Dominant-negative p53 mutant R248Q increases the motile and invasive activities of oral squamous cell carcinoma cells

Seitaro Nakazawa, Ken-ichiro Sakata, Shanshan Liang, Kazuhito Yoshikawa, Hisashi Izasa, Mitsuhiro Tada, Jun-ichi Hamada, Haruhiko Kashiwazaki, Yoshimasa Kitagawa, and Yutaka Yamazaki

1Department of Gerodontology, Division of Oral Health Science, Graduate School of Dental Medicine; 2Department of Oral Diagnosis and Oral Medicine, Division of Oral Pathobiological Science, Graduate School of Dental Medicine, Hokkaido University, N13 W7, Kita-ku, Sapporo, 060-8586, Japan; 3The Key Laboratory of Biomarker High Throughput-Screening and Target Translation of Breast and Gastrointestinal Tumor, Oncology department of Affiliated Zhongshan Hospital of Dalian University, Dalian, Liaoning Province, 116001, China; 4Department of Microbiology, Shimane University Faculty of Medicine, 89-1 Enyacho, Izumo City, Shimane 693-8501, Japan; 5Department of Neurosurgery, Rumoi Municipal Hospital, 2-16-1 Shinonome, Rumoi, Japan; 6Health Sciences University of Hokkaido, School of Nursing and Social Services, 1757 Kanazawa, Ishikari-Tobetsu, Hokkaido 061-0293, Japan; and 7Section of Geriatric Dentistry and Perioperative Medicine in Dentistry, Division of Maxillofacial Diagnostic and Surgical Sciences Faculty of Dental Science, 3-1-1 Maidashi Higashi-ku Fukuoka 812-8582, Japan

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ABSTRACT

The tumor suppressor gene TP53 (gene) codes for a transcription factor which transactivates its target genes responsible for cell cycle arrest, DNA repair, apoptosis, and senescence. TP53 is well known to be the most frequent target of genetic mutations in nearly half of human cancers including oral squamous cell carcinoma (OSCC). Many p53 mutants including R248Q and R248W not only lose its tumor-suppressor activities, but also interfere with the functions of wild-type p53; this is so-called dominant-negative (DN) mutation. The DN p53 mutation is a predictor of poor outcome in patients with various cancers, and also a risk factor for metastatic recurrence in patients with OSCC. Recently it has been reported that DN p53 mutants acquire new oncogenic activities, which is named gain-of-function (GOF). This study aimed at determining whether R248Q and R248W were involved in OSCC cells acquiring aggressive phenotypes, using SAS, HSC4 and Ca9-22 cell lines. First, two mutants p53, R248Q and R248W, were respectively transfected into SAS cells harboring recessive-type p53 (E336X). As a result, SAS cells expressing R248Q showed highly spreading, motile and invasive activities compared to parent or mock-transfected cells whereas those expressing R248W did not increase those activities. Secondly, in HSC4 cells harboring R248Q and Ca9-22 cells harboring R248W, expressions of the mutants p53 were inhibited by the transfection with siRNAs targeting p53. The inhibition of the mutants p53 decreased spreading, motile and invasive activities of HSC4 cells whereas it did not affect those activities of Ca9-22 cells. These findings suggest that R248Q p53 mutation, but not R248W p53 mutation, induces more motile and invasive potentials in human OSCC cells.

Address correspondence to: Seitaro Nakazawa, Ph.D., Department of Gerodontology, Division of Oral Health Science, Graduate School of Dental Medicine, Hokkaido University, N13 W7, Kita-ku, Sapporo, 060-8586, Japan Tel: +81-11-706-4582, Fax: +81-11-706-4582 E-mail: nakazawa@den.hokudai.ac.jp

p53 is a transcription factor, encoded by tumor suppressor gene TP53, also known as “the guardian of the genome”. p53 tetramer can regulate expression of its target genes, through binding the p53 responsive element, located on their promoter region. In response to genomic stress, p53 stabilizes in the nucleus, triggering a transcriptional program of cell
cycle arrest, DNA repair, senescence, autophagy and apoptosis (5). In humans, TP53 mutations are detected among various cancer types, including oral squamous cell carcinoma (OSCC), in which there is a >70% likelihood of p53 mutations occurring (4).

Most of TP53 mutations are point mutations that result from single base substitutions. In particular, Gly143, Arg175, Gly245, Arg248, Arg273, and Arg282 in the p53 DNA-binding domain (102–292 amino acid) have an extremely high mutation frequency, and consequently are referred to as hot spots. In general, p53 with any of these hot-spot mutations will no longer be able to bind to the DNA of target genes of wild-type p53 (wt-p53). Furthermore, because these mutations do not affect the domain for tetramer formation in the C-terminal region, mutations on one allele suppress wt-p53 functions encoded by the other allele as a result of heterotetramer formation with wt-p53. This phenomenon is called dominant-negative (DN) mutation (7) and has been revealed as a risk factor for early OSCC recurrence (3).

It has recently been shown, however, the functions of mutated p53 in hot spots are not only inhibition of wt-p53, but also acquisition of new characteristic as an oncogene (gain-of-function: GOF) (12). They are suggested by finding correlations between DN p53 and poor prognosis even in the absence of the WT p53 allele due to loss of heterozygosity (LOH) (3). DN p53 mutants induce the expression of genes such as Cyclin D3, c-Myc, and MDR by binding to other transcription factors (8, 12). It has also been reported that the invasive activity of the non-small-cell lung cancer cell line H1299 expressing no p53 is enhanced by transfection of the p53 Arg248 mutant R248Q but not R248W (13). These findings indicate that the p53 mutants have a different quality in GOF activities even if the mutations occurred at the same codon. Therefore, this study focused on the p53 R248 mutants R248Q and R248W, and determined what kind of GOF these p53 mutants would confer on OSCC cells from the perspective of tumor cell biology.

MATERIAL AND METHODS

Cell lines and cell culture. The human NSCLC NCI-H1299 (H1299) cell was purchased from American Type Culture Collection (ATCC, Manassas, VA). H1299 cells were grown on Dulbeco’s modified Eagle’s minimum essential medium and Ham’s F12 medium (D-MEM/Ham’s F-12; WAKO, Tokyo, Japan) containing 10% fetal bovine serum (FBS; MP Biomedicals, Solon, OH). Human OSCC cell lines SAS, Ca9-22 and HSC4 were obtained from Japanese Collection of Research Bioresources (JCRB, Sennan, Japan). OSCC cell lines were grown on D-MEM with 10% FBS. All the cell lines were cultured in CO₂ incubator 37°C and 5% CO₂.

Expression and reporter vector. To make wt and mutant TP53-expressing plasmids, the fragments of the coding region of TP53 were cut from expressing vectors for yeast pSS16: wt, pSS16: R248Q, and pSS16: R248W. The fragments were amplified by PCR and integrated in pIRES2-AcGFP1 vector (Clonetech, Mountain View, CA). These expression vectors were named pTP53: wt, pTP53R248Q, and pTP53R248W. As a control, pIRES2-AcGFP1-p53(1–83) was made by removing the coding region between codon 82 and codon 99 from pIRES2-AcGFP1-p53(1–99) and integrating stop codon at the codon 84. All the expressing vectors had been already inserted AcGFP1, which translating depending on IRES (internal ribosomal entry site), on downstream of p53 coding region. The reporter vector p53-Luc, which had a reporter gene of firefly luciferase, was purchased from STRATAGENE (La Jolla, CA). In the p53-Luc, there were p53 binding sites repeated 15 times, on the upstream of HSV-TK promoter. WWP-Luc and HDM2-Luc respectively including p21waf1 and MDM2 promoter sequence were provided by Dr. Bert Vogelstein (Johns Hopkins University). The reporter vector pMO23 including Bax promoter sequence was provided Dr. Moshe Oren (Weizmann Institute of Science). Renilla luciferase (pRL-TK) plasmid was purchased from Promega (Madison, WI).

RNA interference (RNAi). To inhibit p53 proteins expression, the siRNA (small interfering RNA) sequence was designed by BLOCK-iT™ RNAi Designer (Invitrogen, Carlsbad, CA). It was named sip53.

AAGACUCCAGUGUAUCUACTT (Sense)
GUAGAUUACCACUGGAGUCUUTT (Anti-sense)

As a control, siAllStars siRNA (Qiagen, Hilden, Germany) was used.

Transfection and selection of expressing cells. Each of SAS and H1299 cells was transfected with pTP53: wt, pIRES2-AcGFP1-p53 (1–83), pTP53R248Q or pTP53R248W by electroporation using CUY21Pro-Vitro-SH (NEPA GENE, Ichikawa, Japan). The stably transfected cells were selected in the medium...
containing 600 μg/mL G418 sulfate (Cellgro, Herndon, VA). After 4 days, GFP positive cells were selected by FACSAria II (BD Biosciences, San Jose, CA). Ca9-22 and HSC4 cells were transfected with lipofectamine 2000 (Invitrogen). Twenty four hours later, cells were harvested and reacted on Dual-Luciferase Reporter Assay System (Promega). Transcriptional activities were measured by LB9506 luminometer (Berthold, Bad Wildbad, Germany).

**Western blot analysis.** SAS, Ca9-22 and HSC4 monolayer cells at a 100-mm dish were washed with cold PBS, and harvested with a cell scraper in lysis buffer (50 mM Tris-HCl, pH 8.0, 159 mM NaCl, and 1% NP-40) added Complete mini (EDTA-free) Protease inhibitor cocktail tablets (Roche). The cell lysates were centrifuged at 17000 × g for 15 min 4°C. The collected supernatants were measured with DC protein assay (Bio-Rad, Hercules, CA), and added to Laemmli sample buffer (Bio-Rad) for 5 min 95°C. These were subjected to SDS-PAGE in 10% polyacrylamide gel and electro transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore, Billerica, MA). The membranes were blocked in TBST-T (20 mM Tris-HCl, pH 7.5, 0.138 M NaCl, 0.6% Tween 20) with 10% skim milk for 1 h at room temperature, and then incubated with primary antibodies (p53 DO-1; MBL, Nagoya, Japan) for 1 h at room temperature. The membranes were next incubated with peroxidase conjugated antibodies to mouse IgG (GE Healthcare, Pittsburgh, PA) for 1 h at room temperature. Antigen-antibody complexes were visualized with ECL Western blotting extract system (Millipore) and detected with ImageQuant LAS4000 mini (GE Healthcare).

**Cell proliferation assay.** SAS, Ca9-22 and HSC4 cells were seeded on a 96 well plate (500 cells/100 μL). After 2, 3, 4, 5 and 6 days, cells were incubated with Cell Counting Kit-8 (10 μL/well, 2 h; Dojindo, Kumamoto, Japan) and measured fluorescent having an excitation spectrum peak at 450 nm by Micro plate manager III (Bio-Rad).

**Dual luciferase assay.** SAS and H1299 cells were seeded on a 24 well plate and cotransfected with 0.1 μg firefly luciferase vector (P53-Luc, WWP-Luc, HDM2-Luc or pMO23), 1 ng Renilla luciferase vector (pRL-TK) as an internal control and 1 μg p53 expression vector (pTP53 : wt, pIRES2-AcGFP1-p53 (1–83), pTP53R248Q or pTP53R248W) by using Lipofectamine 2000 (Invitrogen). Twenty four hours later, cells were harvested and reacted on Dual-Luciferase Reporter Assay System (Promega). Trancriptional activities were measured by LB9506 luminometer (Berthold, Bad Wildbad, Germany).

**Cell invasion assay.** A cell invasion assay was performed using the following procedures. A sol in which the ratio of type-I collagen solution, 10× DMEM, neutralization buffer, and FBS had been adjusted to 8 : 1 : 1 : 0.5 was injected into the wells of a 24-well plate, attached with pressure to a Transwell® membrane with an 8.0-μm pore size (Costar, Cambridge, MA), and left to stand for 45 min. The slip was then washed twice with DMEM, following which DMEM containing 2% FBS was added. The cells (3 × 10^3) were plated onto the cover slip and cultured for 24 h in an incubator at 37°C and 5% CO₂. They were then observed under an inverted microscope. Five random fields of view were photographed in each well at 40× magnification to measure the area of migration for each cell using Photoshop (Adobe Systems, San Jose, CA). The assay was conducted for three wells per sample.

**Cell attachment assay.** A cell adhesion assay was performed using tissue culture plates that had been separately coated with fibronectin or laminin-1, or with poly D-lysine, BSA, or gelatin as controls. DMEM containing no serum was added to each well, following which SAS, Ca9-22, and HSC4 cells (5 × 10^3) were separately plated into the wells. Their morphological changes were then observed under an inverted microscope after 30, 90, 180, and 360 min. Three random fields of view were observed in each well at 100× magnification. The assay was conducted for three wells per sample.
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**RESULTS**

**Generation of cells expressing the p53 mutants R248Q and R248W**

To investigate the functions of p53 with R248Q and R248W mutations in the same codon in human OSCC cells, vectors forcing the expression of these mutant p53 proteins (pTP53R248Q and pTP53R248W) were separately transfected into SAS cells harboring a recessive p53 mutation (E336X). As a control, SAS cells were transfected with the pTP53 (1–83) vector in which a DNA fragment encoding codons 1–83 of p53 had been inserted. The AcGFP1 gene, which is translated in an IRES-dependent manner, was integrated into each of the vectors downstream of the sequence encoding p53, allowing GFP-positive cells to be sorted by FACS Aria II to confirm the transfection of each vector. Expression of the product of the mutant TP53 gene by cells into which the respective expression vector had been transfected was confirmed by Western blotting (Fig. 1).

**p53-binding sequence-dependent transactivation potential of the p53 mutants R248Q and R248W**

Here, it was determined whether the p53 mutants R248Q and R248W had lost their p53-binding sequence-dependent transactivation potential. First, the reporter vector p53-Luc, which harbored 15 repeats of a p53-binding sequence upstream of the HSV-TK promoter and the firefly luciferase gene, was co-transfected with the expression vector for R248Q, R248W or wtp53 into H1299 and SAS cells to analyze the respective transactivation potential using a luciferase assay. This showed that H1299 and SAS cells into which wtp53 had been transfected showed increased transcriptional activity, whereas those that had been separately transfected with the p53 mutants R248Q and R248W did not (Fig. 2-A and E; \( P < 0.01 \)).

The p21Waf1, MDM2, and Bax genes contain a p53-binding sequence in their promoters and so are transactivated by wtp53. Therefore, the reporter vectors WWP-Luc, HDM2-Luc, and pMO23 containing the respective promoter sequences were cotransfected with an expression vector for R248Q, R248W, or wtp53 into H1299 and SAS cells to analyze the respective p53 transactivation potential using a luciferase assay. It was found that H1299 and SAS cells into which wtp53 had been transfected showed increased transcriptional activity with all of the reporters, whereas those that had been separately transfected with the p53 mutants R248Q and R248W did not (Fig. 2-B–D, and F–H; \( P < 0.05 \)).

**Proliferative activity of SAS cells expressing the p53 mutants R248Q and R248W**

The proliferative activity of SAS cells that were forced to express the p53 mutants R248Q and R248W was examined. Parent SAS cells, mock-transfected cells into which a control vector had been transferred, and p53 mutant (R248Q/R248W)-expressing cells were plated into the wells of a 96-well plate and cultured. On days 2, 3, 4, 5, and 6 after plating, the cells were counted for analysis with WST. It was found that the proliferative activity of cells expressing R248Q or R248W was as high as that of the parent SAS cells and the mock-transfected cells (Fig. 3).

**Invasive and motile activities of SAS cells expressing the p53 mutants R248Q and R248W**

To investigate what kind of effect the p53 mutants R248Q and R248W would have on the invasive activity of SAS cells, the infiltration of the cells into type-I collagen gel was assayed using a Transwell chamber. It was found that the invasive activity of R248Q-expressing cells was approximately 1.5-fold greater than that of the other three groups (Fig. 4; \( P < 0.01 \)). In contrast, no significant enhancement was observed in the invasive activity of R248W-expressing cells compared with the parent and mock-transfected cells. This experiment examined whether the enhanced invasive activity observed in R248Q-expressing cells was caused by an increased motility.
Fig. 2 Analysis of the p53-binding sequence-dependent transactivation potential of the R248Q and R248W mutants. An expression vector for control (mock), R248Q or R248W mutant, or wild-type p53 (wtp53), a reporter vector, and the control reporter vector pRL-TK were cotransfected into H1299 cells (A, B, C, D) and SAS cells (E, F, G, H), and their luciferase activity was measured 24 h later. p53-Luc (A, E), WWP-Luc (B, F), HDM2 (C, G), and pMO23 (D, H) were used as reporter vectors. The vertical axis represents the relative light unit (RLU), which was calculated by dividing the luminescence level of firefly luciferase (reporter) by the luminescence level of Renilla luciferase (control reporter). Data are expressed as the mean and standard deviation (n = 3). H1299 and SAS cells into which wtp53 had been transfected showed increased transcriptional activity (*P < 0.05; wtp53 vs. parent, mock, R248Q, and R248W) in both reporter systems, whereas neither H1299 nor SAS cells into which the p53 mutants R248Q and R248W had been separately transfected showed any transactivation.
Next, the ability of cells to adhere to an extracellular matrix (ECM), which is important for cell invasion/motility, was examined (Fig. 6). Cells were plated into the wells of a 96-well plate at $5 \times 10^2$ cells per well and cultured. On days 2, 3, 4, 5, and 6 after the start of culture, the cells were counted for analysis using WST. The assay was conducted in triplicate, and the results are expressed as the mean and standard deviation ($n=3$). The proliferative activity of cells expressing the p53 mutant R248Q or R248W was as high as that of the parent and mock-transfected SAS cells.

The motile activity was assessed using a phagokinetic track assay. R248Q-expressing cells showed higher motile activity than the other three groups ($P < 0.01$; R248Q vs. parent, mock, and R248W). By contrast, there was no difference in the motility of R248W-expressing cells and the parent and mock-transfected cells.

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**Fig. 4** Analysis of the invasive activity of SAS cells expressing the p53 mutants R248Q and R248W. The invasive activity of cells was assessed by examining their ability to infiltrate type-I collagen gel. Cells that infiltrated the gel 24 h after being plated were counted under a microscope in five random fields of view (100×) per well to calculate the mean number per field. This was performed for three wells, and the results are expressed as the mean and standard deviation per field ($n=15$). The invasive activity of R248Q-expressing cells was higher than that of the other three groups ($*P < 0.01$; R248Q vs. parent, mock, and R248W). By contrast, there was no significant difference in the invasive activity of R248W-expressing cells and the parent and mock-transfected cells.

**Fig. 5** Analysis of the motile activity of SAS cells expressing the p53 mutants R248Q and R248W. The motile activity of cells was assessed using a phagokinetic track assay. Five random fields of view (40×) per well were examined. The assay was performed for three wells per sample, and the results for each cell line are shown as the mean and standard error. R248Q-expressing cells showed a higher motile activity than the other three groups ($**P < 0.01$; R248Q vs. parent, mock, and R248W). By contrast, there was no difference in the motility of R248W-expressing cells and the parent and mock-transfected cells.

Extracellular matrix-adhesive activity of SAS cells expressing the p53 mutants R248Q and R248W

Next, the ability of cells to adhere to an extracellular matrix (ECM), which is important for cell invasion/motility, was examined (Fig. 6). Cells were plated on tissue culture plates coated with either fibronectin or laminin-1, or with poly D-lysine or gelatin as controls, and the morphology of the cells
Fig. 6  Analysis of the extracellular matrix-adhesive activity of SAS cells expressing the p53 mutants R248Q and R248W. Parent cells, mock-transfected cells, and SAS cells expressing the p53 mutant R248Q or R248W were separately plated into plates coated with poly D-lysine (A), gelatin (B), fibronectin (C), or laminin-1 (D), and the percentage of spreading cells was calculated after 30, 90, 180, and 360 min. Three random fields of view (40×) were observed in each well, and the percentage is expressed as the mean and standard deviation (n = 3). R248Q-expressing cells showed significantly enhanced adhesive activity compared with the other three groups on plates coated with fibronectin and laminin-1 (*P < 0.05; R248Q vs. parent, mock, and R248W), whereas no significant difference in adhesive activity was detected among the groups on plates coated with poly D-lysine and gelatin.
was observed after 30, 90, 180, and 360 min. In the case of fibronectin-coated plates, R248Q-expressing cells contained significantly more spreading cells than the other three groups 30 min after cell plating (Fig. 6C; P < 0.01). In the case of laminin-coated plates, R248Q-expressing cells contained more spreading cells 30, 90, and 180 min after plating (Fig. 6D; P < 0.05). In contrast, no significant increase was observed in the adhesive activity of R248Q-expressing cells at any time on the poly D-lysine-coated and gelatin-coated plates. The R248W-expressing cells showed no significant change in adhesive activity compared with the parent and mock-transfected cells under any conditions.

**Suppression of endogenous p53 mutant R248Q and R248W expression**

To analyze the roles of the endogenously expressed p53 mutants R248Q and R248W, mutant p53 expression by OSCC cells (HSC-4 and Ca9-22) was suppressed using RNAi. HSC4 and Ca9-22 cells endogenously express completely R248Q and R248W, respectively (11). To suppress the expression of these mutant p53 proteins, siRNA (sip53) targeting p53 mRNA was transfected into HSC4 and Ca9-22 cells. In addition to these sip53-infected cells, their parent cells and cells that had been subjected to electroporation without the addition of siRNA (“E. only” cells) were also used, along with siAllStars-transfected cells as a control. Expression of the mutant TP53 gene product was confirmed in each cell group by Western blot analysis (Fig. 7A and B).

**Changes in proliferative activity following the suppression of endogenous p53 mutant R248Q and R248W expression**

The proliferative activity of HSC4 and Ca9-22 cells in which the expression of the endogenous p53 mutants R248Q and R248W respectively had been suppressed was examined. Parent cells, E. only cells, and cells transfected with siRNA, i.e., siAllStars or sip53, were plated into the wells of a 96-well plate and cultured. On days 2, 3, 4, 5, and 6 after plating, the cells were counted for analysis using WST. All three treatment groups for both cell types showed lower proliferative activity than the parent cells (Fig. 8A and B; P < 0.01). It was thought that this depression of proliferative activity in all three treatment groups occurred due to electroporation. No difference in proliferative activity was detected among the three groups.

**Changes in invasive and motile activities following the suppression of endogenous p53 mutant R248Q and R248W expression**

The next experiment investigated what kind of effects the suppression of p53 mutant R248Q and R248W expression would have on the invasive activity of HSC4 and Ca9-22 cells. The ability of cells to infiltrate type-I collagen gel was measured 2 days after siRNA transfection. It was found that HSC4 cells in which R248Q expression had been suppressed had less than half the invasive activity of the other three treatment groups (Fig. 9A; P < 0.01). In contrast, Ca9-22 cells with suppressed R248W expression showed the same motile activity as the parent group while no significant effect on their invasive activity compared with the control group (Fig. 9B).

Changes in the motile activity of HSC4 and Ca9-22 cells in which the expression of the p53 mutants R248Q and R248W had been suppressed were analyzed using a phagokinetic track assay. These were compared with the motility of E. only, siAllStars, and sip53 cells 2 days after electroporation. It was found that HSC4 cells in which R248Q expression had been suppressed showed lower motile activity than the other three groups (Fig. 10A; P < 0.01). In contrast, Ca9-22 cells with suppressed R248W expression showed the same motile activity as the
Gain of function of p53 R248Q in OSCC

activity was observed in HSC4 cells in which R248Q expression had been suppressed when plated on BSA- and gelatin-coated plates (Fig. 11A and B). There was no significant change in the adhesive activity of Ca9-22 cells in which R248W expression had been suppressed under any conditions and at any time points compared with the other three treatment groups (Fig. 11A–D).

DISCUSSION
Several studies have demonstrated that TP53 mutations are strongly related to the prognosis of human cancers. For example, it has been reported that DN p53 mutants significantly decrease the vital prognosis of endometrial cancer patients (9) and significantly shorten relapse-free survival in OSCC (3). In control group (Fig. 10B).

Changes in extracellular matrix-adhesive activity following the suppression of endogenous p53 mutant R248Q and R248W expression
The final experiment examined what kind of effect the suppression of p53 mutant R248Q and R248W expression would have on the ECM-adhesive activity of HSC4 and Ca9-22 cells. In the case of HSC4 cells plated into wells coated with fibronectin, only cells in which R248Q expression had been suppressed showed a lower rate of spreading (adhesion rate) 180 min after plating (Fig. 11C; \( P < 0.01 \)). Moreover, HSC4 cells with suppressed R248Q expression also showed a decreased adhesion rate to laminin-1 30 min after plating (Fig. 11D; \( P < 0.05 \)). In contrast, no significant decrease in adhesive activity was observed in HSC4 cells in which R248Q expression had been suppressed when plated on BSA- and gelatin-coated plates (Fig. 11A and B).

There was no significant change in the adhesive activity of Ca9-22 cells in which R248W expression had been suppressed under any conditions and at any time points compared with the other three treatment groups (Fig. 11A–D).
many cancers, one p53 gene allele is often lost when the other allele has a mutation (11). Therefore, DN p53 mutants not only cause loss of function of wtp53, but are also likely to have some new function(s) (i.e., a gain of function, GOF). Indeed, a previous study using the non-small-cell lung cancer cell line H1299 expressing no p53 demonstrated GOF of mutant p53 (13). That study revealed that when H1299 cells were induced to express one of the two types of DN p53 mutants, R248Q and R248W, the R248Q-expressing cells showed higher invasive activity than both the R248W-expressing cells and their parent cells.

This study examined whether the p53 mutants R248Q and R248W would also show a GOF action in OSCC cells. The tongue cancer-derived cell line SAS, which harbored p53 with the recessive mutation E336X, was forced to express one of the two types of DN p53 mutants, R248Q or R248W, and their proliferative, invasive, motile, and adhesive activities were analyzed. An increase in invasive activity was only detected in SAS cells that were forced to express R248Q (Fig. 4), indicating that this mutation is involved in the acquisition of malignant pheno-

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**Fig. 9** Changes in invasive activity following the suppression of endogenous p53 mutant R248Q and R248W expression. The invasive activity of HSC4 (A) and Ca9-22 (B) cells was assessed by examining their ability to infiltrate type-I collagen gel. Five random fields of view (100×) per well were observed 24 h after cell plating to calculate the mean number of cells per field. This was performed across three wells, and the results are expressed as the mean and standard deviation per field (n = 15). The invasive activity of HSC4 cells in which R248Q expression had been suppressed was significantly lower than that in the other three groups (**P < 0.01; sip53 vs. parent, E. only, and siAllStars). By contrast, the suppression of R248W expression in Ca9-22 cells did not have any significant effect on their invasive activity compared with the control group.

**Fig. 10** Changes in motile activity following the suppression of endogenous p53 mutant R248Q and R248W expression. The motile activity of HSC4 (A) and Ca9-22 (B) cells was assessed using a phagokinetic track assay. Five random fields of view (40×) per well were observed. The assay was performed for three wells per sample, and the results by cell line are expressed as the mean and standard error. The motile activity of HSC4 cells in which R248Q expression had been suppressed was lower than that in the other three groups (**P < 0.01; sip53 vs. parent, E. only, and siAllStars). By contrast, the motile activity of Ca9-22 cells in which R248W expression had been suppressed was as high as that of the control group.
Fig. 11 Changes in extracellular matrix-adhesive activity following the suppression of endogenous p53 mutant R248Q and R248W expression. Parent cells, E. only, and siRNA-transfected HSC4 and Ca9-22 cells were plated on plates coated with BSA (A), gelatin (B), fibronectin (C), or laminin-1 (D), and the percentage of spreading cells was calculated after 30, 90, 180, and 360 min. Three random fields of view (40×) per well were observed, and the percentage is expressed as the mean and standard deviation (n = 3). HSC4 cells in which R248Q expression had been suppressed showed decreased adhesion rates to fibronectin- and laminin-1-coated plates (*P < 0.05; sip53 vs. parent, E. only, and siAllStars). By contrast, no significant reduction in adhesive activity of HSC4 cells in which R248Q expression has been suppressed was observed on plates coated with BSA or gelatin. In addition, Ca9-22 cells in which R248W expression had been suppressed showed no significant change in their adhesive activity compared with the other three group cells under any conditions or at any time points.
types in OSCC, as observed in H1299 cells.

The invasion of cancer cells is particularly important at the stage where the cells migrate to the inside or outside of vessels during the establishment of metastasis. This phenomenon consists of three stages: (A) adhesion to ECM, (B) degradation of ECM, and (C) movement of the cancer cells (6). Therefore, the effects of the p53 mutant R248Q on the motile and adhesive activities of cells harboring the mutant were examined. It was found that only SAS cells that were forced to express R248Q showed enhanced motile and adhesive activities (Figs. 5 and 6). Furthermore, neither of the p53 mutants exerted any influence on the proliferative activity of the cells (Fig. 3).

In addition, HSC4 cells endogenously expressing the p53 mutant R248Q and Ca9-22 cells expressing R248W were transfected with the siRNAs to inhibit mutant p53 expression and to investigate changes in their proliferative, invasive, motile, and adhesive activities. This yielded similar results to the experiment in which SAS cells were forced to express p53 mutants. Briefly, the invasive, motile, and adhesive activities were significantly decreased in HSC4 cells in which R248Q expression had been suppressed, whereas these were not affected by the suppression of R248W expression in Ca9-22 cells (Figs. 9, 10, and 11). Once again, suppression of p53 expression did not affect the proliferation of either of these cell lines (Fig. 8). The results of the experiments using these three lines of OSCC cells not only demonstrate that the p53 mutant R248Q is involved in the acquisition of higher invasive activity but also indicate that this enhancement of invasive activity is likely caused by an increase in the cellular motility and adherence to an ECM component.

The exact role that DN p53 mutants play in the mechanism of GOF in OSCC cells, which was demonstrated by their increased invasive, motile, and ECM-adhesive activities, remains unclear. Several reports have considered that DN p53 mutants in OSCC, but also to develop more precise diagnosis for malignancy on the basis of the types of TP53 mutations. In conclusion, we showed that the DN p53 mutant R248Q was involved in the acquisition of malignant phenotypes through the enhancement of invasive, motile, and adhesive activities in oral squamous carcinoma cells.

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