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Alteration of STK11 Expression Associated With Cholangiocarcinoma Progression

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Abstract. *Background/Aim: Serine/threonine kinase 11 (STK11), a tumor suppressor, controls 5' AMP-activated protein kinase (AMPK) signaling in a variety of cellular functions. Mutated STK11 has been identified as a novel driver gene that promotes cancer progression. The purpose of this study was to investigate the alteration of STK11 and its correlation with clinicopathological data in cholangiocarcinoma (CCA). Materials and Methods: Gene mutation and expression analyses were performed using cBioportal and Gene Expression Profiling Interactive Analysis version 2 (GEPIA2). qRT-PCR was performed to measure STK11 mRNA levels and immunohistochemistry was performed to investigate STK11 protein expression in CCA tissues. Results: The results from publicly available cancer datasets showed that 2.7% of CCA cases had STK11 mutations. Most of STK11 gene mutations are of the truncating type and result in low STK11 mRNA and protein expression. We detected a correlation between STK11 mutation status and the tendency for shorter patient survival. The results of qRT-PCR revealed that STK11 mRNA levels were statistically significantly lower in CCA patients with mutated STK11 compared to those with wild-type STK11 (p-value=0.013). Immunohistochemical staining showed high STK11 expression in 43.8% and low expression in 56.2% of CCA tissues examined. Low STK11 protein expression resulted in poor prognosis compared with high STK11 expression, especially in CCA papillary carcinoma. Univariate and multivariate analysis revealed that high STK11 expression was associated with a decreased hazard ratio of patient survival rates (HR=0.696, p-value=0.06 and HR=0.666, p-value=0.04, respectively). Conclusion: Alteration of STK11 mutational or mRNA/protein status might be used as a potential predictive biomarker for the prognosis of the clinical outcomes in CCA patients.*

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Key Words: STK11, prognostic marker, predictive biomarker, cholangiocarcinoma.

Cancer is a complicated disease caused by genetic and environmental factors. In most cancers, the presence of genetic alterations is correlated with unfavorable effects. In order to identify potential biomarkers for prognosis or targeted therapy, a pan-cancer analysis of appropriate genes can be performed using bioinformatics based on publicly accessible datasets.

Cholangiocarcinoma (CCA) is a primary cancer arising from biliary epithelium cells and occurs in the intrahepatic and extrahepatic regions of the bile ducts (1). CCA is a major public health problem in the northeastern region of Thailand, which is the highest endemic area of liver fluke infection (2). Major risk factors of CCA include primary sclerosing cholangitis (PSC), hepatolithiasis, and liver fluke infection especially from *Opisthorchis viverrini* that induces chronic inflammation. The pathogenesis of CCA is strongly influenced by the etiology of inflammation, where increased inducible nitric oxide synthase (iNOS) activity affects the production of nitric oxide which promotes DNA damage and induces genetic alterations (3).

As patients with early stage CCA do not have symptoms, cancer diagnosis is not usually possible until CCA has spread throughout the bile ducts to other tissues, resulting in the late treatment of patients and a poor prognosis. Although

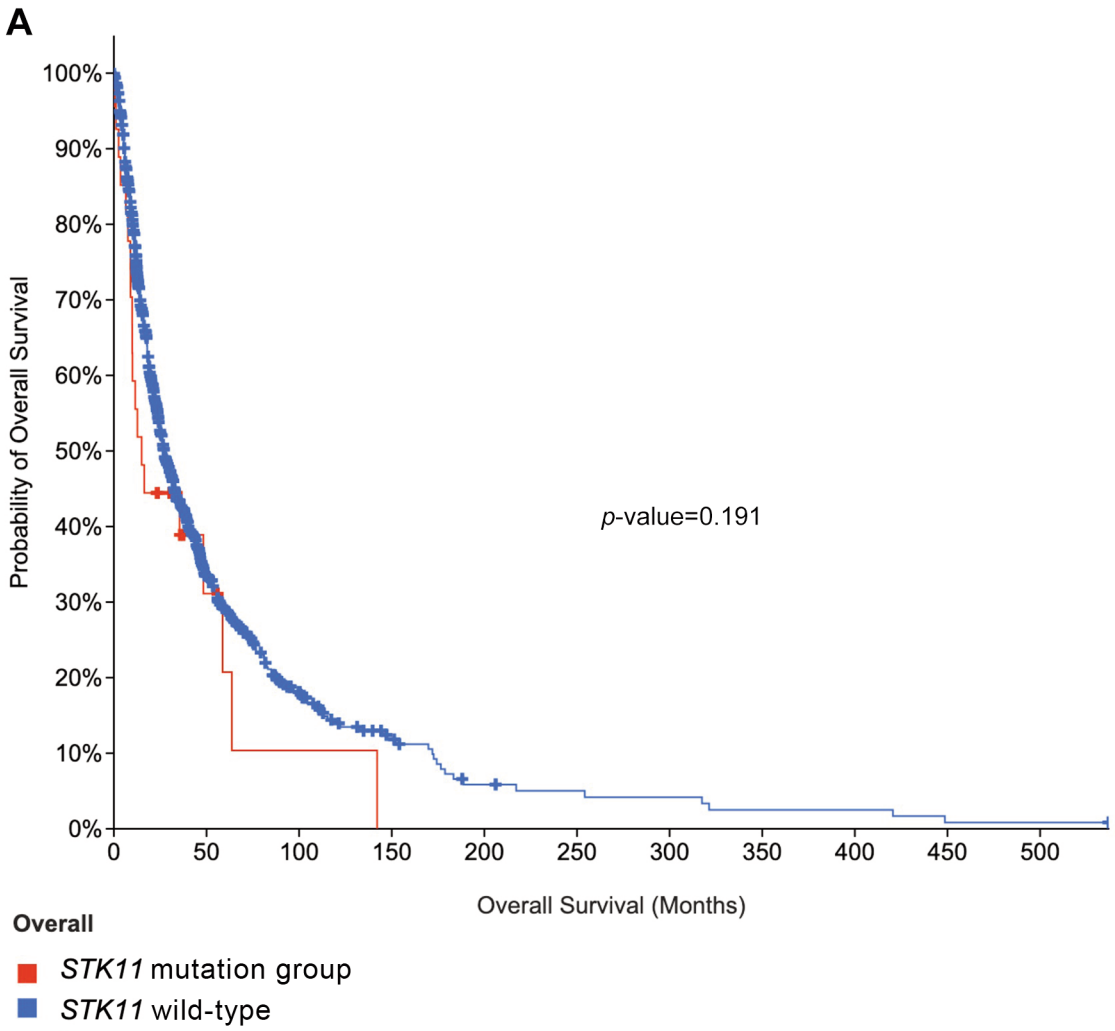

Figure 1. *Continued*

advanced CCA can respond to chemotherapy, there is no effective and reliable standard palliative treatment. Recently, cisplatin and gemcitabine have become the reference regimen for the first-line treatment of CCA (4). The median survival when gemcitabine and cisplatin are combined, however, is still less than a year (5). Immunotherapy including immune checkpoint inhibitors and adaptive T-cell therapy is still early in its development (6). Consequently, identifying possible biomarkers that can be employed as predictive biomarkers or therapeutic targets is critical for improving CCA treatment. STK11, also known as liver kinase B1 (LKB1), acts as a tumor suppressor that regulates 5' AMP-activated protein kinase (AMPK) signaling in multiple cellular processes including apoptosis, energy metabolism, and tumor progression (7). STK11 phosphorylates and activates AMPK, which then phosphorylates enzymes to maintain cellular energy metabolism. Among the critical targets repressed is mTOR, which controls cell growth, cell proliferation, and migration. Loss of STK11/AMPK signaling results in aberrant activation of mTOR, and thereby promotes tumorigenesis (8). *STK11* mutation was found in patients with Peutz-Jeghers syndrome (PJS), lung adenocarcinomas, cervical cancer, and hepatocellular carcinoma (HCC). Previous studies have revealed that mutated genes in CCA include *TP53* (44%), *KRAS* (16.7%), *SMAD4* (16.7%), *ARID1A* (17.6%), and *STK11* (5%) (9). Interestingly, mutated *STK11* has been found as a novel driver gene, which has been associated with enhanced cell survival and invasiveness contributing to cancer progression (10). *STK11* is commonly inactivated by a homozygous deletion (11, 12). STK11 inactivation has been shown to ochttps://www.tvopen.gr/watch/169342/erotasfygas7ckyklos1epeisodio146cur in a variety of clinicopathological diseases, including lung (13), breast (14), and pancreatic cancers (15). Knocking down of STK11 in intrahepatic CCA (iCCA) cell

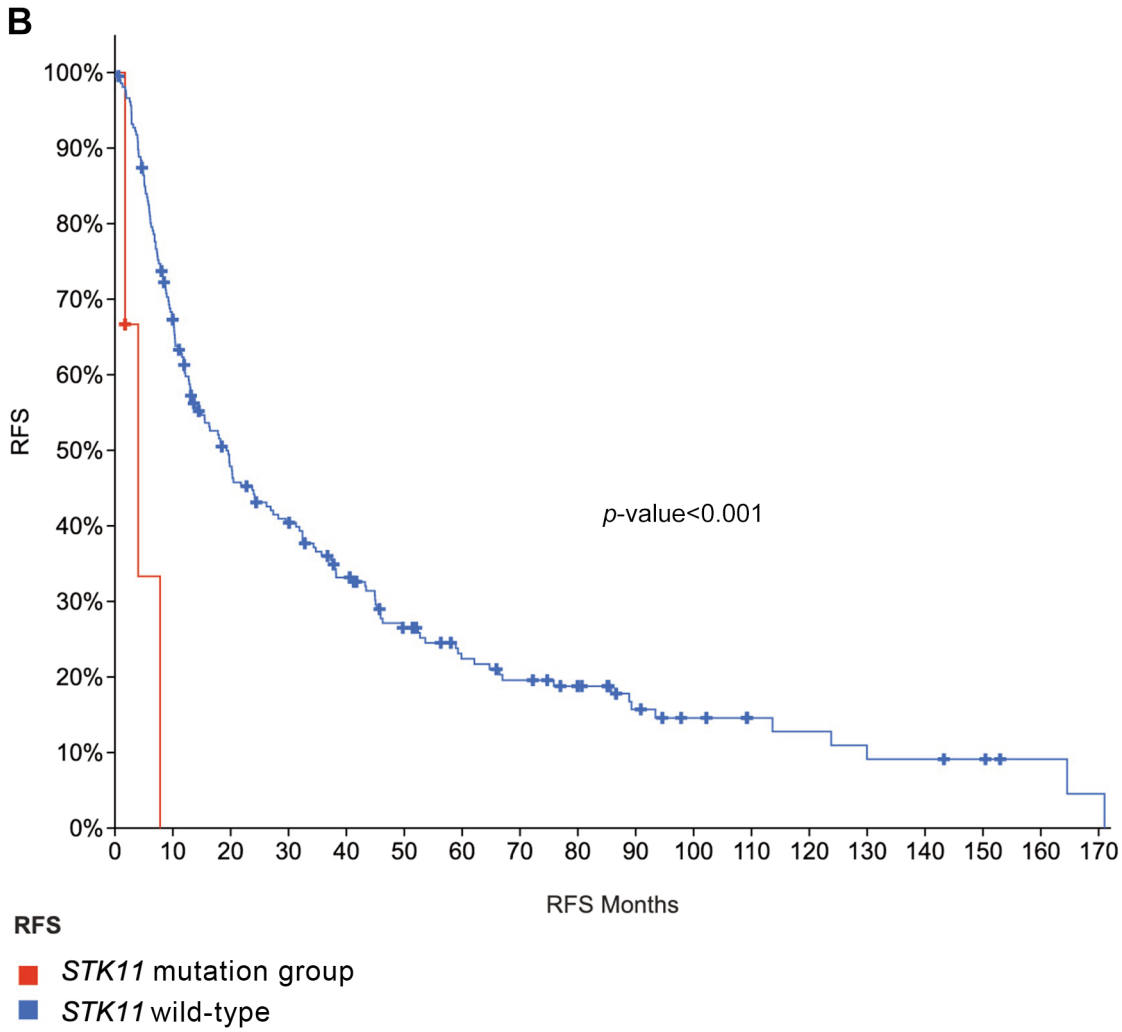

Figure 1. *The overall survival analysis of 1,537 cholangiocarcinoma (CCA) cases from the cBioportal database using the Kaplan-Meier method with a log-rank test shows that median months in the STK11 mutation group (Altered group) were lower than those in the STK11 wild-type group (Unaltered group) (A). Moreover, recurrence-free survival (RFS) in the STK11 mutation group (Altered group) was statistically significantly lower than that in the STK11 wild-type group (Unaltered group) (B).*

lines significantly enhanced proliferation, migration, and invasion (16). In a previous study, STK11 protein expression was investigated in non-small cell lung cancer (NSCLC) tissues. Loss of STK11 expression was an independent factor that negatively impacted prognosis of patients with NSCLC (17).

STK11-mutated cancer cells have been shown to be sensitive to agents that inhibit proliferation-associated proteins up-regulated by *STK11* mutations, such as phenformin (agonists of AMPK) and temsirolimus (mTOR inhibitors) (18). In contrast, *STK11* and *KRAS*-mutated patients with NSCLC show resistance to anti-PD-1/PD-L1 immune checkpoint inhibitors (19). Skoulidis *et al*. also found that in NSCLC patients with *STK11* mutations who were treated with PD-1 or PD-L1 inhibitors showed significantly lower response rates (20). Additionally, the effectiveness of pembrolizumab monotherapy in NSCLC patients with high PD-L 1 expression indicates that the disease progression rate of the low-STK11 group was greater than that of the high-STK11 group (21). As a result, the alteration of STK11 may be used as a predictive marker for choosing the appropriate therapy.

We investigated and analyzed *STK11* gene mutations and expression from publicly accessible datasets and performed qRT-PCR and immunohistochemistry to evaluate STK11 mRNA and protein expression. The correlation between STK11 alteration and the clinicopathological data of the patients was also investigated. The loss of STK11 expression may be useful as a predictive marker for the prognosis of clinical outcomes in patients with CCA.

Materials and Methods

Gene mutation and gene expression analysis. Based on publicly available cancer datasets, *STK11* mutations or mRNA expression were analyzed from cBioPortal for Cancer Genomics. Gene Expression Profiling Interactive Analysis version 2 (GEPIA2) provided powerful assistance in studying the differential expression of *STK11* in The Cancer Genome Atlas (TCGA) database. The online analysis tool is available in the Acknowledgements section. The Kaplan-Meier curves with log rank *p*-value<0.05 were drawn in the "Survival analysis" module *via* cBioportal and GEPIA2.

Human paraffin embedded CCA tissues. CCA tissues (n=112) were obtained at Srinagarind Hospital and stored in the Cholangiocarcinoma Research Institute at Khon Kaen University. All tissue samples were histopathogically ascertained and staged according to the 7th American Joint Committee on Cancer (AJCC). The Human Ethics Committee of Khon Kaen University authorized all procedures based on the National Research Council of Thailand (HE641246), and informed consent was acquired from each patient.

Real-time reverse transcription polymerase chain reaction (qRT-PCR) for STK11 mRNA expression. Total RNA was extracted from CCA tissues using TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. CCA tissues were homogenized using a homogenizer pestle in TRIzol™ Reagent. Chloroform was added and thoroughly mixed by shaking. Subsequently, isopropanol was added to precipitate the RNA, which was then washed with 70% ethanol. The RNA was solubilized in DEPC-treated water (Thermo Fisher Scientific). Total RNA was reverse transcribed using the high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.), and real-time PCR was performed using TaqMan™ probe STK11 (Hs00176092_m1) and TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific). Relative mRNA levels were analyzed using the comparative Ct method quantification ($2^{-\Delta Ct}$). All samples were normalized with internal control (b-actin) levels.

Immunohistochemistry staining for STK11 protein expression. For assessment of the expression levels of STK11 in CCA tissues, the sections were deparaffinized by xylene and a series of ethanol solutions 100%, 90%, 80%, and 70% to rehydrate the sections. The sections were placed in 1 M sodium citrate buffer at 100˚C for antigen retrieval, and 0.3% hydrogen peroxide was used to block endogenous peroxidase activity. Then, skim milk was added to block non-specific binding to tissues. The sections were incubated with anti-STK11 antibody (1:2,000, Abcam, Cambridge, UK #ab185734) at 4˚C overnight. After washing, the sections were incubated with a horseradish peroxidase conjugated Envision™ secondary antibody (DAKO, North America, Inc., Carpinteria, CA, USA). Peroxidase activity was determined using with 3,3' diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA), with Mayer's hematoxylin counterstaining. Then, sections were dehydrated and mounted with permount (Bio-Optica S.p.A., Milan, Italy)

Immunohistochemistry scoring for STK11 protein expression. The Histoscore (H-score) was applied for analysis of immunohistochemical staining for STK11 in the cytoplasm. The expression level of STK11 protein in cancer cells was determined by incorporating both the staining intensity (i) and percentage of stained cells at each intensity level (Pi). Positively stained cells were divided into four intensity types (i), 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). The Pi values range from 0% to 100%. The final H-score was generated from the sum of i multiplied by Pi according to the equation given below. The possible scores were from 0 to 300. The expression level of STK11 protein was classified as low or high according to the mean value of the H-score. STK11 expressed in the nucleus was indicated as positive and negative staining.

$$\text{H-score}=(0\times P0)+(1\times P1)+(2\times P2)+(3\times P3)$$

Statistical analysis. IBM SPSS V.27.0 statistical software (SPSS Inc., Chicago, IL, USA) or GraphPad Prism v.8.0 software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Data are represented as mean±SD. The relationship between STK11 expression and clinicopathological parameters of CCA patients was calculated using the Chi-square test. The Kaplan-Meier survival analysis was used to determine overall survival and low-expression and high-expression groups were compared using the log-rank test. Univariate and multivariate analyses were performed. Values of *p*-value <0.05 were considered statistically significant.

Results

Distribution of STK11 somatic mutation and its correlation with clinicopathological data. When the cBioPortal database was analyzed, 2.7% of 1,537 cases of patients with CCA showed somatic *STK11* mutations. Most *STK11* driver mutations were loss of function mutations. *STK11* mutations appeared to be associated with poor overall survival and a lower recurrence-free survival rate (*p*-value=0.191 and 0.0001, respectively) as shown in Figure 1A and B. Most of the *STK11* mutations were driver mutations, including truncating (24/1,537, 1.56%), missense (5/1,537, 0.31%), SV fusion (3/1,537, 0.2%), and splice-site (3/1,537, 0.2%). There were also variants of uncertain significance including missense (7/1,537, 0.46%) and in-frame mutations (1/1,537, 0.07%). The hotspot mutation is L282Rfs*6/P281Rfs*6 as shown in Figure 2A.

When the GEPIA2 database was analyzed, Kaplan-Meier survival curves with a log-rank test showed that low STK11 mRNA expression in patients with CCA tended to be correlated with shorter survival compared to those with high mRNA expression (Figure 2B).

mRNA and protein expression level of STK11 in human CCA tissues. All the cases in our experiment were included in cBioportal data. A total 20 cases of CCA tissues including 10 cases with *STK11* mutation status (6 truncating, 2 missense, and 2 splice-site) matched with 10 cases with wild-type *STK11* were selected for mRNA determination using qRT-PCR. The results showed that STK11 mRNA levels in CCA patients with mutant *STK11* were statistically significantly lower than those in CCA patients with wild-type *STK11* (*p*-value=0.013), especially those with truncating mutations (Figure 3).

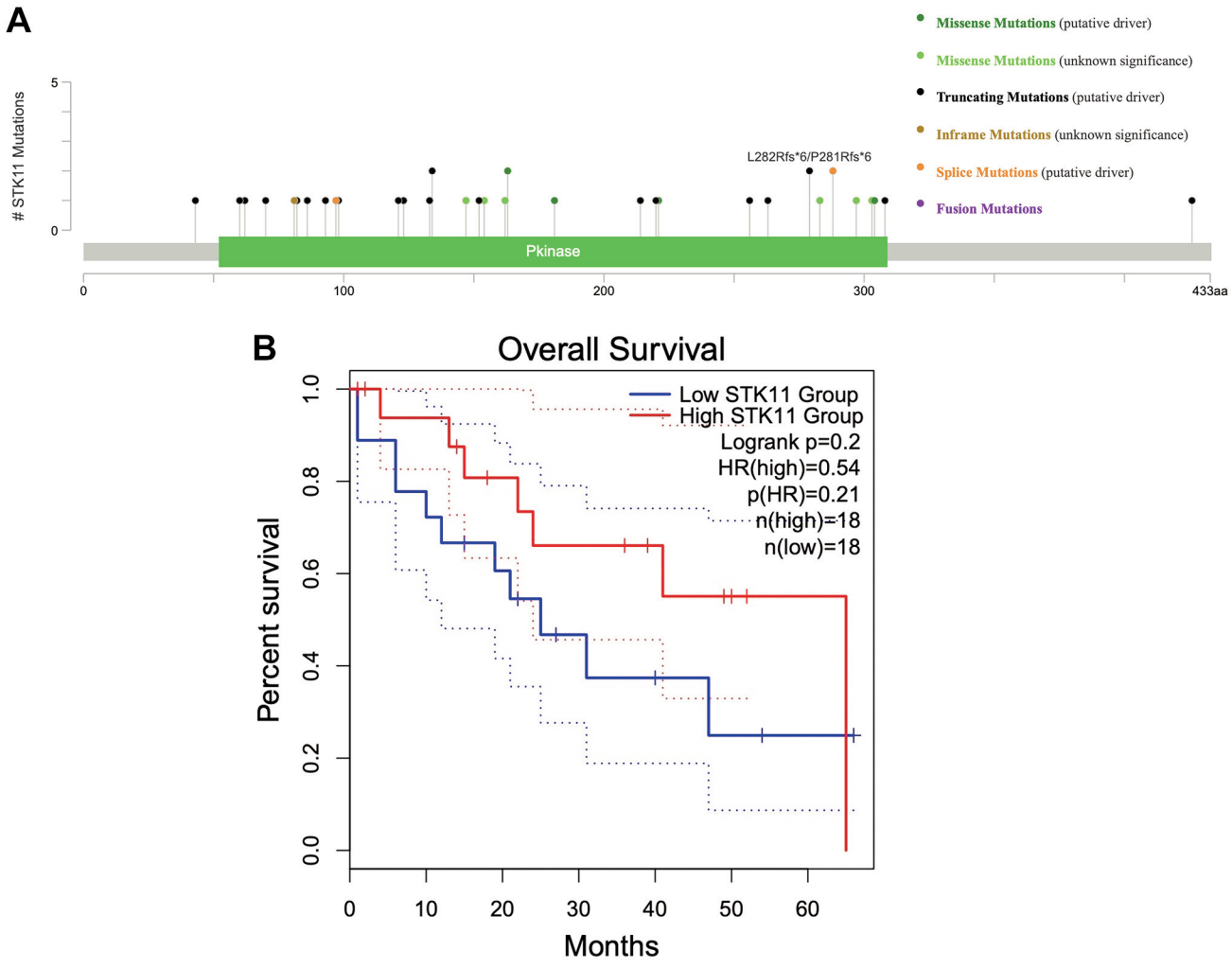

Figure 2. *The distribution of somatic mutation in STK11 in 1,537 cholangiocarcinoma (CCA) cases from the cBioportal database showed that 2.7% of cases were mutated. Most STK11 driver mutations seem to be inactivating (25 truncating, 5 missense, 3 splice and 3 SV/fusion) leading to protein loss of function (A). The overall survival analysis by Kaplan-Meier method with a log-rank test from the GEPIA2 database. CCA patients who had low STK11 expression exhibited a trend for shorter survival and poorer prognosis than those with high STK11 expression (B).*

Immunohistochemical analysis was performed to examine STK11 protein expression. Of 112 cases, high STK11 protein expression was observed in 49 cases (43.8%) whereas low STK11 protein expression was observed in 63 cases (56.2%). The immunoreactivity of STK11 was predominantly found in both cytoplasm and nucleus of bile duct cancer cells. The nucleus grading result indicated that STK11 expression was negative in 21 cases (18.8%) and was not statistically significantly associated with any clinicopathological parameter examined. Cytoplasmic expression of STK11 was higher in normal bile ducts compared with tumor cells (Figure 4).

Alteration of STK11 protein expression and its correlation with clinicopathological data. CCA tissues were obtained from 112 patients, 38 females (34%) and 74 males (66%). The mean age of patients was 57.6 years (range=37-79 years). As shown in Table I, there is a trend for a correlation between low STK11 expression and liver fluke-positive status (p-value=0.084). The univariate analysis revealed that high STK11 expression seemed to be associated with a decreased hazard ratio of patient survival rates (HR=0.696, p-value=0.063). In contrast, TNM stage, metastasis, lymph node and distance metastasis were statistically significantly associated with an increased hazard of patient survival rate (all p-value<0.05). STK11 expression, TNM stage, metastasis, lymph node and distance metastasis were included into the multivariate analysis. The results revealed that high STK11 expression was significantly associated with a decreased hazard ratio of patient survival rates (HR=0.666, 95%CI=0.451-0.985, p-value=0.042), whereas lymph node and distance metastasis had increased hazard ratios of patient survival rates

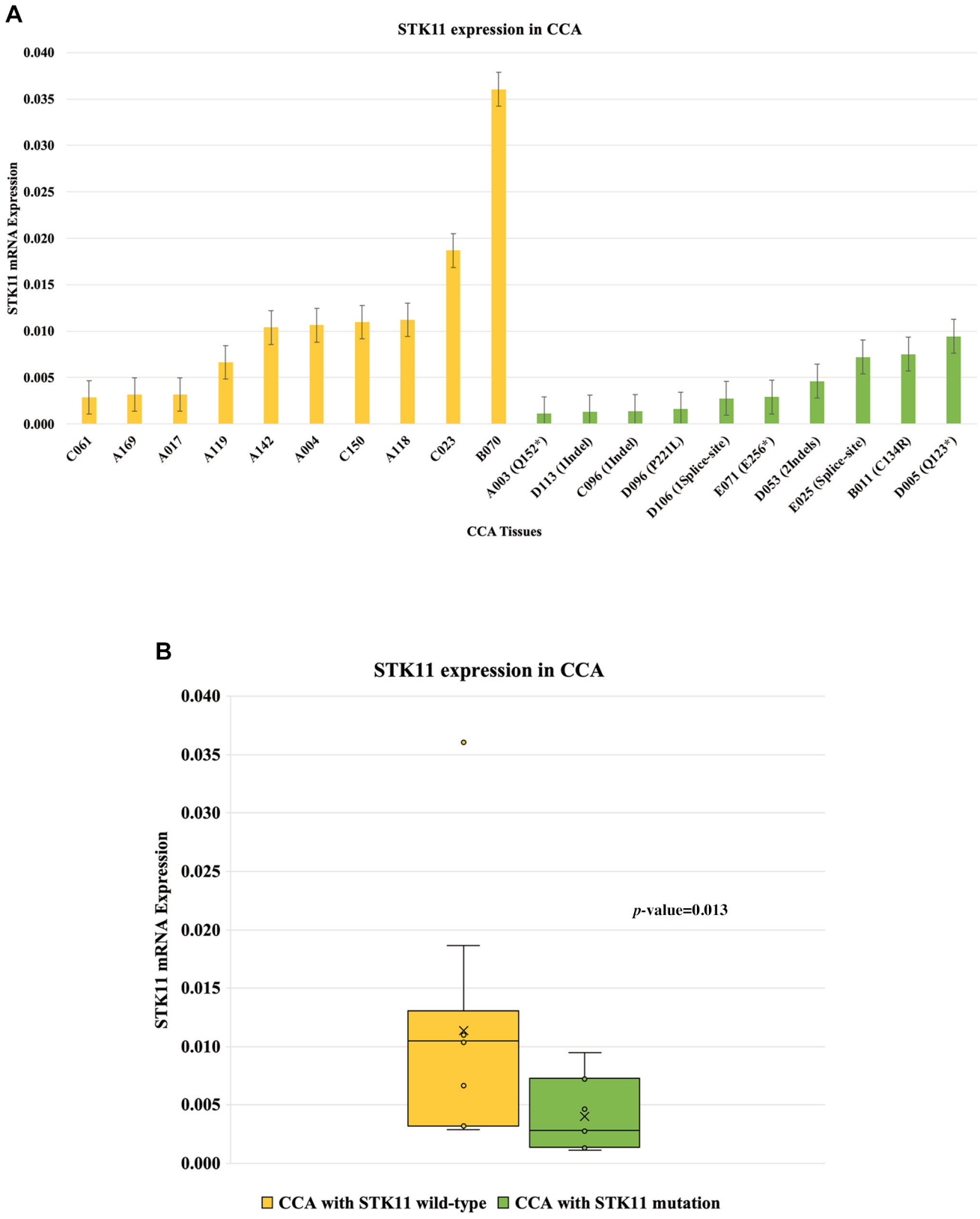

Figure 3. *The qRT-PCR was performed to determine the levels of STK11 mRNA. The STK11 was statistically significantly higher in patients with wild-type STK11 cholangiocarcinoma (CCA) compared with those with mutant STK11 CCA (p-value=0.013), especially for truncated mutations.*

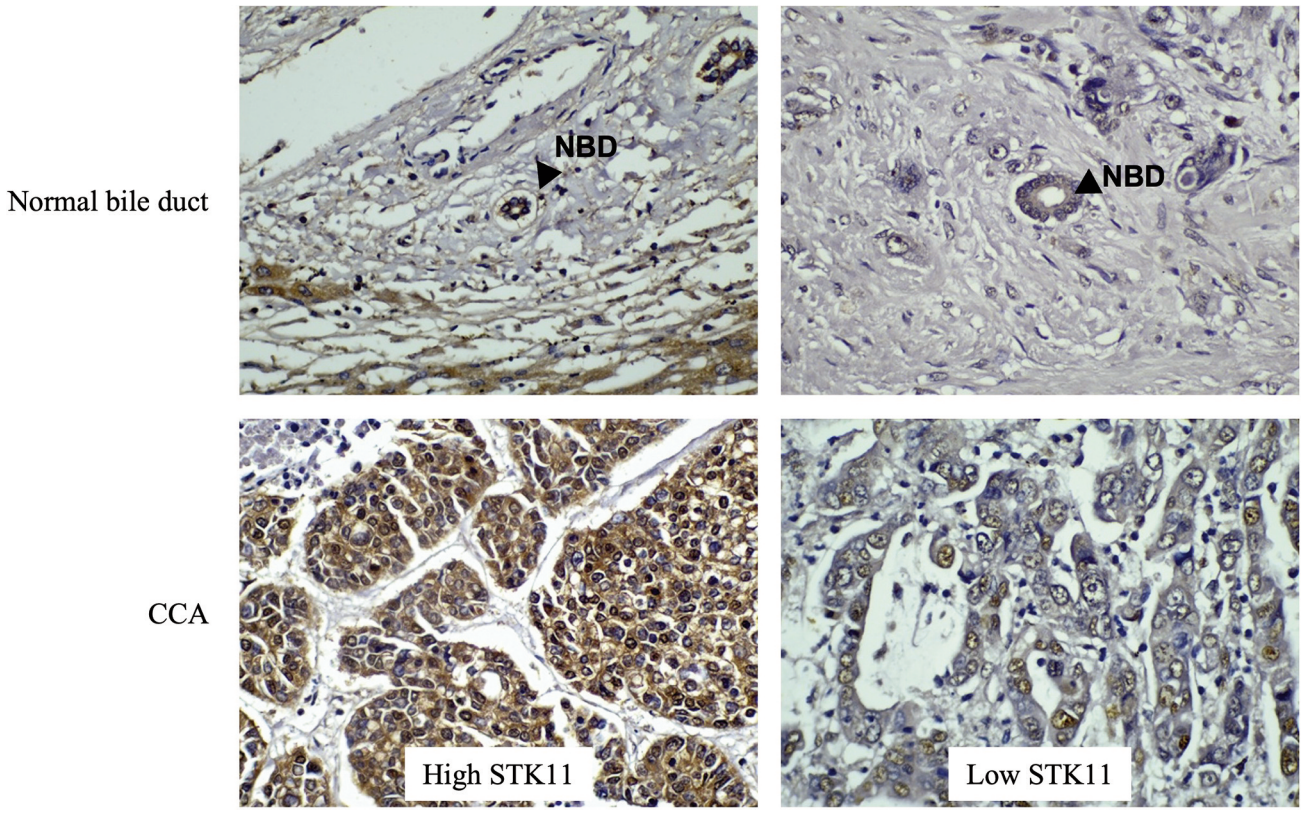

Figure 4. *Immunohistochemical staining for STK11 protein expression in human cholangiocarcinoma tissues. Expression of STK11 in normal bile duct and cancerous area with low and high STK11 levels. Magnification was ×40 for all images.*

(HR=3.760, 95%CI=1.132-12.489, *p*-value=0.031 and HR=4.353, 95%CI=2.048-9.250, *p*-value<0.001, respectively) (Table II).

Figure 5 shows the overall survival analysis using the Kaplan-Meier method. Curiously, CCA patients who had low expression of STK11 tend to exhibit a correlation with shorter survival and poorer prognosis than those with high expression (*p*-value=0.062) (Figure 5A). The mean overall survival times in those with low and high STK11 expression were 451.9 and 629.9 days, respectively. Moreover, low STK11 expression in papillary CCA tissue was significantly correlated with the short-survival time of patients with CCA (*p*-value=0.032) (Figure 5B).

Discussion

STK11, also known as Liver kinase B1 (LKB1), is encoded in humans by the *STK11* gene located on chromosome 19p13.3. It belongs to the calcium calmodulin family, which is ubiquitously expressed in several tissues and highly conserved among eukaryotes. STK11 has been shown to be related to several important biological processes, including cell cycle regulation, cellular energy metabolism, angiogenesis, cell polarity, and the response to DNA damage (22, 23). A previous study found that *STK11* was one of the mutated genes in CCA that has mutations in the kinase-RAS/RAF pathway. *STK11* is mutated in both liver fluke-related and non-liver fluke-related CCA (9). In the analysis of overall survival according to *STK11* gene alteration data, we found a correlation between *STK11* mutation status and a tendency of shorter overall survival of patients with CCA (*p*-value=0.078). The knockdown of *STK11* gene expression enhanced Wnt/β-catenin signaling, which plays a critical role in promoting progression of iCCA (24). Analysis of data from cBioPortal and GEPIA2 databases showed that most *STK11* gene mutations in patients with CCA are driver mutations including truncating, missense, splice-site, and SV/fusion, which result in low STK11 mRNA and protein expression. Likewise, low STK11 mRNA levels exhibited a trend to be correlated with shorter survival and poorer prognosis patients compared to those with high expression.

Analysis of the *STK11* mutational status in our experiment of a total of 10 cases revealed the presence of 6 truncating (3 nonsense and 3 indels), 2 missense, and 2 splice-site mutations that contributed to low STK11 mRNA and protein expression. We then investigated the alteration of STK11

Table I. *Correlation between STK11 and clinicopathological data.*

	STK11 expression in cytoplasm			STK11 expression in nucleus		
Variables, total (n=112)	Low, n=63	High, n=49	*p*-Value	Negative, n=21	Positive, n=91	*p*-Value
Age (year)			0.849			1.000
<58	33	27		11	49	
≥58	30	22		10	42	
Sex			0.843			1.000
Male	41	33		14	60	
Female	22	16		7	31	
Liver fluke			0.091			0.733
Yes	58	39		19	78	
No	5	10		2	13	
Anatomical subtype			0.849			0.630
Intrahepatic	33	27		10	51	
Extrahepatic	30	22		11	41	
Metastasis			1.000			1.000
Yes	31	24		10	45	
No	32	25		11	46	
Lymph node metastasis			0.849			0.813
Yes	28	23		9	42	
No	35	26		12	49	
Distance metastasis			0.772			0.707
Yes	8	5		3	10	
No	55	44		18	81	
Histological types			0.705			1.000
Papillary	31	27		10	43	
Non-papillary	32	22		11	48	
TNM stage			0.705			0.812
0	1	2		0	3	
I	4	2		1	5	
II	6	8		3	11	
III	24	16		6	34	
IV	28	21		11	38	

mRNA and protein levels in CCA tissues using qRT-PCR and immunohistochemistry. The STK11 mRNA levels were statistically significantly lower in CCA patients with mutant STK11 compared to those with wild-type *STK11* (*p*-value=0.013). For STK11 protein expression, results from our study of 112 FFPE CCA tissues (10 cases of mutant *STK11* and 102 cases of wild-type *STK11*) demonstrated low immunoreactions of STK11 in 63 patients with CCA (56%). Additionally, most *STK11* mutant cases showed low STK11 protein expression (7/10, 70%). Also, patients with CCA that expressed a low level of STK11 in tumor cells had greater possibilities of papillary CCA type than those with a high level of STK11 expression.

Furthermore, cumulative OS analysis showed that CCA patients in our study with low expression of STK11 tended to exhibit a poorer prognosis and shorter survival (*p*-value=0.062). The univariate analysis revealed that high STK11 expression seemed to be associated with a decreased hazard ratio of patient survival rates (HR=0.696, *p*-value=0.06) and multivariate analysis revealed STK11 expression was associated with a decreased hazard ratio of patient survival rates (HR=0.666, *p*-value=0.04). Similarly, in non-small cell lung cancer patients, multivariate analyses showed that *STK11* mutation was an independent predictor of shorter progression-free survival (*p*-value=0.02) and OS (*p*-value=0.001) (23). The meta-analysis from Ren YH (25) reveals that decreased STK11 expression is related to shorter OS among patients with a variety of tumor types for instance, lung, gastric, pancreatic, and breast (all *p*-value<0.05). Low STK11 expression is predictive of a shorter OS, poorer tumor differentiation, larger tumor, early lymph node metastasis, and a more advanced TNM stage. Intriguingly, a statistically significant correlation existed between low STK11 expression in papillary CCA' tissue and the short-survival of patients with CCA (*p*-value=0.032).

The existing studies have shown that CCA with primarily intraductal papillary growth was characterized as papillary CCA. Histologically, the invasive lesions associated with papillary CCA were categorized as conventional tubular adenocarcinoma and mucinous carcinoma. Non-papillary

Table II. *The univariate and multivariate analysis of clinicopathological data.*

	Univariate			Multivariate		
Variables, total (N=112)	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Age (>59)	1.126	0.771-1.645	0.539			
Sex (Male)	0.699	0.470-1.039	0.077			
Liver fluke-related status (Positive)	0.807	0.465-1.398	0.444			
Anatomical subtype (Extrahepatic)	0.955	0.657-1.390	0.812			
Metastasis	2.130	1.440-3.151	<0.001	0.437	0.121-1.581	0.201
Lymph node metastasis	2.009	1.361-2.966	<0.001	3.760	1.132-12.489	0.031
Distance metastasis	3.661	1.976-6.600	<0.001	4.353	2.048-9.250	<0.001
Histological types (Papillary)	0.701	0.480-1.023	0.066			
TNM stage 1, 2 *vs*. 3, 4	2.033	1.275-3.243	0.003	1.472	0.856-2.531	0.162
STK11 expression (High)	0.696	0.474-1.020	0.063	0.666	0.451-0.985	0.042

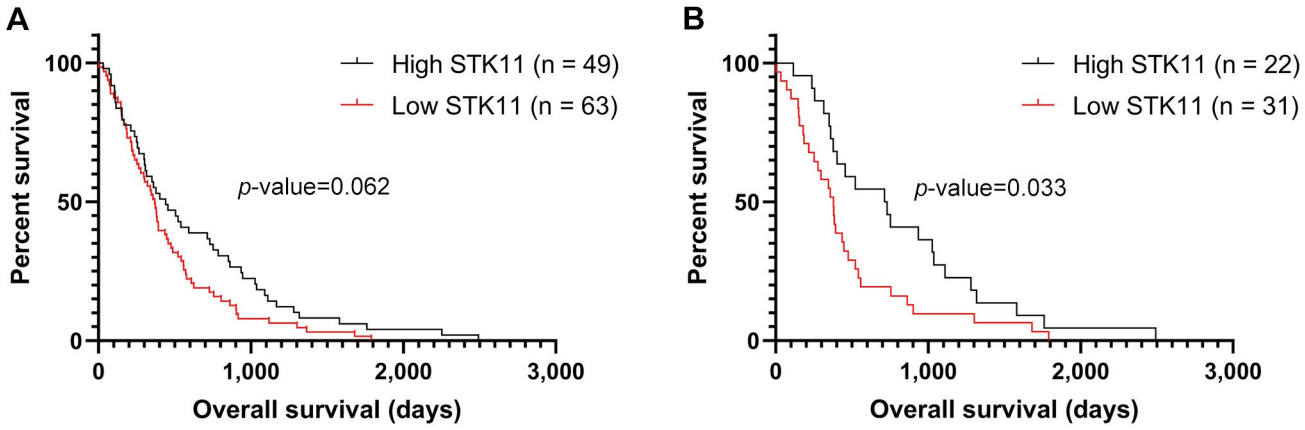

Figure 5. *Overall survival analysis. (A) In all cholangiocarcinoma (CCA) patients according to STK11 cytoplasmic expression and (B) in papillary CCA patients according to STK11 cytoplasmic expression both analyzed cut-off by mean of H-score.*

CCA cases were more likely to have lymph node metastases than papillary CCA cases. Patients with papillary CCA had a considerably better prognosis than those with non-papillary CCA (24). Similarly, our results confirm that patients with papillary CCA have a longer survival than those with non-papillary CCA, although, low STK11 expression in papillary CCA patients contributed to statistically significant shorter survival compared to those with high STK11 expression. This suggests that papillary CCA types with low levels of STK11 have poor prognoses and, therefore, STK11 may be used as the prognostic marker for medical management.

Many studies have found that STK11 is a tumor suppressor and an upstream kinase in the AMPK pathway that regulates tumor energy metabolism enhancing the expression of mesenchymal marker proteins and the cell survival, which causes tumor invasiveness (10). STK11 is an important regulator of cell proliferation *via* a p53-independent manner. Loss of STK11 leads to a reduction of mTORC1 signaling, resulting in the promotion of cell growth and tumorigenesis (26, 27). To develop an effective treatment for patients with CCA, further studies on the role of STK11 in the progression of CCA are necessary.

In summary, truncating mutations in *STK11* seem to result in low STK11 mRNA and protein expression. STK11 alteration in CCA patients tends to be correlated with a shorter survival. Low STK11 protein expression in CCA tissues was associated with the poorest prognostic outcome compared with high STK11 expression, especially in CCA papillary carcinoma type. Therefore, STK11 mutational status and/or low mRNA/protein levels might be used as a potential predictive biomarker for the prognosis of the clinical outcomes of patients with CCA.

Conflicts of Interest

The Authors declare no potential conflicts of interest in relation to this study.

Authors' Contributions

PS: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft. AJ: Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing-review & editing. SK: Conceptualization, Supervision, Validation, Writing-review & editing. MT: Resources, Writing-review & editing. PeS: Formal analysis, Writing-review & editing. SP: Formal analysis, Writing-review & editing. PK: Formal analysis, Writing-review & editing. SJ: Resources, Writing-review & editing. CA: Resources, Writing-review & editing. AT: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing-review & editing. All Authors contributed to the final manuscript.

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