doi:10.4149/neo_2021_201010N1069

DNase1L3 suppresses hepatocellular carcinoma growth via inhibiting complement autocrine effect

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Received October 10, 2020 / Accepted January 14, 2021

Hepatocellular carcinoma (HCC) is one of the most aggressive types of cancer and currently lacks effective treatment strategies. The present study revealed that deoxyribonuclease 1 like 3 (DNase1L3) expression levels were significantly downregulated in numerous types of gastrointestinal cancer, and especially in HCC. Tissue microarrays were further used to illustrate that DNase1L3 expression levels were frequently downregulated in HCC tissues compared with normal liver tissues. In addition, DNase1L3 expression levels were identified to be significantly associated with tumor size ($p=0.0028$), tumor thrombus formation ($p<0.01$), and a poorer overall survival ($p=0.005$) and disease-free survival ($p=0.006$) of HCC. Gene Ontology functional term enrichment analysis of biological processes discovered that DNase1L3 was significantly associated with complement activation. Further studies demonstrated that the ectopic expression of DNase1L3 suppressed cell growth and inhibited the PI3K/AKT signaling pathway activation following C3a receptor agonist treatment. In conclusion, the findings of the present study suggested, for the first time, that DNase1L3 may serve as a biomarker for the prognosis of patients with HCC, and may suppress HCC growth via inhibiting the PI3K/AKT signaling pathway.

Key words: HCC, DNase1L3, complement, PI3K/AKT

Deoxyribonuclease 1 like 3 (DNase1L3) is a 33 kDa Mg^{2+}/Ca^{+}-dependent endonuclease that is located in the nuclear envelope and endoplasmic reticulum [1]. To date, four distinct DNase I-like genes have been identified in humans: DNase1, DNase X/Xib, DNase γ/DNase1L3, and DNase1L2 [2, 3]. Among these, DNase1L3 is homologous to DNase1, which is a secretory DNase that digests DNA in the gastrointestinal tract [4]. DNase1L3 was previously discovered to be more efficient than DNase1 in the internucleosomal cleavage of genomic DNA in isolated cell nuclei, which indicated that it may catalyze nucleosomal DNA fragmentation during cell apoptosis [5, 6]. Interestingly, human osteosarcoma cells, which do not express DNase1L3, failed to undergo internucleosomal DNA fragmentation following the exposure to the chemotherapeutic drug VP-16 [5]. In addition, the forced expression of DNase1L3 was reported to enhance DNA ladder formation when cells were exposed to apoptotic stimuli [4, 7]. Thus, DNase1L3 was hypothesized to promote apoptotic DNA fragmentation during cell apoptosis.

A more recent study identified that a mutation in DNase1L3 was associated with a high incidence of aggressive systemic lupus erythematosus (SLE) in children. In addition, DNase1L3-deficient children developed severe SLE with anti-dsDNA antibodies [8, 9]; in this study, circulating DNase1L3 was produced by dendritic cells and macrophages, and its levels were inversely associated with the DNA levels in the plasma. Thus, DNase1L3 may serve as a genetic determinant of SLE susceptibility and an essential factor protecting humans from SLE.

Hepatocellular carcinoma (HCC) is one of the most aggressive types of cancer worldwide and currently lacks effective treatment options. Tumorigenic cellular processes, including DNA damage, necrosis, and endoplasmic reticulum stress, participate in immune-surveillance and cancer development [10]. However, although the catalytic properties of DNase1L3 in DNA fragmentation during cell apoptosis have been well investigated, to the best of our knowledge, the role of DNase1L3 in HCC remains to be determined.

The complement system is considered the first line of defense and it primarily acts to remove pathogens and injured cells. Complement proteins in the plasma are mainly synthesized in hepatocytes. There are three pathways that initiate the complement system: the classical, lectin, or alternative pathway. In all three complement activation pathways,

the C3 convertase complex cleaves C3 molecules to C3a to generate the membrane attack complex. Systemic increases in C3 levels have been detected in patients with cancer, including liver and lung cancers [11]. Similarly, recent studies have revealed that complement activation can promote cancer [12–14]. These effects are likely mediated through the anaphylatoxins (C3a and C5a) produced during complement activation [15]. Interestingly, the activation of the C3a receptor (R) was discovered to signal through the PI3K/AKT signaling pathway in cancer cells to promote tumor growth by altering cancer cell behavior and modulating the immune response to the tumor [16]. However, the mechanism through which complement is activated in cancer progression remains unknown.

The present study revealed that in patients with HCC, DNase1L3 expression levels were significantly downregulated in the tumor tissues compared with the adjacent non-tumor tissues. Using *in vitro* studies, the administration of a C3aR agonist was discovered to markedly downregulate phosphorylated (p)-PI3K/p-AKT expression levels in DNase1L3-overexpressing cells, suggesting the potential involvement of DNase1L3 in C3a/C3aR-mediated PI3K/AKT pathway activation.

Materials and methods

Patient studies. Primary HCC specimens and their corresponding adjacent non-tumor tissues were obtained from patients who underwent hepatectomy for HCC at the Sun Yat-sen University Cancer Center (Guangzhou, China). A tissue microarray (TMA), containing 124 pairs of matched primary HCC tumor tissues and corresponding non-tumor tissues, was obtained between 2003 to 2010 at the Sun Yat-sen University Cancer Center. The present study was approved by the Committee for Ethical Review of research involving human subjects at the Sun Yat-sen University Cancer Center. Informed consent was obtained from all patients.

Cell lines and culture. Liver cancer cell lines, PLC-8024 and Hep3B were obtained from the Institute of Virology, Chinese Academy of Medical Sciences. All the cell lines used in the present study were authenticated using STR profiling. All cell lines were cultured in high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37 °C with 5% CO_2.

Immunohistochemistry (IHC) staining. IHC staining was performed using a standard streptavidin-biotin-peroxidase complex method. Briefly, the paraffin sections were deparaffinized using xylene, rehydrated using a series of graded ethanol, and rinsed with deionized water. The endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide for 10 min at room temperature. For antigen retrieval, the slides were high-pressure-treated and boiled in 10 mM citrate buffer (pH 6.0) for 4 min. Subsequently, nonspecific binding was blocked with 5% normal goat serum for 30 min at room temperature and then the slides were incubated with a monoclonal rabbit anti-human DNase1L3 antibody (cat. no. 203669; Abcam; 1:5,000) at 4 °C overnight in a humidified chamber. Following the primary antibody incubation, the slides were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody and then stained with the DAB substrate. The following immunoreactivity score system was used to analyze DNase1L3 IHC staining according to the percentage of DNase1L3-positive cells: 0 (<5%), 1 (5–25%), 2 (25–50%), 3 (50–75%), and 4 (75–100%). The intensity of DNase1L3-positive staining was scored using the following intensity scoring system: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The total score was determined using the following formula: Staining index = percentage of DNase1L3-positive staining score x intensity score.

Western blotting. Total protein was extracted from human HCC specimens using RIPA lysis buffer (#9806, Cell Signaling Technology, Inc.) supplemented with a cocktail protease inhibitor (11697498001, Roche) and phosphatase inhibitor PhosSTOP (4906837001, Roche). After being boiled for 5 min, the protein lysates were separated via 10% SDS-PAGE and then transferred onto PVDF membranes (IPVH00010, EMD Millipore). The membranes were subsequently blocked with 5% non-fat milk in TBS-0.05% Tween 20 (TBST) for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. Following the primary antibody incubation, the membranes were washed three times with TBST buffer for 5 min each and incubated with an HRP-conjugated anti-rabbit or mouse secondary antibody for 2 h at room temperature. Protein bands were visualized with enhanced chemiluminescence (32106, Thermo Fisher Scientific, Inc.). DNase1L3 antibody (GTX114363) was purchased from Genetex. β-Tubulin antibody (#2146), PPAR antibody (#9542), Cyclin D1 antibody (#2922), Cyclin E1 antibody (#4129), Cyclin A2 antibody (#4656), CDK4 antibody (#12790), CDK2 antibody (#2546), p-AKT antibody (#9271), AKT antibody (#9272), p21 antibody (#2947), p-cRaf antibody (#9427), and PI3K antibody (#3811) were purchased from Cell Signaling Technology.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from liver cancer cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Expression levels were quantified by qPCR using the FastStart Universal SYBR Green Master mix (Roche) and a 96-well Real-Time PCR Detection system (Roche). The relative expression levels (defined as fold change) of the target genes ($2^{-\Delta\Delta Ct}$) were normalized to the endogenous reference gene (ΔCt).

Construction of DNase1L3-overexpressing cells. For DNase1L3 overexpression, vectors were purchased from GeneCopoeia, Inc. The DNase1L3 overexpression vector and control plasmids were co-transfected together with three lentiviral packaging vectors, pLp1, pLp2, and pLp-VSVG (Invitrogen; Thermo Fisher Scientific, Inc.), into 293FT

cells (Invitrogen; Thermo Fisher Scientific, Inc.) using the HilyMax transfection reagent (Dojindo Molecular Technologies, Inc.), according to the manufacturer's instructions. The viral-containing supernatants from the DNase1L3 overexpression plasmid and empty vector were then collected for subsequent transduction into PLC8024 and Hep3B cells. Puromycin (2 μg/ml; Thermo Fisher Scientific, Inc.) was used to select for stably transduced cells.

ELISAs. C3aR agonist was purchased from Santa Cruz and was dissolved in DMSO. DNAse1L3-transfected and empty vector-transfected PLC-8024 cells were stimulated with C3aR agonist (1 μg/ml) or with DMSO, and the concentration of C3a in the supernatant was determined using a purified anti-human C3a/C3 capture antibody (BioLegend, Inc.) and a biotin-conjugated anti-human C3a/C3 detection antibody (BioLegend, Inc.) in a sandwich ELISA assay. Briefly, 96-well plates were coated with the capture antibody in coating buffer at 4°C overnight. The supernatants were subsequently added to the blocked plate and incubated for 2 h at 37°C. Following the incubation, the biotin-conjugated detection antibody was added to each well and incubated for 1 h at 37°C. Then, avidin-conjugated HRP and TMB substrate solution was added to each well. The reaction was stopped by adding 2 M hydrochloric acid and the plates were read at 450 nm.

Flow cytometric analysis of apoptosis. Cells pre-washed with PBS were incubated with Annexin V and PI (Dojindo Molecular Technologies, Inc.) for 15 min in the dark at room temperature. After washing with PBS, the cells were analyzed by flow cytometry (Beckman Coulter, Inc.).

Statistical analysis. Kaplan-Meier analysis was performed using SPSS software (IBM Corp.), while all other statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc.). Data are presented as the mean ± SD. The mRNA expression levels in non-tumor liver and HCC tissues and the patient's survival curves were obtained from GEPIA, which provides access to publicly available cancer transcriptome data [17]. Gene Ontology (GO) analyses of a Gene Expression Omnibus dataset were analyzed with Coexpedia [18]. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

DNase1L3 expression levels are downregulated in HCC. Disease Ontology analysis of DNase1L3 in humans using Coexpedia indicated that DNase1L3 expression levels were closely associated with cancer development (Figure 1A). The boxplots present DNase1L3 expression data from The Cancer Genome Atlas Program (TCGA) database in multiple types of gastrointestinal cancer, including cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), liver hepatocellular carcinoma (LIHC), and stomach adenocarcinoma (STAD). DNase1L3 expression levels were discovered to be significantly downregulated in the majority of the gastrointestinal cancers compared with the normal tissues, and to the greatest extent in the CHOL and LIHC tissues (Figure 1B). In addition, the DNase1L3 expression levels were consistent with the clinicopathological stages of the five types of gastrointestinal cancer (Figure 1C). DNase1L3 expression levels were further analyzed using RT-qPCR in 43 pairs of HCC and paired adjacent non-tumor tissues. Consistent with the TCGA dataset, the results revealed that DNase1L3 expression levels were significantly downregulated in the HCC cancer samples compared with the non-tumor tissues (Figure 1C). In addition, the protein expression levels of DNase1L3 were also observed to be downregulated in the clinical HCC samples compared with the paired non-tumor tissues, as determined by IHC staining (Figure 1D) and western blotting (Figure 1E).

Downregulated expression levels of DNase1L3 are associated with poor outcomes in HCC. To determine the expression and distribution of DNase1L3 in clinical specimens, a TMA containing 124 pairs of primary HCC tissues and their non-tumor counterparts was used to perform IHC staining and analyze the association between DNase1L3 expression levels and clinicopathological features (Figures 2A–2E). Firstly, IHC staining revealed that the expression levels of DNase1L3 in the HCC tissues were downregulated (Figures 2A, 2B). Notably, these low expression levels of DNase1L3 were identified to be significantly associated with tumor size (p=0.0028) and tumor thrombus formation (p<0.01) (Figures 2C–2E). In addition, Kaplan-Meier survival analysis revealed that the downregulated expression levels of DNase1L3 were significantly associated with poorer overall survival (p=0.005) and disease-free survival (p=0.006) of HCC (Figure 2F). Furthermore, according to the clinical data from the TCGA database, DNase1L3 was one of the most differentially expressed survival genes in HCC (Figure 2H). Kaplan-Meier analysis also revealed that the downregulated expression levels of DNase1L3 were significantly associated with poorer overall survival and disease-free survival in HCC based on the TCGA dataset (Figure 2G). The survival map of the hazard ratio suggested that DNase1L3 might be an important tumor suppressor gene in HCC (Figure 2I).

DNase1L3 overexpression has no effect on HCC cell growth. To investigate the role of DNase1L3 in HCC progression, two HCC cell lines, PLC8024 and Hep3B, were stably transfected with the lentiviral DNase1L3 overexpression construct or empty lentiviral vector. The cell growth assay revealed that the cell growth rates in the DNase1L3-overexpressing cells and control cells were similar (Figure 3A). A previous study suggested that DNase1L3 might catalyze nucleosomal DNA fragmentation during cell apoptosis [19]. Thus, the present study evaluated whether DNase1L3 overexpression could affect tumor cell apoptosis under cisplatin administration. DNase1L3 overexpression significantly increased the Annexin V^+PI^- apoptotic cell population

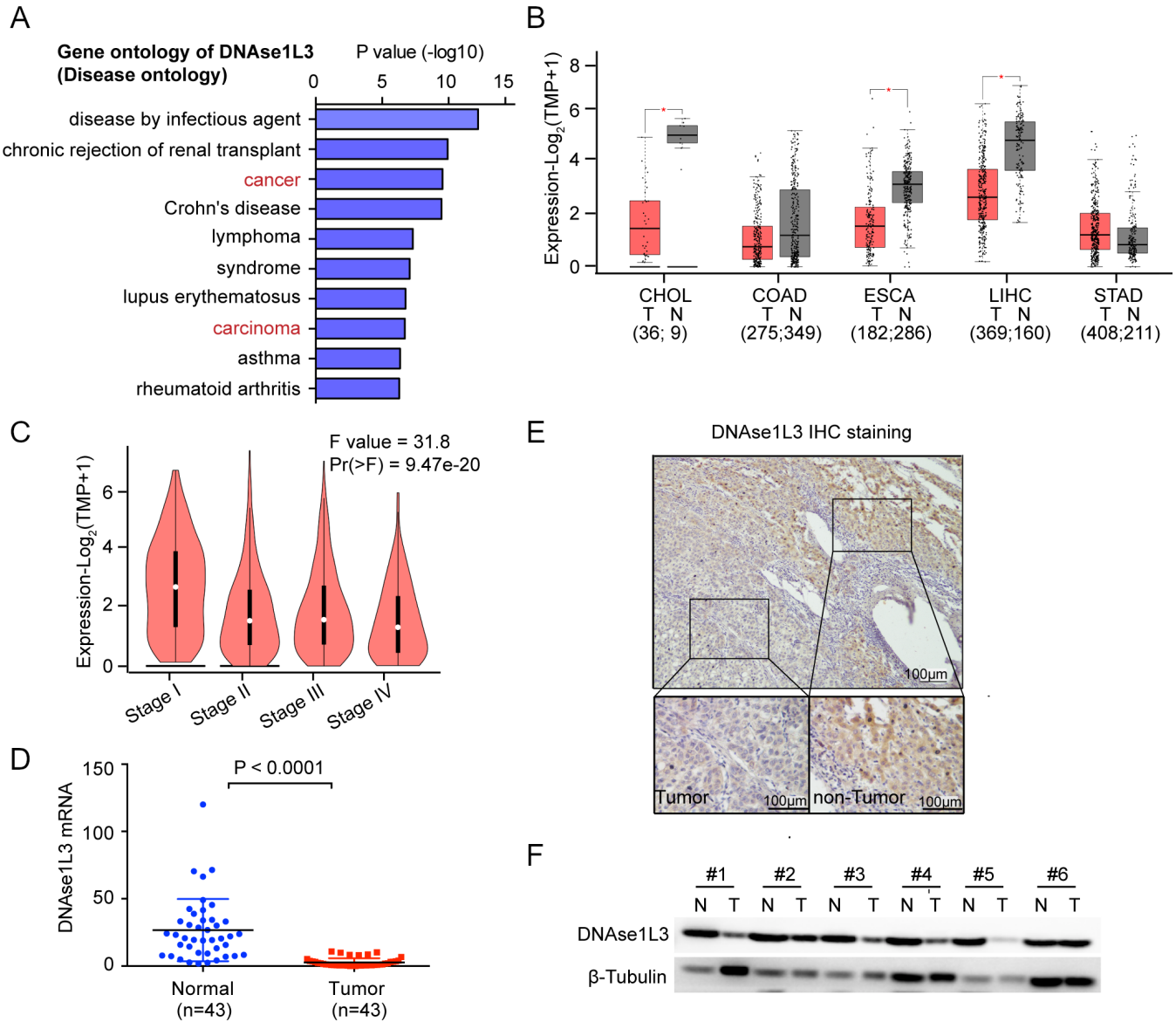

Figure 1. Downregulation of DNAse1L3 in gastrointestinal cancers. A) Gene Ontology Enrichment analysis (Disease Ontology) of DNAse1L3 using Coexpedia internet tool that was based on public GEO database. B) The expression of DNAse1L3 in gastrointestinal cancers was analyzed based on the TCGA database. C) DNAse1L3 expression is related to gastrointestinal cancer stages in the TCGA database. D) DNAse1L3 mRNA expression in HCC and corresponding normal liver tissues (n=43), scale bars = 100 μm. E) DNAse1L3 protein expression in HCC and paired normal liver tissues were tested by IHC staining. F) DNAse1L3 protein expression in 6 pairs of HCC tumor was tested by western blotting.

following cisplatin treatment (Figures 3B, 3C). However, there were no significant differences identified in the proportion of Annexin V^+ cells following cisplatin treatment (Figures 3B, 3D). Western blotting was also used to analyze the expression levels of cell cycle-, apoptosis-, and cell growth-related proteins; interestingly, no significant differences were observed in the expression levels of cleaved PPAR, cyclin D1, cyclin E1, cyclin A2, CDK4, CDK2, phosphorylated-AKT, and p21 between DNase1L3-overexpressing cells and control cells following cisplatin treatment (Figure 3E). These data suggested that DNase1L3 might have a redundant role in HCC cell apoptosis following cisplatin treatment.

DNase1L3 overexpression significantly reduces tumor cell growth and decreases C3a production following C3aR agonist treatment. GO functional term enrichment analysis (biological process) was performed using Coexpedia. The analysis revealed that DNase1L3 was significantly associated with the complement activation pathway (Figure 4A). Therefore, it was hypothesized that DNase1L3 may participate in complement activation in HCC progression. Comple-

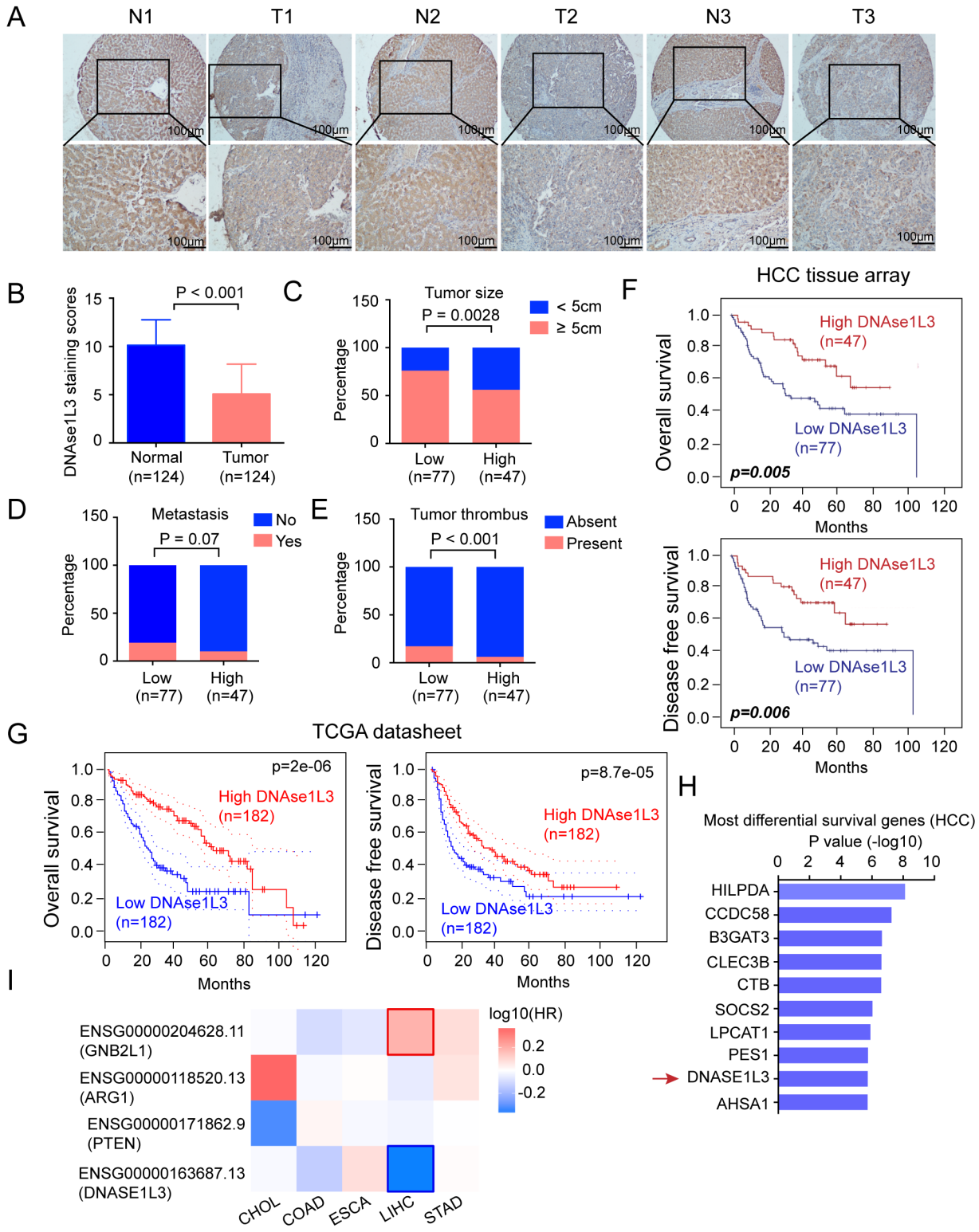

Figure 2. Downregulation of DNAse1L3 predicts the poor survival of HCC. A) IHC staining of DNAse1L3 in HCC tissue microarray (n=124), scale bars = 100 μm. B) DNAse1L3 staining scores in HCC tumors and the corresponding non-tumor tissues (n=124). C–E) Correlation analyses between DNAse1L3 expressions and clinical characteristics in HCCs. F–G) Kaplan-Meier survival curves showed low DNAse1L3 expression was correlated with poor prognosis prediction of HCC analyzed by HCC tissue microarray and TCGA database. H–I) DNAse1L3 was the most differential survival gene and showed a low Hazardous Ratio analyzed by GEPIA based on the TCGA database.

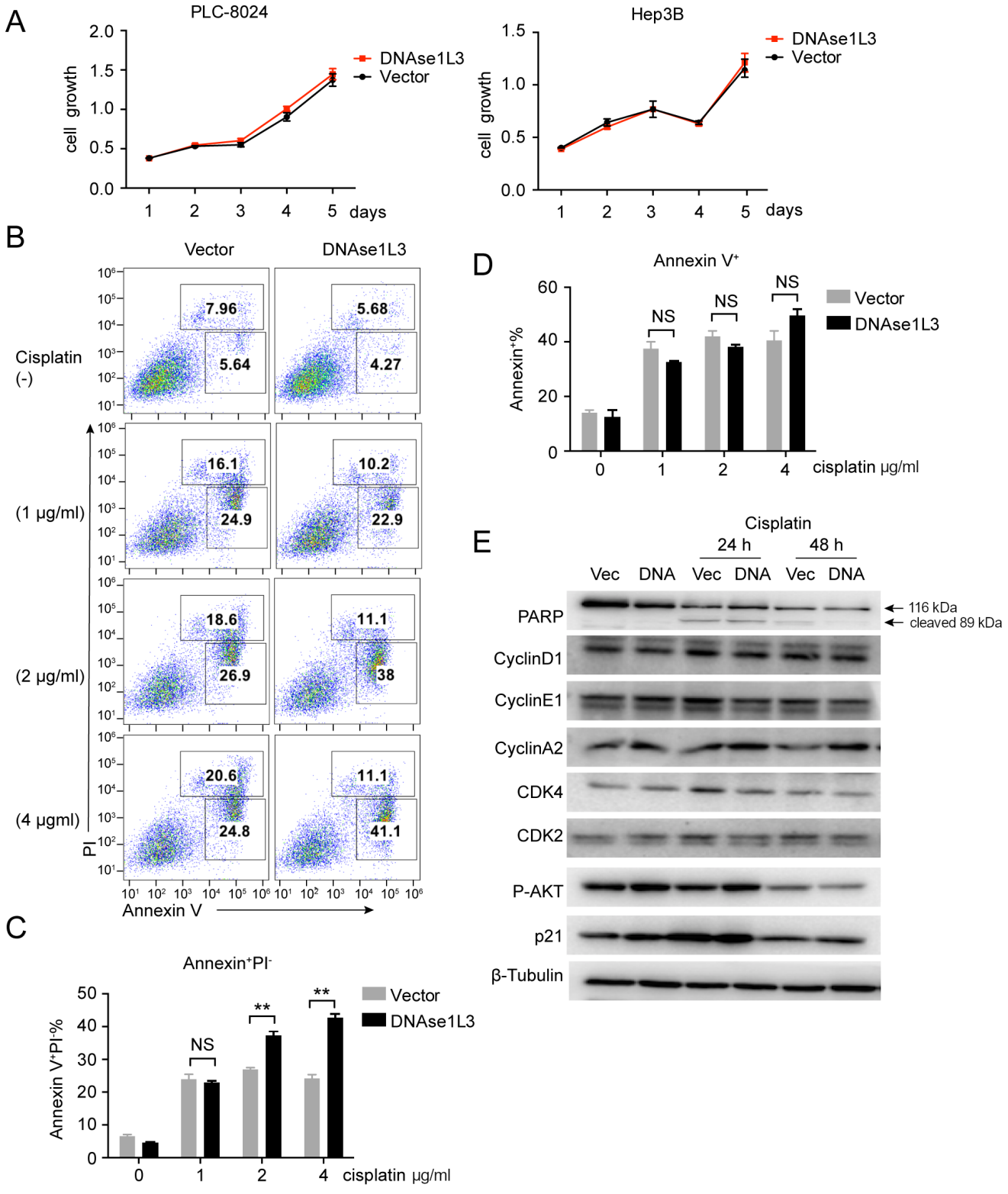

Figure 3. DNAse1L3 overexpression has no effect on HCC cell growth. A) Cell growth rates between DNAse1L3-transfected and empty vector-transfected PLC-8024 and Hep3B cells. B–D) Cell apoptosis was analyzed by flow cytometry in DNAse1L3-transfected and empty vector-transfected PLC-8024 cells after cisplatin treatment. E) Western blotting showed cell cycle and apoptosis-related proteins in DNAse1L3-transfected and empty vector-transfected PLC-8024 cells after cisplatin treatment.

ment can be activated via three di fferent pathways. All three pathways share a common endpoint involving covalent fixation and cleavage of C3 on the surface of targeted cells [20]. Therefore, we used the C3aR agonist to simulate the complement system activation. PLC8024 and Hep3B cells transfected with the lentiviral DNase1L3 overexpression construct or empty lentiviral vector were stimulated with the C3aR agonist. The results revealed that DNase1L3 overexpression significantly decreased cell growth under C3aR agonist treatment (Figure 4B). However, DNase1L3 overexpression exerted no effects on cell apoptosis following C3aR agonist treatment (Figure 4C). The activation of the PI3K/AKT signaling pathway has been associated with poor outcomes in various types of carcinoma [21]. Therefore, to determine whether DNase1L3 overexpression affected the C3aR agonist-induced PI3K/AKT signaling pathway activation, the activation of the PI3K/AKT signaling axis was analyzed using western blotting. DNase1L3 overexpression decreased PI3K, AKT, and cRaf phosphorylation at 48 h following C3aR agonist treatment (Figure 4D). These data suggested that DNase1L3 overexpression might attenuate C3a-induced PI3K/AKT signaling pathway activation.

The complement C3a/C3aR pathway in cancer has recently received significant attention [11]. A previous study reported that complement promoted tumor growth via a direct autocrine effect [16]. Thus, the present study determined whether DNase1L3 regulated complement activation. The mRNA expression levels of C3, C3aR, CFB, and CFD in DNase1L3-overexpressing and control cells were determined using RT-qPCR. DNase1L3-overexpressing PLC-8024 cells were discovered to have significantly downregulated mRNA expression levels of C3, C3aR, CFB, and CFD compared with the control cells (Figure 4E). Subsequently, the supernatant of serum-free media incubated for 72 h with the control and DNase1L3-overexpressing PLC-8024 cells was collected and used to determine the concentration of C3a using ELISAs (Figure 4F). DNase1L3 overexpression significantly reduced the production of C3a in the supernatant, which indicated that DNase1L3 overexpression might inhibit the autocrine effect of C3a on HCC cells.

Discussion

Although the DNase1L3 gene has been cloned and characterized in a few types of human cancer, to the best of our knowledge, the role of DNase1L3 in HCC progression remains unknown. In the majority of circumstances, patients who have a higher pathological stage tend to have a poor prognosis. Therefore, it is important to identify biomarkers associated with the TNM stage and to reveal the molecular mechanisms associated with the development of HCC. The present study demonstrated that DNase1L3 expression levels were significantly downregulated in multiple types of gastrointestinal cancer, including CHOL, ESCA, and LIHC. Importantly, DNase1L3 expression levels were inversely associated with the clinicopathological stages of five types of gastrointestinal cancer, including HCC. Through using TMA analysis, the present study revealed that the average expression levels of DNase1L3 in HCC specimens were significantly lower compared with the adjacent normal liver tissues. In addition, through analyzing the clinical data, the downregulated expression levels of DNase1L3 were discovered to be associated with tumor size, tumor thrombus formation, and adverse outcomes in patients with HCC. Moreover, based on the TCGA database analysis, DNase1L3 was identified as an important tumor suppressor gene through hazard ratio analysis, in addition to being one of the most differentially expressed survival genes in HCC. Therefore, these data indicated that DNase1L3 might be a novel diagnostic and prognostic biomarker for HCC.

The present study demonstrated that the downregulated expression levels of DNase1L3 were associated with a larger tumor volume, which suggested that DNase1L3 might be a negative regulator of tumor growth. However, the overexpression of DNase1L3 in the HCC cell lines had no effect on tumor growth or apoptosis following cisplatin administration. Four distinct DNase I-like genes that degrade DNA have been identified in humans, thus, DNase1L3 may have a redundant role in DNA cleavage during HCC cell apoptosis following cisplatin administration.

GO functional term enrichment analysis (biological process) was performed using Coexpedia [18]. The results revealed that DNase1L3 was significantly associated with complement activation. As previous studies have reported that complement activation promoted cancer, DNase1L3 was hypothesized to regulate HCC cell growth via complement activation. A previous study linked anaphylatoxins (C3a and C5a) to tumor growth and PI3K/AKT signaling pathway activation [16]. In addition, the activation of the PI3K/AKT signaling pathway was associated with poor outcomes in various types of carcinoma [22]. However, to the best of our knowledge, the relationship between DNase1L3 and the C3a/C3aR signaling pathway in HCC pathogenesis remains unknown. Following stimulation of the DNase1L3-overexpressing or control cells with the C3aR agonist, C3aR activation significantly decreased cell growth and attenuated PI3K/AKT signaling pathway activation in the cancer cells. Previous studies have demonstrated that the liver is a central immunomodulator that maintains immune tolerance. Additionally, the deregulation of the liver immunological network is a hallmark of liver disease, including HCC [23]. The complement cascade is an important part of the innate system that acts primarily to remove pathogens. Therefore, our study linked the deregulated expression levels of DNase1L3 to complement activation in HCC and demonstrated that DNase1L3 served as an immunomodulator and participated in C3a-regulated tumor growth and PI3K/AKT signaling pathway activation. However, the underlying mechanism of how DNase1L3 regulates complement cascade activation in HCC requires further investigation.

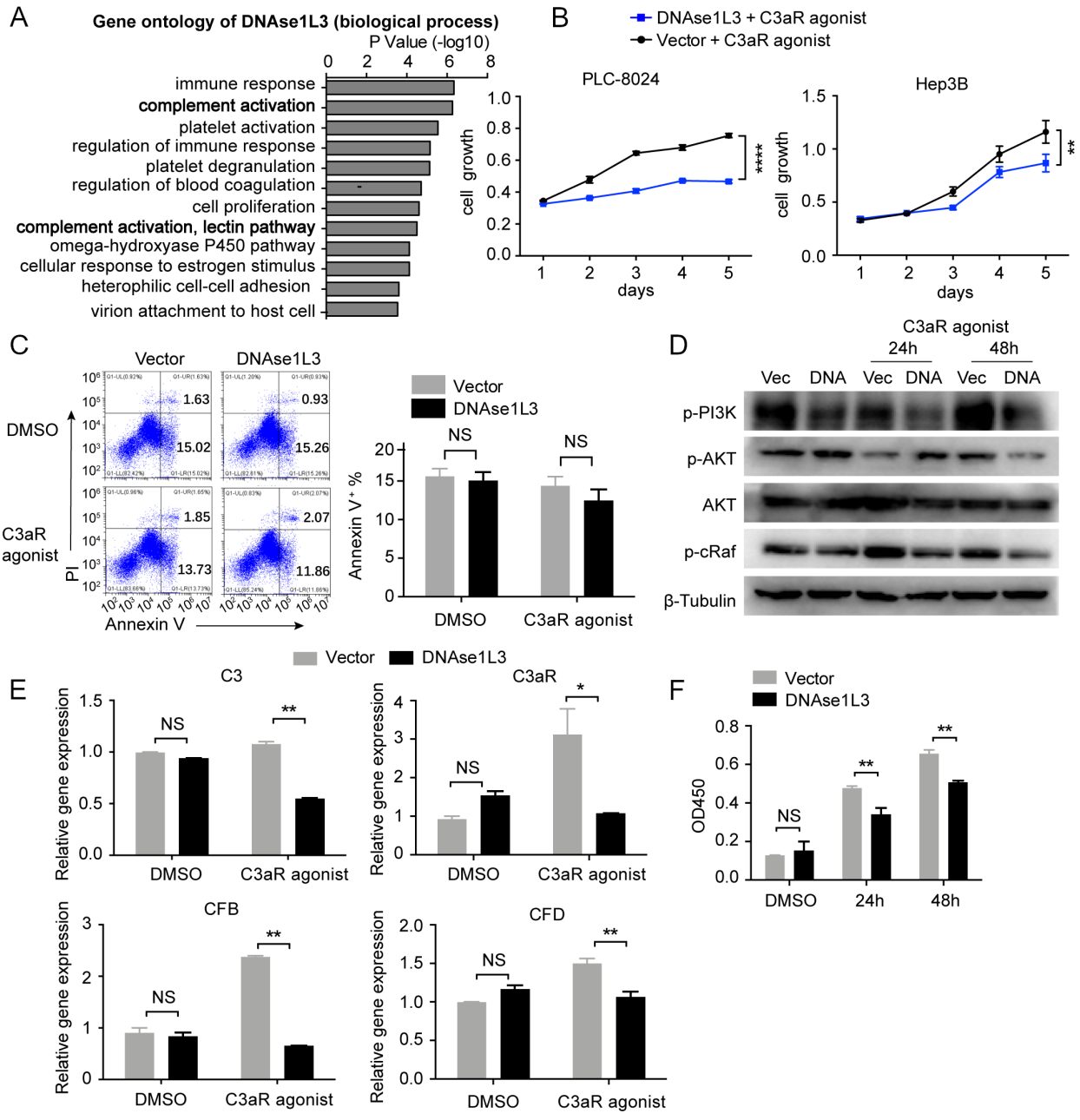

Figure 4. DNAse1L3 overexpression inhibits HCC cell growth and complements productions after C3aR agonist treatment. A) Gene Ontology Enrichment analysis (biological process) of DNAse1L3 using Coexpedia internet tool that was based on public GEO database. B) Cell growth rates between DNAse1L3-transfected and empty vector-transfected PLC-8024 and Hep3B cells after C3aR treatment. C) Cell apoptosis was analyzed by flow cytometry in DNAse1L3-transfected and empty vector-transfected PLC-8024 cells after C3aR agonist treatment. D) Western blotting showed PI3K/AKT pathway proteins in DNAse1L3-transfected and empty vector-transfected PLC-8024 cells after C3aR agonist treatment. E) C3, C3aR, CFB, and CFD mRNA expressions in DNAse1L3-transfected and empty vector-transfected PLC-8024 cells were measured by qRT-PCR. F) The concentration of C3a in the supernatant in DNAse1L3-transfected and empty vector-transfected PLC-8024 cells was analyzed by ELISA.

In conclusion, the present study provided a novel understanding of the role of DNase1L3 as a new biomarker for HCC diagnosis and prognosis. The study also identified a prominent role of DNase1L3 in suppressing C3a production and C3aR activation in HCC growth and development.

Acknowledgments: The present study was supported by the National Nature Science Foundation of China (grant no. 81802410). This research was financially supported by The Pearl River Talent Plan-Overseas Youth Talent Introduction Plan (Postdoctoral Program).

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