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Real-time Reverse Transcription PCR Analysis for Validation of Transketolase Gene in Hepatocellular Carcinoma Tissues

Jongmin Kim[1], Seok Joo Hong[1], Jun Ho Park[1], Chang-Bae Lee[1], HongDu Gu[2], Seung Whan Kim[3], Gyu-Seong Choi[4], Choon Hyuck David Kwon[4], Jae-Won Joh[4] & Dae Shick Kim[5]

[1]CbsBioscience, Inc., 59-5 Jang-Dong, Yuseong-gu, Daejeon 305-343, Korea
[2]Department of Emergency Medicine, National Health Insurance Corporation Ilsan Hospital, Goyang-city, Gyeonggi-do 410-719, Korea
[3]Department of Emergency Medicine, Chungnam National University Hospital, Daejeon 301-721, Korea
[4]Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea
[5]Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea
Correspondence and requests for materials should be addressed to D.S. Kim (oncorkim@skku.edu)

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Abstract

Hepatocellular carcinoma (HCC) is the most common malignant tumor in the adult liver, with high relapse and mortality rates despite diverse treatment modalities. In this study, expression of transketolase (TKT) and transketolase-like 1 (TKTL1) gene, coding for the rate-limiting enzyme in non-oxidative pentose phosphate pathway (PPP), was investigated as a potential prognostic factor of HCC. The expression level of TKT and TKTL1 gene was measured by real-time reverse-transcription PCR (RT-PCR) in 185 primary HCCs and 49 non-cancerous surrounding livers. TKT mRNA level was markedly elevated in HCCs compared to non-cancerous surrounding tissues ($P < 0.0001$). On the other hand, TKTL1 mRNA level was higher in HCCs compared to non-cancerous surrounding tissues but the difference was not statistically significant. TKT expression in tumors was significantly correlated with several clinicopathologic parameters including tumor size and Edmondson grade. Moreover, patients who expressed higher TKT mRNA levels had a significantly shorter overall survival (OS) time ($P=0.00099$) and a significantly shorter disease-free survival (DFS) time ($P=0.0055$). In a multivariate analysis, high TKT expression was found to be an independent prognostic factor for OS both as a discrete variable ($P=0.009$) and as a continuous variable ($P=0.0068$). The results of this study indicated that TKT gene expression is a significant prognostic factor for OS in HCC cases. Therefore, TKT merits further investigation regarding its role as a prognostic factor with a larger cohort of HCC patients.

Keywords: Hepatocellular carcinoma, TKT, TKTL1, Real-time RT-PCR, Prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the most common primary hepatic malignancy, being responsible for 80% of malignant tumors in adult livers. HCC causes more than 600,000 deaths annually worldwide[1], and its endemic prevalence in Asia, including South Korea, makes HCC one of the top causes of death in this region. HCC is resistant to conventional chemotherapy and is rarely amenable to radiotherapy[2], leaving this disease with no effective therapeutic options and a very poor prognosis. Therefore, new developments to identify important prognostic factors and novel molecular targets of HCC are greatly needed.

One of the characteristics of solid, malignant tumors is the strongly enhanced conversion of glucose to lactate even in the presence of adequate oxygen via a process known as aerobic glycolysis or the Warburg effect[3]. Although the role of aerobic glycolysis in tumor development remains controversial[4,5], the widespread clinical use of positron-emission tomography (PET) for the detection of aerobic glycolysis in tumors and recent related findings implicate these physiological changes as important features of tumors. Tumor growth correlates with glucose metabolism and more than 85% of ribose recovered from nucleic acids of certain tumor cells is generated directly or indirectly from the non-oxidative part of the pentose phosphate pathway (PPP)[6] for which transketolase (TKT) is a rate-limiting enzyme. Transketolase gene family coding for transketolase enzyme includes TKT, transketolase-like 1 (TKTL1), and transketolase-like 2 (TKTL2). Recent findings indicate an important role played by TKTL1 in the transketolase metabolism of

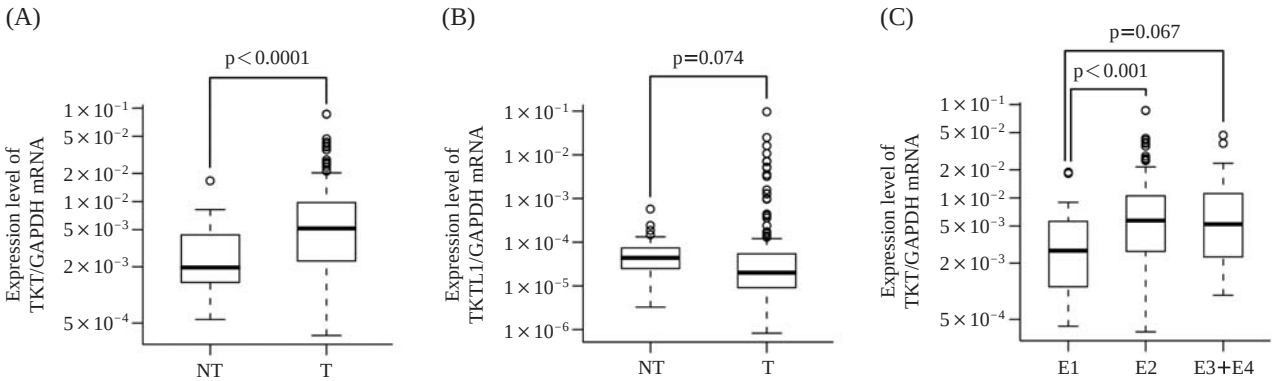

Figure 1. Expression of TKT and TKTL1 gene in HCC. (A) box and whiskers plot for TKT mRNA levels in non-cancerous liver (NT) and HCC (T) determined by real-time RT-PCR. The box is marked by the first and third quartile with the median marked by a thick line. The whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box. (B) box and whiskers plot for TKTL1 mRNA levels in non-cancerous liver (NT) and HCC (T). (C) relationship of TKT mRNA levels and Edmondson grades. E1, Edmondson grade I; E2, Edmondson grade II; E3+E4, Edmondson grade III or IV.

some tumors[7-11]. Thus far, the relative contributions of the three human transketolase genes to the total transketolase enzyme activity in tumors have not been fully determined. In fact, recent studies indicate differential expression of TKT transcript in tumor specimens[12-14], raising the possibility of its close link to tumor biology.

The aim of the present investigation was to examine whether TKT and TKTL1 expression can be used to predict the clinical course of HCC. Using a real-time RT-PCR analysis of TKT and TKTL1 gene expression, a significant degree of correlation was found between the TKT mRNA levels and clinicopathologic features, including poor prognosis of HCC. Thus, potential biological changes related to TKT gene expression require further study, as they may have implications in predicting clinical outcome and choosing treatment modalities due to the central role of TKT in the non-oxidative part of PPP.

Results

Expression of TKT and TKTL1 Gene in Hepatocellular Carcinoma

We performed real-time RT-PCR for TKT and TKTL1 mRNA from frozen paired samples derived from 185 patients with HCC. A total of 185 HCCs (T) and 49 non-cancerous hepatic samples (NT) were assessed by real-time RT-PCR. Expression of TKT and TKTL1 mRNA was measured in triplicate and was then normalized relative to the expression of GAPDH mRNA as an internal control. TKT mRNA was significantly higher in T than in NT tissues (0.0089 vs. 0.0032; mean copy number ratio, $P < 0.0001$) (Figure 1A). On the other hand, TKTL1 mRNA was not significantly higher in T than in NT tissues (0.0011 vs. 0.000066; mean copy number ratio, $P=0.074$) although the fold difference in mean expression levels was much greater than that of TKT (Figure 1B). The average TKT mRNA level was approximately 50-fold higher than the average TKTL1 mRNA level in non-cancerous tissues similar to the previous report in various non-cancerous tissues[15]. TKT mRNA levels in moderately-differentiated tumors (Edmondson grade II) were significantly higher than in well-differentiated tumors (Edmondson grade I) (0.0095 vs. 0.0044; mean copy number ratio, $P < 0.001$) (Figure 1C). Similarly, TKT mRNA levels were higher in poorly-differentiated or undifferentiated tumors (Edmondson grade III or IV) than in well-differentiated tumors although the difference was not statistically significant due to the small number of samples (0.0096 vs. 0.0044; mean copy number ratio, $P=0.067$) (Figure 1C).

Relationship between Tumor TKT and TKTL1 mRNA Level and Clinicopathologic Features

For a better understanding of the significance of TKT expression in HCC, the mRNA expression level was correlated with the major clinicopathologic features. The statistically most significant cutoff value of TKT mRNA level discriminating between patients with a good prognosis and patients with a poor prognosis was used. As shown in Table 1, increased TKT mRNA expression was correlated with old age (P=

Table 1. Relations between TKT and TKTL1 mRNA levels and clinicopathologic features in HCC.

Clinicopathologic parameters	All patients (n=185)			All patients (n=185)		
	Low TKT (n=118)	High TKT (n=67)	P value	Low TKTL1 (n=90)	High TKTL1 (n=95)	P value
Age			0.039			0.529
< 55 years	81	35		59	57	
≥ 55 years	37	32		31	38	
Gender			0.037			0.743
Male	85	58		71	72	
Female	33	9		19	23	
HBV			0.0046			0.775
Absent	22	26		22	26	
Present	96	41		68	69	
HCV			0.023			0.635
Absent	114	58		85	87	
Present	4	9		5	8	
Liver cirrhosis			0.782			0.833
Absent	62	33		45	50	
Present	56	34		45	45	
Tumor stage			0.403			0.981
I & II	94	49		70	73	
III & IV	24	18		20	22	
AFP level			0.868			0.946
< 100 ng/mL	61	33		45	49	
≥ 100 ng/mL	57	34		45	46	
Vascular invasion			0.098			0.652
Absent	53	21		34	40	
Present	65	46		56	55	
Tumor number			0.543			0.875
Single	94	50		70	74	
Multiple	24	17		20	21	
Tumor size			0.036			0.293
< 5 cm	78	33		50	61	
≥ 5 cm	40	34		40	34	
Edmondson grade			0.937			0.890
I & II	103	59		79	83	
III & IV	15	8		11	12	

Cutoff values for TKT and TKTL1 mRNA copy number ratio were 0.0068 and 0.00002, respectively.

0.039), male patients (P=0.037), HBV infection (P=0.0046), HCV infection (P=0.023), and a large tumor size (P=0.036) in 185 HCCs. As shown in Figure 1C, TKT mRNA expression was significantly associated with Edmondson grade when the stratification was grade I, grade II, and grade III-IV (P=0.030). However, no correlation was observed between TKT mRNA level and other clinicopathologic parameters (liver cirrhosis, tumor stage, vascular invasion, tumor number, and AFP level). Because statistically significant cutoff value of TKTL1 mRNA level discriminating between patients with a good prognosis and patients with a poor prognosis was not found using the Kaplan-Meier method, we used a cutoff value close to the median TKTL1 expression level. However, no correlation was observed between TKTL1 mRNA level and clinicopathologic parameters (Table 1).

Impact of Tumor TKT and TKTL1 mRNA Levels on OS and DFS

During the follow-up observation period of up to 93.8 months, locoregional recurrence or distant metastases occurred in 123 patients (66.4%) and death was confirmed in 59 patients (31.9%). To assess the prognostic significance of TKT expression, overall survival (OS) and disease-free survival (DFS) rates were analyzed using the Kaplan-Meier method. At the five-year follow-up, approximately 77% of the patients with low TKT expression (< 0.0068; copy number ratio) survived, whereas 56% of the patients with high TKT expression (≥ 0.0068; copy number ratio) survived (Figure 2A). Similarly, at the five-year follow-up,

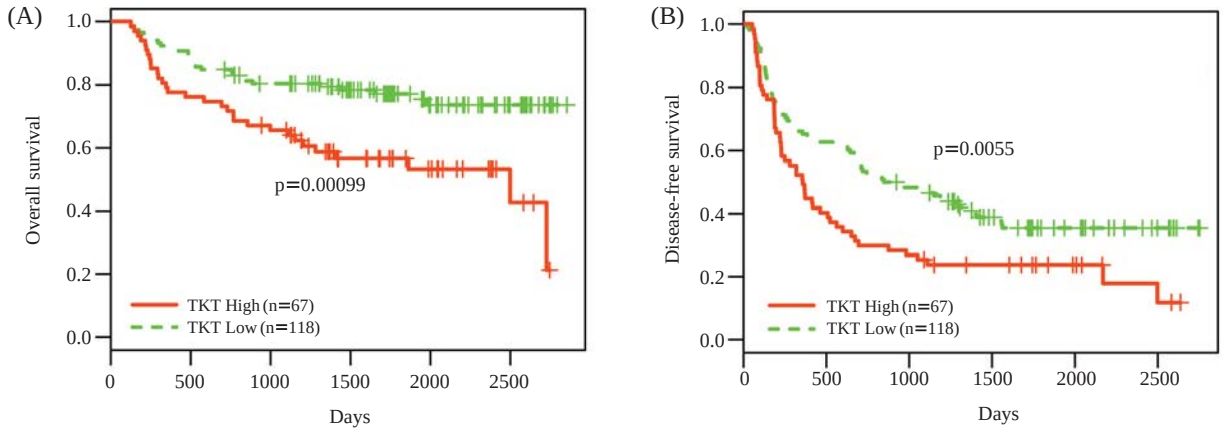

Figure 2. Kaplan-Meier curves for OS and DFS of patients with high and low TKT mRNA levels after surgery. (A) patients with high TKT mRNA levels (≥0.0068; copy number ratio) had a significantly shorter OS time (P=0.00099). Broken lines, patients with low TKT mRNA levels (n=118); thin lines, patients with high TKT mRNA levels (n=67). (B) patients with high TKT mRNA levels had a significantly shorter DFS time (P=0.0055). Broken lines, patients with low TKT mRNA levels (n=118); thin lines, patients with high TKT mRNA levels (n=67).

Table 2. Univariate Cox regression analysis for overall survival and disease-free survival.

Variable	Overall survival		Disease-free survival	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (<55 years vs. ≥55 years)	0.91 (0.54-1.56)	0.74	0.99 (0.69-1.42)	0.96
Gender (male vs. female)	0.55 (0.27-1.11)	0.095	0.85 (0.55-1.29)	0.44
Edmondson grade (I-II vs. III-IV)	1.39 (0.69-2.84)	0.36	1.07 (0.63-1.81)	0.80
HBV (absent vs. present)	1.06 (0.59-1.91)	0.84	1.05 (0.71-1.57)	0.80
HCV (absent vs. present)	1.40 (0.56-3.52)	0.48	1.88 (1.03-3.41)	0.039
AFP level (<100 ng/mL vs. ≥100 ng/mL)	1.92 (1.13-3.27)	0.015	1.55 (1.09-2.20)	0.014
Liver cirrhosis (absent vs. present)	1.18 (0.71-1.97)	0.52	1.07 (0.75-1.51)	0.71
Vascular invasion (absent vs. present)	7.42 (3.19-17.28)	3.4×10^{-6}	2.08 (1.43-3.02)	0.00013
Tumor number (single vs. multiple)	4.87 (2.91-8.16)	1.9×10^{-9}	3.12 (2.12-4.59)	6.7×10^{-9}
Tumor size (<5 cm vs. ≥5 cm)	2.95 (1.75-4.99)	5.2×10^{-5}	1.66 (1.17-2.37)	0.0046
Tumor stage (I-II vs. III-IV)	6.43 (3.83-10.80)	2.0×10^{-12}	4.00 (2.70-5.92)	4.1×10^{-12}
TKT (low vs. high)	2.31 (1.38-3.85)	0.0014	1.65 (1.15-2.35)	0.0059
TKT† ($-\Delta C_T$)	1.28 (1.08-1.52)	0.0046	1.12 (0.99-1.26)	0.063

HR, hazard ratio; CI, confidence interval
† The log ratio of TKT expression ($-\Delta C_T$) was used as a continuous variable.

Table 3. Multivariate Cox regression analysis for overall survival and disease-free survival.

Variable	Overall survival		Disease-free survival	
	HR (95% CI)	P value	HR (95% CI)	P value
TKT (low vs. high)	2.00 (1.19-3.36)	0.009	1.31 (0.90-1.89)	0.16
HCV (absent vs. present)			1.91 (1.02-3.57)	0.041
AFP level (<100 ng/mL vs. ≥ 100 ng/mL)	0.94 (0.53-1.66)	0.84	1.21 (0.83-1.76)	0.33
Vascular invasion (absent vs. present)	4.10 (1.64-10.22)	0.0025	1.28 (0.82-1.99)	0.28
Tumor number (single vs. multiple)	1.47 (0.70-3.08)	0.31	1.66 (1.02-2.73)	0.043
Tumor size (<5 cm vs. ≥ 5 cm)	1.14 (0.61-2.14)	0.69	1.02 (0.68-1.54)	0.92
Tumor stage (I-II vs. III-IV)	2.96 (1.38-6.37)	0.0054	2.55 (1.51-4.32)	4.9×10^{-4}

HR, hazard ratio; CI, confidence interval

Table 4. Multivariate Cox regression analysis for overall survival with log ratio of TKT expression.

Variable	Overall survival	
	HR (95% CI)	P value
TKT† (low vs. high)	1.26 (1.07-1.49)	0.0068
AFP level (<100 ng/mL vs. ≥ 100 ng/mL)	0.91 (0.51-1.60)	0.74
Vascular invasion (absent vs. present)	4.22 (1.69-10.52)	0.002
Tumor number (single vs. multiple)	1.50 (0.72-3.11)	0.28
Tumor size (<5 cm vs. ≥ 5 cm)	1.27 (0.68-2.38)	0.46
Tumor stage (I-II vs. III-IV)	2.86 (1.33-6.15)	0.0072

HR, hazard ratio; CI, confidence interval
† The log ratio of TKT expression ($-\Delta C_T$) was used as a continuous variable.

approximately 36% of the patients with low TKT expression were disease-free, whereas 23% with high TKT expression were disease-free (Figure 2B). The log-rank test showed that patients who expressed higher TKT mRNA levels had a significantly shorter OS time (P=0.00099) and a significantly shorter DFS time (P=0.0055). Statistically significant cutoff value of TKTL1 mRNA level discriminating between patients with a good prognosis and patients with a poor prognosis was not found using the Kaplan-Meier method and log-rank test (data not shown). A univariate Cox regression analysis was used to identify important prognostic factors of OS and DFS. A high TKT mRNA level (P=0.0014), high AFP level (P=0.015), large tumor size ($P=5.2 \times 10^{-5}$), vascular invasion ($P=3.4 \times 10^{-6}$), tumor multiplicity ($P=1.9 \times 10^{-9}$), and high tumor stage ($P=2.0 \times 10^{-12}$) were identified as important risk factors for OS, whereas a high TKT mRNA level (P=0.0059), HCV infection (P=0.039), high AFP level (P=0.014), large tumor size (P=0.0046), vascular invasion (P=0.00013), tumor multiplicity ($P=6.7 \times 10^{-9}$), and high tumor stage ($P=4.1 \times 10^{-12}$) were identified as important risk factors for DFS (Table 2). Interestingly, the log-ratio of relative TKT expression ($-\Delta C_T$) treated as a continuous variable was also identified as an important prognostic factor for OS (P=0.0046). In a multivariate Cox analysis, high TKT expression (P=0.009), vascular invasion (P=0.0025), and high tumor stage (P=0.0054) were found to be independent poor prognostic factors for OS, whereas HCV infection (P=0.041), tumor multiplicity (P=0.043), and high tumor stage ($P=4.9 \times 10^{-4}$) were found to be independent poor prognostic factors for DFS (Table 3). In a multivariate Cox analysis with the log-ratio of relative TKT expression as a continuous variable, high TKT expression (P=0.0068), vascular invasion (P=0.002), and high tumor stage (P=0.0072) were found to be independent poor prognostic factors for OS (Table 4).

Discussion

Cancer cells display high rates of aerobic glycoly-

sis, a phenomenon known as the Warburg effect[3]. Although the role of aerobic glycolysis in tumor development remains controversial[4,5], recent findings implicate these physiological changes as important features of tumors. Tumor progression correlates with increased glucose uptake, and the fully transformed tumor state is most dependent on aerobic glycolysis rather than on the mitochondrial machinery for ATP synthesis[16]. This anaerobic glucose consumption in cancer is related to the non-oxidative part of PPP, which supplies more than 85% of the ribose recovered from nucleic acids of certain tumor cells[6]. Thus, transketolase as a rate-limiting enzyme of non-oxidative PPP is an important molecular target in tumor biology. Despite recent findings which implicate TKTL1 as a major factor in the transketolase metabolism of some tumors[7-11], the relative contributions of three human transketolase genes, TKT, TKTL1, and TKTL2, have not been fully investigated. Recent studies indicate differential expression of the TKT transcript in tumor specimens[12,13] including HCC[14], although the role of TKT in the molecular pathogenesis of HCC has yet to be elucidated.

This study focused on TKT and TKTL1 as potential molecular markers responsible for determining clinicopathologic features and the prognosis of HCC. Utilizing a large number of HCC specimens, a quantitative real-time PCR assay showed that the expression of the TKT transcript was markedly increased in HCCs compared to non-cancerous surrounding tissues. On the other hand, TKTL1 mRNA was not significantly increased in HCCs compared to non-cancerous surrounding tissues. Stratification of HCC specimens based on TKT gene expression levels showed that TKT expression was significantly correlated with age, gender, viral infection, tumor size, and Edmondson grade. Increased TKT expression has been reported in HCC patients with HCV[14], while the mechanism for correlation of HBV related HCC and increased TKT expression is unclear. More importantly, the log-rank test showed that patients who expressed higher TKT mRNA levels had a significantly shorter OS time ($P=0.00099$) and a significantly shorter DFS time ($P=0.0055$). In a multivariate Cox analysis, TKT expression was found to be an independent prognostic factor for OS both as a discrete variable ($P=0.009$) and as a continuous variable ($P=0.0068$). TKT overexpression was not a significant prognostic factor for DFS in a multivariate analysis. While the regulatory mechanisms within HCC that are correlated with high TKT mRNA expression are unknown, we speculate that TKT overexpression influences regulatory processes that control tumor cell proliferation more than tumor invasiveness. The present analysis of TKT expression in correlation with the clinicopathologic features and prognosis of HCC yielded the novel finding that TKT mRNA levels could be used as a prognostic factor for OS.

The inhibition of TKTL1 expression by RNAi in HepG2 cells significantly downregulated the total transketolase activity and inhibited the proliferation of cancer cells. In addition, TKTL1 mRNA was specifically overexpressed in HepG2 cells, whereas TKT and TKTL2 mRNA expression were not upregulated, suggesting that the TKTL1 gene influences cell proliferation by regulating total transketolase activity[17]. In the current study, TKTL1 mRNA level was increased in only a small fraction of HCCs compared to non-cancerous surrounding tissues. Because an identical set of primers for TKTL1 as described in Coy *et al.*[15] and Zhang *et al.*[17] was used with PCR efficiency of greater than 80% observed, differences in experimental procedures would not be an important factor for this apparent discrepancy.

It is unclear why TKTL1 mRNA expression was not significantly correlated with prognosis unlike TKT mRNA expression. It is possible that the level of TKTL1 protein is weakly correlated with TKTL1 mRNA levels in HCC, although an important connection was observed in HepG2 cells using siRNA technique[17]. For instance, while only 10.8% of the gastric cancer tissues revealed a significant TKTL1 mRNA upregulation, 36.9% of the cancer tissues demonstrated a TKTL1 protein overexpression[18]. Similarly, a slight up-regulation of TKTL1 mRNA expression was observed in brain tumor tissues compared to normal brain, while strong overexpression of TKTL1 protein was observed in tumors[10]. The importance of TKT isoforms and their relative contributions at protein levels in HCC is unknown. Unfortunately, antibodies for immunohistochemical analysis against TKT and TKTL2 are not yet available[10]. Further investigations including immunohistochemical staining of TKTL1 in HCC tissues would resolve some of these issues. On the other hand, overexpression of the TKT transcript in tumor specimens[12,13] including HCC[14] have been reported. Recently, increased expression of TKT transcript was associated with unfavorable outcome in neuroblastoma[19]. In the present study, TKT gene was upregulated in HCC tissues and correlated with high recurrence and mortality. This indicates that TKT gene expression may comprise a significant portion of total transketolase activity in HCC tissues and influence tumor cell proliferation and invasiveness.

Current evidence indicates that cancer is a complicated genetic and epigenetic phenomena characterized by abnormalities of cellular growth-regulating genes and their signaling pathways[20]. Studies of the

physiological changes in malignant conversion provided a metabolic signature for tumor progression. Aberrant glucose metabolism is commonly observed in tumor cells, aerobic glycolysis and augmented PPP. Pentoses are required for RNA and DNA synthesis within rapidly growing tumor cells, and access to NADPH generation may be essential for the survival of tumor cells that are challenged by reactive oxygen species. Concomitant with this metabolic switch, high lactate concentrations occur and result in immune protection of cancer cells, acid-mediated matrix degradation, invasiveness and metastasis[21-23]. Furthermore, it was demonstrated that the glycolytic end products lactate and pyruvate also stimulate HIF-1α accumulation[24].

The enhanced metabolism of glucose as a consequence of aerobic glycolysis was successfully applied in clinical setting by the PET technique to visualize tumors and metastases[25,26]. Transketolase enzyme reactions determined cell proliferation in Ehrlich's ascites tumor model[27]. Moreover, a dramatic decrease in tumor cell proliferation and a dose-dependent increase in G_1 phase arrest were observed after the administration of the transketolase inhibitor oxythiamine[28]. Therefore, thiamine supplementation used as a nutritional support for advanced cancer patients can potentially increase transketolase activity because thiamine is metabolized to thiamine pyrophosphate, a cofactor of transketolase[27]. It is important to determine whether the benefits of thiamine supplementation outweigh the risks of tumor proliferation. Fortunately, the development of novel transketolase inhibitors[29] can increase therapeutic options with regard to the aberrant metabolic pathways of tumor cells which could be linked to chemo- and radiation-resistance[30,31].

Conclusions

The results of this study show that TKT gene expression is an independent prognostic factor for OS of patients with HCC. It is important to develop new target molecules and to establish novel therapeutic strategies in malignancies such as HCC, which shows frequent relapse and high mortality despite various treatment modalities. The widespread clinical use of PET for the detection of aerobic glycolysis in tumors in addition to the finding that the inhibition of transketolase enzyme reactions suppresses tumor growth and metastasis suggests that TKT plays an important role in tumor progression and metastasis in general. Therefore, TKT merits further study as a useful marker for prediction of prognosis with a larger cohort of HCC patients. Moreover, TKT as well as other family members, TKTL1 and TKTL2, could be targets for chemotherapeutic agents. Establishing the molecular interactions of TKT with diverse molecular pathogenic factors in HCC will enable new studies as well as the development of effective therapeutic regimens.

Materials and Methods

Patients and Tissue Samples

HCCs and corresponding non-cancerous hepatic tissues were obtained with informed consent from 185 patients who underwent curative hepatectomy for primary HCC between 2001 and 2006 in the Department of Surgery, Samsung Medical Center in Korea. The study protocol was approved by the Institutional Review Board of Samsung Medical Center. Complete clinical data were available in all 185 cases as described in Table 1. The mean age of patients was 51.7 years (ranging from 21 to 80 years). All patients had adequate liver function reserve, and had survived for at least 3 months after hepatectomy (median follow-up, 50 months; range, 3.4-93.8 months). Recurrence or death was evaluated from information obtained from medical records of patients. We defined the recurrence as evidence of an overt new growing mass in the remaining liver or as distant metastasis in Computed Tomography. None of the patients had received treatment prior to surgery such as transarterial chemoembolization or radiofrequency ablation. Immediately after hepatectomy, fresh tumors and background livers were partly snap-frozen in liquid nitrogen and stored at −80°C and were partly embedded in paraffin after fixation in 10% formalin for histological diagnosis. All available hematoxylin and eosin stained slides were reviewed. The tumor grading was based on the criteria proposed by Edmondson and Steiner[32]. The conventional TNM system outlined in the cancer staging manual (6th ed.) by the American Joint Committee on Cancer (AJCC) was used in tumor staging.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from cancerous and surrounding non-cancerous frozen tissues using an RNeasy minikit (Qiagen, Germany) according to the manufacturer's instructions. The integrity of all tested total RNA samples was verified using a Bioanalyzer 2100 (Agilent Technologies, United States). DNase I treatment was routinely included in the extraction step. Residual genomic DNA contamination was assayed by a quantitative real-time PCR assay for GAPDH DNA, and samples with residual genomic DNA were re-subjected to DNase I treatment. Samples contain-

Table 5. Oligonucleotide sequences of PCR primers and probes.

Gene		Sequences
GAPDH	Forward :	5′-CAC ATG GCC TCC AAG GAG TAA-3′
	Reverse :	5′-TGA GGG TCT CTC TCT TCC TCT TGT-3′
	Probe :	5′-CTG GAC CAC CAG CCC CAG CAA G-3′
TKT	Forward :	5′-GAG GCT GTG TCC AGT GCA GTA G-3′
	Reverse :	5′-CCA CTT CTT GGT ACC CGG TTA A-3′
	Probe :	5′-CCT GGC ATC ACT GTC ACC CAC CTG-3′
TKTL1	Forward :	5′-TAA CAC CAT GAC GCC TAC TGC-3′
	Reverse :	5′-CAT CCT AAC AAG CTT TCG CTG-3′
	Probe :	5′-TGC AGC TGC CCT GGA ATT CCC TTC-3′

ing 4 μg of total RNA were incubated with 2 μL of 1 μM oligo $d(T)_{18}$ primer (Genotech, Korea) at 70˚C for 7 min and cooled on ice for 5 min. The enzyme mix was separately prepared in a total volume of 11 μL by adding 2 μL of 0.1 M DTT (Duchefa, Netherlands), 2 μL of 10x reverse-transcription buffer, 5 μL of 2 mM dNTP, 1 μL of 200 U/μL MMLV reverse-transcriptase, and 1 μL of 40 U/μL RNase inhibitor (Enzynomics, Korea). After adding the enzyme mix to the annealed total RNA sample, the reaction was incubated for 90 min at 42˚C prior to heat inactivation of reverse-transcriptase at 80˚C for 10 min. Diethylpyrocarbonate (DEPC)-treated water was added to the cDNA samples to a final volume of 400 μL.

Quantitative Real-time PCR

Real-time PCR amplifications were carried out in 384 well plates using Applied Biosystems PRISM 7900HT instruments. The real-time PCR analysis was performed in a total volume of 10 μL with 5 μL of 2x Taqman gene expression master mix (Applied Biosystems, United States), 1 μL each of 5 μM forward and reverse primers and 1 μM probe (Genotech), and 2 μL of cDNA (or water as a control, which was always included). The amplification steps were as follows: an initial denaturation step at 95˚C for 10 min which was followed by 45 cycles of denaturation at 95˚C for 15 sec and elongation at 60˚C for 1 min. The primer and probe sequences designed using Primer Express 3.0 software (Applied Biosystems) are listed in Table 5. A significant part of TKT gene amplicon (75 bp out of 80 bp) overlaps with the TKT gene amplicon of Coy *et al.*[15] which spanned 176 bp. The primers for TKTL1 gene are identical to that of Coy *et al.*[15]. All probe sequences were labeled with FAM at the 5′ end and with TAMRA at the 3′ end. Expression of TKT and TKTL1 mRNA was measured (the threshold cycle, or C_T) in triplicate and was then normalized relative to the expression of GAPDH mRNA as an internal control. Using the ΔC_T values (TKT C_T or TKTL1 C_T-GAPDH C_T), the mRNA copy number ratio was calculated as $2^{-\Delta C_T}$. Standard curves were constructed by simultaneous amplifications of serial dilutions of the cDNA samples.

Statistical Analysis

All statistical analyses were done with the open source statistical programming environment R. Significant differences between gene expression levels were evaluated by a Student's t test. Correlation between gene expression and clinicopathologic variables was evaluated using a χ^2 test. Kaplan-Meier survival curves were calculated using tumor recurrence or death as the end points. The difference of survival curve was examined by log-rank test. The Cox proportional hazard regression model was used to identify independent prognostic factors. A two-tailed P value test was used with a P value of < 0.05 considered statistically significant.

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