석 사 학 위 논 문

Clinicopathological characteristics and prognosis of TZAP mutation in hepatocellular carcinomas

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이 논문을 석사학위 논문으로 제출함

2019년 2월

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２０１９년 ２월

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1. Introduction

Telomeres are composed of 6 bp TTAGGG repeat sequences and they are nucleoprotein complexes capping each end of eukaryotic chromosomes [1,2]. In somatic cells, telomere is 5 to 15 kilobases and it becomes shorter about 30 to 300 base pairs during each cell division. Its shortening is counteracted by the reverse transcriptase telomerase in cancer and stem cells [3-5]. A telomere trimming mechanism is induced in the presence of overly long telomeres, which are cut as little length by rapid telomere trimming [6-8]. Though the detail mechanism of this process has not been clarified, recent studies have discovered a novel protein that is necessary for this process [9]. They identified the zinc finger protein ZBTB48, as a telomere-associated factor, and renamed it telomeric zinc finger-associated protein (TZAP). The study showed that overexpression of TZAP caused progressive telomere shortening. TZAP localizes to chromosome 1p36, a region that is cytologically changed in many cancers [10-12]. Genetic change in TZAP suggested be correlated with cancers, but TZAP has not been studied in any specific type of cancer.

Hepatocellular carcinoma (HCC) comprises the majority of primary hepatic tumors as a leading cause of cancer-related deaths worldwide [13,14]. Many researches focused on the clinical and prognostic values of telomeres in HCC [15,16]. They suggested that the regulation of telomere has an association with survival results in patients with HCC.

Here, I analyzed TZAP mutation and expression in patients with HCC for the first time by determining their respective clinicopathological and prognostic characteristics. Furthermore, based on previous studies [17], I also confirmed whether genetic changes of TZAP induce the

deregulation of telomere length (TL).

2. Materials and Methods

2.1. Patients and tissue samples:

A total of 123 cases were identified from pathology reports of patients HCC surgery at the Kyungpook National University Hospital (KNUH) from January 2006 to October 2011. The clinical characteristics of each patient were analyzed by reviewing their medical records and slides. Patients who received preoperative therapy, such as transarterial chemoembolization, radiofrequency ablation and systemic chemotherapy with sorafenib (Nexavar, Pittsburgh, PA, USA) were excluded from this study. In addition, patients with other malignancies or incomplete survival data were also excluded. This study was approved by the institutional review board (KNUH-2014-04-056-001).

Tumor tissues and matched non-malignant liver samples were fixed with formalin and paraffin. And then, paraffin blocks harboring the representative tumor tissues were selected by specialized pathologists for HCC diagnosis. Representative tissues were marked on the slide block and cored manually with a 3.0 mm diameter cylindrical device.

Total DNA samples of the tumor tissue and adjacent non-tumoral tissue were extracted using an extraction kit according to the instructions of manufacturer (AbsoluteTM DNA extraction Kit, BioSewoom, Korea). Total RNA was also extracted from same samples by TRIzol® Reagent (Molecular Research Center, Cincinnati, OH, USA). The quantity and quality of extracted DNA and RNA were measured by using NanoDrop 1000 (Thermo Fisher Scientific, Pittsburgh, PA,

USA).

2.2. TZAP mutation analysis:

TZAP exons were amplified by isolated DNA performing the polymerase chain reaction (PCR). It was carried out by using AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The sequences of primer for all exons are presented in Table 1. Thermocycling was conducted under the following conditions: 40 cycles of 94 °C for 40 sec, 55-57 °C for 40 sec, and 72 °C for 50 sec. And then, the product was separated on a 2.0% agarose gel as electrophoresis, and then it was stained with ethidium bromide to confirm PCR result. Direct DNA sequencing of the TZAP was subsequently performed using an ABI 3730 DNA sequencer (Bionics, Seoul, South Korea).

2.3. Telomere length analysis:

Each telomere length (TL) was examined by quantitative real-time PCR. To analyze quantitative TL relative to nuclear DNA (S), primers were selected for telomere length using specific amplification (T) and ß-globin for nuclear DNA (S). The primer sequences are presented in Table 1. Real-time PCR was performed by LightCycler 480 II system (Roche Diagnostics, Mannheim, Germany). TL was relatively presented by calculating T/S values using the formula: T/S = 2 - ΔCt, where Δ Ct = average Ct telomere - average Ct ß-globin. Each measurement was repeated three times and five serially diluted control samples were included in each experiment.

2.3. TZAP expression analysis:

cDNA from each sample was synthesized from 1 μg of total RNA by using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the protocol of manufacturer. To clarify the quality of mRNA extraction and reverse transcription, the products were subjected to qPCR amplification with the β actin primers (Bionics Inc., Seoul, Korea) using a LightCycler® 480 II system (Roche Diagnostics, Basel, Switzerland) with SYBR® Green PCR Master Mix (Toyobo, Osaka, Japan). The TZAP qPCR assay reactions were performed using primers as described in Table 1. TZAP expression levels were calculated as above method. Each experiment was repeated three times.

2.4. The Cancer Genome Atlas (TCGA) data analysis:

To investigate the clinical significance of TZAP, we used the TCGA database from OncoLnc and cBioPortal. TZAP mRNA expression data were downloaded from TCGA data portal on November, 2018 [18]. Its clinicopathological and prognostic values were analyzed.

2.5. Statistical analyses:

Chi-square test, the Mann - Whitney U test, Fisher's exact test, and simple correlation analysis were performed to analyze the associations between the variables. Survival result by using the univariate Kaplan-Meier estimators, were compared using the log-rank test.

Overall survival (OS) was set as the period between first diagnosis and mortality. Disease-free survival (DFS) was defined as the period between first diagnosis, and disease recurrence or the progression of distant metastasis. The correlations between clinicopathologic parameters were assessed with Pearson's correlation coefficient analysis. A P value of < 0.05 denoted significance for all statistical analysis performed in this study.

Table 1. Primer Sequences Used in This Study

Name	Primer sequences
TZAP exon 1	F: CCAGACCTCAACAGCACAGA R: CACAGCCCACGAACCTAGTG
TZAP exon 2	F: ATCCCATTGGCCGTTCTCT R: CCGGCACAGTGAGAGGAT
TZAP exon 3	F: TAGAGGCCAACTTCCCGTTT R: CCTGGGCACAGTACCTCATT
TZAP exon 4	F: CCTGCTGATTCATTTGGTGA R: GGAATGGCAGACAGGAAAAG
TZAP exon 5-1	F: GGAGGTGAGGAAGTTGACCA R: CCCTTCTAAGGGGAACAAGTG
TZAP exon 5-2	F: GCTTGTCCCTGCACCTTAAC R: GGAGAGGGCAACACATAACC
TZAP exon 5-3	F: AGTCTGTCTGGGCCTGAGAA R: CCCTCCCTGTCACTTACTGC
TZAP exon 6	F: CCCTTCCCTGCTCTCACC R: AAGAGAGAACGGGCGACAC
TZAP exon 7	F: GTCACTTCCCTTGGTGATGG R: GAGGGGACCAGTGGTTTACA
TZAP exon 8	F: CTGGGTGGCACTGGAGAG R: CACGGGAACAGACTGTCAGG
TZAP	F: AAGGCCCTTAGAGGCTGAAG R: GACTCCCTCCTGGTCAGCAC
β-Actin	F: ACCCACACTGTGCCCATCTAC R: TCGGEGAGGATCTTCATGAGG
Telomere length	F: CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT R: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
β-globin	F: TGTGCTGGCCCATCACTTTG R: ACCAGCCA-CCACTTTCTGATAGG

3. Results

3.1. TZAP mutation and TZAP expression in HCC:

The sequences of TZAP regions were successfully analyzed in the 123 HCCs tissue samples. I found TZAP mutations in 10.6% (13/123) of these samples, all of which were c.1272G>A (L424L) (Figure 1). TZAP expression was analyzed in only 43 HCCs due to sample shortage, and as its expression in the HCC and paired non-cancerous tissues was similar (P = 0.53). The average TZAP expression in the HCC tissue was 1.64 ± 0.58, which contrasted with its expression in normal tissues. To further clarify the correlation between TZAP expression and the clinicopathological characteristics of HCC, patients were divided into two groups according to the median value of the Tumor/Non-tumor ratio (1.64). Clinicopathological significances associated with the TZAP mutation and expression are summarized in Table 2. There was no association between TZAP mutation and TZAP expression (P = 0.53). Other clinicopathological parameters showed no association with the TZAP mutation and expressions.

I also performed a quantitative correlation analysis with the clinical and genetic parameters including age, AST, ALT, tumor size, telomere length, and TZAP expression (Table 3). An association between age and tumor size was shown, however, it did not get a statistical significance. And then, no quantitative correlation was observed with the other parameters.

3.2. Prognostic value of TZAP mutation and TZAP expressions in HCC:

I assessed the survival results to confirm the prognostic value of the TZAP mutation and TZAP expression in patients with HCC. For survival analysis, the median follow-up term of HCC patients was 71.86 months (range: 3—105 months). The survival results of the univariate analysis was carried out by Kaplan - Meier curve, presented a poorer overall survival in HCC patients with TZAP expression (49.99 vs. 69.48 months, χ^2 = 2.83, P = 0.092), albeit this result was not statistical significant (Figure 2). TZAP expression was also associated with poorer disease-free survival (26.60 versus 45.75 months, χ^2 = 3.59, P = 0.058). However, TZAP mutations did not have any prognostic value for the patients with HCCs (overall survival: 65.92 vs. 73.42 months, χ^2 = 0.001, P = 0.995; Disease-free survival: 51.24 versus 47.86 months, χ^2 = 0.348, P = 0.555) (Figure 3). The Cancer Genome Atlas (TCGA) data analysis showed no prognostic value of TZAP expression in HCC (P = 0.576) (Figure 4).

Table 2. Clinicopathological Characteristics of TZAP Mutation and TZAP mRNA Expressions and Telomere Length in HCCs

	TZAP mutation			TZAP expression		
	(+)	(−)	*P*	(+)	(−)	*P*
Total	13 (10.7)	109 (89.3)		15 (34.9)	28 (65.1)	
Age			.213			.606
<60	10 (13.3)	65 (86.7)		9 (32.1)	19 (67.9)	
≥60	3 (6.3)	45 (93.8)		6 (40.0)	9 (60.0)	
Gender			.356			.863
Male	8 (9.0)	81 (91.0)		12 (34.3)	23 (65.7)	
Female	5 (14.7)	29 (85.3)		3 (37.5)	5 (62.5)	
AST			.629			.196
<40	9 (11.7)	68 (88.3)		9 (29.0)	22 (71.0)	
≥40	4 (8.9)	41 (91.1)		6(50.0)	6(50.0)	
ALT			.591			.711
<40	10 (11.6)	76 (88.4)		12 (36.4)	21(63.6)	
≥40	3(8.3)	33(91.7)		3(30.0)	7(70.0)	
Tumor size			.669			.377
<5 cm	7 (9.6)	66 (90.4)		7 (29.2)	17 (70.8)	
≥5 cm	6 (12.0)	44 (88.0)		8(42.1)	11 (57.9)	
T stage			.762			.112
T1	1(9.1)	10(90.0)		2(100)	0(0)	
T2	8 (9.4)	77 (90.6)		10 (29.4)	24(70.6)	
T3	4 (16.0)	21 (84.0)		3 (34.9)	4(57.1)	
T4	0(0)	2(100)				
Telomere length			.721			.811
Short	9 (11.4)	70 (88.6)		8 (33.3)	16 (66.7)	
Long	4 (9.3)	39 (90.7)		7 (36.8)	12 (63.2)	

Table 3. Correlation Between TZAP Expression and The Clinical Parameters in Patients with HCC

		Age	Size	TZAP expression	Telomere length
Age	R	1	.141	-.073	.114
	P		.071	.642	.148
Size	R	.141	1	-.078	-.019
	P	.071		.626	.813
TZAP expression	R	-.073	-.078	1	-.053
	P	.642	.626		.736
Telomere length	R	.114	-.019	-.0053	1
	P	.148	.813	.736	

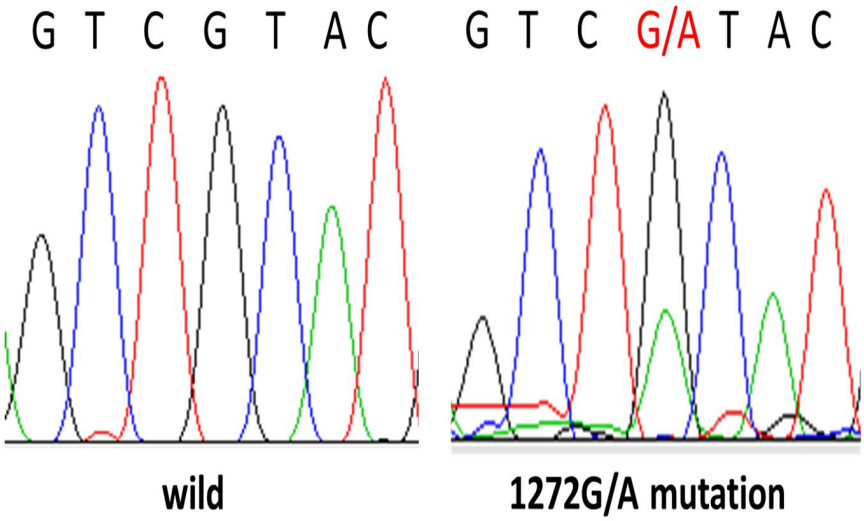

Figure 1. TZAP mutation (1272G/A) in HCCs.

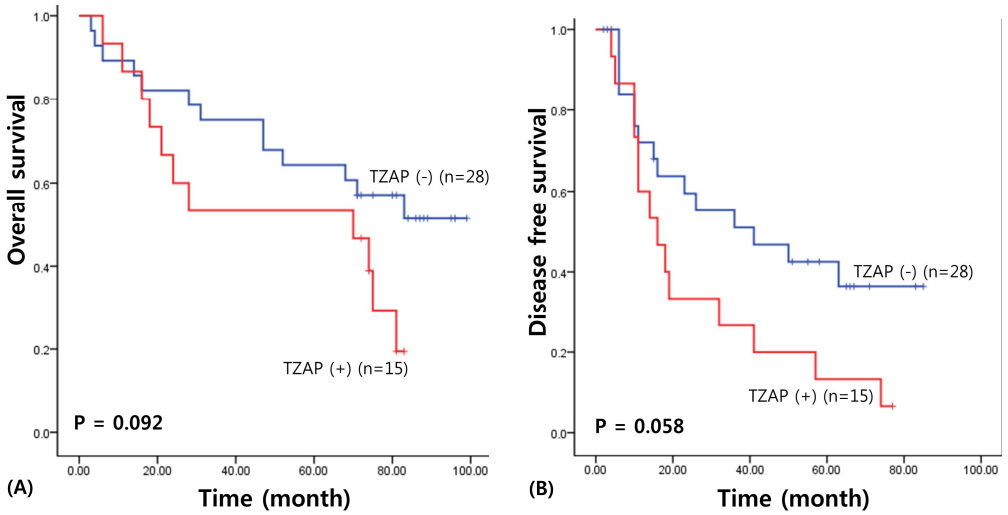

Figure 2. Survival analysis of TZAP expression in HCCs. (A) Overall survival (B) Disease free survival.

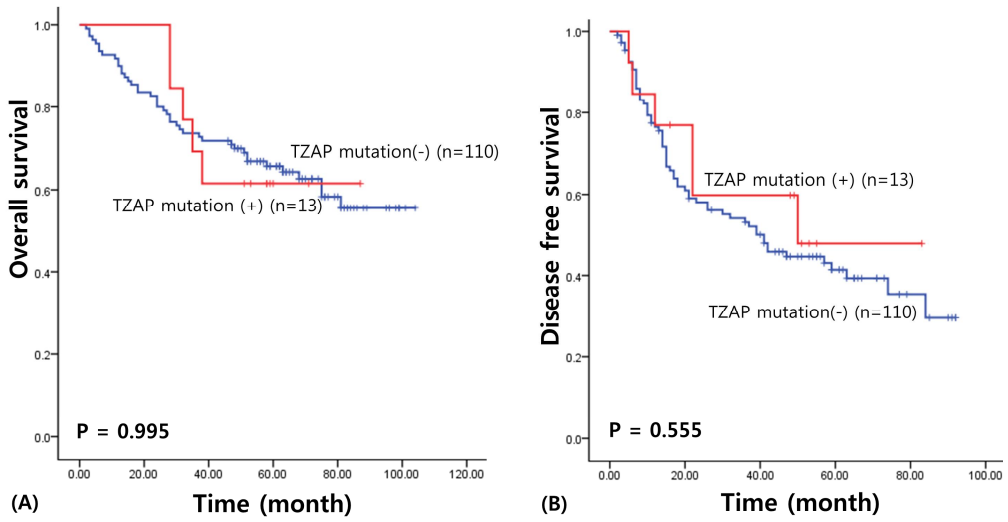

Figure 3. Survival analysis of TZAP mutation in HCCs. (A) Overall survival (B) Disease free survival.

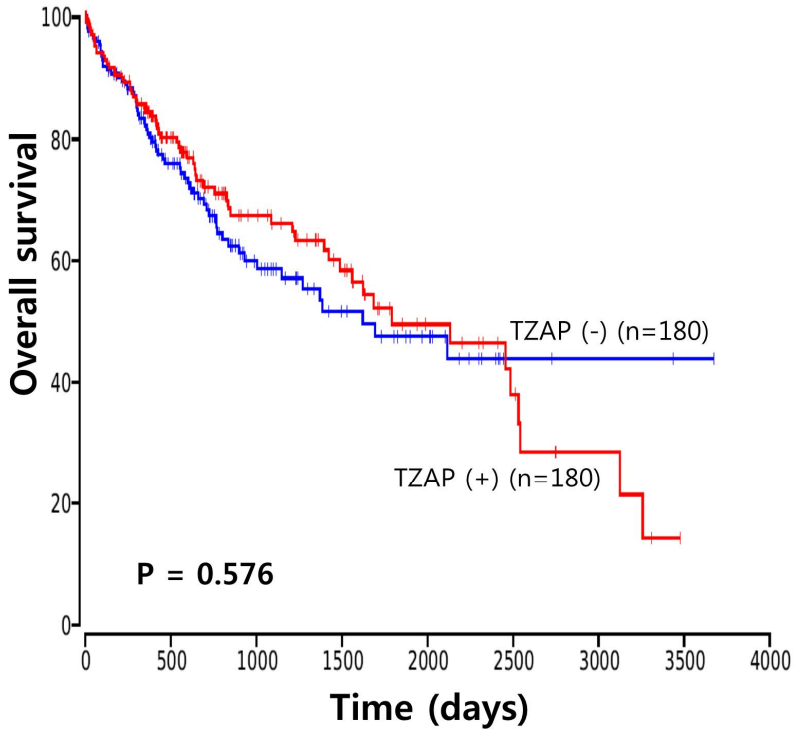

Figure 4. TCGA data of TZAP expression (ZBTB48) in HCCs.

4. Discussion

In present study, I demonstrated gene mutation and expression of TZAP in cancer specifically HCC for the first time. The study about TZAP mutations have not been performed and little mutations were only reported in the International Cancer Genome Consortium data [19]. I showed that the c.1272G>A mutation in TZAP was frequently found in patients with HCC, which has been reported previously by studies; however, this mutation was not found in 200 tissue samples of lung cancer and non-cancerous tissue, respectively. Therefore, TZAP mutation suggested to be HCC specific, though the hypothesis should be further confirmed.

TZAP expression was slightly increased in the HCC tissue samples. To understand TZAP biology and the associated molecular mechanisms of HCC, telomere length were also analyzed and then, their correlations investigated based on the suggestions of previous research [17]. A previous study demonstrated that TZAP acts as a transcriptional activator for the mitochondrial fission regulator, MTFP1, providing evidence that a strong connection exits between telomere and mitochondrial homeostasis [17]. However, the study showed that there is no difference between HeLa TZAP knockout and TZAP wild type cells in terms of mitochondrial DNA level. More studies about the molecular mechanisms of telomere and TZAP with other cellular processes are warranted.

This study has presented a poorer survival result in HCC patients with TZAP expression. However, TZAP mutation did not have any

clinical and prognostic values. The TCGA data also showed how overexpression of TZAP gene had a prognostic significance in some cancers, as colorectal, cervical and pancreatic cancers [18]. Interestingly, TZAP expression has an association with poorer prognosis in colorectal cancers, but a better prognosis in pancreatic and cervical cancers. TCGA data demonstrated no prognostic value of TZAP expression in HCC [18-22]. I suggested that TZAP may have a different role and produce a different effect on telomere regulation and cancer cell survival according to cancer type.

In conclusion, I studied the clinicopathological and prognostic characteristics of a TZAP mutation and TZAP expression in patients with HCC. The result suggested that TZAP mutation and expression may have an important role in hepatocellular carcinogenesis. The results of my study warrant further study with larger samples to clarify the underling mechanisms and signal pathway of TZAP gene and to suggest its potential utility for clinicians.

5. Summary

A telomere is a repetitive sequences located at all chromosome end. Average of telomere length in normal humans is from 10 kilobases at birth to less than 5 kilobases in older. Many studies suggest that telomere is an possible target for cancer treatment.

Zinc finger and BTB domain containing 48 (ZBTB48), renamed as telomeric zinc-finger associated protein (TZAP) binds directly to the double-stranded repeat sequence of telomeres. It has a competition with the telomeric repeat binding factors, such as TRF1 and TRF2. TZAP expression induced telomere trimming via telomere shortening for normal telomere length. Therefore, I examined genetic study about TZAP mutation and expression in hepatocellular carcinoma (HCC). The TZAP mutation (c.1272G>A) was frequently shown in HCCs. TZAP expression may be associated with a shorter overall survival, but, telomere length did not show any predictable potential for HCCs. This result suggests that mutation and expression of TZAP have a important role in hepatocellular pathogenesis. It warrant future study to clarify signal pathway and functional mechanisms of TZAP and to confirm its potential role as a therapeutic target for HCC.

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Clinicopathological characteristics of TZAP mutation and TZAP expression in hepatocellular carcinomas

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(Abstract)

The zinc finger protein ZBTB48 is a telomere-associated factor and renamed it as telomeric zinc finger-associated protein (TZAP). It binds preferentially to long telomeres competing with telomeric repeat factor 1 and 2 (TRF1 and TRF2). However, its expression in cancers has not been performed. In the present study, I analyzed TZAP mutation and expression in 123 hepatocellular carcinomas (HCC) and their association with telomere length was also investigated. TZAP mutations (c.1272G>A, L424L) was found in 10.6% (13/123) and TZAP expression level was not different between HCC and paired non-cancerous tissues. There was no association between TZAP mutation and TZAP expression (P = 0.53). TZAP mutation did not have any clinical and prognostic values in HCC. And, TZAP expression tended to induce

poorer survival result (overall survival, χ^2 = 2.83, P = 0.092; disease-free survival, χ^2 = 3.59, P = 0.058). However, The Cancer Genome Atlas (TCGA) survival analysis showed no prognostic value of TZAP expression in HCC (P = 0.576). This result suggested that TZAP expression appears to be a possible prognosis marker in HCC.

간암에서 TZAP 돌연변이의 임상병리학적, 예후적인 특징

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(초록)

아연 집게 단백질 ZBTB48은 텔로미어 아연 집게 단백질(TZAP)로 불리는 텔로미어 관련 인자이다. 이는 정상보다 길이가 긴 텔로미어에 붙는 편이며, 텔로미어 반복인자 1 또는 2와 경쟁한다. 하지만 아직까지 암 발생과정에서 이 단백질의 역할에 대해서 밝혀진 바가 없다. 본 연구에서 TZAP의 돌연변이를 123개의 간암환자 표본에서 확인했다. 더불어 이 단백질이 텔로미어의 길이에 연관이 있는 지에 대해 연구하였다. 간암에서 TZAP의 돌연변이(c.1272G>A, L424L)는 약 10.6%에서 발견되었으며, 간암 조직과 정상 조직사이에 TZAP의 발현 정도의 차이는 발견되지 않았다. TZAP의 돌연변이와 TZAP의 발현은 서로 관련이 없는 것으로 관찰되었다(P = 0.53). 간암에서 TZAP의 돌연변이는 임상적, 예후적인 가치를 보이지 않았다. 그리고 생존율 분석에 따르면 TZAP의 발현이 있는 군에서 전반적으로 예후가 좋지 않은 것으로 나타났다(전체생존, χ^2 = 2.83, P = 0.092; 무질병

생존, χ^2 = 3.59, P = 0.058). 그러나 암유전체지도 자료에 따르면 간암에서의 TZAP의 발현은 예후적인 가치를 가지지 않았다(P = 0.576). 이 연구의 결과는 TZAP의 발현이 간암에서 예후를 예측할 수 있는 표지자가 될 가능성을 보여준다.