SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 46: 148, 2021

Oncogenic E6 and/or E7 proteins drive proliferation and invasion of human papilloma virus-positive head and neck squamous cell cancer through upregulation of Ror2 expression

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Received December 2, 2020; Accepted April 15, 2021

DOI: 10.3892/or.2021.8099

Abstract. Ror2 (receptor tyrosine kinase like orphan receptor 2) is highly expressed in various types of cancers; in the majority of these cancers, Ror2 expression is associated with more aggressive disease states. Recently, it has been reported that Ror2 is highly expressed in human papilloma virus (HPV)-positive head and neck squamous cell cancer (HNSCC) cell lines, presumably indicating that Ror2 plays a critical role in HPV-related cancers. However, the function of Ror2 in HPV-positive HNSCC is currently unknown. Here, we first examined the expression levels of Ror2 in clinical specimens from patients with HPV-negative and HPV-positive oropharyngeal squamous cell cancer (OPSCC) via immunohistochemical analysis. We found that Ror2 was expressed in both HPV-negative and HPV-positive OPSCC tissues. We then confirmed that HPV-positive HNSCC cell line, UPCI:SCC152 cells, express Ror2 higher than HPV-negative cell lines as previously reported. Suppressed expression of HPV *E6/7* resulted in reduced expression levels of Ror2. We also revealed that Ror2 downregulation significantly inhibited the proliferation of UPCI:SCC152 cells without inducing apoptosis. Moreover, Ror2 knockdown decelerated G_1/S phase progression and abrogated invasive migration of UPCI:SCC152 cells. These results provide strong evidence that E6 and/or E7 oncoproteins regulate the progression of HPV-positive HNSCC by upregulating Ror2 expression, suggesting that Ror2 could potentially be a novel target in HPV-related cancers.

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Abbreviations: HPV, human papilloma virus; HNSCC, head and neck squamous cell cancer; OPSCC, oropharyngeal squamous cell cancer

Key words: human papilloma virus, E6, E7, head and neck squamous cell cancer, oropharyngeal squamous cell cancer, Ror2, G_1/S transition, cell cycle, invasion

Introduction

Tobacco and alcohol consumption are well-recognized risk factors for head and neck squamous cell cancer (HNSCC). However, over the last few decades, oncogenic human papilloma virus (HPV) has emerged as another major etiologic factor for HNSCC and some other cancers (1). HPV is responsible for 4.5% of all human malignancies and is associated with 30% of cases of HNSCC (2). HPV is an ancient, nonenveloped, circular double-stranded DNA virus consisting of approximately 220 distinct HPV genotypes that have been identified to date (3,4). The HPV genome is comprised of the early genes E1, E2, E4, E5, E6, and E7, and late genes L1 and L2. HPV E6 and E7 are considered to be the main oncoproteins that manipulate cellular signaling pathways to promote HPV-mediated carcinogenesis (3,4). HPV-associated HNSCC has been shown to differ from alcohol- and tobacco-associated HNSCC in terms of molecular pathophysiology, presentation and prognosis. Patients with HPV-related HNSCC generally have less exposure to alcohol and tobacco, tend to be younger, and have a better prognosis compared to HPV-negative HNSCC (5). However, the molecular and cellular alterations underlying the pathobiology of HPV-related cancers remains ambiguous. While the prevalence of patients with HPV-related cancers is rapidly increasing, there are still many questions that need to be addressed for proper control of HPV-positive HNSCC. Further studies comparing the cellular pathways between HPV-negative and HPV-positive HNSCC in more detail would help to elucidate strategies targeting cellular pathways unique to HPV-positive HNSCC.

A recent study which employed comprehensive molecular and histoepigenetic analysis with epigenomic deconvolution has identified potential novel biomarkers and therapeutic targets

for HPV-positive HNSCC (6). In this report, receptor tyrosine kinase like orphan receptor 2 (Ror2) was proclaimed as a novel therapeutic target for HPV-positive HNSCC. Ror2 belongs to the group of Ror-family receptors that regulate cellular proliferation, differentiation, polarity, and migration (7). The Ror-family receptors, consisting of Ror1 and Ror2, act as receptors for Wnt5a to mediate the noncanonical Wnt signaling which plays essential roles during developmental morphogenesis (7). Although it has been shown that Ror2 is highly expressed in various malignant tumor cells and is involved in tumor progression by promoting proliferation, migration, and invasion of tumor cells (8-10), the mechanisms by which Ror2 mediates the promotion of tumor progression remain poorly understood. Recently, we have shown that Ror2 plays a crucial role in regulating the G_1/S phase transition of bFGF-stimulated NIH/3T3 fibroblasts (11); however, it remains unclear whether Ror2 regulates the G_1/S transition of tumor cells. We have also shown that Ror2 expression in oral cancer was significantly higher than that in normal oral mucosa, and that Ror2 is associated with cellular polarity, motility, and tumor aggressiveness (12). However, it is currently unknown whether Ror2 is involved in the aggressiveness of HPV-related HNSCC. Considering the apparent difference in cellular properties between HPV-negative and HPV-positive HNSCC, the function of Ror2 in HPV-positive HNSCC might offer a unique platform for exploring novel diagnostic and therapeutic targets.

Hence, the aim of the present study was to investigate the role of Ror2 in the regulation of cellular proliferation, apoptosis, and invasive migration of HPV-positive HNSCC.

Materials and methods

Cell culture and transfection. The human HPV16-positive HNSCC cell line UPCI:SCC152 was obtained from the American Type Culture Collection (ATCC). The human HPV-negative HNSCC cell lines UM-SCC-22B and Ca9-22 were obtained from RIKEN Bio Resource Center (The Institute of Physical and Chemical Research, Tsukuba, Japan). The short-tandem repeat profiles of UM-SCC-22B and Ca9-22 were analyzed, and we confirmed that these cell lines are not contaminated. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Corp.) supplemented with 10% FBS at 37˚C. For siRNA transfection, UPCI:SCC152 cells were transfected with the respective siRNAs using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. siRNAs targeting human HPV16 *E6/7* (Ambion, Thermo Fisher Scientific, Inc.), *Ror2* (Sigma-Aldrich; Merck KGaA), and their control siRNA (Silencer Select Negative Control No. 1, cat. no. 4390843, Thermo Fisher Scientific Inc.; Mission siRNA Universal Negative Control, cat. no. SIC_001: Sigma-Aldrich; Merck KGaA) were used. The following target sequences were used for the respective siRNAs: si-*Ror2*#1, 5'-GCAAUGUGCUAGUGUACGA-3'; si-*Ror2*#2, 5'-CCUUUGAGAAGACACCAUA-3'; si-*E6/7*#1, 5'-CCGGACAGAGCCCAUUACA-3'; si-*E6/7*#2, 5'-CACCUACAUUGCAUGAAUA-3'.

Flow cytometric analysis. For cell cycle analysis, cells were synchronized using single thymidine block. UPCI:SCC152 cells were seeded onto 6-cm (ϕ) dishes and were transfected with *Ror2* siRNA or control siRNA. Forty-eight hours after transfection, the cells were arrested at the G_1/S phase by treatment with 2 mM thymidine for 24 h. Synchronized cells were released by two washes with phosphate-buffered saline (PBS) and adding fresh medium. Cells were fixed in 70% (v/v) ethanol and treated with 100 μg/ml RNase A (Thermo Fisher Scientific, Inc.) and 50 μg/ml propidium iodide (PI) (Sigma-Aldrich; Merck KGaA). DNA contents were analyzed using a BD-LSRFortessa™ X-20 flow cytometer (BD Biosciences).

For the apoptosis assay, cells transfected with either *Ror2* siRNA or control siRNA were synchronized using a single thymidine block as described above. Synchronized cells were resuspended in 100 μl of binding buffer containing 5 μl FITC-Annexin V and 5 μl PI, following the manufacturer's instructions (BD Biosciences). Following a 15-min incubation period at room temperature in the dark, 400 μl of binding buffer was added to the cell suspension and the samples were analyzed using a BD-LSRFortessa X-20 flow cytometer (BD Biosciences) within 1 h.

WST-8 assay. The WST-8 assay was carried out using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. In brief, 2 days after transfection with the indicated siRNAs, 1,000 cells were seeded onto a 96-well plate in triplicate. After the cells were cultured for the indicated periods of time, WST-8 reagent was added to the culture media. After incubation for 3 h, the absorbance at 450 nm was measured.

Invasion assay. The Matrigel invasion assay was carried out as described previously (13). Briefly, 2.5×10^4 cells in serum-free DMEM were loaded into the upper wells of a Transwell chamber (8 μm ϕ pore size, 24 well; Corning, Inc.) precoated with Matrigel (1:30 in DMEM). After 24 h, the invasive cells on the lower surface of the membrane were stained with DAPI and observed by using a fluorescence microscope BZ-X710 (Keyence, Osaka, Japan). The number of DAPI-stained cells was counted by using ImageJ software 1.53c (National Institutes of Health, Bethesda, MD, USA).

Semi-3D invasion assay. Semi-3D invasion assay was carried out as described previously (14). In brief, the 2-well culture insert with 0.5 mm gap between wells (ibidi, Germany) was placed on a fibronectin coated 24-well plate. Cells transfected with the respective siRNAs were seeded onto the culture insert and grown to confluent monolayers. After removing the insert, the monolayers were overlaid with Matrigel (BD Biosciences), followed by incubation for 4 h before an addition of medium. Cells were then cultured for 48 h to allow invasion toward the space between the monolayers. The invasion ratio indicates the percentage of filled space in a 48 h incubation.

Patient samples and immunohistochemical analysis. Primary oropharyngeal squamous cell cancer (OPSCC) tissue specimens were resected from 10 patients (mean age: 68.6, age range: 53-86, sex distribution: Number of males is 8 and females is 2) at Kobe University Hospital, fixed, and embedded in paraffin for sectioning. All patients met the following inclusion

criteria: i) histologically diagnosed stage 1 OPSCC (15) and ii) complete resection, indicating a homogeneous study population. Patients with a history of cancer or any form of anticancer treatment were excluded from this study (see the clinical profile of the patients in Table SI). Five cases were positive for p16 staining and five were negative. The resultant tissue sections were incubated with antibodies against Ror2 (AF2064, 1:50; R&D Systems) overnight at 4˚C and then with anti-goat IgG antibodies conjugated with HRP-labeled polymer (ImmPRESS Reagent kit Peroxidase; Vector Laboratories) for 30 min at room temperature. Secondary antibodies were visualized with DAB Chromogen (Dako Cytomation) and nuclei were counterstained with hematoxylin. Clinical tissue specimens were obtained and analyzed in accordance with procedures approved by the Institutional Review Board of Kobe University Hospital (no. B200096).

Extraction of DNA. Paraffin blocks were processed under strict conditions to avoid contamination. Formalin-fixed, paraffin-embedded (FFPE) tissues were cut into 5-μm thick sections on glass slides, and DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen GmbH) according to the manufacturer's protocol. The quantity and quality of extracted DNA was determined via NanoDrop ND-1000 spectrophotometry (Thermo Fisher Scientific Inc.). Isolated DNA was stored at -20˚C until further use.

HPV DNA detection. As an internal control of the reaction, samples with DNA were subjected to polymerase chain reaction (PCR) for human β-globin gene amplification using PCO3 (5'CTTCTGACACAACTGTGTTCACTAGC3') and PCO4 (5'TCACCACAACTTCATCCACGTTCACC3') oligonucleotides, which flank a sequence of approximately 110-bp (16). HPV DNA detection was carried out using general primers GP5+/6+ (16). PCR was performed with a final reaction volume of 50 μl, containing 5 μl of isolated DNA, 25 μl 2X buffer, 10 μl each dNTP, 0.3 μl each of forward and reverse primers, and 1.0 unit DNA polymerase (Kod Fx Neo; Toyobo Co.). The PCR conditions were as follows: Preheating for 10 min at 94˚C followed by 40 cycles of 1 min at 94˚C, 2 min at 40˚C, and 1.5 min at 72˚C, and a final extension of 7 min at 72˚C. The amplified products were subjected to electrophoresis in 3% agarose gels and observed using nucleic acid dye.

Western blotting. Cells were solubilized in ice-cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40 (NP-40), 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM p-APMSF] . Proteins (10 μg in total per lane) was separated by SDS-PAGE (10 to 16% polyacrylamide gels were used) and transferred onto an Immobilon-P membrane (Merck Millipore). Membranes were immunoblotted with the following antibodies: Anti-Ror2, anti-E6 (GTX132686, 1:500, GeneTex), anti-E7 (sc-65711, 1:200, Santa Cruz Biotechnology, Inc.), anti-α-tubulin (PM054, MBL). Immunoreactive bands were visualized using Western Lightning Plus-ECL (Perkin Elmer). The relative intensities of immunoblotted bands were determined with ImageJ software.

Real-time quantitative RT-PCR. Total RNA was extracted from cultured cells using Sepasol-RNA I SuperG (Nacalai Tesque). cDNAs were synthesized from these RNAs as templates using PrimeScript RT Reagent (Takara Bio). Expression levels of the respective genes of interest were measured using the LightCycler 480 II system (Roche). The qPCR thermocycling conditions were as follows: 95˚C for 5 min followed by 60 cycles at 95˚C for 10 sec, 60˚C for 15 sec, 72˚C for 15 sec, and final extension step at 72˚C for 5 min. The amounts of mRNAs were normalized relative to those of *18S ribosomal RNA*. Analysis of relative gene expression was performed using the $2^{-\Delta\Delta Cq}$ method (17). The sequences of the primer pairs were as follows: *Ror2* (5'-ATGTGGACTCCCTCCAGATG-3' and 5'-GAAGACGAAGTGGCAGAAGG-3'); *E6/E7* (5'-CAATGTTTCAGGACCCACAGG-3' and 5'-CTCACGTCGCAGTAACTGTTG-3').

Statistical analysis. Data were analyzed using Bell Curve for Excel (Social Survey Research Information Co., Ltd.) and are presented as the mean ± standard deviation. Significance was determined as $^{*}P<0.05$, $^{**}P<0.01$, or $^{***}P<0.001$ compared with the control and indicated with the relevant symbols in the figures. Data were analyzed using a Student's t-test when two groups were compared or a one-way analysis of variance (ANOVA) with Tukey's honest significance difference test when more than three groups were analyzed.

Results

E6 and/or E7 oncoproteins enhance Ror2 expression in HNSCC. Ror2 is highly expressed in HPV-positive HNSCC cell lines (6). However, the mechanism underlying the enhanced expression of Ror2 in HPV-positive HNSCC remains unclear. We examined the expression levels of Ror2 in clinical specimens from patients with OPSCC using immunohistochemical analysis. First, we confirmed the p16 expression status and examined HPV DNA expression via PCR using G5+/6+ consensus primers for the L1 region of the viral genome. Among the five p16-positive cases, we detected HPV DNA in four of them, and, as expected, HPV DNA was not detected in the p16-negative cases (Fig. 1A and B). Among the 10 cases examined, all cases contained OPSCC cells that were positive for Ror2 (Fig. 1C and D). Ror2 expression was much stronger in cancer tissues than in normal adjacent tissues (Fig. S1). Expression of Ror2 was localized in the cytoplasm of tumor cells. Interestingly, in one HPV-positive patient (Patient 2), metastatic lesions in the neck lymph node showed higher expression of Ror2 than in the primary lesion, suggesting that expression levels of Ror2 might be correlated with the invasive properties of cancer cells (Fig. 1C). We further confirmed that UPCI:SCC152 cells (HPV-positive HNSCC cell line) expressed higher levels of Ror2 than UM-SCC-22B and Ca9-22 cells (HPV-negative HNSCC cell lines) as assessed by qPCR (Fig. 1E) and western blot analyses (Fig. 1F), as previously reported (6). These results suggest that HPV enhances the expression levels of Ror2 in HNSCCs and that Ror2 might be involved in the progression of HNSCC.

E6 and E7 oncoproteins encoded by the HPV genome within infected HNSCC have been shown to induce the expression of various genes in the host genome, thereby promoting tumor progression (18). Thus, we next examined whether E6 and E7 are involved in the enhanced expression of Ror2 in

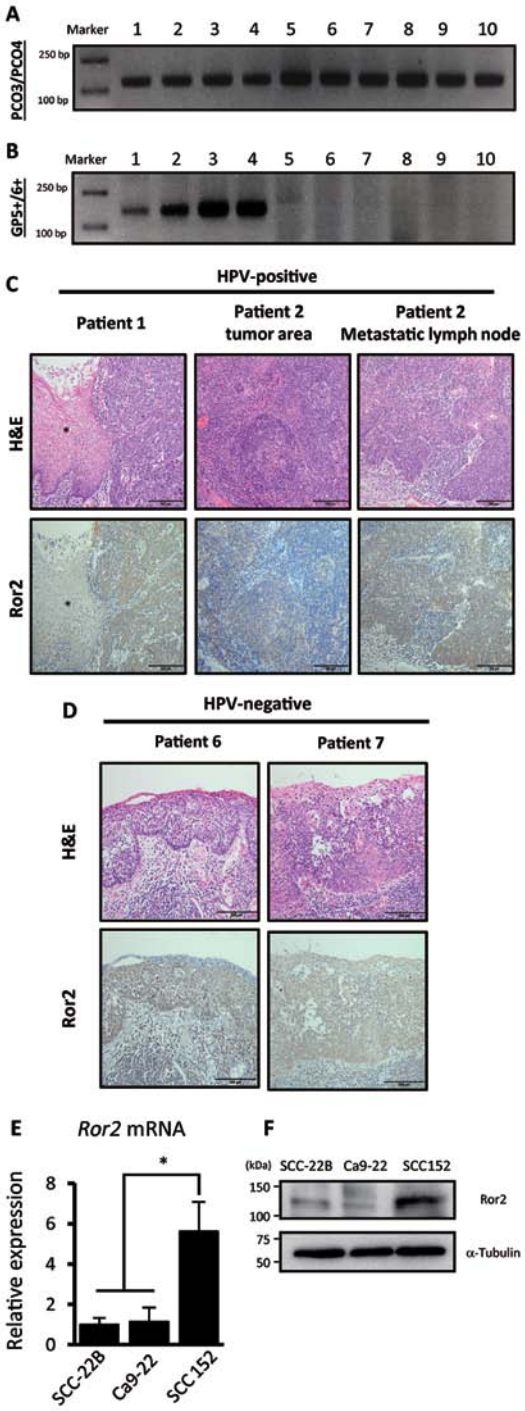

Figure 1. Ror2 is highly expressed in HNSCC. (A and B) Representative electrophoresis results of the amplicons from tissues of patients with OPSCC for detecting the β-globin gene (PCO3/PCO4 primer) and human papilloma virus L1 DNA (Gp5+/6+ primer). The images show the polymerase chain reaction (PCR) products of 10 samples. (C and D) Immunohistochemical analyses of Ror2 with surgical specimens from patients with HPV-positive (C) and negative (D) OPSCC. Representative images of hematoxylin and eosin (H&E) staining and anti-Ror2 immunohistochemistry of HNSCC tissue sections are shown. The asterisks indicate the non-tumor area. Scale bars,e 200 μm. (E) Expression levels of Ror2 in HNSCC cell lines UM-SCC-22B (HPV-negative), Ca9-22 (HPV-negative), and UPCI:SCC152 (HPV-positive); cells were measured by qRT-PCR analysis. Relative expression levels were determined by defining the expression level of Ror2 in UM-SCC-22B as 1. Data are expressed as the mean ± standard deviation (n=3; *P<0.05, Tukey's honest significance difference test). (F) Expression levels of *Ror2* and α-tubulin proteins in HNSCC cell lines UM-SCC-22B, Ca9-22 and UPCI:SCC152; cells were evaluated by western blot analysis. Ror2, receptor tyrosine kinase like orphan receptor 2; HNSCC, head and neck squamous cell cancer; HPV, human papilloma virus; OPSCC, oropharyngeal squamous cell cancer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

UPCI:SCC152 cells using siRNAs against the HPV16 *E6* and *E7* transcripts (si-*E6*/*E7*). The expression of Ror2 was significantly inhibited by suppressing the expression of *E6* and *E7* oncoproteins with si-*E6*/*E7* (Fig. 2A-C). These results indicate that E6 and/or E7 oncoproteins enhance expression levels of Ror2 in HNSCC. However, the role of Ror2 upregulation in HNSCC remains unclear.

Ror2 plays an important role in regulating proliferation of HPV-positive HNSCC cells by promoting S phase entry. E6 and E7 oncoproteins promote the progression of HPV-positive HNSCC cells by regulating their proliferative activities (19). Indeed, we confirmed that *E6* and *E7* knockdown resulted in suppressed proliferative ability of UPCI:SCC152 cells (Fig. S2A). We performed an WST-8 assay to examine whether Ror2 induced by E6 and/or E7 oncoproteins can regulate the proliferative activities of UPCI:SCC152 cells. We found that suppressed expression of Ror2 resulted in significantly decreased proliferation of UPCI:SCC152 cells (Fig. 3A and B). To confirm whether the inhibitory effect of *Ror2* knockdown on the proliferation of UPCI:SCC152 cells was partly due to induction of apoptosis, the FITC-Annexin V apoptosis assay was performed. Both early and late apoptotic rates of cells were unaffected by suppressed expression of Ror2 (Fig. 3C and D). These findings indicate that Ror2 plays an important role in regulating the proliferation of UPCI:SCC152 cells.

We previously showed that Ror2 is required for the G_1/S phase transition of NIH/3T3 fibroblasts (11). We thus investigated whether Ror2 is involved in the G_1/S transition of UPCI:SCC152 cells. For this purpose, the effect of *Ror2*-knockdown on cell cycle profiles of UPCI:SCC152 cells was monitored by flow cytometric analysis with PI under non-synchronized conditions and we re-started the cell cycle after synchronization. Since a double thymidine block exhibited strong cell toxicity (data not shown), we employed a single thymidine blockade to achieve overall synchronization at the G_1 phase. Any apparent effects of *Ror2*-knockdown on the cell cycle profile were observed under non-synchronized conditions (conventional cell culture conditions), unlike the previous report (8,9) (data not shown). UPCI:SCC152 cells transfected with either si-*Ror2* or control siRNA were subjected to one thymidine treatment to synchronize the cell cycle at the G_1 phase. Subsequently, cell cycle progression of the resultant cells was monitored every 3 h after the release of thymidine blockade. Following synchronization, a vast majority of the cells were arrested at the G_1 phase. In total, 54.88% of control cells entered the S phase within 3 h after release, while 40.98% of si-*Ror2#1* treated cells and 42.46% si-*Ror2#2* treated cells entered the S phase (Fig. 4A and B). This significant deceleration continued for si-*Ror2#1* at 6 h after release, but lessened for si-*Ror2#2* (P=0.011 and P=0.55, respectively). These findings indicate that Ror2 plays an important role in regulating the proliferation of HPV-positive HNSCC cells by promoting the G_1/S phase transition.

Ror2 is required to promote the invasive ability of HPV-positive HNSCC cells. HPV has been shown to promote the invasive ability of HNSCC cells (16). In fact, it was confirmed that knockdown of *E6* and *E7* resulted in significant inhibition of invasive ability of UPCI:SCC152 cells (Fig. S2B). Thus, we examined

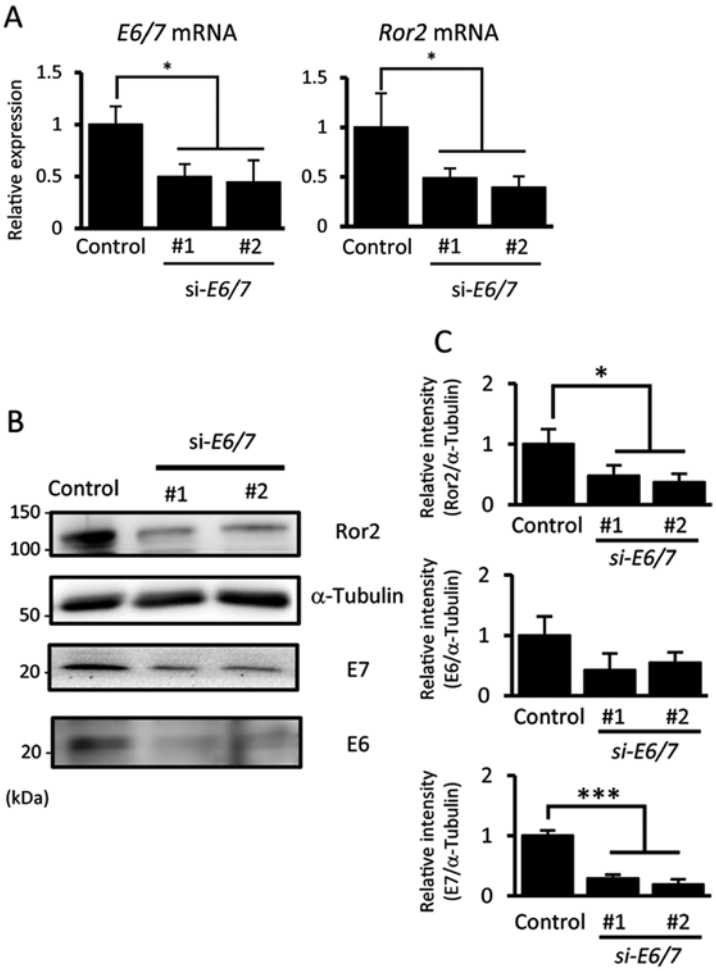

Figure 2. Expression of Ror2 is regulated by oncogenic E6 and/or E7 proteins in HPV16-positive HNSCCs. (A) Expression levels of *E6/7* and *Ror2* mRNAs in UPCI:SCC152 cells were measured by qRT-PCR analysis. Relative expression values were determined by defining the expression level in the control as 1. Data are expressed as the mean ± standard deviation (n=3; *P<0.05, Tukey's honest significance difference test). (B) Expression levels of Ror2, E6, E7 and α-tubulin proteins in HPV-positive UPCI:SCC152 cells transfected with siRNA Control, si-*Ror2#1* or si-*Ror2#2*, were evaluated by western blotting. (C) Relative band intensities of Ror2, E6 and E7 proteins normalized by α-tubulin were determined. Data are expressed as mean ± standard deviation (n=3; *P<0.05, ***P<0.001 Tukey's honest significance difference test). Ror2, receptor tyrosine kinase like orphan receptor 2; HNSCC, head and neck squamous cell cancers; HPV, human papilloma virus; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

whether Ror2, induced by E6 and/or E7 oncoproteins, is also involved in the invasive ability of HPV-positive HNSCC cells. To this end, we employed Semi-3D and Matrigel invasion assays to evaluate the invasive abilities of UPCI:SCC152 cells transfected with control *siRNA, si-Ror2#1*, or si-*Ror2#2*. *Ror2*-knockdown significantly inhibited the invasive ability of UPCI:SCC152 cells (Fig. 5A-C), indicating that Ror2 is required to promote the invasive ability of HPV-positive HNSCC cells. Moreover, in agreement with the undetectable expression of Ror2 in UM-SCC22B and Ca9-22 cells, siRNAs against Ror2 failed to affect significantly their invasiveness (Fig. S3). Interestingly, *Ror2*-knockdown resulted in suppressed expression of Slug, an epithelial-to-mesenchymal transition (EMT)-related transcription factor, in UPCI:SCC152 cells (Fig. 5D), indicating that Ror2 might mediate the invasive ability of HPV-positive HNSCC cells through epithelial-mesenchymal transition (EMT).

Discussion

Recently, the expression of several possible target cancer cell surface proteins for human papilloma virus (HPV)-negative and HPV-positive head and neck squamous cell cancer (HNSCC) has been established. Ror2 has been found to be more highly expressed in HPV-positive HNSCC cell lines than in HPV-negative cell lines (6). Thus, Ror2 (receptor tyrosine kinase like orphan receptor 2) signaling pathways have become a focus in the cancer community because of their critical roles in cancer progression. In the present study, we used molecular biology and cell biology methods to determine the role of Ror2 in the regulation of HPV-positive HNSCC.

It has been suggested that Ror2 is important for the regulation of cancer cell proliferation. However, the precise role of Ror2 in the regulation of cancer cell proliferation, as well as its underlying mechanism, remains unclear. Huang *et al* demonstrated that knockdown of *Ror2* led to cell cycle arrest in osteosarcoma cells (8). Similarly, Jiang *et al* found that miR-208b inhibited the proliferation of osteosarcoma cell lines by targeting Ror2 (20). Conversely, it has been shown that the expression levels of Ror2 were reduced in gastric carcinoma tissues compared with that noted in matched normal adjacent tissues. In addition, overexpression of Ror2 inhibited gastric cancer cell proliferation, while inducing cell apoptosis and cell

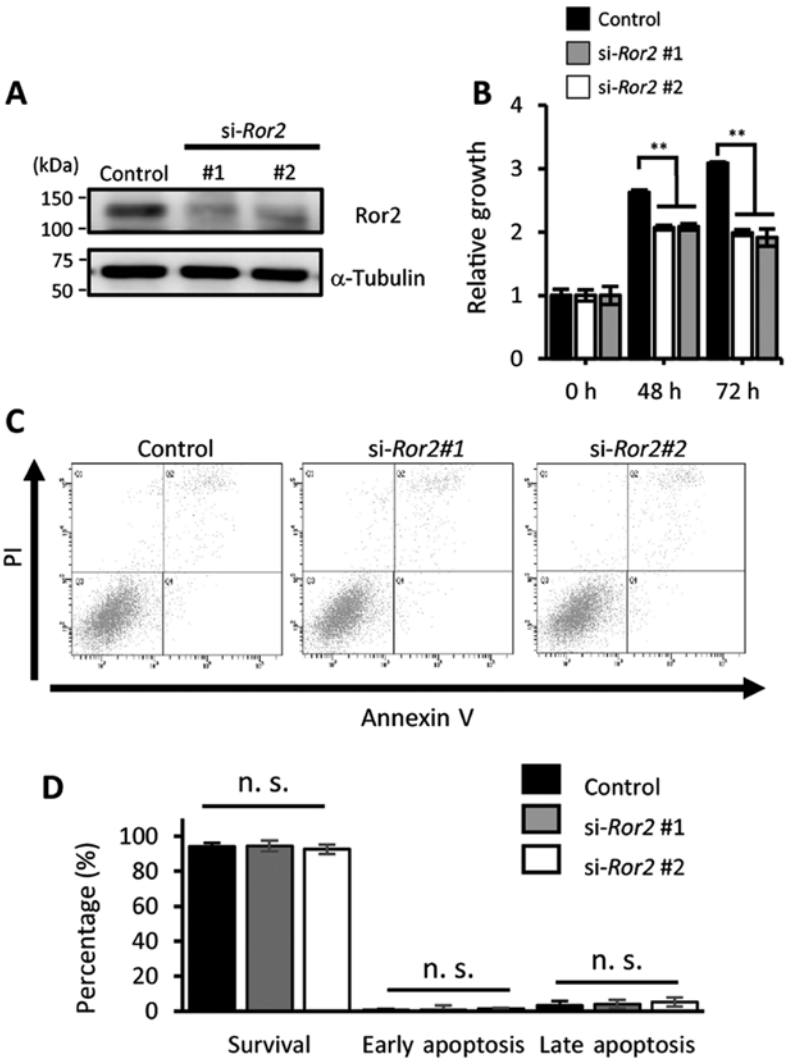

Figure 3. Ror2 plays an important role in regulating the proliferation of HPV-positive HNSCCs. (A) Expression levels of Ror2 and α-tubulin proteins in UPCI:SCC152 cells treated with the indicated siRNA control, si-*Ror2#1*, or si-*Ror2#2* were evaluated by western blot analysis. (B) Viable cell numbers of HPV-positive UPCI:SCC152 cells transfected with the indicated siRNA were measured using the WST-8 assay at the indicated time points. Data are expressed as the mean ± standard deviation (n=3; **P<0.01, Tukey's honest significance difference test). (C) Proportion of apoptotic UPCI:SCC152 cells, transfected with the indicated siRNAs, were examined by flow cytometric analysis with PI and anti-Annexin V staining. (D) Quantification of the proportion of apoptotic UPCI:SCC152 cells transfected with the indicated siRNA. Data are expressed as the mean ± standard deviation (n=3; n.s., not significant, Tukey's honest significance difference test). Ror2, receptor tyrosine kinase like orphan receptor 2; HNSCC, head and neck squamous cell cancer; HPV, human papilloma virus.

cycle arrest at the G_1 phase (9). In the present study, *Ror2* knockdown decreased the proliferation of UPCI:SCC152 cells without inducing apoptosis. Furthermore, we also confirmed that Ror2 plays an important role in G_1/S phase transition. The findings of this study are consistent with our previous study, which showed that Ror2 promotes G_1/S phase transition in bFGF-stimulated NIH/3T3 fibroblasts by regulating E2F-target genes (11).

Interestingly, HPV-related HNSCC has a higher risk for lympho-vascular invasion and lymph node metastasis (21), in spite of a better prognosis compared with HPV-negative HNSCC (21). This indicates an essential role of HPV in carcinogenic processes, although its role in tumor metastasis to the lymph nodes remains unclear. Considering that lymph node metastasis is a major risk factor related to poor outcomes in patients with HNSCC, identification of the underlying mechanism associated with HPV infection may reveal potential strategies for managing lymph node metastasis in these patients. Here, we found that the invasive ability of UPCI:SCC152 cells was downregulated by *Ror2* knockdown. Although some reports showed that Ror2 promotes invasion by acting as a receptor for Wnt5a (22), others have indicated that Ror2 regulates invasion of cancer cells independently of Wnt5a (23,24). It is presently unclear whether Ror2 can promote invasion of UPCI:SCC152 cells by acting as a receptor for Wnt5a. Therefore, it is important to understand the molecular mechanism of Ror2-mediated invasion of HPV-positive HNSCC in future studies.

Our results uncovered a novel relationship between E6/7 oncoproteins and Ror2, which may be relevant for cancer progression. We found that HPV16-E6/7 oncogenes can upregulate the expression of Ror2 in UPCI:SCC152 cells, suggesting a possible interaction between E6/7 and Ror2 during carcinogenesis. It is well known that the HPV oncogenic potential is associated with the E6 and E7 oncoproteins which mediate the degradation of p53 and Rb tumor suppressor proteins, respectively. We previously showed that E2F1 induces Ror2

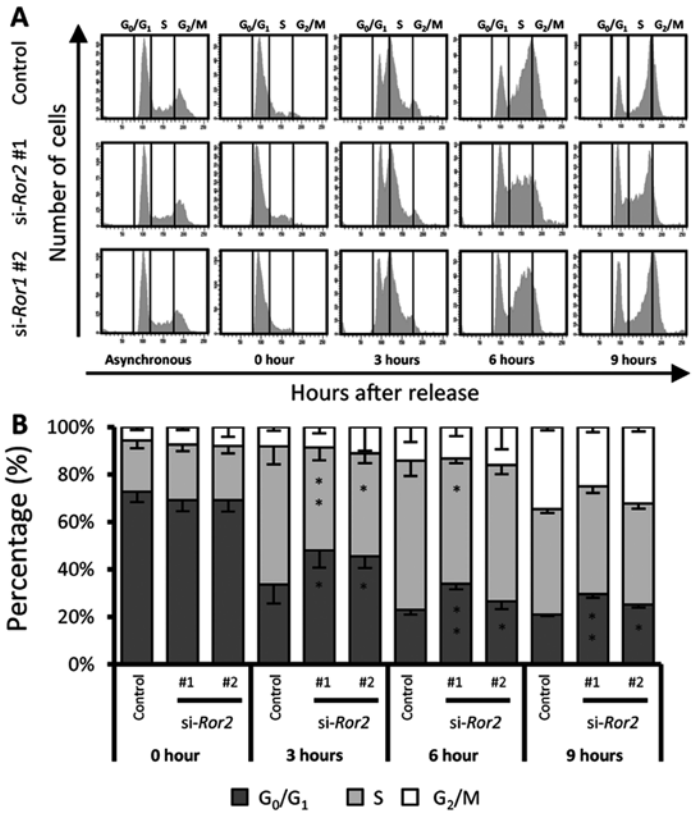

Figure 4. The S phase entry of HPV-positive HNSCCs is regulated by Ror2. (A) HPV-positive UPCI:SCC152 cells, transfected with siRNA control, si-*Ror2#1*, or si-*Ror2#2*, were cultured asynchronously or were synchronized by treatment with thymidine. Twenty-four hours after treatment with thymidine, the thymidine in the medium was removed and the DNA contents of UPCI:SCC152 cells at the indicated time points were determined by flow cytometric analysis. The DNA content histograms reveal the proportions of cells that reside in the different cell cycle phases at the respective time points. (B) Quantification of the percentages of cells in each cell cycle phase at the indicated time points. Data are expressed as the mean ± standard deviation (n=3; *P<0.05, **P<0.01, Tukey's honest significance difference test). Ror2, receptor tyrosine kinase like orphan receptor 2; HNSCC, head and neck squamous cell cancer; HPV, human papilloma virus.

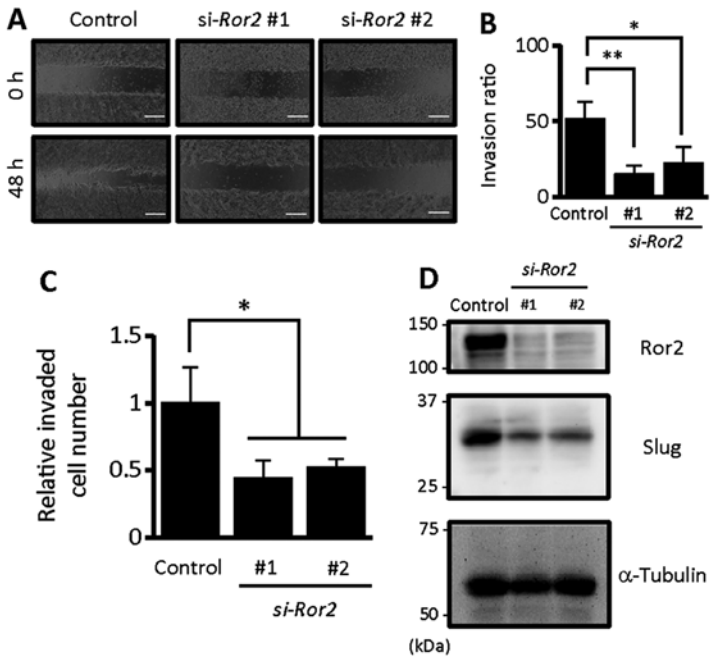

Figure 5. Ror2 plays a critical role in the invasive ability of UPCI:SCC152 cells. (A) Representative phase contrast images of the Semi-3D invasion assay of UPCI:SCC152 cells transfected with the indicated siRNAs before (0 h) and after incubation for 48 h are shown. Scale bars, 300 μm. (B) Invasion ratios of the respective cells after incubation for 48 h were determined. Data are expressed as mean ± standard deviation (n=3; *P<0.05, **P<0.01, Tukey's honest significance difference test). (C) Relative number of invaded HPV-positive UPCI:SCC152 cells transfected with the indicated siRNAs were analyzed using the Matrigel invasion assay. Data are expressed as the mean ± standard deviation (n=3; *P<0.05, Tukey's honest significance difference test). (D) Expression levels of Ror2, Slug and α-tubulin proteins in HPV-positive UPCI:SCC152 cells transfected with the indicated siRNAs were evaluated by Western blotting. Ror2, receptor tyrosine kinase like orphan receptor 2; HPV, human papilloma virus.

expression in bFGF-treated NIH/3T3 fibroblasts by binding to the promoter region of the *Ror2* gene. Therefore, the E7 oncoprotein might be sufficient to induce Ror2 expression through E2F1 by degradation of the Rb protein. Moreover, it has recently been shown that E6 and E7 promote cancer progression through p53- and Rb-independent pathways (25-27). Therefore, the expression of Ror2 might not depend on the degradation of p53 and Rb. Future studies investigating how E6/7 regulate the expression level of Ror2 are required.

We found that Ror2 is expressed in both HPV-negative and HPV-positive oropharyngeal squamous cell cancer (OPSCC) patients. In contrast, we and others showed that expression levels of Ror2 are significantly higher in HPV-positive cancer cell lines (UPCI:SCC152) than in HPV-negative cancer cell lines (UM-SCC22B, Ca9-22). It can be assumed that this discrepancy is at least partly due to the difference between *in vitro* and *in vivo* microenvironmental factors. HPV-negative cancer cell lines might lose some carcinogenic features such as Ror2 expression with the loss of tumor stroma, while HPV-positive cell lines do not. This might be explained by the cell-autonomous interaction between E6/7 and Ror2 expression in the HPV-positive cell line. Although the present study indicates that E6/7 induces the expression of Ror2 in UPCI:SCC152 cells, the molecular mechanism of Ror2 upregulation in HPV-negative HNSCC remains unclear. The tumor microenvironment plays a critical role in regulating cancer progression. We previously showed that CXCL16 derived from mesenchymal stem cells, one of the components of the tumor microenvironment, induces the expression of Ror1 in gastric cancer cells (28). Therefore, this might indicate the possibility that the tumor microenvironment regulates Ror2 expression in HPV-negative HNSCC through an E6/7 independent pathway. Future studies investigating the relationship between the tumor microenvironment and Ror2 upregulation in HPV-negative HNSCC are required.

Recently, treatment for HPV-positive cases has been changed to biopsy followed by chemoradiotherapy. Thus, the incidence of excisional HPV-positive cancer tissues is declining. The main limitation of the present study was that the number of OPSCC tissues was not sufficient to compare the association between the expression levels of Ror2 in HPV-negative and HPV-positive samples. Furthermore, we were not able to examine the clinical characteristics of patients, such as the tumor grade, TNM stage, and metastasis. Consequently, future studies with more HPV-negative and HPV-positive cancer cell lines and larger sample sizes are required to compare Ror2 expression in HPV-negative and HPV-positive cases.

Although it has been four decades since the association between HPV and human cancer was first proposed (29), the epidemic of HPV-positive disease still requires the identification of novel biomarkers. Our findings indicate that: i) E6/7 induce the expression of Ror2 to promote proliferation and invasion, suggesting a novel mechanism of Ror2 function in HPV-related carcinogenesis and ii) E6/7 have the potential to be novel targets in HPV-related cancers. Since HPV-induced cancers depend on the expression of E6/7, targeting E6/7 may alter Ror2 signaling pathways. Advances in understanding the role and characteristics of Ror2 in HPV-related cancers and understanding how to manipulate Ror2 signaling by HPV oncogenes may serve as novel approaches to treat HNSCC and other HPV-related cancers.

Acknowledgements

Not applicable.

Funding

The present study was supported by Tokyo Biochemical Research Foundation.

Availability of data and materials

Original data and material will be made available upon reasonable request.

Author's contributions

MOA, KK and YM designed the study. MOA, KK and NJ performed the experiments and analyzed the data. MOA, KK and YM interpreted the data. NJ, HS, KIN and MN were involved in the interpretation and evaluation of the data and findings. MOA, KK and YM prepared the manuscript. NJ, HS, KIN and MN reviewed and edited manuscript. All authors approved the final manuscript.

Ethics approval and consent to participate

This study is approved by the institutional review board of Kobe University Hospital (no. B200096). The Opt-out method has been used.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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