Anterior gradient 2 regulates cancer progression in *TP53*-wild-type esophageal squamous cell carcinoma

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Abstract. Anterior gradient 2 (AGR2) reportedly promotes tumor growth and has an unfavorable impact on survival in several cancers. However, no comprehensive functional analysis of AGR2 in esophageal squamous cell carcinoma (ESCC) has been performed. In the present study, the function and clinical significance of AGR2 were examined using ESCC cell lines and clinical samples. AGR2 was upregulated in EC tissue and ESCC cell lines. The downregulation of AGR2 suppressed cell proliferation and increased the proportion of G2/M-phase cells and phosphorylation of p53 in *TP53*-wild-type ESCC and osteosarcoma cells. However, these changes were not observed in *TP53*-mutant ESCC cells. In addition, immunohistochemistry results demonstrated that high AGR2 and low p53 expression levels in ESCC tissues were correlated with a worse prognosis. These results suggested that although AGR2 enhanced cell proliferation by inhibiting p53 phosphorylation in *TP53*-wild-type ESCC, the same mechanism did not regulate cell functions in *TP53*-mutant ESCC. Thus, AGR2 served an important role in ESCC progression and might be a useful prognostic marker in patients with *TP53*-wild-type ESCC.

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Abbreviations: ACTB, β-actin; AGR2, anterior gradient 2; EC, esophageal cancer; ER, endoplasmic reticulum; ESCC, esophageal cell carcinoma; HNSCC, head and neck squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA

Key words: anterior gradient 2, esophageal squamous cell carcinoma, *TP53* mutation, phosphorylation of p53, prognostic factor

Introduction

Esophageal cancer (EC) is the eighth commonest malignancy and sixth leading cause of cancer-related mortalities worldwide (1). In Eastern countries, esophageal squamous cell carcinoma (ESCC) is the commonest histological type of EC and one of the most aggressive malignancies (2). Despite the development of multidisciplinary treatments, the prognosis of patients with EC remains poor owing to its high invasiveness and metastatic potential (3). This limited improvement in the prognosis of patients with EC has prompted the search for novel candidates that can act as clinically useful biomarkers and treatment targets.

Anterior gradient 2 (AGR2), the human homolog of the *Xenopus laevis* cement gland gene (XAG-2), is a member of the protein disulfide isomerase family and usually localizes to the endoplasmic reticulum (ER) (4,5). Although it primarily functions as a modulator of ER homeostasis, it has been reported to have a number of functional roles, such as in cell proliferation, migration and differentiation, in some types of human cancer (6). Among human cancers, the overexpression of AGR2 was first reported in breast cancer (7) and similar results were subsequently reported in several human adenocarcinomas, including prostate, pancreas, ovary and lung carcinomas (8-11). A recent review reported that the overexpression of AGR2 has an unfavorable impact on overall survival in breast, prostate and gastric cancers (12). AGR2 is also found to be highly expressed in head and neck squamous cell carcinoma (HNSCC) (13-15) and its downregulation in HNSCC cells decreases cell proliferation and induces apoptosis (15,16). In addition, in esophageal adenocarcinoma, AGR2 has been reported to promote tumor growth and cell migration (17).

AGR2 suppresses p53 phosphorylation and worsens prognosis in estrogen receptor-positive *TP53*-wild-type breast cancer (18). In addition, upregulated AGR2 expression in Barrett's epithelium and precancerous lesions suppresses the phosphorylation of p53 (19). These studies show that AGR2 suppresses p53-related pathways via the inhibition of p53 phosphorylation, which is the main step in p53 activation (20). It has been reported that p53 serves multiple roles in a number of cellular processes, such as cell cycle regulation,

apoptosis induction, differentiation and senescence (21,22) and a major role of p53 is the maintenance of genomic stability and integrity (23). The phosphorylation of the p53 protein is related to malignancy and poor prognosis in some types of cancer (4,6,18,19). p53 serine 15 is generally phosphorylated in response to DNA damage and this phosphorylation activates p53 (20). Therefore, the inactivation of p53 via the inhibition of phosphorylation represents a critical step in preventing tumor development and progression.

To the best of the authors' knowledge, no comprehensive functional analyses on the role of AGR2 in ESCC have been conducted, although several studies have focused on AGR2 in other cancers. The present study examined the effects of AGR2 downregulation on tumor progression and the phosphorylation of p53 in ESCC cell lines. In addition, the associations between AGR2 and p53 expression, as well as clinical outcomes, were analyzed in ESCC tissue.

Materials and methods

Bioinformatics analysis. Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html) was used to show the expression of AGR2, which was the target gene, in esophageal cancer.

Cell lines and culture. Human ESCC cell lines TE2, TE5, TE8, TE11 and TE15 and human osteosarcoma cell lines U2OS and SAOS2 were obtained from the RIKEN BioResource Center Cell Bank. Human ESCC cell lines KYSE70, KYSE150 and KYSE170 were obtained from the Japanese Collection of Research Bioresources Cell Bank. The immortalized esophageal epithelium cell line Het-1A and mesothelial cell line MeT-5A were obtained from the American Type Culture Collection. The ESCC cell lines Het-1A and MeT-5A were maintained in Roswell Park Memorial Institute medium (Nakalai Tisque) and U2OS and SaOS2 cells were cultured in HyClone McCoy's 5A medium (Cytiva). The two media were supplemented with 10% fetal bovine serum (System Biosciences), 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines were cultured in a humidified 37˚C incubator with 5% carbon dioxide.

RNA extraction and quantification of mRNA expression. Total RNA was extracted from the cell lines using a miRNeasy Mini kit (Qiagen) as per the manufacturer's instructions. Reverse transcription reactions were performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), as per the manufacturer's given procedure, at 25˚C for 10 min, followed by 37˚C for 120 min and 85˚C for 5 min. The level of mRNA expression was measured using TaqMan Gene Expression Assays (Hs_00356521_m1 for AGR2 and Hs_02758991_g1 for GAPDH; Applied Biosystems; Thermo Fisher Scientific, Inc.), as per the manufacturer's protocol. Reverse transcription-quantitative (RT-q) PCR was performed using a StepOnePlus PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the cycle threshold (Ct) value was calculated using StepOne Software v2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. The results were examined using the $2^{-\Delta\Delta Cq}$ method relative to the expression of GAPDH (24).

Western blot analysis. The cultured cells were lysed using the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.) and protein was extracted. The protein concentration was adjusted using the Protein Assay Rapid kit Wako II (FUJIFILM Wako Pure Chemical Corporation). The prepared lysate containing 20 μg of total protein was subjected to 12 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene difluoride membrane (Cytiva). After blocking by 5% skimmed milk powder or 5% bovine serum albumin (Sigma-Aldrich, Merck KGaA) at room temperature for 1 h, the membrane was subsequently probed with each candidate antibody at 4˚C overnight. Next day, the secondary antibody was added into the membranes at room temperature for 1 h and the level of protein expression in each case was visualized via SuperSignal West Dura (Thermo Fisher Scientific, Inc.) and evaluated using an Amersham Imager 680, software version 2.0.0 (Cytiva). β-actin (ACTB) was used as a loading control.

The antibodies were purchased and diluted as follows: anti-AGR2 antibody (rabbit, 1:5,000; cat. no. Ab76473) from Abcam; anti-phospho-p53 antibody (serine 15) (mouse, 1:1,000; cat. no. 9286S) from Cell Signaling Technology, Inc.; anti-p53 antibody (DO1, Mouse, 1:1,000; cat. no. sc-126) from Santa Cruz Biotechnology, Inc. and anti-ACTB antibody (Mouse, 1:20,000; cat. no. A5441) from Sigma-Aldrich. The secondary antibodies were: Anti-mouse IgG, HRP-linked anti body (horse; cat. no. 7076) and anti-rabbit IgG, HRP-linked anti body (goat; cat. no. 7074) both from Cell Signaling Technology, Inc. The dilution rate of the secondary antibody varied according to the dilution rate of the primary antibody.

Downregulation of AGR2 by small interfering RNA (siRNA). The expression of *AGR2* was downregulated using small interfering RNA (siRNA) targeting AGR2 (Stealth RNAi™ siRNA, Thermo Fisher Scientific, Inc.; cat. no. HSS116220; UUUCUU UAAAGCUUGACUGUGUGGG). ESCC and osteosarcoma cells were transfected either with control siRNA (Stealth RNAi™ siRNA Negative Control, Thermo Fisher Scientific; cat. no. 12935112) or siRNA targeting AGR2 at 10 nmol/l in a six-well culture plate using Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection for 72 h at 37˚C, AGR2 mRNA and protein expression was confirmed using RT-qPCR and western blotting, respectively.

Proliferation assay. Cells were seeded on 96-well plates, incubated for 24 h and transfected with either control or AGR2-targeting siRNA. After transfection, cells were incubated for 24 h at 37˚C, and the number of viable cells 0, 24, 48, 72 and 96 h after treatment was determined using absorbance data at a wavelength of 450 nm using Cell Count Reagent SF (Nakalai Tesque).

Colony formation assay. TE2, TE5 and TE15 cells transfected with either control or AGR2-targeting siRNA were seeded in six-well culture plates at a density of 1,000 cells per well.

After 2 weeks, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min and briefly stained with crystal violet (Nakalai Tesque) at room temperature until all cells were stained enough. The areas of all stained cells were measured using the NIH ImageJ System (v1.53) (http://rsb.info.nih.gov/ij/).

Cell cycle assay. Cell cycles were analyzed 72 h after transfection using flow cytometry. The cells were detached from the plate using trypsin-EDTA, treated with 0.2% Triton X-100 and stained with PI RNase staining buffer (Becton-Dickinson and Company). The cells were analyzed using a BD Accuri C6 (BD Biosciences) and cell cycle distribution was recorded using the BD Accuri Software (BD Biosciences). At least 10,000 cells were assessed for each measurement.

Apoptosis assay. The cells were stained and evaluated using an Annexin V-FITC kit (Beckman Coulter, Inc.) 72 h after transfection according to the manufacturer's instructions. The proportions of early and late apoptotic cells were measured by flow cytometry using the BD Accuri C6. At least 10,000 cells were assessed for each measurement.

Patients and tissue samples. A total of 81 patients with ESCC who had undergone esophagectomy between 1999 and 2013 at the Kyoto Prefectural University of Medicine were enrolled in the present study. Written informed consent for the research was obtained from all patients prior to their respective treatments. Patients who had undergone non-curative resection or preoperative chemo- or radiation-therapy were excluded from the present study. The median length of the follow-up period for the censored cases was 5.98 years (range, 0.38-16.7 years). A total of 35 patients (43.2%) had recurrence within 5 years of surgery and 26 patients (32.0%) died of primary ESCC. The clinicopathological features were evaluated using the 8th edition of the International Union Against Cancer (UICC)/tumor-node-metastasis (TNM) Classification of Malignant Tumors (25). The present study was approved by the Faculty of Science Ethics Committee of the Kyoto Prefectural University of Medicine (approval no. ERB-C-1414-1).

Immunohistochemistry (IHC). IHC staining was performed using the labeled streptavidin-biotin method using a Vectastain Universal Quick kit (Vector Laboratories, Inc.) as per the manufacturer's protocol. The method can be briefly described as follows: antigen retrieval was performed using heated Dako Target Retrieval Solution (Dako; Agilent Technologies, Inc.) at 95˚C for 60 min and endogenous peroxidase activity was disrupted by incubation in 0.3% H_2O_2 at room temperature for 20 min; then, 2.5% normal horse serum (Vector Laboratories, Inc.) was used to block non-specific binding at room temperature for 10 min. The slides were incubated with either the anti-AGR2 antibody 1:500 (cat. no. Ab76473; Abcam) or the anti-p53 antibody 1:200 (mouse, NCL-L-p53-DO7; Novocastra Laboratories Ltd.) at 4˚C overnight. They were then incubated with a biotinylated pan-specific universal antibody at room temperature for 10 min and a streptavidin/peroxidase complex (Vector Laboratories, Inc.) at room temperature for 5 min, followed by incubation with 3,3'-diaminobenzidine tetrachloride. Counterstaining was performed using hematoxylin. A formalin-fixed ESCC cell line overexpressing AGR2 (TE15 cells) was used as the positive control.

The AGR2 staining was evaluated based on a score depicting the intensity and proportion of stained cancer cells. The intensity was scored as 3 (strong), 2 (intermediate), 1 (weak), or 0 (no staining). The proportion was evaluated as the ratio of stained areas in the cancer tissue and was scored from 0 to 1. Cancer tissues with an intensity score of 3 and a proportion ≥0.3 or an intensity score of 2 and a proportion ≥0.5 were considered the high expression group and the others comprised the low expression group.

p53 staining was evaluated in a manner similar to that of AGR2. The intensity was scored as 3 (strong), 2 (intermediate), 1 (weak), or 0 (no staining) and the proportion was scored from 0 to 1. Cancer tissues with an intensity score of 3 or 2 and a proportion ≥0.3 were considered the high expression group and the others comprised the low expression group.

Statistical analysis. Statistical analysis was performed using JMP Version 14 (ASA Institute). The Wilcoxon signed-rank test or Student's t-test was used to compare differences between the paired and unpaired samples. Differences between survival curves were examined using the log-rank test. Multivariate analysis of survival was carried out using the Cox regression method. Relationships between clinicopathological factors were assessed using Fisher's exact test or the Chi-square test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of AGR2 in tissue and cell lines. The mRNA expression of *AGR2* was significantly higher in EC tissue compared with the normal counterpart($P<0.001$; Fig. 1A), according to GEPIA (26). Among the nine ESCC cell lines and two non-cancerous cell lines, the mRNA and protein expression of AGR2 was higher in the TE2, TE5 and TE15 cells compared with the other cells (Fig. 1B). p53 protein expression was also confirmed, a result that was partly consistent with the previously reported mutation status (Fig. 1B) (27).

Effects of AGR2 downregulation on the functions of ESCC cells. AGR2-expressing cell lines TE15 and TE5, which are reported to be *TP53*-wild-type and mutant cell lines, respectively (27,28), were selected for the subsequent experiments. The transient introduction of the siRNA targeting AGR2 efficiently downregulated AGR2 mRNA and protein expression levels (Fig. S1). As determined using the proliferation assay, the downregulation of AGR2 expression inhibited the growth of TE15 cells but not TE5 cells (Fig. 1C). The downregulation of AGR2 also reduced colony formation in TE15 cells but not TE5 cells (Fig. 1D).

In addition, cell cycle analysis demonstrated that AGR2 downregulation increased the proportion of TE15 cells, but not TE5 cells, in the G2/M phase (Fig. 2A). The apoptosis assay demonstrated that early and late apoptosis was increased only for the TE15 cells (Fig. 2B). The TE2 cell line, which is a *TP53*-wild-type cell line with AGR2 expression, demonstrated

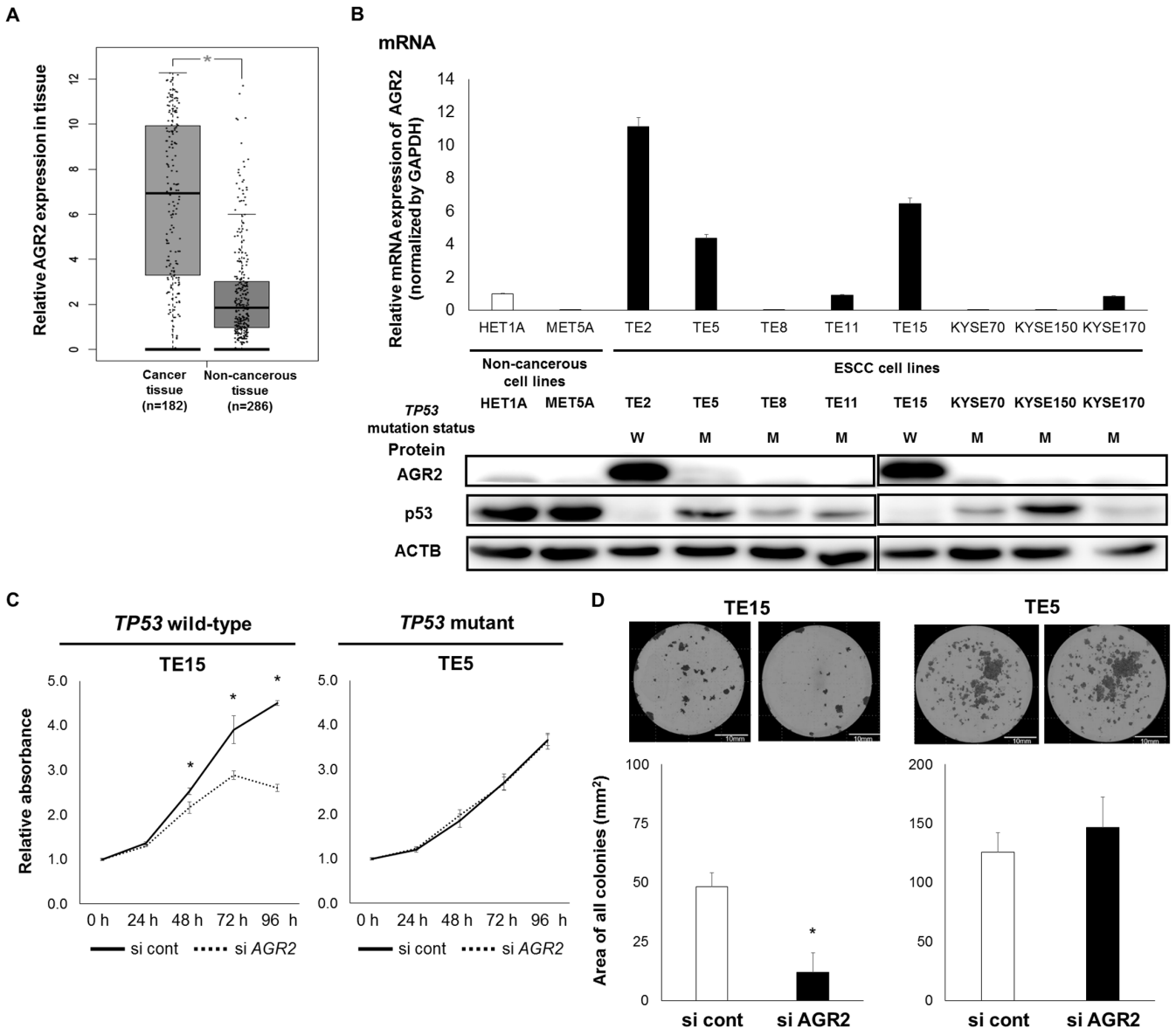

Figure 1. Expression of AGR2 in ESCC and effects of AGR2 downregulation on ESCC cell proliferation. (A) mRNA expression of AGR2 from the Gene Expression Profiling Interactive analysis dataset for esophageal cancer tissue vs. the normal counterpart shown as raw data. (B) mRNA and protein expression of AGR2 and protein expression of p53 in nine ESCC cell lines. The levels of AGR2 mRNA were determined by quantitative real-time polymerase chain reaction. The levels of AGR2 and p53 protein were determined by western blotting. The figure shows representative western blots. (C and D) Effects of AGR2 downregulation on cell proliferation at the indicated times and colony formation. Results are mean ± standard deviation. Experiments were performed in triplicate. *P<0.05. AGR2, anterior gradient 2; ESCC, esophageal squamous carcinoma; ACTB, β-actin; si AGR2, AGR2 specific small interfering RNA; si cont, control small interfering RNA.

similar changes in cell function as TE15 cells owing to AGR2 downregulation (Fig. S2A-E).

Relationships between AGR2 expression and phosphorylation of p53. Based on the aforementioned results, the associations between AGR2 expression and the phosphorylation or mutation status of *TP53* were examined. The phosphorylation of p53 Ser15 was markedly enhanced by the downregulation of AGR2 in *TP53*-wild-type TE15 cells (Fig. 2C). By contrast, in *TP53*-mutant TE5 cells, the phosphorylation of p53 was already high and not enhanced by the downregulation of AGR2 (Fig. 2C).

For further verification of these associations, similar examinations were performed using the osteosarcoma cell lines U2OS, which is a *TP53*-wild-type cell line and SAOS2, which is a *TP53*-deficient cell line (29). AGR2 and p53 expression was confirmed in U2OS cells, whereas in SAOS2 cells, AGR2 expression was very low and p53 expression was not detected (Fig. 3A and B). The present study therefore only examined the effects of AGR2 downregulation in U2OS cells. The downregulation of AGR2 also suppressed the proliferation of and enhanced p53 Ser15 phosphorylation in U2OS cells (Fig. 3C-E).

Immunohistochemistry of AGR2 and p53 in ESCC tissues. AGR2 was expressed in the esophageal gland but not the non-cancerous esophageal epithelium (Fig. 4A and B). In ESCC tissue, AGR2 was detected in the cell membrane and

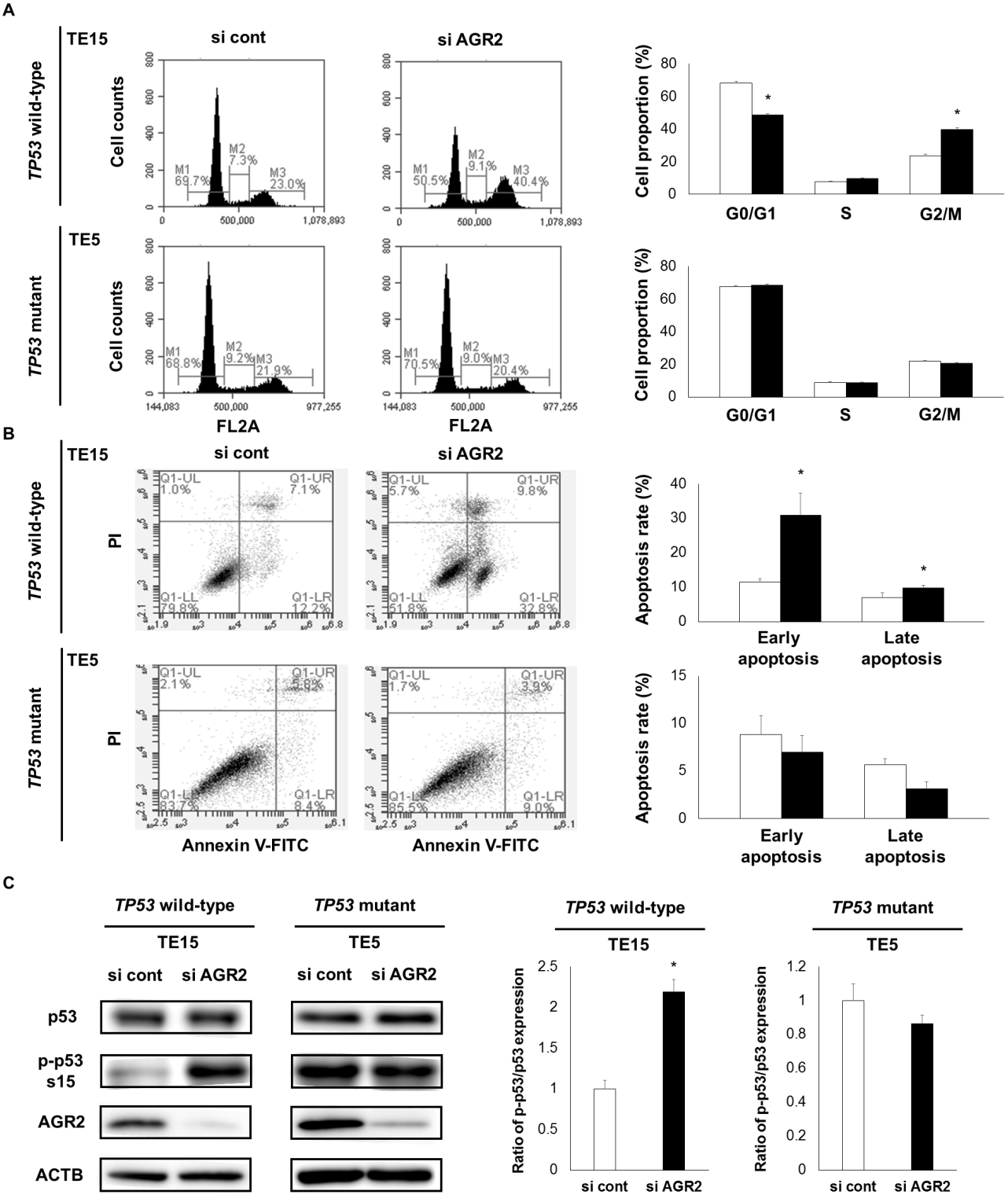

Figure 2. Effects of AGR2 downregulation on cell cycle, apoptosis and p53 and phosphorylation of p53 serine 15 in ESCC cells. (A) Effects of AGR2 downregulation on cell cycle. The cells were transfected with the control or AGR2-targeting siRNA. They were stained with PI and analyzed by flow cytometry 72 h after transfection. (B) Effects of AGR2 downregulation on apoptosis. The cells were transfected with the control or AGR2-targeting siRNA. They were stained with PI and Annexin V and then analyzed by flow cytometry 72 h after transfection. (C) Effects of AGR2 downregulation on p53 and phosphorylation of p53 serine 15 in *TP53*-wild-type TE15 cells and *TP53*-mutant TE5 cells. The figure shows representative western blots of ESCC cells transfected either with the control or AGR2-targeting siRNA. Results are mean ± standard deviation. Experiments were performed in triplicate. *P<0.05. AGR2, anterior gradient 2; ESCC, esophageal squamous carcinoma; p-p53 s15, phosphorylation of p53 serine 15; PI, propidium iodide; siRNA, small interfering RNA; ACTB, β-actin; si AGR2, AGR2 specific small interfering RNA; si cont, control small interfering RNA.

cytoplasm and the level of AGR2 expression was categorized as either low or high (Fig. 4C and D). No significant differences were observed in the 5-year overall survival rate was between the high and low expression groups (60.5 vs. 71.2%; P=0.32; Fig. 4E). In addition, no significant differences were observed in the clinicopathological features between these groups (Table I).

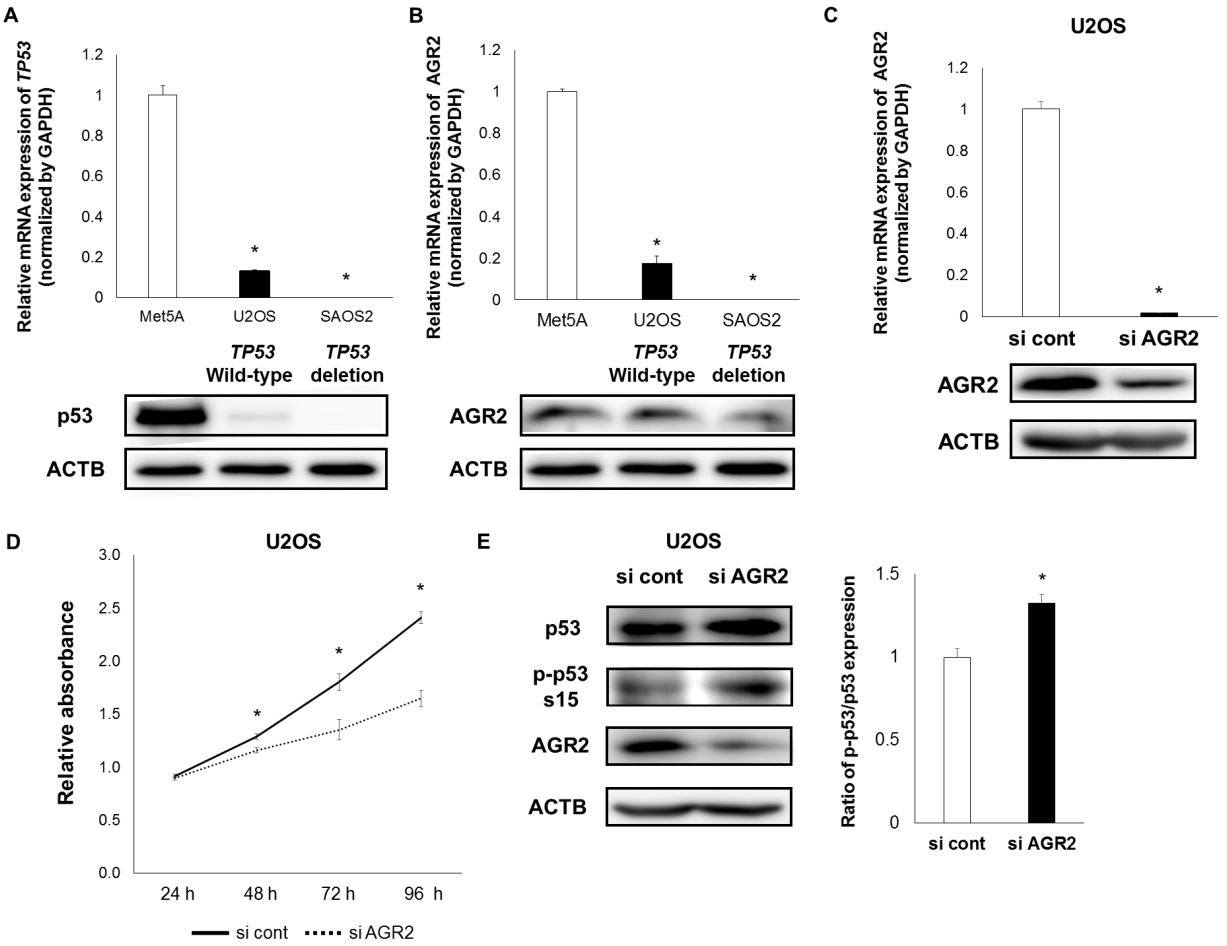

Figure 3. Expression of AGR2 in osteosarcoma cells and effects of AGR2 downregulation on cell function. (A and B) Expression of p53 and AGR2 in two osteosarcoma cell lines and a non-cancerous cell line. The levels of AGR2 mRNA and protein and p53 protein expression were determined by RT-qPCR and western blotting, respectively. (C) RT-qPCR and western blots reveal that AGR2-targeting siRNA effectively reduced the mRNA and protein levels of AGR2 in U2OS. (D) Effects of AGR2 downregulation on cell proliferation at the indicated times. (E) Effects of AGR2 downregulation on p53 and phosphorylation of p53 serine 15 in U2OS. The figure shows representative western blots of U2OS transfected either with the control or AGR2-targeting siRNA. Results are mean ± standard deviation. Experiments were performed in triplicate. *P<0.05. ACTB, β-actin; AGR2, anterior gradient 2; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA; ESCC, esophageal squamous carcinoma; p-p53 s15, phosphorylation of p53 serine 15; si AGR2, AGR2 specific small interfering RNA; si cont, control small interfering RNA.

The result of IHC for p53 was also examined and categorized into low and high expression groups (Fig. S3A and B). No significant differences were observed in the 5-year overall survival rate between the high and low p53 expression groups (73.7 vs. 61.9%, P=0.28; Fig. S3C). However, with respect to AGR2 and p53 expression in combination, patients with high-AGR2/low-p53 expression demonstrated significantly worse prognosis than other patients. The 5-year overall survival rate was 40.0% in the high-AGR2/low-p53 expression group, whereas it was 70.3% in the low-AGR2/low-p53 expression group (P=0.046; Fig. 4F). The survival analysis of patients with low p53 expression demonstrated that a large tumor of >40 mm in size (HR=3.82, P=0.03) and high AGR2 expression (HR=3.66; P=0.03) were independent prognostic factors (Table II).

Discussion

Several studies have shown that the downregulation of AGR2 decreases cell proliferation (15-17,30) and induces cell cycle arrest (31) or apoptosis (32,33) in some types of cancer. The present study demonstrated that AGR2 downregulation in *TP53*-wild-type ESCC cells suppressed cell proliferation and increased the proportion of G2/M-phase and apoptotic cells. In addition, it enhanced the phosphorylation of p53 Ser15. However, these changes were not observed in *TP53*-mutant ESCC cells. Similar findings were also verified in *TP53*-wild-type osteosarcoma cells. In addition, the IHC results demonstrated that high AGR2 and low p53 expression in ESCC tissue was associated with worse prognosis. These results suggest that AGR2 expression enhances cell proliferation via the inhibition of p53 phosphorylation in *TP53*-wild-type ESCC and that this mechanism does not regulate cell functions in *TP53*-mutant ESCC because the function of p53 is already impaired.

The correlation between AGR2 overexpression in solid tumors and poor prognosis has been reported (12) and it is particularly clear in adenocarcinoma tissue (8,17,34). There are some reports on SCC of the lung or head and neck, but the potential of AGR2 as a prognostic biomarker would be weaker in those cells compared with adenocarcinoma (6,8,13-15,34). Indeed, AGR2 is reported to be a useful positive marker of esophageal

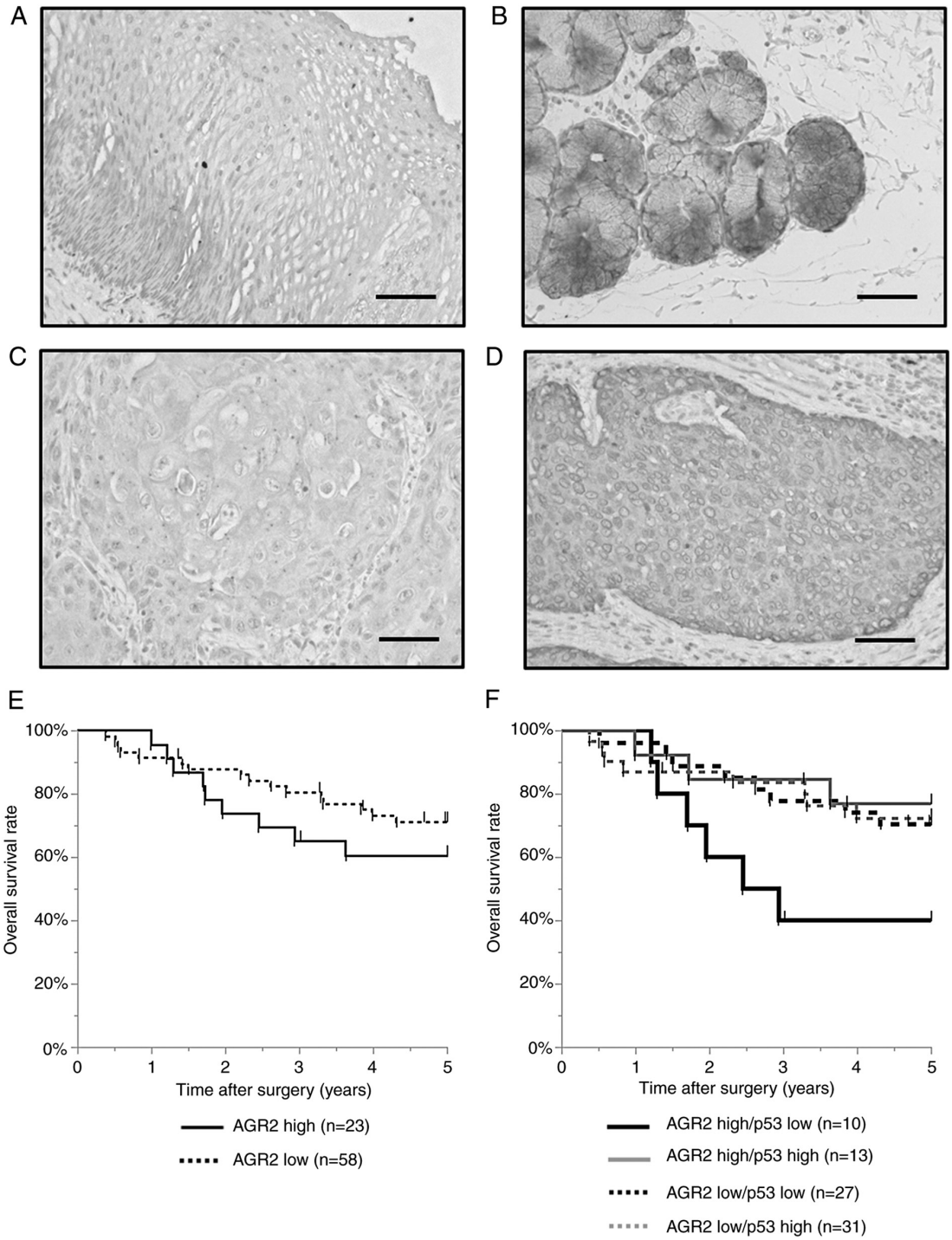

Figure 4. Expression of AGR2 in ESCC cells and survival after curative resection according to AGR2 and/or p53 expression. (A and B) IHC staining of the non-cancerous esophageal epithelium and esophageal gland with the AGR2 antibody. Magnification, x400. Scale bar, 50 μm. (C and D) IHC staining of the primary human ESCC samples with the AGR2 antibody. Magnification, x400. Scale bar, 50 μm. (E) Patients were classified into two groups with high or low tumor expression of AGR2. (F) Patients were classified into four groups with high or low tumor expression of AGR2 and p53. AGR2, anterior gradient 2; ESCC, esophageal squamous carcinoma; IHC, Immunohistochemistry.

adenocarcinoma (35). However, there are no reports of AGR2 functions in ESCC; to the best of the authors' knowledge, the present study is the first report on the molecular mechanism and clinical significance of AGR2 expression in SCC of the esophagus.

Phosphorylation regulation as a protein disulfide isomerase is an important mechanism in the function of AGR2 as an oncogenic factor. Pohler *et al* (19) report that AGR2 attenuates p53 activity, suppressing its phosphorylation even in preneoplastic Barrett's esophageal epithelium. Hrstka *et al* (18) mention that AGR2 upregulates DUSP10 expression, which results in the inhibition of p38 mitogen-activated protein kinase and p53 activation. In addition, Sicari *et al* (36) report that ER stress in cancer cells causes AGR2 migration to the cytoplasm and the inhibition of p53 signaling via the binding of AGR2 to p53 protein.

Table I. Relationship between AGR2 expression and clinicopathological features.

Factors	AGR2 (-) n=58	AGR2 (+) n=23	P-value
Age (years)			0.63
≥65	29	10	
<65	29	13	
Sex			1.00
Female	9	4	
Male	49	19	
T factor[a]			0.11
T3	22	4	
T1/2	36	19	
N Factor[a]			0.78
N2/N3	13	6	
N0/N1	45	17	
Stage[a]			0.80
III, Iva	21	9	
I, II	37	14	
Histopathological type[b]			0.78
Well, moderate	42	15	
Poorly	15	7	
Tumor size (mm)			0.81
≥40	33	12	
<40	25	11	
Lymphatic invasion			0.09
Present	28	16	
Absent	30	7	
Venous invasion			1.00
Present	26	10	
Absent	32	13	
Adjuvant chemotherapy			0.34
Present	28	14	
Absent	30	9	
IHC status of p53			1.00
High	31	13	
Low	27	10	

[a]According to the 8th edition of UICC/TNM staging system; [b]poorly, poorly differentiated squamous cell carcinoma; well, moderate, well or moderately differentiated squamous cell carcinoma; AGR2, anterior gradient 2; IHC, immunohistochemistry.

TP53, one of the most well-known tumor-suppressive genes, controls cell proliferation via the regulation of the cell cycle and apoptosis (21,22). A mutation in *TP53* is reported to reduce the anti-proliferative and apoptotic functions of p53 and occasionally even exert an oncogenic function (37). In the present study, AGR2 downregulation increased the phosphorylation of p53 Ser15 and decreased malignant potential in the *TP53*-wild-type cell lines TE15, TE2 and U2OS, but not in the *TP53*-mutant cell line TE5 because the phosphorylation of p53 is frequently induced without AGR2 expression in p53-mutant cells.

In the present study, prognosis, as determined by AGR2 expression in ESCC tissues, demonstrated no significant differences between groups. However, higher AGR2 expression was correlated with worse prognosis in patients with lower p53 expression. The presence of a *TP53* gene mutation generally induces the accumulation of the p53 protein because the half-life of a mutant p53 protein is extended (38-40). Thus, the level of mutant p53 protein expression can be evaluated by IHC, but wild-type p53 cannot be detected. Therefore, as in the present study, lower expression of p53 based on IHC closely reflected wild-type p53 protein expression. These IHC results are consistent with altered AGR2 regulation in p53-wild-type cell lines. The present study also performed IHC of phosphorylated p53; however, almost all tissues were negative (data not shown). This is due to the fact that the expression level of phosphorylated p53 in p53-wild-type cells was much lower compared with p53-mutant cells, as shown in the present study, indicating that the expression of phosphorylated p53 is unstable and difficult to detect by IHC.

Regarding AGR2 overexpression, Hrstka *et al* (41) report on the AKT-dependent pathway in tamoxifen-resistant cancer. Ondrouskova *et al* (42) mention that HER2 expression upregulates AGR2 expression in a hormone-independent manner in breast cancer. In addition, Pohler *et al* (19) speculate that the activity of the AGR2 pathway in suppressing p53 could be related to the *TP53* mutation status. In the present study, AGR2 overexpression was detected in 33% (3/9) of the cell lines and 28% (23/81) of the tissue samples. These rates were consistent with the results of a previous report showing that AGR2 overexpression could be detected in 36% (15/41) of ESCC samples (35). However, the level of AGR2 expression was not significantly related to that of p53 expression in the ESCC tissue samples (Table I). There might be several mechanisms that upregulate AGR2 expression in p53-wild-type cancer cells to attenuate the active tumor-suppressive functions of p53. In this case, AGR2 inhibition, such as blocking with an antibody targeting this protein (43), might become a potential therapeutic option for *TP53*-wild-type ESCC.

There are some limitations to the present study. The number of patients was small and it was difficult to arrive at a definite conclusion. Therefore, examinations based on more samples, particularly for patients with SCC, are needed. No definitive *TP53* mutation status in cell lines and tissues was confirmed in the present study. However, the results obtained from the cell lines are similar to previous findings (27). The frequency of the *TP53* mutation in the ESCC tissues was reported to be in the range of 40 to 80% (27,44). These results are consistent with the data from the present study showing that the high p53 expression group comprised 54% (44/81) of cases, although some functionally suppressed p53 mutants might be mixed in with the low-p53 expression group and it remains unclear whether p53 activity in p53-wild-type cells is a clinically effective marker for cancer progression.

In conclusion, AGR2 serves an important role in the progression of *TP53*-wild-type ESCC by inhibiting the phosphorylation of p53. AGR2 might be a useful prognostic marker and potential therapeutic target in patients with *TP53*-wild-type ESCC.

Table II. Survival analysis of patients with low p53 expression.

Factors	n	5-year OS (%)	Univariate P-value	Multivariate HR	Multivariate 95% CI	Multivariate P-value
Age (years)			0.82			
≥65	16	62.5				
<65	21	61.9				
Sex			1.00			
Female	5	60.0				
Male	17	62.2				
T factor[a]			0.69			
T3	13	69.2				
T1/2	24	57.7				
N Factor[a]			**0.04**	1.98	0.63-5.92	0.23
N2/N3	10	40.0				
N0/N1	27	70.1				
Stage[a]			0.19			
III, IVa	12	50.0				
I, II	25	67.7				
Histopathological type[b]			0.86			
Well, moderate	26	61.3				
Poorly	11	63.6				
Tumor size (mm)			**0.03**	3.82	1.17-14.83	**0.03**
≥40	18	50.0				
<40	19	78.6				
Lymphatic invasion			0.75			
Present	22	63.0				
Absent	15	60.0				
Venous invasion			0.36			
Present	18	55.0				
Absent	19	68.4				
Adjuvant chemotherapy			0.64			
Present	21	66.6				
Absent	16	55.5				
IHC status of AGR2			**0.047**	3.66	1.12-11.56	**0.03**
High	10	40.0				
Low	27	70.3				

Bold indicates P<0.05. [a]According to the 8th edition of UICC/TNM staging system; [b]poorly, poorly differentiated squamous cell carcinoma; well, moderate, well or moderately differentiated squamous cell carcinoma. AGR2, anterior gradient 2; CI, confidential interval; HR, hazard ratio; IHC, immunohistochemistry; OS, overall survival.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors conceived and planned the experiments. KT carried out the experiments. KT and HK confirm the authenticity of all the raw data. KT, HK, TA and KS performed data analysis. KT, HK, TA, SK, JS, HF, WT, KS, HS, YY, SK and AS contributed to the interpretation of the results. OK and OE assisted in research planning based on the results obtained. KT and HK wrote the first draft of the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participant

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The present study was approved by the Faculty of Science Ethics Committee of Kyoto Prefectural University of Medicine. Informed consent was obtained from all individual participants included in the study (approval no. ERB-C-1414-1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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