due to Cks1 not being expressed in meiotic cells, leaving them completely deficient for CKS protein expression (7).

CKS proteins are essential for the G2- to M-phase cell cycle transition, as they are vital for efficient transcription of a number of crucial cell cycle control genes (8). Cell cycle phase transitions are regulated by cyclin-dependent protein kinases (cdks) and their cyclin binding partners.

Cell cycle regulatory failure is associated with the development or the progression of cancer (9,10). In addition, CKS proteins have been found frequently overexpressed in a broad spectrum of other human malignancies (11-14). However, the precise molecular rules of CKS proteins remain largely unknown in this context. In addition, the mechanistic link between CKS protein overexpression and oncogenesis remains unknown. To elucidate the roles of CKS2 proteins and to determine whether CKS2 expression may be a prognostic marker in ESCC, we investigated CKS2 expression in 121 patients and analyzed the significance.

Materials and methods

Esophageal cancer cell lines. The human esophageal cancer cell line KYSE70 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). It was maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% humidified CO_2 atmosphere.

Tissue sampling. In order to verify the presence of CKS2 proteins in esophageal cancer tissues, we initiated immunohistochemical studies of tissue microarray from surgical specimens from 121 patients with ESSC (109 men and 12 women). These patients had undergone esophagectomy with lymph node dissection between 1987 and 1998 at Kagoshima University Hospital, Japan. The median age of the patients was 65 years (range, 47-87 years). Postoperative follow-up data were obtained from all patients, with a median follow-up period of 24 months (range, 1-181 months).

To verify the presence of *CKS2* mRNA in esophageal cancer tissues, we conducted studies of surgical specimens from 62 esophageal cancer patients (58 men and 4 women) who underwent esophagectomy with lymph node dissection between 1998 and 2005 at Kagoshima University Hospital and the Medical Institute of Bioregulation Hospital, Kyushu University, Japan. The median age of the patients was 65 years (range, 47-87 years). Postoperative follow-up data were obtained from all patients, with a median follow-up period of 17 months (range, 1-75 months).

All samples of tissues were collected from patients after informed consent had been obtained in accordance with the institutional guidelines of our hospital. Using the tumor node metastasis classification of the International Union Against Cancer (15), all of the M1 tumors exhibited distant lymph node metastases.

Immunohistochemical study of CKS2 expression. The tissue microarray was cut into 3-micron-thick sections, that were mounted on glass slides. Immunohistochemical staining was carried out using the avidin-biotin-peroxidase complex method (Vectastatin Elite ABC kit; Vector, Burlingame, CA, USA), following the manufacturer's instructions. Briefly, the immunostaining was performed manually at room temperature. The sections were deparaffinized in xylene and dehydrated in ethanol; endogenous peroxidase activity was blocked by incubating sections for 10 min in 3% hydrogen peroxide in methanol. Then, the sections were autoclaved in citrate buffer (0.01 mol/l, pH 6.5) at 121°C for 15 min to reveal the antigen. After cooling, the sections were pre-incubated in 1% BSA for 20 min. Next, sections were incubated with anti-CKS2 mouse monoclonal antibody (1:50, CKS2; LifeSpan Biosciences, Inc., Seattle, WA, USA) for 60 min. After rinsing with phosphate-buffered saline (PBS) for 15 min, the sections were incubated with secondary antibody for 20 min and washed again. After washing with PBS for 10 min, sections were incubated with avidin-biotin complex for 30 min and washed again, and reactions were visualized using diaminobenzidine tetrahydrochloride for 2 min. All samples were lightly counterstained with hematoxylin for 1 min. No antigen retrieval was performed. Positive and negative controls were used for each section.

Evaluation of immunohistochemistry was independently performed by two investigators. Positive expression of CKS2 was defined as detectable immunoreaction in nuclear regions of >20% of the cancer cells. To evaluate expression of CKS2, 10 fields (within the tumor and at the invasive front) were selected and expression in 1,000 tumor cells (100 cells/field) was evaluated using high-power (x200) microscopy.

The negative controls consisted of sections treated with the same protocol but with PBS instead of the primary antibody. The human esophageal cancer cell line KYSE150 was used as a positive control.

Quantitative reverse transcription-PCR. Sixty-two paired malignant and normal specimens of esophageal mucosa were homogenized and total RNA was extracted according to the manufacturer's instructions (16). Complementary DNA (cDNA) was immediately synthesized from the extracted RNA. The oligonucleotide primers for *CKS2* were: sense primer, 5'-TGTCTGAAGAGGAGTGGAGGA-3' and antisense primer, 5'-CATGCACAGGTATGGATGAAA-3'. The length of the amplified fragment was 241 bp. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. The primers for GAPDH were: sense primer, 5'-TTGGTATCGTGGAAGGACTCA-3' and antisense primer, 5'-TGTCATCATATTTGGCAGGTT-3'. The lengths of the amplicons were 249 bp. Real-time monitoring of PCR reactions was carried out using the LightCycler System (Roche Applied Science, Indianapolis, IN, USA) and SYBR-Green I dye (Roche Diagnostics) (17). Monitoring was performed according to the manufacturer's instructions.

We determined the levels of *CKS2* and *GAPDH* mRNA expression by comparing results to cDNA from the human esophageal cancer cell line KYSE150. After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was distinguished from the baseline, and that cycle number (threshold cycle) was used as a crossing-point value. The standard curve was produced by measuring the crossing-point of each standard value (4-fold serially diluted cDNAs of KYSE150) and

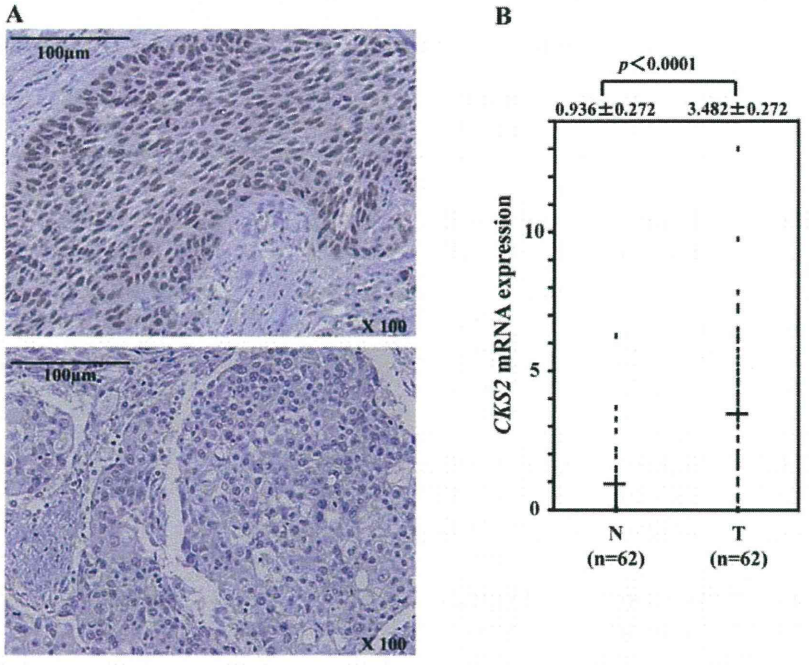

Figure 1. The expression of CKS2 protein and *CKS2* mRNA in ESCC. (A) Immunohistochemical identification of CKS2 protein in ESCC at x100 original magnification. The upper image represents a CKS2 high expression case. Most positively stained cells were associated with the tumor and the expression of CKS2 protein was restricted to the nuclei. The lower image shows a low expression case. (B) *CKS2* mRNA expression in ESCC patients as assessed by real-time quantitative PCR (n=62). Horizontal lines indicate the mean value of each group (N, non-cancerous tissue; T, cancerous tissue). CKS2, cyclin-dependent kinase subunit 2; ESCC, esophageal squamous cell carcinoma.

plotting them against the logarithmic value of the concentrations. Standard curve samples were included in each run. The concentrations of all samples were then calculated by plotting their crossing-points against the standard curve.

Calculated concentrations of all samples were relative to the concentration of the cDNA of KYSE150, and the amount of the target molecule was then divided by the amount of the endogenous reference (*GAPDH*) to obtain normalized CKS2 expression values (17). Each assay was performed three times to verify the results, and the mean mRNA expression value was used for subsequent analysis.

CKS2 RNA interference. CKS2-specific siRNA (Stealth™ siRNA duplex oligoribonucleotides) and negative control RNAi (Stealth™ Negative Control siRNA duplex oligoribonucleotides) were purchased from Invitrogen. Logarithmically growing cells (KYSE70) were seeded at either $1.0x10^5$ or $2.0x10^3$ cells/well in a final volume of 2 ml or 100 μl in 6- or 96-well flat bottom microplates, respectively. The cells were cultured overnight for adherence. RNAi oligomer was diluted with OPTI-MEM I reduced serum medium (Invitrogen Corp.) and incubated for 5 min at room temperature. The diluted RNAi oligomer was mixed with diluted Lipofectamine™ RNAiMAX (Invitrogen Corp.). The RNAi-Lipofectamine™ RNAiMAX complexes were added to each well at a final concentration of 30 pmol/ml. The cells were incubated for 5 h followed by replacement of the media. The assays were performed after 48 h incubation.

In vitro proliferation assays. Proliferation was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics Corp., GmbH). Logarithmically growing cells were seeded at $5.0x10^3$ cells/well in flat-bottomed 96-well microtiter plates in a final volume of 100 μl culture medium/well, and incubated in a humidified atmosphere (37°C and 5% CO_2). MTT labeling reagent (10 μl at a final concentration 0.5 mg/ml) was then added to each well. The microtiter plate was incubated for 4 h in a humidified atmosphere, after which solubilization solution (100 μl) was added to each well. The plate was then incubated overnight in a humidified atmosphere. Once complete solubilization of the purple formazan crystals was confirmed, the absorbance of the samples was measured using a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA), at a wavelength of 570 nm corrected to 655 nm. Each independent experiment was performed in triplicate.

Statistical analysis. The statistical analysis of group differences was performed using the χ^2 test, the Student's t-test and the repeated-ANOVA test. Overall survival curves were plotted according to the Kaplan-Meier method, with the Wilcoxon test applied for comparisons and $p<0.05$ was considered statistically significant. Variables with a value of $p<0.05$ by univariate analysis were used in subsequent multivariate analyses based on Cox's proportional hazards model. All statistical analyses were performed using JMP™ for Windows (version 5.0.1; SAS Institute Inc., Cary, NC, USA).

Results

Clinicopathologic significance of the expression of CKS2 protein and CKS2 mRNA in ESCC. CKS2 protein

Table I. Relationship between CKS2 mRNA, CKS2 protein and clinicopathological factors.

Factors	Total	CKS2 protein expression Negative (n=69) no. (%)	Positive (n=52) no. (%)	P-value	Total	CKS2 mRNA expression Negative (n=31) no. (%)	Positive (n=31) no. (%)	P-value
Gender								
Male	109	61 (56.0)	48 (44.0)	0.472	58	28 (48.3)	30 (51.7)	0.291
Female	12	8 (66.7)	4 (33.3)		4	3 (75.0)	1 (25.0)	
Tumor location								
Upper	18	10 (55.6)	8 (44.4)	0.957	8	4 (50.0)	4 (50.0)	0.593
Middle	57	32 (56.1)	25 (43.9)		32	12 (37.5)	20 (62.5)	
Lower	46	27 (58.7)	19 (41.3)		22	10 (45.6)	12 (54.4)	
Histology								
Well	51	26 (51.0)	25 (49.0)	0.345	14	8 (57.1)	6 (42.9)	0.593
Moderate	53	31 (58.5)	22 (41.5)		34	15 (44.1)	19 (55.9)	
Poor	17	12 (70.6)	5 (29.4)		14	8 (57.1)	6 (42.9)	
pT								
T1,2	48	33 (68.8)	15 (31.2)	**0.033**	32	20 (62.5)	12 (37.5)	**0.041**
T3,4	73	36 (49.3)	37 (50.7)		30	11 (36.7)	19 (63.3)	
pN								
N0	52	33 (63.5)	19 (36.50)	0.213	18	12.0 (66.7)	6 (33.30)	0.091
N1	69	36 (52.2)	33 (47.80)		44	19.0 (43.2)	25 (56.80)	
pM								
M0	87	56 (64.4)	31 (35.60)	**0.009**	59	29.0 (49.2)	30 (50.80)	0.091
M1	34	13 (38.2)	21 (61.80)		3	2.0 (66.7)	1 (33.30)	
p-Stage								
I, II	58	39 (67.2)	19 (32.80)	**0.028**	28	17.0 (60.7)	11 (39.30)	0.125
III, IV	63	30 (47.6)	33 (52.40)		34	14.0 (41.2)	20 (58.80)	
Lymphatic invasion								
Negative	45	31 (68.9)	14 (31.1)	**0.041**	19	14 (73.7)	5 (26.3)	**0.012**
Positive	76	38 (50)	38 (50)		43	17 (39.5)	26 (60.5)	
Vascular invasion								
Negative	92	53 (57.6)	39 (42.4)	0.817	16	10 (62.5)	6 (37.5)	0.240
Positive	29	16 (55.2)	13 (44.8)		46	21 (45.7)	25 (54.3)	

CKS2, cyclin-dependent kinase subunit 2.

was expressed and distributed in the nuclei of cancer cells (Fig. 1A). Positive expression of CKS2 protein was observed in 52 patients (43.0%). CKS2 protein expression was significantly associated with the following clinicopathologic parameters: depth of tumor invasion (category T of TNM classification), distant lymph node metastasis, stage and lymphatic invasion (Table I).

In the mRNA analysis, 58/62 patients (93.5%) showed higher expression levels of *CKS2* mRNA in cancerous tissues than in non-cancerous tissues by real-time quantitative reverse transcription-PCR. The mean expression level of *CKS2* mRNA in tumor tissues was 3.482±0.272 (means ± SD), which was significantly higher than the value obtained from the corresponding normal tissues (0.936±0.272, $p<0.0001$) (Fig. 1B). The patients with values below the median expression level in tumor tissues were assigned to the low expression group (n=31), whereas those with values above the median were assigned to the high expression group (n=31). *CKS2* mRNA expression was significantly associated with the depth of tumor invasion (category T of TNM classification) and the incidence of lymphatic invasion (Table I).

Relationships between CKS2 protein expression, mRNA expression and prognosis. The 5-year overall survival rates of patients with high *CKS2* mRNA levels and those with low CKS2 mRNA levels were 42.5 and 53.9%, respectively. The CKS2 high expression group tended to have a poorer prognosis than the low expression group; however, the survival difference between these two groups was not statistically significant ($p=0.550$) (Fig. 2A). The 5-year overall survival

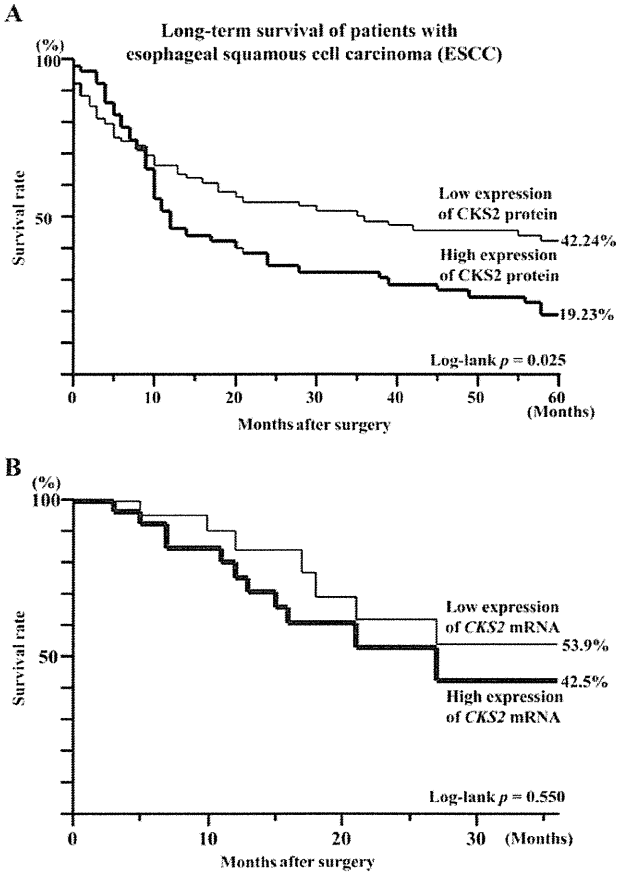

Figure 2. Long-term survival of patients with ESCC. (A) Survival of patient groups with high and low levels of CKS2 protein expression in cancerous tissue. Patients in the high CKS2 protein group had a significantly poorer prognosis than those in the low CKS2 expression group (p=0.025). (B) Survival of patient groups with high and low levels of *CKS2* mRNA expression in malignant tissue. Patients in the *CKS2* mRNA expression group tended to have poorer prognosis than those in the low *CKS2* mRNA expression group. The survival difference between these two groups was not statistically significant (p=0.550). ESCC, esophageal squamous cell carcinoma; CKS2, cyclin-dependent kinase subunit 2.

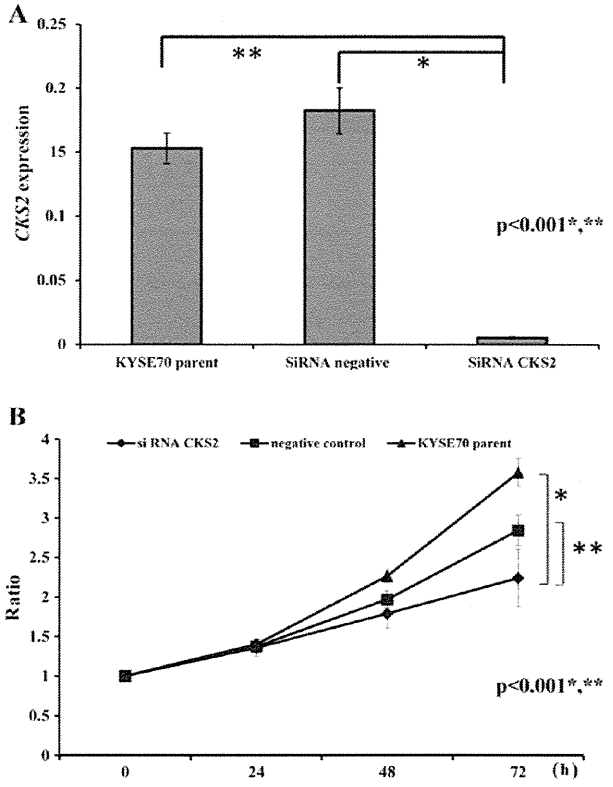

Figure 3. (A) CKS2 expression is suppressed by CKS2 specific-siRNA in KYSE170 cells. Forty-eight hours after siRNA addition, CKS2 expression was measured by real-time quantitative PCR. (B) Effect of CKS2 suppression on KYSE170 cell proliferation as assessed by the MTT assay. CKS2-suppressed cells were less proliferative than control cells (p<0.01). CKS2, cyclin-dependent kinase subunit 2.

rates of patients with high CKS2 protein levels and those with low CKS2 protein levels were 19.23 and 42.24%, respectively. The 5-year survival rate was significantly lower in patients with positive-CKS2 protein expression than in those with negative-CKS2 protein expression (p=0.035; Fig. 2B).

Univariate and multivariate analyses of survival. Table II shows univariate and multivariate analyses of factors related to patient prognosis according to CKS2 protein expression. Multivariate regression analysis indicated that the depth of invasion and lymph node metastasis were independent prognostic factors, but that lymphatic invasion and CKS2 protein expression were not independent prognostic factors.

Effect of CKS2 gene silencing. KYSE70 cells normally express mRNA at a high level. Suppression of *CKS2* mRNA was confirmed by real-time quantitative PCR (Fig. 3A), with subsequent reduction in the proliferation rate of KYSE70 cells (p<0.001) (Fig. 3B).

Discussion

We previously analyzed genes related to lymph node metastasis in ESCC. Using laser microdissection techniques and cDNA and oligo nucleotide microarray, several genes were identified simultaneously in comparisons of lymph node-positive and -negative primary tumors or primary tumors and lymph node metastasis. CKS2 was among the genes of interest that were identified (18,19). In the present study, CKS2 expression was associated with lymphatic invasion and distant metastasis. This finding is consistent with our previous findings as a category of distant metastasis contains distant lymph node metastasis which arises frequently in ESCC (15). Moreover, in gastric cancer, we previously showed that CKS2 mRNA expression was associated with lymph node metastasis (20). Li *et al* reported that CKS2 was overexpressed in cancer tissues compared to non-cancer tissues of the colon. A similar observation was made in cancers with liver metastasis compared to those without liver metastasis by means of genome-wide cDNA microarray (21). This result suggests that CKS2 also plays an important role in lymph node metastasis as well as hematogenous metastasis. In addition, the expression of CKS2 (both mRNA and protein) correlated with the depth of tumor invasion. Kawakami *et al* and Chen *et al* demonstrated that bladder cancer of the invasive type has higher expression of

Table II. Univariate and multivariate analysis of clinicopathological factors affecting overall survival rate.

Variables	N	5-year survival rate (%)	Univariate analysis P-value	Multivariate analysis Relative risk (CI)	Multivariate analysis P-value
Tumor depth					
pT1,2	48	50.3	**<0.0001**	1.680	**0.0001**
pT3,4	73	18.4		(1.297-2.224)	
p-Stage					
I, II	48	58.8	**<0.0001**	0.876	0.238
III, IV	73	7.9		(0.702-1.092)	
Lymph node metastasis					
Negative	52	55.4	**<0.0001**	1.639	**0.0001**
Positive	69	14.0		(1.274-2.139)	
Lymphatic invasion					
Negative	45	47.6	**<0.0001**		
Positive	76	22.9			
Venous invasion					
Negative	92	39.1	**0.002**	1.168	0.217
Positive	29	10.3		(0.910-1.483)	
Distant metastasis					
Negative	87	42.5	**<0.0001**		
Positive	34	23.5			
CKS2 protein expression					
Negative	69	42.4	**0.025**	1.146	0.228
Positive	52	19.2		(0.918-1.431)	

N, number of patients; CI, confidence interval; CKS2, cyclin-dependent kinase subunit 2.

CKS2 than that of the superficial type (22,23). The previous reports are in line with our recent study.

Our previous study of gastric cancer revealed that CKS2 mRNA expression was an independent prognostic factor (20). In the present study of ESCC, CKS2 protein expression correlated well with depth of invasion or lymph node metastasis and was predicted to be a prognostic factor, although it was not independent in multivariate analysis. However, this is the first study reporting a correlation between CKS2 expression and prognosis in a large scale study of ESCC. Further study of CKS2 expression is required to better understand the clinical and prognostic significance in ESCC.

In the patients studied here, *CKS2* mRNA was overexpressed in ESCC tissues compared to normal esophageal tissues. Similar results have been reported in the analyses of esophagus (24), colon (21), cervical cancers (12), malignant melanomas (25) and human gliomas (26,27). Moreover, we analyzed both the expression of mRNA and protein. While the trend of expression of mRNA was similar to that of protein, differences were apparent. We hypothesized that the reason for this difference is post transcriptional regulation including epigenetic changes such as DNA methylation and histone modification (28). For instance, microRNAs function throughout post-transcriptional gene silencing by modulating the translation of mRNAs into proteins (29) and there are recent reports on epigenetic regulation by non-coding RNAs (30). Indeed, Lv *et al* reported that miR26a targets and represses CKS2 expression in papillary thyroid carcinoma (31).

The suppression of CKS2 expression by siRNA reduced cellular proliferation (Fig. 3). Several reports have demonstrated that the inhibition of CKS2 decreases cell proliferation and increases caspase 3 and Bax expression at the protein level concerning apoptosis (13,20,26). In ESCC, CKS2 may play an important role by inhibiting apoptosis as observed in other cancers. Liberal *et al* showed that overexpression of CKS2 in malignancy overrode the intra-S-phase checkpoint that blocks aberrant DNA replication in response to stress (32). These results may explain why inhibition of CKS2 increased the proliferation of cancer cells in our *in vitro* study.

In conclusion, we demonstrated that the expression of CKS2 in ESCC was elevated relative to levels in normal tissue, and that CKS2 overexpression is associated with the depth of tumor invasion, lymphatic invasion, clinical stage, distant metastasis and poor prognosis. Therefore, the expression profile of CKS2 may contribute to the creation of a new clinical classification predicting an aggressive tumor phenotype. The evaluation of CKS2 expression may be useful for predicting the malignant properties and prognosis of patients with ESSC.

Acknowledgements

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

Nrf2 is Useful for Predicting the Effect of Chemoradiation Therapy on Esophageal Squamous Cell Carcinoma

Yota Kawasaki, MD, Hiroshi Okumura, MD, Yasuto Uchikado, MD, Yoshiaki Kita, MD, Ken Sasaki, MD, Tetsuhiro Owaki, MD, Sumiya Ishigami, MD, and Shoji Natsugoe, MD

Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, Kagoshima University, Kagoshima, Japan

ABSTRACT

Background. The transcription factor NF-E2-related factor 2 (Nrf2) was originally identified to be a critical regulator of intracellular antioxidants and phase II detoxification enzymes. Recent studies have shown that high Nrf2 expression gives cancer cells an advantage for survival from anticancer chemotherapy and radiation therapy. The aims of this retrospective study were to examine the expression of Nrf2 in biopsy specimens of esophageal squamous cell carcinoma (ESCC) and to evaluate whether such expression is useful for predicting the response to chemoradiation therapy (CRT).

Methods. A total of 46 patients with ESCC who received curative surgery after CRT from 1997 to 2011 were enrolled in the current study. Nrf2 expression in the biopsy specimens before CRT was examined immunohistochemically using anti-Nrf2 antibody. The correlations between Nrf2 expression and clinical factors and histological and clinical response to CRT were analyzed.

Results. The rate of Nrf2-positive expression was 39 %. Both clinically and histologically, significant correlations were found between positive Nrf2 expression and unfavorable response to CRT. Furthermore, Nrf2 was significantly correlated with clinical lymph node metastases and patients' postoperative outcomes. Multivariate analysis showed that Nrf2 expression status was an independent prognostic factor.

Conclusions. Nrf2 expression was found to be closely related to the effect of CRT and could predict the CRT outcome in patients with ESCC.

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive tumors of the gastrointestinal tract. Since postoperative relapse often occurs even when patients with ESCC undergo curative resection, the prognosis of patients with ESCC remains poor.[1] Various types of aggressive therapy, such as extended lymphadenectomy, radiotherapy, and chemotherapy, are being used to improve patients' prognosis.[1–3] Chemoradiation therapy (CRT) for ESCC started in the late 1960s using bleomycin. In the 1980s CRT using cisplatin started, and it is currently considered to be one of the most useful treatments.[3] The most important fact is that ESCC patients who respond to CRT survive longer than any other patients; therefore, it would be useful to preselect responders.[4]

Oxidative stress has been shown to play important roles in the carcinogenesis and progression of many cancers, including ESCC.[5] The transcription factor NF-E2-related factor 2 (Nrf2), a basic redox-sensitive bZIP transcription factor, was originally identified to be a critical regulator of intracellular antioxidants and phase II detoxification enzymes by the transcriptional upregulation of many antioxidant response element (ARE)-containing genes.[4–6] Under basal conditions, Nrf2 is bound to the Kelch-like ECH-associated protein 1(Keap1), which is a cul3-based E3 ubiquitin ligase adapter that regulates Nrf2 ubiquitination and proteasome-dependent degradation.[7] On exposure of cells to oxidative stress or chemopreventive compounds, Nrf2 translocates to the nucleus, forms a heterodimer with its obligatory partner Maf, binds to the ARE DNA

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H. Okumura, MD
e-mail: hokumura@m.kufm.kagoshima-u.ac.jp

sequences, and activates the transcription of downstream genes, such as antioxidants and phase II detoxification enzymes.[4,8–10] Therefore, Nrf2 has been viewed as a good transcription factor that is essential in protecting us from oxidative stress-related disease and substances and therapies that produce reactive oxygen species.[11] Indeed, accumulating evidence has been provided recently indicating that Nrf2 has a protective role of against many human pathologic conditions.[12,13] However, new emerging data have revealed the dark side of Nrf2.[14] Recently, it has been revealed that aberrant activation of the Nrf2 pathway occurs frequently in cancer cells. Nrf2 protects not only normal cells, but also cancer cells from cellular stress and enhances cancer cell survival.[15] Many reports have shown that high Nrf2 expression gives cancer cells an advantage for survival from anticancer chemotherapy and radiation therapy.[13,14,16–23]

The aims of this retrospective study were to examine the expression of Nrf2 in biopsy specimens of ESCC and to evaluate whether such expression is useful for predicting the response to CRT.

MATERIALS AND METHODS

Study Groups

There were 46 patients diagnosed with ESCC (45 males and 1 female) who underwent CRT between 1997 and 2011 at Kagoshima University Hospital, Kagoshima, Japan. The median age of the patients was 61.1 years (range, 43–74 years).

They underwent CRT followed by esophagectomy with lymph node dissection 4–6 weeks after completing CRT. After all patients gave their informed consent, biopsy specimens of the primary tumors were endoscopically collected. Clinical factors were assessed by the International Union against Cancer tumor-node-metastasis (TNM) classification system.[24] According to this classification, 2 patients had cT1 tumors, 1 patient had cT2 tumor, 33 patients had cT3 tumors, and 10 patients had cT4 tumors (Supplemental Table 1). The cT4 tumors in this study were resectable tumors that invaded to lung, pleura, or the recurrent nerve. Follow-up data after surgery were available for all patients with a median follow-up period of 33 months (range, 3–136 months).

The study was approved by the Institutional Review Board of Kagoshima University and performed according to the Helsinki Declaration.

Chemoradiation Therapy

A total radiation dose of 40 Gy was applied; 2-Gy fractions were delivered 5 days per week for 4 weeks, to the mediastinum and neck. In the same period, chemotherapy was performed intravenously using two anticancer agents: cisplatin (7 mg/m^2 over 2 h) and 5-FU (350 mg/m^2 over 24 h). Basically, areas supraclavicular to lower mediastinal LN and cardiac LN areas were irradiated as a long T-shaped field for upper-to-lower thoracic tumors, and perigastric LN areas were additionally irradiated for lower tumors. The clinical response to CRT was evaluated by the findings of esophagography, esophagoscopy, endoscopic ultrasonography, and computed tomography.

The clinical criteria for the response to CRT against the primary ESCC site were evaluated by endoscopic examination.[25] The criteria were as follows.[26,27] A complete response (CR) was defined as disappearance of tumor lesion, disappearance of ulceration, and absence of cancer cells in biopsy specimens. Existence of erosion, a granular protruded lesion, ulcer scar, and a Lugol-voiding lesion did not prevent a CR evaluation. Progressive disease (PD) was defined as obvious enlargement of the tumor lesion or progression of esophageal stenosis by tumor enlargement. Incomplete response/stable disease (IR/SD) was defined as not satisfying CR criteria without obvious enlargement of the tumor lesion.

The histological criteria for the response to CRT were: grade 0, neither necrosis nor cellular or structural changes can be seen throughout the lesion; grade 1, necrosis or disappearance of the tumor is present in no more than 2/3 of the whole lesion; grade 2, necrosis or disappearance of the tumor is present in more than 2/3 of the whole lesion, but viable tumor cells still remain; and grade 3, the whole lesion falls into necrosis and/or is replaced by fibrosis, with or without granulomatous changes, and no viable tumor cells are observed.[26,27] In patients whose histological response was grade 2 or 3, the CRT was considered effective. On the other hand, in patients whose histological response was grade 1, the CRT was considered ineffective.

Immunohistochemical Staining and Evaluation of Nrf2 in ESCC

Paraffin-embedded sections (4 μm), including tumor, were deparaffinized and soaked in PBS prior to immunohistochemical analysis. Sections were treated with 3 % H_2O_2 for 10 min in order to block endogenous tissue peroxidase. For staining with Nrf2 antibodies, sections were pretreated with citrate buffer for 10 min at 121 °C in a microwave oven. The sections were washed with PBS and then blocked by treatment with PBS containing 3 % skim milk. The blocked sections were incubated with the diluted primary antibody: Nrf2 (sc-365949, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), with PBS at 4 °C overnight, followed by staining with a streptavidin–biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were washed in PBS, and the immune complex was visualized by incubating the sections with diaminobenzidine tetrahydrochloride. They were rinsed briefly in

water, counterstained with hematoxylin, and mounted. Nrf2 expression was determined by counting the number of cancer cells in which the nucleus was stained with the anti-Nrf2 antibody. Normal human placenta tissue was used as positive control of Nrf2, and the primary antibody was replaced with PBS for negative control. Evaluation of immunohistochemistry was independently carried out by 2 investigators (Y.K. and H.O.). To evaluate this, 10 fields within the tumor were selected, and expression in 1,000 cancer cells (100 cells per field) was evaluated using high-power (200×) microscopy. The average Nrf2 labeling index was assessed according to the proportion of positive cells in each field. Nrf2 expression was assessed using the proportion of positive cells and intensity. Nuclear Nrf2 expressions were quantified using a 3-value intensity score (0, 1+, or 2+) and the percentage (0–100 %) of the extent of reactivity. An immunohistochemical score was obtained by multiplying the intensity and reactivity extent values (range, 0–200), and these expression scores were used to determine expression levels. Positive nuclear Nrf2 expression was defined as a score >50, which represents the median expression for gastric cancer evaluated using whole tissue sections. The evaluation method used was an improved version of the method of Solis et al.[28]

Statistical Analysis

Statistical analysis of group differences was performed using the χ^2 test or the Mann–Whitney *U* test. The Kaplan–Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards regression model). A p value <0.05 was considered to indicate significance. All statistical analyses were performed using the StatFlex version 6.0 for Windows software (StatFlex version 6.0; Artec Inc., Osaka, Japan).

RESULTS

Expression of Nrf2 in ESCC

Immunohistochemically, in human ESCC, Nrf2 expression was identified mainly in cellular nuclei. According to the immunohistochemical evaluation, 18 of 46 patients (39.1 %) were placed in the Nrf2-positive expression group (Fig. 1).

Relationship Between Nrf2 Expression and Clinicopathological Findings

About the correlations between Nrf2 expression and clinicopathological characteristics, there was a significant correlation between the Nrf2-positive group and clinical lymph node metastases ($p = 0.006$), while no significant differences were observed regarding age, sex, histology, tumor depth, and clinical stage (Supplemental Table 2).

Relationship Between Nrf2 Expression and Clinical Response to CRT

In the Nrf2-negative and Nrf2-positive groups, the clinical response was CR in 11 and 2 cases, respectively, IR/SD in 17 and 15 cases, respectively, and PD in 0 and 1 cases, respectively. There was a significant difference in the clinical effect of CRT between the Nrf2-negative and Nrf2-positive groups ($p = 0.02$, Table 1).

Relationship Between Nrf2 Expression and Histological Response to CRT

In the Nrf2-negative and Nrf2-positive groups, there were 9 and 12 grade 1 cases, respectively, and 19 and 6 grade 2 and 3 cases, respectively. There was a significant difference in the histological effect of CRT between the Nrf2-negative and Nrf2-positive groups ($p = 0.02$, Table 2). There is significant correlation between clinical and pathological response of the CRT ($p < 0.01$).

Clinical Outcomes According to Nrf2 Expression or CRT Response

In analyzing clinical outcomes according to Nrf2 and pathological response to CRT in 46 patients who underwent surgery, the 5-year survival rates were 65.4 % in the Nrf2-negative group and 23.2 % in the Nrf2-positive group ($p = 0.0037$, Fig. 2). On univariate regression analyses, clinical lymph node metastasis (cN) and Nrf2 expression significantly affected postoperative outcome. On multivariate analysis, Nrf2 expression was a significant prognostic factor (Table 3).

DISCUSSION

Cancer cell apoptosis is believed to occur as a result of the antitumor efficacy of chemotherapy and radiotherapy that induces reactive oxygen species (ROS) within cancer cells.[21,29,30] Genetic or functional inhibition of Nrf2 results in repressed cellular Nrf2-regulated antioxidant enzymes, including cellular glutathione, thioredoxin, and nonprotein thiols. Finally, these alterations can restore the sensitivity of human cancer cells to anticancer chemotherapy and radiation therapy.[11,14,20,23,28,31] These studies revealed that the antioxidant system plays an important role in the development of resistance to chemotherapy and radiation therapy. In fact, Cho et al.[23] reported that functional

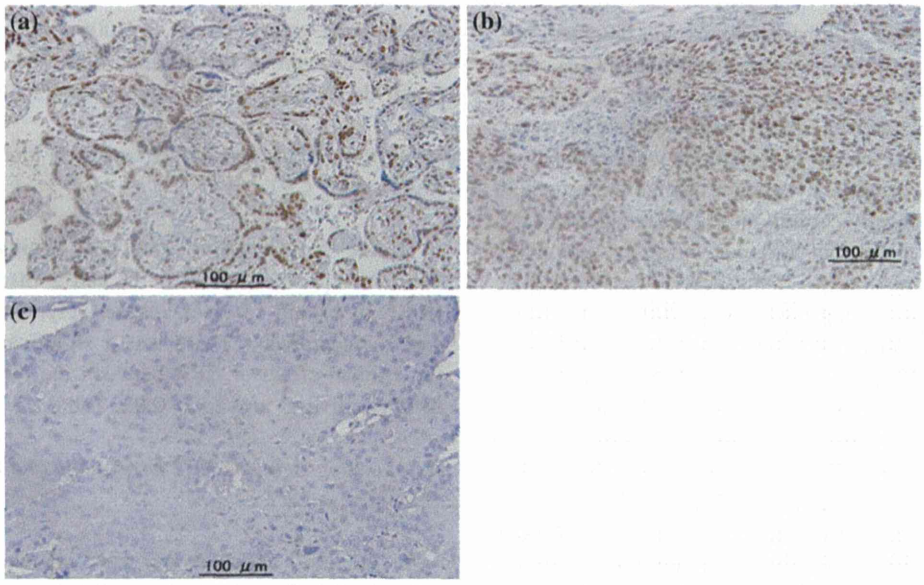

FIG. 1 Expression of Nrf2 in clinical samples. Immunostaining of Nrf2 (original magnification, ×400). **a** Example of noncancerous placental tissue as a positive control. **b** Nrf2-positive ESCC. **c** Nrf2-negative ESCC. Positive staining is detected in the cell nucleus

TABLE 1 Correlation between Nrf2 expression and clinical response to CRT

	Clinical response to CRT ($n = 46$)			Total	p value
	CR	IR/SD	PD		
Nrf2 (−)	11	17	0	28	0.02
Nrf2 (+)	2	15	1	18	

CR complete response, *PD* progressive disease, *IR/SD* incomplete response/stable disease

TABLE 2 Correlation between Nrf2 expression and histological response to CRT

	Histological response to CRT ($n = 46$)			p value
	Grade 1	Grade 2 and 3	Total	
Nrf2 (−)	9	19	28	0.02
Nrf2 (+)	12	6	18	

Grade 1 necrosis or disappearance of the tumor is present in no more than 2/3 of the whole lesion, *Grade 2* necrosis or disappearance of the tumor is present in more than 2/3 of the whole lesion, but viable tumor cells are still remaining, *Grade 3* the whole lesion falls into necrosis and/or is replaced by fibrosis, with or without granulomatous changes. No viable tumor cells are observed

inhibition of Nrf2 leads to sensitization of cancer cells to alkylating anticancer agents. Also, Ma et al.[20] reported that not only cisplatin treatment combined with Nrf2 knockdown, but also Nrf2 knockdown alone inhibited tumor growth significantly in vivo. Therefore, it was suggested that Nrf2, which is a critical regulator of intracellular

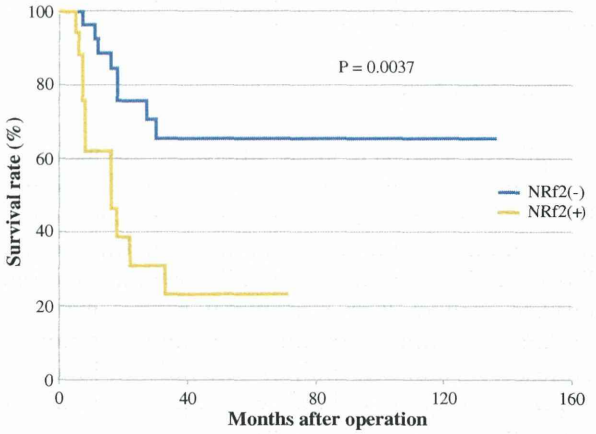

FIG. 2 Cause-specific survival curves for ESCC patients treated by CRT and surgery according to Nrf2 expression ($n = 46$).The 5-year survival rates are indicated for each curve. The p values were calculated using log-rank tests

TABLE 3 Univariate and multivariable analyses of prognostic factors in ESCC

Clinical factors	Univariate analysis p value	Multivariate analysis		
		p value	Hazard ratio	95 % CI
Nrf2	0.0037	0.044	2.674	1.024–6.979
cT	0.5293	0.487	2.068	0.266–16.065
cN	0.0087	0.073	3.998	0.875–18.253

antioxidants, is an important factor that affects antitumor efficacy.

A cisplatin-based regimen and radiation therapy, which are common major therapies for ESCC, show effectiveness by inducing ROS.[9] Thus, this may explain the difference in the efficacy of CRT for the treatment of ESCC between the high Nrf2 expression group and the low Nrf2 expression group. In the present study, the expression of the protein Nrf2 was examined in biopsy specimens of ESCC to determine whether such expression was useful for predicting the response to CRT. As shown in Table 1, there was a significant correlation between Nrf2 expression and the clinical effects of CRT. Furthermore, as shown in Table 2, not only the clinical effect, but also the histological effects of CRT showed significant correlations with Nrf2 expression. These data imply that, in a tumor with low expression of Nrf2, depression of antioxidants, which is the target gene of Nrf2, leads to depression of ROS scavenging ability, resulting in more apoptosis in tumors with low expression of Nrf2, leading to high sensitivity to CRT. With this viewpoint, we examined the Nrf2 expression in the surgical specimens after treating CRT. Although the percentage of Nrf2 positive in the biopsy specimens before CRT was 39.1, 80.4 % of surgical specimens had positive Nrf2 expression in their nuclei (data not shown). All cases with Nrf2 positive expression before CRT in the biopsy specimens never changed their positivity in the surgical specimens after surgery. This phenomenon implied that residual cancer cells might have a high antioxidant ability that is able to overcome ROS produced by CRT and survive (Supplemental Fig. 1). Taken together, it was suggested that evaluation of the Nrf2 expression level in ESCC can be useful to predict the effectiveness of CRT.

As another finding in this study that involved patients who underwent radical resection after CRT, a significant relationship was seen between the expression level of Nrf2 and clinical lymph node metastases. In patients who underwent CRT before radical surgical resection, it has been proposed that Nrf2 expression status in biopsy specimens of ESCC could be used to help identify patients who are at high risk of developing lymph node metastasis.[32]

Concerning the survival analysis, Nrf2 was a good prognostic factor in the patients of this study, and Nrf2 expression was an independent prognostic factor. Thus, the Nrf2 expression level could be used as a useful prognostic parameter for predicting the survival of patients who underwent radical resection after CRT.

It was suggested that, in ESCC, high expression of Nrf2 is associated with high antioxidant ability, which is responsible for natural immunity against not only CRT, but also the ROS produced by macrophage or leukocyte as tumor immunity against cancer cells. In this study group, the patients who responded to CRT survived longer than any other patients, as in other reports.[33] Therefore, in ESCC patients with high Nrf2 expression, knockdown of Nrf2 in the cancer cells before CRT should contribute to converting nonresponders to responders, resulting in a more favorable prognosis. From this perspective, Nrf2 could be a new therapeutic target.

In conclusion, Nrf2-negative expression in biopsy specimens of primary tumors is associated with not only a favorable effect of CRT, but also the prognosis of ESCC. Patients with Nrf2-negative expression may be good candidates for CRT. Since immunohistochemical analysis of biopsy specimens for Nrf2 expression is a simple and inexpensive test, Nrf2 expression should be evaluated before treatment.

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Arsenic Trioxide Prevents Osteosarcoma Growth by Inhibition of GLI Transcription via DNA Damage Accumulation

Shunsuke Nakamura[1], Satoshi Nagano[1], Hiroko Nagao[1], Yasuhiro Ishidou[2], Masahiro Yokouchi[1], Masahiko Abematsu[1,3], Takuya Yamamoto[1], Setsuro Komiya[1], Takao Setoguchi[3*]

1 Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan, **2** Department of Medical Joint Materials, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan, **3** The Near-Future Locomotor Organ Medicine Creation Course (Kusunoki Kai),Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

Abstract

The Hedgehog pathway is activated in various types of malignancies. We previously reported that inhibition of SMO or GLI prevents osteosarcoma growth in vitro and in vivo. Recently, it has been reported that arsenic trioxide (ATO) inhibits cancer growth by blocking GLI transcription. In this study, we analyzed the function of ATO in the pathogenesis of osteosarcoma. Real-time PCR showed that ATO decreased the expression of Hedgehog target genes, including *PTCH1*, *GLI1*, and *GLI2*, in human osteosarcoma cell lines. WST-1 assay and colony formation assay revealed that ATO prevented osteosarcoma growth. These findings show that ATO prevents GLI transcription and osteosarcoma growth in vitro. Flow cytometric analysis showed that ATO promoted apoptotic cell death. Comet assay showed that ATO treatment increased accumulation of DNA damage. Western blot analysis showed that ATO treatment increased the expression of γH2AX, cleaved PARP, and cleaved caspase-3. In addition, ATO treatment decreased the expression of Bcl-2 and Bcl-xL. These findings suggest that ATO treatment promoted apoptotic cell death caused by accumulation of DNA damage. In contrast, Sonic Hedgehog treatment decreased the expression of γH2AX induced by cisplatin treatment. ATO re-induced the accumulation of DNA damage attenuated by Sonic Hedgehog treatment. These findings suggest that ATO inhibits the activation of Hedgehog signaling and promotes apoptotic cell death in osteosarcoma cells by accumulation of DNA damage. Finally, examination of mouse xenograft models showed that ATO administration prevented the growth of osteosarcoma in nude mice. Because ATO is an FDA-approved drug for treatment of leukemia, our findings suggest that ATO is a new therapeutic option for treatment of patients with osteosarcoma.

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* E-mail: setoro@m2.kufm.kagoshima-u.ac.jp

Introduction

Osteosarcoma is the most common malignant bone tumor in children and adolescents [1,2]. Osteosarcoma is a highly aggressive neoplasm that is resistant to current therapeutic approaches, including radiation, chemotherapy, and surgical treatment. The survival rate of patients treated with neoadjuvant chemotherapy and local control therapy is 60–80% [3]. The predicted outcome is poor in patients with lung metastasis at first diagnosis, with long-term survival rates ranging between 10% and 40% [4]. Therefore, more effective treatments and more personalized therapies (i.e., treatments targeting a specific signaling pathway or gene) are essential for patients with osteosarcoma.

The Hedgehog pathway is involved in various aspects of development. The Hedgehog pathway is activated via the PATCHED (PTCH1) and SMOOTHENED (SMO) Hedgehog receptors. Activation of SMO promotes the activation of GLI family transcription factors (GLI1, GLI2, and GLI3) to regulate the transcription of target genes [5–7]. Aberrant activation of the Hedgehog pathway is associated with malignant tumors (reviewed in ref [8].). We have previously reported that aberrant

activation of the Hedgehog pathway is involved in the pathoetiology of osteosarcoma. Inhibition of the Hedgehog pathway by knockdown of SMO or GLI2 prevents osteosarcoma growth in vitro and in vivo [9,10]. Although several SMO inhibitors have been developed, they have several limitations, including constitutive activation of SMO, spontaneous mutation of SMO that impairs its binding to the drug, and constitutive activation downstream of SMO [11–21]. Arsenic trioxide (ATO) is an FDA-approved drug used for the treatment of patients with acute promyelocytic leukemia (APL) who show relapse after first-line chemotherapy (reviewed in 22. ATO promotes complete remission without myelosuppression and causes few adverse reactions. Recently, it has been reported that ATO prevents human cancer cell growth by inhibiting activation of the Hedgehog pathway [23–25]. In the present study, we examined the effect of ATO treatment on GLI transcription and osteosarcoma growth in vitro and in vivo. Our findings show that ATO inhibits Hedgehog pathway signaling and prevents human osteosarcoma cell growth via accumulation of DNA damage.

Materials and Methods

Cell culture

The osteosarcoma cell line 143B, Saos-2, and U2OS were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HsOs1 cell line was purchased from the Riken cell bank (Tsukuba, Japan). Osteosarcoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). For analyzing DNA damage, recombinant Sonic Hedgehog protein (R&D Systems, Minneapolis, MN, USA), ATO (Nihon Shinyaku, Kyoto, Japan), and cisplatin (CDDP) (LKT Laboratories, Minneapolis, USA) were used. Cell lines were cultured in a humidified incubator with 5% CO_2 at 37°C.

Real-time polymerase chain reaction

Human osteosarcoma cells were cultured with or without 1 μM ATO. A vehicle (aqueous sodium hydroxide and hydrochloric acid to adjust to pH 7.5) was used as the control. Primer sets amplified amplicons of 150 to 200 bp in size. Polymerase chain reactions (PCRs) were performed using SYBR Green (BIO-RAD) on a MiniOpticon™ machine (BIO-RAD). The comparative Ct (ΔΔCt) method was used to evaluate the fold change in mRNA expression using *β-actin* as the reference gene. All PCR reactions were performed in triplicate, with 3 different concentrations of cDNA. All primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). The following primers were used: *PTCH1*: 5′-TAACGCTGCAACAACTCAGG-3′, 5′-GAAGGCTGTGACATTGCTGA-3′; *GLI1*: 5′-GTGCAAGTCAAGCCAGAACA-3′, 5′-ATAGGGGCCTGACTGGAGAT-3′, *GLI2*: 5′-CGACACCAGGAAGGAAGGTA-3′, 5′-AGAACGGAGGTAGTGCTCCA-3′; *β-actin*: 5′-AGAAAATCTGGCACCACACC-3′, 5′-AGAGGCGTACAGGGATAGCA-3′.

Each experiment was performed in triplicate, and all experiments were performed 3 times.

WST-1 assay

Human osteosarcoma cells were cultured with or without 1 μM or 3 μM ATO. An equivalent volume of vehicle (aqueous sodium hydroxide and hydrochloric acid to adjust to pH 7.5) was used as the control. The cells were treated with WST-1 substrate (Roche, Basel, Switzerland) for 4 h, washed with phosphate-buffered saline, and lysed to release formazan. Then, the cells were analyzed on a microplate reader (BIO-RAD, Hercules, CA, USA). Each experiment was performed in triplicate, and all experiments were performed 3 times.

Colony formation assay

Cells were cultured in DMEM containing 0.33% soft agar and 5% fetal bovine serum, and plated on 0.5% soft agar layer. Cells were cultured in 6-well plates at a density of 5×10^3 cells per well. Human osteosarcoma cells were cultured with or without 3 μM ATO. An equivalent volume of vehicle was used as the control. Fourteen days later, the number of colonies was evaluated. Each experiment was performed in triplicate, and all experiments were performed 3 times.

Cell cycle analysis

Human osteosarcoma cells were cultured with or without 1 μM ATO. An equivalent volume of vehicle was used as the control. Cell cycle analysis was performed as previously reported [9]. Cells were collected, fixed with 70% ethanol for 2 h at 4°C, washed with phosphate-buffered saline, and treated with 500 μL staining buffer containing RNase A and 50 μg/mL propidium iodide (Wako Chemicals, Kanagawa, Japan). The DNA content was examined by flow cytometry using CyAn™ ADP (Beckman Coulter, CA, USA) and Summit software (Beckman Coulter). Each experiment was performed in triplicate, and all experiments were performed 3 times.

Comet assay

Human osteosarcoma cells were cultured with or without 3 μM ATO. An equivalent volume of vehicle was used as the control. Cells were trypsinized and electrophoresed on agarose gels as previously reported [26]. Tail moment (TM) and tail length (TL) were used to evaluate DNA damage in individual cells. Image analysis and quantification were performed using NIH ImageJ software. TM = % DNA in the tail × TL, where % of DNA in the tail = tail area (TA) × tail area intensity (TAI) × 100/(TA × TAI) + [head area (HA) × head area intensity (HAI)].

Western blotting

Human osteosarcoma cells were cultured with or without 3 μM ATO. An equivalent volume of vehicle was used as the control. The cells were dissolved in NP40 buffer containing 0.5% NP40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 3 mM pAPMSF (Wako Chemicals, Kanagawa, Japan), 5 mg/mL aprotinin (Sigma, St. Louis, MO, USA), 2 mM sodium orthovanadate (Wako Chemicals), and 5 mM EDTA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and

immunoblotting were performed subsequently. The following antibodies were used: phospho-histone H2AX (Ser139) (γH2AX) (Cell Signaling Technology, MA, USA), cleaved caspase-3 (Asp175) (Cell Signaling Technology), poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology), Bcl-2 (Cell Signaling Technology), Bcl-xL (Cell Signaling Technology), SAPK/JNK (Cell Signaling Technology), Phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology), NF-κB p65 (Cell Signaling Technology), phospho-NF-κB p65 (Ser468) (Cell Signaling Technology), and tubulin (Santa Cruz, California, USA). Bands were visualized using the ECL chemiluminescence system (Amersham, Giles, UK).

Xenograft model

143B cells (1×10^6) and 100 μL Matrigel (BD, NJ, USA) suspension were subcutaneously inoculated into 5-week-old nude mice. The mice were randomly allocated to treatment with either ATO (10 μg/g) or an equivalent volume of vehicle (30 mM NaOH, pH 7.0). ATO and vehicle were administered intraperitoneally every day. ATO and vehicle treatment was started at 1 week after inoculation, at which time, the tumors had grown to a visible size. The tumor size was measured using the formula $LW^2/2$ (L and W represent the length and width of tumors, respectively). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Kagoshima University. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee, Graduate School of Medical and Dental Sciences, Kagoshima University (Permit Number: MD11017). All surgeries were performed under general anesthesia, and every effort was made to minimize the number of animals used and animal pain.

Immunohistochemistry

ApopTag® Peroxidase In Situ Apoptosis Detection Kit was used for TUNEL staining according to the supplier's protocol (MerckMillipore, Billerica, MA, USA). The sections were stained with methyl green (Merck-Chemicals, Darmstadt, Germany) to identify nuclei.

Statistical analysis

All examinations were performed 3 times, except where otherwise stated, and all samples were analyzed in triplicate. All results are presented as mean (SD). Statistical differences between groups were assessed by Student's *t*-test for unpaired data using Microsoft Office Excel (Microsoft, Albuquerque, NM, USA) and Kaplan 97.

Results

ATO prevents GLI transcription and proliferation of osteosarcoma cells

To determine whether ATO prevents GLI transcription in osteosarcoma cells, real-time PCR was performed for ATO-treated cells. Four human osteosarcoma cell lines showing upregulation of GLI transcription were examined [9,10]. The human osteosarcoma cell lines were treated with ATO at previously reported concentrations, which inhibit human cancer cell proliferation by inhibiting activation of the Hedgehog pathway [25]. Real-time PCR revealed that ATO prevented the transcription of GLI target genes, including *PTCH1*, *GLI1*, and *GLI2*, in human osteosarcoma cell lines (Figure 1). The WST-1 assay showed that proliferation of the 143B, Saos2, HsOs1, and U2OS cell lines was inhibited by ATO (Figure 2). We next evaluated the effects of ATO on anchorage-independent growth of osteosarcoma cells. The colony formation assay showed that ATO treatment decreased the number of colonies in soft agar (Figure 3). These findings showed that ATO treatment prevents GLI transcription and growth of osteosarcoma cells in vitro.

ATO promotes DNA damage and apoptotic cell death

To examine whether ATO treatment promoted cell death or cell cycle arrest, we performed flow cytometric analysis. The results showed that ATO treatment increased the population of sub-G1 cells (Figure 4). These findings show that ATO treatment promotes apoptotic cell death in osteosarcoma cells. To examine whether ATO promotes DNA damage, we performed a comet assay, which can be used to detect single cell DNA damage by the cellular elution pattern through agarose gels. The comet assay showed that ATO treatment altered the elution profiles (Figure 5). These findings show that ATO treatment promotes the accumulation of DNA damage in osteosarcoma cells. In addition, we used western blotting to examine the expression of DNA damage markers and apoptosis-related proteins after ATO treatment. Western blot analysis showed that ATO treatment increased the expression of γH2AX, a marker of double-strand breaks, cleaved poly (ADP-ribose) polymerase (PARP), and cleaved-caspase 3. In contrast, ATO treatment decreased the expression of Bcl-2 and Bcl-xL (Figure 6A). These findings suggest that ATO treatment promotes apoptotic cell death caused by accumulation of DNA damage.

It has been reported that ATO promotes apoptotic cell death and phosphorylation of JNK [27]. Although western blot analysis showed that ATO treatment increased the amount of phosphorylated JNK, inhibition of JNK activity had no effect on osteosarcoma cell proliferation with or without ATO, as seen with Ewing sarcoma cells (Figure S1) [23]. It has been reported that ATO treatment decreases the phosphorylation of NF-κB and promotes cell death [28]. Our findings showed that ATO treatment did not affect the status of NF-κB phosphorylation (Figure S1).

Hedgehog signaling prevents DNA damage caused by CDDP treatment

To examine whether activation of Hedgehog signaling affects accumulation of DNA damage, we performed western blot analysis after cisplatin (CDDP) treatment. Western blotting showed that CDDP treatment upregulated the expression of γH2AX. Treatment with Sonic Hedgehog attenuated the upregulation of γH2AX (Figure 6B). In addition, we examined the effect of ATO treatment on the attenuation of DNA damage by Hedgehog activation. The attenuation of DNA damage caused by Hedgehog activation was reversed by ATO

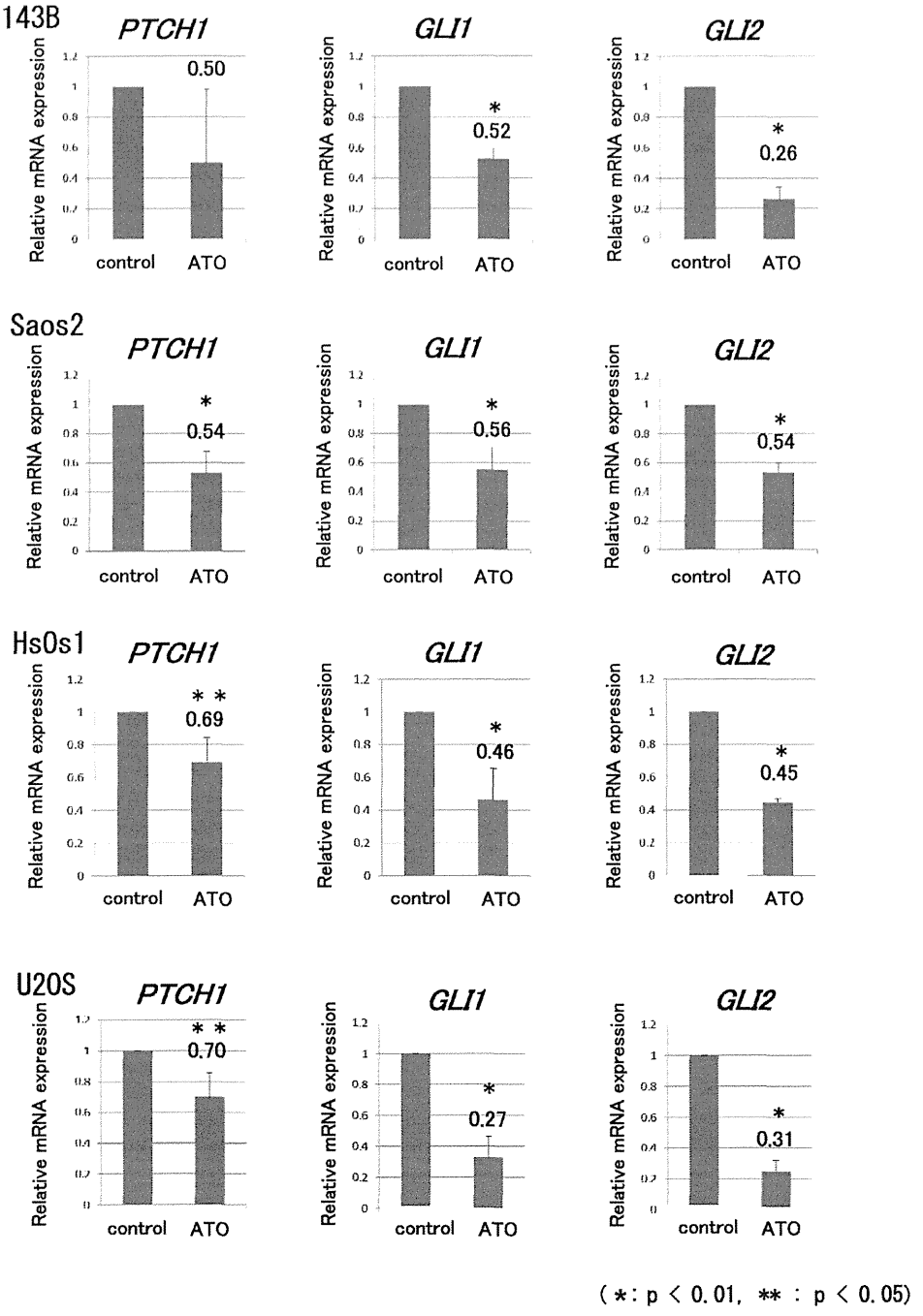

Figure 1. ATO prevents the transcription of GLI target genes. Human osteosarcoma cells were cultured with or without 1 μM ATO. An equivalent volume of vehicle was used as the control. Total RNA collected from osteosarcoma cell lines was examined by real-time polymerase chain reaction (PCR). A comparative Ct (ΔΔCt) analysis was performed to examine fold changes in mRNA expression compared with *β-actin*. Real-time PCR showed that ATO decreased the transcription of GLI target genes, including *PTCH1*, *GLI1*, and *GLI2*, in 143B, Saos2, HsOs1, and U2OS cells. The experiment was performed in triplicate with similar results (error bars represent mean [SD]) (*P < 0.01, **P < 0.05).

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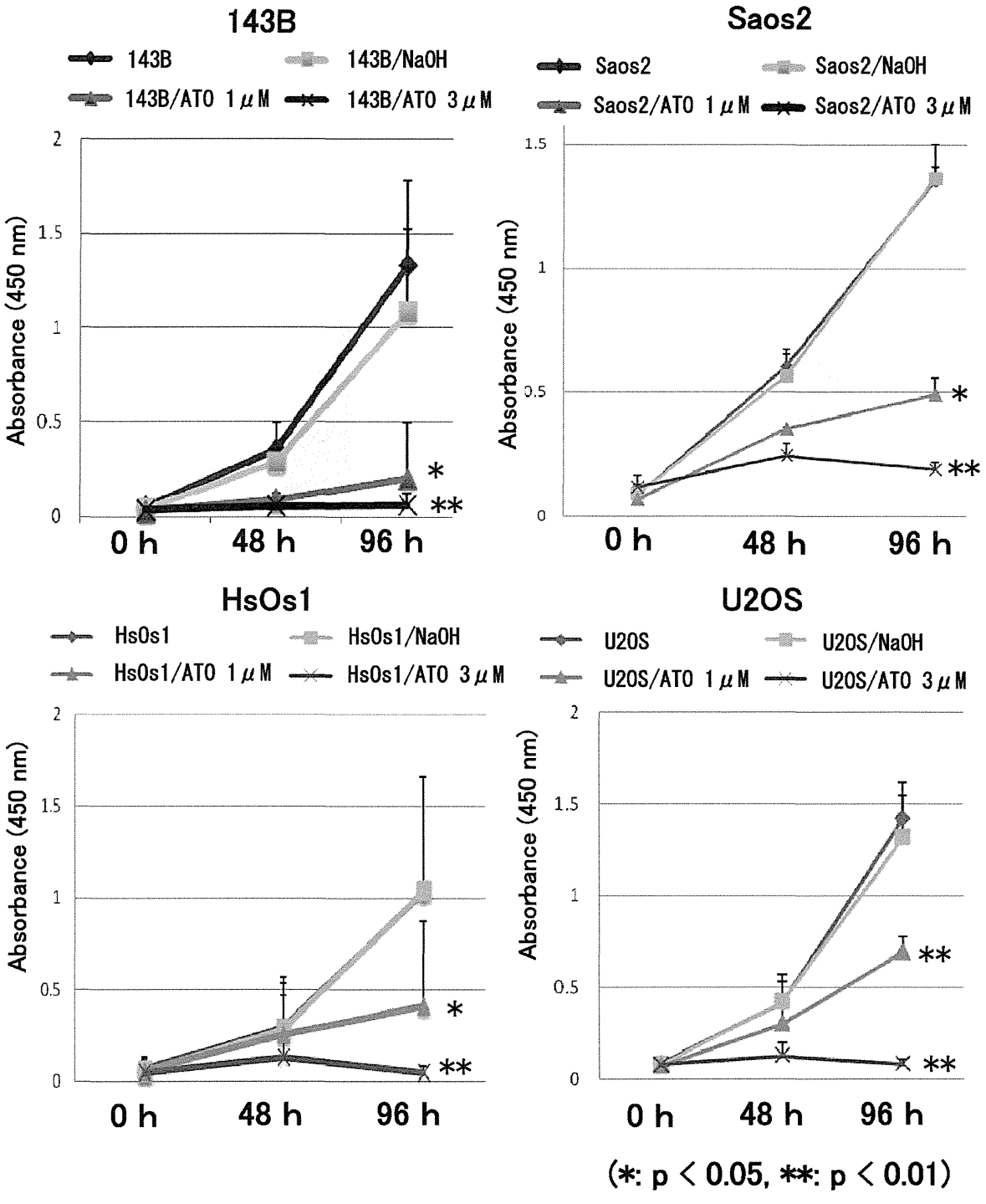

Figure 2. ATO prevents human osteosarcoma cell proliferation. WST assay showed that the growth of 143B, Saos-2, HsOs1, and U2OS cells was prevented by 1 μM or 3 μM ATO treatment for 96 h. An equivalent volume of vehicle was used as the control. The experiment was performed in triplicate with similar results (*P < 0.05, **P < 0.01) (error bars represent mean [SD]).

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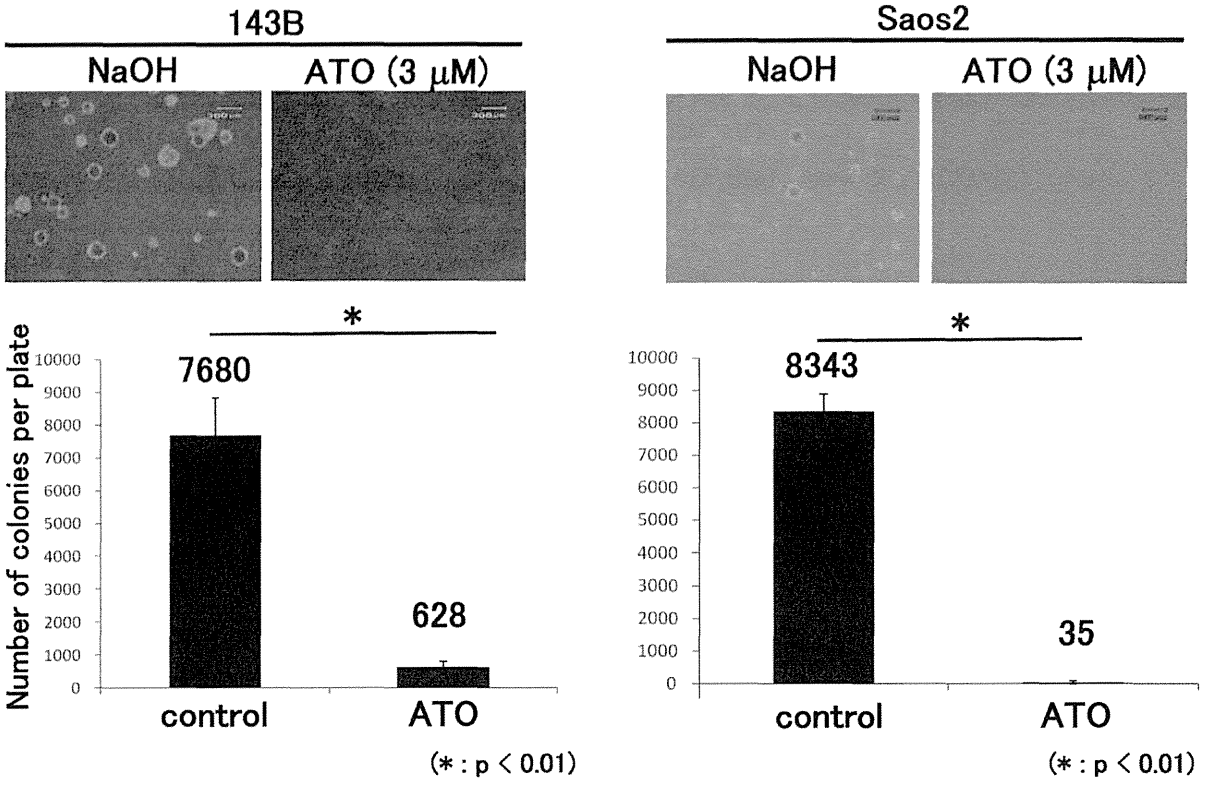

Figure 3. ATO inhibits anchorage-independent osteosarcoma growth. Treatment of 143B and Saos2 cells with 3 μM ATO reduced the number of colonies in soft agar at 14 days. An equivalent volume of vehicle was used as the control. These experiments were performed in triplicate with similar results (*P < 0.01) (error bars represent mean [SD]).

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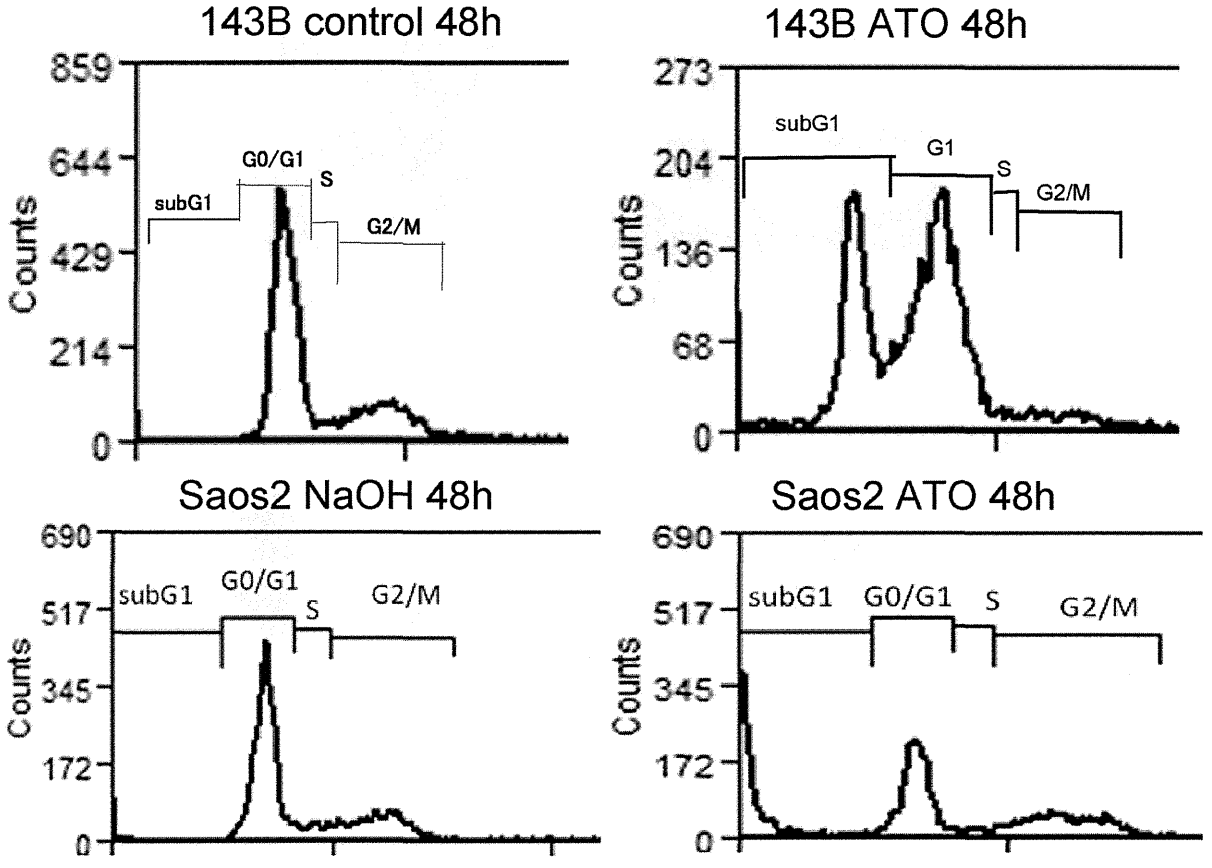
treatment (Figure 6C). These findings suggest that ATO promotes the accumulation of DNA damage by inhibiting Hedgehog signaling.

ATO prevents osteosarcoma growth in vivo

143B osteosarcoma cells were intradermally inoculated into nude mice, and palpable tumors were formed within 7 days. Then, ATO or an equivalent volume of vehicle was injected intraperitoneally. The injections were administered every day. Compared with vehicle treatment, treatment with ATO significantly prevented tumor growth (Figure. 7). Kaplan-Meier analysis showed that ATO treatment provided a significant survival benefit (Figure 7A). TUNEL staining showed that ATO treatment induced apoptotic cell death. The number of apoptotic cells was significantly increased in ATO-treated tumors (Figure 7B).

Discussion

We and other researchers have previously reported that inhibition of the Hedgehog pathway prevented the growth of osteosarcoma cells [9,10,29]. In particular, we showed that knockdown of GLI2 prevented osteosarcoma cell growth in vitro and in vivo [9]. ATO prevents Ewing sarcoma, medulloblastoma, and basal cell carcinoma growth by inhibition of GLI transcription [23–25]. To apply our previous findings in clinical settings, we examined the effects of ATO in human osteosarcoma. We showed that ATO prevents the transcription of GLI target genes and promotes apoptotic cell death in osteosarcoma cells as a result of accumulation of DNA damage. In addition, ATO re-induces the accumulation of DNA damage attenuated by recombinant Sonic Hedgehog treatment. These findings suggest that ATO inhibits the activation of Hedgehog signaling and promotes apoptotic cell death in osteosarcoma cells as a result of accumulation of DNA damage. In addition, our findings showed that ATO decreased the expression of Bcl-2 and Bcl-xL. GLI1 and GLI2 upregulate the transcription of Bcl-2 and Bcl-xL [30–33]. Inhibition of the Hedgehog pathway by ATO treatment may downregulate Bcl-2 and Bcl-xL to promote apoptotic cell death in osteosarcoma cells. Singh et al. reported that ABCG2, a drug transporter protein, is a direct transcriptional target of Hedgehog signaling [33]. These findings suggest that activation of Hedgehog



	SubG1	G1	S	G2/M
143B control	1.5±1.6 %	68.7±3.4 %	6.0±1.3 %	23.5±2.4 %
143B (ATO: 3 μM)	24.5±10.0 % **	45.2±21.7 %	2.9±1.1 % **	5.7±2.6 % *
Saos2 control	2.7±2.8 %	56.0±15.3 %	7.4±2.1 %	34.0±10.9 %
Saos2 (ATO: 3 μM)	25.7±6.4 % **	33.0±9.2 %	5.0±3.0 %	24.1±6.0 %

(*: $P < 0.01$, **: $P < 0.05$)

Figure 4. ATO promotes apoptotic cell death in human osteosarcoma cells. Human osteosarcoma cells were cultured with or without 1 μM ATO. An equivalent volume of vehicle was used as the control. Flow cytometric analysis was performed after ATO treatment for 48 h. ATO treatment significantly increased the Sub-G1 population of 143B and Saos2 cells. These experiments were performed in triplicate with similar results (*$P < 0.01$, **$P < 0.05$).

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signaling promoted the export of CDDP by the ABCG2 transporter and reduced the accumulation of DNA damage in osteosarcoma cells. Inhibition of the Hedgehog pathway by ATO treatment may be useful as an adjunct treatment to conventional chemotherapy for osteosarcoma. In addition, several molecular mechanisms have been reported for inhibition of the Hedgehog pathway by ATO. Kim et al. reported that ATO prevented growth of medulloblastoma by reducing stability of GLI2 protein and ciliary accumulation of GLI2 [25]. Elspeth et al. reported that ATO prevents growth of cancer cell

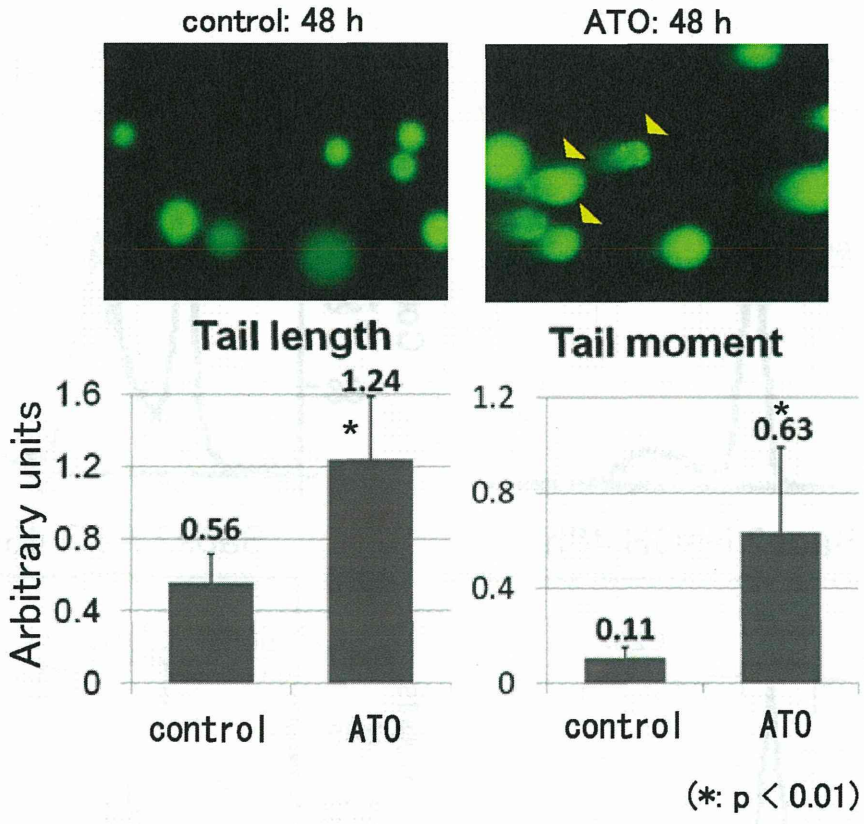

Figure 5. ATO elicits DNA damage in human osteosarcoma. COMET assay was performed to detect DNA damage in single cells after ATO treatment. 143B cells were treated with ATO (3 μM) or an equivalent volume of control vehicle for up to 48 h and analyzed by performing the COMET assay. Graphs represent DNA damage by tail length and tail moment, evaluated as described in the Materials and Methods section. These experiments were performed in triplicate with similar results (*P < 0.01).

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lines and Ewing sarcoma by inhibiting GLI transcription through direct binding to GLI [23]. Although there were some discrepancies related to the mechanism of Hedgehog pathway inhibition by ATO, these studies independently suggest that ATO inhibits malignant tumor growth by inhibition of the Hedgehog pathway at the level of GLI transcription factors. These mechanisms may prevent osteosarcoma growth after ATO treatment. Because aberrant activation of the Hedgehog pathway has been implicated in several malignant tumors, the pharmaceutical industry has invested in the development of Hedgehog pathway inhibitors. SMO inhibitors have been evaluated in recent clinical trials [34,35]. However, treatment with SMO inhibitors showed a lack of efficacy in a portion of patients. Investigation of the underlying mechanism revealed that the patient tumors showed a mutation in SMO that prevented binding of the SMO inhibitors to SMO [15]. Several genes with potential mutations within SMO and downstream of SMO have been found [16–21,36]. In addition, non-Hedgehog pathway-mediated activation of GLI transcription has been reported [37–41]. In this regard, direct GLI inhibition by ATO is likely to be useful for treating tumors with mutations within or downstream of SMO. For example, inhibition of GLI, but not SMO, inhibited tumor growth in myeloid leukemia, colon carcinoma, hepatocellular carcinoma, and osteosarcoma [9,42–44]. Originally, arsenic was used in the 17th century to treat leukemia. ATO has been approved for the treatment of intractable acute promyelocytic leukemia in Japan. Our findings suggest that ATO is one of the most suitable molecular target reagents for inhibiting the Hedgehog pathway in human osteosarcoma. We have now obtained approval from the ethics committee for clinical research, Kagoshima University, to use ATO for treating patients with intractable osteosarcoma.

We examined whether the inhibitory effect of ATO on osteosarcoma growth is mediated, at least in part, by JNK or NF-κB [45–47]. As previously reported, treatment with ATO increased JNK phosphorylation. However, treatment with a JNK inhibitor did not prevent osteosarcoma growth. In contrast, treatment with ATO did not affect NF-κB activation. These