学位論文

FOXM1-mediated regulation of reactive oxygen species and radioresistance in oral squamous cell carcinoma cells
（FOXM1 を介した口腔扁平上皮癌細胞における活性酸素の制御と放射線抵抗性）

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FOXM1-mediated regulation of reactive oxygen species and radioresistance in oral squamous cell carcinoma cells

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Abstract

Radioresistance is a major obstacle to the successful treatment of oral squamous cell carcinoma (OSCC). To help overcome this issue, we have developed clinically relevant radioresistant (CRR) cell lines generated by irradiating parental cells over time, which are useful for OSCC research. In the present study, we conducted gene expression analysis using CRR cells and their parental lines to investigate the regulation of radioresistance in OSCC cells. Based on gene expression changes over time in CRR cells and parental lines subjected to irradiation, forkhead box M1 (FOXM1) was selected for further analysis in terms of its expression in OSCC cell lines, including CRR cell lines and clinical specimens. We suppressed or upregulated the expression of FOXM1 in OSCC cell lines, including CRR cell lines, and examined radiosensitivity, DNA damage, and cell viability under various conditions. The molecular network regulating radiotolerance was also investigated, especially the redox pathway, and the radiosensitizing effect of FOXM1 inhibitors was examined as a potential therapeutic application. We found that FOXM1 was not expressed in normal human keratinocytes but was expressed in several OSCC cell lines. The

expression of FOXM1 was upregulated in CRR cells compared with that detected in the parental cell lines. In a xenograft model and clinical specimens, FOXM1 expression was upregulated in cells that survived irradiation. FOXM1-specific siRNA treatment increased radiosensitivity, whereas FOXM1 overexpression decreased radiosensitivity, and DNA damage was altered significantly under both conditions as well as the levels of redox-related molecules and reactive oxygen species production. Treatment with the FOXM1 inhibitor thiostrepton had a radiosensitizing effect and overcame radiotolerance in CRR cells. According to these results, the FOXM1-mediated regulation of reactive oxygen species could be a novel therapeutic target for the treatment of radioresistant OSCC; thus, treatment strategies targeting this axis might overcome radioresistance in this disease.

Introduction

The survival rate of patients with oral squamous cell carcinoma (OSCC), a common cancer of the oral cavity, has not improved despite advances in diagnostic modalities and treatments [1]. Thus, the prognosis of patients with advanced OSCC remains poor, e.g., the five-year survival

rate is ~50% [2]. This lack of improvement in the OSCC survival rate is mainly due to the existence of high-grade malignant cells with properties such as resistance to chemotherapy or radiotherapy, abnormal proliferation, and invasion or metastasis abilities [3]. Among these properties, radioresistance is a serious problem that prevents the improvement of radiotherapy treatment outcomes in OSCC [4].

Used as a treatment for head and neck cancers, including OSCC, radiotherapy can effectively kill cancer cells by damaging their DNA through the generation of reactive oxygen species (ROS) [5]. However, cancer cells that possess antioxidant abilities can escape the damaging effects of radiation by scavenging ROS, which leads to radioresistance [6]. Radioresistance is a major clinical obstacle to the successful treatment of OSCC; therefore, understanding the regulation of ROS and subsequent control of DNA damage by radioresistant cancer cells is important. To aid research in this area, we established clinically relevant radioresistant (CRR) cell lines by irradiating cells with >60 Gy for 5 weeks at 2 Gy per day, replicating the irradiation used in actual clinical practice [7]. Based on preclinical research in which the CRR cell lines were employed to investigate the molecular mechanism underlying

radioresistance [8], these cells can be considered a useful experimental resource.

Forkhead box M1 (FOXM1), a member of the FOX transcription factor family, serves as a transcription factor for many genes and plays critical roles in cell cycle progression, cell proliferation, and the inhibition of apoptosis [9, 10]. The overexpression of FOXM1 has been associated with tumor development in various cancers [11]. Although the involvement of FOXM1 in the regulation of radiosensitivity has been reported in several malignancies, including OSCC [12-17], the role of FOXM1 in radioresistant OSCC has not been clarified.

Drug repositioning is a drug development strategy in which new drugs are sought to be established from existing approved drugs by determining the novel effects or targets of the approved drugs. Drug repositioning has substantial benefits over the traditional development of new drugs (*de novo* drugs) as it minimizes development risks and facilitates the development process [18]. In oncology, drug repositioning could help meet the demand for new therapies and do so faster and in a more cost-effective manner than is possible with traditional drug development [19]. Thiostrepton, a FOXM1 inhibitor, is a natural product

with antibiotic properties that is isolated from *Streptomyces azureus*. It interacts directly with FOXM1 and inhibits FOXM1 binding to genomic target sites [20]. Furthermore, thiostrepton has been shown to inhibit cell proliferation and induce apoptosis in various human cancer cell lines by inhibiting FOXM1 expression [21].

In the present study, we investigated the role played by FOXM1 in radiosensitivity in OSCC using *in vitro* analyses and tested the ability of the FOXM1 inhibitor thiostrepton to overcome the radioresistant phenotype of OSCC.

Materials and Methods

Cell lines

Human OSCC cell lines derived from oral cancer (HSC-2, HSC-3, Ca9-22, and SAS) were purchased from the National Institute of Biomedical Innovation (Osaka, Japan). Human HaCaT nonmalignant keratinocytes were kindly provided by Dr. P. Boukamp (DKFZ, Heidelberg, Germany). Human normal oral keratinocyte cells (PCS-200-014™) were purchased from the American Type Culture Collection (Manassas, VA, USA). SAS-R and HSC-2-R cells established from SAS and HSC-2 cells

were used as the CRR cell lines, which were produced by exposing the latter cells to gradually increasing X-ray doses [7]. The OSCC cell lines and HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; D6429; Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37°C and 5% CO_2. Human normal oral keratinocytes were cultured in Dermal Cell Basal Medium (PCS-200-030™; American Type Culture Collection) supplemented with Keratinocyte Growth Kit media (PCS-200-040™; American Type Culture Collection).

Preparation of OSCC cell lines for DNA microarray analysis

To prepare samples for DNA microarray analysis, total RNA was extracted from each cell over time following the method described below. SAS and SAS-R cells were irradiated with X-rays at 2 Gy per day for 5 days, and total RNA was collected 1, 3, 6, and 12 h after irradiation. Total RNA was also extracted from nonirradiated SAS and SAS-R cells that were cultured for use as control samples.

Total RNA isolation

Total RNA was isolated from the cerebellum of individual animals using TRIzol Reagent (nitrogen) and purified using an SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. RNA concentration was quantified using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA quality was confirmed using an Experion System (Bio-Rad Laboratories, Hercules, CA, USA).

Gene expression microarrays

Using the total RNA, cRNA was amplified, labeled (Low Input Quick Amp Labeling Kit, Tokyo, Japan), and hybridized to a 60K Agilent 60-mer oligomicroarray (SurePrint G3 Human GE microarray 8×60K Ver. 2.0, Agilent) according to the manufacturer's instructions. All hybridized microarray slides were scanned using an Agilent scanner (Agilent Microarray Scanner G2505B, Agilent). Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1).

Data analysis and filter criteria

Raw signal intensities and flags for each probe were calculated using the hybridization intensities (gProcessedSignal) and spot information (gIsSaturated) according to the procedures recommended by Agilent (flag criteria in Agilent GeneSpring software: “Absent”: “Feature is not positive and significant” and “Feature is not above background”; “Marginal”: “Feature is not Uniform,” “Feature is Saturated,” and “Feature is a population outlier”; “Present”: others). The raw signal intensities of two samples were $\log_2$-transformed and normalized using a quantile algorithm via the “preprocessCore” library package [22] of Bioconductor [23]. We selected probes that were called the “present” flag in at least one sample, excluding lincRNA probes. To identify upregulated and downregulated genes, we calculated Z-scores [24] and ratios (nonlog-scale fold-change) using the normalized signal intensities of each probe and compared the resultant data between the control and treatment samples. We then established the criteria for regulated genes as follows: upregulated genes: Z-score ≥ 2.0, ratio ≥ 1.5-fold; downregulated genes: Z-score ≤ -2.0, ratio ≤ 0.66. Microarray expression profiling is available on the NCBI GEO website

(https://www.ncbi.nlm.nih.gov/geo/); data accession number GSE210922.

Ingenuity® Pathway Analysis

Upstream regulators were analyzed using Ingenuity® Pathway Analysis (IPA; Qiagen). *P*-values were calculated using a right-tail Fisher's exact test, reflecting the association or overlap between a set of significant molecules from the experiment and a given pathway. In this analysis, the Z-score is used to infer the activation states of the predicted transcriptional regulators (z-score definition: http://pages.ingenuity.com/rs/ingenuity/images/0812%20upstream_regulator_analysis_whitepaper.pdf). In practice, Z-scores >2 or <−2 are considered significant.

Clinical samples from patients

To conduct histopathological analysis, primary OSCC tissue samples were obtained from 12 patients with advanced OSCC treated at Kumamoto University Hospital (detailed patient characteristics are shown in Supplementary Table S1). To analyze the variation in FOXM1

expression before and after recurrence or metastasis after radiotherapy, 12 cases were used in which tissue samples could be obtained before and after treatment. Tissue samples were collected before radiotherapy, and recurrence or metastasis was confirmed. The post-treatment tissue samples were obtained from recurrent or metastatic regions when tissue sampling could be performed safely. However, in addition to the 12 cases analyzed in the histopathological analysis, 14 cases for which tumor tissue could be obtained before treatment were used for clinicopathological analysis. The clinicopathological characteristics of the 14 additional cases are presented in Supplementary Table S3 (Supplementary Table S3). Each patient was treated with a total chemoradiotherapy or radiotherapy dose of 66 Gy, and radiotherapy was administered at a daily dose of 2 Gy five times per week for 33 days. These processes were conducted in accordance with the guidelines of the Ethics Committee of Kumamoto University (project identification code: SENSHIN No. 2389 and RINRI No. 1427). Informed consent was obtained from all patients before biopsy and surgery based on the guidelines of Kumamoto University (SENSHIN No.2389). In this study, a retrospective analysis was performed, which did not require individual

consent; nevertheless, patients were provided with an opportunity to refuse participation in an opt-out format (RINRI No. 1427).

Animals and the in vivo *experimental protocol*

BALB/c-nu/nu female mice (6 weeks old) were purchased from Charles River Japan (Yokohama, Japan) and maintained at the Center for Animal Resources and Development of Kumamoto University. The animals were handled in accordance with the animal care policy of Kumamoto University. OSCC and CRR cells were trypsinized, washed with serum-free medium, resuspended in PBS, and adjusted to a density of 1×10^7 cells/100 μl in PBS. Then, the cell suspensions were subcutaneously injected into the backs of nude mice (n = 3/cell line). When tumor volumes approached 100–150 mm^3, the experiments were started (day 0). Mice were exposed to a single X-ray dose of 30 Gy. Excluding the tumor regions, the bodies of the mice were protected from radiation by a lead shield. Seven days after final irradiation, tumor tissues were excised, placed in sterile tubes, and immediately fixed in 10% formalin.

Irradiation

Irradiation was performed using a 150 KVp X-ray generator with total filtration through a 0.5 mm aluminum plus 0.1 mm copper filter (MBR-1520R; Hitachi, Tokyo, Japan). The dose rate (1.01 Gy per min) was measured using a thimble ionization chamber (IC 17A; Far West Technology, Goleta, CA, USA).

Western blotting

Whole-cell proteins (Minute Cytoplasmic and Nuclear Extraction Kits; Invent Biotechnologies, Inc., Plymouth, MN, USA) were separated using 10%–20% SDS-PAGE, transferred to nitrocellulose membranes and blocked for 1 h. The membranes were then incubated in primary antibody cocktails [diluted in TBS-T containing 5% bovine serum albumin (BSA)] overnight at 4°C, washed three times for 10 min per wash in TBS-T, and incubated in a secondary antibody cocktail for 60 min at room temperature. The membranes were again washed three times for 10 min per wash in TBS-T, after which they were developed using an ECL Prime Detection Kit (GE Healthcare, Chicago, IL, USA). The emitted light was measured using a C-DiGit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA), and captured images were analyzed

using Image Studio for C-Digit (LI-COR Biosciences). The list of antibodies used in this study is shown in Supplementary Table S2.

RT-PCR

Total RNA was isolated using a FastGene™ RNA Basic Kit (NIPPON Genetics, Tokyo, Japan) and reverse-transcribed into cDNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo). Data obtained from RT-qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method [25], with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) used as a housekeeping gene. Each sample was run in triplicate. The following primers were used: *FOXM1* forward: 5′ -TGGGGAGGAAATGCCACACTTAG-3′; *FOXM1* reverse: 5′-TAGGACTTCTTGGGTCTTGGGGTG-3′; *GAPDH* forward: 5′-CAACAGCCTCAAGATCATCAGC-3′; *GAPDH* reverse, 5′-TTCTAGACGGCAGGTCAGGTC-3′. The PCR products were analyzed using agarose gel electrophoresis and visualized using staining with ethidium bromide.

Immunofluorescent staining and evaluation

Cells (2×10^4) were seeded onto glass slides (Merck Millipore, Tokyo, Japan) and incubated in DMEM with 10% fetal bovine serum (FBS) for 24 h. FOXM1 siRNA (30 nM) was then added to the cells, which were subsequently exposed to X-rays at 6 Gy. After 1, 3, 6, and 12 h incubation, the cells were fixed in 4% paraformaldehyde in a phosphate-buffered saline (PBS) solution for 10 min. The cells were then washed with phosphate-buffered saline with Tween 20 (PBS-T), and transmittance processing was conducted with PBS-T containing 0.2% TritonX-100. Subsequently, the cells were washed with PBS-T and blocked with PBS-T containing 1% BSA and 0.15% glycine for 60 min. After blocking, the cells were incubated overnight at 4℃ for 24 h and then with anti-human antibody 53BP1 (1:100 dilution; #NB100-304; Novus Biologicals, CO, USA) in PBS-T containing 0.1% BSA at 4℃ overnight. Subsequently, the glass slides were washed with PBS-T, and the cells were incubated for 1 h at room temperature with the secondary antibodies (Alexa Fluor 488 and 594 donkey anti-rabbit and/or anti-mouse IgG; Life Technologies, Tokyo, Japan) in PBS-T containing 0.1% BSA. After the cells were washed with PBS, they were

counterstained and mounted using Vectashield® (Vector Laboratories, Inc., Burlingame, CA, USA), after which they were observed under a fluorescent microscope (Model BZ-X700; KEYENCE, Osaka, Japan). If the observed cell had 10 × 53BP1 foci per nucleus, it was considered positive for 53BP1 [26].

Transfection with siRNA

SAS, SAS-R, and HSC-2-R cells were transfected with siRNA. At 24 h before siRNA transfection, the cells were diluted in fresh medium without antibiotics and transferred to 60 mm dishes (Nunc, Waltham, MA, USA). The cells were grown and transfected with FOXM1-specific siRNA and Stealth™ RNAi Universal Negative Control (Stealth siRNA, 40 nM; Invitrogen) using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instructions. The siRNA sequences were as follows: s5249 sense strand: 5′ -CACUAUCAACAAUAGCCUATT-3′; s5249 antisense strand: 5′-UAGGCUAUUGUUGAUAGUGCA-3′; s5250 sense strand: 5′-GGAUCAAGAUUAUUAACCATT-3′; s5250 antisense strand: 5′-

UGGUUAAUAAUCUUGAUCCCA-3′. The cells were harvested 48 h after transfection for use in *in vitro* assays.

Plasmids

The human FOXM1 expression plasmid, Flag-FOXM1, was gifted by Stefan Koch (Addgene plasmid #153136; http://n2t.net/addgene:153136; RRID:Addgene_153136) [27]. The control vector, pCS2FLAG, was gifted by Peter Klein (Addgene plasmid #16331; http://n2t.net/addgene:16331; RRID:Addgene_16331).

Transfection with plasmids

HSC-2 cells were transfected with plasmids. At 24 h before siRNA transfection, the cells were diluted in fresh medium without antibiotics and transferred to 60 mm dishes (Nunc). The cells were grown and transfected with FLAG-FOXM1 (Addgene) and pCS2FLAG (Addgene) using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions.

Immunohistochemical staining analysis

Immunohistochemical staining analysis was performed as described previously [28]. Briefly, formalin-fixed and paraffin-embedded specimens prepared from patients with OSCC and samples obtained from mouse experiments were sliced into sections with a thickness of 4 μm. Following deparaffinization and rehydration with ethanol, the sections were heated in an autoclave with 0.01 mol/L citrate buffer solution (pH 6.0) at 121℃ for 15 min to activate antigens. Endogenous peroxidase was inactivated using methanol containing 3% hydrogen peroxide, and then the sections were reacted with Protein Block Serum-Free Reagent (Dako, Glostrup, Denmark) for 15 min. After overnight incubation at 4℃ with anti-human FOXM1 (1:100 dilution; ORIGENE), all specimens were contrast-stained with hematoxylin for 1 min and subjected to dehydration and inclusion. The level of FOXM1 expression was determined according to the system introduced by Allred *et al*. [29]. The proportion of FOXM1-positive cells among the total number of cancer cells and the staining intensity for FOXM1 were semi-quantified to calculate the immunostaining score of FOXM1. The proportion and intensity scores were summed to produce a FOXM1 score of 0–6.

Measurement of cell proliferation activity

OSCC cell lines (SAS and HSC-2) and CRR cell lines (SAS-R and HSC-2-R) in the logarithmic growth phase were seeded in 96-well microplates (1×10^3 cells per well). At 24 h after seeding, the cells were treated with FOXM1-specific siRNA or thiostrepton (0, 0.50, 0.75, 1.00, or 1.50 μM). Thiostrepton (sc-203412) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Every 24 h after incubation, Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was added to each well, and the color reaction was allowed to occur for 2 h. Finally, the absorbance at 450 nm was measured using an iMark™ Microplate Reader (Bio-Rad Laboratories, Inc.).

Clonogenic assay

After irradiating SAS, HSC-2, SAS-R, or HSC-2-R cells with 2 or 6 Gy of X-rays, the cells (1×10^3) were seeded in a 60 mm gelatin-coated culture dish (Asahi Techno Glass Co., Ltd) and treated with control agents, FOXM1-specific siRNA, or thiostrepton (0.5 μM), after which they were incubated in DMEM with 10% FBS for 10 days.

Subsequently, the cells were fixed with 99.5% methanol and stained with Giemsa solution (Wako, Osaka, Japan).

Cellular ROS/superoxide detection assay

Cells (2×10^4) were seeded onto 35 mm glass dishes (Asahi Techno Glass Co., Ltd) and incubated in DMEM with 10% FBS for 24 h. FOXM1 siRNA (30 nM) was then added to the cells, and they were exposed to 6 Gy of X-rays. After 6 h of incubation, the presence of ROS/superoxide in the cells was detected using a Cellular ROS/Superoxide Detection Assay Kit (Abcam, Tokyo, Japan). The acquisition and measurement of cell images were performed using a fluorescent microscope (Model BZ-X700, KEYENCE).

Statistical analysis

Statistical analyses were performed using JMP 9 software (SAS Institute Inc., Cary, NC, USA). Differences in the means between two groups were analyzed using a Wilcoxon rank-sum (Mann–Whitney) test. Differences in the means between multiple groups were analyzed using one-way ANOVA with a Bonferroni/Dunn test. Progression-free

survival (PFS) was defined as the time from the initiation of CRT treatment to the date of recurrence/metastasis or regrowth of cancer. The Kaplan–Meier method was used to estimate the probability of PFS as a function of time. Statistical differences in the survival of patients in subgroups were compared using the log-rank test. All *P*-values were calculated based on two-tailed statistical analysis, and $P < 0.05$ was considered statistically significant.

Results

Identification of the transcription factors involved in the radioresistance of OSCC

To elucidate the molecular mechanism underlying radioresistance in OSCC, gene expression analysis was performed using SAS and SAS-R cells with the aim of identifying the transcriptional regulators of the molecules involved in radioresistance, particularly the molecules for which transcriptional activity decreased over time in the parental cells compared with that in the CRR cells (Figure 1A). This analysis revealed that the gene expression of *FOXM1* varied substantially over time after a

specific dose of radiation (Figure 1B). Thus, FOXM1 was chosen for further analysis.

Expression analysis of FOXM1

FOXM1 expression was analyzed to confirm its involvement in the regulation of malignant phenotypes in OSCC. First, we investigated FOXM1 expression in normal keratinocytes (HaCaT: skin-derived; human normal oral keratinocytes: oral cavity derived) and OSCC cell lines. FOXM1 was not expressed in normal keratinocytes but was expressed in three of the four OSCC cell lines (Figure 1C). Next, we compared the expression of FOXM1 in CRR cells and their parental lines, finding that FOXM1 expression was enhanced in the CRR cells (Figure 1D). Thus, we further examined FOXM1 expression in clinical specimens obtained from twelve OSCC patients for whom tissue samples were collected before and after radical radiation therapy. Interestingly, FOXM1 expression was elevated in eight of the twelve cases; moreover, in cases where expression was initially high, it remained high in the post-treatment samples. Statistical comparison of immunostaining scores for FOXM1 in tumor tissue before and after

treatment showed a statistically significant increase in the post-treatment samples ($P < 0.01$, Figure 1E). In addition, the expression of FOXM1 significantly increased in irradiated tissues obtained from the mouse xenograft model using SAS and SAS-R ($P < 0.05$, Figure 1F). Furthermore, the expression of FOXM1 in radiation-naïve tissue was higher in SAS-R than in SAS. Finally, we used the OSCC cell lines and CRR cells to determine changes in FOXM1 expression at 1, 3, 6, and 12 h after exposure to 2 Gy irradiation per day for 5 days. In the OSCC parental cell lines and SAS-R, FOXM1 expression was enhanced following irradiation, but the expression peaked at 6 h. In HSC-2-R cells, however, FOXM1 expression was enhanced following irradiation and remained high at 12 h (Figure 1G). All cells were cultured until 24 h after irradiation ended, at which point there was no significant difference in the number of dead cells among the cell lines (data not shown). In addition to the expression analysis, we attempted to clarify the relationship between FOXM1 and the effect of radiotherapy using clinical specimens. Although the limited number of patients who received RT or CRT did not allow for adequate analysis, we observed a trend toward prolonged PFS in the low FOXM1 expression group

compared to the high FOXM1 expression group (P = 0.065, Supplementary Figure S1).

Effect of FOXM1 expression on radiosensitivity in OSCC cells

To determine the effect of FOXM1 expression on radiosensitivity in OSCC cells, we evaluated the survival of OSCC cells after their exposure to 6 Gy irradiation using a clonogenic assay under various conditions. Initially, changes in radiosensitivity were observed in SAS, SAS-R, and HSC-2-R cells, which each expressed FOXM1, after FOXM1-specific siRNA treatment. Specifically, the SAS, SAS-R, and HSC-2-R cells treated with FOXM1-specific siRNA showed a significant increase in radiosensitivity compared with that of negative control-treated cells (Figure 2A–C). Conversely, the forced expression of FOXM1 in HSC-2 cells, which exhibited low expression levels of FOXM1, significantly reduced radiosensitivity (Figure 2D). The proliferative capacity of cells may affect radioresistance; however, FOXM1 had only a slight effect on the proliferative activity of OSCC cells under each of the experimental conditions (Supplementary Figure S2).

Effect of FOXM1 expression on DNA damage in irradiated OSCC cells

To determine whether FOXM1 expression affects the number of double-stranded DNA breaks in irradiated OSCC cells, 53BP1 was used to quantify the double-stranded DNA breaks attributed to X-ray irradiation [26]. The cells with X10 53BP1 foci were counted at 1, 3, 6, and 12 h after 6 Gy irradiation. DNA damage was increased significantly in SAS, SAS-R, and HSC-2-R cells treated with FOXM1-specific siRNA compared with the DNA damage detected in the control group (Figure 3A–C). In contrast, DNA damage was decreased significantly in HSC-2 cells in which the expression of FOXM1 was forced (Figure 3D).

Effect of FOXM1 expression on the regulation of ROS in irradiated OSCC cells

Radiation is known to make a substantial contribution to the generation of ROS in various cells and thereby induce DNA damage [30]; hence, we hypothesized that FOXM1 regulates radiosensitivity via the regulation of ROS in OSCC cells. Therefore, we examined the

expression of superoxide dismutase (SOD), a ROS scavenger [31], in cells exposed to 6 Gy irradiation under FOXM1-specific siRNA treatment or the forced expression condition using a Cellular ROS/Superoxide Detection Assay Kit. The number of SODpositive cells (scavengers of radiation-generated ROS) decreased, resulting in an increase in ROS production in SAS, SAS-R, and HSC-2-R cells treated with FOXM1-specific siRNA to suppress FOXM1 expression (Figure 4A–C). Conversely, the number of SOD–positive cells increased, and ROS production decreased in HSC-2 cells in which the expression of FOXM1 was forced (Figure 4D). In addition, we examined the expression changes of the ROS scavengers, superoxide dismutase 2 (SOD2), catalase (CAT), and peroxiredoxin 3 (PRDX3), which are downstream targets of FOXM1 [32], using western blotting. Supporting the results of Cellular ROS/Superoxide Detection Assay analysis (Figure 4A–D), SOD2 expression levels were decreased in FOXM1-specific siRNA-treated SAS, SAS-R, and HSC-2-R cells according to western blots (Figure 5A–C), whereas SOD2 expression levels were increased in HSC-2 cells in which FOXM1 expression was forced (Figure 5D). Although there were differences among individual cells, the changes in

the expression levels of the ROS scavengers CAT and PRDX3 were correlated with the changes in FOXM1 expression (Figure 5A–D).

Effects of the pharmacological-based downregulation of FOXM1 expression on radiosensitivity in OSCC cells

To determine whether the clinical application of radiotherapy with FOXM1 inhibition is a feasible treatment approach, we investigated the existing drugs that inhibit FOXM1. Specifically, we used thiostrepton, which is a natural cyclic oligopeptide thiazole antibiotic, the inhibitory activity of which is largely due to its action as a proteasome inhibitor [20]. Thiostrepton inhibited the proliferative activity of FOXM1-expressing OSCC cells in a concentration-dependent manner (Supplementary Figure S3). Because the proliferative activity of cells can affect their radiosensitivity, concentrations of thiostrepton that did not affect the proliferative activity of cells were used in subsequent experiments. In a subsequent clonogenic assay, thiostrepton markedly suppressed FOXM1 expression and significantly enhanced the radiosensitivity of SAS, SAS-R, and HSC2-R cells (Figure 6A–C). Additionally, while not exactly the same, the variation in the expression

of FOXM1 and ROS scavengers under each condition when OSCC cells were exposed to thiostrepton was similar to that of the experimental system in which FOXM1 was knocked down (Figure 6D).

Discussion

Previous studies have aimed to identify radioresistance-related molecules using radioresistant OSCC cell lines, and expression analysis has also been used to identify such molecules and elucidate radioresistance mechanisms [33-35]. However, studies focusing on the mechanisms underlying radioresistance in OSCC remain rare, especially those employing multiple CRR cells established via routine clinical irradiation of OSCC cell lines. In the present study, we focused on the effects of FOXM1 expression on radioresistance in OSCC using CRR cells to investigate changes in gene expression over time following exposure to irradiation. Consequently, we are the first to elucidate a molecular mechanism underlying radioresistance in OSCC using such a method.

The overexpression of FOXM1 has been reported in various malignancies, including breast, ovarian, prostate, colorectal, lung, and

gastric cancers [36]. In head and neck squamous cell carcinoma (HNSCC), including OSCC, the overexpression of FOXM1 was first reported by Gemenetzidis *et al*. [37] in 2009, and additional studies on the expression and biological significance of FOXM1 in HNSCC have been published subsequently [38, 39]. In the present study, *in vitro* expression analysis revealed that the expression of FOXM1 was upregulated in OSCC cells compared with that in normal keratinocytes, consistent with the aforementioned studies. Thus, FOXM1 is a possible therapeutic target in OSCC with relatively high tumor specificity.

Interestingly, our expression analysis revealed that (*i*) FOXM1 expression was upregulated in radioresistant OSCC cells compared with their parental cell lines, (*ii*) there was an apparent association between FOXM1 expression and radioresistance in human clinical and mouse xenograft specimens, and (*iii*) FOXM1 expression increased for a specific period after irradiation, including in OSCC cells with low expression levels of FOXM1, and FOXM1 expression levels remained high in some CRR cells over time. Furthermore, although there was no statistically significant difference, OSCC with high FOXM1 expression showed a trend toward a shorter disease control time after radiotherapy.

In other words, the results suggest that FOXM1 may correlate with radioresistance not only experimentally but also clinically. FOXM1 has been associated with regulating tumor cell radiosensitivity in cervical cancer, breast cancer, and glioblastoma [12, 13, 40]. Eckers *et al*. [16] reported that FOXM1 is involved in regulating radiosensitivity during cellular quiescence in HNSCC. They found that FOXM1 has a much lower basal expression level in quiescent tumor cells than that in proliferating tumor cells, but significant FOXM1 expression changes were only observed upon irradiation. In contrast, some OSCC and CRR cells in the present study showed constant FOXM1 expression, even under normal culture conditions. Kambach *et al*. reported that FOXM1 protein expression levels were elevated in response to irradiation in breast cancer cells [41]. Moreover, FOXM1 expression is induced by ROS in brain tumors [42]. It is possible that the expression of FOXM1 is regulated as a self-defense mechanism against irradiation in OSCC cells following the production of ROS due to irradiation. However, it is also possible that the so-called cancer stem cell fraction is increased by daily exposure to 2 Gy of irradiation, as reported previously by Osuka *et al*. [43]. FOXM1 expression may not necessarily fluctuate characteristically

in OSCC cells during the quiescent phase; however, the relationship between cancer stem cells and FOXM1 in OSCC should be addressed in future studies.

FOXM1 was initially found to be expressed in proliferating cells and has since been associated with the proliferative potential of tumor cells [11]. In the present study, only slight changes in proliferative activity were detected when FOXM1 expression was either suppressed or upregulated, and these may represent cell type-dependent effects. In previous studies on keratinocytes and HNSCC cells, proliferative activity did not decrease markedly when FOXM1 expression was suppressed [38, 44-47]. FOXM1 has also been associated with various malignant phenotypes, such as the epithelial-mesenchymal transition, regulation of hormone signaling, chemoresistance, cancer metabolism, and cancer stem cell properties in tumor cells [48]. Although these malignant phenotypes have been studied in various malignancies [10], a limited number of studies have been conducted on radioresistance, and these were mainly studies on brain tumors [12-15, 40, 49]. Indeed, only one study has been published on head and neck cancers, including OSCC [16]. In the current study, we found for the first time that FOXM1

expression in radioresistant OSCC cells is associated with radiosensitivity. Collectively, our results indicate the potential of FOXM1 as a therapeutic target in the treatment of OSCC and other carcinomas with radioresistant tumors.

Although radiation is thought to exert therapeutic effects on cancer cells by inducing various types of cell death (i.e., apoptosis, mitotic catastrophe, necrosis, and autophagy) [50], based on the changes in FOXM1 expression over time detected here, we hypothesized that FOXM1 functions in OSCC in the early stages of irradiation to confer radioresistance. To test this hypothesis, we analyzed ROS production and DNA damage after irradiation and found that both were significantly altered when FOXM1 expression was regulated. In general, irradiation causes DNA damage via ROS production, and the regulation of ROS production in radioresistance has been studied extensively [5, 6]. Kambach *et al.* [41] reported that FOXM1 is involved in the regulation of the radiation-responsive cell invasion of breast cancer cells through the regulation of ROS production. Artem *et al.* [17] reported that FOXM1 is a key regulator of ROS in normal dividing skin epithelial cells and that squamous cell carcinoma cells can escape early senescence

and apoptosis using FOXM1, with which the cells regulate oxidative stress [17]. We recently reported that the regulation of the antioxidant pathway plays an important role in radioresistance in OSCC [51]. Building on this finding, our present data indicate that FOXM1 regulates radiation-induced ROS production and, consequently, DNA damage and may contribute to overcoming radioresistance in OSCC.

FOXM1 is a transcription factor that regulates the expression of various downstream molecules [48]. In the present study, we found that the expression of ROS scavenger molecules involved in ROS regulation was altered in response to FOXM1 expression. A similar result was reported by Smirnov *et al.* [17], suggesting that ROS scavengers might contribute to FOXM1-mediated ROS regulation [17]. Notably, the ROS scavengers that showed altered expression levels differed among cell lines, which may have been due to FOXM1 regulating ROS through crosstalk with various molecules and the regulation of downstream molecules [10]. Therefore, further research on the redox system associated with FOXM1 in OSCC is required.

Quinoline compounds and the thiazole antibiotic thiostrepton are known as FOXM1 inhibitors [20]. In the present study, we found that

pharmacological-based downregulation of FOXM1 via thiostrepton treatment abrogated the proliferative capacity and radiosensitivity of OSCC cells, including CRR cell lines, as reported previously [12, 16]. Furthermore, since the suppression of FOXM1 expression and subsequent changes in ROS scavenger expression were observed in thiostrepton-treated OSCC cells, thiostrepton may be directly involved in ROS regulation. Conversely, the concentrations of thiostrepton that did not affect cell proliferation efficiently increased radiosensitivity in OSCC cells. Collectively, these results indicate that thiostrepton could be combined with radiotherapy, possibly at a low dose that does not affect cell proliferation, to overcome the radioresistance of OSCC.

Recently, therapies targeting ROS regulation have attracted attention as novel treatments for treatment-resistant cancers, as reported by Nagano et al [52]. Given that ROS is involved in the regulation of FOXM1, as already mentioned, targeting ROS production therapeutically may control the emergence of radioresistant OSCC. Therefore, studies focusing on ROS regulation in radioresistant OSCC are needed to elucidate this issue.

In summary, our findings suggest that FOXM1 can be considered a novel therapeutic target for the treatment of radioresistant OSCC, and future research should focus on treatment strategies that could target this axis and potentially overcome radioresistance in OSCC (Figure 7).

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this article.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Kumamoto University (approval number: 2389, 1427) and performed in accordance with the Good Clinical Practice and the Declaration of Helsinki guidelines. Informed consent was obtained from all patients before biopsy and surgery based on the guidelines of Kumamoto University

(SENSHIN No.2389). In this study, retrospective analysis was performed, which did not require individual consent; nevertheless, patients were provided with an opportunity to refuse participation in an opt-out format (RINRI No. 1427).

Author Contributions

Conceptualization: H.T., Y.M., and R.Y.; conducted the experiments: K.S, H.T., J.I., M.H., T.O., R.K., K.Y., Y.N., and S.G.; investigation: J.S., R.Y., K.K., M.H., and N.T.; data curation: K.I., M.N., A.H., and Y.K.; writing—original draft preparation: H.T. and R.Y.; writing review and editing: M.F., R.T., and H.N; supervision: Y.K., M.F., and R.M.; project administration: R.Y. and H.N.; funding acquisition: R.Y. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The datasets generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. Investigating radioresistance *in vitro* in CRR cells and human clinical specimens via expression analysis.

(A) SAS and SAS-R cells were irradiated with X-rays at 2 Gy per day for 5 days, and total RNA was collected 1, 3, 6, and 12 h after irradiation. Nonirradiated SAS and SAS-R cells were used as controls in the cDNA microarray analysis. Ingenuity Pathway Analysis (IPA) was used to identify radioresistance-associated molecules. (B) The top 10 molecules of interest, i.e., those with the highest variability between

SAS and SAS-R cell lines according to IPA, are shown. The expression variability of the Z-score is indicated by color (see the bar above the table): red, upregulated genes; green, downregulated genes. (C) The protein expression levels of FOXM1 in OSCC cell lines and human normal keratinocytes were determined via western blotting. The expression of β-actin was used as the internal control. (D) The protein expression levels of FOXM1 in OSCC cell lines (SAS and HSC-2) and their clinically relevant radioresistant cell lines, SAS-R and HSC-2-R, were determined via western blotting. The expression of β-actin was used as the internal control. (E) Representative immunohistochemical staining of FOXM1 in clinical specimens. Immunohistochemical staining was performed on tissue samples before (Pre-RT) and after (Post-RT) irradiation. Scale bar: 100 μm (upper). Immunostaining scores among samples before (Pre-RT) and after (Post-RT) irradiation (lower). (F) Representative immunohistochemical staining of FOXM1 in tissue obtained from a mouse xenograft model. Immunohistochemical staining was performed on tissue samples nonirradiated (Non-IR) and after 30 Gy irradiation (IR 30 Gy). Scale bar: 100 μm (upper). Immunostaining scores among Non-IR and IR 30 Gy samples (lower). All data shown are

the means ± standard deviations of three independent experiments. Student's t-test: *$P < 0.05$; **$P < 0.01$. (G) After irradiation (IR) of SAS and HSC-2 cells and their clinically relevant radioresistant cell lines, SAS-R and HSC-2-R, with X-rays at 2 Gy per day for 5 days, proteins were collected at the indicated times, and changes in FOXM1 expression levels were detected via western blotting (top). The expression of β-actin was used as the internal control. Western blotting results were analyzed semi-quantitatively using densitometry (bottom). IR, irradiation; Non-IR, non-irradiation, NT, no treatment. Data are the means ± standard deviations of three independent experiments.

Figure 2. Effects of FOXM1 expression status on radiosensitivity in OSCC cell lines.

(A) SAS cells were incubated with either control (si-control) or FOXM1-specific siRNA (si-FOXM1 #1 and #2) for 24 h. Total RNA and protein were collected, and the expression of FOXM1 was measured using real-time RT-PCR (upper left) and western blotting (upper right). The expressions of GAPDH and β-actin were used as internal controls in real-time RT-PCR and western blotting, respectively. After exposure to

0, 2, and 6 Gy of X-rays (IR), the SAS cells were seeded in gelatin-coated culture dishes (60 mm) and treated with FOXM1-specific siRNA. After 10 days of culture, the cells were fixed and visualized, and the survival fraction was determined using a clonogenic assay. A representative image of the cell survival fractions (lower left) and the corresponding graphs (lower right) are shown. (B, C) Results of the analysis described in (A) for (B) SAS-R cells and (C) HSC-2-R cells. (D) HSC-2 cells were incubated with either control (Mock) or FOXM1-expression vector (FOXM1-OE) for 24 h. Total RNA and protein were collected, and the expression of FOXM1 was measured via real-time RT-PCR (upper left) and western blotting (upper right). The expressions of GAPDH and β-actin were used as internal controls in real-time RT-PCR and western blotting, respectively. At 24 h after transfection with FOXM1-OE, the cells were exposed to 0, 2, and 6 Gy of X-rays, and the HSC-2 cells were seeded in gelatin-coated culture dishes (60 mm) and treated with FOXM1-OE. The cells were cultured for 10 days, fixed and visualized, and the survival fraction was determined using a clonogenic assay. A representative image of the cell survival fraction (lower left) and the corresponding graph (lower right) are shown. GAPDH,

glyceraldehyde-3-phosphate dehydrogenase. All data shown in (A–D) are the means ± standard deviations of three independent experiments. Student's t-test: *$P < 0.05$; **$P < 0.01$.

Figure 3. Effects of FOXM1 expression status on DNA damage in OSCC cell lines.

Representative images and quantitative analysis of the immunofluorescence of 53BP1 foci under FOXM1 knockdown or overexpressing conditions after exposure to 6 Gy of X-rays. (A) Images of 53BP1 foci in SAS cells shown at 12 h under control siRNA (si-control) or FOXM1-specific siRNA (si-FOXM1) treatments after exposure to 6 Gy of X-rays (top). Number of 53BP1-positive cells detected at the indicated time points after exposure to 6 Gy of X-rays under si-control or si-FOXM1 treatments (bottom). (B, C) Results of the analysis described in (A) for (B) SAS-R cells and (C) HSC-2-R cells. (D) Representative images of the immunofluorescence of 53BP1 foci under FOXM1-overexpressing conditions after exposure to 6 Gy of X-rays. Images of 53BP1 foci in HSC-2 cells were captured at 12 h under treatment with the control (Mock) or FOXM1-expression vector

(FOXM1-OE) after exposure to 6 Gy of X-rays (top). Number of 53BP1-positive cells detected at the indicated time points after exposure to 6 Gy of X-rays under Mock or FOXM1-OE treatments (bottom). DAPI, 4',6-diamidino-2-phenylindole. All data in (A–D) are the means ± standard deviations of three independent experiments. Student's t-test: *$P < 0.05$; ** $P < 0.01$.

Figure 4. Effects of FOXM1 expression status on ROS regulation in OSCC cell lines.

Representative images and quantitative analysis of the immunofluorescence of ROS/SOD under FOXM1 knockdown or overexpressing conditions after exposure to 6 Gy of X-rays (IR). (A) At 12 h after exposure to 6 Gy of X-rays, SAS cells treated with control siRNA (si-control) or FOXM1-specific siRNA (si-FOXM1) were examined for their ROS/superoxide content using a Cellular ROS/Superoxide Detection Assay Kit (top). Results were quantified by counting the number of cells positive for ROS (green) and SOD (red) from all identified cells (blue, Hoechst staining) (bottom). NT, no treatment. (B, C) Results of the analysis described in (A) but for (B)

SAS-R cells and (C) HSC-2-R cells. (D) At 12 h after exposure to 6 Gy of X-rays, HSC-2 cells treated with the control (Mock) or FOXM1-expression vector (FOXM1-OE) were examined for their ROS/superoxide content using a Cellular ROS/Superoxide Detection Assay Kit (top). Results were quantified as described in (A) and the resultant data are shown (bottom). ROS, reactive oxygen species; SOD, superoxide dismutase; IR, irradiation; Non-IR, non-irradiation; NT, no treatment. All data in (A–D) are the means ± standard deviations of three independent experiments.

Figure 5. Analysis of FOXM1 and ROS regulation-related components in OSCC cell lines.

(A–D) Western blots used to determine the expression of FOXM1 and ROS regulation-related molecular components (SOD2, CAT, and PRDX3) in (A) SAS, (B) SAS-R, (C) HSC-2-R, and (D) HSC-2 cells at 12 h after exposure to 6 Gy of X-rays under the indicated treatment conditions (left). The expression of β-actin was used as the internal control. Quantitative analysis of the expression levels of FOXM1, SOD2, CAT, and PRDX3 under the indicated treatment conditions

(right). ROS, reactive oxygen species; SOD2, superoxide dismutase 2; CAT, catalase; PRDX3, peroxiredoxin 3; IR, irradiation; Non-IR, non-irradiation; NT, no treatment. All data in (A–D) are the means ± standard deviations of three independent experiments.

Figure 6. Effects of the pharmacological-based downregulation of FOXM1 on radiosensitivity in OSCC cells.

(A) SAS cells were incubated with either vesicles or thiostrepton for 48 h, after which proteins were collected and the expression of FOXM1 was measured using western blotting. The expression of β-actin was used as the internal control in western blotting (left). After exposure to 0, 2, and 6 Gy of X-rays, the SAS cells were seeded in gelatin-coated culture dishes (60 mm) and treated with FOXM1-specific siRNA. After 10 days of culture, the cells were fixed and visualized, and the survival fraction was calculated using a clonogenic assay. A representative image of the cell survival fraction (middle) and corresponding graph (right) are shown. (B, C) Results of the analysis described in (A) but for (B) SAS-R cells and (C) HSC-2-R cells. All data in (A–C) are the means ± standard

deviations of three independent experiments. Student's t-test: ** $P <$ 0.01.

(D) Analysis of FOXM1 and ROS regulation-related components in OSCC cell lines under thiostrepton treatment. Western blots were used to determine the expression of FOXM1 and ROS regulation-related molecular components (SOD2, CAT, and PRDX3) in (left) SAS, (middle) SAS-R, and (right) HSC-2-R cells at 12 h after exposure to 6 Gy of X-rays under the indicated treatment conditions. The expression of β-actin was used as the internal control. Quantitative analysis of the expression levels of FOXM1, SOD2, CAT, and PRDX3 under the indicated treatment conditions. ROS, reactive oxygen species; SOD2, superoxide dismutase 2; CAT, catalase; PRDX3, peroxiredoxin 3; IR, irradiation; Non-IR, non-irradiation; NT, no treatment.

Figure 7. Schematic representation of the speculative mechanism underlying FOXM1-mediated radioresistance in OSCC based on the present results.

When OSCC cells are irradiated, ROS production occurs, leading to DNA damage and cell death. However, some cells survive due to a

transient increase in FOXM1 expression caused by ROS as a redox response. After repeated fractionated irradiation, cells with permanently high expression of FOXM1 emerge and become radioresistant cells. Thus, FOXM1 appears to be a promising therapeutic target for the treatment of radioresistant OSCC. ROS, reactive oxygen species; IR, irradiation.