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Citation
Oral Oncology, 45(6), pp.521-525; 2009

Issue Date
2009-06

URL
http://hdl.handle.net/10069/20064

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Title: Ribonucleotide reductase small subunit p53R2 promotes oral cancer invasion via the E-cadherin/β-catenin pathway

Running title: p53R2 promotes oral cancer invasion

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**Abstract:** The p53-inducible p53R2 gene has been isolated and shown to play a crucial role in DNA repair and synthesis after DNA damage. Moreover, the expression and activity of p53R2 has been reported to be associated with the anticancer agent resistance of human cancer cells. Previously, we reported that the presence of p53R2 expression was a predictive factor for regional lymph node metastasis in oral squamous cell carcinoma; however, the mechanism of cancer metastasis by p53R2 expression is still unclear. In the present study, we analyzed the correlation of p53R2 expression with cancer invasion in vitro. Three human oral cancer cell lines (SAS, HSC-3 and Ca9-22) were cultured, and the invasive potential of these cancer cells was evaluated using Matrigel invasion assay. To investigate the effect of p53R2 on cancer invasion, the down-regulation of p53R2 was examined by small interfering RNA (siRNA). Moreover, we examined the intracellular localization of cell adhesion molecules (E-cadherin and β-catenin) in subcellular extractions of cancer cells by immunoblotting. The proteolytic activity of matrix metalloproteinases (MMPs) was assessed by gelatin zymography. Down-regulation of p53R2 significantly enhanced the invasion potential (p<0.01), and enhanced nuclear translocation of β-catenin with loss of total cellular E-cadherin.
expression in p53 mutant cancer cells, but not in p53 wild-type cancer cells. These changes in the invasion index by p53R2 siRNA transfection were not accompanied by alterations in MMP activity and expression. These results suggested that the expression of p53R2 could be associated with the invasion of cancer cells, and indicated that p53R2 might promote cancer invasion via the E-cadherin/β-catenin pathway without the alteration of MMP activity.

**KEYWORDS:** p53R2; invasion; E-cadherin; β-catenin; small interfering RNA
Introduction

The p53-inducible p53R2 gene has been isolated and shown to play a crucial role in DNA repair and synthesis after DNA damage.\(^1\,^2\) The p53R2 gene is localized on human chromosome 8q23.1\(^1\), and some investigators have suggested that the genomic instability often seen in cancers lacking wild-type p53 may reflect the dysfunction of ribonucleotide reductase (RR) due to the failure of p53R2 induction.\(^1\,^3\,^4\) In cancer cells, it is suggested that overexpression of p53R2 interferes with regulation of the p53-dependent DNA repair pathway and increase the sensitivity of cells to anticancer drugs.\(^2\,^3\) In fact, the expression and activity of p53R2 has been reported to be associated with the anticancer agent resistance of human malignancies, including oral cavity\(^5\) and esophageal cancers.\(^6\) Recently, we have reported that the inhibition of p53R2 by small interfering RNA (siRNA) technology enhances 5-fluorouracil sensitivity of oral cancer cells in vitro.\(^7\) These reports support the idea that p53R2 is a potential target for cancer gene therapy; however, the effect of p53R2 on the cellular biological characteristics of cancer cells is still unclear.

The presence of neck regional lymph node metastasis is strongly related to a poor prognosis in oral squamous cell carcinoma.
Some investigators have demonstrated that high malignancy grading of the deep invasive front had predictive value for neck regional lymph node metastasis and prognosis in OSCC. Previously, we reported that the presence of p53R2 expression was a predictive factor for regional lymph node metastasis in OSCC. Some investigators have reported that overexpression of p53R2 was related with cancer progression. On the other hand, a recent report suggested that p53R2 had metastasis-suppressing ability in colon cancer, and down-regulation of p53R2 by siRNA increased the invasion potential of cancer cells. Thus, the correlation of p53R2 expression with cancer metastasis or invasion is still controversial. Here, we analyzed the correlation of p53R2 expression with oral cancer invasion in vitro.

**Materials and Methods**

**Cells**

The human OSCC cell lines, SAS (p53 wild type), HSC-3 (p53 mutant), and Ca9-22 (p53 mutant) were obtained from the Human Science Research Resource Bank (Osaka, Japan). All cells were cultured in a 1:1 mixture of Ham’s F-12/DMEM supplemented with 10% FBS (Trace
Scientific, Melbourne, Australia). All cells were maintained under humidified 5% CO₂ incubation at 37°C.

Small interfering RNA (siRNA) treatment
All siRNAs were purchased from Takara (Otsu, Japan). Cells were transfected with double-strand RNA using CodeBreaker™ siRNA transfection reagent (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, 5 × 10⁴ cells were plated in 6-well plates and allowed to grow for 24 h (until they were approximately 50% confluent). Cells were then transfected with siRNA at a final concentration of 30nM. The p53R2 siRNA sequences were 5’-GAACAAGCUUAAAGCAGAUdTdT-3’ and 5’-AUCUGCUUUAAGCUUGUUCdTdT-3’. The scrambled control siRNA sequences were 5’-AGCAGACGAAUAUAACGAUdTdT-3’ and 5’-AUCGUUAUAUUCGUCUGCdTdT-3’. All sequences were submitted to the National Institutes of Health Blast program to ensure gene specificity.

RNA isolation and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad,
CA) and first-strand cDNA was synthesized from 1 μg total RNA using Oligo d (T) primer (Invitrogen) and ReveTra Ace (TOYOBO, Osaka, Japan). For PCR analysis, cDNA was amplified by Taq DNA polymerase (TAKARA, Otsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Each PCR program involved a 3-min initial denaturation step at 94°C, followed by 28 cycles for p53R2, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), or 18 cycles for GAPDH at 94°C for 30 s, 57°C for 30 s and 72°C for 1 min, on a PCR Thermal Cycler MP (TAKARA). Each primer was designed to encompass an exon junction to prevent templating from possibly contaminated genomic DNA. Primer sequences were, for p53R2: F, TAAACAGGCACAGGCTTCCT and R, CGCTCCACCAAATTTTCATT; for MMP-2: F, CACTTTCCTGGGCAACAAAT and R, TGATGTCATCCTGGGACAGA; for MMP-9: F, TTCATCTTCCAAGGCAAATC and R, CAGAAGCCCACTTCTTGT; for GAPDH: F, ATGTCGTGGAGTCTACTGGC and R, TGACCTTGCCCACAGCCTTG. The amplified products were separated by electrophoresis on ethidium bromide-stained 1.2% agarose gels. Band intensity was measured by Image J version 1.37.
Western blot analysis

Total cell lysates were purified using a Mammalian cell extraction kit (BioVision, Mountain View, CA) whereas the membrane, cytoplasmic and nuclear proteins were purified using a ProteoExtract subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany). All subsequent manipulations were performed on ice. The protein concentration of each sample was measured with micro-BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). The samples were denatured in SDS sample buffer and loaded onto 12.5% polyacrylamide gels. After electrophoresis, the proteins were transferred onto a polyvinylidine difluoride membrane and immunoblotted with polyclonal rabbit anti-p53R2 (GeneTex, San Antonio, TX), polyclonal rabbit anti-β-catenin (Cell Signaling Technology, Danvers, MA), polyclonal rabbit anti-E-cadherin (Cell Signaling Technology), or polyclonal rabbit anti-actin (Sigma-Aldrich, Tokyo, Japan). The signal was detected using the horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ), and then visualized using an ECL Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).
Invasion assay

The BioCoat Matrigel invasion chamber (Becton Dickinson, Bedford, MA) contains an internal chamber with an 8-μm porous membrane bottom and is coated with Matrigel. Twenty-four-well cell culture inserts and a 24-well multiwell companion plate were used for this experiment. Cells were transfected with p53R2 siRNA, transfection reagent alone (mock), or scrambled control siRNA, followed by incubation for 24 h. The transfected cells were collected by tripsinization, followed by seeding in the internal chamber at $2.5 \times 10^4$ cells in medium containing 5% FBS. The lower chamber was filled with medium containing 10% FBS as a chemoattractant. Cells were incubated for 48 h at 37°C in a 5% CO$_2$ atmosphere. After incubation, non-invading cells were removed from the top of the wells with a cotton swab, and cells transferred to the inverse surface of the membrane were subjected to Diff-Quick staining. The cells were counted under a microscope at 100× magnification. For the control cell count, cells that passed through a control chamber without Matrigel were counted. All experiments were completed in triplicate, and at least 4 fields/well were counted. The percentage of the cell count that passed through the Matrigel chamber to the control count was calculated as the invasion index.
Gelatin zymography

The proteolytic activity of MMPs was assessed by SDS-PAGE using Novex zymogram gel (Invitrogen) containing 0.1% (m/v) gelatin. Cells were transfected with p53R2 siRNA, mock, or scrambled control siRNA, followed by incubation for 24 h, and were rinsed PBS and then incubated in serum-free medium for 48 h. Conditioned media were subjected to zymography. Following electrophoresis at 4°C, the gels were incubated in Novex zymogram renaturation buffer (Invitrogen) for 30 minutes at room temperature and agitated with Novex zymogram development buffer (Invitrogen) overnight at 37°C. Clear bands indicative of gelatinolytic activity were visualized by staining the gels with Coomassie blue.

Statistical analysis

Statistical analyses were performed using StatMate III (Atms Co., Tokyo, Japan). Continuous data are given as the mean ± standard deviation. Data sets were examined by one-way analysis of variance (ANOVA) followed by Scheffe’s post-hoc test. P values less than 0.05 were considered significant.
Results

Down-regulation of p53R2 significantly enhanced the invasion potential in p53 mutant cell lines, but not in the p53 wild-type cell line

To determine the effect of p53R2 siRNA treatment on the invasion potential, we transfected SAS, HSC-3, and Ca9-22 cells with p53R2 siRNA, mock, and scrambled siRNA. Mock and scrambled siRNA transfection had no effect on p53R2 expression. An obvious reduction in p53R2 was observed with p53R2 siRNA at a final concentration of 30 nM (Fig. 1A), and analyzed using Matrigel invasion assay (Fig. 1B). Transfection with p53R2 siRNA significantly decreased invasive cells in the p53 wild-type cell line (SAS) whereas, in p53 mutant cell lines (HSC-3 and Ca9-22), transfection with p53R2 siRNA significantly increased the invasion index (p<0.01, Fig. 1C).

Down-regulation of p53R2 increased the levels of total cellular E-cadherin in p53 wild-type cell lines, but not in p53 mutant cell lines

We assessed the effect of p53R2 siRNA treatment on the total
cellular content of cell-cell adhesion molecules. Transfection with p53R2 siRNA increased the levels of total cellular E-cadherin in the p53 wild-type cell line (SAS) whereas, in p53 mutant cell lines (HSC-3 and Ca9-22), transfection with p53R2 siRNA significantly decreased the levels of total cellular E-cadherin. The down-regulation of p53R2 did not alter the levels of total cellular β-catenin in both p53 wild-type (SAS) and p53 mutant (HSC-3 and Ca9-22) cells (Fig. 2A). Furthermore, we examined the influence of the down-regulation of p53R2 on MMPs. Analysis by gelatin zymography showed that the proteolytic activities of MMP-2 and MMP-9 were not altered in the down-regulation of p53R2 in both p53 wild-type (SAS) and p53 mutant (HSC-3 and Ca9-22) cells. Moreover, the comparison of MMP mRNA levels in the transfection of p53R2 by RT-PCR demonstrated that MMP-2 and MMP-9 were not affected by the down-regulation of p53R2 (Fig. 2B).

**Down-regulation of p53R2 enhanced nuclear translocation of β-catenin in p53 mutant cell lines, but not in the p53 wild-type cell line**

To examine the effect of p53R2 siRNA treatment on the localization of β-catenin protein, the fractionated membrane, cytoplasmic and...
nuclear proteins were analyzed by immunoblotting. The nuclear fraction of β-catenin clearly decreased in response to p53R2 siRNA treatment in the p53 wild-type cell line (SAS). In contrast, in p53 mutant cell lines (HSC-3 and Ca9-22), the nuclear fraction of β-catenin increased in response to p53R2 siRNA treatment (Fig. 2C).

Discussion
Tumor invasion and metastasis in various cancers including oral squamous cell carcinoma (OSCC) are regulated by various genetic instabilities. These genetic instabilities involve mutation of the tumor suppressor gene p53 and loss of function of adhesion molecules. RR plays an essential role in DNA replication and repair. In particular, p53-inducible RR small subunit 2 homologue p53R2 is a direct target for p53, and its induction in response to DNA damage assists in G2 arrest and provides DNA precursors for DNA repair; therefore, activation of the invasive potential caused by genetic instabilities is association with disruption of the p53-p53R2 DNA repair system.

The RR large subunit R1 (M1) has been known to possess metastasis-suppressing activity, while the RR small subunit R2 (M2)
has been demonstrated to play a critical role in enhancing invasive potential in human cancer cells.\textsuperscript{24} However, few studies have examined the relationship with p53R2 and the invasive potential in human cancer cells. Although a recent study demonstrated that p53R2 had metastasis-suppressing potential in human cancer cells as well as M1, the mechanism by which p53R2 regulated the invasion potential was not concluded.\textsuperscript{15} In our series, down-regulation of p53R2 significantly enhanced the invasion potential in p53 mutant cell lines (HSC-3 and Ca9-22). Conversely, the down-regulation of p53R2 in the p53 wild-type cell line significantly inhibited the invasion potential. In contrast with our results, Liu et al. reported that down-regulation of p53R2 enhanced the invasion potential in both p53 wild-type and p53 mutant cells.\textsuperscript{15} However, they reported that p53R2 would be negatively related to the metastasis of colon adenocarcinoma only in the p53 mutant group and not in the p53 wild-type group, and indicated that the invasion-suppressing ability of p53R2 could be more apparent in cancer cells with a dysfunction of p53.\textsuperscript{15} Other reports suggested that wild-type p53 could not enhance the metastasis-suppressing ability of p53R2.\textsuperscript{22,25} Likewise, our results indicated that the inhibition of p53R2 by siRNA technology decreased the invasion
potential in p53 wild-type cancer cells, but not in p53 mutant cells.

In p53 mutant cells, we demonstrated that the down-regulation of p53R2 decreased the levels of total cellular E-cadherin accompanied by the nuclear accumulation of β-catenin. These results suggested that the down-regulation of p53R2 would decrease cellular adhesion mediated by the cadherin-catenin complex. Catenin links cadherin with the actin cytoskeleton, and can also form a complex with epidermal growth factor receptor.26 β-catenin, a multifunctional protein, is involved in major functions, such as cell adhesion and mediation of the Wnt signal pathway,27,28 which consists of highly conserved secreted ligands that bind cell-surface receptors called frizzled and lipoprotein receptor-related protein.29,30 In the presence of Wnt signaling, β-catenin is accumulated in the cytoplasm, translocated into the nucleus, interacts with the DNA-binding T-cell factor complex and acts as a transcriptional activator, thus turning on target genes, including c-myc, vascular endothelial growth factor, cyclooxgenase 2, and others, which are involved in development and oncogenesis.32 Moreover, a recent study reported that dysfunction of the Wnt signaling pathway caused by microsatellite and chromosomal instability was associated with high nuclear β-catenin
Thus, our results suggested that the down-regulation of p53R2 increased genetic instability and thereby brought about dysfunction of the Wnt pathway with the nuclear accumulation of β-catenin in p53 mutant cancer cells, and the loss of E-cadherin resulted in enhancing invasion potential.

We further investigated the effect of p53R2 down-regulation on MMP activities. MMPs are a family of zinc dependent proteinases, which are classically recognized to play a critical role in the degradation of many components of the extracellular matrix. Among the many MMPs, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) have a critical function in the degradation of extracellular matrix. The activation of MMP-2 and MMP-9 expression has been well correlated with an invasive phenotype of OSCC cells. In our experiments, proteolytic activity and mRNA expression were analyzed by gelatin zymography and RT-PCR, respectively; however, our results indicated that changes in invasion index by p53R2 siRNA transfection were not accompanied by alterations in MMP activity and expression.

In conclusion, we suggest that p53R2 might be associated with oral cancer invasion, and possessed invasion-suppressing ability in p53 mutant cancer cells, but not in p53 wild-type cells. Moreover,
we suggest that p53R2 might promote cancer invasion via the E-cadherin/β-catenin pathway without altering MMP activity.

**Acknowledgement**
This study was partially supported by Grant 20791534 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.
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Figure legends

Figure 1 (A) Effect of p53R2 siRNA on the expression of p53R2 was evaluated by RT-PCR or Western blot of SAS, HSC-3 and Ca9-22 cells. Cells were transfected for 72 hours with transfection reagent alone (Mock), scrambled (SCR) siRNA, or p53R2 siRNA. (B) Representative Diff-Quick staining of Matrigel invasion chambers (x 200). (C) Down-regulation of p53R2 significantly increased the invasion index in p53 mutant cell lines (HSC-3 and Ca9-22), but not in p53 wild-type cell line (SAS). Data are presented as the means of three separate experiments, each performed in triplicate. Bars, SD. *P<0.01, compared with Mock and Scrambled siRNA, respectively.

Figure 2 Effect of p53R2 siRNA on the expression of E-cadherin, β-catenin, and MMPs. (A) Western blot analyses show E-cadherin and β-catenin expressions in SAS (p53 wild-type), HSC-3 (p53 mutant), and Ca9-22 (p53 mutant) cells. (B) Analysis by zymography of gelatinolytic activities in conditioned media as described in Materials and Methods. Representative RT-PCR of MMP-2 and MMP-9 for the inhibition of p53R2. (C) Representative Western blot for β-catenin expression in membrane, cytoplasmic, and nuclear fractions of SAS, HSC-3, and Ca9-22 cells treated with transfection
reagent alone (Mock), scrambled (SCR) siRNA, or p53R2 siRNA.