Clinical Significance of Methylation and Reduced Expression of the Quaking Gene in Colorectal Cancer

NORIKO IWATA¹, TOSHIAKI ISHIKAWA², SATOSHI OKAZAKI¹, KAORU MOGUSHI³,
HIRONOBU BABA¹, MEGUMI ISHIGURO⁴, HIROTOSHI KOBAYASHI¹, HIROSHI TANAKA³,
TATSUYUKI KAWANO¹, KENICHI SUGIHARA² and HIROYUKI UETAKE²

¹Division of Surgical Gastroenterology, ²Division of Surgical Specialties,
and ⁴Department of Translational Oncology, Graduate School of Medical and Dental Sciences,
and ³Department of Systems Biology, Graduate School of Biochemical Science,
Tokyo Medical and Dental University, Tokyo, Japan

Abstract. *Background:* This study investigated abnormal methylation in colorectal cancer (CRC) and the potential role of the Quaking RNA-binding protein (*QKI*) gene in tumorigenesis. *Materials and Methods:* Oligonucleotide microarray expression profiling was carried out on a panel of primary CRC specimens ($n=17$) and CRC cell lines ($n=5$), followed by methylation analysis using methylation-specific polymerase chain reaction. *QKI* expression levels were assessed in 156 primary CRCs by qRT-PCR and immunohistochemistry. *Results:* Low *QKI* expression was observed in 47.7% in CRCs. *QKI* promoter methylation was detected in 32.1% of patients with CRC, and in these patients mRNA expression in tumor tissue was significantly down-regulated compared to matched normal tissues ($p=0.049$). There was a significant relationship between low *QKI* expression and recurrence after surgery ($p=0.004$). Low *QKI* expression was an independent risk factor for recurrence after surgery in 153 patients with CRC without distant metastases ($p=0.036$). *Conclusion:* Patients with tumors expressing low levels of *QKI* experienced significantly higher rates of tumor recurrence after curative surgery and worse prognoses. Methylation of the *QKI* promoter and concomitant reduced expression of *QKI* mRNA may be important for CRC initiation and progression. Low *QKI* expression may be a useful clinical biomarker for predicting recurrence and prognosis.

Correspondence to: Toshiaki Ishikawa, Division of Surgical Specialties, Tokyo Medical and Dental University, Graduate School of Medical and Dental Sciences, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan. Tel: +81 358035261, e-mail: ishi.srg2@tmd.ac.jp

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Colorectal cancer (CRC) is one of the best understood neoplasms from a genetic perspective, yet it remains the second most common cause of cancer-related death. Although the diagnosis and treatment of CRC has significantly improved during recent decades, leading to a substantial reduction in cancer-related mortality (1), CRC continues to be a major public health problem worldwide.

It is now well established that widespread epigenetic changes, including alterations in DNA methylation profiles relative to non-neoplastic tissue, are a characteristic of many cancer types (2, 3). Epigenetic alterations, such as aberrant DNA hypermethylation are now recognized as one of the crucial mechanisms underlying tumor-suppressor gene inactivation in cancer (4). In addition to identifying genes with a potential role in oncogenesis, methylation of specific gene promoters is a hallmark of different cancer types and can be used in cancer diagnosis and classification (5). For example, in CRC, coordinated methylation of a specific set of genes classifies cancers as the CpG island methylator phenotype (CIMP), and this classification is associated with B-Raf proto-oncogene *BRAF* mutations (6).

Advances in genome-wide methylation analysis technology have revealed that methylation regulates many genes that control apoptosis, and cell-cycle regulation of other fundamental cellular processes (7, 8). DNA microarray and high-throughput DNA sequencing are two powerful techniques that enable the large-scale analysis of DNA methylation (9-12). In this study, we searched for clinically significant tumor-suppressor genes in CRC by screening for candidate genes suspected to be silenced by DNA methylation using microarray analysis. We focused our study on the Quaking RNA-binding protein gene (*QKI*) gene, which encodes a RNA-binding protein that was reported to be inactivated in CRC (13), and demonstrated the clinical significance of *QKI* expression in CRC specimens.

Materials and Methods

Clinical sample collection. We collected primary CRC specimens and matched corresponding normal mucosa from 156 patients who had undergone surgery at Tokyo Medical and Dental University Hospital (Tokyo, Japan) between 2005 and 2009. All patients provided written informed consent, and the study was approved by the Institutional Review Boards of all institutions involved (approval number: 831). Clinical data were obtained from the medical records of each patient. The patients were monitored every 3 months after surgery, and their median follow-up was 60 months.

Cell culture and demethylation treatment of cell lines. We used five CRC cell lines (RKO, HCT116, HT29, colo320DM, SW480). These were purchased through the American Type Culture Collection (Manassas, VA, USA) or Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan), minimum essential medium, Dulbecco's modified Eagle's medium, or McCoy's 5A medium (Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 10 mM of HEPES and 1.0 mM of sodium pyruvate and incubated at 37°C in 5% CO₂. Cells were plated in culture plates on day -2. On day 0, the culture medium was removed and new medium containing 0.5 µM of the DNA methylase inhibitor 5-aza-2-deoxycytidine (5-aza-DC) was added. The cells were treated with 0.5 µM of 5-aza-DC for 72 h, according to a previous report (14). The cells were then rinsed twice with fetal bovine serum-free medium and collected with trypsin-ethylenediaminetetra-acetic acid after 72 h of 5-aza-DC treatment.

Oligonucleotide microarray analysis. RNA from five human CRC cell lines (RKO, HCT116, HT29, colo320DM, SW480) was extracted before and after treatment with 5-aza-DC. Matched samples of primary cancer and adjacent normal tissues were obtained from 17 patients with CRC, and total RNA was extracted from each using a RNeasy mini-kit (Qiagen, Hilden, Germany). The integrity of the total RNA was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples had an RNA integrity number of at least 5.0 prior to gene-expression analysis. Complementary RNA was prepared from 2 µg cell line total RNA, and 100 ng tissue sample total RNA by one-cycle and two-cycle target labeling, respectively, using a control reagents kit (Affymetrix, Santa Clara, CA, USA). Hybridization to Human Genome (HG) U133 Plus 2.0 arrays (Affymetrix) and signal detection were performed according to the manufacturer's protocol. The gene -expression data are deposited in the Gene Expression Omnibus ([http:// www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) under accession number GSE32323.

For each of the 54,613 probes on the HGU133 Plus 2.0 array, fold-change (FC) values were calculated using R2.11.1 statistical software together with a Bioconductor package (R Foundation for Statistical Computing, Vienna, Austria) as previously described (15).

Probe sets from cell lines were selected using a combination of the following criteria: i) FC>2.0 compared with the CIMP RKO cell line, and ii) up-regulation of gene expression in at least two CRC cell lines. For the paired clinical samples, probe sets were selected for FC of normal compared to tumor tissue (N/T)>1.5 (*i.e.* those with higher expression in normal than tumor tissue) (Figure 1).

mRNA expression assay. We assessed the QKI mRNA expression level in each cell line and clinical sample by quantitative Reverse

Transcription Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted using an RNeasy mini kit (Qiagen) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. cDNA was then amplified by polymerase chain reaction PCR using a fluorescence-based real-time detection method on an ABI Prism 7300 Real-time PCR System according to the manufacturer's protocol. TaqMan QKI and β-actin (ACTB) Gene Expression Assays-on-demand (QKI Assay ID Hs00287641_m1, ACTB Assay ID Hs99999903_m1; Applied Biosystems) were used for qRT-PCR. QKI mRNA expression levels in each sample were normalized to those of ACTB as an internal standard. Relative quantification of QKI mRNA expression was calculated by the ΔΔCt method using SDSv1.2 with RQv1.0 software (Applied Biosystems). Each analysis was performed in triplicate.

Methylation analysis. Total genomic DNA was isolated from both tumor and normal colon epithelium samples using the phenol/chloroform method. Bisulfite treatment was performed using an EpiTect Plus DNA Bisulfite kit (Qiagen) according to the manufacturer's instructions. Bisulfite-modified DNA was then used as template DNA for PCR amplification with the PCR primers corresponding to the region affected by the methylation. Methylated primer sequences were based on previous reports (13). Methylation-specific PCR (MSP) was performed using an EpiTect MSP kit (Qiagen). PCR conditions were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, and finally 5 min at 72°C. Epitect control DNA (human), methylated/unmethylated and bisulfite-converted gDNA (Qiagen, Hilden, Germany) was used as a positive control for the methylated/unmethylated determinations. After amplification, PCR products were electrophoresed on a 1.5% agarose gels.

Immunohistochemistry. IHC studies of QKI expression were conducted using FFPE tissue blocks from each of 156 patients. Paraffin-embedded tumor tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by autoclave sterilization in 0.01 M sodium citrate buffer (pH 9.0) for 30 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 15 min. Sections were blocked in goat serum for 30 min at room temperature, and incubated with a 1:100 dilution of rabbit antibody to QKI (IHC-00574, 9444, AAH19917.1; BETHYL, Montgomery, AL, USA) at 4°C overnight, then sections were incubated with Histofine simple stain MAX-PO (MULTI; Nichirei Bioscience, Tokyo, Japan) secondary antibody for 30 min at room temperature. QKI staining was visualized after incubation with 3,3'-diaminobenzidine tetrahydrochloride (Nichirei Bioscience) and was followed by counterstaining with hematoxylin. Phosphate-buffered saline was used instead of the primary antibody as a negative control. Staining results were assessed by two pathologists independently.

Staining intensity and proportion were both considered in the scoring system (Figure 2): Intensity grade (IG): Negative: 0; weak staining: 1; moderate staining: 2; strong staining: 3. Extent grade (EG): <10% positively stained: 1, 10-50% positively stained: 2, >50% positively stained: 3. Then the immunoreactive score (IRS) was calculated as: IRS = (IG0 × EG) + (IG1 × EG) + (IG2 × EG) + (IG3 × EG).

Statistical analysis. All analyses were performed with the SPSS software package version (version 17.0;SPSS Inc., Chicago, IL,

Figure 1. Outline of selection of candidate genes in colorectal cancer. 5-Aza-DC: 5-aza-2-deoxycytidine; CIMP: CpG island methylator phenotype.

USA). To estimate the differences between groups, the Chi-square test, Fisher's exact test, and Mann-Whitney *U*-test were used where appropriate. The IRS was used to classify the samples into two groups depending on a cut-off score of 5.5 based on the ROC curve analysis (Figure 3). Survival curves were plotted according to the Kaplan-Meier method, and differences between the curves were analyzed by log-rank test. Univariate and multivariate analysis for risk factor of postoperative recurrence were undertaken using the logistic regression model. A value of $p < 0.05$ was considered as significant.

Results

Candidate genes suppressed by DNA methylation. We treated colo 320DM, HCT116, HT29, RKO and SW480 CRC cell lines with 5-aza-DC and compared mock- and treated cell lines using microarray analysis to identify genes that were reactivated in response to demethylation (methodology summarized in Figure 1). Genes which were re-expressed by more than 2.0-fold after 5-aza-DC treatment compared with the CIMP RKO CRC cell line, and up-regulated more than 1.5-fold in cancer tissues compared to normal mucosa were selected as candidate genes. In this way, we identified 99 genes (123 probes) that appeared to be suppressed by DNA

methylation. We examined the published literature for additional analysis of these genes. In order to identify epigenetically affected genes with methylation, we first excluded genes in neoplasm not affected by methylation (51 genes). In addition, we removed genes with high or unknown expression in neoplasm (14 genes), genes with hypomethylation or unknown methylation status in neoplasm (23 genes). Finally, a list of 11 genes was determined which includes genes previously reported as being hypermethylated in neoplasms (Table I), some of which have tumor-suppressor functions, such as *RASSF2* (16-18) and *KLF4* (19, 20) and *AKAP12* (21). Among these candidate genes, we focused on *QKI* because it was reported to be down-regulated in CRC (13, 22, 23), but the clinical significance and the mechanism of inactivation remain unclear.

***QKI* expression in primary CRC specimens.** We investigated the association between methylation status and gene expression in tumor and normal tissue to determine whether the down-regulation of *QKI* expression was related to hypermethylation of the promoter. The methylation status of *QKI* in 115 CRC specimens was investigated by MSP (Table

Figure 2. Representative immunohistochemical analysis showing negative (a), weak (b), moderate (c) and strong (d) staining of Quaking RNA-binding protein expression in primary colorectal cancer specimens. Main images, $\times 40$, insets: $\times 200$.

II and Figure 4) and *QKI* promoter methylation was detected in 32.2% of specimens (Table II). *QKI* mRNA expression was significantly down-regulated in CRC specimens with promoter methylation compared with normal tissue ($p=0.049$). On the other hand, *QKI* mRNA expression levels in normal tissue and unmethylated CRC specimens were not significantly different ($p=0.397$) (Figure 4).

***QKI* protein expression in CRC specimens.** Following antibody optimization and staining, the cellular localization of the *QKI* protein was investigated using cancer tissue from 156 patients. *QKI* protein was observed in the nucleus of cancer cells. The staining tended to be weak at the invasive tumor front, however, the entire tumor cross-section showed heterogeneous staining (Figure 2). Therefore, we assessed the entire tumor cross-section, and graded the *QKI* expression level using the IRS.

The median of IRS was 6 (range=0-14). The cutoff value of IRS was 5.5, as determined from ROC curve for predicting recurrence after surgery (Figure 3). The patients

Figure 3. Receiver operating characteristic (ROC) curve of immunoreactivity score for detecting colorectal cancer recurrence after surgery. Based on the drawn ROC curve, the cut-off point for IRS was 5.5, area under the ROC curve: 0.651, $p=0.009$.

Figure 4. Box and whiskers plot of the methylation status and mRNA expression levels of Quaking RNA-binding protein gene (*QKI*) in 115 colorectal cancer specimens (T) and normal mucosa (N) with unmethylated (a) and methylated (b) *QKI* by Mann–Whitney U-test. M: Mean.

Figure 5. Relapse-free (a) and overall (b) survival curves of 153 patients with stage I-III colorectal cancer according to level of Quaking RNA-binding protein gene expression in tumor. *p*-Value from log-rank test.

were thereby divided into two groups based on tumor IRS: IRS <5.5: low expression group, IRS ≥5.5: high expression group. There were 75 patients in the low expression group and 81 patients in the high expression group.

The relationships between QKI expression and clinicopathological features. We analyzed the association between *QKI* expression level and age, gender, tumor location, histological type, depth of tumor invasion, lymph node metastasis,

Table I. *Candidate genes in colorectal cancer.*

Gene symbol	Probes	Gene name	Locus	Function (Ref)
<i>QKI</i>	212262_at	RNA binding protein Quaking	6q26	Protein binding, splicing (13, 22-24, 30)
<i>TIMP2</i>	203167_at	TIMP metalloproteinase inhibitor 2	17q25	Suppress the proliferation of endothelial cells (43)
<i>RET</i>	211421_s_at	Ret proto-oncogene	10q11.2	Transduce signals for cell growth and differentiation (44)
<i>TFPI2</i>	209278_s_at	Tissue factor pathway inhibitor2	7q22	Inhibit a variety of serine proteases, tumor suppressor, alternative splicing (45, 46)
<i>KLF4</i>	221841_s_at	Kruppel-like factor 4	9q31	Tumor suppressor (19, 20)
<i>AKAP12</i>	210517_s_at	A kinase (PRKA) anchor protein 2	9q31.3	Binds to the regulatory subunit of protein kinase A and is found associated with the actin cytoskeleton (21)
<i>PEG3</i>	209242_at	Paternally expressed 3	19q13.4	Alternative splicing cell proliferation and p-53-mediated apoptosis, tumor suppressor (47)
<i>NT5E (CD73)</i>	203939_at	5'-Nucleotidase,ecto (CD73)	6q14-q21	A plasma membrane protein, determinant of lymphocyte differentiation (48)
<i>RASSF2</i>	203185_at	Ras association (RalGDS/AF-6) domain family member 2	20q13	Tumor suppressor (16-18)
<i>GPX3</i>	214091_s_at	Glutathione peroxidase 3	5q33.1	Tumor suppressor (49)
<i>UCHL1</i>	201387_s_at	Ubiquitin carboxyl-terminal esterase L1	4q14	A key role in proliferation, apoptosis and metastasis of osteosarcoma cells (50)

lymphatic or venous invasion, distant metastasis, TNM stage, and recurrence rate after surgery. The relationships between *QKI* expression and clinicopathological features are summarized in Table III. A significant relationship was detected between low *QKI* expression and high recurrence rate ($p=0.004$, chi-square test). We then investigated whether *QKI* expression was a predictor of tumor recurrence after surgery. The analysis of risk factors for postoperative recurrence in patients with stage I, II and III disease is shown in Table IV. Univariate analysis revealed that depth of tumor invasion ($p=0.004$), lymph node metastasis ($p=0.001$) and low *QKI* expression ($p=0.002$) were possible risk factors for tumor recurrence after surgery. Multivariate analysis also showed that low *QKI* expression was an independent risk factor for tumor recurrence after surgery ($p=0.015$). Furthermore, we found that low *QKI* expression was an independent risk factor in patients with stage II and III CRC ($p=0.025$) (data not shown). The 5-year relapse-free survival (RFS) rate for stage I, II and III CRC with low and high *QKI* expression was 66.5% and 85.5%, respectively. Patients with low tumor *QKI* expression had significantly shorter RFS than those with high *QKI* expression ($p=0.007$) (Figure 5a). In addition, patients with low tumor *QKI* expression had significantly shorter overall survival than those with high *QKI* expression ($p=0.042$) (Figure 5b).

Discussion

In this study, we carried out genome-wide expression screening in order to identify abnormally methylated genes in CRC with a potential role in tumorigenesis. From this analysis we chose to focus on the *QKI* gene, which encodes a RNA-binding protein that belongs to the signaling transduction and activation

Table II. *Methylation status and mRNA expression in 115 patients with colorectal cancer.*

<i>QKI</i> mRNA expression	Methylation status		Total
	Methylated	Unmethylated	
Tumor<normal	24	34	58
Tumor>normal	13	44	57
Total	37	78	115

QKI: Quaking RNA-binding protein gene.

of RNA (STAR) family (24). Aberrant expression of the STAR proteins is associated with a number of developmental defects and human diseases (25, 26). *QKI* appears to be relevant to brain biology, as evidenced by its down-regulation in glioblastoma multiforme (27) and schizophrenia (28, 29). *QKI* produces a diverse set of proteins by alternative splicing (30), and is implicated in the regulation of cellular processes such as the cell cycle and differentiation (31), apoptosis (32), cell fate decisions, development and angiogenesis (33, 34). There is evidence that the tumor-suppressive actions of *QKI* are linked to: i) regulation of alternate splicing, for example, regulating the levels of alternate splice forms of the histone Macro-H2A1 (22), ii) stabilization of *mir-20a*, which regulates genes in the transforming growth factor β pathway (23) and iii) regulation of splicing in lung cancer and regulation of the NOTCH signaling pathway.

In the context of CRC, *QKI* is thought to be a tumor-suppressor gene that functions as a principal regulator of the

Table III. Relationship between *Quaking* RNA-binding protein gene expression and clinicopathological factors.

Factor	Low expression (n=75)	High expression (n=81)	<i>p</i> -Value*
Age at surgery (median=68 years)			
≥Median	39	37	0.480
<Median	36	44	
Gender			
Male	49	50	0.686
Female	26	31	
Location			
Colon	45	53	0.551
Rectum	30	28	
Right-sided colon	21	21	0.731
Left-sided colon	54	60	
Histological type			
Well-differentiated	32	28	0.268
Not well-differentiated	43	53	
Tumor depth of invasion			
T1, 2	12	23	0.070
T3, 4	63	58	
Lymph node metastasis			
Absent	43	55	0.207
Present	32	26	
Distant metastasis			
Absent	74	79	0.614
Present	1	2	
Lymphatic invasion			
Absent	28	32	0.831
Present	47	49	
Venous invasion			
Absent	13	14	0.963
Present	62	67	
TNM stage			
I, II	42	55	0.152
III, IV	33	26	
Recurrence after surgery			
Absent	52	72	0.004*
Present	23	9	

* χ^2 test.

differentiation of colon epithelium, and a suppressor of carcinogenesis by coordinately targeting multiple genes associated with cell growth and differentiation. Yang *et al.* found that *QKI* expression was greatly reduced in or absent from CRC cells, and down-regulation of *QKI* was associated with deregulation of β -catenin and p27^{kip1} signaling (13). Hence, the down-regulation of *QKI* expression in the colon may be involved in cancer onset and progression (13). Consistent with this possibility, we found that low *QKI* expression occurred in about 47.4% of CRC specimens, and multivariate analysis showed that low *QKI* expression was an independent risk factor for tumor recurrence after surgery. Furthermore, the RFS and overall survival of patients with

stage I, II and III CRC with low *QKI* expression was significantly shorter than those with high *QKI* expression.

Yang *et al.* reported that *QKI* down-regulation in some CRCs was at least partially due to promoter hypermethylation. Indeed, consistent with this suggestion, we found that *QKI* was one of the candidate genes for which mRNA expression levels were increased after demethylation treatment. *QKI* mRNA expression levels were significantly decreased in tumor tissues in which promoter methylation was detected compared with normal tissues ($p=0.049$). Interestingly, the link that we identified between *QKI* promoter methylation and reduced *QKI* mRNA expression in CRC, is distinct from the mechanisms underlying *QKI* deregulation in glioma (27, 35), suggesting that the regulation of *QKI* expression is context specific, and diverse aberrations have an impact on *QKI* expression in cancer cells.

We did not detect a relationship between *QKI* protein expression measured by immunostaining and *QKI* promoter methylation status (data not shown). One possible explanation for this unexpected result is that there might be another pathway regulating *QKI* expression.

This study showed that low *QKI* expression was an independent risk factor for tumor recurrence after surgery, and patients with low *QKI* expression had a significantly poorer prognosis than those with high *QKI* expression. Postoperative adjuvant chemotherapy for patients with stage III CRC is internationally accepted as standard care for improving survival (36). For stage II CRC, the major Western guidelines recommend adjuvant chemotherapy when patients have risk factors including T4 lesions, fewer than 12 lymph nodes examined, perforation, poorly differentiated histology, and lymphovascular involvement, even though the efficacy of adjuvant chemotherapy for stage II CRC remains controversial (37-39). Our results suggest that patients with stage II and III CRC with low *QKI* expression are at high-risk for recurrence after surgery and might be candidates for adjuvant chemotherapy. In addition, if methylation-related mechanisms contribute to the inactivation of *QKI*, demethylation could be an appropriate therapeutic strategy. Recent studies have shown the clinical efficacy of demethylating agents in the treatment of patients with hematological malignancies (40-42).

In conclusion, our study revealed that methylation of the *QKI* promoter and concomitant reduced expression of *QKI* mRNA may be important for CRC initiation and progression. In addition, patients with tumors expressing low levels of *QKI* experienced significantly higher rates of tumor recurrence after curative surgery and had worse prognoses. Our results suggest that low *QKI* expression may be a useful clinical biomarker for predicting recurrence and prognosis. Further validation or prospective studies are needed to assess the clinical utility of *QKI* as a biomarker.

Table IV. Analysis of risk factors for postoperative recurrence in 153 patients with stage III colorectal cancer.

Factor	Univariate analysis		p-Value	Multivariate analysis	
	Recurrence (n=31)	No recurrence (n=122)		HR (95% CI)	p-Value
Age at surgery (median=68 years)					
≤Median	14	59	0.750		
>Median	17	63			
Gender					
Male	19	78	0.785		
Female	12	44			
Location					
Colon	16	79	0.178		
Rectum	15	43			
Histological type					
Well-differentiated	11	48	0.693		
Not well-differentiated	20	74			
Tumor depth					
T=1,2	1	88	0.004	4.004 (0.94716.936)	0.059
T=3,4	30	34			
Lymph node metastasis					
Absent	12	85	0.001	2.453 (1.237-4.863)	0.010
Present	19	37			
Lymphatic invasion					
Absent	10	50	0.374		
Present	21	72			
Venous invasion					
Absent	3	24	0.192		
Present	28	98			
<i>QKI</i> expression					
Low	23	51	0.002	2.158 (1.052-4.439)	0.036
High	8	71			

QKI: Quaking RNA-binding protein gene, HR: hazard ratio, CI: confidence interval.

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