Acknowledgments

The authors thank Ms Makiko Naka Mieno for her advice on the statistical analysis.

References

[1] Matsubara D, Kishaba Y, Ishikawa S, et al. Lung cancer with loss of BRG1/BRM, shows epithelial mesenchymal transition phenotype and distinct histologic and genetic features. Cancer Sci 2013;104:266-73.

[2] Yauch RL, Januario T, Eberhard DA, et al. Epithelial versus mesenchymal phenotype determines in vitro sensitivity and predicts clinical activity of erlotinib in lung cancer patients. Clin Cancer Res 2005;11:8686-98.

[3] Matsubara D, Ishikawa S, Sachiko O, Aburatani H, Fukayama M, Niki T. Co-activation of epidermal growth factor receptor and c-MET defines a distinct subset of lung adenocarcinomas. Am J Pathol 2010;177:2191-204.

[4] Takeuchi T, Tomida S, Yatabe Y, et al. Expression profile-defined classification of lung adenocarcinoma shows close relationship with underlying major genetic changes and clinicopathologic behaviors. J Clin Oncol 2006;24:1679-88.

[5] Jones S, Wang TL, Shih IM, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science 2010;330:228-31.

[6] Gui Y, Guo G, Huang Y, et al. Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. Nat Genet 2011;43:875-8.

[7] Varela I, Tarpey P, Raine K, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 2011;469:539-42.

[8] Zang ZJ, Cutcutache I, Poon SL, et al. Exome sequencing of gastric adenocarcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. Nat Genet 2012;44:570-4.

[9] Dacwag CS, Ohkawa Y, Pal S, Sif S, Imbalzano AN. The protein arginine methyltransferase Prmt5 is required for myogenesis because it facilitates ATP-dependent chromatin remodeling. Mol Cell Biol 2007;27:384-94.

[10] Dacwag CS, Bedford MT, Sif S, Imbalzano AN. Distinct protein arginine methyltransferases promote ATP-dependent chromatin remodeling function at different stages of skeletal muscle differentiation. Mol Cell Biol 2009;29:1909-21.

[11] Mallappa C, Hu YJ, Shamulailatpam P, Tae S, Sif S, Imbalzano AN. The expression of myogenic microRNAs indirectly requires protein arginine methyltransferase (Prmt)5 but directly requires Prmt4. Nucleic Acids Res 2011;39:1243-55.

[12] Scoumanne A, Zhang J, Chen X. PRMT5 is required for cell-cycle progression and p53 tumor suppressor function. Nucleic Acids Res 2009;37:4965-76.

[13] Cho EC, Zheng S, Munro S, et al. Arginine methylation controls growth regulation by E2F-1. EMBO J 2012;31:1785-97.

[14] Ren J, Wang Y, Liang Y, Zhang Y, Bao S, Xu Z. Methylation of ribosomal protein S10 by protein-arginine methyltransferase 5 regulates ribosome biogenesis. J Biol Chem 2010;285:12695-705.

[15] Zhou Z, Sun X, Zou Z, et al. PRMT5 regulates Golgi apparatus structure through methylation of the golgin GM130. Cell Res 2010;20: 1023-33.

[16] Kim JM, Sohn HY, Yoon SY, et al. Identification of gastric cancer-related genes using a cDNA microarray containing novel expressed sequence tags expressed in gastric cancer cells. Clin Cancer Res 2005;11:473-82.

[17] Pal S, Baiocchi RA, Byrd JC, Grever MR, Jacob ST, Sif S. Low levels of miR-92b/96 induce PRMT5 translation and H3R8/H4R3 methylation in mantle cell lymphoma. EMBO J 2007;26:3558-69.

[18] Wang L, Pal S, Sif S. Protein arginine methyltransferase 5 suppresses the transcription of the RB family of tumor suppressors in leukemia and lymphoma cells. Mol Cell Biol 2008;28:6262-77.

[19] Powers MA, Fay MM, Factor RE, Welm AL, Ullman KS. Protein arginine methyltransferase 5 accelerates tumor growth by arginine methylation of the tumor suppressor programmed cell death 4. Cancer Res 2011;71:5579-87.

[20] Gu Z, Li Y, Lee P, Liu T, Wan C, Wang Z. Protein arginine methyltransferase 5 functions in opposite ways in the cytoplasm and nucleus of prostate cancer cells. PLoS One 2012;7:e44033.

[21] Gu Z, Gao S, Zhang F, Wang Z, Ma W, Davis RE. Protein arginine methyltransferase 5 is essential for growth of lung cancer cells. Biochem J 2012;446:235-41.

[22] Matsubara D, Kanai Y, Ishikawa S, et al. Identification of CCDC6-RET fusion in a human lung adenocarcinoma cell line, LC-2/ad. J Thorac Oncol 2012;7:1872-6.

[23] Yoshizawa A, Motoi N, Riely GJ, et al. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases. Mod Pathol 2011;24:653-64.

[24] Shilo K, Wu X, Sharma S, et al. Cellular localization of protein arginine methyltransferase-5 correlates with grade of lung tumors. Diagn Pathol 2013;8:201.

[25] Friesen WJ, Paushkin S, Wyce A, et al. The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. Mol Cell Biol 2001;21:8289-300.

[26] Abell AN, Jordan NV, Huang W, et al. MAP3K4/CBP-regulated H2B acetylation controls epithelial-mesenchymal transition in trophoblast stem cells. Cell Stem Cell 2011;8:525-37.

[27] Tee WW, Pardo M, Theunissen TW, et al. Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. Genes Dev 2010;24:2772-7.

Stromal fibroblasts are predictors of disease-related mortality in esophageal squamous cell carcinoma

SHIN SAITO[1], KAZUE MORISHIMA[1], TAKASHI UI[1], DAISUKE MATSUBARA[2], TOMOKO TAMURA[2], SACHIKO OGUNI[2], YOSHINORI HOSOYA[1], NAOHIRO SATA[1], ALAN T. LEFOR[1], YOSHIKAZU YASUDA[1] and TOSHIRO NIKI[2]

Departments of [1]Surgery and [2]Integrative Pathology, Jichi Medical University, Shimotsuke-City, Tochigi 329-0498, Japan

Received March 27, 2014; Accepted April 28, 2014

DOI: 10.3892/or.2014.3216

Abstract. The growth, invasiveness and metastasis of human cancers are determined not only by cancer cells, but also by their microenvironment. Activated stromal fibroblasts promote tumor progression by secreting growth factors. In the present study, we focused on interrelations between cancer and fibroblasts, the main component of tumor stroma. We retrospectively analyzed the relations of mortality to clinical, pathological, and α-smooth muscle actin (α-SMA) characteristics in 97 consecutive patients with esophageal squamous cell carcinoma (ESCC). *In vitro*, we used TE-11, KYSE150 and KYSE220 ESCC cell lines and isolated esophageal stromal fibroblasts, some of which were immortalized. Migration assays were conducted to assess the effects of fibroblasts on cancer-cell migration and 3-dimensional organotypic cultures. *In vivo*, TE-11 and KYSE220 cells plus immortalized fibroblasts were co-transplanted subcutaneously in Nod/Scid mice to assess the effects of fibroblasts on tumorigenicity. Clinicopathologically, the α-SMA expression of cancer stroma was correlated with venous invasion ($p<0.01$), nodal involvement ($p=0.02$), recurrence ($p=0.01$), and was a predictor of survival in patients with stage I and II ESCC ($p=0.04$). *In vitro*, the presence of fibroblasts strongly promoted the migration of TE-11, KYSE150 and KYSE220 cells. On organotypic culture, stromal invasion was observed only in the presence of immortalized fibroblasts. *In vivo*, tumors developed or grew in a fibroblast-dependent manner after implantation. Our findings provide evidence that stromal fibroblasts and tumor cells interact to promote tumor progression in ESCC. In patients with earlier stage ESCC, α-SMA may be a predictor of mortality. Inhibition of paracrine systems associated with tumor fibroblasts may slow or reverse tumor progression, potentially leading to the development of new targeted therapies.

Correspondence to: Professor Toshiro Niki, Department of Integrative Pathology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-City, Tochigi 329-0498, Japan
E-mail: tniki@jichi.ac.jp

Key words: esophageal cancer, SMA, stroma, myofibroblast, prognosis

Introduction

Worldwide, esophageal cancer is the sixth leading cause of cancer-related mortality (1). Squamous cell carcinoma (SCC) is the predominant histologic type of esophageal cancer worldwide (2), and esophageal squamous cell carcinoma (ESCC) is often encountered in Asia (3-5), including Japan. ESCC is one of the least studied and most fatal cancers (6).

The microenvironment of cancer cells has recently been shown to strongly influence the biologic properties of cancer (7). In fact, tumors consist of tumor cells, fibroblasts, endothelial cells, immune cells and extracellular matrix. In addition, many types of solid tumors contain smooth muscle actin (SMA)-positive myofibroblasts [i.e., activated fibroblasts, peritumor fibroblasts, and carcinoma-associated fibroblasts (CAFs)] within the stroma (8). Molecular features thought to influence outcomes are generally related to the characteristics of carcinoma cells (9); however, it has become increasingly apparent that the components of the tumor stroma play an important role in promoting tumor progression (10,11). Myofibroblasts have been reported to be related to poor outcomes in several types of carcinoma (12-14). Fibroblasts are associated with cancer cells at all stages of disease progression, and their production of growth factors, chemokines, and extracellular matrix facilitates the angiogenic recruitment of endothelial cells and pericytes (15).

Marsh *et al* reported that an SMA-positive myofibroblastic stroma is the strongest predictor of mortality in patients with oral SCC (9). In the present study, we focused on the complex interrelations between ESCC cells and fibroblasts, the main component of cancer stroma. The levels of α-smooth muscle actin (α-SMA) were determined immunohistochemically in ESCC lesions, and immunoreactivity was observed in stromal fibroblasts. We also prepared *in vitro* and *in vivo* experimental systems to evaluate interactions between ESCC and fibroblasts.

Materials and methods

Tissue collection and processing. From January 1st 2001 to December 31st 2010, a total of 347 patients underwent resection of ESCC in Jichi Medical Hospital. We identified and studied patients with tumor invasion beyond the muscularis mucosa who received neither preoperative chemotherapy nor

radiotherapy. We then retrospectively analyzed the relations of clinical, pathological and molecular (α-SMA and vimentin) features to outcomes in 97 patients with ESCC. Data were collected on age, gender, tumor stage (16,17), outcomes, the presence or absence of recurrence, lymph node metastasis, depth of tumor invasion, and the presence or absence of venous invasion.

Immunohistochemistry (IHC). Representative paraffin blocks were selected, and tissue sections (4-μm thick) of the specimens were deparaffinized in xylene, rehydrated in a graded series of alcohol, and transferred to phosphate-buffered saline (PBS). The slides were stained immunohistochemically for α-SMA and vimentin according to conventional protocols. The antibodies used were α-SMA (clone 1A4) and vimentin (clone V9) (both from Dako, Ely, UK). α-SMA was scored according to the extent of stromal positivity as low (<5% stroma stained positive), moderate (patchy/focal expression, 5-50% stroma stained positive) or high (diffuse expression throughout tumor, >50% stroma stained positive) (Fig. 1) (9). In addition to α-SMA, vimentin immunostain was used as an ancillary method to help identify stromal fibroblasts.

Cell lines and media. We used three ESCC cell lines and esophageal stromal fibroblasts. TE-11 was purchased from RIKEN Cell Bank (Tsukuba, Japan). KYSE150 and KYSE220 were purchased from Health Science Research Resources Bank (Osaka, Japan). All cancer cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Autogen-Bioclear, Wiltshire, UK), glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 and 95% air. These three cell lines were selected from among 22 ESCC cell lines on the basis of the results of cell proliferation induced by fibroblast supernatant (unpublished observation). Primary human esophageal fibroblasts designated as HEF75 were isolated from normal human esophageal tissue specimens resected surgically in the Department of Surgery, Jichi Medical Hospital. The patients had not received any neoadjuvant chemotherapy or radiotherapy irradiation. The study was approved by the Jichi Medical University Ethics Committee, and written informed consent was obtained from the patients.

To isolate fibroblasts (18,19), the epithelial tissue was washed twice in PBS and cut into 1- to 2-mm^3 pieces. A couple of pieces were placed into the well of a 6-well plate. The explants were cultured for 48 h in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% FBS, antibiotics, and glutamine at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. After removing the explants and the non-adherent cells, the remaining cells were incubated for 1 to 2 weeks. The adherent cells were then trypsinized and passaged into a new culture flask at a ratio of 1:3 for further expansion. The cells were used for subsequent experimental studies at the third passage. Some of the human esophageal fibroblasts were immortalized. Briefly, to induce cell immortalization, transforming DNA (pCLXSN-hTERT and pVSV-G) was added to competent cells (*Escherichia coli*), which were then spread on LB plates and incubated at 37˚C to produce colonies. Next, in accordance with the Qiagen Plasmid Midi/Maxi kit (Qiagen KK, Tokyo, Japan) protocol, bacterial

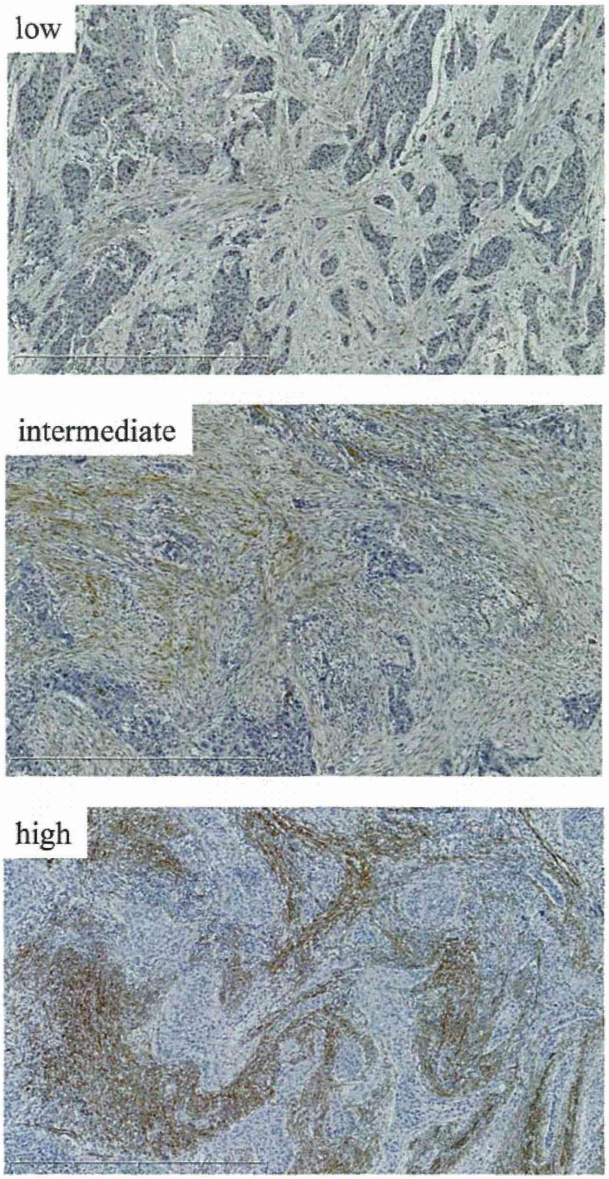

Figure 1. α-SMA immunohistochemistry. α-SMA immunostained sections of esophageal squamous cell carcinoma (ESCC). Representative images of ESCC with low α-SMA expression, intermediate α-SMA expression, and high α-SMA expression in stroma are shown. α-SMA, α-smooth muscle actin.

cells were collected from the LB plate colonies. Plasmid DNA was eluted from the bacterial pellets. The concentration of DNA was adjusted to 1.0 μg/μl. The two DNA products were digested by adding the restriction enzymes *Hind*III and *Eco*RI, and 1% agarose gel electrophoresis was performed to confirm that pVSV-G was cut with *Eco*RI.

In accordance with the protocol of Lipofectamine 2000 reagent (Invitrogen Co., Ltd.), plasmid DNA was transfected into GP2-293 cell line. After 48 h, the culture supernatant including the retrovirus was added to fibroblasts to induce transduction. Selection was performed with G418 (Geneticin®; Invitrogen) 48 h after transduction. In the present study, cells between the 20 and 25th passages after viral transduction were used. Immortalized human esophageal fibroblasts were designated as human embryonic fibroblast (HEF) 75-human telomerase reverse transcriptase (HEF75-hTERT).

Migration assay and 3D organotypic culture. Migration and invasion assays were conducted as described previously (20,21). For migration assay, 24-well cell culture inserts with a pore size of 8-μm (Falcon) were used. The inserts were placed in a 24-well plate containing medium with HEF75. Cancer cells in serum-free medium (containing 5-20x10^4 cells) were placed in each insert. Migratory cells pass through membrane and cling to the bottom side. After cells remaining on the membrane were removed, migratory cells were stained and captured in 5 different high-power fields. The numbers of migratory cells were then automatically counted with the use of a VH analyzer (Keyence, Osaka, Japan). For invasion assay, HEF75-hTERT fibroblasts were mixed with collagen gel at a concentration of 1x10^6 cells/ml and allowed to contract. Then, 2x10^5 of cancer cells were plated, using a cloning ring. The cells were allowed 4 h to attach to the upper surface of the contracted collagen lattice. The cultures were submerged for 4 days, then subsequently placed into 6-well inserts, raised to an air-liquid interface, and cultured for an additional 10-14 days. The schemata of these experiments are shown in Fig. 2.

Co-injection in Nod/Scid mice. Cancer cells and fibroblasts were co-transplanted subcutaneously in Nod/Scid mice to assess the effects of fibroblasts on tumorigenicity. ESCC cells alone or together with human esophageal fibroblasts (HEF75) were suspended in PBS and injected subcutaneously into the flanks of the mice (two injection sites per mouse). We subcutaneously injected 200 μl of a cancer cell suspension with a concentration of 1x10^6 cells/ml into the right side of the mice and 200 μl of a suspension containing a 1:1 mix of cancer cells (2x10^6 cells/ml) and HEFs (6x10^6 cells/ml) into the left side of the mice. The mice (n=3) were sacrificed 6 weeks after the injections. We measured the minor axis and major axis and calculated the tumor volume as an oval sphere shape. The following formula was used to calculate tumor volume: Volume = 0.5236 ddD (d, minor axis; D, major axis).

Statistical analysis. The primary end point was death from ESCC. Survival time was measured from the date of the surgery until the date of mortality due to ESCC or the date on which the patient was last confirmed to be alive. Data were censored at the time of death for patients who died of causes other than ESCC.

Kaplan-Meier survival curves and log-rank tests were used to assess the statistical significance of differences in survival. Analyses were performed using the JMP 9 statistical software package (SAS Institute Inc., Cary, NC, USA). Pearson's Chi-square test was used to analyze relations between α-SMA expression and clinicopathological characteristics. Student's t-test was used to compare differences between 2 groups and to analyze the results of migration assays. Both of these analyses were performed with the use of JMP 9 software. $p<0.05$ was considered to indicate a statistically significant difference.

Table I. Characteristics of 97 patients with ESCC.

	No.	%
Age (years)		
40-59	18	18.5
60-79	76	78.4
80-99	3	3.1
Average	67.2±7.7	
Gender		
Female	15	15.5
Male	82	84.5
Recurrence		
Present	34	35.1
Absent	63	64.9
Outcome		
AWOD	62	63.9
AWD	4	4.1
DOAD	1	1.1
DOD	30	30.9
Disease stage[a]		
I	29	29.9
II	32	33.0
III	32	33.0
IV	4	4.1
Disease stage[b]		
I (A, B)	36 (29, 7)	37.1
II (A, B)	26 (9, 17)	26.8
III (A, B, C)	31 (18, 8, 5)	32
IV	4	4.1
Depth of invasion		
T1b	44	45.4
T2	15	15.5
T3	37	38.1
T4	1	1.0
Nodal involvement		
Present	51	52.6
Absent	46	47.4
α-SMA		
Low	46	47.4
Intermediate	24	24.7
High	27	27.8

DOD, died of disease; AWOD, alive without disease; AWD, alive with disease; DOAD, died of another disease. [a]Japanese Classification of Esophageal Cancer, 10th edition; [b]TNM Classification of Malignant Tumours, Union for International Cancer Control, 7th edition. ESCC, esophageal squamous cell carcinoma; α-SMA, α-smooth muscle actin.

Results

α-SMA expression is associated with ESCC mortality in stage I and II. During the period from 2001 to 2010, data were available for 97 patients with ESCC. Table I shows the characteristics of these patients. There were 15 women (15.4%) and 82 men (84.5%). The mean age at surgery (± standard deviation) was 67.2±7.7 years (range, 44-82 years). The median follow-up was 1,244 days, and the minimum follow-up was 94 days. Of the 97 patients, 30 were confirmed to have succumbed to

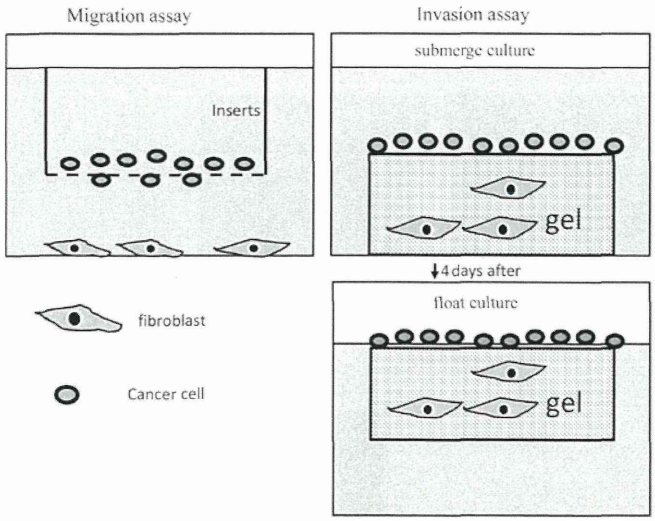

Figure 2. Migration and invasion assay schema. For migration assays, 24-well cell culture inserts with a pore size of 8 μm (Falcon) were used. Inserts were placed in a 24-well plate containing medium with fibroblasts. Cells in serum-free medium were placed in each insert. Migratory cells pass through the membrane and cling to the bottom side. Migratory cells were stained and counted with a VH analyzer (Keyence). For 3-dimensional organotypic cultures, immortalized fibroblasts were mixed with collagen and allowed to contract. Cancer cells were then plated, using a cloning ring. The cells were allowed 4 h to attach to the upper surface of the contracted collagen lattice. Cultures were submerged for 4 days and were then placed in 6-well inserts. Cultures were raised to an air-liquid interface and cultured for 10-14 days.

Table II. Relationship between SMA expression and clinicopathological features.

		α-SMA expression				
Clinicopathological features	n	Low	Intermediate	High	r	P-value
Venous invasion					0.33	<0.01
v0	24	21	2	1		
v+	73	25	22	26		
Nodal involvement					0.18	0.02
pN0	46	28	10	8		
pN+	51	18	14	19		
Recurrence					0.2	0.01
-	64	37	13	14		
+	33	9	11	13		

pN+, positive for pathological lymph node metastasis; v+, positive for pathological venous invasion; n, number; r, correlation coefficient; SMA, smooth muscle actin; α-SMA, α-smooth muscle actin.

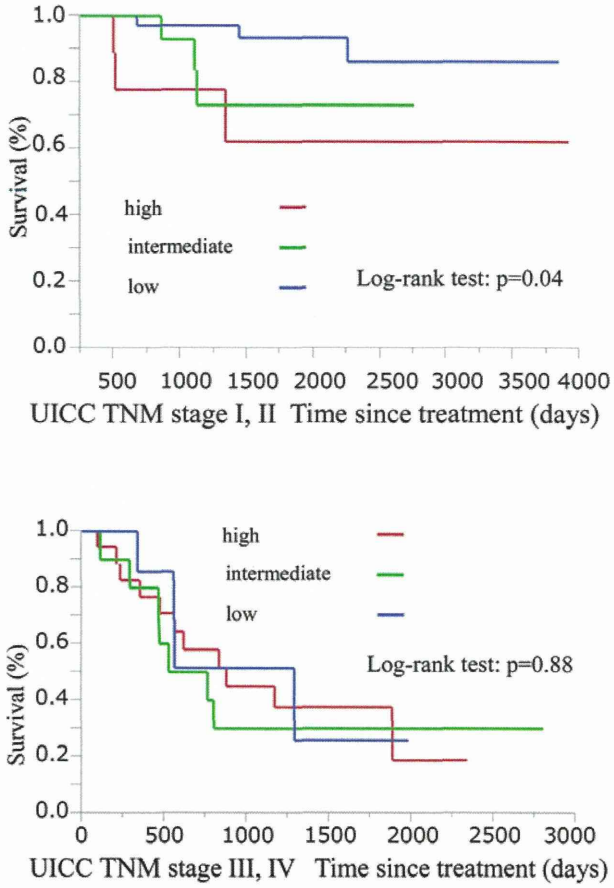

Figure 3. Kaplan-Meier survival curves according to α-SMA expression. In stage I and II disease, survival rate correlated with α-SMA expression (log-rank test, p=0.04), whereas there was no correlation between survival rate and α-SMA expression in stage III and IV disease. α-SMA, α-smooth muscle actin.

ESCC. More than half of the patients (62/97, 64%) presented with relatively early-stage disease (stage I-II according to the TNM classification) (17). Fig. 1 shows typical examples of tumors with low, intermediate and high SMA expression. The percentages of patients with low, intermediate and high α-SMA expression were 47.4, 24.7 and 27.8%, respectively. Clinicopathologically, α-SMA expression correlated with venous invasion, nodal involvement and recurrence (p<0.01, p=0.02 and p=0.01, respectively) (Table II). The mean survival time of the 97 patients with ESCC was 1,720±83 days.

Among patients with stage I and II disease, the Kaplan-Meier survival curves differed significantly according to α-SMA expression (p=0.04). In patients with advanced stage III and IV disease, however, there was no difference in survival according to SMA expression (p=0.88) (Fig. 3).

Isolated human esophageal fibroblast promotes ESCC cell line progression in vitro and in vivo. We used *in vitro* and *in vivo* experimental systems to evaluate interactions between ESCC cells and stromal fibroblasts. In the migration assay, we compared stained images acquired from the bottom membrane of the cell culture inserts according to the presence and absence of fibroblasts. The presence of fibroblasts (HEF75) significantly promoted migration of KYSE150 and TE-11 cells (Fig. 4). For KYSE220, there was virtually no migration of cancer cells through the membrane in the absence of fibroblasts (data not shown). The KYSE150 and KYSE220 cell lines showed invasion into the extracellular matrix gel mixed with human immortalized esophageal fibroblasts (HEF75-hTERT), while cancer cells alone showed no such effect (Fig. 5). Finally, ESCC cells (TE-11 and KYSE220)

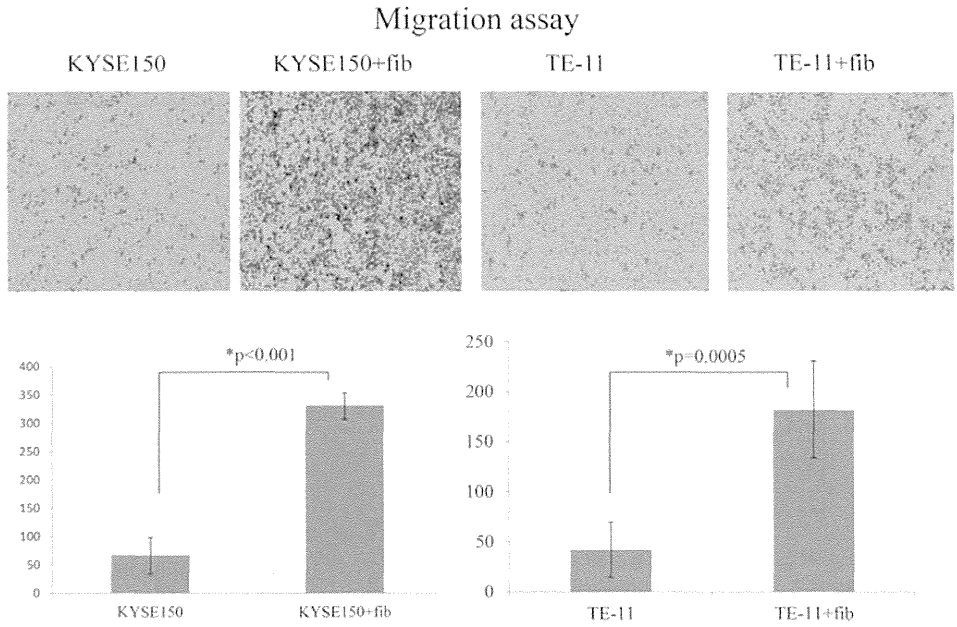

Figure 4. Migration assay. Comparison of stained images acquired from the bottom of the membranes of the cell culture inserts in the presence and absence of fibroblasts. The presence of fibroblasts (HEF75) significantly increased migration of specific cancer cells.

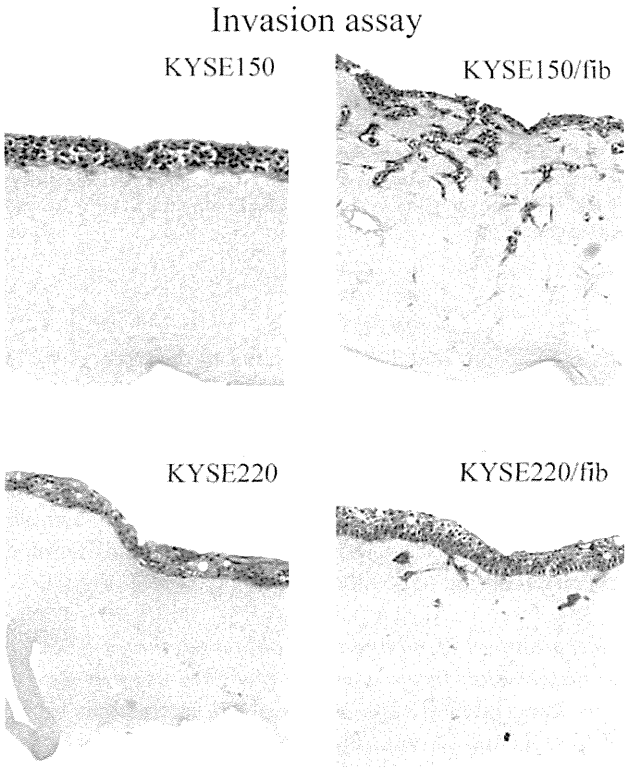

Figure 5. Invasion assay. Cultures were harvested by fixing in 10% formaldehyde and were later embedded in paraffin. KYSE150 and KYSE220 cell lines invaded into the extracellular matrix gel mixed with human immortalized esophageal fibroblasts (HEF75-hTERT), while cancer cells alone did not.

alone or together with immortalized human esophageal fibroblasts (HEF75-hTERT) were suspended in PBS and injected subcutaneously into the flanks of the Nod/Scid mice to assess the effects of fibroblasts on tumorigenicity. Tumors developed in a fibroblast-dependent manner after the implantation of the two ESCC cell lines (Fig. 6).

Discussion

Cancer results from the accumulated effects of many genetic alterations, and the specific combination of changes is reflected in the unique characteristics of each tumor. However, the microenvironment of cancer cells has recently been shown to strongly influence the biologic properties of cancer (7). Activated fibroblasts in tumor stroma, referred to as CAFs, have been associated with poor outcomes in several types of carcinoma (9). α-SMA is the most widely used marker of CAFs (22,23). In the present study, we initially examined expression levels of α-SMA in tumor stroma of resected ESCC specimens. Clinicopathologically, lymph node metastasis, venous invasion and recurrence were significantly related to α-SMA expression levels. Furthermore, increased α-SMA expression was associated with poorer outcomes in patients with earlier stage ESCC. In contrast, there was no relationship between α-SMA expression and outcomes in patients with more advanced disease. These findings suggested that increased stromal α-SMA expression may be a predictor of mortality in patients with earlier stage ESCC.

Next, we conducted migration and invasion assays to assess the effects of stromal fibroblasts. The presence of fibroblasts (HEF75) strongly promoted migration of KYSE150, KYSE220 and TE-11 cells (Fig. 4). Furthermore, the KYSE150 and KYSE220 cell lines invaded into the extracellular matrix gel mixed with human immortalized esophageal fibroblasts (HEF75-hTERT) (Fig. 5). These assays showed that isolated esophageal fibroblasts could promote cancer-cell migration and invasion. Tumor stroma has been suggested to play a critical role in the progression of human ESCC (24). Growth

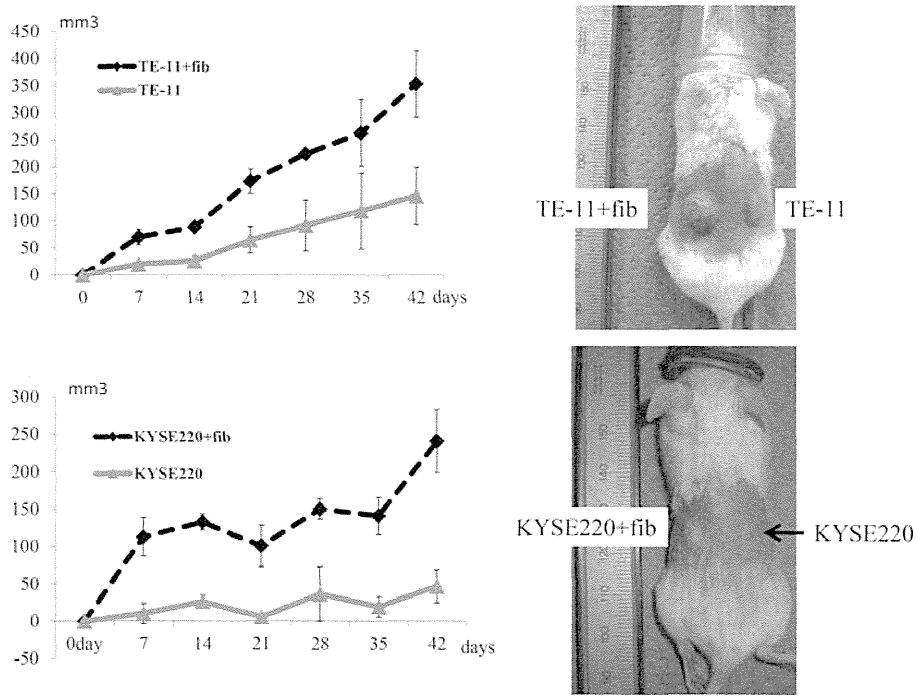

Figure 6. Co-injection into Nod/Scid mice. TE-11 cells plus fibroblasts and KYSE220 cells plus fibroblasts were co-transplanted subcutaneously in Nod/Scid mice. ESCC cells alone or together with HEF75-hTERT were suspended in PBS and injected subcutaneously into the flanks of the mice (two injection sites per mouse). A cancer cell suspension ($2x10^5$ cells/200 μl) was injected subcutaneously into the right side of the mice and a suspension of $2x10^5$ cancer cells plus $6x10^5$ HEF75-hTERT/200 μl into the left side of the mice. The mice were sacrificed after 6 weeks. We measured the minor axis and major axis and calculated the tumor volume as an oval sphere shape. The following formula was used to calculate tumor volume: Volume = 0.5236 ddD (d, minor axis; D, major axis). The data represent means ± standard deviation of three independent experiments.

factors such as hepatocyte growth factor (HGF) and fibroblast growth factors (FGF) secreted by cancer stroma can promote ESCC development and progression (7,25-28). In the present study, growth factors and cytokines (29) secreted by isolated fibroblasts may have played an important role in cancer-cell migration and invasion. Gaggioli *et al* reported that fibroblasts may generate a track within the matrix that SCC cells then use to invade in organotypic culture model experiments (30). Not only paracrine systems induced by growth factors secreted by fibroblasts, but also the ability of fibroblasts to create tracks in the matrix may have promoted cancer-cell invasion in our organotypic assay. *In vivo*, fibroblasts may also have helped cancer cells grow in a fibroblast-dependent manner after the implantation of two ESCC cell lines (Fig. 6). We also subcutaneously transplanted HEF75 and HEF75-hTERT into the Nod/Scid mice and sacrificed the animals 2, 4, and 6 weeks after injection. Immunohistochemical studies of vimentin (clone V9; Dako), which does not react with cells of mice (31), showed that the survival periods of transplanted normal fibroblasts and immortalized fibroblasts were 2 and 4 weeks, respectively (data not shown). These findings suggested that interactions between ESCC cells and fibroblasts play a critical role in the relatively early period after co-transplantation. This hypothesis may be consistent with our finding that higher stromal α-SMA expression was related to poorer survival in patients with earlier stage ESCC.

In conclusion, our data provide evidence that stromal fibroblasts and tumor cells interact to promote tumor progression in ESCC. In patients with earlier stage ESCC, α-SMA may be a predictor of mortality. Our findings also suggest that inhibition of paracrine systems related to fibroblasts and cancer cells may slow or reverse tumor progression.

References

1. Pisani P, Parkin DM, Bray F and Ferlay J: Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 83: 18-29, 1999.
2. Cook MB: Non-acid reflux: the missing link between gastric atrophy and esophageal squamous cell carcinoma? Am J Gastroenterol 106: 1930-1932, 2011.
3. Higuchi K, Koizumi W, Tanabe S, *et al*: Current management of esophageal squamous-cell carcinoma in Japan and other countries. Gastrointest Cancer Res 3: 153-161, 2009.
4. Chang CY, Cook MB, Lee YC, *et al*: Current status of Barrett's esophagus research in Asia. J Gastroenterol Hepatol 26: 240-246, 2011.
5. Hongo M, Nagasaki Y and Shoji T: Epidemiology of esophageal cancer: Orient to Occident. Effects of chronology, geography and ethnicity. J Gastroenterol Hepatol 24: 729-735, 2009.
6. Enzinger PC and Mayer RJ: Esophageal cancer. N Engl J Med 349: 2241-2252, 2003.
7. Mueller MM and Fusenig NE: Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4: 839-849, 2004.
8. Radisky DC, Kenny PA and Bissell MJ: Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? J Cell Biochem 101: 830-839, 2007.
9. Marsh D, Suchak K, Moutasim KA, *et al*: Stromal features are predictive of disease mortality in oral cancer patients. J Pathol 223: 470-481, 2011.
10. De Wever O and Mareel M: Role of tissue stroma in cancer cell invasion. J Pathol 200: 429-447, 2003.
11. Liotta LA and Kohn EC: The microenvironment of the tumour-host interface. Nature 411: 375-379, 2001.
12. Surowiak P, Murawa D, Materna V, *et al*: Occurence of stromal myofibroblasts in the invasive ductal breast cancer tissue is an unfavourable prognostic factor. Anticancer Res 27: 2917-2924, 2007.

13. Kellermann MG, Sobral LM, da Silva SD, *et al*: Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. Histopathology 51: 849-853, 2007.
14. Tsujino T, Seshimo I, Yamamoto H, *et al*: Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res 13: 2082-2090, 2007.
15. Kalluri R and Zeisberg M: Fibroblasts in cancer. Nat Rev Cancer 6: 392-401, 2006.
16. Japanese Esophageal Society: Japanese Classification of Esophageal Cancer, tenth edition: part I. Esophagus 6: 1-25, 2009.
17. Sobin LH GM, Gospodarowicz MK and Wittekind CH (eds): International Union Against Cancer (UICC) TNM Classification of Malignant Tumors. 7th edition. Wiley-Blackwell, 2009.
18. Andl CD, Mizushima T, Nakagawa H, *et al*: Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo. J Biol Chem 278: 1824-1830, 2003.
19. Underwood TJ, Derouet MF, White MJ, *et al*: A comparison of primary oesophageal squamous epithelial cells with HET-1A in organotypic culture. Biol Cell 102: 635-644, 2010.
20. Aoki S, Takezawa T, Uchihashi K, Sugihara H and Toda S: Non-skin mesenchymal cell types support epidermal regeneration in a mesenchymal stem cell or myofibroblast phenotype-independent manner. Pathol Int 59: 368-375, 2009.
21. Okawa T, Michaylira CZ, Kalabis J, *et al*: The functional interplay between EGFR overexpression, hTERT activation, and p53 mutation in esophageal epithelial cells with activation of stromal fibroblasts induces tumor development, invasion, and differentiation. Genes Dev 21: 2788-2803, 2007.
22. Erez N, Truitt M, Olson P, Arron ST and Hanahan D: Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-κB-dependent manner. Cancer Cell 17: 135-147, 2010.
23. Sugimoto H, Mundel TM, Kieran MW and Kalluri R: Identification of fibroblast heterogeneity in the tumor microenvironment. Cancer Biol Ther 5: 1640-1646, 2006.
24. Noma K, Smalley KS, Lioni M, *et al*: The essential role of fibroblasts in esophageal squamous cell carcinoma-induced angiogenesis. Gastroenterology 134: 1981-1993, 2008.
25. Yoshino M, Ishiwata T, Watanabe M, *et al*: Expression and roles of keratinocyte growth factor and its receptor in esophageal cancer cells. Int J Oncol 31: 721-728, 2007.
26. Ren Y, Cao B, Law S, *et al*: Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas. Clin Cancer Res 11: 6190-6197, 2005.
27. Grugan KD, Miller CG, Yao Y, *et al*: Fibroblast-secreted hepatocyte growth factor plays a functional role in esophageal squamous cell carcinoma invasion. Proc Natl Acad Sci USA 107: 11026-11031, 2010.
28. Zhang C, Fu L, Fu J, *et al*: Fibroblast growth factor receptor 2-positive fibroblasts provide a suitable microenvironment for tumor development and progression in esophageal carcinoma. Clin Cancer Res 15: 4017-4027, 2009.
29. Lederle W, Depner S, Schnur S, *et al*: IL-6 promotes malignant growth of skin SCCs by regulating a network of autocrine and paracrine cytokines. Int J Cancer 128: 2803-2814, 2011.
30. Gaggioli C, Hooper S, Hidalgo-Carcedo C, *et al*: Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol 9: 1392-1400, 2007.
31. Bohn W, Wiegers W, Beuttenmüller M and Traub P: Species-specific recognition patterns of monoclonal antibodies directed against vimentin. Exp Cell Res 201: 1-7, 1992.

The HSP90 inhibitor 17-N-allylamino-17-demethoxy geldanamycin (17-AAG) synergizes with cisplatin and induces apoptosis in cisplatin-resistant esophageal squamous cell carcinoma cell lines via the Akt/XIAP pathway

TAKASHI UI[1], KAZUE MORISHIMA[1], SHIN SAITO[1], YUJI SAKUMA[2], HIROFUMI FUJII[3], YOSHINORI HOSOYA[1], SHUMPEI ISHIKAWA[4], HIROYUKI ABURATANI[5], MASASHI FUKAYAMA[6], TOSHIRO NIKI[2] and YOSHIKAZU YASUDA[1]

Departments of [1]Surgery, [2]Pathology and [3]Clinical Oncology, Jichi Medical University, Shimotsuke, Tochigi 329-0498; [4]Division of Genomic Pathology, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8510; [5]Division of Genome Science, Research Center for Advanced Science and Technology, the University of Tokyo, Meguro-ku, Tokyo 153-8904; [6]Department of Pathology, Graduate School of Medicine, the University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

Received October 9, 2013; Accepted November 20, 2013

DOI: 10.3892/or.2013.2899

Abstract. Although cisplatin (CDDP) is a key drug in the treatment of esophageal squamous cell carcinoma (ESCC), acquired chemoresistance remains a major problem. Combination therapy may represent one strategy to overcome this resistance. Heat shock protein 90 (HSP90) is known to be overexpressed in several types of cancer cells, and its inhibition by small molecules, either alone or in combination, has shown promise in the treatment of solid malignancies. In the present study, we evaluated the synergistic effects of combining CDDP with the HSP90 inhibitor 17-N-allylamino-17-demethoxy geldanamycin (17-AAG) on two CDDP-resistant human esophageal squamous cancer cell lines, KYSE30 and KYSE150. The results obtained demonstrated the synergistic inhibitory effects of CDDP and 17-AAG on the growth of KYSE30 and KYSE150 cells. Cell growth and cell number were more effectively reduced by the combined treatment with CDDP and 17-AAG than by the treatment with either CDDP or 17-AAG alone. Western blotting revealed that the combined action of CDDP and 17-AAG cleaved poly (ADP-ribose) polymerase (PARP) and caspase-3, which demonstrated that the reduction in both cell growth and cell number was mediated by apoptosis. Time-course experiments showed that reduction in X-linked inhibitor of apoptosis protein (XIAP) and phosphorylated Akt were concomitant with apoptosis. The results of the present study demonstrate that 17-AAG synergizes with CDDP and induces apoptosis in CDDP-resistant ESCC cell lines, and also that modulation of the Akt/XIAP pathway may underlie this synergistic effect. Combination therapy with CDDP and an HSP90 inhibitor may represent a promising strategy to overcome CDDP resistance in ESCC.

Correspondence to: Professor Toshiro Niki, Department of Pathology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi, 329-0498, Japan
E-mail: tniki@jichi.ac.jp

Key words: cisplatin, tanespimycin, HSP90 heat shock protein inhibitor, esophageal squamous cell carcinoma

Introduction

Esophageal cancer is one of the most aggressive types of cancer of the gastrointestinal tract. The estimated incidence of esophageal cancer was 3.8% of all cancers and the estimated cancer mortality worldwide was 5.4% in 2008 (1). The incidence of esophageal cancer is particularly high in both males and females in East Asia, with squamous cell carcinoma being the predominant histologic type in this region, accounting for more than 90% of all esophageal cancer cases (1). Multimodality therapy, including surgery, chemotherapy and radiotherapy, is required for the effective management of advanced esophageal cancer. However, the 5-year survival rates of patients treated using surgery, chemotherapy alone, radiotherapy alone and concurrent chemoradiotherapy were reported to be 50.2, 8.6, 15.5 and 26.4%, respectively (2). Therefore, further improvements in outcomes are urgently required (2-5).

Cisplatin (CDDP) is a platinum drug that is widely used to treat esophageal squamous cell carcinoma (ESCC) (6,7). CDDP, either alone or in combination with other agents, has been shown to improve patient outcomes. However, acquired chemoresistance develops during the course of treatment and is often the reason for treatment failure. Therefore, overcoming chemoresistance is essential for improving the outcomes of patients.

Molecular chaperone proteins function to ensure the proper conformation of client proteins when cells experience stress or damage (8). Heat shock protein 90 (HSP90) is a molecular chaperone that participates in stabilizing and activating more than 200 proteins, including serine/threonine kinases (Akt, Raf-1, and Cdk4), and the transcription factors hypoxia-inducible factor 1α (HIF1α) and p53, receptor/non-receptor kinases (HER2, EGFR, and Src family kinases), and steroid hormone receptors (9). Since many of these client proteins have been shown to significantly contribute to tumor growth and survival, abrogating their function with an HSP90 inhibitor is an attractive prospect (10).

In this study, we explored the potential synergistic effect of CDDP and the HSP90 inhibitor, 17-N-allylamino-17-demethoxy geldanamycin (17-AAG), on human ESCC cell lines. We also attempted to identify the molecular mechanism involved in this synergistic effect.

Materials and methods

Cell lines and culture. The TE series (TE1, TE4, TE5, TE6, TE8, TE9, TE10, TE11, TE14 and TE15) and EC-GI-10 were obtained from the Riken BioResource Center (Saitama, Japan). The KYSE series (KYSE30, KYSE70, KYSE140, KYSE150, KYSE170, KYSE180, KYSE220 and KYSE270), TT, and TTn were obtained from the Health Science Foundation (Tokyo, Japan). These cell lines were derived from human esophageal squamous cancer. Cells were cultured in a 5% CO_2 atmosphere at 37°C in RPMI-1640 (R8758; Sigma-Aldrich, St. Louis, MO, USA) complete medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Chemicals and antibodies. CDDP and 17-AAG were purchased from Sigma-Aldrich. CDDP was dissolved in 0.9% sodium chloride solution and 17-AAG was dissolved in dimethyl sulfoxide (DMSO). All drugs were stored in aliquots at -20°C.

Antibodies to caspase-3 (#9662), PARP (#9542), XIAP (3B6) (#2045), c-IAP1 (#4952), c-IAP2(58C7) (#3130), livin (D61D1) XP (#5471), survivin (71G4B7) (#2808), phospho-Akt (Ser473) (D9E) (#4060), Akt (#9272), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9101), and p44/p22 MAPK (Erk1/2) (#9102) were from Cell Signaling Technologies (Beverly, MA, USA). The antibody to β-actin was from Sigma-Aldrich. Antibodies to Bcl-2 (#610391), Bcl-xL (#60982), Bid (#61158), Bad (#610391), Bax (#610982), Beclin (#612112), and BAG-1 (#611868) were from BD Biosciences (San Jose, CA, USA).

Drug sensitivity assay. Cells were suspended in RPMI/10% FBS and seeded at between 2,000 and 4,000 cells/well in quintuplicate in 96-well plates. Cells were treated with varying doses of CDDP and 17-AAG 24 h after plating and were allowed to grow for an additional 72 h. Viable cell density was determined by a water-soluble tetrazolium salt (WST-8, Cell Counting kit-8; Dojindo, Japan) according to the manufacturer's instructions using a microplate absorbance reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

Determination of IC_{50} and combination index (CI). IC_{50} was calculated using the CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). CI values were calculated for 50% toxicity based on the equation below (11):

$$CI = D_1 / Dx_1 + D_2 / Dx_2 + \alpha \times [(D_1 \times D_2) / (Dx_1 \times Dx_2)]$$

where, Dx_1 = Dose of drug 1 to produce 50% cell kill alone; D_1 = Dose of drug 1 to produce 50% cell kill in combination with D_2; Dx_2 = Dose of drug 2 to produce 50% cell kill alone; D_2 = Dose of drug 2 to produce 50% cell kill in combination with D_1; α=0 for mutually exclusive or 1 for mutually non-exclusive modes of drug action.

Time-dependent cell growth assay. Equal numbers of cells were seeded in quintuplicate in 96-well plates and cell growth was measured using WST-8 Cell Counting kit-8 at 0, 24, 48 and 72 h. The results were expressed as percentages relative to the absorbance at 0 h.

Western blot analysis. KYSE30 and KYSE150 cells were seeded on 100 mm plates and were exposed to CDDP with/without 17-AAG after 24 h. Cells were subsequently cultured for 24, 48 and 72 h; adherent and floating cells were then pelleted, washed with cold PBS and lysed in RIPA buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 50 mM NaF, 20 μM Na_3VO_4 and protease inhibitor]. Lysates were centrifuged at 15,000 rpm at 4°C for 10 min and the protein concentration in each sample was determined by the BCA Protein Assay kit (Takara Bio, Inc., Shiga, Japan). Cell lysates or their fractions containing equal amounts of protein (12 μg) were resolved by the Mini-Protean TGX Precast Gel (Bio-Rad Laboratories) and transferred to nitrocellulose membranes. Membranes were probed with the primary antibody, followed by the secondary antibody conjugated to HRP, developed using western blot detection reagents (Amersham Biosciences, Uppsala, Sweden), and then detected by the ChemiDoc SRS image analysis system (Bio-Rad Laboratories).

Statistical analysis. Statistical analyses were performed with IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). P-values of <0.05 were considered to indicate a statistically significant difference. We used Dunnett's test for the time-dependent cell growth assay. Data are presented as means ± standard deviation.

Results

Determination of IC_{50} for CDDP and 17-AAG across a panel of ESCC cell lines. Treating esophageal cell lines with CDDP and 17-AAG resulted in dose-dependent cytotoxicity. Fig. 1 shows IC_{50} values for CDDP (Fig. 1A) and 17-AAG (Fig. 1B) across our panel of ESCC cell lines. IC_{50} values ranged from 0.983 to 14.9 μM for CDDP, and 0.0128 to 2.37 μM for 17-AAG, respectively. Based on these results, we decided to use KYSE30, KYSE150, EC-GI-10 and TE6 as representative CDDP-resistant cell lines in subsequent experiments.

CDDP and 17-AAG exhibit synergistic inhibitory effects on the growth of KYSE30 and KYSE150 esophageal squamous carcinoma cell lines. To evaluate the impact of the combined treatment with CDDP and 17-AAG on CDDP-resistant cell