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Overexpression of TONSL might be an independent unfavorable prognostic indicator in hepatocellular carcinoma

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ABSTRACT

Background: TONSL has been suggested to function as an oncogene in lung, esophageal and cervical cancer. This study was aimed to identify the expression of TONSL and its role in hepatocellular carcinoma (HCC).

Methods: By data mining in the Cancer Genome Atlas (TCGA) and Human Protein Atlas (HPA) databases, the expression profile of TONSL, its clinical significance, the potential mechanisms of its dysregulation and its underlying biological function in HCC were investigated.

Results: TONSL was significantly upregulated in HCC tissues relative to normal liver tissues ($P < 0.05$). High TONSL expression was significantly correlated with advanced TNM stage, poorly differentiated tumors, vascular invasion, elevated serum alpha-fetoprotein expression and a worse prognosis (all $P < 0.05$). Multivariate analysis further confirmed that TONSL overexpression was an independent risk factor for poor overall survival (OS) and recurrence-free survival (RFS) in HCC (all $P < 0.05$). Additionally, 16% of HCC cases ($n = 370$) had TONSL DNA amplification. The total methylation level of TONSL was moderately and negatively correlated with its mRNA expression ($P < 0.05$). TONSL was predictively targeted by miR-133b, which was downregulated in HCC and negatively related to TONSL mRNA expression (all $P < 0.05$). Kaplan-Meier analyses demonstrated that low miR-133b expression was significantly associated with poor OS and RFS (all $P < 0.05$). Moreover, gene set enrichment analysis revealed that cases with TONSL overexpression were enriched in cell cycle regulation pathways (all $P < 0.05$).

Conclusions: TONSL holds promise for serving as a prognostic biomarker for HCC. DNA amplification, hypomethylation and miR-133b downregulation could be the mechanisms associated with TONSL upregulation in HCC. TONSL might function as an oncogene via cell cycle regulation pathways in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent malignancy and the third leading cause of cancer-related death worldwide [1]. Despite progress in early detection and comprehensive treatment of HCC, the relatively high recurrence rate and poor prognosis highlight the importance of further clarifying the biological features of HCC as well as exploring the mechanisms by which tumors evade treatment [2]. Several studies have revealed that the tumorigenesis and progression of HCC are complicated courses that are subject to numerous genes, and aberrant gene-expression profile is the triggering factor on which the tumor biology behaviors depend [3]. Thus, recognition of genes with potential clinical implications may be beneficial to HCC diagnosis and therapy.

TONSL (also named NFKBIL2), located on chromosome 8q24.3, was initially identified as the human IκB-R (the inhibitors of NF-κB related) gene. It encodes tonsoku-like DNA repair protein (TONSL), which was thought to be a negative regulator of NF-κB mediated transcription [4]. However, a subsequent study demonstrated that TONSL exhibited significant differences from the genomic structures of other IκB members, such as the region of the ankyrin repeats and its chromosomal localization, suggesting that TONSL might actually not belong to the IκB family [5]. TONSL is characterized by several typical domains, including a ubiquitin-like fold, six leucine-rich repeats in C-terminal, three central ankyrin repeats and seven N-terminal tetratricopeptide repeats [6]. Interestingly, due to its diversity of functional domains, previous studies revealed that TONSL was not only involved in diverse physiological processes including homologous recombination, but also implicated in carcinogenesis [6–8]. For example, upregulation of TONSL has been observed in several cancers, such as lung, esophageal

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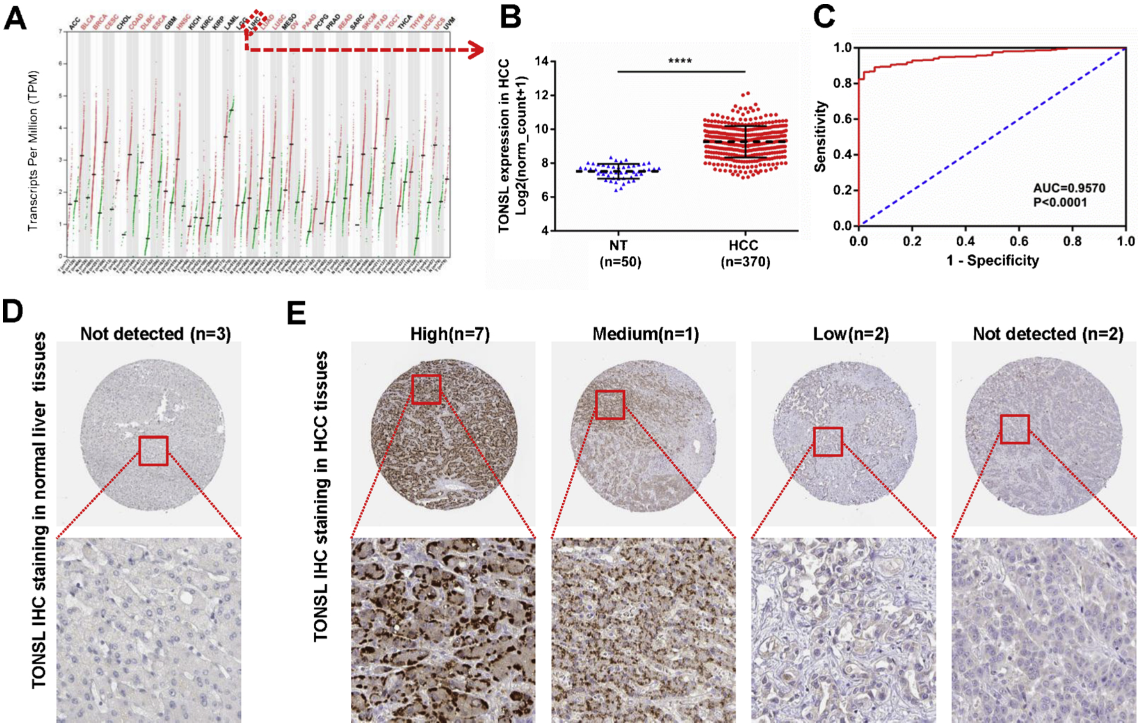

Fig. 1. TONSL expression profile both at mRNA and protein level in human tissues. (A) TONSL mRNA expression in different normal human tissues and cancer tissues. (B) Comparison of TONSL expression in HCC tissues (n = 370) and in normal liver tissues (n = 50). (C) Validation of TONSL overexpression for HCC prediction using ROC curve. Representative IHC images of TONSL in normal liver tissues (D) and in HCC tissues (F).

and cervical cancer [6,8]. Furthermore, Nguyen et al. reported that siRNA-mediated reduction of TONSL could significantly suppress lung cancer cell (LC319) growth [8]. However, the expression profile of TONSL and its role in HCC remain unclear.

In this study, by data mining in the Cancer Genome Atlas (TCGA), the expression profile of TONSL, its clinical significance, the potential mechanisms of its dysregulation and its underlying biological function in HCC were investigated.

2. Materials and methods

2.1. Data mining in the TCGA database

The mRNA-seq, miRNA-seq and clinical data (level 3) of 50 normal liver tissues and 377 primary HCC tissues were drawn from the TCGA database (TCGA-LIHC data set) (https://cancergenome.nih.gov/). Among them, 370 patients had both mRNA-seq data and clinical data, whereas 366 patients, in addition to clinical data, had both mRNA-seq data and miRNA-seq data. The package edgeR of R was used to normalize the raw count data and identify the differential expression genes (mRNA and miRNA) between the HCC samples and normal controls. $|\log2$ (fold change)$| > 1$ and adjusted P-value < 0.05 were set as the cut-off criteria. TONSL mRNA expression level in several common cancers (including normal tissues and tumor tissues) were reviewed by using GEPIA (http://gepia.cancer-pku.cn) [9]. TONAL DNA copy number alterations (CNA) and methylation alterations in TCGA-LIHC were analyzed by using cBioPortal (http://www.cbioportal.org/) [10].

2.2. Data mining in the human protein atlas

The protein expression of TONSL in normal liver tissues and in HCC tissues were examined using immunohistochemistry (IHC) data from the Human Protein Atlas (HPA) database (http://www.proteinatlas.org/) [11].

2.3. Predicting the regulatory miRNAs of TONSL

The regulatory miRNAs of TONSL were predicted using two prediction databases: DIANA-microT (http://diana.imis.athena-innovation.gr) and TargetScan v7.1 (http://www.targetscan.org). Validated regulatory miRNAs of TONSL were obtained from three algorithms: MiDRB (http://www.mirdb.org/), miRwalk (http://129.206.7.150/) and Tarbase v8.0 (http://carolina.imis.athena-innovation.gr). The predicted cohort was defined as the validated miRNAs plus miRNAs predicted by both DIANA-microT and TargetScan. Furthermore, based on the standard mentioned above, a list of downregulated miRNAs in TCGA-LIHC samples was collected. The common miRNAs in both the predicted cohort and TCGA cohort were then defined as potential regulatory miRNAs of TONSL in HCC.

2.4. Gene set enrichment analysis (GSEA)

GSEA was performed to evaluate the correlations between TONSL expression (high vs low) and cancer-related pathways using the TCGA data set. C2 (c2.cp.kegg.v6.0.symbols.gmt) from Molecular Signatures Database (MSigDB) was selected as the reference gene sets. By analyzing with permutations for 1000 times, a normalized enrichment score (NES) was obtained, and a gene set was regarded as significantly enriched when a normal P-value was less than 0.05.

2.5. Statistical analyses

The comparison of TONSL expression between different clinicopathological groups was conducted using Student's t-test. The diagnostic value of TONSL in HCC was judged by receiver operating characteristic (ROC) curves. Area under the curve (AUC) and P-value were calculated. The TONSL expression level in HCC tissues were then divided into 2 groups (low and high TONSL expression) based on the association with death or HCC-recurrence using the X-tile software version 3.6.1, which can voluntarily choose the optimum cutoff value

Table 1
The association between TONSL expression and the clinical parameters in patients with hepatocellular carcinoma in TCGA.

Variables	n	TONSL expression	*t*	*P**
Age (year)				
< 65	221	9.330 ± 0.911	1.711	0.088
≥65	149	9.162 ± 0.950		
Gender				
Male	249	9.347 ± 0.945	2.527	**0.012**
Female	121	9.089 ± 0.875		
TNM stage				
I-II	256	9.204 ± 0.911	−2.464	**0.014**
III-IV	90	9.483 ± 0.960		
Unknown	24	–		
Histologic grade				
G1-G2	232	9.092 ± 0.938	−4.886	**< 0.001**
G3-G4	133	9.572 ± 0.840		
Unknown	5	–		
Ishak score				
0-4	133	9.126 ± 0.870	0.250	0.803
5-6	79	9.094 ± 0.880		
Unknown	158	–		
Child-Pugh grade				
A	216	9.216 ± 0.878	0.242	0.809
B-C	22	9.168 ± 0.883		
Unknown	132	–		
Vascular invasion				
No	206	9.128 ± 0.862	−2.231	**0.026**
Yes	108	9.365 ± 0.960		
Unknown	56	–		
Alpha fetoprotein				
Negative	147	9.056 ± 0.829	−3.906	**< 0.001**
Positive	130	9.463 ± 0.906		
Unknown	93	–		
Recurrence status				
No	145	9.088 ± 0.917	−2.560	**0.011**
Yes	174	9.348 ± 0.897		
Unknown	51	–		
Living status				
Alive	240	9.169 ± 0.910	−2.660	**0.008**
Dead	130	9.436 ± 0.943		

* statistically significant P-values are given in bold, $P < 0.05$.

based on the maximum χ^2 value and lowest P-value calculated by Kaplan-Meier method [12]. Survival analyses for overall survival (OS) and recurrence-free survival (RFS) were performed utilizing the Kaplan–Meier method and log-rank test. A Cox proportional hazards model was performed to evaluate the relative risk factors associated with OS or RFS, and hazard ratios (HR) with 95% confidence intervals (CI) were obtained for each variable. Linear regression analyses were performed to assess the correlation between TONSL expression and the methylation of CpG sites in its DNA or the miRNAs expression. Statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). A two-tailed P-value below 0.05 was considered statistically significant.

3. Results

3.1. TONSL was upregulated in HCC tissues relative to normal liver tissues

Using mRNA-seq data from GEPIA, we reviewed TONSL expression in different normal human organs in TCGA. Compared with other normal tissues, normal liver tissues displayed extremely low TONSL expression at the mRNA level, which was merely higher than that in the normal thymus and pancreas (Fig. 1A). However, TONSL mRNA expression in HCC tissues ($n = 370$) was significantly higher than that in normal liver tissues ($n = 50$) ($P < 0.0001$) (Fig. 1B). The AUC value of TONSL overexpression for HCC diagnosis was 0.9570 ($P < 0.0001$) (Fig. 1C). Consistent with it, the different TONSL expression level ($P < 0.05$) and its diagnosis value ($AUC = 0.9328$, $P < 0.0001$) were also confirmed between the HCC tissues ($n = 50$) and its paired-normal tissues (Supplementary Fig. 1). To further examine the protein expression of TONSL, we retrieved the IHC staining data from the HPA. In normal liver tissues, hepatocytes usually had low TONSL staining (Fig. 1D). In contrast, most of the HCC tissues had moderate (1/12) and strong (7/12) TONSL staining, which mainly distributed in the cytoplasm (Fig. 1E). These results suggested that TONSL was significantly upregulated both at the mRNA and protein levels in HCC tissues relative to normal liver tissues.

3.2. High TONSL expression independently predicted poor OS and RFS in HCC

A total of 370 HCC patients, with complete TONSL mRNA-seq data and clinical data in the TCGA-LIHC, were enrolled to explore the clinical significance of TONSL. As shown in Table 1, the expression level of TONSL was not associated with patients' age, Ishak score and Child-Pugh grade (all $P > 0.05$). However, high TONSL expression in HCC was significantly correlated with male patients, advanced TNM stage, poorly differentiated tumors, vascular invasion, elevated serum alpha-fetoprotein (AFP) expression, recurrence and death (all $P < 0.05$).

We further assessed the prognostic value of TONSL in HCC patients. Base on the best performing threshold, Kaplan-Meier analyses indicated that high TONSL expression was generally associated with poor OS and RFS (all $P < 0.05$) (Fig. 2). Subsequently, a Cox regression model was performed to evaluate the independent risk factors of OS and RFS. In the univariate model, TONSL expression, TNM stage and AFP were significantly associated with OS (all $P < 0.05$), while TONSL expression, TNM stage, vascular invasion and AFP were associated with RFS (all $P < 0.05$). After adjusting those prognostic values, the following multivariate analysis confirmed that high TONSL expression was an

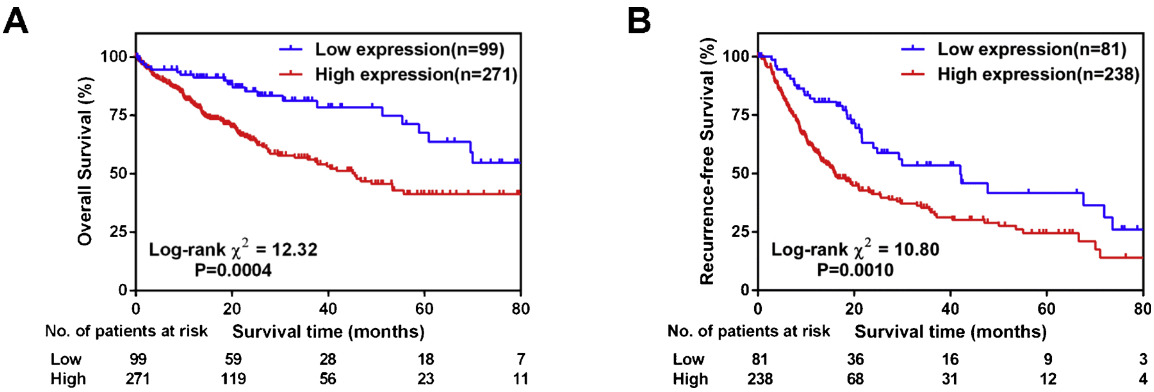

Fig. 2. Kaplan-Meier survival analysis by X-tile plots cut-off point. (A) Kaplan–Meier curves of overall survival (OS). (B) Kaplan–Meier curves of recurrence-free survival (RFS).

Table 2
Cox proportional hazards regression model analysis of overall survival.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P*	HR (95%CI)	P*
TONSL(High vs Low)	2.206(1.402,3.470)	**0.001**	2.208(1.379,3.536)	**0.001**
Age(≥65 vs < 65)	1.237(0.875,1.748)	0.228	–	–
Gender(Female vs Male)	1.226(0.860,1.747)	0.260	–	–
TNM stage (III-IV vs I-II)	2.483(1.713,3.599)	< **0.001**	1.916(1.297,2.831)	**0.001**
Histologic grade(G3-G4 vs G1-G2)	1.123(0.783,1.610)	0.528	–	–
Ishak score(5-6 vs 0-4)	0.818(0.477,1.401)	0.464	–	–
Child-Pugh grade(B-C vs A)	1.632(0.807,3.298)	0.173	–	–
Vascular invasion(Yes vs No)	1.342(0.886,2.032)	0.165	–	–
Alpha fetoprotein(Positive vs Negative)	1.670(1.080,2.582)	**0.021**	1.379(0.885,2.150)	0.156

HR: Hazard ratio; CI: Confidence interval.
* Statistically significant P-values are given in bold, $P < 0.05$.

Table 3
Cox proportional hazards regression model analysis of recurrence-free survival.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P*	HR (95%CI)	P*
TONSL(High vs Low)	1.865(1.279,2.722)	**0.001**	1.690(1.146,2.490)	**0.008**
Age(≥65 vs < 65)	0.950(0.700,1.289)	0.743	–	–
Gender(Female vs Male)	1.154(0.842,1.583)	0.372	–	–
TNM stage (III-IV vs I-II)	2.380(1.711,3.310)	< **0.001**	1.814(1.268,2.594)	**0.001**
Histologic grade(G3-G4 vs G1-G2)	1.106(0.811,1.508)	0.524	–	–
Ishak score(5-6 vs 0-4)	1.122(0.762,1.652)	0.561	–	–
Child-Pugh grade(B-C vs A)	1.347(0.720,2.519)	0.351	–	–
Vascular invasion(Yes vs No)	1.974(1.399,2.787)	< **0.001**	1.611(1.131,2.296)	**0.008**
Alpha fetoprotein(Positive vs Negative)	1.469(1.039,2.076)	**0.029**	1.290(0.907,1.834)	0.156

HR: Hazard ratio; CI: Confidence interval.
* Statistically significant P-values are given in bold, $P < 0.05$.

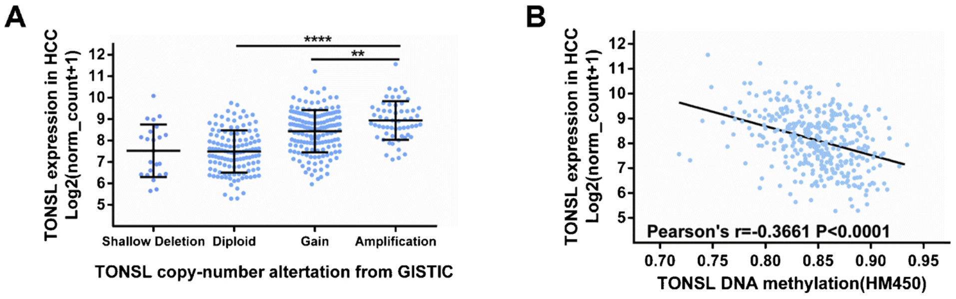

Fig. 3. DNA amplification and hypomethylation contribute to TONSL upregulation in patients with HCC. (A) Box plots of TONSL mRNA expression in HCC tissues with indicating genetic status. (B) Correlation analysis between TONSL mRNA expression and TONSL DNA methylation.

independent indicator of poor OS (High vs Low, HR = 2.208(1.379, 3.536), P = 0.001) and RFS (High vs Low, HR = 1.690(1.146, 2.490), P = 0.008). All independent risk factors and their HR with 95% Cl are listed in Tables 2 and 3.

3.3. DNA amplification, DNA hypomethylation and miR-133b dysregulation contributed to TONSL upregulation in HCC

To further explore the mechanism of TONSL dysregulation in HCC, we examined its genetic and epigenetic alterations in TCGA-LIHC. According to the tumor samples with mRNA data and copy number alterations (CNA) data (n = 370), 16% patients with HCC had TONSL amplification, and amplification was significantly associated with increased TONSL mRNA expression ($P < 0.05$) (Fig. 3A). Furthermore, we examined the correlation between TONSL expression and its DNA methylation. Linear regression analyses indicated that the total methylation level of TONSL was moderately and negatively correlated with its mRNA expression (Pearson’s r = -0.3661, $P < 0.0001$) (Fig. 3B).

In addition, we tried to identify the regulatory miRNAs of TONSL. The potential regulatory miRNAs (n = 432) of TONSL were obtained from both prediction algorithms and experimentally supported databases. Moreover, data from the TCGA database revealed that 49 miRNAs were significantly downregulated in HCC tissues compared to normal liver tissues. Between the prediction cohort and the TCGA cohort, miR-133b was screened as a candidate for further analysis and validation (Fig. 4A**, B, and C**). Linear regression analyses indicated that miR-133b expression level was negatively correlated with TONSL mRNA expression (Pearson’s r = -0.1353, P = 0.0095) (Fig. 4D). Furthermore, Kaplan-Meier analyses revealed that low miR-133b expression was correlated with poor OS and RFS (all $P < 0.05$) (Fig. 4E**, F**).

3.4. Gene set enrichment analysis

GSEA was conducted to search cancer-related pathways enriched in the samples with high TONSL expression. Six gene sets, “KEGG_

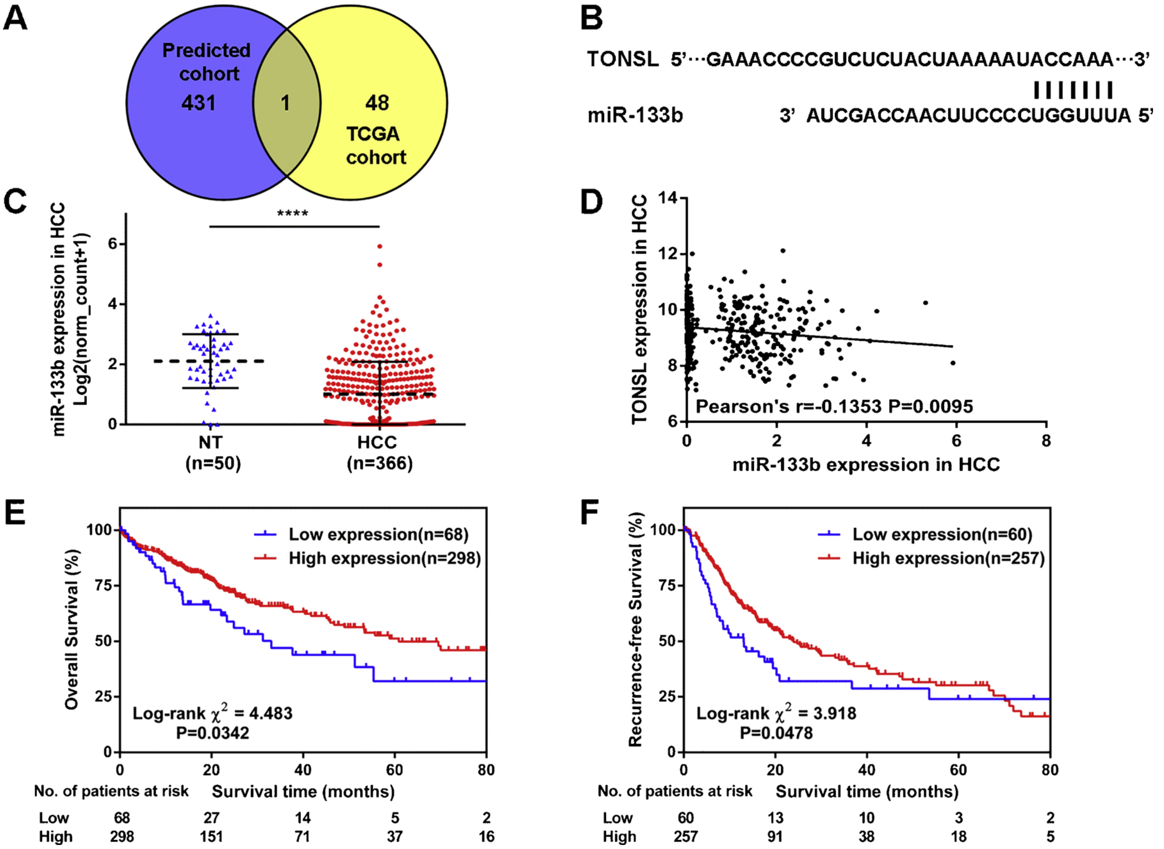

Fig. 4. TONSL is targeted by miR-133b in HCC. (A) Selection of potential regulatory miRNAs of TONSL both in the predicted cohort and down-regulated in HCC tissues in TCGA dataset. (B) The putative binding site of TONSL 3′UTR by miR-133b. (C)The expression of miR-133b in HCC tissues compared to normal liver tissues. (D) The correlation between the miR-133b expression and TONSL mRNA expression. Low miR-133b expression is correlated with poor OS (E) and RFS (F) in HCC.

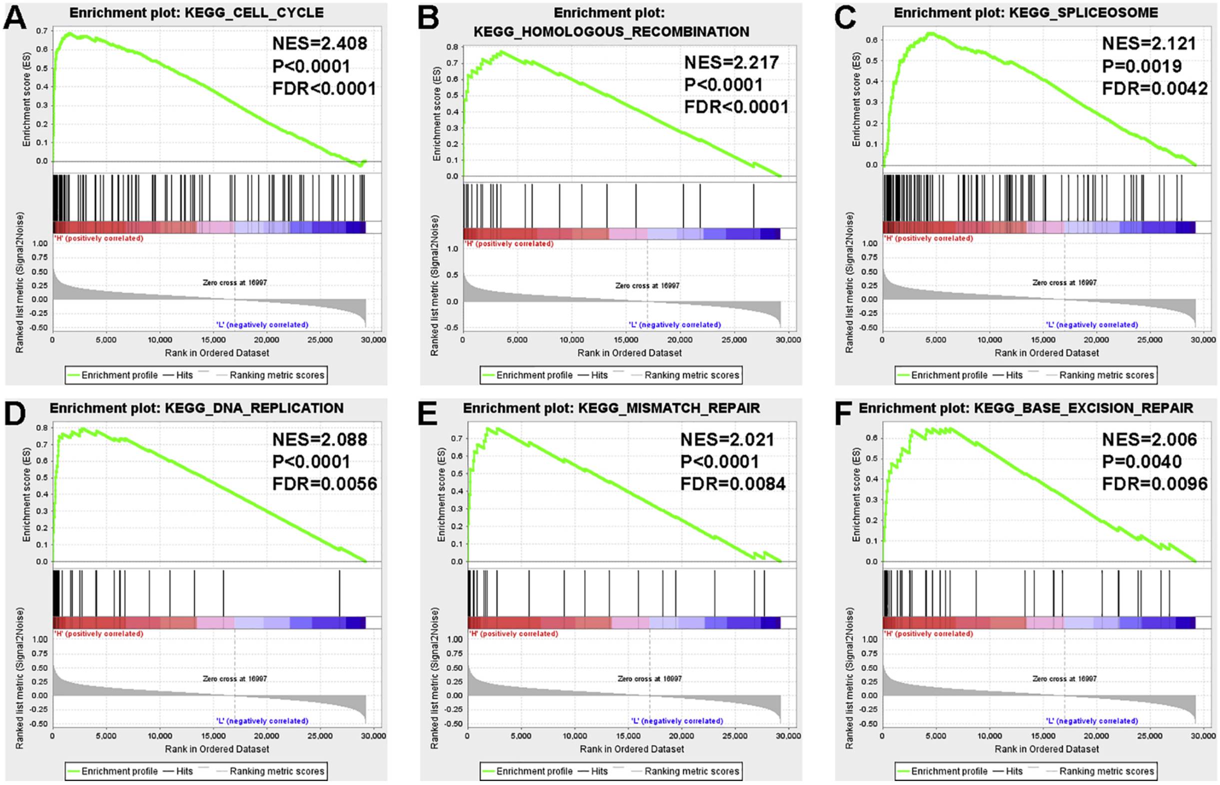

Fig. 5. Gene set enrichment analysis (GSEA) using TCGA dataset. TONSL overexpression was significantly correlated with “cell cycle” (A), “homologous recombination” (B), “spliceosome” (C), “DNA replication” (D), “mismatch repair” (E) and “base excision repair” (F) pathways.

CELL_CYCLE", "KEGG_ HOMOLOGOUS_RECOMBINATION", "KEGG_ SPLICEOSOME", "KEGG_DNA_REPLICATION", "KEGG_MISMATCH_ REPAIR", "KEGG_ BASE_EXCISION_REPAIR", were enriched (all $P < 0.05$) (Fig. 5).

4. Discussion

Progresses in genetics and molecular biology have recently enabled the rapid development of personalized tumor management. Identifying crucial biomarkers may contribute to improved diagnosis and more individualized treatment of HCC. Previous studies have reported that TONSL is upregulated in several solid tumors and that it functions as an oncogene in lung cancer [6,8]. However, no study had yet investigated the expression profile of TONSL and its clinical significance in HCC. In the current study, we initially demonstrated that TONSL was significantly upregulated in HCC tissues relative to normal liver tissues both at the mRNA and protein levels. Based on ROC curves, the diagnostic value of TONSL mRNA expression achieved high performance ($AUC = 0.9570$, $P < 0.0001$), which indicated that TONSL may hold promise for becoming a biomarker used for the pathological diagnosis of HCC.

Furthermore, we explored the clinical significance of TONSL expression in HCC. The results showed that high TONSL expression was related to advanced TNM stage, poorly differentiated tumors, vascular invasion and higher serum AFP expression, suggesting that TONSL might be involved in the progression of HCC. Accordingly, Kaplan-Meier analyses indicated that TONSL upregulation was associated with unfavorable OS and RFS (all $P < 0.05$). Multivariate analysis further confirmed that TONSL upregulation was an independent risk factor of poor OS ($HR = 2.208(1.379, 3.536)$, $P = 0.001$) and RFS ($HR = 1.690(1.146, 2.490)$, $P = 0.008$) in HCC patients. These findings indicated that the TONSL expression level might serve as a valuable prognostic indicator in HCC, which can improve the strategies for post-surgery surveillance of patients with HCC, especially for tumor recurrence. Currently, due to the relatively deficient sensitivity, specificity and accuracy of existing serum biomarkers (such as AFP and GP73), there has been growing interest searching for substitutes which are useful for early noninvasive screen and early detection of the post-operative recurrence of HCC [13,14]. In this study, we observed a significant correlation between TONSL expression in HCC tissues and serum AFP expression. As far as we know, whether abnormal expression of serum TONSL exists in HCC patients remains unknown. Thus, determining the independent or combined (with AFP, GP73, etc.) diagnostic and prognostic values of serum TONSL in HCC warrants further study.

The underlying mechanisms of TONSL dysregulation in cancers have not been fully elucidated. TONSL is located on chromosome 8q24.3, which is often amplified in various malignancies [15–17]. Genetic aberrations in this region have been considered a breakpoint in tumorigenesis. Consistent with this, we found that 16% of HCC patients in the TCGA cohort had TONSL DNA amplification. Additionally, DNA methylation alteration has been observed in various cancers, and hypomethylation is typically related to chromosomal instability and gene upregulation [18]. We further examined the association between TONSL DNA methylation and its mRNA expression. The results indicated that hypomethylation was significantly associated with TONSL upregulation. Moreover, miRNAs are the key regulators of gene expression, which can downregulate target genes either by inducing mRNA degradation or by translation obstruction through binding the target mRNA within the 3′UTR region [19]. The dysregulation of miRNA, occurring in cancer, might result in the up- or downregulation of gene expression. In this study, we found that miR-133b was significantly downregulated in HCC tissues relative to normal liver tissue, and that miR-133b expression was negatively correlated with TONSL mRNA expression. The tumor suppressor role of miR-133b has been identified in several cancers including breast, gastric, esophageal and liver cancer [20–25]. Li et al. found that miR-133b was significantly downregulated during HCC progression and overexpression of miR-133b in HepG2 cells could significantly inhibit tumor growth [23,24]. What's more, in line with our findings, Wang et al. demonstrated that miR-133b downregulation in HCC tissues was correlated with patients' poor OS and RFS [25]. Considering the putative binding site of TONSL 3′UTR by miR-133b, our results suggested that miR-133b might be the upstream regulator of TONSL in HCC, which provided a new mechanism for the upregulation of TONSL. These findings indicate that both genetic and epigenetic alterations contribute to TONSL dysregulation in HCC.

Our findings demonstrated that TONSL was significantly upregulated in HCC tissues and might be a potential prognostic biomarker in HCC with respect to OS and RFS. However, the mechanism of how TONSL functions as an oncogene in HCC remains to be further explored. The enrichment analysis implied that gene sets related to cell cycle regulation were significantly enriched in the high TONSL expression group. Due to its multi-protein interaction domain structure, TONSL functions as a scaffolding component responsible for assembling a larger DNA repair complex, such as the MMS22L-TONSL complex, which is the key component of homologous recombination involved in cell cycle regulation [26]. In a wide range of tumor types including HCC, dysregulation of the cell cycle has been proved to be implicated in tumorigenesis and progression, such as cell proliferation and differentiation [27]. Interestingly, it was reported that siRNA-mediated knockdown of TONSL could significantly suppress lung cancer LC319 cells proliferation [8]. Furthermore, our results showed that elevated expression of TONSL was significantly correlated with higher pathological grade and advanced TNM stage of HCC. Until now, no research has been conducted to investigate the carcinogenesis of TONSL via cell cycle regulation pathways in HCC. Hence, it would be of great interest to further clarify the underlying mechanisms by which TONSL regulates the cell cycle in HCC in the future.

5. Conclusions

In summary, TONSL expression was significantly upregulated in HCC tissues relative to normal liver tissues. TONSL overexpression could independently predict poor OS and RFS in patients with HCC. DNA amplification, hypomethylation and miR-133b downregulation could be the mechanisms associated with TONSL dysregulation in HCC. Furthermore, TONSL overexpression was significantly correlated with cell cycle regulation pathways. Additional studies are warranted to further elucidate the exact role of TONSL in HCC.

Conflicts of interest

There are no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.01.044.

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