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RESEARCH ARTICLE

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Over-expression of TNNI3K is associated with early-stage carcinogenesis of cholangiocarcinoma

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Cholangiocarcinoma (CCA) is a devastating disease with very poor prognosis due to late diagnosis and resistance to traditional chemotherapies and radiotherapies. Herein, thioacetamide (TAA)-induced rat CCA model and CGCCA cell line were used; we aim to study the cytogenetic features during tumoral development of CCA and uncover the mystery regarding carcinogenesis of CCA. The Array comparative genomic hybridization analysis, in silico method, gene knockdown, Western blot, cell count proliferation assay, clonogenecity assay, and IHC staining were applied in this study. Array comparative genomic hybridization analysis was performed on all different TAA-induced phases of rat tissues to reveal the certain pattern, +2q45, +Xq22, −12p12, have been identified for the tumor early stage, where involve the gene *TNNI3K*. In addition, 16 genes and 3 loci were associated with rapid tumor progression; JAK-STAT signaling pathway was highly correlated to late stage of CCA. In silico database was used to observe TNNI3K was highly express at tumor part compared with normal adjacent tissue in CCA patients from TCGA dataset. Furthermore, the growth of *TNNI3K*-knockdown SNU308 and HuCCT1 cells decreased when compared with cells transfected with an empty vector cell demonstrated by proliferation and colonogenecity assay. Besides, over expression of TNNI3K was especially confirmed on human CCA tumors and compared with the intrahepatic duct stone bile duct tissues and normal bile duct tissues ($P < 0.001$). Our findings might uncover the mystery regarding carcinogenesis of CCA, and provide the potential genetic mechanism to the clinicians some ideas for the patients' treatment.

KEYWORDS
array comparative genomic hybridization, carcinogenesis, cholangiocarcinoma, chromosomal alterations

1 | INTRODUCTION

Being one of the deadliest cancers, cholangiocarcinoma (CCA) comes with a very low survival rate—more than 90 percent of patients die within 5 years, and the majority of patients have a survival of less than 12 months after diagnosis.[1,2] CCA commonly presents an arduous diagnostic and treatment challenge; neither the primary tumor nor metastases can be fully resected or successfully treated with radiotherapy or chemotherapy.[2–5]

The value of present tumor markers for the diagnosis of CCA remains controversial. So far the most common used markers are carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen

(CEA). The combined use of CEA and CA 19-9 was reported to be able to improve the diagnosis of CCA.[6] However, CA 19-9 only identifies patients with advanced and unresectable CCA.[7–10]

Although the genetic association of great complexity in CCA have been discovered from time to time, including specific genetic mutations (eg, *KRAS*, *IDH1* and *IDH2*, and *p53*) might play an important role in CCA, and might be correlated with a more aggressive phenotype; the epigenetic silencing, aberrant signaling pathway activation (eg, interleukin (IL)-6/signal transducer and activator of transcription 3 [*STAT3*], tyrosine kinase receptor-related pathways) and molecular subclasses with unique alterations (eg, proliferation and inflammation subclasses) have been mentioned as well.[11–13] However, there were no consistent trends of tumorigenic findings of CCA so far.

In our previous studies, we have successfully built a TAA-induced CCA rat model and established a rat CCA cell line, CGCCA. We have also revealed the molecular features of late stage CCA.[14] Based on it, in order to seek markers for early diagnosis, as well as the therapeutic targets, array comparative genomic hybridization (aCGH) was used in current study. Herein, we compared the cytogenetic alterations among the different stages of TAA-induced CCA (an animal model recapitulating the multi-stage progression of CCA) in quest of initial genetic alterations, which might be responsible for tumor initiation as well as gene copy number variations to reveal the possible tumorigenesis of CCA. Furthermore, a panel of normal part of bile duct tissue from hepatocellular carcinoma (HCC), hepatolithiasis inflammatory hepatic bile duct (IHD) tissues, and intrahepatic cholangiocarcinoma (CCA) were also collected and provided for further confirmation.

2 | MATERIALS AND METHODS

2.1 | TAA-induced CCA rat tissue samples preparation

The TAA-induced rat CCA was induced as previously reported and followed the animal experiment guideline of Chang Gung Memorial Hospital, Chang Gung University, Taoyuan, Taiwan with approval code: IACUC: 2010121409[14]; in which, the TAA-induced CCA tissues were derived from seventeen Male Sprague-Dawley (SD) rats, and CGCCA cell line developed by ourselves and published at previous study[15] were used for current experiments. In brief, the CCA tumor were induced by feeding TAA 300 mg/L in rats drinking water every day up to the time they were sacrificed; during the period, we regularly collected the TAA-induced CCA tissues from the time points from the 4th week ($n = 3$), 8th week ($n = 3$), 12th week ($n = 3$), 16th week till 25th week ($n = 3$), the CGCCA cell line was established at 25th week of induced CCA rat cells. The normal bile ducts were harvest from five SD rats feeding with normal drinking water, after extraction the DNAs were then mixed and used as control samples. All experimental samples were snap freezing and stored at −80°C until used for genomic DNA (gDNA) extraction.

2.2 | Genomic DNA isolation

All tissue samples and CGCCA cells were extracted using the illustra Tissue and Cells GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire HP7 9NA, UK) according to the manufacturer's instructions. In brief, the samples were treated with proteinase K and lysis buffer type 1 for an hour at 56°C, then followed with RNase A for 15 min at room temperature. After applied with lysis buffer type 4 and centrifugation, the samples were washed by wash buffer type 6 and centrifugation again then resuspended in ddH_2O. Genomic DNA quantity and quality was determined by 0.8% agarose gel electrophoresis and a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). The size of DNA bands should be greater than 10 kb; the optical density of gDNA should have an A260/A280 greater than 1.8 and A260/A230 greater than 1.9 then were used for array-based comparative genomic hybridization.

2.3 | Array comparative genomic hybridization (aCGH)

A genome-wide analysis of rat tissue samples and CGCCA cells were performed by using Rat CGH 385 K Whole-Genome Tiling Array chips, which contained 385000 probes with an average spatial resolution of ~5.3 kb (Roche NimbleGen, Inc, Madison, WI). The protocol was conducted according to the instruction of NimbleGen arrays user's guide, CGH and CNV Arrays with slightly modification. In brief, the equal amounts of gDNA (0.5 μg) were labeled with Cy5 and Cy3 on the control DNA and tumor DNA, respectively. The labeled DNAs were then quantified using the NanoDrop spectrophotometer (ND-1000, Thermo Scientific), after mixed well the 6 μg of each labeled control and tumor DNA, the probes were hybridized onto microarray chip of Rat CGH 385 K Whole-Genome Tiling Array, and incubated for 16-20 h at 42°C in a humid chamber. The dual-color digital images were captured by MS 200 Microarray Scanner (Roche NimbleGen, Inc) in combination with the MS 200 Data Collection Software (Roche NimbleGen) then the images were analyzed with the NimbleScan v2.6 Software (Roche NimbleGen) then carried out using SignalMap v1.9 (Roche NimbleGen). The Cy5/Cy3 ratios of probes were converted into log2 ratios. The threshold values above or below log2 ratio of 0.2 were considered as gains or losses, respectively. The significant targets were further searched by Rat Genome Database (http://rgd.mcw.edu/).

2.4 | In silico study

The clinical information and genomic matrix file of The Cancer Genome Atlas (TCGA) database were download form the USCS cancer browser website (https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/).The expression level of N/T pairs and groups were performed using Prism software, respectively. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL). The differences between two groups were analyzed using paired *t*-test or Mann-Whitney U test. The *P* values of less than 0.05 were considered statistically significant.

2.5 | Cell culture and reagents

The CCA cell line SNU-308 and HuCCT1 were obtained from Korean Cell Line Bank (KCLB) (Seoul, Korea) and Japanese Collection of Research Bioresources (JCRB) (Tokyo, Japan), respectively. The cell line was authenticated and identified by KCLB and JCRB using morphology, karyotyping, and PCR-based approached. SNU-308 were cultured in RPMI1640 medium (Gibco/Thermo, CA) and HuCCT1 were cultured in DMEM (Sigma-Aldrich, St Louis, MO). SNU-308 and HuCCT1 were supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, 100 μg/mL penicillin, and 2 mM L-glutamine in a humidified atmosphere containing 5% CO_2 at 37°C.

2.6 | Transient transfection

For transfection, SNU308 and HuCCT1 cells were plated at a confluence of 50-60% in 6 cm culture dishes. Different quantities of DNA (0.5, 1, or 3 μg) from the pCMV-SPORT6 backbone were then incubated with serum-free medium in a total volume of 500 μL, and then mixed with 500 μL of X-tremeGENE™ HP DNA transfection reagent (Roche) plus serum-free medium solution (ratio 1:3) to bring the final volume to 1 mL. This pre-mixture was then incubated at room temperature for 30 min and then added to SNU308 cells for 3 h. The cells were further treated with RPMI medium overnight and analyzed after 72 h.

2.7 | Western blot analysis

Whole cell lysates from CCA cell lines were obtained by using Pierce immunoprecipitation assay buffer (Thermo Scientific, Rockford, IL). Protein samples were separated on 8-12% gradient dodecyl sulfate-polyacrylamide gels and transferred to Immobilion-PVDF membranes (Millipore, Billerica, MA). Antigen-antibody complexes were detected using an electrochemiluminescence blotting analysis system (Millipore). The following primary antibodies were used: TNNI3K (Abbexa, Cambridge, United Kingdom), and β-actin (Abcam). The working dilution of primary antibodies was 1:1000.

2.8 | Cell count proliferation assay

1×10^5 cells of SNU308 and HuCCT1 cells with or without transfected with shRNAs were seeded into 6-cm plates separately. After 10 days of incubation, cells were fixed with 4% paraformaldehyde and stained with 0.01% crystal violet. Purple colonies of cells were captured.

2.9 | Clonogenecity assay

1000 cells of SNU308 cells with or without transfected with shRNAs were seeded into 6-cm plates separately. After 10 days of incubation, cells were fixed with 4% paraformaldehyde and stained with 0.01% crystal violet. Purple colonies of cells were captured.

2.9.1 | Human cohort for validation of TNNI3K expression by immunohistochemical staining of normal hepatic duct, inflammatory hepatic duct, and CCA

In total 80 patients with formalin-fixed paraffin-embedded (FFPE) tissues selected from the archives of Chang Gung Memorial Hospital were involved in the current study (IRB approval code: 99-3810B and 104-9367B). Twenty patients in each group (blunt abdominal trauma with liver injury, hepatocellular carcinoma (HCC), hepatolithiasis and intrahepatic cholangiocarcinoma) who all had undergone hepatectomy, and based on the availability of sufficient quantities of bile duct cells. The bile duct in the non-tumor part of the HCC was as normal control in this study. Hematoxylin and eosin (H&E)-stained slides from each case were reviewed (Representative case presented as supplementary Figure S1). A 4-μm section of each specimen was stained for TNNI3K. The primary antibody against TNNI3K (mouse anti-TNNI3K monoclonal antibody, MS-1185-P1, NeoMarkers, Fremont, CA.) was diluted (1:200) and added to the slides that were then incubated overnight at 4°C. The slides were then washed three times for 5 min in TBST before visualization with the DAKO LSAB2 System, Peroxidase (DAKO A/S, No K0675). Control slides were incubated with a secondary antibody only. After washing three times in TBST for 5 min each, the slides were mounted. We analyzed the slides blindly under the microscope and labeled the cholangiolar epithelium as negative (<1% cytoplasmic staining), one positive (1-20% cytoplasmic staining), two positive (21-50% cytoplasmic staining), or three positive (>50% cytoplasmic staining). Negative and one positive immunostaining were arbitrarily classified as negative.

3 | RESULTS

3.1 | Cytogenetic change of TAA-induced rat CCA in different stages of tumorigenesis

To clarify the cytogenetic change of TAA-induced rat CCA in the process of tumoral progression, a series of different stage TAA-induced CCA tissue was used to detect the genetic aberrations. Four groups (denoted by group I-IV) of aCGH data were assembled based on the harvested weeks (followed by 4th(I), 8th(II), 12th (III), and 16th till 25th (IV) week) were analyzed (Table 1).

Notably, there are two loci, +2q45 and +Xq22, represented the genetic alterations in the very early stage (4th week), and loss the region of 12p12 appeared at 8th week, all the alterations were then remaining till late stage; in which, one region involved a suppress apoptotic gene – *TNNI3K* (+2q45), and two regions were identified unknown function of predicted loci – *LOC680159* and *LOC498132*, respectively (Table 1) (Figure 1). Interestingly, after continued feeding TAA water to rats till 12th week, the genetic altered numbers dramatically increasing and showed complexity of gains and losses on chromosomes; at least fourteen regions of chromosomal aberrations, where involved more than 19 genes were assembled as group III (Tables 1 and 2) (Figure 2). The frequent gains were observed on the

TABLE 1 The genetic alterations related with the induced-CCA tumoral progression detected by array CGH

Alter genes groups	Cytoband	Candidate genes				Biological function in cancer categories
		4th week	8th week	12th week	16th-25th week	
I		→	→	→	→	
	+2q45	TNNI3K				Suppress apoptosis
	+Xq22	LOC680159				-
II			→	→	→	
	-12p12		LOC498132			-
III				→	→	
	+5q22			TESK1		Motility
				CD72		-
				RGD1560723		-
	+5q36			IPP		-
	+10q24			CLDN7		Proliferation, drug resistance, motility
				ELP5		Motility
				CTDNEP1		-
				GABARAP		TNFα resistance
				PHF23		Transcription dysrugulation
				LOC100361515		-
	+20p12			RT1-DOB		-
				TAP1		Drug resistance
				TAP2		Drug resistance
				PSMB8		Drug resistance
	-1q32			OLR11		-
	-13p11			CNTNAP5A		-
	-16q12.5			FAM90A1		-
				LOC100362525		-
				FAM90A13-PS1		-
					→	
IV					>1000 of genes, selected genes:	
	+1q51-q53				JAK2	Upregulate interlulin-6
	+10q32.1				STAT3	Upregulate interlulin-6
	+10q32.1				STAT5A	Suppress apoptosis, proliferation, motility

The arrows represented the genetic alteraions remain during the time periods.

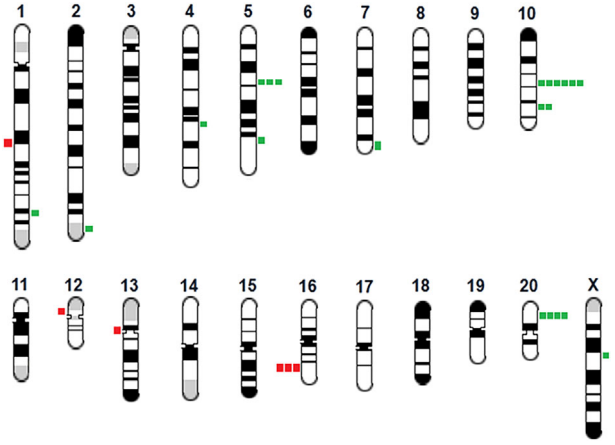

FIGURE 1 Schematic representations of genes in 12th week of TAA-induced rat CCA. The squares show the variation regions that exhibited gains (green) or losses (red) [Color figure can be viewed at wileyonlinelibrary.com]

chromosomes 5q22, 5q36, 10q24, and 20p12. Besides, more than a thousand of genes alterations involved in total 712 regions of genome have been detected in the 16th till 25th week, in which, gene numbers of gains were much more than losses, with 1074 to 157 were assembled as group IV (Table 2) (Figure 2). After analyzed based on database, we further concluded the pattern of over expression in the grouping genes *JAK2*, *STAT3*, and *STAT5* might play an important role in late stage of tumoral progression (Group IV). Further, the most common gain regions occurred on chromosome 10q24, and then follow by chromosomes 20p12 and 5q22; besides the most common loss region 16q12.5 was observed by 12th week of tumor progression (Figure 1 and Table 1).

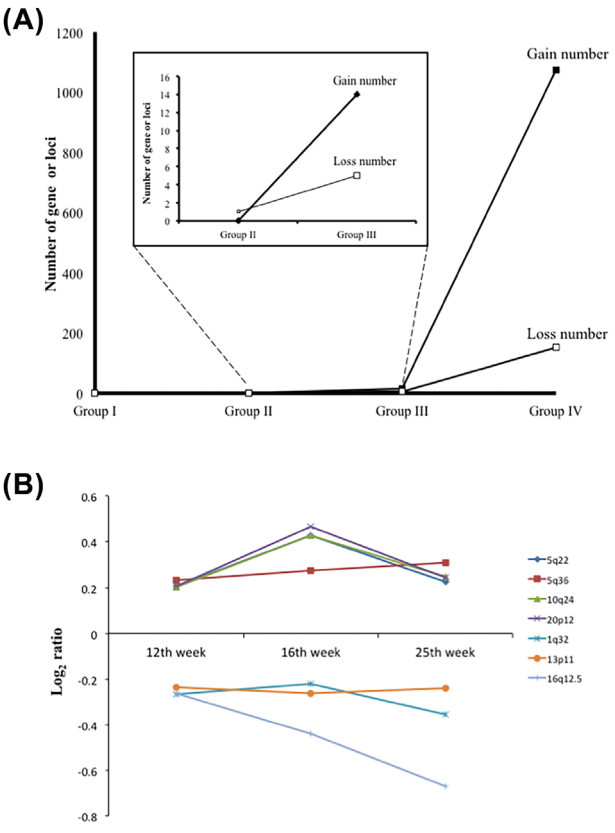

FIGURE 2 Increasing number of altered genes or loci followed by the tumor development. (A) Gain and loss of genes occurred from 4th week to 25th week. Limited genes altered during group I to II; dot line enlarge the figure from group II to III; massive amount of genetic alterations occurred from group III to IV; (B) The altered genes in group III represented different expression level during 12th week to 25th week [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | The growth of human CCA cells were suppressed by inhibiting TNNI3expression

To explore the role of *TNNI3K* in cholangiocarcinoma, we utilized in silico database to predict the expression level of TNNI3K. We observed TNNI3K was highly express at tumor part compared with normal adjacent tissue in cholangiocarcinoma patients from TCGA dataset (Figure 3). We further used nine pairs of TCGA cohort to validate our finding. Seven patients had highly TNNI3K express in tumor compare with that adjacent normal tissue (Figure 3B). Tumor part had >1.5-fold express level more than normal group (Figure 3C). Therefore, we established knockdown model by shRNA in SNU-308 and HuCCT1 cells (Figure 3D, Supplementary Figure S2A). Cell count proliferation assay and colonogenecity assay were used to observe the proliferative activity of these cells. The growth of *TNNI3K*-knockdown SNU308 and HUCCT1 cells both decreased when compared with cells transfected with an empty vector (Figure 3E and 3F, Supplementary 2B and 2C). All these results indicate the oncogenic role of TNNI3K in CCA.

3.3 | Validation of TNNI3expression by immunohistochemical staining of normal hepatic duct, inflammatory hepatic duct, and CCA

The demographic data of these eighty patients who all had undergone hepatectomy (twenty in each group) were described as follows: twenty patients with blunt abdominal trauma with liver injury (11 male and 9 female with median age 41 [ranging from 17 to 67]), 20 HCC (13 male and 7 female with median age 64 [ranging from 21 to 87]), 20 hepatolithiasis (7 male and 13 female with median age 61 [ranging from 29 to 80]) and 20 intrahepatic cholangiocarcinoma (8 male and 12 female with median age 64 [ranging from 52 to 72]) who all had undergone hepatectomy. To elucidate the significance of TNNI3K in human CCA, we performed immunohistochemical staining on liver tissues with CCA, hepatolithiasis, and normal bile ducts. As expected, most CCA (18/20, 90%) exhibited diffuse TNNI3K immunostaining (Figure 4A). TNNI3K was also frequently expressed (14/20, 70%) in the inflamed intrahepatic bile ducts of patients with hepatolithiasis (Figure 4B). In contrast, expression of TNNI3K was uncommon in normal intrahepatic bile ducts in either normal liver (0/20, 0%) or liver with HCC (2/20, 10%), whereas diffuse TNNI3K immunostaining was noted in hepatocytes (Figures 4C and 4D). Overall, TNNI3K expression was significantly more frequently observed in malignant and inflamed bile ducts than in normal intrahepatic bile ducts ($P < 0.001$).

TABLE 2 Number of the gain or loss genes, and number of variant regions on each chromosome

	No. of gain genes (Regions)				No. of losses genes (Regions)			
	I	II	III	IV	I	II	III	IV
Chr1	–	–	–(1)	249 (98)	–	–	1 (1)	21 (28)
Chr2	1 (1)	–	–(1)	47 (33)	–	–	–	4 (13)
Chr3	–	–	–	13 (7)	–	–	–	5 (8)
Chr4	–	–	–(1)	21 (17)	–	–	–	23 (18)
Chr5	–	–	4 (2)	14 (9)	–	–	–	6 (7)
Chr6	–	–	–	12 (8)	–	–	–	3 (11)
Chr7	–	–	–(1)	240 (107)	–	–	–	–(1)
Chr8	–	–	–	48 (32)	–	–	–	4 (5)
Chr9	–	–	–	11 (7)	–	–	–	3 (3)
Chr10	–	–	6 (2)	105 (46)	–	–	–	3 (3)
Chr11	–	–	–	1 (2)	–	–	–	–(1)
Chr12	–	–	–	114 (60)	–	1 (1)	–	–
Chr13	–	–	–	32 (16)	–	–	1 (1)	–(6)
Chr14	–	–	–	7 (5)	–	–	–	11 (10)
Chr15	–	–	–	9 (3)	–	–	–	9 (18)
Chr16	–	–	–	10 (6)	–	–	3 (1)	13 (13)
Chr17	–	–	–	3 (1)	–	–	–	6 (5)
Chr18	–	–	–	3 (4)	–	–	–	1 (3)
Chr19	–	–	–	14 (11)	–	–	–	–
Chr20	–	–	4 (1)	121 (43)	–	–	–	20 (6)
ChrX	1 (1)	–	–(1)	–	–	–	–(1)	20 (38)
Total	2 (2)	–	14 (10)	1074 (515)	–	1 (1)	5 (4)	157 (197)

4 | DISCUSSION

To reveal the cytogenetic alteration for tumorigenesis of CCA, a series of TAA-induced rat CCA tissue at different time point has been investigated by aCGH in current study. To clarify the correlation between genetic variations and tumoral development, we dissect formation of rat CCA into three stages, including early stage, tumor rapid progression stage, and late stage (Table 1).

Firstly, we identified the possible early genetic pattern change of TAA-induced rat CCA including gain on the chromosomal regions of 2q45, Xq22 (group I), and loss on the region 12p12 (group II), where the associated genes−*TNNI3K* and predicted loci *LOC680519 and LOC498132*. The three loci might play a role for the initiation of CCA tumoral formation and might be regarded as diagnostic markers. Despite the two unknown function of predicted loci-*LOC680159* and *LOC498132*, *TNNI3K* has been studied in the cardiac myogenesis process, and involved in the repair of ischemic injury. *TNNI3K* was also known belonging to a tyrosine kinase-like branch in the kinase tree of the human genome, and may play a key role in the anti-apoptosis by inhibiting the phosphorylation of p38 and JNK.[16,17] CCA growth has been reported to require functional p38 MAPK signaling.[18] Herein, we further mapped the candidate gene *TNNI3K* on human chromosome; it located on chromosome 1p31.1.[19] The functional study of TNNI3K demonstrated TNNI3K induced growth of CCA by in-vitro study. Two different CCA cells were used to illustrate its general impact on proliferation of CCA by TNNI3K over-expression. The fact of further validation of TNNI3K expression by in silico data and clinical cohort by immunohistochemical staining. Interestingly, patients with inflammatory hepatic duct and CCA revealed significantly increased expression of TNNI3K than that of human normal hepatic duct with significance *P*-value <0.0001 (positive rate: 14/20 [70%] vs 18/20 [90%] vs 0/20 [0%] vs 2/20 [10%], respectively) (Figure 3). So we hypothesized that over expression of *TNNI3K* might trigger suppression of apoptosis in the early stage of rat CCA, in turn results in initiation of tumorigenesis of rat CCA. Besides, we strongly suggested the genetic pattern of +2q45, +Xq22, and −12p12 in rat CCA and involved gene *TNNI3K* were supposed as trunk genes regarded as early events occurred in the CCA tomoral formation, since all the genetic alterations were remaining till very late stage (Table 2). Taking together, over-expression of TNNI3K might be associated with early-stage carcinogenesis of cholangiocarcinoma.

In addition, sixteen genes and three predicted loci involved in 10 regions of 9 chromosomes, which were derived from the 12th week of TAA-induced rat CCA tissues, were considered to be associated with rapid tumor progression stage of rat CCA (Table 2) (Figures 2A and 2B). Similarly, all genetic aberrations combined with (Groups I-II) were remained the changes till the late stage of tumor progression. We further made a detailed investigation on the correlation between the aberration regions of group III and time scale of tumor progression, the obvious over expression of certain genetic alterations showed even higher gain

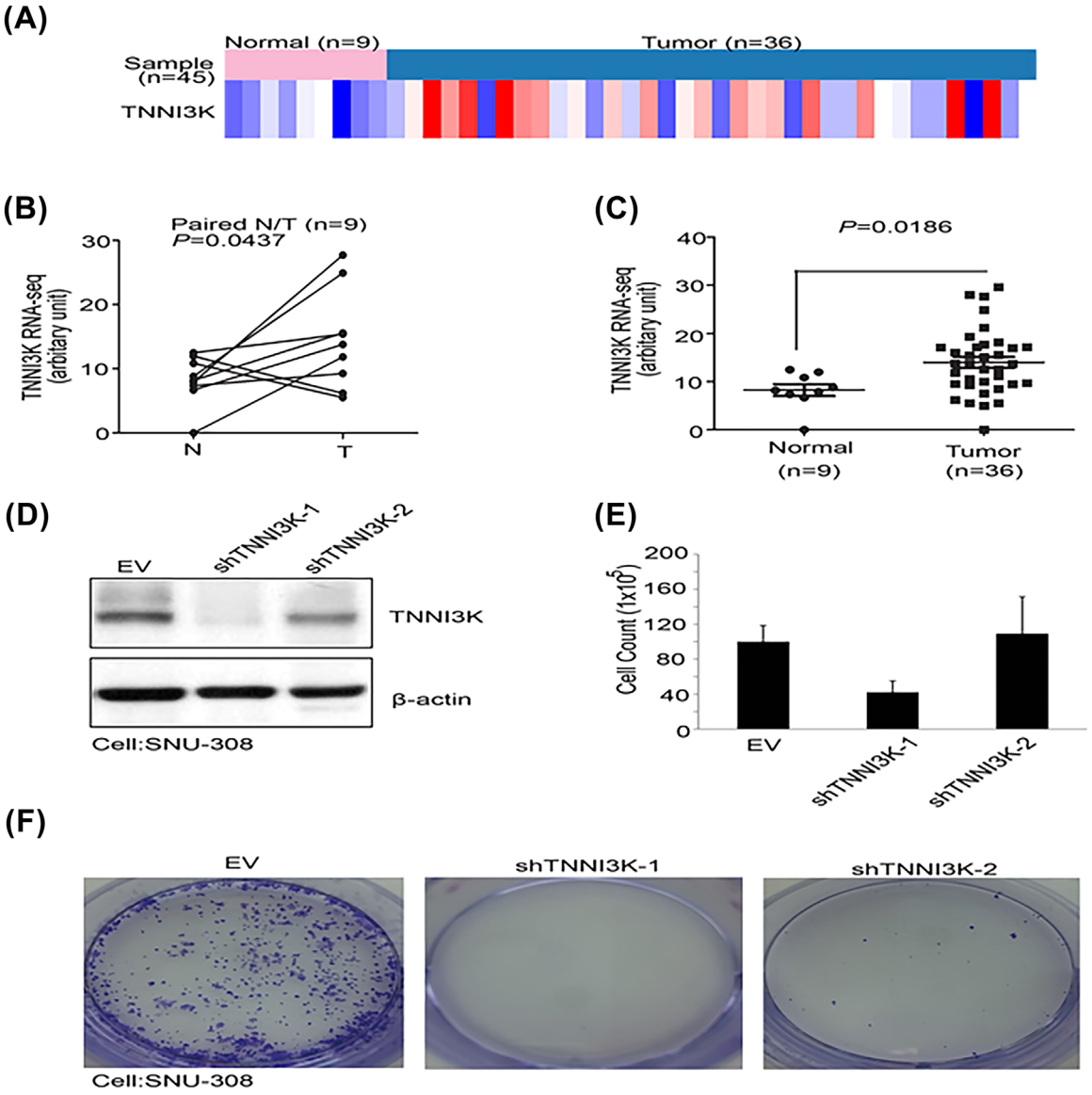

FIGURE 3 High TNNI3K expression in cholangiocarcinoma patients and enhance cell growth and colony formation ability in vitro. (A) Heatmap of TNNI3K gene expression from the TCGA RNA sequencing database in non-tumor and tumor tissues derived from clinical patients with cholangiocarcinoma. (B) TNNI3K gene expression from the TCGA RNA sequencing database in paired non-tumor and tumor tissues derived from cholangiocarcinoma patients. (C) TNNI3K gene expression from the TCGA RNA sequencing database of each group from A ($P = 0.0186$). The significance of the difference was analyzed using the non-parametric Mann-Whitney *U*-test. (D) Knockdown of TNNI3K in SNU308 cell lines with two different shRNAs was confirmed by Western blot assays. (E) The cell proliferation rate of SNU308 with or without knockdown TNNI3K was determined by cell count using trypan blue exclusion assay. (F) Colony formation assay in SNU308 cells with or without knockdown TNNI3K were incubated at 10 days. * $P < 0.05$ [Color figure can be viewed at wileyonlinelibrary.com]

folds of amplification at 16th week but regressed to the level as same as 12th week and the late stage of tumor (except the region 5q36). The occurred loci included +5q22, +10q24, and +20p12 (Figure 2B). On the other hand, the loss of regions of 1q32 and 13p11 were remaining similar level of changes. Such genetic pattern might harbor potential key genes to trigger tumor rapid progression. In which, the most common gains located on chromosome 10q24, where several genes have been identified by mapping on NCBI database, for example, *CLDN7*, *CTDNEP1*, *ELP5*, *GABARAP*, *PHF23*; and common losses loci occurred on chromosome 16q12.5, where involved genes *FAM90A1* have been identified (Table 1) (Figure 1).

CLDN 7 is overexpressed in the stage of tumor rapid progression status of rat CCA. *CLDN7* is one of the members of claudin family that encodes a protein responsible for formation the tight junctions in epithelial and endothelial cells.[20,21]. Many malignancies has been observed the expression of this gene, including breast cancer, ovarian cancer, hepatocellular carcinomas, urinary tumors, prostate cancer, lung cancer, thyroid carcinomas, gastric cancer and so on.[22–26] In addition, when tight junction protein CLDN7 interact with EpCAM might trigger tumor cell migration, proliferation, and drug resistance,[27] and the over expression of EpCAM is commonly observed in CCA.[28] Besides, *GABARAP* encodes gamma- aminobutyric acid A (GABA (A))

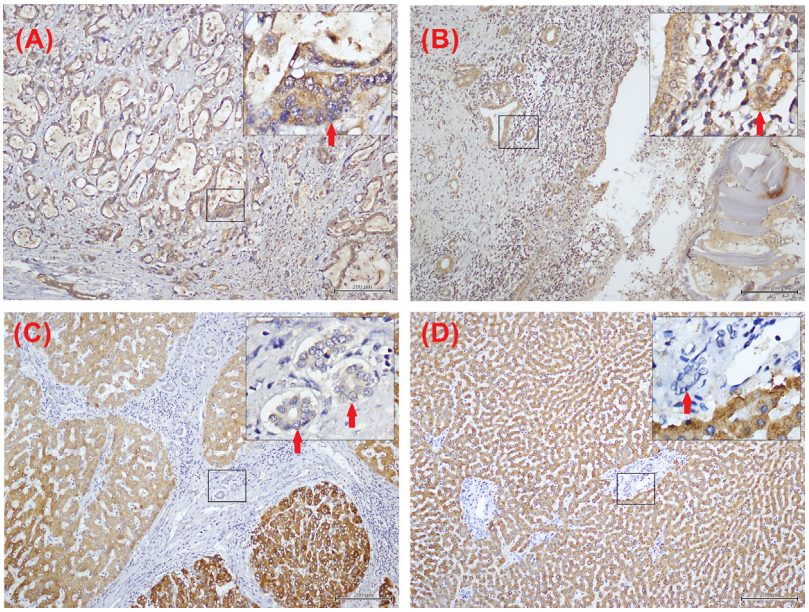

FIGURE 4 The different expression level of TNNI3K protein presented in human cholangiocarcinoma (CCA), inflammatory bile duct and normal bile duct presented with 100 fold magnification and highlighted with 400 fold (200 μm scale bar) (A) Positive cytoplasmic immunostaining of TNNI3K of CCA (X100). (B) Positive cytoplasmic immunostaining of TNNI3K of inflammatory bile duct (stone: intrahepatic bile duct stone) (X100). (C, D) Negative immunohistochemical staining of normal bile duct (X100) [Color figure can be viewed at wileyonlinelibrary.com]

receptor-associated protein, and it was known may play a role in intracellular transport of GABA(A) receptors and its interaction with the cytoskeleton,[29] and also involved in apoptosis and autophagy.[30,31] *ELP5* encodes elongator complex subunit that responsible for promote RNA polymerase II to transcription in cells, it has been proven in melanoma cells might play a role in regulating tumorigenicity and migration.[32] Furthermore, translocation of *PHF23* and fusion to *NUP98* form a novel fusion gene *NUP98-PHF23* has been reported in acute myeloid leukemia.[33] However, we did not investigate whether the gene structure alteration or not in current CCA cells. Based on it, we strongly suggested that the certain group III genetic instability pattern occurred in 12th week could be considered as a crucial time-point and highly associated with tumor rapid development of CCA.

We were highly surprised at the vast amount of the altered genes increasing in group IV, which more than a thousand of genes or loci (in total 1074 of gains and 157 of losses changes) were noted at the 16th week of TAA-induced CCA rat tissues analysis (Table 2) (Figure 2A). We further concluded that *JAK2* and *STAT3* and *STAT5A* might be important genes involve in the late stage of tumor development. *JAK2* gene produces protein tyrosine kinase, which involved in a specific subset of cytokine receptor signaling pathways. Phosphorylation of STAT can be stimulated by JAK, in turn JAK/STAT signaling pathway may trigger cell proliferation and prohibit apoptosis has been reported in Hodgkin Lymphoma study.[34,35]

Further, an in vitro model of epidermal growth factor receptor (EGFR) inhibitor-Cetuximab (Erbitux) has been tested in CGCCA cell line in our studies, however, there was no significant genetic alteration regarding EGFR could be found in this study (data not shown).

Herein, we concluded our study contributed to identification of copy number variations and candidate genes in serial rat CCAs developed in different stage. All the findings might uncover the mystery about CCA pathogenesis and provide the potential genetic mechanism responsible for CCA tumorigenesis.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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