysis. Patients with high NNT expression showed significantly better overall survival (OS; **Figure 2A**, $P = 0.0035$) and disease-specific survival (DSS; **Figure 2B**, $P = 0.032$) than those in the low expression group. Among the 93 patients in the TCGA cohort with microvascular invasion of the cancer, those with high NNT expression also had a significantly better OS than patients with low NNT expression (**Figure 2C**, $P = 0.025$). Similarly, among the 30 patients in the TCGA cohort treated with sorafenib, a multitarget inhibitor, those with low NNT expression had a shorter OS than those with high NNT expression (**Figure 2D**).

NNT expression was downregulated in HCC tissues

Paired tissue samples collected from 12 patients with HCC at our institute were further evaluated for NNT expression. In line with the public data, *NTT* mRNA expression levels were significantly lower in the HCC tissues than in paired adjacent tissues (**Figure 3A**, $P < 0.05$). Western blot of 11 paired tissues further indicated reduced NNT protein expression in the

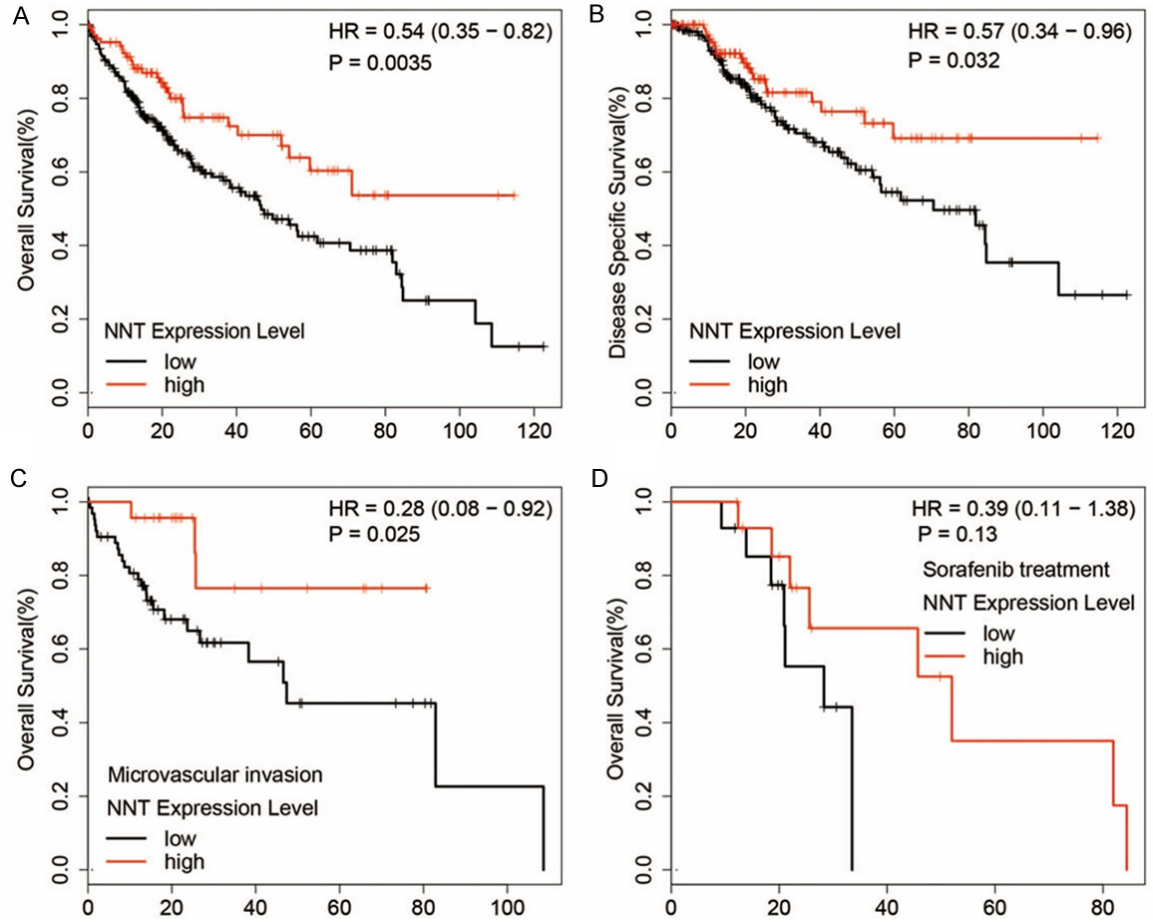

Figure 2. Low NNT expression was correlated with a poor prognosis. A. Overall survival (OS) analysis of hepatocellular carcinoma (HCC) patients in the TCGA cohort according to high and low NNT expression (P = 0.0035). B. Disease-specific survival (DSS) analysis of HCC patients in the TCGA cohort according to high and low NNT expression (P = 0.032). C. OS analysis of microvascular invasion-positive HCC patients with high and low NNT expression in the TCGA cohort (P = 0.025). D. OS analysis of patients treated with sorafenib with high and low NNT expression in the TCGA cohort.

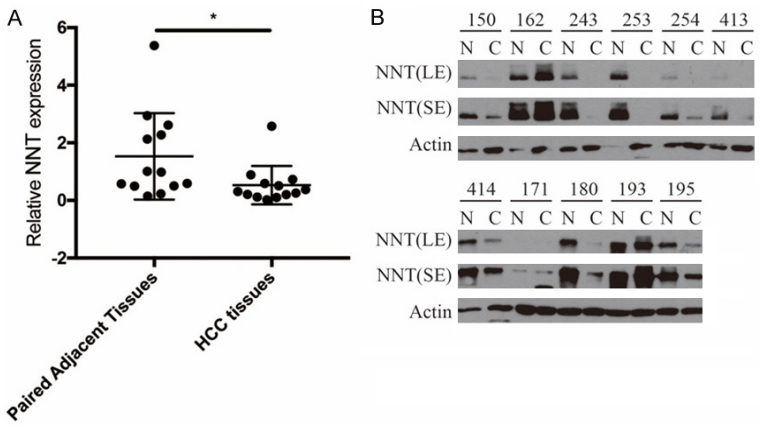

Figure 3. NNT expression levels are reduced in cancer tissues in patients with hepatocellular carcinoma (HCC) at our institute. A. The mRNA expression of *NNT* in 12 HCC tissues and paired adjacent normal tissues measured using quantitative polymerase chain reaction (P < 0.05). B. The NNT protein level evaluated using western blot in 11 paired non-cancer and cancer tissues.

cancer tissues than in the non-cancer tissues in the majority of the patients (**Figure 3B**).

NNT is associated with lipid metabolism

Gene set enrichment analysis (GSEA) of the TCGA cohort demonstrated that the expression level of *NNT* was inversely correlated with gene signatures of bile acid metabolism

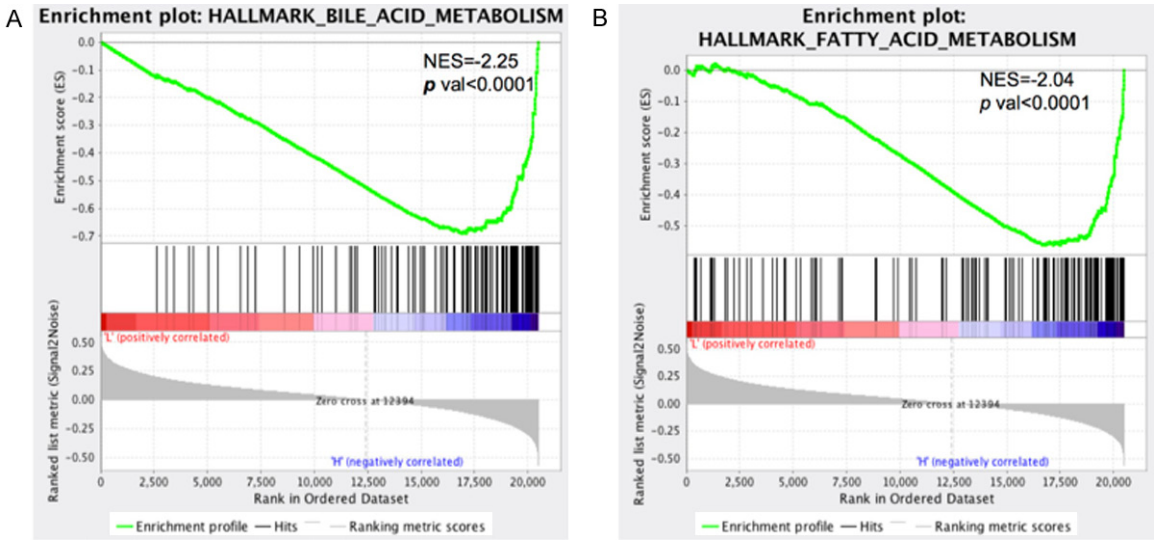

Figure 4. NNT was associated with lipid metabolism. A. Gene set enrichment analysis (GSEA) results plotted to visualize the correlation between the expression of NNT and gene signatures of bile acid metabolism ($P < 0.0001$) in the TCGA cohort. B. GSEA results plotted to visualize the correlation between the expression of NNT and gene signatures of fatty acid metabolism ($P < 0.0001$) in the TCGA cohort.

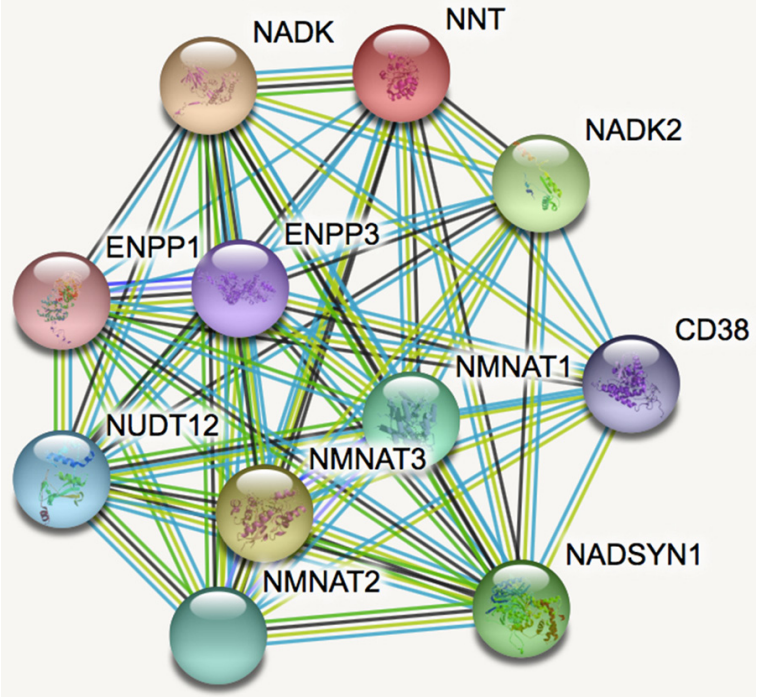

Figure 5. Network analysis of NNT. The protein-protein interaction network was determined by the String database with a confidence cut-off of 0.9. Disconnected nodes are not displayed.

(**Figure 4A**, $P < 0.0001$) and fatty acid metabolism (**Figure 4B**, $P < 0.0001$), suggesting a role in lipid metabolism.

Network analysis of NNT

The protein-protein interaction network of NTT is shown in **Figure 5**. A total of 11 proteins constituted a complex of associations in a multi-centered interaction network with interaction scores >0.9. All of these genes (*NNT, NADK, NADK2, CD38, NADSYN1, NMNAT1, NMNAT2, NMNAT3, ENPP1, ENPP3,* and *NUDT12*) were significant hub genes interacting with other genes in the interaction network.

Discussion

HCC is a prevalent malignant induced within an established background of chronic liver disease and cirrhosis after long latency [24]. Metabolic processes dysfunction, HCC in particular, is common in the pathogenesis of cancer [16]. NNT has been shown to be altered in several types of cancer, implicating a role of lipid metabolism in cancer development and progression. Despite the fact that metabolic

dysfunction is a known risk factor for HCC, the role of NNT in HCC has not been investigated.

Here, we demonstrate that NNT expression is downregulated in HCC patients compared to that in various controls from public databases. Moreover, patients with high NNT levels had a better prognosis than those with low NNT levels. We further confirmed these findings from public cohorts in our own cohort of HCC patients, in which NNT expression was reduced in HCC tissues compared to that in paired normal adjacent tissues at both the mRNA and protein levels. Bioinformatics analysis further indicated that NNT is associated with functions of bile acid metabolism and fatty acid metabolism. NNT was also correlated with a large number metabolism-related genes. More detailed mechanisms of these interactions should be experimentally investigated in the future, which may highlight additional treatment targets and cancer mechanisms.

Although there are limitations of the present study, the results suggest the potential of NNT as a novel prognostic biomarker for HCC patients. However, more detailed investigations with larger samples are needed to fully elucidate the role of NNT in HCC.

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Disclosure of conflict of interest

None.

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