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BASIC RESEARCH

Amplification of chromosome 21q22.3 harboring trefoil factor family genes in liver fluke related cholangiocarcinoma is associated with poor prognosis

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Abstract

AIM: To determine allelic imbalance on chromosomal region 21q22-qter including trefoil factor family genes (*TFF*) in cholangiocarcinoma (CCA) patients and analyze the correlation between allelic imbalances and clinicopathological parameters.

METHODS: Quantitative PCR amplification was performed on four microsatellite markers and trefoil factor family genes (*TFF1*, *TFF2*, and *TFF3*) using a standard curve and SYBR Green I dye method. The relative copy number was determined by DNA copy number of tested locus to reference locus. The relative copy number was interpreted as deletion or amplification by comparison with normal reference range. Associations between allelic imbalance and clinicopathological parameters of CCA patients were evaluated by χ^2-tests. Kaplan-Meier method was used to analyze survival.

RESULTS: The frequencies of amplification at D21S1890, D21S1893, and *TFF3* were 32.5%, 30.0%, and 28.7%, respectively. Patients who had amplification at regions covering D21S1893, D21S1890, and *TFF* showed poor prognosis, whereas patients who had deletion showed favorable prognosis (mean: 51.7 wk *vs* 124.82 wk, $P = 0.012$). Multivariate Cox regression analysis revealed that amplification of D21S1893, D21S1890 and *TFF*, blood vessel invasion, and staging were associated with poor prognosis.

CONCLUSION: D21S1893-D21S1890 region may harbor candidate genes especially *TFF* and serine protease family, which might be involved in tumor invasion and metastasis contributing to poor survival. The amplification in this region may be used as a prognostic marker in the treatment of CCA patients.

Key words: Cholangiocarcinoma; Amplification on chromosome 21; Trefoil factor family; Quantitative PCR; Liver fluke

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INTRODUCTION

Cholangiocarcinoma (CCA) or intrahepatic bile duct cancer (ICC) is a malignant tumor in the biliary tree peripheral to the bifurcation of the right and left hepatic duct[1,2]. Incidence rates of ICC vary substantially worldwide, reflecting the distribution of local geographic risk factors, in addition to genetic differences among various populations. In Western countries, the disease is rare, however, it is highly frequent in Southeast Asia, especially in Khon Kaen, Northeast Thailand. Truncated age-standardized incidence of CCA at ages > 35 years varied by three fold between districts, from 93.8 to 317.6 per 100 000 population[3]. In Western countries, primary sclerosing cholangitis is the commonest known predisposing condition for this cancer. Eight percent to 40% of CCA have been reported in patients with primary

Table 1 Locations and sequences of microsatellite markers, trefoil factor family genes and reference loci

Primer name	Chromosome	Product size (bp)	Forward primer	Reverse primer
D21S1253	21q21.3	174-190	GAAGAATCTCCCGAACCAGG	AAGACCAGTGTTATTCAGAGCC
D21S1255	21q22.2	112-126	AGCTCTTTATTTTGCCACATAG	CTGCATGTTTGCCTGG
D21S1893	21q22.2	111-119	GTATGCACACCACACGG	TAACAAAATCCGCCACG
D21S1890	21q22.3	143-173	GGTCTGACCACAGATTTCC	AAAAACACTCTGAACGATTAAGG
Trefoil factor family 1	21q22.3	219	CAGGGATCTGCCTGCATC	ATCGATCTCTTTTAATTTTTAGGCC
Trefoil factor family 2	21q22.3	123	GAAGAATCTCCCGAACCAGG	GTCACACTTCAAAAACTAGAGG
Trefoil factor family 3	21q22.3	129	CAGGCACTGTTCATCTCAGC	TATTCGTTAAGACATCAGGCTCC
β-actin	7p15	375	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG
GAPDH[1]	12p13	250	ACAGTCCATGCCATCACTGCC	GCCTGCTTCACCACCTTCTTG

[1]Glyceraldehyde-3-phosphate dehydrogenase.

sclerosing cholangitis[4]. Several clinical studies and animal model experimental studies suggest that the interaction between chemical carcinogens, especially nitrosamines and *Opisthorcis viverrini* infestation may play an important role in the development of CCA in Thailand[5-7]. Thus, either the chemical carcinogen nitrosamine or liver fluke infection alone does not produce cancer. Food derived exogenous and *in situ* nitrosamine formation may lead to DNA alkylation and also deamination in predisposed and inflamed tissues. Furthermore, chronic irritation caused by the fluke results in hyperplasia and adenomatous change of bile duct epithelium[6]. The DNA damaged biliary epithelium may then be transformed to malignant CCA[7-9]. To date, the molecular basis of carcinogenesis and pathogenesis of cholangiocarcinoma is still unclear.

Allelic imbalance at specific genomic loci is an important step in the molecular genetic analysis of human cancers. Allelic imbalance at chromosome 21, especially region 21q22-qter, was found in several types of human cancers such as gastric cancer, breast cancer, ovarian clear cell adenocarcinoma, and primary colorectal cancer[10-13]. Furthermore, chromosome 21q22.3 harbors a cluster of trefoil factor family (*TFF*) genes consisting of *TFF1*, *TFF2*, and *TFF3*[14]. TFF functions include mucus stabilization and stimulation of normal epithelial cell restitution during wound repair through motogenic and antiapoptotic activities. However, TFF peptides are overexpressed in several human solid tumors such as prostate, esophagus, breast, and pancreas and also function as tumor progression factor[15-18]. Prolonged inflammation caused by parasitic infection frequently occurs in liver fluke related CCA. *TFF* and its neighborhood located at 21q22 may be involved in tumor development and progression. Moreover, our data on comparative genomic hybridization (CGH) in CCA showed the alteration of DNA copy number at 21q22-qter at 28%.

Taken these data together, the chromosomal region 21q22-qter may harbor candidate genes, which are involved in carcinogenesis and pathogenesis of CCA. Therefore, this study attempted to determine allelic imbalance on chromosomal region 21q22-qter including *TFF* genes to define affected sites for candidate genes which are involved in molecular carcinogenesis and pathogenesis of CCA. The associations between allelic imbalance and clinicopathological parameters were also determined.

MATERIALS AND METHODS

Samples and DNA preparation

This project was approved by the Ethical Committee of Khon Kaen University. Informed consents were obtained from patients who were willing to participate in the project. Frozen liver tissues were obtained from 80 CCA patients undergoing surgical resection at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. CCA cases were diagnosed by physicians according to clinical finding, laboratory investigation and histological examination. Neural, blood vessel and lymphatic invasion were assessed by standard method[19]. The clinicopathological data such as age, gender, histological type, and TNM stage[20] were evaluated by reviewing the medical charts and pathology records. DNA was prepared from frozen liver tissues containing 80% of tumor cells by using a Puregene™ DNA purification system (Gentra System, USA) according to manufacturer's instructions. In addition, DNA was prepared from placental tissue collected from a normal labor (postpartum) woman and used for setting a standard curve. Normal leukocyte DNA derived from 50 healthy donors was prepared into 14 pooled normal DNA and generated for normal reference range.

Quantitative PCR assay

Quantitative PCR amplification was performed on a Rotor Gene 2000 Real-time Amplification (Corbett Research, Australia) using four microsatellite markers (telomere-D21S1890-D21S1893-D21S1255-D21S1253-centromere) and trefoil factor family genes (*TFF1*, *TFF2*, and *TFF3*) covering chromosomal region 21q21-qter. Reference primers were chosen in the region of the housekeeping genes that usually are not altered alteration in CCA, β-*actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primer sequences were obtained from the Genome Data Base. Locations of selected oligonucleotides and their sequences are shown in Table 1. PCR reaction was performed in a 25 μL reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris HCl pH 8.3, 100 μmol/L each of deoxynucleoside triphosphate (dNTP), 2.5 mmol/L $MgCl_2$ or 3.0 mmol/L (D21S1253, *TFF1*, and β-*actin*), 1.25 μL SYBR® Green I dye solution (Amresco, USA) (1:10 000 in DMSO), and 10 μmol/L or 5 μmol/L (D21S1253 and

D21S1255) or 15 μmol/L (D21S1890) of each primer with 1.5 units or 2.0 units (D21S1253, D21S1255, and *GAPDH*) of *Taq* DNA polymerase. The PCR was performed at 95℃ for 5 min followed by 95℃ for 15 s, 45℃ -60℃ for 15 s and 72℃ for 15 s for 35 cycles with an additional cycle of 72℃ for 10 min.

Quantitative PCR amplification was performed using a standard curve and SYBR Green I dye method as described previously[21]. The standard curve for each primer was generated using serial dilutions of placental DNA. The standard curve was constructed in each PCR run and the copy numbers of genes in each sample were interpolated using these standard curves. Placental DNA with known concentration was used for precision control. A coefficient of variation (CV) of each sample was determined based on triplicate test. The sample with a CV higher than 15% was re-tested. DNA copy number of each locus was calculated based on triplicate determination and duplicate PCR run.

Analysis of allelic imbalance

The relative copy number was determined by DNA copy number of tested locus to DNA copy number of reference locus. The DNA copy numbers of reference loci consisting of β-*actin* and *GAPDH* were averaged before calculation. The normal reference range was generated from the relative copy numbers of 14 pooled normal leukocyte DNA of 7 markers (n = 98). If the relative copy number of sample calculated differed significantly from normal reference range (mean ± 2SD), the sample was verified as loss or gain. The relative copy number was interpreted as loss when the ratio was less than mean -2SD of normal reference range. On the other hand, the relative copy number was interpreted as gain when the ratio was more than mean + 2SD of normal reference range.

Statistical analysis

Associations between allelic imbalance and clinicopathological parameters of 80 CCA patients were evaluated by means of the χ^2-tests. Survival curves for patients with allelic imbalance versus those without were calculated using the Kaplan-Meier method. Only 69 cases were available for follow-up. Six patients were lost for follow-up and five cases were perioperative death (patients who died within 4 wk after surgery). Differences in survival between these two groups were assessed by the log-rank test. Cox proportional hazards model was used in univariate and multivariate analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Allelic imbalance on chromosomal region 21q22-qter

The normal reference range generated from relative copy numbers of pooled normal leukocyte DNA was 0.54-1.34 with 95% confidence interval (mean ± 2SD). Allelic imbalance of 7 loci showed percentages of amplification at D21S1890 (32.5%), D21S1893 (30.0%), *TFF3* (28.8%), D21S1253 (26.3%), D21S1255 (23.8%), *TFF1* (22.5%), and *TFF2* (7.5%) and of deletion at *TFF3* (3.8%), D21S1255 (2.5%), *TFF2* (2.5%), and D21S1890 (1.3%).

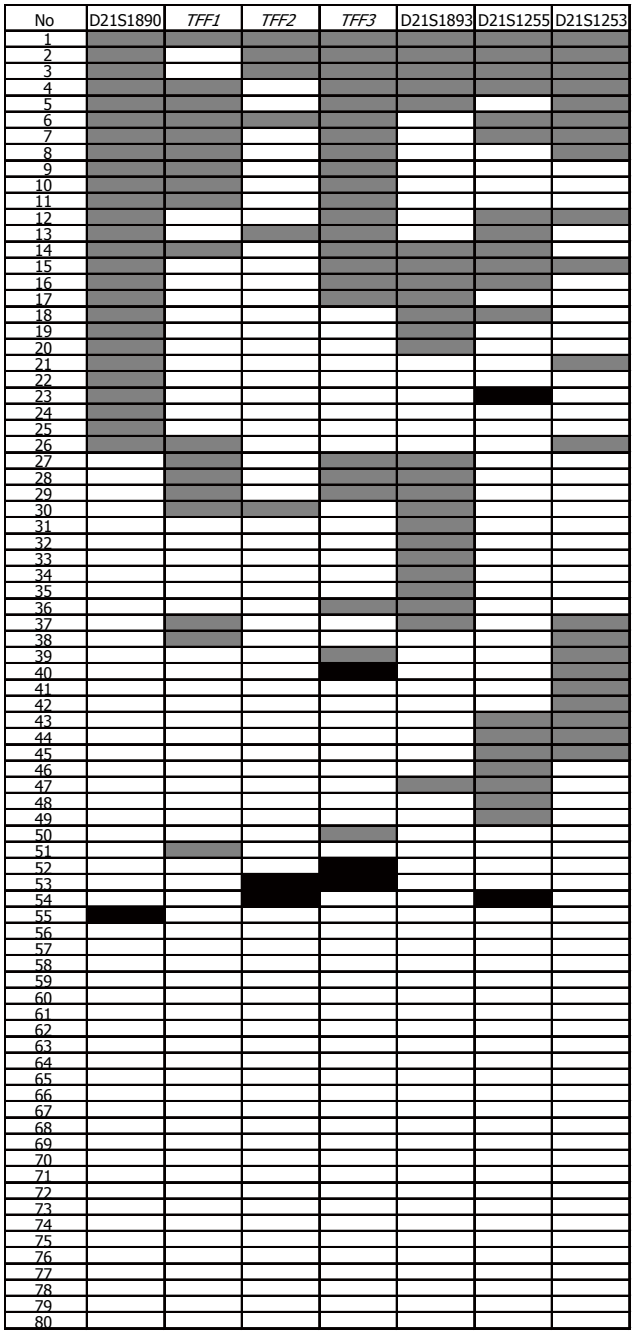

Figure 1 Fine mapping of allelic imbalance on chromosomal region 21q22-qter. D21S1890 is located at telomeric end while D21S1253 is located toward centromeric end. There are two common amplification regions at D21S1890 and the region between D21S1893 and *TFF*3.

The relative copy number of amplification of these loci ranged between 1.35-4.24 and of deletion between 0.39-0.52. Fine mapping of these regions is shown in Figure 1. Two regions of common amplification were D21S1890 and the region between D21S1893 and *TFF3*.

Associations between allelic imbalance and clinicopathological parameters of patients

The associations between allelic imbalance and clinicopathological parameters of patients were analyzed. The result showed no differences in age, sex, histological type, invasion (blood vessel, lymphatic, and nerve), and survival time between patients with and without

Table 2 Clinicopathological parameters of patients with and without allelic imbalance

Clinical parameters	*n*	Gene/microsatellite makers D21S1893, D21S1890 and *TFF*			
		Normal *n* (%)	Amplification *n* (%)	Deletionl *n* (%)	*P* value
Age (yr)	80				
⩽ 54	39	18 (46)	20 (51)	1 (3)	NS
> 54	41	15 (36)	22 (54)	4 (10)	
Gender	80				
Male	57	22 (39)	32 (56)	3 (5)	NS
Famale	23	11 (48)	10 (43)	2 (9)	
Histological type	80				
Non-Papillary adenocarcinoma	58	27 (46)	30 (52)	1 (2)	0.023
Papillary adenocarcinoma	22	6 (27)	12 (55)	4 (18)	
Staging	80				
II & III	10	5 (50)	5 (50)	-	NS
IVA & IVB	70	28 (40)	37 (52.8)	5 (7.2)	
Blood vessel invasion	79				
Non-invasion	28	8 (29)	16 (57)	4 (14)	NS
Invasion	51	24 (47)	26 (51)	1 (2)	
Lymphatic invasion	79				
Non-invasion	15	4 (27)	11 (73)	-	NS
Invasion	64	28 (43)	31 (49)	5 (8)	
Nerve invasion	79				
Non-invasion	36	14 (39)	19 (53)	3 (8)	NS
Invasion	43	18 (42)	23 (54)	2 (4)	
Survival time (wk)	69				0.012
Mean		54.12	51.7	124.82	
Median		49	27.4	84.28	
Minimum-maximum		7.14-119.28	4.85-242.85	67.71-143.85	

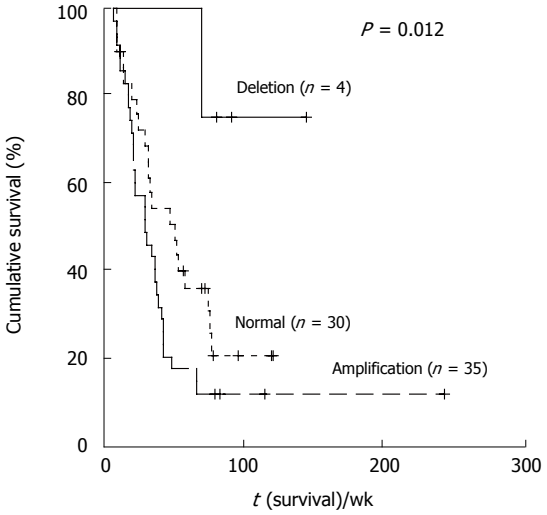

Figure 2 The association between survival time and allelic imbalance on loci D21S1893, D21S1890 and TFF was analyzed by Kaplan-Meier. The patients who had amplification at D21S1893, D21S1890 and TFF showed poor prognosis, whereas patients who had deletion at D21S1893, D21S1890 and TFF showed favorable prognosis.

allelic imbalance in almost all loci ($P > 0.050$). However, significant difference was observed in histological type at loci covering D21S1893 through D21S1890 including *TFF* ($P = 0.023$). Deletion at D21S1893, D21S1890, and *TFF* found in a papillary type was higher than that in a non-papillary one (Table 2). Furthermore, patients who had deletion in this region tended to show non-invasion of blood vessel ($P = 0.052$) (Table 2). Kaplan-Meier survival curves demonstrated that patients who had amplification at D21S1893, D21S1890, and *TFF* showed poor prognosis, whereas patients who had deletion at D21S1893, D21S1890, and *TFF* showed favorable prognosis ($P = 0.012$) (Figure 2). Multivariate analysis showed significant effects of amplification at D21S1893, D21S1890, and *TFF*, blood vessel invasion, and staging on prognosis (Table 3).

DISCUSSION

Many techniques have been used to detect genetic alterations. The real-time qPCR technique is an alternative method to determine allelic imbalance because most allelic imbalances also result in changes of relative copy numbers. In our study, allelic imbalance determined by qPCR using SYBR Green I system showed CV less than 15%, suggesting good consistency and reliability[21]. The house keeping genes, β-*actin* and *GAPDH,* were used as reference loci in our study. To assess the validity of these reference genes, we determined the copy number ratios of β-*actin*/*GAPDH* in 14 pooled normal leukocytes and 80 CCA samples. The observed ratios measured from normal DNA (1.01 ± 0.14SD) and tumor DNA (1.06 ± 0.15SD) were similar ($P = 0.271$) and both were significantly equal to 1, thus, confirming their validity as appropriate reference genes in our work.

Our finding of allelic imbalance on chromosomal region 21q22-qter in CCA patients showed predominant amplifications at markers D21S1890 (32.5%) and D21S1893 (30%). This region has length about 3.5 Mb in physical map distance and contains about 25 identified genes. Trefoil factor family (*TFF1, TFF2*, and *TFF3*) and serine protease family (*TMPRSS2* and *TMPRSS3)* are candidate genes located in this region and have a potential tumor progression activity. In this study, all CCA tissues were obtained from patients who were residents of northeastern region of Thailand where liver fluke infection remains highly endemic. Increased gene amplification at chromosome 21q22.3 especially *TFF* genes in liver fluke related CCA may result from healing process of inflamed tissues. Normally, TFF peptides are involved in the normal mucosal defense and epithelial restitution in cell injury. However, in chronic inflammation, the overproduction of TFF peptides may result in tumor progression. TFF peptides might exert beneficial effects during the early step of mucosal injury and inflammation and subsequently undesirable effects during chronic inflammation and neoplastic progression. TFF peptides function as a tumor progression factor by increasing cell scattering, invasion, survival and proangiogenesis[22-25]. TFF3 stimulates cell motility by inducing a rapid phosphorylation of β-catenin, which is associated with perturbation of the functional integrity of E-cadherin/catenin system. The promotion of cell motility in association with epidermal growth factor receptor (EGFR) leads to the enhancement of tumor cell invasion and metastasis[26,27]. However, exogenous TFF peptides

Table 3 Univariate and multivariate analysis of overall survival in cholangiocarcinoma

Variable	*n*	Univariate		Multivariate	
		Relative risk (95%CI)	*P* value	Relative risk (95%CI)	*P* value
Age (yr)	69	0.831 (0.483-1.430)	NS		
≤ 54	34				
> 54	35				
Gender	69	0.443 (0.231-0.849)	0.014	0.628 (0.312-1.263)	NS
Male	49				
Famale	20				
Histological type	69	0.622 (0.332-1.167)	NS		
Non-Papillary adenocarcinoma	50				
Papillary adenocarcinoma	19				
Staging	69	2.265 (0.899-5.708)	0.083	3.320 (1.270-8.681)	0.014
II & III	9				
IVA & IVB	60				
Blood vessel invasion	69	2.108 (1.139-3.902)	0.018	2.183 (1.088-4.382)	0.028
Non-invasion	23				
Invasion	46				
Lymphatic invasion	69	0.981 (0.479-2.012)	NS		
Non-invasion	12				
Invasion	57				
Nerve invasion	69	1.129 (0.654-1.950)	NS		
Non-invasion	32				
Invasion	37				
D21S1893, D21S1890, and TFF	69		0.026		0.002
Normal	30	Reference		Reference	
Amplification	35	1.707 (0.974-2.991)	NS	2.473 (1.342-4.557)	0.004
Deletion	4	0.187 (0.025-1.394)	NS	0.224 (0.029-1.701)	NS

alone are not sufficient to induce the invasive phenotype in premalignant human colonic adenoma cells PC/AA/C1 and kidney MDCK epithelial cells, but require the priming and permissive action of src and RhoA to exert their proinvasive activity[22]. These observations suggest that trefoil peptides elicit a coordinated cellular response enabling cell migration without triggering the programmed cell death response usually precipitated by cell detachment from a stationary anchored state. TFF1 protects cells from anoikis, chemical-, or Bad-induced apoptosis by reducing caspase-3, caspase-6, caspase-8, and caspase-9 activities[25]. TFF3 may act as anti-apoptosis by preventing p53-dependent and p53-independent apoptosis pathways[28]. The anti-apoptotic effects of TFF3 are associated with activation of the PI3K-Akt signaling pathway. Thus, TFF functions as an anti-apoptosis factor, resulting in an increase in number of survived cancer cells through proliferation *via* Ras/MEK/MAP kinase signaling transduction pathway. Angiogenic activity of TFF is comparable to that induced by vascular endothelial growth factor (VEGF), leptin, and transforming growth factor-alpha. Stimulation of angiogenesis by TFF1 in the chick chorioallantoic membrane (CAM) assay was COX-2- and EGFR-dependent, but independent of the VEGF receptor KDR/flk-1 and the thromboxane A2 receptor (TXA-2-R). These results implicate a role of TFF in the formation of new blood vessels during normal and pathophysiological processes linked to wound healing, inflammation, and cancer progression[23].

Transmembrane protease, serine 2 *(TMPRSS2)* and transmembrane protease, serine 3 (*TMPRSS3*) are members of serine protease family. Proteases have been increasingly recognized as important factors in the pathophysiology of tumorous diseases. Members of the endopeptidases, such as serine protease family, mediate the proteolytic degradation of the extracellular matrix, which is an indispensable step in tumor invasion and metastasis[29]. *TMPRSS2* was highly expressed in prostate cancer and correlated with the metastatic potential and involved in microvascular endothelial cell reorganization and capillary morphogenesis[30,31]. *TMPRSS3* is strongly expressed in a subset of pancreatic cancer and various other cancer tissues, and its expression correlates with the metastatic potential of the clonal SUIT-2 pancreatic cancer cell lines[32]. The data suggested that both *TMPRSS2* and *TMPRSS3* may be important for the processes involved in metastasis, invasion, and angiogenesis in tumor cells. Our study showed high amplifications of markers D21S1893 and D21S1890, suggesting that existence of candidate genes might be involved in pathogenesis of CCA. The data regarding the involvement of serine protease and *TFF* in aggressive feature and metastasis supported our finding that amplifications of candidate genes at regions D21S1893, D21S1890, and *TFF* were found in poor prognostic CCA patients. Clinical data of CCA patients supported our hypothesis that TFF stimulated cell motility via E-cadherin/catenin and APC complexes and promoted tumor cell survival by anti-apoptosis, while serine protease mediated vascular endothelial invasion and angiogenesis leading to poor survival in CCA patients. Although the differences in genetic alterations between liver fluke related and non-liver fluke related CCA have been observed previously[33,34], the conclusion regarding the difference between these two groups in allelic imbalance on chromosome 21q22 cannot be drawn.

As far as we know, allelic imbalance on the chromosomal region 21q22-qter including *TFF* in CCA patients is first reported by our group. The protein expression and functions of *TMPRSS2, TMPRSS3,* and *TFF* related to cancer invasion and metastasis in liver fluke related CCA patients require further study. The application of allelic imbalance on D21S1893, D21S1890, and *TFF* may be of value as a prognostic marker and a selection for CCA patient treatment.

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