TUMORIGENESIS AND NEOPLASTIC PROGRESSION

The Gain-of-Function Mutation p53R248W Suppresses Cell Proliferation and Invasion of Oral Squamous Cell Carcinoma through the Down-Regulation of Keratin 17

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Keratin 17 (KRT17) expression promotes the proliferation and invasion of oral squamous cell carcinoma (OSCC), and mutations in *TP53* have been reported in 65% to 85% of OSCC cases. We studied the correlation between KRT17 expression and *TP53* mutants. Ca9-22 cells, which exhibit low KRT17 expression, carried mutant p53 (p53R248W) and p53R248W knockdown promoted KRT17 expression. p53R248W knockdown in Ca9-22 cells promoted migration and invasion activity. In contrast, in HSC3 cells, which have p53 nonsense mutations and exhibit high KRT17 expression, the overexpression of p53R248W decreased KRT17 expression, cell size, proliferation, and migration and invasion activities. In addition, p53R248W significantly suppressed *MMP2* mRNA expression and enzyme activity. Moreover, s.c. and orthotopic xenografts were generated from p53R248W- or p53R248Q-expressing HSC3 cells. Tumors formed from p53R248W-expressing HSC3 cells grew more slowly and had a lower Ki-67 index than those derived from the control or p53R248Q-expressing HSC3 cells. Finally, the survival rate of the mice inoculated with p53R248W-expressing HSC3 cells was significantly higher than that of the control mice. These results indicate that the p53R248W mutant suppresses proliferation and invasion activity through the suppression of KRT17 expression. We propose that OSCC with p53R248W-expressing cells may be classified as a new OSCC type that has a good prognosis. (*Am J Pathol* 2021, 191: 555–566; <https://doi.org/10.1016/j.ajpath.2020.11.011>)

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer. It is estimated that approximately 300,000 new OSCC cases and 140,000 deaths occur worldwide each year.¹ OSCC is reported to be caused by mutations in several genes such as *TP53*, *NOTCH*, and *PTEN*, which occur in oral epithelial keratinocytes.^{2–8} Although various therapies, including surgery, chemotherapy, biologic therapy, and radiotherapy, have progressed over the years, the overall survival of OSCC remains poor.^{3,9}

OSCC expresses keratin 17 (KRT17) but not keratin 13, whereas normal epithelial cells of the oral mucosa express keratin 13 but not KRT17. On the basis of this OSCC-specific expression of keratin 13 and KRT17, immunohistochemistry with antibodies against keratin 13 and KRT17 is used for standard pathologic diagnosis to distinguish responsive hyperplasia from carcinoma.¹⁰

Keratins are intermediate filament proteins that not only provide structural support for cells but also regulate metabolic processes, cellular growth, apoptosis, and motility,^{11,12} and stimulate intracellular signaling pathways. KRT17 induced in wounded stratified epithelia regulates cell growth¹³ and that expressed in OSCC and gastric cancer promotes cell proliferation and migration.^{14–17} In addition, it has been indicated that the *KRT17* mRNA level may be a prognostic marker for OSCC.¹⁸ High levels of KRT17 expression in cervical cancer correlate with poor outcome and cancer cell differentiation.^{19–21} However, the

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mechanisms by which KRT17 expression is regulated in OSCC are largely unknown.

TP53 encodes p53, which is one of the most common tumor suppressors; p53 transactivates target genes that are responsible for cell cycle arrest, DNA repair, apoptosis, and cell death. Mutations in *TP53* are often found in human cancers and have been detected in 65% to 85% of OSCC cases.^{22–24} More than one-half of mutations in *TP53* are missense mutations that have been identified at the following sites in the DNA-binding domain: R175, G245, R248, R249, R273, and R282.^{22,23,25} It has become apparent that these different *TP53* mutations alter p53 functions, as mutations can result in loss of function, dominant negative, or gain of function (GOF).^{25–27}

p53 Mutants have different kinds of *de novo* functions in different cancer cell lines.²⁸ For example, some p53 mutants bind to the AMP-activated protein kinase α subunit, leading to the inhibition of AMP-activated protein kinase activation and promotion of cell growth and metabolism in head and neck squamous cell carcinoma cell lines.²⁹ In addition, another p53 mutant promotes epidermal growth factor receptor/integrin signaling, causing the invasion and metastatic behavior of lung cancer cells.³⁰ Moreover, it has been indicated that mutant p53 may be a molecular indicator of recurrence in OSCC patients.^{24,31}

Two p53 mutants, p53R248W and p53R248Q, in which an arginine in the 248th amino acid residue of p53 is substituted with tryptophan and glutamine, respectively, result in different levels of transcriptional activity of their downstream target genes, even though the mutations occur in the same codon.^{32,33} The current study aimed to demonstrate how two types of GOF mutants, p53R248W and p53R248Q, regulate KRT17 expression in OSCC lines, which, in turn, influences cell proliferation, migration, and invasion.

Materials and Methods

Cell Culture

Ca9-22, SAS, HSC3, and SAT cell lines, which were derived from oral squamous cell carcinomas of the gingiva, tongue, oral cavity, and tongue, respectively, were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). These cells were seeded at 1.0 to 2.0×10^5 cells/mL. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere.

RNA Preparation and Real-Time PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and single-stranded cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturers' instructions. Real-time PCR was performed using TB Green Premix EX Taq II (TaKaRa Bio Inc.) and a Thermal Cycler

Dice Real Time System (TaKaRa Bio Inc.), according to the manufacturers' instructions. The cDNA concentration used was 500 ng/ μ L. The $\Delta\Delta$ threshold cycle method³⁴ was used for quantifying the quantitative RT-PCR (RT-qPCR) results. The primers used for PCR were as follows: *KRT17*, 5'-ACACTGAGCTGGAGGTGAAGAT-3' (forward) and 5'-CATTGTCAATCTGTAGCAGGATGT-3' (reverse); *MMP2*, 5'-AGCTCCCGGAAAAGATTGATG-3' (forward) and 5'-CAGGGTGCTGGCTGAGTAGAT-3' (reverse); *MMP9*, 5'-ACGACGTCTTCCAGTACCGA-3' (forward) and 5'-TTGGTCCACCTGG TTCAACT-3' (reverse); and *GAPDH*, 5'-CGACCACTTTGTCAAGCTCA-3' (forward) and 5'-AGGGGTCTACATGGCAACTG-3' (reverse). Relative gene expression was determined and normalized to *GAPDH* expression.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (TAAB Laboratories, England, UK) in phosphate-buffered saline (PBS; TaKaRa Bio Inc.) for 15 minutes at room temperature. After rinsing with PBS three times, the cells were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO) for 30 minutes at room temperature and then blocked with 1% bovine serum albumin (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) for 30 minutes at room temperature. The cells were incubated with primary antibodies overnight at 4°C. The primary antibodies used were anti-KRT17 (EPR1624Y; Abcam, Cambridge, UK; diluted 1:100) and p53 (DO-7; Dako, Glostrup, Denmark; diluted 1:100). These antibodies were then allowed to react with secondary antibodies for 1 hour at room temperature. The secondary antibodies used were Alexa Fluor 488 rabbit anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA; diluted 1:500) and Cy3 goat anti-rabbit IgG (Thermo Fisher Scientific; diluted 1:200). After washing with PBS, the cells were mounted in VECTA-SHIELD mounting medium for detecting the fluorescence of DAPI (Vector Laboratories, Burlingame, CA). Immunostaining was visualized using an all-in-one microscope (Keyence, Osaka, Japan).

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay buffer (FUJIFILM Wako Pure Chemical Corp.) containing 1% protease inhibitor cocktail. The protein concentration was quantified by Bradford assay. The protein samples were separated by 10% SDS-PAGE gels and electrotransferred onto polyvinylidene difluoride membranes (Merck, Darmstadt, Germany). The membranes were incubated overnight with primary antibodies at 4°C, allowed to react with horseradish peroxidase-conjugated secondary antibodies, and visualized using ECL Prime Western blotting detection reagents (GE Healthcare, Boston, MA). Images of the immunoreaction were collected using a cooled charge-

coupled device camera (ATTO Corp., Tokyo, Japan). β -Actin protein was used as an internal control. Densitometric analysis of the proteins was performed using a CS analyzer (ATTO Corp.).

Generation of p53-Knockdown and p53-Overexpressing Cells

The p53 shRNA and a control shRNA were purchased from Sigma-Aldrich. The shRNAs were transfected into Ca9-22 cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific), according to the manufacturer's protocol, and puromycin (1 μ g/mL)-resistant clones were isolated. For overexpressing cells, human *TP53* cDNA was purchased from DNAFORM (Yokohama, Japan), and p53R248W and p53R248Q mutant plasmids were synthesized using a Quikchange Lightning site-directed mutagenesis kit (Agilent, Santa Clara, CA). After confirmation of the desired mutations by sequencing, p53R248W and p53R248Q vectors were transformed into competent cells using a gateway system (Thermo Fisher Scientific). cDNA-expressing plasmids were transfected into HSC3 cells using Lipofectamine 3000 reagent, and cell colonies resistant to G418 (1500 μ g/mL) were isolated.

Proliferation, Migration, and Invasion Assays

Cell proliferation rates were evaluated by measuring the metabolic activity using Cell Counting Kit-8 (Dojindo Molecular Technologies, Tokyo, Japan). The cells were counted using a TC automated cell counter (BIO RAD, Irvine, CA). Cell migration was evaluated by the Transwell method; 6.5-mm-diameter inserts with 8.0- μ m pore size (Corning, Kennebunk, ME) were used. Cells of each lineage (2.0×10^5 cells/mL) were suspended in serum-free medium and seeded into the three upper chambers. The lower chamber was filled with serum-containing medium. Forty-eight hours later, the insert membrane was fixed with 4% paraformaldehyde and then stained with crystal violet. The stained cells were manually counted in three microscopic fields per sample. For the invasion assay, insert membranes were coated with Corning Matrigel basement membrane matrix (Corning) that was diluted 1:50 in PBS.

Gelatin Zymography

The cells were cultured in a 6-well plate, and the medium was changed to serum-free medium when the cells reached 70% to 80% confluency. After 48 hours, the medium was collected, centrifuged, and concentrated using a Vivaspin6 centrifugal concentrator (Sartorius, Göttingen, Germany). Each sample was mixed with the same volume of sample buffer and incubated for 15 minutes at room temperature. Gelatin zymography was performed using a gelatin-zymography kit (Cosmo Bio Co, Ltd, Tokyo, Japan), according to the manufacturer's protocol.

ChIP Assay

Chromatin immunoprecipitation (ChIP) assays were performed using a SimpleChIP enzymatic Chromatin IP Kit (magnetic beads; Cell Signaling Technology, Danvers, MA). The antibodies used were anti-p53 (1C12; Cell Signaling Technology; diluted 1:200), anti-histone H3 (Cell Signaling Technology; diluted 1:50), and normal rabbit IgG (Cell Signaling Technology; diluted 1:250). DNA fragments that were precipitated with antibodies were amplified by PCR with the following primers: Number 1, 5'-AGTCTGAAACATGACCACATTCC-3' (forward) and 5'-CACAGGAAACCTAAAGGACATTG-3' (reverse); Number 2, 5'-GGGTTCAAGAGCAGAAGGAG-3' (forward) and 5'-CAAACAGGATGCCCCCTAC-3' (reverse); Number 3, 5'-TGGGGAAAGGTTTACACGAA-3' (forward) and 5'-GAGACCAACCCACATCACAG-3' (reverse); and Number 4, 5'-AGAGTGCATTCTGCGGTGT-3' (forward) and 5'-GGAGACCGAGAGCCTATCCT-3' (reverse). Quantification of the PCR products was performed using ImageJ software version 1.52 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

Xenograft and Immunohistochemical Staining

All animal experiments were performed in accordance with the guidelines of the Animal Studies Committee of Wakayama Medical University (Wakayama, Japan). For the s.c. xenograft model, cells (1.0×10^5) suspended in 100 μ L of Matrigel were subcutaneously injected into the back skin of nude mice (BALB/c Slc-nu/nu) at three sites. For the orthotopic xenograft model, cells (1.0×10^6) suspended in 100 μ L of PBS were injected into the tongues of nude mice ($n = 8$, for each cell line). Five weeks after inoculation, tongues and cervical lymph nodes were dissected and fixed with 4% paraformaldehyde. The tissues were embedded in paraffin, and the sections were used for histologic examination, including immunohistochemical analysis of KRT17, marker of proliferation Ki-67, CD31, and D2-40. The primary antibodies used were anti-KRT17 (EPR1624Y; Abcam; diluted 1:100), D2-40 (Dako; diluted 1:200), MKI67 (Dako; diluted 1:150), and anti-CD31 (Abcam; diluted 1:50). For antibodies derived from the mice, a M.O.M (mouse-on-mouse) immunodetection kit (Vector Laboratories) was used. The percentage of MKI67-positive nuclei in the cancer cells was calculated by counting at least 1000 total tumor cells in five $\times 400$ fields. Mice were humanely sacrificed by cervical dislocation after administration of anesthesia via isoflurane inhalation at the end of the experiments, which was in accordance with the Regulations for Animal Experiments at Wakayama Medical University.

Statistical Analysis

Data are presented as means $\pm$ SEM. The *t*-test or *U*-test was performed to compare the means of two groups. Data from

more than three groups were analyzed by one-way analysis of variance followed by Tukey-Kramer or Dunnett test or by Kruskal-Wallis test followed by Dunnett test. Data for lymph node metastasis were analyzed by Fisher exact test. Survival was analyzed by Kaplan-Meier survival curves and Wilcoxon tests using JMP pro13 software (SAS Institute, Inc., Cary, NC). $P < 0.05$ was considered significant.

Results

Differences in the Mutations in the *TP53* Gene Influence KRT17 Expression in OSCC Cell Lines

To investigate the expression of p53 and KRT17 in OSCC, we used four different OSCC cell lines, HSC3, SAS, Ca9-22, and SAT cells. Western blot analysis showed that the Ca9-22 cells express higher levels of p53 than the other cell lines, whereas the KRT17 protein was virtually undetectable in Ca9-22 cells. In contrast, HSC3, SAS, and SAT cells expressed high levels of the KRT17 protein, whereas p53 protein levels were low compared with those of the Ca9-22 cells (Figure 1A). These results suggest that differences in p53 expression levels may influence KRT17 expression.

Immunocytochemical analysis showed that the Ca9-22 cells express high levels of p53 and low levels of KRT17 protein, whereas the HSC3 cells express low levels of p53 and high levels of KRT17 protein, findings in line with the results of the Western blot analysis (Figure 1B). In addition, RT-qPCR experiments showed that the *KRT17* mRNA expression levels are lower in the Ca9-22 cells than in other cell lines (Figure 1C).

It has been reported that SAS and HSC3 cells carry *TP53* with nonsense mutations (E336X and R306X, respectively), whereas Ca9-22 and SAT cells carry missense mutations (R248W and R110L, respectively).⁴ Taken together, these results suggest that different mutations in *TP53* may explain the different levels of KRT17 expression between Ca9-22 and the other cell lines.

The p53R248W Mutant Suppresses KRT17 Expression, which Promotes the Proliferation and Invasion of OSCC Cells

We generated a Ca9-22 cell line with p53 knocked down (Ca9-22 p53KD), in which the p53 expression was constitutively down-regulated (Figure 2A). Western blot analysis and immunocytochemical analysis showed that KRT17 expression was highly increased in the Ca9-22 p53KD cells compared with that of the control Ca9-22 cells (Ca9-22 Con) (Figure 2A and B). In addition, RT-qPCR showed that the *KRT17* mRNA levels were increased in the Ca9-22 p53KD cells (Figure 2C). As it has been reported that KRT17 modulates cell size in OSCC,¹⁷ KRT17-expressing Ca9-22 p53KD cells were larger than Ca9-22 Con cells (Figure 2D). To investigate the function of KRT17 in Ca9-22 p53KD cells, their proliferation, migration, and invasion

Figure 1 Expression of p53 and keratin 17 (KRT17) in oral squamous cell carcinoma (OSCC) cell lines. **A:** Western blot analysis of p53 and KRT17 in OSCC cell lines. β -Actin was used as a loading control. **B:** Dual immunohistochemical staining for p53 (green) and KRT17 (red). **C:** *KRT17* mRNA expression in OSCC cell lines. Data are expressed as means $\pm$ SEM (C). * $P < 0.05$, *** $P < 0.001$. Scale bars = 30 μ m (B).

were examined. Proliferation assays showed that p53KD Ca9-22 cell numbers increased more quickly compared with those in Ca9-22 Con cells (Figure 2E). Furthermore, migration and invasion assays revealed that the Ca9-22

Figure 2 Effect of p53R248W knockdown on keratin 17 (KRT17) expression and invasion activity. **A:** Western blot analysis of p53 and KRT17 in control Ca9-22 cells (Ca9-22 Con) and Ca9-22 cell line with p53 knocked down (Ca9-22 p53KD). β-Actin was used as a loading control. **B:** Dual immunohistochemical staining for p53 (green) and KRT17 (red). **C:** Comparison of *KRT17* mRNA expression between Ca9-22 Con and Ca9-22 p53KD cells. **D:** Microscopic bright-field images showing the cell shape of Ca9-22 Con and Ca9-22 p53KD cells. Cell size was measured by NIS-Elements D 4.30 software (Nikon, Tokyo, Japan; 100 cells were counted for each cell line). **E:** Proliferation assay showing that Ca9-22 p53KD cells grew significantly faster than Ca9-22 Con cells 2 days after inoculation. **F:** Transwell migration and invasion assays for comparison of Ca9-22 Con and Ca9-22 p53KD cells. * $P < 0.05$, *** $P < 0.001$; † $P < 0.05$ versus control. Scale bars = 100 μm (**B**, **D**, and **F**).

p53KD cells migrated and invaded significantly faster than the Ca9-22 Con cells (Figure 2F). These results are consistent with previous reports showing that KRT17 promotes cell proliferation, migration, and invasion.¹⁴

p53 Mutants R248W and R248Q Regulate KRT17 Expression Levels, Resulting in Changes in Cell Proliferation and Cell Size

Considering the aforementioned experiments, we hypothesized that the p53R248W mutant is a GOF mutant that suppresses KRT17 expression. Both R248W and R248Q are common mutations of *TP53* that occur in the same codon; however, these two mutants reportedly have different GOF effects in other cancers. To examine the case of OSCC, we established two HSC3 cell lines overexpressing p53R248W and p53R248Q (HSC3-R248W and HSC3-R248Q, respectively).

Western blot analysis indicated that KRT17 expression was significantly decreased in the HSC3-R248W cells. In contrast, intriguingly, KRT17 expression levels increased in the HSC3-R248Q cells compared with those in the control HSC3 cells (HSC3-Con) (Figure 3A). Immunocytochemical analysis showed that KRT17 expression was greatly suppressed in cells with the p53R248W mutant, whereas those with the p53R248Q mutant showed increased KRT17 immunoreactivity, as indicated by greater intensity (Figure 3B). In addition, the *KRT17* mRNA levels were significantly decreased in the HSC3-R248W cells, as shown by RT-qPCR (Figure 3C). HSC3-R248W cells were significantly smaller than the HSC3-Con cells, whereas HSC3-R248Q cells were larger than the HSC3-Con cells (Figure 3D), indicating that HSC3 cell size changes depending on the KRT17 expression level. Next, we compared the proliferation activity between the HSC3-Con,

Figure 3 p53R248W decreases keratin 17 (KRT17) expression, cell size, and proliferation, whereas p53R248Q increases KRT17 expression and cell size. **A:** Western blot analysis of p53 and KRT17 in control HSC3 (Con), HSC3-p53R248W (W), and HSC3-p53R248Q (Q) cells. β -Actin was used as a loading control. **B:** Dual immunohistochemical staining for p53 (green) and KRT17 (red). **C:** Comparison of KRT17 mRNA expression levels between Con, W, and Q cells, as assessed by real-time PCR. **D:** Microscopic bright-field images showing the cell shape of Con, W, and Q cells. Cell size was measured by NIS-Elements D 4.30 software (Nikon; at least 75 cells were counted for each cell line). **E:** Cells (1000 cells per well) were seeded in a 96-well plate, and the number of cells was counted after 4 days. $n = 4$ (**E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 50 μ m (**B**); 100 μ m (**D**).

HSC3-R248W, and HSC3-R248Q cells. Cell growth trended to decrease in HSC3-R248W cells, whereas there was no change in cell growth of HSC3-R248Q and HSC3-Con cells (Figure 3E).

p53R248W Decreases Cell Migration and Invasion by Suppressing the Enzyme Activity of MMP2 and MMP9

There was a significant decrease in the migration of HSC3-R248W cells compared with that in other cell lines (Figure 4A). In addition, the invasion assays revealed that the number of HSC3-R248Q cells that crossed the Transwell membrane was significantly greater than the number of HSC3-Con cells, whereas a significant decrease in HSC3-R248W cell invasion was observed (Figure 4A). These results suggest that cell proliferation and invasion in OSCC are regulated by KRT17 expression levels.

To explore the mechanisms by which invasion activity is regulated by GOF in p53 mutants, we examined the mRNA

expression levels and enzyme activity of matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), which are thought to affect the invasiveness and aggressiveness of OSCC.³⁵ RT-qPCR showed that the MMP2 and MMP9 mRNA levels were significantly decreased in both the HSC3-R248W and HSC3-R248Q cells (Figure 4B). In addition, gelatin zymography assays demonstrated that the enzyme activity of MMP2 was clearly decreased compared with that of the HSC3-Con and HSC3-R248Q cells (Figure 4C). In contrast, the enzyme activity of MMP9 was greatly increased in the HSC3-R248Q cells, although the mRNA levels were decreased (Figure 4C). Taken together, these results indicate that the difference in invasion activity between the p53R248W and p53R248Q cells may be caused by the difference in MMP2 and MMP9 enzyme activities. It can be concluded that p53R248W suppresses the enzyme activity of MMP2, resulting in a significant decrease in the number of invading cells, as observed in the invasion assay (Figure 4A and C).

Figure 4 p53R248W decreases migration and invasion activities by reducing the expression levels of matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9). **A:** Migration and invasion activities of control HSC3 (Con), HSC3-p53R248W (W), and HSC3-p53R248Q (Q) cells, as assessed by Transwell assay. **B:** Comparison of *MMP2* and *MMP9* mRNA expression levels in Con, W, and Q cells, as assessed by real-time PCR. **C:** Gelatin zymography showing the enzyme activity of MMP2 and MMP9 in Con, W, and Q cells. The **arrow** and **arrowhead** indicate MMP2 and MMP9, respectively. Quantification of MMP2 and MMP9 bands was performed using ImageJ software version 1.52. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = 100 μ m (A).

p53R248W and p53R248Q Bind to Several Putative p53-Binding Sites in the *KRT17* Gene

Because p53 is a direct transcriptional repressor of *KRT17* in radiation dermatitis,³⁶ we hypothesized that p53R248W may also function as a direct transcriptional repressor of the *KRT17* gene. We performed a ChIP assay on four putative p53-binding sites that we identified in the promoter region of the *KRT17* gene; we used three different HSC3 cell lines: control DNA, p53R248W, and p53R248Q (Figure 5A). The ChIP assays showed a positive binding signal with either p53R248W or p53R248Q at putative binding site Number 1 or Number 4, respectively (Figure 5B and C, and Supplemental Figure S1). In addition, positive binding signals with both p53R248W and p53R248Q were observed at putative binding sites Number 2 and Number 3. Thus, it is possible that putative binding sites Number 1 and Number 4 may be unique to p53R248W and p53R248Q, respectively,

whereas putative binding sites Number 2 and Number 3 may be common to both p53R248W and p53R248Q. Considering that *KRT17* expression levels were decreased in the HSC3-R248W cells and increased in the HSC3-R248Q cells, it is possible that p53R248W may down-regulate *KRT17* expression by directly binding to putative binding site Numbers 1, 2, and 3, causing a gain of function, whereas p53R248Q may up-regulate *KRT17* expression by directly binding to putative binding site Numbers 2, 3, and 4.

p53R248W Inhibits Tumor Growth in Xenografts

To examine the behavior of cancer cells in tissue, we subcutaneously injected Ca9-22 Con and Ca9-22 p53KD cells into the backs of nude mice. Two weeks after inoculation, the tumors formed from the Ca9-22 p53KD cells were significantly larger than those formed by the Ca9-22 Con cells (Figure 6A). s.c. xenograft tumors were generated from

Figure 5 p53R248W and p53R248Q bind to several putative binding sites in the *KRT17* gene. **A:** A schematic depiction of putative binding sites that were found in the *KRT17* promoter region. **B:** Sequences of four putative p53 binding sites (Numbers 1, 2, 3, and 4) in the promoter region of the *KRT17* gene. The numbers indicate the nucleotide number, which is counted from the translation start site. **C:** PCR products of the chromatin immunoprecipitation assay. **Asterisks** indicate binding sites where antibodies against p53 specifically bind compared with control IgG in control HSC3 (Con), HSC3-p53R248W (W), and HSC3-p53R248Q (Q) cells.

HSC3-Con, HSC3-R248W, and HSC3-R248Q cells. Four weeks after inoculation, the tumors that formed from the HSC3-R248W cells were significantly smaller than those formed from the HSC3-Con or HSC3-R248Q cells (Figure 6B). Tumors formed from the HSC3-Con and HSC3-R248Q cells had more vascular formation in the surrounding tissue than those formed from the HSC3-R248W cells (Supplemental Figure S2).

In addition, to examine the incidence of metastasis to cervical lymph nodes, HSC3-Con, HSC3-R248W, and HSC3-R248Q cells were injected into the tongues of nude mice. Histologic examination showed that the HSC3-R248W-derived tumors had more differentiated features than the HSC3-Con- or HSC3-R248Q-derived tumors in the orthotopic xenografts. In addition, the HSC3-Con- and HSC3-R248Q-derived tumors showed aggressive invasion, whereas the HSC3-R248W-derived tumors had mild invasion of the surrounding tissue, which is a feature of the tumor invasive front (Figure 6C). The HSC3-R248Q-derived tumors exhibited high-grade atypia and larger cells than the other tumors, which is consistent with the results shown in Figure 3D (Figure 6C).

The immunohistochemistry results indicate that KRT17 was less intensely stained in the HSC3-R248W-derived tumors than in the tumors derived from HSC3-Con and HSC3-R248Q cells (Figure 6C). Immunohistochemical examination of MKI67 show that the Ki-67 index was significantly decreased in the HSC3-R248W-derived tumors compared with the HSC3-Con and HSC3-R248Q-derived tumors (Figure 6C). Moreover, the survival rate of the HSC3-R248W-injected mice was significantly higher than that of the HSC3-Con-injected mice (Figure 6D). Although HSC3-R248W cells metastasized to fewer cervical lymph nodes than HSC3-Con cells, statistically significant differences in metastasis to cervical lymph nodes between the three cell lines were not observed (Table 1).

Discussion

It has been reported that mutations in *TP53* not only disrupt its tumor suppressor function but also endow it oncogenic GOF properties independent of wild-type *TP53*. Various reports have shown that all p53 mutants do not have the

Figure 6 p53R248W inhibits tumor growth in xenografts. **A:** Image showing the difference in the sizes of the tumors derived from Ca9-22 cell line with p53 knocked down (Ca9-22 p53KD) and control Ca9-22 cells (Ca9-22 Con). The cells were inoculated subcutaneously, and tumors were removed after 2 weeks. **B:** Image showing the difference in the size of tumors derived from control HSC3 (Con), HSC3-p53R248W (W), and HSC3-p53R248Q (Q) cells. Cells were inoculated subcutaneously, and tumors were removed after 4 weeks. **C:** Histologic analysis with low and high magnification of hematoxylin and eosin staining (HE L. mag and HE H. mag, respectively) and immunohistochemistry staining for keratin 17 (KRT17) and MKI67 of tumors derived from Con, W, and Q cells, which were removed from orthotopic xenografts after 4 weeks. **Arrowheads** indicate the tumor invasive front. The Ki-67 index was calculated as the percentage of MKI67-positive nuclei in cancer cells by counting at least 1000 total tumor cells. **D:** Survival analysis of orthotopic xenograft models of Con, W, and Q cells. $n = 5$ for each group (**D**). * $P < 0.05$, ** $P < 0.01$; $^{\dagger}P < 0.05$ versus control. Scale bars: 10 mm (**A** and **B**); 100 μ m (**C**).

Table 1 Comparison of the Number of Cervical Lymph Nodes with or without Metastasis from Tumors Formed in Mouse Tongues from HSC3-Con, HSC3-R248W, and HSC3-R248Q Cells

Cell type	Metastasis		Total	P value
	+	–		
HSC3-Con	3	5	8	0.569
HSC3-R248W	1	7	8	0.569
HSC3-R248Q	3	5	8	
Total	7	7	24	

+, Present; –, absent; HSC3-Con, control HSC3.

same activity or biological properties.^{27,28} In this study, we focused on p53 mutants R248W and R248Q in OSCC cells because one OSCC cell line, Ca9-22, had endogenous p53R248W and expressed less KRT17 protein than the other OSCC cell lines. We found that p53R248W suppressed KRT17 expression by binding the promoter region of the *KRT17* gene, resulting in decreased proliferation and invasion activities of OSCC cells. To our knowledge, this is the first report to show the relationship between the p53R248W mutation and KRT17 suppression in OSCC, which results in a GOF of p53.

Several articles have previously reported on the GOF impacts of p53R248W. In human colon carcinoma, p53R248W increases the number of vessels in tumors via the activation of the hypoxia-inducible factor 1/vascular endothelial growth factor-A pathway, resulting in the promotion of tumor growth with reactive oxygen species–induced angiogenesis.³⁷ Cancer cells expressing both wild-type p53 and p53R248W exhibited restored wild-type p53 activity and induced apoptosis and/or proliferation arrest following the knockdown of only p53R248W.³⁸ In addition, in pancreatic cancer cells, knockdown of p53R248W reduced cell proliferation and migration.³⁸ These reports basically show an effect of p53R248W that is opposite to the one we observed in OSCC cells in this study.

This study demonstrated that p53R248W reduced KRT17 expression and cell size and suppressed proliferation, migration, and invasion in OSCC cell lines. Although a direct correlation between decreased KRT17 expression and the altered characteristics of OSCC cells was not demonstrated, these results are in good agreement with previous reports showing that KRT17 increases cell size and promotes proliferation, migration, and invasion of cancer cells.^{14,15,17}

In addition, orthotopic xenograft experiments with HSC3-R248W and HSC3-R248Q cells in mouse tongues revealed that tumors formed from p53R248W cells grew slowly and that the survival rate of mice inoculated with p53R248W significantly increased compared with that of control and p53R248Q cells. When we observed tumors from the inside of the skin, we found that the tumors formed with HSC3-Con and HSC3-R248Q cells in s.c. xenograft models showed more vascular formation from the surrounding tissue than those formed with HSC3-R248W cells

(Supplemental Figure S2). In addition, when we examined the histology of the tumors, there was a trending decrease in vascular formation in the tumors formed with HSC3-R248W cells compared with those formed with HSC3-Con and HSC3-R248Q cells. However, there was no obvious difference in vascular or lymph vessel formation between tumors formed with HSC3-Con, HSC3-R248Q, and HSC3-R248W cells in the orthotopic xenograft models (Supplemental Figure S3). We failed to find differences in the expression levels of molecules critical for vascular formation, such as hypoxia-inducible factor 1 and vascular endothelial growth factor-A (data not shown).

More important, lower levels of KRT17 and MKI67 expression were observed in tumors formed from the HSC3-R248W cells than in tumors formed from the HSC3-R248Q and HSC3-Con cells. These results suggest that p53R248W represses tumor growth through down-regulation of KRT17 expression in OSCC. We speculate that p53R248W may function as a suppressor of KRT17 in OSCC, whereas it is a promoter in other tumors; these differences probably depend on the type of tumor and tissue.

Nakazawa et al³³ reported that p53R248Q induced motile and invasive potential in OSCC cells, whereas p53R248W had no influence on malignancies. In our study, although the p53R248Q-overexpressing cell line showed higher levels of KRT17 expression and invasion activity than the control cell line, no significant difference between tumors that formed from the control or p53R248Q cell lines was observed in our xenograft experiments. The discrepancy between the findings of this previous study and our present study might be due to different types of OSCC cell lines being used for each experiment. Further study using different types of OSCC cell lines could solve the discrepancy.

In the invasion experiment, HSC3-p53R248W cells showed significantly decreased invasion activity. To explore the molecular causes behind this observation, we examined the enzyme activity of MMP2 and MMP9, which are zinc-dependent endopeptidases with collagenase and gelatinase activity; these enzymes are involved in tumor invasion and metastasis.³⁹ As observed in previous reports on MMP2 and MMP9 from sections of OSCC tissue,³⁵ we found that p53R248W decreased the expression levels and enzyme activity of MMP2, which may result in a significant decrease in invasion of the HSC3-p53R248W cells compared with that of the HSC3-p53R248Q and HSC3-Con cells (Figure 4). In addition, Lawal et al⁴⁰ reported that poorly differentiated OSCC strongly expressed MMP2 compared with highly or moderately differentiated OSCC. In the xenograft model in Figure 6, the histology of the tumors formed from the p53R248W cells showed well-differentiated features compared with those formed from p53R248Q and control cells. It is therefore possible that p53R248W may differentiate OSCC cells into cells with low-grade invasiveness by suppressing MMP2 activity. In this study, we did not explore the direct correlation between

KRT17 and MMP2 at the molecular level. It is necessary to further investigate the mechanism of how MMP2 activity is suppressed by the p53R248W mutation.

To date, the relationship between p53 and KRT17 expression has been shown only in radiation dermatitis. In this setting, ionizing radiation activates wild-type p53, which in turn down-regulates KRT17 transcription by directly binding to the promoter region of the *KRT17* gene.³⁶ Thus, we examined whether p53R248W could also be a direct transcriptional repressor of *KRT17* in radiation dermatitis. ChIP assays showed direct binding of p53R248W to several putative p53 binding sites that we identified in the promoter region of the *KRT17* gene, which is consistent with the fact that p53R248W suppresses KRT17 expression and that knocking down p53R248W restores KRT17 expression. On the other hand, p53R248Q, which up-regulates KRT17 expression, also showed positive binding in the *KRT17* promoter. As indicated by the ChIP assay, p53R248Q and p53R248W bind to different putative p53-binding sites, in addition to binding to two common sites. We hypothesize that *KRT17* transcription is regulated by the binding of different p53 mutants to different binding sites: p53R248W down-regulates KRT17 expression by directly binding to putative binding site Numbers 1, 2, and 3, and p53R248Q up-regulates KRT17 expression by directly binding to putative binding site Numbers 2, 3, and 4, as shown in Figure 5. This regulation of KRT17 expression by p53R248 and p53R248Q needs to be validated by performing a promoter assay, such as a luciferase assay.

We found that *KRT17* mRNA expression levels were decreased in the HSC3-R248Q cells, although KRT17 protein levels were significantly increased in HSC3-R248Q cells (Figure 3A and C). It may be possible that the high KRT17 protein expression observed in the p53R248Q cells is not due to an increase in transcription and translation but a decrease in the degradation of the KRT17 protein.⁴¹

On the other hand, glioma-associated oncogene transcription factor-mediated KRT17 expression has been reported as one of the mechanisms of KRT17 emergence in OSCC.¹⁵ In addition, Yoon et al⁴² reported that p53 down-regulated GLI oncogene activity through interactions with TATA-box binding protein associated factor 9. They also demonstrated that p53R248W reduced GLI1 activity, although the decrease was weak compared with the levels observed in the presence of wild-type p53.⁴² These reports suggest that p53R248W may suppress KRT17 expression by down-regulating GLI1.

Finally, although the true incidence of the p53R248W mutation in OSCC is not known, it will be important to detect the mutant in patients with OSCC because of the indication that p53R248W actually suppresses tumor growth and invasion, leading to a good prognosis. As with the diagnosis of glioblastoma using *IDH1* mutations, including R132H,⁴³ it would be useful to detect p53R248W mutations in OSCC using a specific antibody and

immunohistochemistry. We emphasize that OSCCs with p53R248W mutations may be classified as a specific type of OSCC associated with a good prognosis.

In summary, we show that p53R248W represses KRT17 expression in OSCC cells, resulting in decreased proliferation and invasiveness. Repression of KRT17 by p53R248W may become a new therapeutic for OSCC. We hope that the findings of this study will be applied to the development of a new treatment for OSCC in the future. Further investigation of the precise mechanisms of induction of KRT17 expression in OSCC is needed to develop a new therapeutic strategy for OSCC treatment.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.11.011>.

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