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Downregulation of tumor suppressor QKI in gastric cancer and its implication in cancer prognosis

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ABSTRACT

Gastric cancer (GC) is the fourth most common cancer and second leading cause of cancer-related death worldwide. RNA-binding protein Quaking (QKI) is a newly identified tumor suppressor in multiple cancers, while its role in GC is largely unknown. Our study here aimed to clarify the relationship between QKI expression with the clinicopathologic characteristics and the prognosis of GC. In the 222 GC patients' specimens, QKI expression was found to be significantly decreased in most of the GC tissues, which was largely due to promoter hypermethylation. QKI overexpression reduced the proliferation ability of GC cell line *in vitro* study. In addition, the reduced QKI expression correlated well with poor differentiation status, depth of invasion, gastric lymph node metastasis, distant metastasis, advanced TNM stage, and poor survival. Multivariate analysis showed QKI expression was an independent prognostic factor for patient survival.

1. Introduction

Although the incidence of GC declines, it remains the fourth most common cancer and second leading cause of cancer-related death worldwide [1], accounting for 989,600 new cancer cases and 738,000 deaths worldwide annually [2]. Infection with *Helicobacter pylori* is considered as the strongest risk factor of GC [3,4]. Besides, Epstein-Barr virus infection [5,6], unhealthy dietary [7,8] and irregular lifestyle [9,10] are also important risk factors. It is well established that these extrinsic factors activate or act synergistically with the intrinsic oncogenic pathways, resulting in initiation and development of GC. Determining the expression profiles of key molecules involved in cell differentiation and proliferation control in GC progression may aid in diagnosing and predicting disease progression.

Nowadays, miRNAs and RNA binding proteins have emerged as critical regulators in cell biology, which posttranscriptionally regulate the mRNA stability, splicing, translation efficiency and transport. Defects in these mRNA regulators have been linked to a number of human disorders, including cancers [11]. RNA binding protein QKI (Quaking) belongs to the evolutionarily conserved signal transduction and activation of RNA (STAR) family. As an RNA binding protein, QKI regulates the target mRNAs by binding the bipartite consensus sequence NACUAAY-$N_{(1-20)}$-UAAY (denoted as QKI response element (QRE)) located in the 3′UTR[12]. Multiple cancer related genes are clarified to be QKI targets, such as p27 [13–15], β-catenin [16], c-fos [17]. Recently aberrant expression of QKI is believed to be involved in the initiation and progression of many kinds of tumors [16,18,19]. Taken together, all of these suggest a ubiquitous tumor suppressor role of QKI in multiple cancers. In addition, 6q26, where QKI locates, has shown a high risk of LOH in GC [20,21], further implicating a role of QKI in GC.

It is thus interesting to test the expression of QKI and its role in GC. In this study, we for the first time clarify that QKI is downregulated in cancer tissues due to aberrant hypermethylation of the promoter, and the reduced QKI expression correlated well with poor differentiation status, depth of invasion, gastric lymph node metastasis, distant metastasis, advanced TNM stage and poor survival. Our findings suggest that QKI is an independent prognostic factor for survival of GC patients, shedding light on QKI as diagnosis and therapeutic target for GC.

2. Materials and methods

2.1. Study cohort and tissue samples

This study was approved by the ethics committee of the Fourth Military Medical University. All patients involved in the study have

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offered full consent for the study. The study cohort included 222 participants, who were randomly selected from patients continuously diagnosed with GC between January 2005 and December 2006 in Department of Gastrointestinal Surgery, Xijing Hospital. Patients who received treatment prior to surgery including neoadjuvant chemotherapy or postoperative adjuvant chemotherapy were excluded from the cohort. Every 3 months, participants have been sent follow-up scheme or telephone visit to update the information. Thirty noncancerous, healthy gastric mucosa tissues obtained from patients who underwent endoscopy without malignancy served as controls. All the fresh tissues, after surgical removal, were immediately put into liquid nitrogen for 10 min and then into a −80 °C ultra freezer. The cancer tissues and the matched adjacent normal tissues of every participant had been reviewed and confirmed by Department of Pathology, Xijing Hospital. Pathological information was collected from patient clinical database, and the information was blind to study physicians who reviewed all the records of GC and recorded data into database. The clinicopathologic characteristics of patients are shown in Table 2.

2.2. Measurement of the overall survival time

The follow-up deadline was December 2011, and every patient had follow-up records more than 5 years. The survival time is defined from the date of surgery to the date of death or follow-up deadline. The physicians assigned to record the status of overall survival were blind to clinicopathologic data and QKI mRNA expression information.

2.3. RNA extraction and real-time PCR

The RNA from all the GC tissues, matched adjacent normal tissue specimens, and the 30 noncancerous gastric mucosa tissues were purified as recommended by the manufacturer using Trizol reagent (Invitrogen). Real-time PCR was done as described before [22]. The primers of QKI and GAPDH were shown in Table 1. QKI mRNA levels were normalized to GAPDH and the relative expression of QKI was analyzed using the $2^{-\Delta\Delta Ct}$ method. Thermal dissociation plots were examined for biphasic melting curves.

2.4. Immunohistochemistry

The patient paraffin blocks were treated as follows: 4 μm sections were prepared for H&E staining and also for immunohistochemistry examination. For immunohistochemistry treatment, endogenous peroxidases were blocked using 0.75% H_2O_2 in phosphate-buffered saline (PBS) for 30 min, followed by incubation with 5% bovine serum albumin blocking buffer. Incubation with primary antibody anti-QKI (Sigma) (1:300) was performed for 24 h at 4 °C. Immunodetection was performed in a 3-step protocol, using streptavidin–horseradish peroxidase complex, with visualization by 3,3-diaminobenzidine.

Table 1
Primer sequences used in this study.

Gene/primer	Sequence
QKI	
Forward	TAGCAGAGTACGGAAAGACATG
Reverse	GGGTATTCTTTTACAGGCACAT
GAPDH	
Forward	GACCTGACCTGCCGTCTA
Reverse	AGGAGTGGGTGTCGCTGT
MSP external	
Forward	GATTTAGTTTTTGTGTTTAGGTT
Reverse	AAAATCTCTCTAAACTAATCCC
MSP internal 1	
Forward	GCGTCGGCGGTTGTTTCGGTCGCG
Reverse	CGCCGCGCTCCGACTACGCTCCTC
MSP internal 2	
Forward	GGGGAGGTAGGGAGGAGGGGG
Reverse	AAATTCACCTCAATTCAAAC

2.5. Protein extraction and Western blot

Samples of the cancer and matched adjacent normal tissues were lyzed by lysis buffer as described before SDS–PAGE assay. The expression of QKI and the internal control GAPDH was analyzed using standard techniques with primary antibodies as follows: rabbit anti-QKI (Sigma) and mouse anti-GAPDH (Santa Cruz). The secondary antibodies anti-rabbit IgG and anti-mouse IgG were both from Odyssey.

2.6. DNA extraction and methylation assay

Total genomic DNA of the specimens was isolated from both tumor and matched adjacent normal tissues, using the DNA extraction kit according to the manufacturer's instruction (Tiangen, Beijing). Genomic DNA (2 μg) was then modified with bisulfite as described before. Bisulfite-modified DNA (2 μl) was then used as template DNA for amplification with the external nest PCR primers corresponding to the region unaffected by the methylation status. Equal aliquots of the amplicon were then amplified with internal nest PCR primers corresponding to methylated promoter region only (Primer methylation (Primer MSP internal 1) (Table 1) or the region unaffected by the methylation status (Primer MSP internal 2) (Table 1). The latter 2 internal resulting PCR products were then electrophoresed on a 2% agarose gel. To compare the methylation proportion, the band of the MSP internal 2 was served as a loading control.

2.7. MTT assay

MTT assay was done as described before [16]. Briefly, cells (2×10^3 cells/well) were seeded to 96-well culture plate in a final volume of 200 μl and treated as indicated. After treatment, 20 μl of

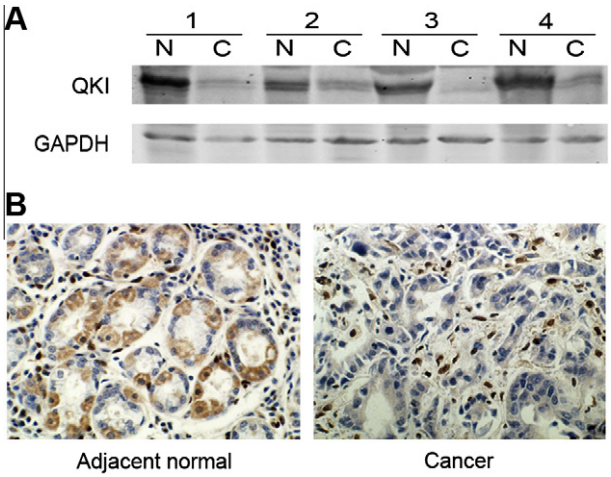

Fig. 1. Expression of QKI protein in GC samples. (A) Western Blot assay of QKI expression in fresh clinical samples. Data presented here is a representative of all the samples. (B) Representative data of the expression of QKI in adjacent normal and GC samples assayed by immunohistochemistry. QKI expression was much lower in most cancerous tissues than that in the adjacent normal tissues.

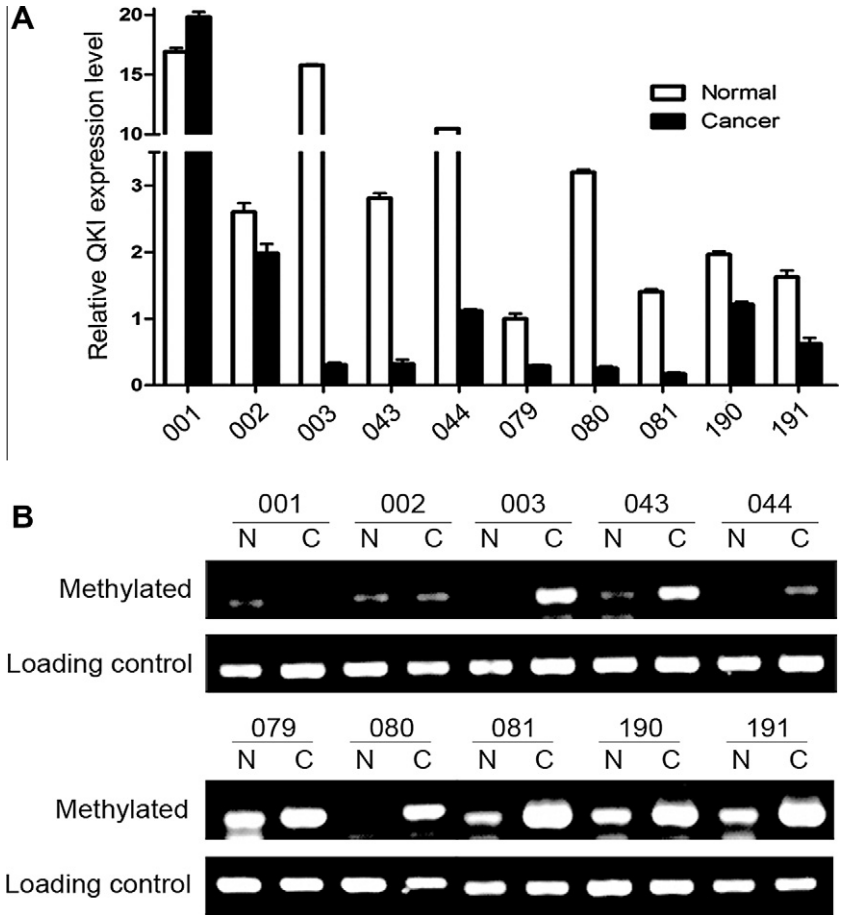

Fig. 2. Promoter methylation contributes to the deregulation of QKI in GCs. (A) Relative expression of QKI in both matched adjacent normal and cancer samples from the selected patients. QKI expression was expressed as the $2^{-\Delta\Delta Ct}$. (B) Methylation status of QKI promoter region in normal and cancerous tissues from the 5 representative patients at stage I–II and III–IV. The upper panel represents the early stage (I–II), and the lower panel represents the advanced stage (III–IV). Methylated promoter and the total level (served as a loading control) of each sample were amplified with specific primers.

MTT (5 mg/ml of PBS) was added to the medium. After 4 h incubation at 37 °C, the culture medium was carefully removed and 100 μl of DMSO was added to each well and plates were agitated for 1 min. Absorbance was read at 570 nm on a multi-well plate reader.

2.8. Flow cytometry assay

To analyze the cell cycle distribution, cells with indicated treatment were washed twice in PBS and fixed for at least 2 h in PBS containing 70% ethanol. Cells were spun down gently in 200 μl extraction buffer (0.1% Triton X-100, 45 mM Na2HPO4, and 2.5 mM sodium citrate) at 37 °C for 20 min and then re-suspended in PBS containing 40 mg/ml PI, 0.1 mg/ml RNase (Sigma) and 0.1% Triton X-100 at 37 °C for 30 min in the dark. Cell cycle distribution was detected by FACS (Becton–Dickinson San Jose, CA).

2.9. Statistical analysis

Statistical analysis was carried out using SPSS 13.0. Associations between QKI mRNA expression and categorical variables were assessed by Pearson's χ^2 test or Mann–Whitney *U* test, as appropriate. Correlation coefficients were assessed by Spearman correlation analysis. The survival curve was estimated by the Kaplan–Meier method, and the log-rank test was used to compute differences in survival distribution. Cox's proportional hazards regression model was done to assess HRs and identify factors which might have a significantly independent value on survival. Differences with a p value of 0.05 or less were considered to be statistically significant and all p values were two-sided.

3. Results

3.1. Expression of QKI in tumor samples

To elucidate the role of QKI in the initiation and progression of GC, we first analyzed the expression of QKI in GC and the matched adjacent normal tissues at both mRNA and protein levels.

At the mRNA level, relative expression of QKI was normalized to GAPDH. The lowest level of QKI in cancerous sample was set as 1. The RQs of QKI mRNA in GC and the matched adjacent normal samples were 1.53 ± 0.41 and 2.35 ± 0.64 (mean ± SD), respectively. While, in the 30 noncancerous mucosa samples the relative QKI mRNA expression was 3.97 ± 1.13. The difference among the three groups was statistically significant ($p < 0.001$). The data above indicated that the expression of QKI in GC was aberrantly reduced. Similar results of QKI protein expression were obtained by western blot analysis and immunohistochemistry analysis. Representative data are shown in Fig. 1A and B, respectively. All the data above indicated that expression of QKI in GC was aberrantly decreased at both RNA and protein level.

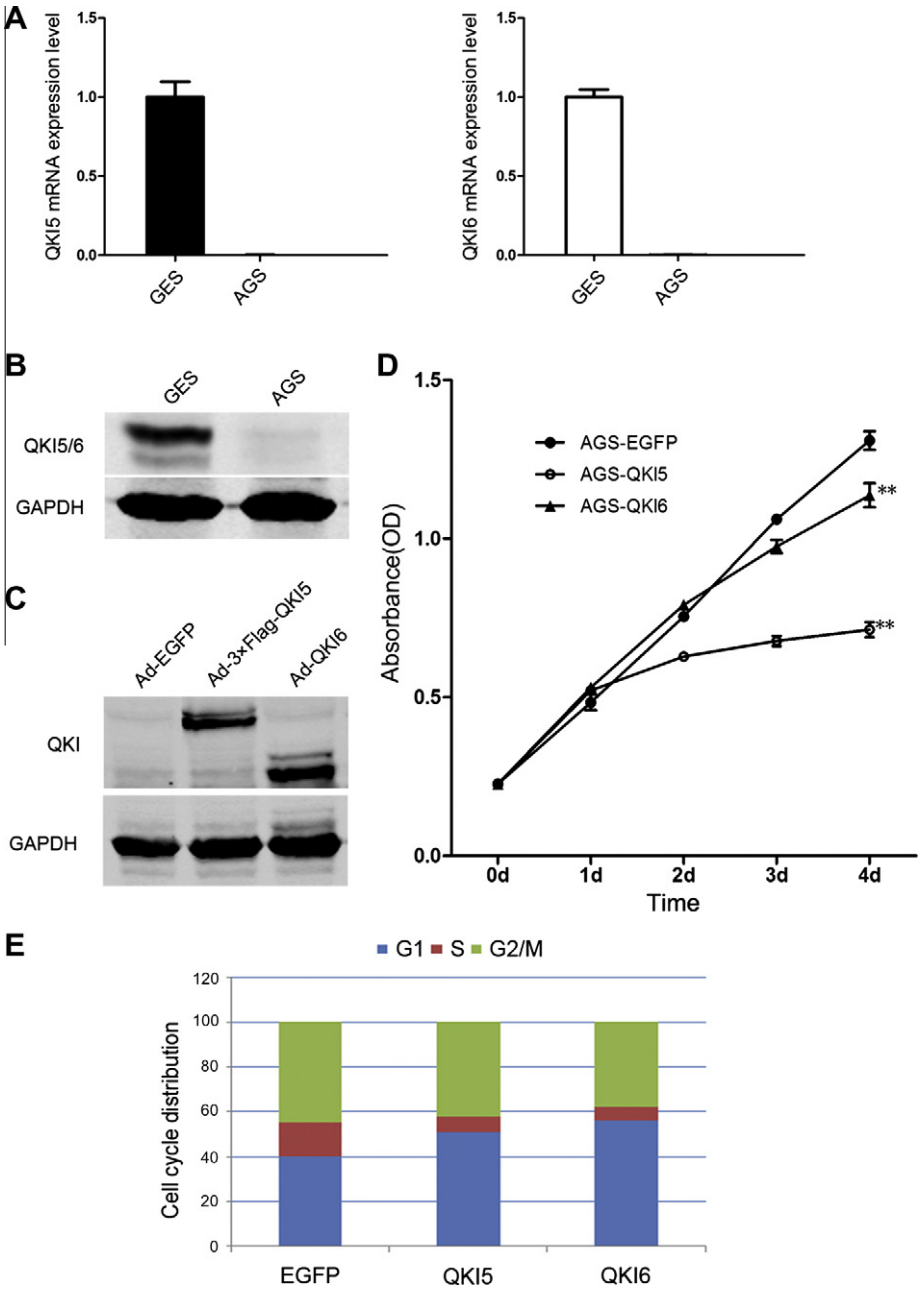

Fig. 3. QKI inhibits the GC cell growth. (A) Expression of QKI at mRNA level in both normal GES and cancerous AGS cells. Cells growing in exponential phase were harvested for qRT-PCR analysis. (B) Expression of QKI at protein level in both normal GES and cancerous AGS cells. Cells same as above were harvested for Western blot assay. (C) Overexpression of QKI5 or QKI6 in AGS cells. (D) Overexpression of QKI5 or QKI6 significantly inhibited the growth of AGS cells. AGS cells with either control (EGFP), QKI5 or QKI6 overexpression were cultured for indicated time and cell number were analyzed by MTT ($n = 3$, $^{*}p < 0.05$). (E) Cell cycle distribution of cells with QKI overexpression. Cells were treated same as above and the cell cycle analysis was done by FACS. Data presented here is a representative of three different experiments.

3.2. QKI promoter methylation contributes to the deregulation of QKI in GC

To explore the potential mechanisms accounting for deregulation of QKI in GC samples, we analyzed the promoter region of QKI. We found that QKI promoter region is rich in CpG islands, especially in the 500 base pairs region upstream of the putative transcription start site. The interesting phenomenon led us to ask whether CpG hypermethylation is responsible for the low expression of QKI.

In the samples with higher QKI expression, there was higher percentage of promoter methylation. Furthermore, the QKI promoter methylation level in samples from the advanced stages (III–IV) was higher than that from the early stages (I–II). Representative data are given in Fig. 2A and B. All of these suggest that DNA methylation was the reason for the low expression of QKI in gastric cancer samples.

3.3. QKI inhibits the growth of GC cells

From the above data, we suppose that QKI might play a tumor suppressor role in GC. To this end, we screened the QKI expression in available normal and cancerous gastric cell lines. As shown in Fig. 3A and B, normal GES cells had abundant expression of QKI both at mRNA and protein level, while the GC cell AGS displayed significantly lower expression of QKI. Thus, we overexpressed

Table 2
Correlation of QKI with clinicopathologic characteristics of patients with GC.

Variable	N	QKI mRNA expression				P
		Reduced	%	Unreduced	%	
Sex						0.847[a]
Male	144	96	66.7	48	33.3	
Female	78	51	65.4	27	34.6	
Age at diagnosis						0.881[a]
⩾60	105	69	65.7	36	34.3	
<60	117	78	66.7	39	33.3	
Location						0.464[a]
Cardia of stomach	57	33	57.9	24	42.1	
Body of stomach	42	30	71.4	12	28.6	
Antrum of stomach	93	63	67.7	30	32.3	
Whole	30	21	70.0	9	30.0	
Differentiation status						<0.001[b]
Well	57	27	47.4	30	52.6	
Moderately	93	60	64.5	33	35.5	
Poor and undifferentiatedly	72	60	83.3	12	16.7	
Lauren classification						0.200[a]
Intestinal type	138	87	63.0	51	37.0	
Diffuse type	84	60	71.4	24	28.6	
Depth of invasion						<0.001
T1 + T2	60	27	45.0	33	55.0	
T3 + T4	162	120	74.1	42	25.9	
Lymph node metastasis						0.001
Absent (N0)	75	39	52.0	36	48.0	
Present (N1–3)	147	108	73.5	39	26.5	
Distant metastasis						0.040
Absent (M0)	189	120	63.5	69	36.5	
Present (M1)	33	27	81.8	6	18.2	
TNM stage						<0.001[b]
I	42	12	28.6	30	71.4	
II	36	24	66.7	12	33.3	
III	111	84	75.7	27	24.3	
IV	33	27	81.8	6	18.2	

[a] *P* value when expression levels were compared using Pearson's χ^2 test.
[b] *P* value when expression levels were compared using Mann–Whitney *U* test.

Table 3
Correlation coefficient of QKI with clinicopathological characteristics GC.

Variable	Correlation coefficient (*r*)	*P*
Sex	−0.013	0.848
Age at diagnosis	0.010	0.882
Location	0.075	0.268
Differentiation status	0.289	<0.001
Lauren classification	0.086	0.202
Depth of invasion	0.273	<0.001
Lymph node metastasis	0.215	0.001
Distant metastasis	0.138	0.040
TNM stage	0.342	<0.001

QKI in AGS cells (Fig. 3C) and as expected, either QKI5 or QKI6 overexpression reduced the cell proliferation (Fig. 3D), further suggesting the tumor suppressor role of QKI in GC. Cell cycle analysis further indicated that QKI overexpression increased the G1 phase percentage (Fig. 3E), suggesting that QKI decreased the cell proliferation through delaying the S phase entry.

3.4. Correlation between expression of QKI mRNA and clinicopathologic characteristics of GCs

Next, correlation of QKI mRNA levels with different clinicopathologic factors was analyzed. Because of the inaccurate quantization of the western blot analysis and the immunochemistry analysis, the correlation was analyzed by the results of real-time PCR. According to the RQ of the matched adjacent normal tissues which was 2.35 ± 0.64, we separated the GC tissues into three groups: increased (>2.99), normal (1.71–2.99), and reduced (<1.71). Owing to the limited number of the increased and normal expression of QKI in the GC tissues, we combined the two groups into a single group, which defined as unreduced expression of QKI. The correlation of QKI mRNA levels in GC samples with different clinicopathologic factors was shown in Table 2. Statistically significant correlations could be found between QKI mRNA and differentiation status ($p < 0.001$), depth of invasion ($p < 0.001$), lymph node metastasis ($p = 0.001$), distant metastasis ($p = 0.040$), and TNM stage ($p < 0.001$). The correlation coefficients between QKI mRNA and clinicopathologic characteristics were shown in Table 3.

3.5. QKI mRNA expression predicts the overall survival of GC patients

The correlation of QKI mRNA expression with the survival of GC patients was estimated by the Kaplan–Meier analysis. A significant correlation was obtained between reduced QKI mRNA expression and poor survival, with a mean survival time of 52.58 months (95% CI: 48.82–56.34) in patients with unreduced QKI mRNA expression, compared with 28.46 months (95% CI: 25.99–30.94) in patients with reduced QKI mRNA expression (Fig. 4, log-rank test: $p < 0.001$). In addition, differentiation status, depth of invasion, lymph node metastasis, distant metastasis, and TNM stage were also proved to be correlated with overall survival of patients with GC. Patients with poor differentiation, gastric lymph node metastasis, or advanced TNM stage had poor survival rate. However, survival did not correlate with sex, age at diagnosis, tumor location, and Lauren classification.

Multivariate Cox proportional hazards model showed that distant metastasis and TNM stage remained to be significant risk

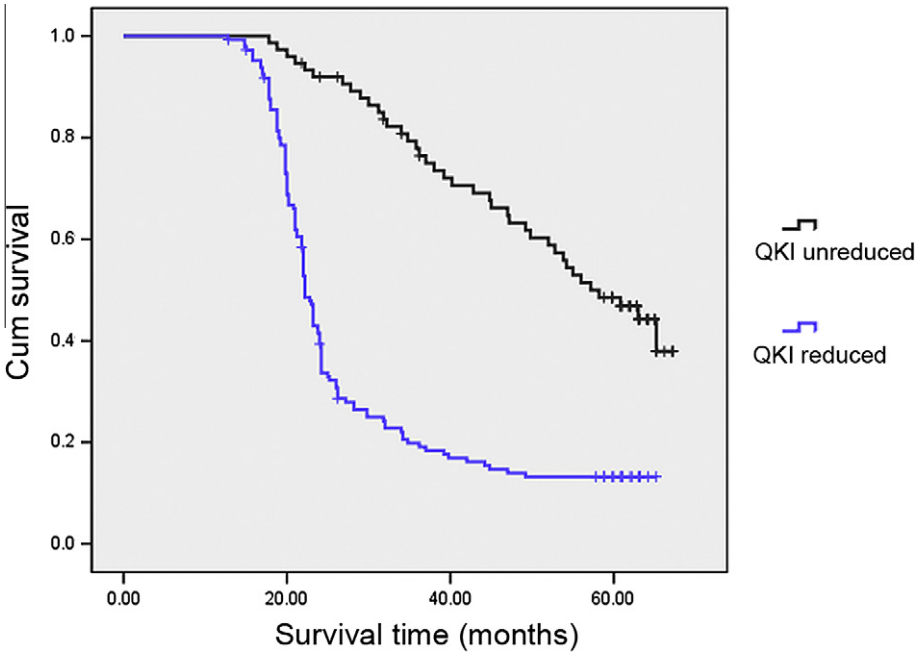

Fig. 4. Kaplan-Meier postoperative survival curve for GC patients with different QKI expression levels.

Table 4
Univariate and multivariate analysis of survival in patients with GC according to clinicopathologic factors and QKI mRNA expression.

Variable	Unadjusted HR[a] (95% CI)	*p*	Adjusted HR[b] (95% CI)	*p*
Sex	0.814 (0.585–1.131)	0.220	1.308 (0.903–1.893)	0.156
Age at diagnosis	1.008 (0.737–1.380)	0.959	0.826 (0.592–1.153)	0.261
Location	1.094 (0.933–1.281)	0.268	0.924 (0.786–1.085)	0.333
Differentiation status	1.831 (1.446–2.318)	<0.001	1.117 (0.854–1.460)	0.421
Lauren classification	0.950 (0.688–1.311)	0.756	1.049 (0.748–1.471)	0.781
Depth of invasion	1.907 (1.324–2.748)	0.001	0.594 (0.331–1.066)	0.081
Lymph node metastasis	2.638 (1.821–3.820)	<0.001	1.387 (0.805–2.388)	0.239
Distant metastasis	5.058 (3.217–7.952)	<0.001	2.426 (1.114–5.284)	0.026
TNM stage	2.195 (1.781–2.706)	<0.001	1.850 (1.112–3.075)	0.018
QKI expression	4.116 (2.804–6.042)	<0.001	3.657 (2.440–5.481)	<0.001

[a] Hazard ratios in univariate models.
[b] Hazard ratios in multivariable models.

factor. The QKI mRNA expression was still seen to provide independent prognostic value adjusted for sex, age, tumor location, differentiation status, Lauren classification, depth of invasion, lymph node metastasis, distant metastasis, and TNM stage (Table 4). The results above suggested that the reduced QKI expression correlates with poor prognosis of patients with GC.

4. Discussion

Gastric cancer is one of the most common malignant cancers worldwide. In China, there are over 400,000 new diagnosis cases and more than 300,000 deaths from GC every year, which ranks the third most common cancer and the first cancer-related deaths. The major dilemma is that majority of patients suffering from GC are diagnosed at advanced stages [23]. Although great improvement has been made in imaging detection, surgery skill, chemotherapy, and radiotherapy, the prognosis of the patients with advanced GC is still poor.

The patients with residual micro-lesions and micrometastases after surgical resection have a high risk of recurrence and metastasis, which is one of most fundamental reasons for the poor prognosis of the patients. At present, postoperative chemotherapy, which targets to the residual micro-lesions and micrometastases, is still a classic therapy given to patients with poor prognosis. Identification of patients who may benefit from the postoperative chemotherapy would be of significant clinical importance, because it might be harmful for the patients receiving unnecessary or insufficient adjuvant treatment. However, the prognosis of each patient is still difficult to predict. The TNM staging system is now still the most commonly used method to predict the prognosis of the patients with GC. But its accuracy is challenged by more and more doctors, owing to its limited information to individualized therapy. Therefore, it is of great value to find the potential markers which could predict the outcome of the patients with GC.

Our study demonstrates that QKI expression is very lowly expressed in most of GC tissues compared with the matched adjacent normal tissues. In addition, the reduced QKI expression correlated well with poor differentiation status, depth of invasion, gastric lymph node metastasis, distant metastasis, and advanced TNM stage. Kaplan–Meier analysis showed that there is a significant correlation obtained between reduced QKI mRNA expression and poor survival. Multivariate Cox proportional hazards model showed the QKI mRNA expression was seen to provide independent prognostic value. All the results suggest that QKI plays an important role in the initiation and progression of GC.

Nowadays, aberrant DNA methylation is reported to play an essential role in many kinds of diseases, especially in the various cancers [24]. Our study identifies that the low expression of QKI is mainly because of the hypermethylation in its promoter region, which is different from the reported mechanism in glioma [19,25], but nearly the same with the reported mechanism in colon cancer [16]. Furthermore, our study also demonstrates that QKI promoter methylation increases with the progression of the TNM stages, which is consistent with the gradually decreased expression of QKI in the advanced tumors tissues. It is thus reasonable to deduce that QKI promoter methylation status (extent and site) could be a marker of GC progression stages. Further study to see whether QKI promoter methylation status could discriminate different subtypes of GC is worthy of study.

To validate the tumor suppressor role of QKI in GC, MTT assay and FACS analysis were carried out. MTT assay showed either QKI5 or QKI6 overexpression reduced cell proliferation, and FACS assay further suggested QKI inhibited the cell proliferation through delaying the S phase entry. All of these suggest that QKI plays a tumor suppressor role in GC.

In summary, our study reveals that lower expression of QKI in GC tissues leads to aberrant cell growth and thus contributes to cancer progression. In addition, the reduced QKI expression correlated well with poor differentiation status, depth of invasion, gastric lymph node metastasis, distant metastasis, advanced TNM stage and poor survival. All of these shed light on QKI as a novel diagnostic and therapeutic target of GC.

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