QKI, a miR-200 target gene, suppresses epithelial-to-mesenchymal transition and tumor growth

Eun Ju Kim, Jeong Seon Kim, Sieun Lee, Heejin Lee, Jung-Sook Yoon, Ji Hyung Hong, Sang Hoon Chun, Der Sheng Sun, Hye Sung Won, Soon Auck Hong, Keunsoo Kang, Jeong Yeon Jo, Minyoung Choi, Dong Hoon Shin, Young-Ho Ahn and Yoon Ho Ko

1Department of Molecular Medicine and Tissue Injury Defense Research Center, College of Medicine, Ewha Womans University, Seoul, South Korea
2Cancer Research Institute, College of Medicine, The Catholic University of Korea, Seoul, South Korea
3Clinical Research Laboratory, Uijeongbu St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Uijeongbu, Gyeonggi, South Korea
4Division of Oncology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, South Korea
5Department of Pathology, College of Medicine, Chung-Ang University, Seoul, South Korea
6Department of Microbiology, College of Natural Sciences, Dankook University, Cheonan, Chungnam, South Korea
7Research Institute and Department of Cancer Biomedical Science, Graduate School of Cancer Science and Policy, National Cancer Center, Goyang, Gyeonggi, South Korea

The microRNA-200 (miR-200) family plays a major role in specifying epithelial phenotype by preventing expression of the transcription repressors ZEB1 and ZEB2, which are well-known regulators of the epithelial-to-mesenchymal transition (EMT) in epithelial tumors including oral squamous cell carcinoma (OSCC). Here, we elucidated whether miR-200 family members control RNA-binding protein quaking (QKI), a newly identified tumor suppressor that is regulated during EMT. We predicted that miR-200a and miR-200b could recognize QKI 3′-UTR by analyzing TargetScan and The Cancer Genome Atlas head and neck squamous cell carcinoma (HNSCC) dataset. Forced expression of miR-200/a/429 inhibited expression of ZEB1/2 and decreased cell migration in OSCC cell lines CAL27 and HSCC. QKI expression was also suppressed by miR-200 overexpression, and the 3′-UTR of QKI mRNA was directly targeted by miR-200 in luciferase reporter assays. Interestingly, shRNA-mediated knockdown of QKI led to pronounced EMT and protumor effects in both in vitro and in vivo studies of OSCC. Furthermore, high expression of QKI protein is associated with favorable prognosis in surgically resected HNSCC and lung adenocarcinoma. In conclusion, QKI increases during EMT and is targeted by miR-200; while, it suppresses EMT and tumorigenesis. We suggest that QKI and miR-200 form a negative feedback loop to maintain homeostatic responses to EMT-inducing signals.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the seventh leading cancer by incidence, with approximately 600,000 new cases worldwide every year. Oral squamous cell carcinoma (OSCC), the most common type of HNSCC, has early recurrence or metastasis and high mortality rate even after curative treatment. Despite the increasing knowledge of tumor biology, HNSCC treatment efficacy has not significantly improved over the past decade. Thus, it is crucial to clarify the molecular mechanisms for OSCC progression to identify optimal targets and predictive biomarkers for treatment.

Key words: miRNA-200, QKI, epithelial-to-mesenchymal transition, oral cancer, lung cancer

Abbreviations: 3′-UTR: 3′-untranslated region; EMT: epithelial-to-mesenchymal transition; GSEA: gene set enrichment analysis; HNSCC: head and neck squamous cell carcinoma; LUAD: lung adenocarcinoma; miRNA: microRNA; OS: overall survival; OSCC: oral squamous cell carcinoma; qRT-PCR: quantitative reverse transcription-PCR; TCGA: The Cancer Genome Atlas; TMA: tissue microarray

Additional Supporting Information may be found in the online version of this article.

Conflict of Interest: The authors declare that they have no competing interests.

Grant sponsor: Catholic Medical Center Research Foundation; Grant sponsor: National Research Foundation of Korea (Ministry of Science and ICT); Grant numbers: NRF-2015R1C1A1A01054591, 2010-0027945; Grant sponsor: National Research Foundation of Korea (Ministry of Education); Grant number: NRF-2016R1D1A1B03930068

4E.J.K. and J.S.K. contributed equally to this work

DOI: 10.1002/ijc.32372

History: Received 26 Oct 2018; Accepted 17 Apr 2019; Online 26 Apr 2019

Correspondence to: Young-Ho Ahn, Department of Molecular Medicine, College of Medicine, Ewha Womans University, 808-1 Magok-dong, Gangseo-gu, Seoul 07804, South Korea, Tel.: +82-2-6986-6268, E-mail: yahn@ewha.ac.kr; Yoon Ho Ko, Division of Oncology, Department of Internal Medicine, Uijeongbu St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, 271 Cheonbo-ro, Uijeongbu-si, Gyeonggi 11765, South Korea, Tel.: +82-31-820-3985, Fax: +82-31-847-2719, E-mail: koyoonho@catholic.ac.kr

Int. J. Cancer: 00, 00–00 (2019) © 2019 UICC
Epithelial-to-mesenchymal transition (EMT), thought to play a fundamental role during tumorigenesis, is associated with poor histologic differentiation, local invasiveness and distant metastasis in various cancers, and it renders resistance to cancer treatment. In HNSCC, the EMT-related gene expression signature is also associated with metastatic potential and cancer progression. EMT is a reversible cellular process that is elaborately controlled by extracellular matrix (e.g., collagens and laminins), adhesion molecules (e.g., E-/N-cadherin and integrins), cytoskeletal proteins (e.g., vimentin), growth factor receptors (e.g., TGFβ receptors and EGFR), transcription factors (e.g., ZEB1/2 and SNAIL1/2), and noncoding RNAs including microRNAs (e.g., miR-200 and miR-34).

MicroRNAs (miRNAs) are a class of small (19–22 nucleotides), endogenous and noncoding RNAs that suppress gene expression by forming base pairs with target mRNAs and thereby regulate various pathophysiological processes implicated in cancer development and progression. Among miRNAs, miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) act as key inhibitors of EMT by directly targeting transcriptional repressors of E-cadherin, ZEB1 and ZEB2. Interestingly, ZEB1 binds to the promoter regions of both miR-200 genomic clusters (miR-200b/200a/429 and miR-200c/141) and suppresses their transcription, suggesting that miR-200 and ZEB1 form a double-negative feedback loop to control cell migration, invasion and EMT. miR-200 also targets VEGF and VEGF-R1, which play important roles during angiogenesis in the tumor microenvironment. Interplay between miR-200 and its target genes has been reported to control cancer cell migration, invasion, EMT and cancer progression in HNSCC. Therefore, identification of novel targets of miR-200 and elucidation of their functions during tumorigenesis and metastasis would provide an opportunity to achieve long-term survival for patients diagnosed with metastatic HNSCC.

TargetScan (http://www.targetscan.org), a common miRNA target prediction tool, was used to identify novel miR-200 target genes. Putative targets whose expression patterns showed inverse correlations with miR-200 in The Cancer Genome Atlas (TCGA) HNSCC data were then filtered out. Through these approaches, 20 candidate target genes of miR-200 were obtained, and most of them were confirmed to be downregulated by forced expression of miR-200 in OSCC cells. Among these candidates, QKI was selected for further investigation. QKI is a member of the signal transduction and activation of RNA (STAR) protein family and is a key posttranscriptional regulator. With a series of experiments, we elucidated the mechanism of QKI regulation by miR-200 and the effects of QKI and miR-200 on cancer cell invasion, EMT and tumor development.

Materials and Methods

miRNA target prediction

Processed (level 3) miRNA and mRNA expression data of HNSCC tumors were downloaded from the TCGA portal (http://cancergenome.nih.gov). Expression data for miRNA and mRNA were log2-transformed and mean-centered, and the standard deviation was normalized to 1 per array. Spearman's correlation coefficients were calculated for all possible combinations of entries between miR-200 and genes. Values of p were also generated and adjusted with Benjamini and Hochberg corrections (q-values). The cutoff for negative correlations between miRNA and gene pairs was determined to be q-value of 0.01. A sequence complementarity approach was then used to exploit the direct interaction between miR-200 and its mRNA targets using predicted target lists identified by TargetScan (http://www.targetscan.org).

Cell culture

Human OSCC cell lines (YD8 and YD10B) and human lung cancer cell lines (H1299, A549 and HCC827) were cultured in RPMI 1640 (Welgene, Gyeongsan, Korea) and CAL27, HSC3 and SCC25 OSCC cell lines were cultured in Dulbecco’s modification of Eagle’s medium (Welgene) with 10% fetal bovine serum (HyClone, Logan, UT) at 37°C in the presence of 5% CO2. YD8 and YD10B were purchased from Korean Cell Line Bank (Seoul, Korea), and HSC3 was from JCRB Cell Bank (Osaka, Japan). CAL27 and SCC25 were from ATCC (Manassas, VA). For cell growth analyses, cells (1–2 × 10⁴/well) were plated in 24-well plates and counted after the indicated number of days using a LUNA™ automated cell counter (Logos Biosystems, Anyang, Korea). For scratch assays, artificial scratches were made when cells became confluent in six-well plates, and wound area was measured using Image J (http://imagej.nih.gov/ij) at the indicated time. For actin staining, cells were cultured on coverslips coated with collagen (0.2 mg/ml) and then stained with Alexa Fluor® 594–phalloidin (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. A fluorescence microscope was used to capture fluorescent images of cell morphology. For miR-200 overexpression, genomic DNA
sequence containing pre-miR-200b/200a/429 was obtained from pLenti4.1_mirR-200b/200a/429 vector and subcloned into pLVX-NeO vector, which was modified from pLVX-Puro vector (Clontech, Mountain View, CA). pLVX-NeO_mirR-200b/200a/429 was introduced into cells by lentiviral infection. Lung cancer cells (A549 and HCC827) were transiently transfected with mir-200b mimic (Bioneer, Daejeon, Korea) using TransIT-X2 Dynamic Delivery System (Mirus Bio, Madison, WI) according to the manufacturer’s protocol. QKI shRNAs (MISSION® shRNA lentiviral vectors) were purchased from Sigma-Aldrich (St. Louis, MO) and introduced into cells by lentiviral infection. Human QKI cDNA (QKI-5 isoform, pCMV-SPORT6_QKI) was purchased from Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea, and inserted into pLVX-Puro vector with a FLAG-tag at the N-terminus.

Quantitative reverse transcription-PCR
Total RNA was isolated from cells with WelPrep Total RNA Isolation Reagent (Welgene) according to the manufacturer’s protocol. To analyze mRNA levels, quantitative reverse transcription-PCR (qRT-PCR) assays were performed using BioFACT A-Star Real-time PCR Kit including SFgreen® I (BioFACT, Daejeon, Korea) after reverse transcription with ELPIS RT Prime Kit (Elpis-Biotech, Daejeon, Korea). mRNA levels were normalized to that of mRNA for ribosomal protein L32 (RPL32). See Supporting Information Table S1 for primer sequences. mRNA levels were quantified using HB miR Multi Assay Kit™ (Heimbiotek, Seongnam, Korea) according to the manufacturer’s protocol and were normalized to level of RNU6B snoRNA.

Western blot
Cells were lysed with lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease/phosphatase inhibitors (Sigma-Aldrich). Cell lysates were separated by SDS-PAGE, transferred onto PVDF membrane, and then incubated with primary antibodies and HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA). Protein bands were visualized with PicoEPD (Enhanced Peroxidase Detection) Western Reagent Kit (Elpis-Biotech). Antibodies against QKI-5 isoform (Bethyl Laboratories, Montgomery, TX; #A300-183A-T), ZEB1 (Santa Cruz Biotechnology, Santa Cruz, CA; #sc-25388), vimentin (Santa Cruz Biotechnology, #sc-5565), E-cadherin (Santa Cruz Biotechnology, #sc-8426), N-cadherin (Santa Cruz Biotechnology, #sc-7939), FLAG-tag (MBL, Nagoya, Japan; #M185-3L) and actin (Bioworld Technology, St. Louis Park, MN; #BS6007M) were purchased.

Spheroid invasion assay
To create spheroids, cells (1–2 × 10^5 cells/5 ml) in 20% METHOCHEL™ (Sigma-Aldrich) and 1% Matrigel™ (BD Biosciences, Franklin Lakes, NJ) were hung on the lid of 15 cm dishes and incubated at 37°C for 2 days. Spheroid mixture (spheroids in 0.5× PBS, 0.01 N NaOH, 3 mg/ml collagen and spheroids) was then implanted in the center of each well of a 12-well plate. After gels were polymerized, wells were filled with cell culture media. A phase contrast microscope was used to capture images of invading cells. The invasion ratio was calculated by dividing total invading area by central spheroid area measured using Image J at the indicated time.

3’-UTR luciferase assay
A part of human QKI-5 isoform 3’-UTR (2,418 bp) containing putative mir-200-binding sites was amplified by PCR from HEP293T genomic DNA and ligated into pcI-neo-hRL vector. miR-200-binding sites of QKI 3’-UTR were predicted by TargetScan and mutated using a PCR-based site-directed mutagenesis method. Then, 3’-UTR reporters (500 ng) and pGL3-control (50 ng; Promega, Madison, WI) were cotransfected into CAL27 cells seeded on 24-well plates (1 × 10^5 cells/well) in the presence or absence of pre-miR-200 precursor (20 nM; Ambion-Thermo Fisher Scientific, Waltham, MA) with the TransIT-X2® (Mirus Bio).

RNA sequencing
RNA quality was assessed by analyzing rRNA band integrity on an Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). Before cDNA library construction, 2 µg of total RNA and magnetic beads with oligo-dT were used to enrich poly-A mRNA. Then, purified mRNAs were disrupted into short fragments, and double-stranded cDNAs were immediately synthesized. The cDNAs were subjected to end-repair, poly-A addition and connected with sequencing adapters using the TruSeq RNA sample prep Kit (Illumina, San Diego, CA). Suitable fragments automatically purified by BluePippin 2% agarose gel cassette (Sage Science, Beverly, MA) were selected as templates for PCR amplification. The final library sizes and qualities were electrophoretically evaluated with an Agilent High Sensitivity DNA kit (Agilent Technologies), and the fragment was between 350 and 450 bp. Subsequently, the library was sequenced using an Illumina HiSeq2500 sequencer (Illumina). Then, RNA-seq data were analyzed using Octopus-toolkit. StringTie™ was used to build a comprehensive transcriptome for all RNA-seq samples. Cufflinks was used to estimate the abundance of transcripts via fragments per kilobase of exon per million fragments mapped (FPKM). Cuffdiff was used to calculate p values for identification of differentially expressed genes.

Gene set enrichment analysis
Gene set enrichment analysis (GSEA) was conducted using the GSEAprogrammed tool in the GSEA application (version 3.0). The log2 ratio of FPKM values (QKI knock-down over wild-type) was used as an input for GSEAanalysis.

Mouse experiments
Before initiation, all proposed mouse studies were submitted to and approved by the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University College of
Medicine (ESM16-0357). Mice were cared for and euthanized according to standards set forth by the IACUC. BALB/c nude mice (4 weeks, female) were purchased from Central Lab Animal (Seoul, Korea), and CAL27 cells (2 × 10^6 cells in 100 μl of serum-free DMEM) were subcutaneously injected into flanks. Mice were monitored twice a week for tumor growth, sacrificed at 8 weeks, and necropsied to isolate primary tumors for hematoxylin & eosin (H&E) staining and Western blotting. For orthotopic injection, BALB/c nude mice were anesthetized with 4% isoflurane, and CAL27 cells (5 × 10^6 cells in 50 μl of serum-free DMEM) were injected submucosally into the tongue. For tail-vein injection, BALB/c nude mice were injected with CAL27 cells (1 × 10^6 cells in 300 μl of serum-free DMEM) via tail vein. Mice were sacrificed at the first sign of morbidity, or at day 40 (orthotopic injection) or day 53 (tail-vein injection). The tongues or the lungs with tumors were carefully isolated and stained with Bouin’s fixative (Polysciences, Warrington, PA).

**Tissue microarray**
Representative tumor areas were selected on H&E-stained slides; corresponding areas with 2 mm diameters were obtained from each tumor paraffin block using a manual tissue arrayer (SuperBioChips Laboratories, Seoul, Korea), and selected areas were assembled in a tissue microarray (TMA) format. One section from each TMA block was stained with H&E and subjected to histological examination. Sections (4 μm in thickness) were used for immunohistochemical staining.

**Immunohistochemistry**
Immunohistochemistry was performed using the Ventana Benchmark XT platform (Ventana Medical System, Tucson, AZ). Formalin-fixed paraffin-embedded tissue sections mounted on slides were stained for QKI (1:800, Bethyl Laboratories), according to the manufacturer’s recommendation. The UltraView DAB Detection Kit (Ventana Medical System) was used to visualize bound anti-QKI primary antibody. The expression of QKI in tissue cores was evaluated by a board-certified pathologist (SAH) blinded to the clinical and pathological data. Nuclear QKI expression was graded as negative, weak, moderate and strong by comparing to the intensity of stromal fibroblasts (moderate expression). Expression was dichotomized into low (negative and weak) and high (moderate and strong).

**Statistical analysis**
Data were analyzed with Student’s t-test, Spearman’s rank correlation test and log-rank test using GraphPad Prism (La Jolla, CA) unless otherwise noted.

**Results**

**QKI level negatively correlates with miR-200 family members**
To predict miR-200 target genes, TargetScan and HNSCC TCGA data (Fig. 1a) were exploited. Using TargetScan, a total of 744 genes for miR-200a (and miR-141) and 1,057 genes for miR-200b (and miR-200c/429) were predicted as putative targets. In HNSCC TCGA data, mRNA levels of 337 and 390 genes negatively correlated with those of miR-200a and miR-200b, respectively (p-value < 0.01). Combining the lists from TargetScan and TCGA data, we predicted 20 common target genes of both miR-200a and miR-200b. To see if these genes are actually targeted and inhibited by miR-200, a miR-200 cluster (miR-200b/200a/429) was overexpressed in two human OSCC (CAL27 and HSC3) cell lines. miR-200a and miR-200b were highly overexpressed, and their target genes, ZEB1 and ZEB2, were suppressed in miR-200-transfected cells (Supporting Information Figs. S1A and S1B). The mRNA expression levels of all 20 miR-200 putative targets were measured in these miR-200-overexpressing cells. Most genes were downregulated by miR-200; however, among 20 genes, only QKI was included within the top three genes that were highly suppressed by miR-200 in both CAL27 and HSC3 cells (Fig. 1b and Supporting Information Fig. S1C). QKI is an important regulator of EMT-associated RNA processing (i.e., circular RNA formation) and RNA splicing, and it has been reported to increase during EMT and modulate EMT-related phenotypes. On the basis of these findings, QKI was selected for further analysis in our study. QKI mRNA expression was negatively correlated with those of miR-200a (Spearman r = −0.4554, p < 0.0001) and miR-200b (r = −0.4851, p < 0.0001) in HNSCC TCGA data (Fig. 1c). In lung adenocarcinoma (LUAD) TCGA data, QKI and miR-200a/200b expression also showed negative correlations (Supporting Information Fig. S2). To confirm this negative correlation at the cellular level, five OSCC cell lines were chosen (YD8, YD10B, CAL27, HSC3 and SCC25), and expression of miR-200 and QKI was measured (Figs. 1d–1f). In cells with low miR-200 levels (e.g., YD8), QKI protein level was high, and vice versa (e.g., SCC25). In addition, QKI mRNA correlated positively with ZEB1 mRNA and EMT scores calculated based on the expression of 130 EMT-core genes in both HNSCC and LUAD TCGA data (Supporting Information Figs. S3A–S3F). Collectively, all these data demonstrate that QKI and miR-200 levels have a negative correlation in cancer cells.

**QKI is a direct target of miR-200**
As reported previously, miR-200 overexpression inhibited cancer cell migration in wound healing assays (Fig. 2a and Supporting Information Fig. S4A) and suppressed cancer cell invasion in spheroid invasion assays (Fig. 2b and Supporting Information Figs. S4B–S4F). As mentioned above, both QKI mRNA and protein expression were suppressed by miR-200 in OSCC and LUAD cells (Figs. 1b and 2c and Supporting Information Figs. S1C and S3A–S3E). All three isoforms of QKI (QKI-5, QKI-6 and QKI-7) were decreased after miR-200 overexpression, but QKI-5 is the most abundant isoform in OSCC cells (Supporting Information Fig. S6); therefore, we performed subsequent studies on QKI-5. To test whether miR-200 directly targets QKI through binding to QKI 3’-untranslated region (UTR), a QKI-5 3’-UTR luciferase construct containing
miR-200-binding sites was cloned (Fig. 2d). When cotransfected with miR-200 mimic, the luciferase reporter activity of wild-type QKI 3'-UTR was inhibited. This effect was abolished by mutation of the miR-200b-binding sites on QKI 3'-UTR (Fig. 2d). On the basis of these findings, we suggest that QKI is a direct target of miR-200.

QKI knock-down induces EMT and enhances cancer cell invasion

QKI was next knocked-down using shRNAs in cancer cells to investigate the role of QKI in cancer development and progression (Figs. 3a and 3b). Because QKI is a target gene of miR-200, QKI knock-down (KD) would naturally inhibit EMT and invasion of cancer cells, just like miR-200. QKI-KD in CAL27 OSCC cells, however, promoted cancer cell growth (Fig. 3c) and induced EMT-like morphologic changes (Figs. 3d and 3e). QKI-KD also increased cancer cell invasion in spheroid invasion assays (Fig. 3f). Similar results were observed in other OSCC (HSC3; Supporting Information Figs. S7A–S7C) and LUAD cell lines (A549 and HCC827; Supporting Information Figs. S8A–S8F). To further examine the effect of QKI on EMT, levels of EMT markers in QKI-KD cells were measured. Mesenchymal markers (ZEB1, N-cadherin and vimentin) were increased and epithelial markers (E-cadherin and miR-200) were decreased by QKI-KD (Figs. 3g and 3h and Supporting Information Fig. S7D). Conversely, overexpression of QKI decreased vimentin (Fig. 3i), increased miR-200 expression levels (Fig. 3j), and suppressed cancer cell invasion (Supporting Information Fig. S7F). These
data imply that QKI-KD induces EMT and enhances cancer cell invasion, an unexpected opposition. To further investigate the role of QKI in migration and invasion inhibition by miR-200, QKI and ZEB1 were added back to miR-200-overexpressing cells (Supporting Information Figs. S9A and S9D). ZEB1 restored the migratory ability of miR-200-overexpressing cells; however, QKI had no effect on cell migration (Supporting Information Fig. S9B). Rather, QKI further reduced the invasive ability of miR-200-overexpressing cells (Supporting Information Figs. S9C and S9E), suggesting that QKI suppresses cancer cell migration and invasion despite being a miR-200 target.

\section*{QKI knock-down promotes tumor growth in vivo}

Control and QKI-KD CAL27 cells were injected subcutaneously into BALB/c nude mice to assess the effect of QKI on tumorigenic potential in a xenograft model (Fig. 4a). After 2 months, QKI-KD greatly increased tumor volume and weight (Fig. 4b). As the results described above, tumors developed from QKI-KD cells expressed more mesenchymal markers (ZEB1, N-cadherin and vimentin) and less epithelial markers (E-cadherin and miR-200) than control tumors (Figs. 4c–4e). Mice were also injected with control or QKI-KD CAL27 cells submucosally into the tongue for an orthotopic model (Figs. 4f–4h). Mice injected with QKI-KD cells died earlier than the control group (Fig. 4f) and the average volume of QKI-KD tumors was larger than that of control tumors (Figs. 4g and 4h). To further examine the effect of QKI on metastatic colonization, we injected control or QKI-KD cells intravenously into the tail vein of BALB/c nude mice. As expected, all mice injected with QKI-KD cells developed lung metastatic nodules (12/12), but control group did not form any visible tumor nodules on the lungs (0/12; Fig. 4i). These results strongly demonstrate that QKI-KD promotes tumor progression and metastasis in vivo.

\section*{QKI knock-down elicited a proinflammatory gene expression signature}

To gain insight into how QKI influences EMT and cancer cell invasiveness, whole transcriptome analysis with total RNA sequencing was performed in QKI knock-down CAL27 cells.
After QKI knock-down, 1,627 genes were upregulated and 662 genes were downregulated (fold change >1.5, q < 0.05). Gene set enrichment analyses revealed that RNA splicing (normalized enrichment score, NES = -10.36) gene signature was depleted in QKI knock-down cells, as expected\(^{20,26}\); however, gene signatures including cytokine production (NES = 8.57), interferon gamma response (NES = 7.71) and inflammatory response (NES = 5.70) were enriched (Figs. 5a and 5b). Gene ontology analysis also showed that genes associated with interferon signaling (q = 1.43E−18), cytokine signaling in immune system (q = 1.63E−14) and inflammatory response (q = 1.20E−09) were significantly enriched in QKI knock-down cells (Supporting Information Fig. S10).

Cancer cells undergoing EMT actively produce proinflammatory factors, and in inflammatory stimuli conversely promote EMT.\(^{27,28}\) Indeed, qRT-PCR analyses confirmed that inflammation-related genes were upregulated in QKI knock-down cells (Fig. 5c), which suggests that inflammatory...
signaling activated by QKI knock-down may promote EMT and invasiveness of cancer cells.

**High expression of QKI is associated with good prognosis in HNSCC and LUAD patients**

Finally, to validate the clinical role of QKI, tissue microarrays of 51 HNSCC and 81 LUAD tumor samples collected from patients with surgical resections were generated. QKI was consistently expressed in stromal fibroblasts. In HNSCC, high expression of QKI was found in 38 cases (74.5%), while low expression was found in 13 cases (25.5%; Fig. 6a). In LUAD, 44 cases (54.3%) and 37 cases (45.7%) showed high and low expression (Fig. 6c), respectively. In patients with HNSCC, low QKI expression resulted in a significantly shorter overall survival (OS) duration (5-year OS rate: 21.5%) compared to high QKI expression (5-year OS rate: 65.9%, p = 0.0004; Fig. 6b). Also in patients with LUAD, low QKI expression was associated with shorter overall survival time (5-year OS rate: 66.1% vs. 89.2%, p = 0.048; Fig. 6d). In HNSCC and LUAD TCGA database, patients with low QKI gene expression tend to have worse survival rates than those with high QKI gene expression (Supporting Information Figs. S11A and S11B). Taken together,
these data suggest that QKI inhibits tumor progression in patients with cancer.

Discussion

Epithelial tumor cells acquire mobility and invasiveness through the EMT process, which is controlled by various transcription factors (e.g., ZEB1/2, SNAIL1/2 and TWIST1) and noncoding RNAs including miRNAs. miR-200 family members, consisting of miR-200a, miR-200b, miR-200c, miR-141 and miR-429, are well-known EMT-suppressive miRNA targets of EMT-inducing transcription factors ZEB1 and ZEB2. EMT is an initial step for tumor cells to disseminate to other distant sites and form metastatic colonies. Therefore, many efforts have been made to understand the underlying mechanisms of EMT to develop therapeutic approaches for treatment of metastatic cancer.

In our study, QKI was identified as a novel target gene of miR-200 family members in oral and lung cancer cells. QKI mRNA and protein expression are inversely correlated with miR-200 expression in a cancer cell line panel and TCGA mRNA/miRNA data. miR-200 suppressed QKI mRNA and protein expression levels and QKI 3'-UTR reporter activity. QKI knock-down, however, enhanced cell growth, invasion, EMT and xenograft tumor growth. Furthermore, QKI knock-down inhibited, and QKI overexpression promoted miR-200 expression, suggesting that QKI and EMT/miR-200 form a negative feedback loop (Fig. 6).

miRNAs are key factors playing important roles in posttranscriptional regulation of gene expression. A single miRNA can target a variety of genes at the same time. Thus, identifying target genes of a miRNA is a major obstacle to revealing the pathophysiologic function of that miRNA. In our study, two independent approaches were adopted to identify novel miR-200 target genes: (i) screening of genes that are negatively correlated with miR-200 expression and (ii) prediction of miR-200 target genes using TargetScan. In addition, to increase the probability of identifying target genes, two sets of candidate target genes were recombined. These target genes were obtained from analyses using both miR-200a/141 and miR-200b/c/429, which differ slightly in their seed sequences and have different predictive target lists. After confirming that most candidates...

Figure 5. QKI knock-down prompted proinflammatory gene expression signature. (a) Heatmap showing RNA sequencing results in CAL27_NTC and CAL27_shC cells. Cytokine- or inflammation-related genes (upregulated) and RNA splicing-related genes (downregulated in shC cells; fold change >1.5, q < 0.05) were selected on the basis of gene ontology and gene set enrichment analysis. Yellow: increased expression; blue: decreased expression. (b) Gene set enrichment analysis of differentially expressed genes in CAL27_NTC and shC cells. "Cytokine production," "inflammatory response" and "interferon-gamma response" gene sets were significantly enriched, but the "RNA splicing" gene set was depleted in QKI knock-down (shC) cells. (c) qRT-PCR of cytokine/inflammation- and RNA splicing-related genes in CAL27_NTC and CAL27_shC cells. Relative values to those of CAL27_NTC cells (set at 1.0) are presented. Data are mean + SD (n = 3). *p < 0.05, **p < 0.01; two-tailed Student’s t-test.
are downregulated by miR-200 in vitro, QKI was chosen for further experiments. Even though several miRNAs such as miR-143-3p and miR-155 have been reported to target QKI, our approach starting with in silico methods revealed that QKI can be targeted by miR-200 family members that are EMT-suppressive miRNAs. During the preparation of this article, a new paper showing that miR-200c can target QKI was published, which further supports our findings.

QKI, a STAR family RNA-binding protein, regulates mRNA splicing, miRNA stabilization and circular RNA formation. Although QKI functions as a tumor suppressor in many cancer types including colon, stomach, kidney, brain and lung, its expression is known to increase during the EMT process. Similar to our data presented here, QKI has been shown to play tumor suppressive roles and suppress EMT in lung cancer. However, recent reports in breast cancer show that QKI promotes EMT through the modulation of alternative splicing. These opposing effects of QKI may be dependent on the cellular context or the relative levels of QKI isoforms expressed in each cancer types. QKI-5 is mainly found in the nucleus, while QKI-6 and QKI-7 are localized to the cytoplasm, and they have somewhat different functions. Furthermore, similar to our result, QKI-5 is more abundant that QKI-6 and QKI-7 in breast cancer cells. In our study, we found that QKI is targeted by miR-200 in HNSCC and LUAD; however, instead of promoting EMT and cancer growth, QKI suppresses cancer cell invasion, EMT and cancer progression. For these reasons, we assume that there is a negative feedback loop between QKI expression and the EMT phenomenon.

Whole transcriptome analysis with RNA sequencing provided a clue as to how the EMT process is regulated by QKI. Genes associated with cytokine production, inflammatory response and interferon gamma signaling were significantly enriched in QKI knock-down cells, implying that inflammation-related signaling was activated after QKI knock-down. In addition, QKI deficiency amplifies proinflammation signaling via modulating STAT1-NFκB and p38 MAPK pathways. It has been widely accepted that inflammation and EMT are closely linked. A number of proinflammatory factors, such as TNFα, TGFβ and IL6, have been reported to promote EMT in epithelial cancer cells. Conversely, EMT-initiating stimuli also activate the transcriptional machinery producing these proinflammatory factors.
factors. Although further detailed analyses were required, it is possible that QKI modulates mRNA splicing, splicing and stabilization of inflammation-related genes. Thus, QKI suppresses activation of the inflammation-EMT axis.

In our study, we found that high QKI expression was associated with good clinical outcome in patients with resected HNSCC and LUAD. QKI is believed to suppress cancer development through regulation of alternative splicing of its downstream target genes. In a bioinformatics analysis, over 1,400 putative targets with QKI-responsive elements were presented, and 24% of them were annotated as involved in cell growth and maintenance pathways, implying the crucial role of QKI in cancer development. QKI mediates alternative splicing of a histone variant macroH2A1 and suppresses cancer-specific splicing of NUMB to perform tumor suppressive effects. QKI also associates with and stabilizes miR-20a, a negative regulator of TGFβRII and TGFβ signaling, to suppress gliomagenesis. Further research into the mechanisms of QKI expression and biological functions will guarantee the development of novel cancer treatment strategies.

Acknowledgements

Our study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2015R1C1A1A01054591 to YHK and 2010-0207945 to YHA) and by the Ministry of Education (NRF-2016R1D1A1B03930068 to YHA) and was also supported by the Catholic Medical Center Research Foundation made in the program year of 2016 (to YHK).

References