Original Article

RNA-binding protein QKI suppresses breast cancer via RASA1/MAPK signaling pathway

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Background: RNA-binding protein Quaking (QKI) has been linked with the pathogenesis and development of various human malignancies. Herein, we explored the particular role of QKI in breast cancer (BC) progression.

Methods: The methods employed in the study included public dataset analysis, western blot, quantitative real-time PCR (qRT-PCR), cell count kit-8 (CCK8) assay, colony formation assay, flow cytometric analysis, RNA immunoprecipitation (RIP), messenger RNA (mRNA) stability assay, QKI overexpression and knockdown, and Ras p21 protein activator 1 (RASA1) knockdown.

Results: Aberrant expression levels of QKI and RASA1 were detected in BC and compared with those in noncancerous tissues. A moderately positive correlation between QKI and RASA1 was verified within BC tissues. Low expression of QKI was associated with positive estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status, non-triple-negative breast cancer (TNBC), non-basal-like BC, and poor clinical outcomes in BC patients. QKI overexpression suppressed BC cell proliferation and colony formation, and arrested cell cycle at G1 phase. RIP assay and mRNA stability assay confirmed that QKI directly bound to RASA1 transcript and increased its stability, thus inactivating the MAPK pathway and inhibiting BC progression. RASA1 knockdown could partly attenuate the inhibitory effect of QKI on BC cell proliferation via activating the mitogen-activated protein kinase (MAPK) pathway.

Conclusions: QKI, which was frequently downregulated in BC, could significantly inhibit cell proliferation and arrest cell cycle at G1 phase by binding and enhancing RASA1 mRNA expression. Low expression of QKI was prominently associated with unfavorable clinical outcomes in BC patients, indicating the prognostic value of QKI in BC.

Keywords: Breast neoplasms; cell cycle; cell proliferation; RNA-binding proteins

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Introduction

Breast cancer (BC) is the most commonly diagnosed malignancy and the main cause of cancer-associated mortality among women worldwide (1). Despite the great achievements in screening, diagnosis and treatment regimens, prognosis for a high percentage of BC patients remains poor. In this regard, identification of genes and underlying mechanisms in BC progression is urgent. It has been clarified that post-transcriptional regulation can alter the expression and function of cancer driver genes, and regulate the biological properties of cancer cells. RNA-binding proteins are key players in post-transcriptional events. They interact with their RNA targets via specific RNA-binding domains and enable swift and robust adjustments of protein expression, leading to cancer initiation, progression and metastasis (2).

Quaking (QKI) belongs to the signal transduction and activation of RNA (STAR) family of RNA-binding proteins (3). The human *QKI* gene expresses three major, alternatively spliced, transcripts, namely QKI-5, QKI-6 and QKI-7. QKI-5 is the only isoform that is exclusively located in the nucleus, while QKI-6 and QKI-7 are distributed in the cytoplasm (4). As an RNA-binding protein, QKI post-transcriptionally regulates precursor messenger RNA (pre-mRNA) processing, mRNA export, mRNA stability and protein translation of target genes via binding to QKI response element (QRE; ACUAAY[N1-20] UAAY) (5-8). Recent studies employing crosslinking-immunoprecipitation and high-throughout sequencing (CLIP-seq) and bioinformatic analysis identified thousands of putative mRNA targets containing at least one QRE. Among these mRNA targets, *RAS*, *JUN*, *FOS* and *TP53* were implicated in cancers (6). Meanwhile, increasing evidence demonstrates that QKI might be a tumor suppressor in multiple malignancies, including colon, lung, oral and prostate cancers (9-12). These findings suggest a role for QKI protein in tumorigenesis and development. However, the role of QKI in BC remains to be elucidated.

In this study, we assayed QKI expression in BC by comparison to that in noncancerous tissues and examined the correlation of QKI expression with clinicopathological characteristics and patient survival. The biological function and molecular mechanism of QKI in BC cells were then determined. Our results shed new light on the fundamental role of QKI in BC, and identified QKI as a novel prognostic marker for BC patients. We present the following article in accordance with the Materials Design Analysis Reporting (MDAR) reporting checklist (available at http://dx.doi.org/10.21037/atm-20-4859).

Methods

Clinical sample collection

Ninety-six BC tissues paired with adjacent mammary epithelial tissues were collected from female patients who underwent surgery at Huashan Hospital from January 2017 to December 2018. These patients ranged in age from 18 to 75 years old. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Ethics Committee of Huashan Hospital (approval number: 2016-013). Every patient had provided written informed consent.

BC expression and survival analysis in public datasets

The Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) is an interactive web server for analyzing RNA-sequencing data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects (13). GEPIA was used to identify the differential expression of QKI in BC and normal mammary tissues. The BC Gene-Expression Miner v4.3 (bc-GenExMiner v4.3; http://bcgenex.centregauducheau.fr/) is a statistical mining tool of published annotated cancer transcriptomic data and RNA-seq (14). The bc-GenExMiner v4.3 was used to analyze the correlation between QKI mRNA expression and clinicopathological parameters of BC patients. The PrognoScan database (http://www.prognoscan.org/) is a web-based platform. It was utilized to evaluate the relationship between QKI expression and prognosis in BC patients (15). Hazard ratios (HR), 95% confidence intervals (CI) and Cox P values were automatically calculated by the website.

Cell culture and transfection

Human BC cell lines MCF-7 and T-47D were purchased from the Chinese Institute of Biochemistry and Cell Biology (Shanghai, China). MCF-7 and T-47D cells were respectively maintained in Dulbecco's Modified Eagle's Medium and RPMI 1640 Medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37 ℃ with 5% CO_2. QKI overexpression and knockdown lentivirus vectors, along with the scramble controls, were purchased from GeneChem (Shanghai, China) and named as lv-QKI, lv-

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NC, sh-QKI and sh-NC. The target sequence of sh-QKI was as follows: 5'-CTGATGCTGTGGGACCTATTG-3'. Cells were transfected with lentivirus vectors for 48 hours and further selected using 2 μg/mL puromycin (Selleckchem, Houston, TX, USA). The efficiency of QKI overexpression and knockdown was validated by western blot and quantitative real-time PCR (qRT-PCR).

Western blot analysis

Total protein was harvested using RIPA lysis buffer (Beyotime, Shanghai, China), separated by sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membranes. Blots were incubated with antibodies and then visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The primary and secondary antibodies used in this study were listed as follows: anti-GAPDH (60004-1-Ig, Proteintech, Rosemont, IL, USA), anti-QKI (A300-183A, Bethyl Laboratories, Inc., Montgomery, TX, USA), anti-Flag (F1804, Sigma-Aldrich, St. Louis, MO, USA), anti-RASA1 (ab40677, Abcam, Cambridge, MA, USA), anti-CDK2 (2546), anti-CDK4 (12790), anti-CDK6 (13331), anti-cyclin D1 (2978), anti-cyclin E1 (20808), anti-p21 Waf1/Cip1 (2947), anti-p27 Kip1 (3686), anti-Erk1/2 (4695), anti-phospho-Erk1/2 (Thr202/Tyr204) (4370), anti-p38 MAPK (8690), anti-phospho-p38 MAPK (Thr180/Tyr182) (4511) (Cell Signaling Technology, Danvers, MA, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized using PrimeScript RT Master Mix (TaKaRa, Dalian, China) and qRT-PCR was subsequently performed on StepOne Plus PCR System using SYBR Green kit (Yeasen, Shanghai, China). GAPDH gene was detected for normalization of data. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta ct}$ method.

Cell proliferation assay

Cell growth was detected by cell count kit-8 (CCK8) assay and colony formation assay. For the CCK8 assay, 3×10^3 cells were seeded per well in quintuplicate in 96 well plates; 10 μL CCK8 reagent (Yeasen, Shanghai, China) was added to each well and incubated for 2 hours at 37 ℃. Absorbance of each well was measured at 450 nm at indicated time points. For the colony formation assay, cells were plated into 35 mm dishes at a density of 5×10^2 cells per dish and cultured for 2 weeks. Colonies were fixed in methanol, stained with 0.1% crystal violet solution (Beyotime, Shanghai, China), and counted using a microscope at 40× magnification.

Cell cycle distribution

Cells were starved for 24 hours and then cultured in fresh medium with 10% FBS for 24 hours. Cells were harvested and fixed in 75% ethanol overnight at 4 ℃. Then cells were suspended and incubated in propidium iodide (PI)/RNase staining buffer (BD Biosciences, San Jose, CA, USA) for 15 minutes in the dark. Cell cycle distribution was analyzed using flow cytometer (BD Biosciences, San Jose, CA, USA).

Cell apoptosis analysis

Apoptotic rates were determined using a phycoerythrin (PE)-labeled Annexin V/7-aminoactinomycin D (7-AAD) apoptosis detection kit (Yeasen, Shanghai, China) following the standard procedure. Cells were gently trypsinized, washed and suspended in 500 μL Annexin V binding buffer, and then treated with 5 μL Annexin V and 2.5 μL 7-AAD. After incubation for 15 minutes in the dark, samples were assessed using the same flow cytometer.

RNA immunoprecipitation (RIP) assay

Detailed procedure was described elsewhere (16). Briefly, cell lysates were obtained and incubated with Protein A Dynabeads (Life Technologies, Carlsbad, CA, USA) precoated with anti-Flag antibody (Sigma-Aldrich, St. Louis, MO, USA) or control mouse immunoglobulin G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). RNA was extracted using TRIzol reagent, followed by detecting of RASA1 mRNA via qRT-PCR.

mRNA stability assay

QKI expression was upregulated via transfecting with lentivirus vectors for 48 hours. Then *de novo* RNA synthesis was blocked with 5 μg/mL actinomycin D (Apexbio, Houston, TX, USA). Total RNA was harvested at 0, 2, 4, 6, 8 and 10 hours. RASA1 mRNA expression at indicated times was measured by qRT-PCR and then compared

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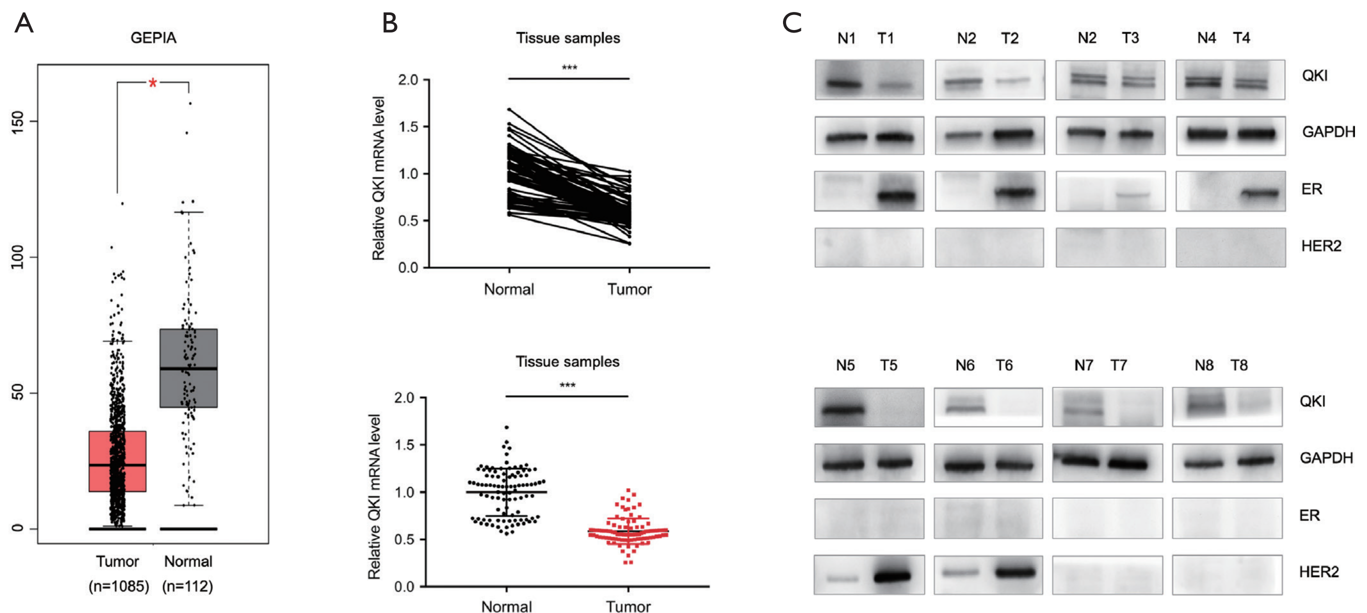

Figure 1 Downregulation of QKI in BC and its clinical significance. (A) Expression profile of QKI in BC and normal mammary tissues (data from GEPIA). (B) QKI mRNA expression in 96 paired primary BC samples and adjacent normal tissues (C) QKI protein level in 10 BC tissues comparing to paired noncancerous tissues. *, P<0.05; ***, P<0.001. GEPIA, Gene Expression Profiling Interactive Analysis; QKI, Quaking; N, normal; T, tumor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

to RASA1 mRNA level before adding actinomycin D. The half-life ($T_{1/2}$) of the RASA1 transcript was further calculated via curve fit analysis.

RNAi treatment

Small interfering RNA (siRNA) was purchased from GenePharma (Shanghai, China). The target sequences were listed as follows: si-RASA1-1 sense: 5'-CCACCGACAUUGAGAUAUATT-3', antisense: 5'-UAUAUCUCAAUGUCGGUGGTT-3'; si-RASA1-2 sense: 5'-CUGGCGAUUAUUCACUUUATT-3', antisense: 5'-UAAAGUGAAUAAUCGCCAGTT-3'. Cells were treated with 100 nM siRASA1 solution and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 6 hours, and then incubated in fresh medium.

Statistical analysis

Each experiment was performed in triplicate and repeated 3 times. Quantitative data was presented as mean ± standard deviation (SD) and analyzed with GraphPad Prism 7.0 (La Jolla, CA, USA). Student's *t*-test and one-way analysis of variance were conducted for comparisons among the groups. Pearson's correlation coefficient was calculated to analyze the association between QKI and RASA1 mRNA. A P value <0.05 was considered statistically significant.

Results

Downregulation of QKI predicted poor prognosis in BC

We first explored the expression of QKI in BC using the GEPIA database. QKI was significantly downregulated in BC by comparison to that in noncancerous tissues (*Figure 1A*). We then detected the QKI expression level in 96 pairs of tissue samples using qRT-PCR and western blot. The results revealed that both QKI mRNA and protein levels were markedly reduced in BC tissues (*Figure 1B,C*). Survival curve analyses illustrated that low QKI level was remarkably correlated with unfavorable clinical outcomes in BC patients (*Figure 2*, *Table 1*), suggesting that QKI might be predictive of prognosis in BC patients. To unveil the association between QKI expression and patients' clinicopathological characteristics, data from

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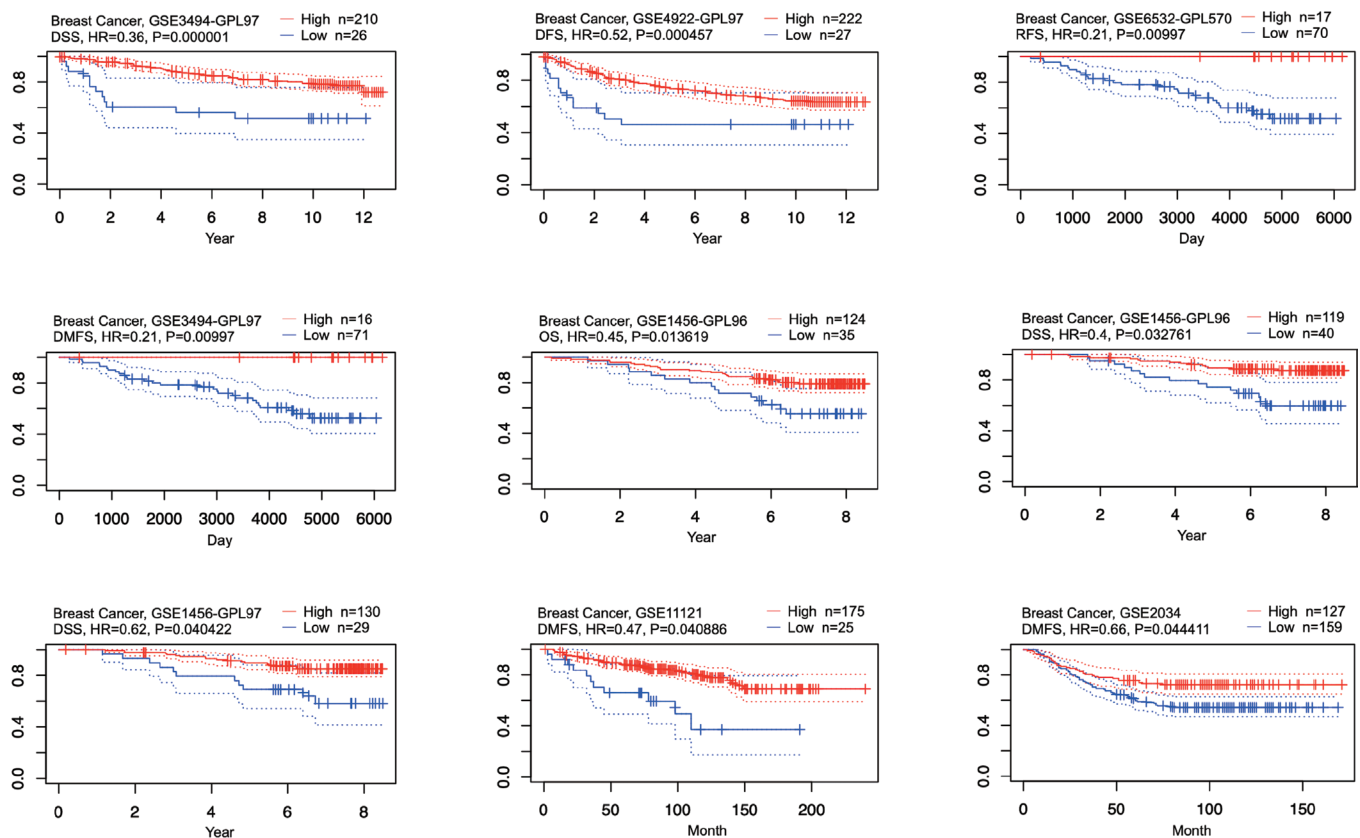

Figure 2 Low expression of QKI indicated poor prognosis of BC patients (data from PrognoScan). HR, hazard ratio; DSS, disease-specific survival; DFS, disease-free survival; RFS, relapse-free survival; DMFS, distant metastasis-free survival; OS, overall survival.

Table 1 The expression of QKI and survival rates in BC patients

Dataset	Endpoint	Probe ID	Sample number	Cox P value	HR	95% CI
GSE3494-GPL97	Disease specific survival	236154_at	236	0.000001	0.36	0.24–0.54
GSE4922-GPL97	Disease free survival	236154_at	249	0.000457	0.52	0.36–0.75
GSE6532-GPL570	Relapse free survival	236154_at	87	0.009970	0.21	0.06–0.69
GSE6532-GPL570	Distant metastasis free survival	236154_at	87	0.009970	0.21	0.06–0.69
GSE1456-GPL96	Overall survival	212265_at	159	0.013619	0.45	0.24–0.85
GSE1456-GPL96	Disease specific survival	212636_at	159	0.032761	0.40	0.18–0.93
GSE1456-GPL97	Disease specific survival	236154_at	159	0.040422	0.62	0.39–0.98
GSE11121	Distant metastasis free survival	212262_at	200	0.040886	0.47	0.23–0.97
GSE2034	Distant metastasis free survival	212263_at	286	0.044411	0.66	0.44–0.99

QKI, Quaking; BC, breast cancer; HR, hazard ratio; CI, confidence interval.

bc-GenExMiner v4.3 was obtained and analyzed. The results showed that QKI expression level was significantly decreased in patients with positive estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status, non-basal-like BC and non-triple-negative BC (TNBC) (*Figure 3*, *Table 2*). Thus,

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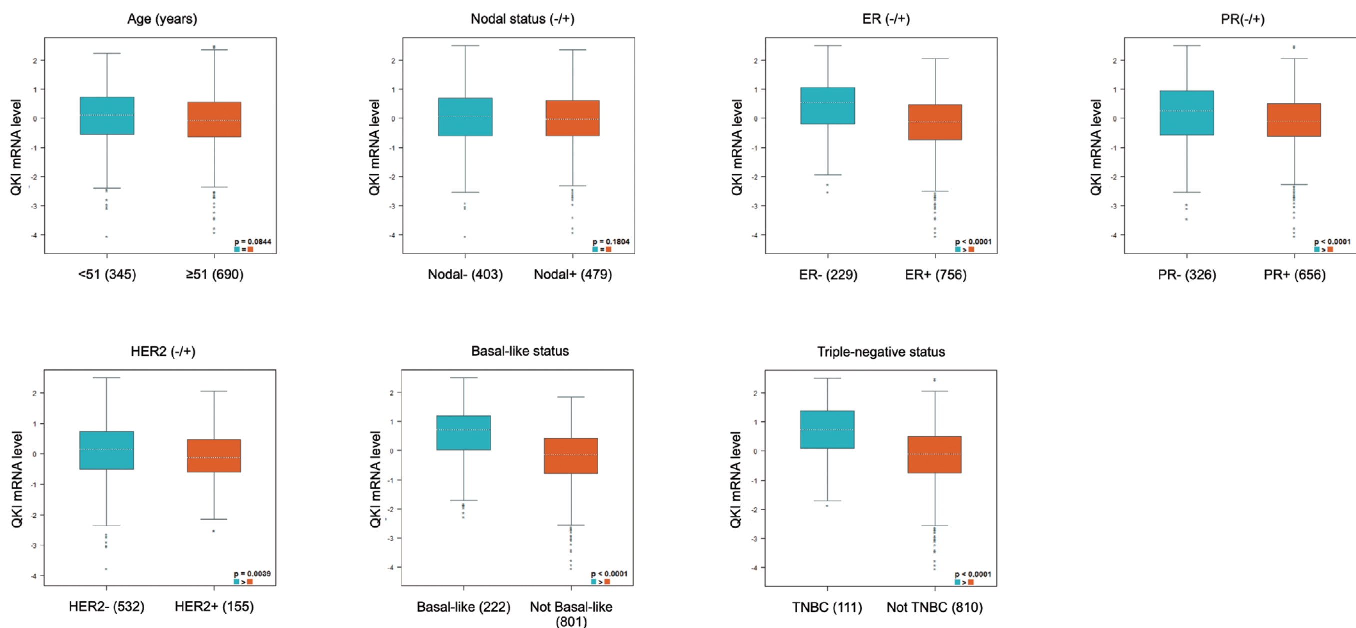

Figure 3 The correlations between QKI expression and clinicopathological features in BC patients were evaluated using bc-GenExMiner v4.3. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer.

we reached the tentative conclusion that QKI was one of the key molecules playing a pivotal role in BC.

QKI inhibited cell proliferation and blocked cell cycle in BC

To determine the biological functions of QKI in BC progression, QKI was stably overexpressed and knocked down in human BC cell lines MCF-7 and T-47D. The elevated and decreased expression of QKI was verified via western blot and qRT-PCR (*Figure 4A,B*). The CCK8 curve showed that exogenous expression of QKI remarkably suppressed the viability of BC cells. Consistently, cell growth was significantly promoted when QKI was silenced (*Figure 4C*). QKI overexpression prominently reduced both number and size of cell colonies, while QKI downregulation resulted in more colony formation (*Figure 4D,E*). Our findings demonstrated that QKI inhibited the proliferation of BC cells. To further elaborate the effect of QKI on cell growth, flow cytometry was performed to explore cell cycle distribution in QKI-overexpressed cells. Ectopic expression of QKI caused cell cycle arrest and increased the percentage of cells in G1 phase (*Figure 5A,B*). The expression levels of key regulators in G1/S phase transition were detected via western blot and qRT-PCR. The protein levels of CDK2, CDK4, CDK6, cyclin D1 and cyclin E1 were reduced in QKI-overexpressed cells, while p27 Kip1 and p21 Waf/Cip1 protein levels were elevated (*Figure 5C*). The results of qRT-PCR were in parallel with those of western blot (*Figure 5D*). Subsequently, the effect of QKI on cell apoptosis was analyzed and there was no significant difference in apoptotic rates between the overexpression groups and control groups (data not shown). These results suggested that QKI might suppress cell growth through cell cycle blockage rather than cell apoptosis induction. Taken together, the aforementioned results confirmed the suppressive role of QKI in BC.

QKI physically interacted with RASA1 mRNA and enhanced its stability

Acting as an RNA-binding protein, QKI modulates the expression of mRNA targets at the post-transcriptional level. Previous studies identified cell growth and/or maintenance as the primary categories of potential mRNA targets of QKI via gene ontology annotation (6), emphasizing QKI as a key regulator in cell proliferation and cell cycle. Therefore, we scrutinized both coding and noncoding regions of cell proliferation- and cell cycle-associated genes, which contained putative QREs. Ras p21 protein activator 1 (RASA1) was identified as an appealing candidate that met the above criteria. RASA1 is a member

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Table 2 The association between QKI mRNA expression and clinicopathological parameters in BC

Variable	Case	mRNA expression	P value
Age (years)			0.0844
≤51	345	–	
>51	690	↓	
Nodal status			0.1804
Positive	479	↓	
Negative	403	–	
ER (IHC)			<0.0001
Positive	756	↓	
Negative	229	–	
PR (IHC)			<0.0001
Positive	656	↓	
Negative	326	–	
HER2 (IHC)			0.0039
Positive	155	↓	
Negative	532	–	
Basal-like status			<0.0001
Not basal-like	801	↓	
Basal-like	222	–	
Triple-negative status			<0.0001
Not TNBC	810	↓	
TNBC	111	–	

QKI, Quaking; BC, breast cancer; ER, estrogen receptor; IHC, immunohistochemistry; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer.

of GAP1 family of GTPase activating proteins and a negative modulator of Ras protein. It stimulates the weak intrinsic GTPase activity of Ras protein, and results in the inactive GDP-bound form of Ras, thereby regulating cellular proliferation and differentiation (17). Western blot and qRT-PCR analyses were performed to examine whether the expression of RASA1 was altered in accordance with QKI overexpression. Ectopic expression of QKI increased both mRNA and protein levels of RASA1 in MCF-7 and T-47D cells (*Figure 6A*). Further, the RASA1 level was detected by qRT-PCR in paired tissue samples, and the relevance between QKI and RASA1 was confirmed. The expression of RASA1 mRNA was 2.09-fold higher in BC comparing with noncancerous tissues (*Figure 6B*), indicating that QKI and RASA1 had similar expression patterns in BC. Meanwhile, the level of RASA1 mRNA was moderately correlated with QKI, with a relevance ratio of 0.4568 (*Figure 6C*) (P<0.001). These findings suggested that RASA1 might be a downstream target of QKI in BC. RIP assay was then carried out and the result demonstrated that QKI protein directly bound to RASA1 mRNA (*Figure 6D,E*). In addition, *de novo* mRNA synthesis was blocked in QKI-overexpressed cells with actinomycin D treatment. The decay rate of RASA1 mRNA in QKI-overexpressed cells was slower than that in the corresponding control cells (*Figure 6F*) ($T_{1/2}$=5.01 *vs.* 7.89 hours, P<0.001). Collectively, these results revealed that QKI upregulated the expression of RASA1 in BC cells, at least in part, via stabilizing the mRNA of RASA1.

Knockdown of RASA1 abolished QKI-inhibited cell proliferation via MAPK pathway

Previous studies reported the inhibitory effect of RASA1 in numerous solid tumors (18,19); however, its precise role in BC is unclear. Based on the results that QKI elevated the expression of RASA1 via stabilizing its mRNA, we further investigated the role of QKI-driven RASA1 expression in BC progression. QKI-overexpressed cells and control cells were treated with siRNA targeting RASA1, and the knockdown efficiency was validated by western blot and qRT-PCR.

The mitogen-activated protein kinase (MAPK) pathway, a crucial transmitter of extracellular signals from cell surface to intracellular components, participates in various cellular activities, including cell proliferation, differentiation, apoptosis and metastasis (20,21). Previous studies revealed that MAPK pathway promoted cell growth and metastasis in BC, and agents targeting the MAPK pathway might be effective for BC treatment (22-24). Given that RASA1 was a negative modulator of the MAPK pathway, we assessed several critical kinases in the MAPK pathway. Overexpression of QKI suppressed the phosphorylation of Erk1/2 and p38 MAPK, while total Erk1/2 and p38 MAPK expression was hardly changed. RASA1 silencing largely restored the attenuated phosphorylation level of Erk1/2 and p38 MAPK in QKI-overexpressed cells (*Figure 7A,B*). Cell proliferation capacity was further determined. The CCK8 and colony formation assays demonstrated that RASA1 silencing abrogated the inhibitory effect of QKI on cell growth (*Figure 7C,D,E*). Taken together, QKI suppressed

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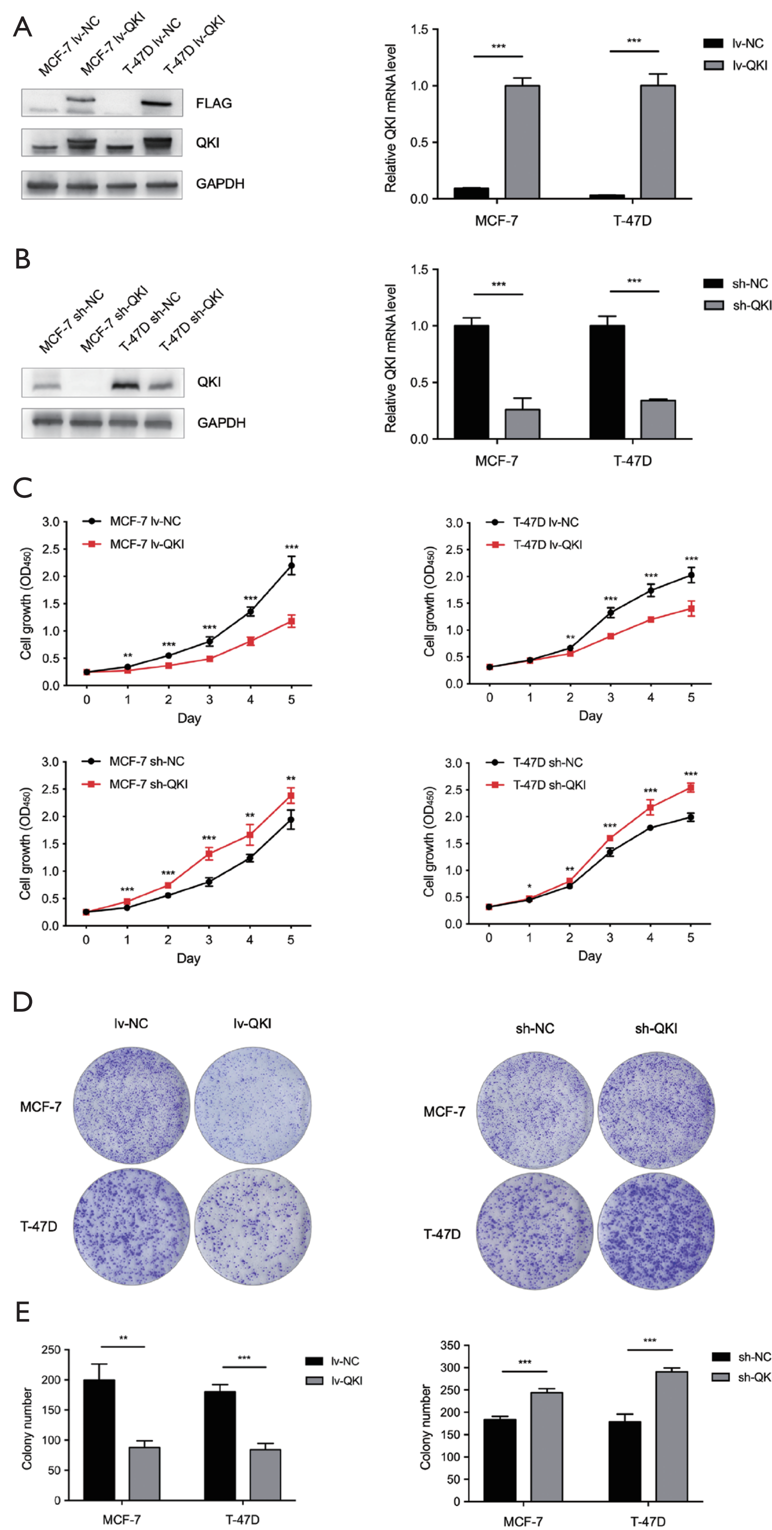

Figure 4 QKI suppressed the proliferation capacity of BC cells. (A,B) Overexpression and knockdown efficiency of QKI in MCF-7 and T-47D cells was determined by western blot and qRT-PCR. (C,D) QKI significantly repressed cell growth and colony formation of MCF-7 and T-47D cells (×40). (E) Quantification of colonies in colony formation assay. Colonies over 100 cells were scored. *, P<0.05; **, P<0.01; ***, P<0.001. NC, negative control; QKI, Quaking; BC, breast cancer; OD_{450}, optical density at 450 nm.

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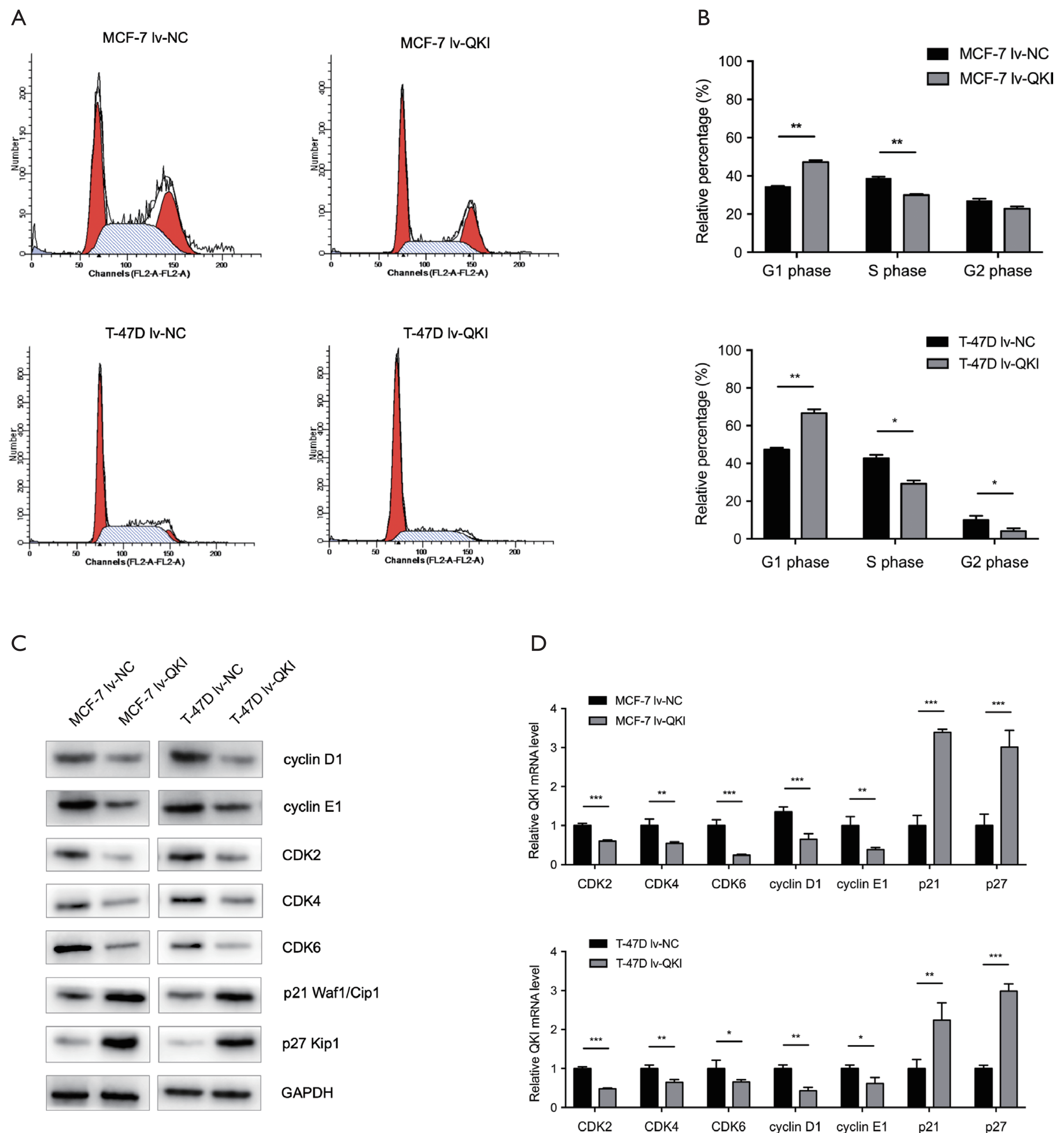

Figure 5 QKI blocked BC cell cycle progression at G1 phase. (A) Effect of QKI on cell cycle progression was detected by flow cytometry. (B) Cell cycle distribution showed that QKI overexpression induced cell cycle arrest at G1 phase. (C,D) Western blot and qRT-PCR analysis of cyclin-dependent kinases and cyclins in indicated BC cells. Data were plotted relative to expression levels in control groups. *, P<0.05; **, P<0.01; ***, P<0.001. NC, negative control; QKI, Quaking; CDK, cyclin-dependent kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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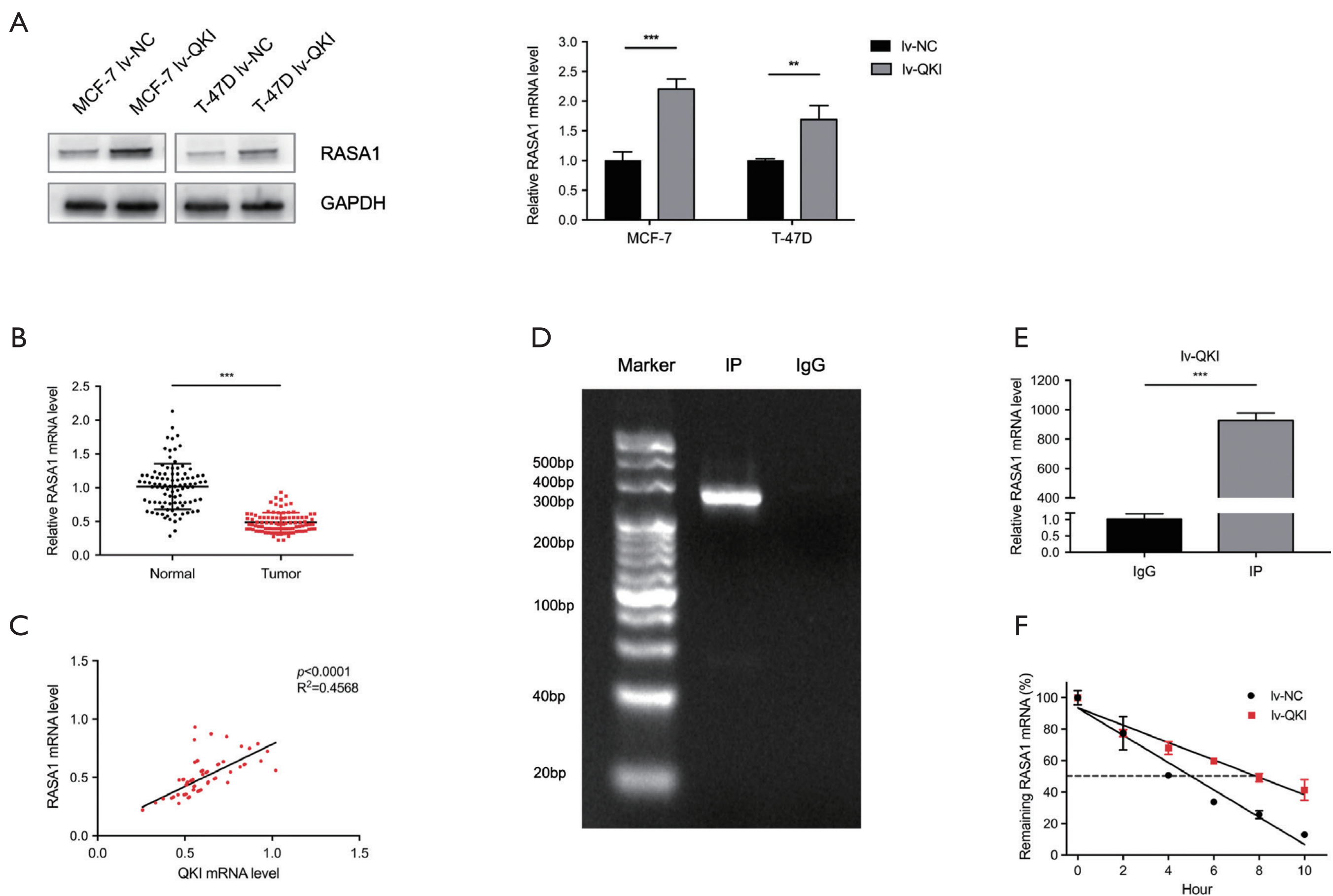

Figure 6 QKI exerted inhibitory effects through RASA1-mediated inactivation of MAPK pathway. (A) QKI overexpression elevated RASA1 mRNA and protein levels in BC cells. (B,C) Expression of RASA1 and clinical relevance between QKI and RASA1 were examined in 96 paired tissue samples. (D,E) Cells with QKI overexpression were immunoprecipitated using anti-Flag antibody or negative control IgG. The presence of RASA1 mRNA in the immunoprecipitate was determined via ethidium bromide staining and qRT-PCR. (F) QKI-overexpressed cells and corresponding controls were treated with 5 μg/mL actinomycin D for the indicated times. RASA1 mRNA level was detected by qRT-PCR and the decay rates were calculated. **, P<0.01, ***, P<0.001. NC, negative control; QKI, Quaking; RASA1, Ras p21 protein activator 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitate.

the malignant phenotype of BC cells, at least in part, via QKI/RASA1/MAPK pathway.

Discussion

Post-transcriptional regulation can alter the expression and function of cancer-associated genes, therefore modulating multiple cancer traits. RNA-binding proteins are vital components in the post-transcriptional regulatory network. They modulate the expression and function of target genes via their RNA-binding domains, and are involved in virtually all steps of RNA metabolism (2). Perturbations in interactions between RNA-binding proteins and their RNA targets are closely related to cancer progression. Therefore, deciphering the interaction network of RNA-binding proteins and their RNA targets will help in better understanding tumor biology.

QKI is an RNA-binding protein which was firstly identified in the central and peripheral nervous system, where it regulates pre-mRNA processing, mRNA stability, subcellular localization and translation of target genes (5-8). Aberrant expression of QKI was reported in numerous tumors (9-12,25), and indicated poor survival in lung, prostate and gastric cancers (10,12,26). QKI impaired

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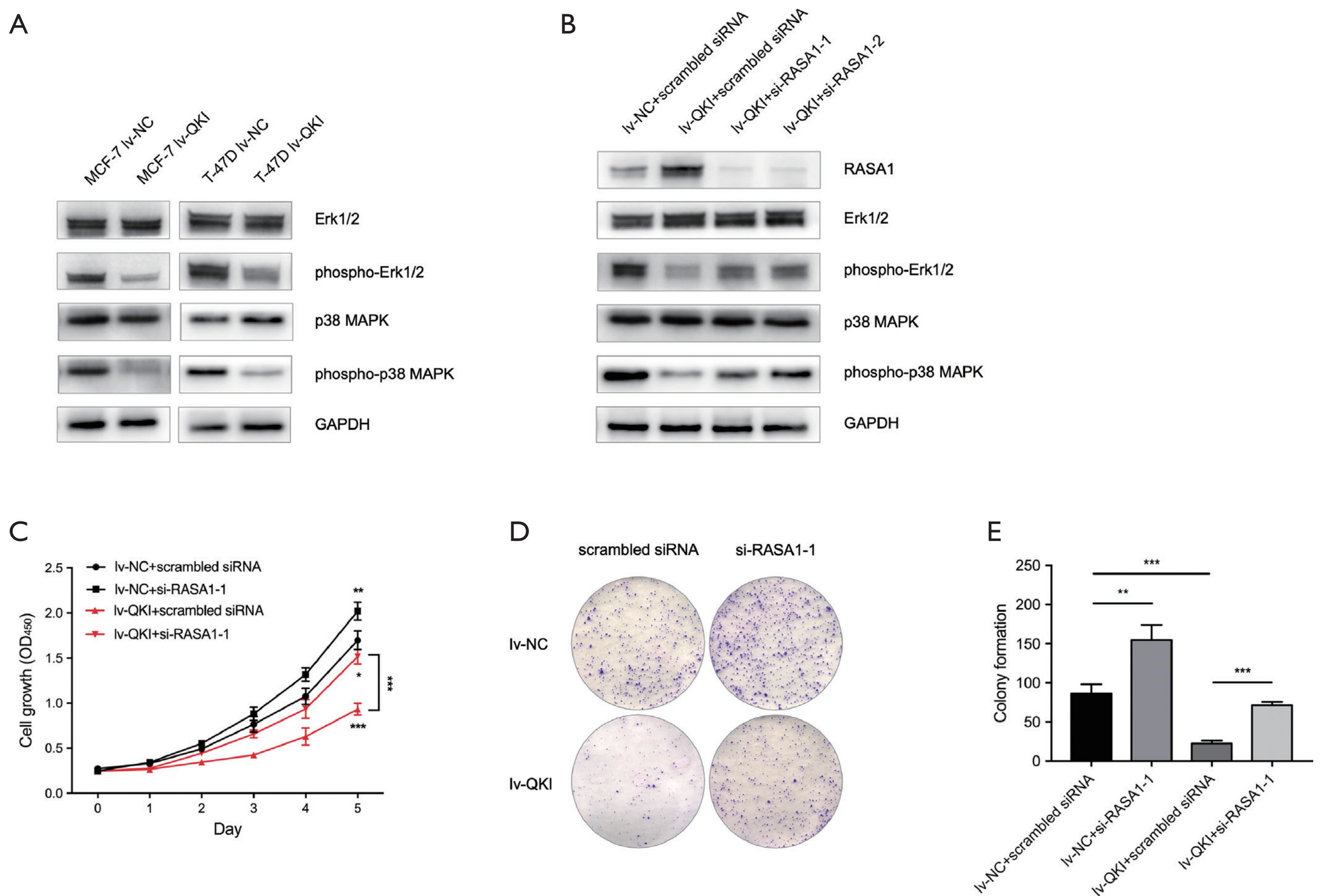

Figure 7 Knockdown of RASA1 eliminated QKI-inhibited cell proliferation in BC. (A) Expression levels of phosphorylated Erk1/2, total Erk1/2, phosphorylated p38 MAPK and total p38 MAPK in QKI-overexpressed cells and control cells. (B) RASA1 knockdown restored the phosphorylation level of the MAPK pathway in QKI-overexpressed MCF-7 cells. (C,D) Cell proliferation and colony formation ability were examined in QKI-overexpressed MCF-7 cells with RASA1 knockdown (×40). (E) Quantification of colonies in colony formation assay. Colonies over 100 cells were scored. *, P<0.05; **, P<0.01; ***, P<0.001. NC, negative control; QKI, Quaking; MAPK, mitogen-activated protein kinase; RASA1, Ras p21 protein activator 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OD450, optical density at 450 nm.

self-renewal of cancer cells and inhibited epithelial-to-mesenchymal transition in oral squamous call carcinoma (11,27). QKI regulated cell cell contact inhibition and suppressed the progression of clear cell renal cell carcinoma (28). However, the particular role of QKI in BC development remains unclear.

The Ras family members belong to a class of small GTPase proteins. They switch between the active GTP-bound and inactive GDP-bound states in response to intracellular signals, thus accelerating tumor progression and promoting its aggressiveness. As a Ras family member, RASA1 acts as a negative regulator of Ras. Declined expression or activity of RASA1 can activate the MAPK signaling pathway by reducing the GTPase activity of Ras protein, thus increasing cell proliferation, suppressing cell apoptosis, and modulating cell cycle distribution (17). Altered RASA1 expression is implicated in various human cancers, and accumulative evidence suggests that RASA1 functions as a tumor suppressor (18,19). A previous study identified RASA1 as an unfavorable prognosis marker in BC (29); however, the biological function and detailed mechanism of RASA1 in BC requires further investigation.

In the present study, we measured QKI expression and determined its clinical significance in BC. The effect of QKI on BC biology and the underlying mechanism were further investigated. We first demonstrated the downregulation

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of QKI mRNA and protein in BC. Decreased expression of QKI was remarkably associated with positive ER, PR and HER2 status, non-basal-like BC and non-TNBC; meanwhile, patients with low QKI expression had poorer survival, suggesting the prognostic value of QKI in BC patients. Ectopic expression of QKI suppressed cell growth and colony formation capacity, but induced cell cycle arrest at G1 phase. In contrast, QKI knockdown possessed the opposite effect on BC cells *in vitro*. In contrast to previous research in which QKI was identified as an oncogene in esophageal squamous cell cancer (30) and pancreatic cancer (31), our current data suggested the suppressive role of QKI in BC. Considering that QKI is an RNA-binding protein and its function greatly depends on the cellular environment and the accessibility of its RNA targets, it is reasonable to assume that QKI functions distinctly in different tumors.

RASA1 was selected as a potential downstream target of QKI due to the putative QRE. Herein, we observed the downregulation of RASA1 in BC clinical samples and a positive correlation between QKI and RASA1 expression. Both mRNA and protein levels of RASA1 could be elevated by the overexpression of QKI. QKI overexpression also attenuated the phosphorylation of MAPK signaling pathway. RIP and actinomycin D assay provided substantial evidence that QKI protein directly bound to RASA1 transcript and increased its stability. Accordingly, knockdown of RASA1 attenuated the growth inhibitory effect of QKI overexpression in BC cells via activation of MAPK pathway.

Conclusions

In summary, our current data provided initial evidence that QKI directly bound to RASA1 mRNA and enhanced RASA1 expression by stabilizing its transcript, thus inhibiting the activation of MAPK pathway and BC progression. Low expression of QKI was significantly associated with poor survival in BC patients, indicating the predictive utility of QKI in BC progression.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Ethics Committee of Huashan Hospital (approval number: 2016-013). Every patient had provided written informed consent.

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