厚生労働科学研究費補助金（萌芽的先端医療技術推進研究事業）

（分担）研究報告書

食道癌生検標本の遺伝子発現プロファイル解析による放射線化学療法感受性予測の臨床導入を目指した基盤的研究　（H18-ファーマコ一般-003）

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研究要旨

食道癌治療において化学放射線療法は次第に位置を占めるようになってきている。しかし、事前にその奏効を予測する方法はなく、無効の場合には通常の手術に比して数倍のリスクを負った救済手術を施行せざるを得なくなる。治療の個別化を目的として、マイクロアレイを用いた遺伝子プロファイル解析による治療感受性予測研究を計画した。

A．研究目的

化学放射線療法を予定した病期Ⅱ・Ⅲ（T4除く）の胸部食道扁平上皮癌患者を対象として、高感度・高再現性DNAチップを用いて治療前生検標本の発現遺伝子プロファイルを解析する。化学放射線療法の治療効果を予測する可能性が高い遺伝子群を同定し、食道癌における化学放射線療法感受性予測アルゴリズムの作成を目的とする。

B．研究方法

本研究の適格基準を満たす食道癌患者のうち、CDDP/5FU:70(2)/700(2):d1/d1-4と外照射60GyかCDDP/5FU:75(2)/1000(2):d1/d1-4と外照射50.4Gyの化学放射線療法を予定したものから、治療開始前に内視鏡生検により腫瘍組織2個、正常組織1個を採取する。検体はRNA-later液に浸透保存するかマイナス80℃で保管し、京都大学薬学研究科に移送、マイクロアレイ法により遺伝子発現レベルの測定を行う。化学放射線療法の臨床効果とのマッチングを行い、感受性または抵抗性関与遺伝子をスクリーニングする。

（倫理面への配慮）

個人が特定できる様な情報は検体から削除し、個人の検定には検体番号を付すものの、「匿名化番号対照表」にて一元化管理することにより、個人情報は秘匿される。本研究は各施設の倫理審査委員会（IRB）の承認を得たうえで開始する。また、患者本人から文書によるインフォームド・コンセントを得ることを前提としている。

C．研究結果

現在、当センターでは倫理審査委員会により承認が得られたところで、研究は端緒についたばかりである。適格症例があれば今後積極的に登録を行う予定である。

D．E．考察と結論

本研究により化学放射線療法感受性予測アルゴリズムが完成すれば、Ⅱ・Ⅲ期に限らず、あらゆる病期の食道癌の個別化治療戦略に繋がる可能性がある。集中的な症例集積により、早急に結論を出すことが望まれる。

G．研究発表

1．論文発表

Shinoda M
Clinical Aspects of Multimodality Therapy for Resectable Locoregional Esophageal Cancer.
Ann Thorac Cardiovasc Surg
12(4)：234-241　2006

2．学会発表

なし

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tanaka E, Shimada Y et al.	The suppression of Aurora-A/STK15/BTAK expression enhances chemo-sensitivity to Docetaxel in human esophageal squamous cell carcinoma.	Clin Cancer Res	13	1331-1340	2007
嶋田裕	分子生物学を考慮した食道癌３領域郭清の適応	外科治療	95	337-342	2006
Kondo K, Shimada Y et al.	Cisplatin-dependent upregulation of death receptors 4 and 5 auguments induction of apoptosis by TNF-related apoptosis-inducing ligand against esophageal squamous cell carcinoma.	Int J Cancer	118	230-242	2006
Umeoka S , Shimada Y et al.	Esophageal Cancer: Evaluation with triple-phase Dynamic CT-Initial Experience	Radiology	239	777-783	2006
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研究成果の刊行物・別刷

The Suppression of *Aurora-A/STK15/BTAK* Expression Enhances Chemosensitivity to Docetaxel in Human Esophageal Squamous Cell Carcinoma

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Abstract

Purpose: We previously reported that the expression of Aurora-A was frequently up-regulated in human esophageal squamous cell carcinoma (ESCC) tissues as well as cell lines and the up-regulation contributed to a poor prognosis. In this study, we assessed the possibility of Aurora-A suppression as a therapeutic target for ESCC using ESCC cell lines.

Experimental Design: We established subclones using vector-based short hairpin RNA (shRNA). Then, we investigated the effect of Aurora-A suppression on proliferation and cell cycle changes *in vitro*. Next, chemosensitivity against docetaxel was investigated by tetrazolium salt–based proliferation assay (WST assay) and cell number determinations, and furthermore, the type of cell death induced by docetaxel was analyzed by flow cytometry. Finally, to examine the effect of Aurora-A shRNA on proliferation and chemosensitivity against docetaxel *in vivo*, a s.c. tumor formation assay in nude mice was done.

Results: We established two genetically different stable cell lines (510 A and 1440 A) in which levels of Aurora-A were reduced. Cell growth was inhibited by 38.7% in 510 A and by 24.3% in 1440 A *in vitro* compared with empty vector–transfected controls (510 m and 1440 m), and this growth inhibition was mediated through G_2-M arrest as confirmed by flow cytometry. Next, in a WST assay, the IC_{50} for Aurora-A shRNA-transfected cells was lower than that of empty vector–transfected cells (510 A, 2.7×10^{-7} mol/L; 510 m, 4.8×10^{-7} mol/L; 1440 A, 2.6×10^{-7} mol/L; 1440 m, 4.9×10^{-7} mol/L). In addition, 0.3 nmol/L docetaxel induced a notable level of apoptosis in Aurora-A shRNA-transfected cells compared with empty vector–transfected cells. In the assay of s.c. tumors in nude mice, tumor growth in 510 A was inhibited by 36.1% compared with that in 510 m, and in tumors treated with docetaxel, the suppression of Aurora-A resulted in 44.0% tumor growth suppression *in vivo*.

Conclusions: These results indicated that Aurora-A might play an important role in chemosensitivity to docetaxel, and the suppression of its expression might be a potential therapeutic target for ESCC.

Several proteins strictly regulate the process of cellular division. Defects in chromatid segregation cause genetic instability, a condition associated with tumorigenesis. During the proliferation of normal cells, the centrosome ensures the equal segregation of chromosomes to the postmitotic daughter cells by organizing the bipolar mitotic spindle. In contrast, in cancer cells, multipolar mitotic spindles and various centrosomal anomalies, such as supernumerary centrosomes, centrosomes of abnormal size and shape, aberrantly phosphorylated centrosomic proteins, and prematurely split centrosomes, are frequently observed (1–7).

Aurora-A, a member of the Aurora/Ipl1p family of cell cycle–regulating serine/threonine kinases, is expressed at interphase mitotic centrosomes and the spindle poles in the nucleus where it regulates segregation of chromosomes and cytokinesis. Recent studies have shown that the ectopic expression of Aurora-A in mouse NIH/3T3 cells and Rat 1 fibroblasts causes centrosome amplification and transformation *in vitro* as well as tumorigenesis *in vivo* (8, 9). Furthermore, the up-regulation of Aurora-A expression in diploid human breast epithelial cells leads to abnormal numbers of centrosomes and the induction of aneuploidy (8). A correlation between the up-regulated expression of Aurora-A and clinical aggressiveness has also been reported for several cancers (10–14). Moreover, recent reports showed that the up-regulation of Aurora-A resulted in resistance

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to apoptosis induced by taxanes in a human cancer cell line (15, 16) and inhibition of the expression of Aurora-A resulted in potent antitumor activity and chemosensitizing activity to taxanes in pancreatic cancer (17). These findings suggest that *Aurora-A* is a critical kinase-encoding gene and a potential chemotherapeutic target.

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies in the world, including Japan, despite the recent refinement of various therapeutic strategies, including surgery, chemotherapy, radiotherapy, and combined therapy (18, 19). Docetaxel is used as monotherapy or in combination with other agents to treat ESCC, but its activity is far from satisfactory (20, 21). Therefore, identifying and targeting genes conducive to the treatment of ESCC, such as enhancement of conventional chemotherapy, is necessary to improve the survival of patients with this type of refractory cancer.

Using comparative genomic hybridization, we previously investigated changes in the copy number of chromosomes in 29 ESCC cell lines and found that a chromosome gain of the proximal part of 20q, where the *Aurora-A* gene is located, is one of the most common sites of aberrations (19 of 29, 65.5%; ref. 22). We also reported that the expression of Aurora-A was frequently up-regulated in ESCC tissues as well as cell lines, and this contributed to a poor prognosis (14). In the present study, to further elucidate the possibility of using *Aurora-A* in the treatment of human ESCC, we analyzed the phenotypic changes of cultured ESCC cells induced by suppression of Aurora-A expression using a plasmid vector-mediated short hairpin RNA (shRNA) expression system, especially synergistic enhancement of the cytotoxicity of docetaxel.

Materials and Methods

Cell culture. All tested ESCC cell lines of the KYSE series were established in our laboratory and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) and Ham's F12 (Nissui Pharmaceutical, Tokyo, Japan) mixed (1:1) medium containing 2% fetal bovine serum (23). HeLa cells were purchased from the American Type Culture Collection (Rockville, MD), cultured in DMEM (Life Technologies) with 10% FCS, and used as a positive control (24, 25).

Western blot analysis. Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100] containing Complete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). Cell lysates were sonicated and the protein concentration was estimated by the Bradford method using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Cell lysates (15 μg) were electrophoresed on 2% to 15% gradient polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a semidry transfer blot system (Bio-Rad, Hercules, CA). After blocking with TBS containing 1% Tween 20 and 5% skim milk for 1 h, the membranes were incubated at 4°C overnight with anti-human Aurora-A polyclonal antibody (diluted 1:100; TransGenic, Inc., Kumamoto, Japan) or anti-human β-actin monoclonal antibody (1:2,000; Sigma, Inc., St. Louis, MO). The membranes were subsequently incubated at room temperature for 1 h with secondary antibody and analyzed using enhanced chemiluminescence plus reagent (Amersham, Buckinghamshire, United Kingdom). Quantitative analysis was done on a Macintosh computer using the public domain NIH Image program version 1.61 (developed at the NIH and available on the Internet).[3]

Immunofluorescent staining. 510 A, 510 m, 1440 A, and 1440 m cells were cultured onto collagen-coated glass coverslips (BD Biosciences, Bedford, MA). Then, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were incubated with 0.3% Triton X-100 solution for 15 min at room temperature, and the cells were blocked for 1 h with 2% fetal bovine serum in PBS at room temperature. Subsequently, the cells were incubated with anti-human Aurora-A polyclonal antibody (diluted 1:50; TransGenic) for 1 h at room temperature. After washing twice with PBS, the cells were incubated with goat anti-rabbit FITC-conjugated secondary antibody (Invitrogen Co., Carlsbad, CA) for 1 h at room temperature. The cells were washed and mounted in glycerol and viewed under a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany).

Construction of an Aurora-A shRNA expression vector. To construct a vector for Aurora-A shRNA, the pSUPERIOR-puro (OligoEngine, Seattle, WA) was digested with *Bgl*II and *Hin*dIII (TaKaRa Bio, Shiga, Japan). A chemically synthesized oligonucleotide encoding an Aurora-A short hairpin small interfering RNA, including a loop motif, was inserted downstream of the H1 promoter of the plasmid using a DNA ligation kit (TaKaRa Bio) and cloned. The sequence of the oligonucleotide targeted at Aurora-A is 5′-ATGCCCTGTCTTACTGTCA-3′ for KYSE 510, corresponding to positions 853 to 871 within the Aurora-A mRNA sequence (17). To confirm the result, we designed another sequence for KYSE 1440, 5′-GCCGGTTCAGAATCAGAAG-3′, corresponding to positions 335 to 353 within the Aurora-A mRNA. For the negative control vector, an empty pSUPERIOR-puro was used. We checked the internal stabilities of each sequences using Oligo 4.0-s software (National Biosciences, Inc., Plymouth, MN) and found that the 3′-end in the sense strand was less stable than the 5′-end, which is known to cause less off-target effects according to literature (26). Furthermore, we checked each sequence using BLAST Web site software[4] and found that each sequence was specific to *Aurora-A*. Moreover, we also stably transfected each sequence to HeLa cell, and preliminary experiments were undertaken to further confirm their effects (Supplementary Data 1).

Transfections. The ESCC cell lines KYSE 510 and KYSE 1440 were stably transfected with the Aurora-A shRNA expression vector or the empty pSUPERIOR-puro vector using Lipofectin reagent (Invitrogen) as suggested by the manufacturer's instruction. Briefly, 2 μg of each plasmid DNA and 20 μL of Lipofectin reagent together with Opti-MEM I medium (Invitrogen) were used with serum-free medium for 8 h. Cells were incubated for another 48 h with normal growth medium, and subsequently, the cell clones were selected against 1.0 μg/mL puromycin (Nacalai Tesque, Kyoto, Japan) for 3 weeks, and we picked up single colonies originated from single cells and expanded to obtain stably transfected cell lines.

WST assay for sensitivity to doxetaxel. Cytotoxic activity against docetaxel was measured by the tetrazolium salt–based proliferation assay (WST-8 assay; Wako Chemicals, Osaka, Japan) following the manufacturer's instructions. Briefly, cells were cultured in 96-well microtiter plates in 90 μL of growth medium (4,000 cells per well) and incubated for 24 h for sufficient cell growth. Then, 10 μL of a graded concentration of docetaxel (10^{-5} to 10^{-10} mol/L) were added into each well and cultured for 48 h. Control cultures received normal growth medium only. After 48 h, 10 μL of WST-8 solution were added to each well and the plates were incubated at 37°C for another 3 h. Absorbance at 450 and 640 nm was measured using the Delta Soft ELISA analysis program, and cell viability was measured and compared with that of control cells. Each experiment was carried out independently and repeated at least thrice. The IC_{50} value was defined as the concentration that reduced the absorbance in each test by 50%.

Cell proliferation assay. Cells were cultured in 6-cm dishes (2×10^4 per dish) and incubated for 24 h for sufficient cell growth and then treated with medium containing 0.3, 0.6, or 1.0 nmol/L of docetaxel for

[3] http://rsb.info.nih.gov/nih-image/

[4] http://www.ddbj.nig.ac.jp/search/blast-j.html

48 h. Then, they were cultured for another 48 h with normal growth medium. Control cultures received normal growth medium only. Cells were harvested with trypsin/EDTA every 48 h for 4 days and enumerated using a cell counter (Coulter Z1, Beckman Coulter, Fullerton, CA). A comparison was made with the control culture to examine the effect of suppressing the expression of Aurora-A on cell proliferation and to investigate the effect of the suppression on chemosensitivity to docetaxel. The experiment was repeated at least thrice.

Flow cytometry for analyzing the cell cycle. A flow cytometric analysis of DNA content was done to assess the cell cycle phase distribution. Cells were harvested at the 70% confluent stage and fixed in 70% ethanol at −20°C. After being washed with PBS, the cells were treated with PBS containing RNase A (100 mg/mL) at 37°C for 30 min. After centrifugation, the cells were resuspended in PBS containing propidium iodide (50 μg/mL) and stained at room temperature for 30 min. DNA content was evaluated using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest software (Becton Dickinson). The experiment was repeated thrice.

Flow cytometry for detection of apoptotic cells. KYSE cells transfected with empty vector or shRNA against Aurora-A were cultured in 6-cm dishes (2×10^4 per dish) and treated as already described for the proliferation experiment. Briefly, cells were incubated for 24 h and treated with medium containing 0, 0.3, 0.6, or 1.0 nmol/L of docetaxel for 48 h. Then, they were cultured for another 48 h with normal growth medium. Subsequently, floating cells in the medium and adherent cells were collected. Using an Annexin V-FITC Apoptosis Detection kit (Medical & Biological Laboratories Co. Ltd., Woburn, MA), cells were stained with Annexin V-FITC and propidium iodide according to the manufacturer's instructions. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained) from early apoptotic cells (stained only with Annexin V) and late apoptotic or necrotic cells (stained with both Annexin V and propidium iodide). Untreated cells and cells treated with 3% formaldehyde for 30 min served, respectively, as negative and positive controls for double staining. Cells were analyzed immediately after staining by using a FACScan flow cytometer and the CellQuest software. For each measurement, >10,000 cells were counted.

Tumor formation assay in nude mice. Suspensions of 1.0×10^6 KYSE 510-derived cells (Aurora-A shRNA-transfected cells, 510 A; empty vector-transfected cells, 510 m) in PBS (50 μL) were injected s.c. into the left flanks of 5-week-old male BALB/c *nu/nu* mice (Japan SLC, Shizuoka, Japan) at day 0. The inoculation was conducted in five mice, and tumor growth was estimated from the average volume of tumors. Tumor volume was calculated by the formula $1/2 \times L^2 \times W$ (L = length and and W = width of the tumor). At 46 days after inoculation, all mice were sacrificed, and s.c. tumors were resected and fixed in 10% formaldehyde/PBS. The tumors were paraffin embedded and stained with H&E and for Aurora-A. Immunohistochemical staining for Aurora-A was done as reported previously (14). All the animal experiments were done in accordance with institutional guidelines of the Kyoto University.

Treatment with docetaxel in nude mice. 510 m and 510 A tumors were generated as above. Briefly, suspensions of 1.0×10^6 cells in PBS (50 μL) were injected s.c. into the left flanks of 5-week-old male BALB/c *nu/nu* mice at day 0. The inoculation was conducted in five mice, and mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly) or with 0.1 mL PBS (pH 7.4; i.p., thrice weekly) as described previously with modifications (27). Tumor growth was estimated from the average volume of tumors, and tumor volume was calculated as already described. At 39 days after inoculation, all mice were sacrificed, and s.c. tumors were resected, and tumor size was compared. All the animal experiments were done in accordance with institutional guidelines of the Kyoto University.

Statistical analysis. All experiments were done in duplicate or triplicate. The Bonferroni multiple comparison test and t test were used for the statistical analysis of comparative data using StatView version 5 (SAS Institute, Cary, NC). Values of $P < 0.05$ were considered significant and are indicated by asterisks in the figures.

Results

Expression of Aurora-A and sensitivity to docetaxel in ESCC cell lines. Levels of Aurora-A protein expression in three cancer cell lines were determined by Western blotting (Fig. 1A). KYSE 510 and KYSE 1440 had higher levels of expression than KYSE 110. Next, the sensitivity of the cell lines to docetaxel was checked by WST assay. As shown in Fig. 1B, IC_{50} of KYSE 510 and KYSE 1440 ($4.9 \times 10^{-7} \pm 0.20 \times 10^{-7}$ mol/L and $4.6 \times 10^{-7} \pm 0.46 \times 10^{-7}$ mol/L, respectively) was higher than that of KYSE 110 ($2.4 \times 10^{-8} \pm 0.21 \times 10^{-8}$ mol/L; Fig. 1B). Thus, the cell lines with the higher levels of Aurora-A protein (KYSE 510 and KYSE 1440) were more resistant to docetaxel than the cell line with the lower level (KYSE 110).

Vector-based Aurora-A shRNA decreased proliferation in ESCC cell lines. To assess the role of the overexpression of Aurora-A in ESCC cells, we first established subclones via the transfection of vector-based shRNA for Aurora-A in KYSE 510 and KYSE

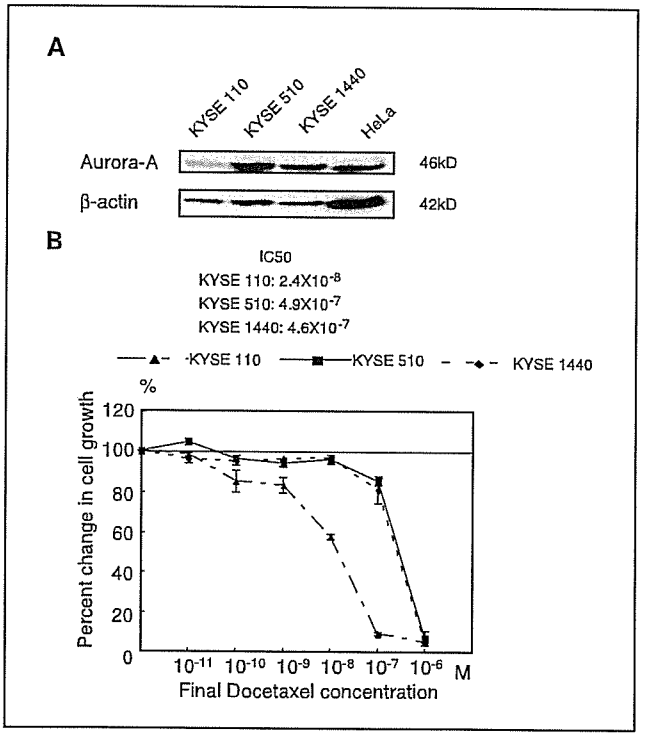

Fig. 1. Aurora-A expression and chemosensitivity to docetaxel in ESCC cell lines. *A*, expression of Aurora-A protein in ESCC cell lines. Immunoblots were probed with the anti-Aurora-A antibody and the anti-β-actin antibody. The HeLa cell line was included as a positive control of Aurora-A expression. *B*, cytotoxic activities of docetaxel in ESCC cell lines were measured by WST-8 assay. Graded concentrations of docetaxel (10^{-5} to 10^{-10} mol/L) were added to the wells, and the cells were cultured for 48 h. Control cultures received normal growth medium only. Cell viability was measured as absorbance at 450 nm, and values were compared with the control. The IC_{50} value was defined as the concentration that reduced the absorbance in each test by 50%. X axis, final docetaxel concentration (10^{-6} to 10^{-11} mol/L). The experiments were repeated thrice.

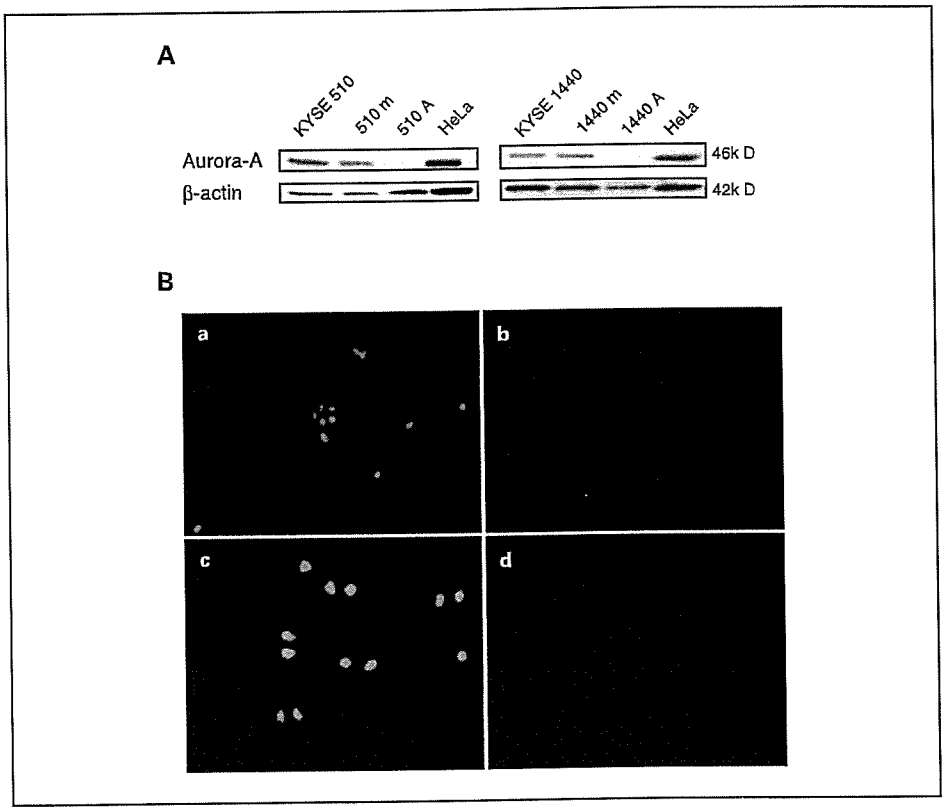

Fig. 2. Vector-based Aurora-A shRNA decreased proliferation of ESCC cell lines. *A*, suppression of Aurora-A protein expression with the Aurora-A shRNA vector. The KYSE 510 and KYSE 1440 cells were stably transfected with an empty vector (510 m or 1440 m) or an Aurora-A shRNA vector (510 A or 1440 A). The expression of Aurora-A protein in these clones was examined by Western blotting. Bottom, protein expression of β-actin. *B*, representative immunofluorescent staining of Aurora-A in an Aurora-A shRNA-transfected cells (510 A or 1440 A) or an empty vector–transfected cells (510 m or 1440 m). a, immunofluorescent staining of Aurora-A in empty vector–transfected cells (510 m). Magnification, ×200. Strong nuclear staining was observed. b, immunofluorescent staining of Aurora-A shRNA-transfected cells (510 A). Magnification, ×200. Weak nuclear staining was observed. c, immunofluorescent staining of Aurora-A in empty vector–transfected cells (1440 m). Magnification, ×200. Strong nuclear staining was observed. d, immunofluorescent staining of Aurora-A in Aurora-A shRNA-transfected cells (1440 A). Magnification, ×200. Weak nuclear staining was observed.

1440 cells. The levels of Aurora-A expression were efficiently reduced by 87% in the stable subclone 510 A and by 90% in 1440 A but were not reduced in empty vector–transfected clones (510 m and 1440 m; Fig. 2A). We also confirmed suppression of Aurora-A expression by immunofluorescent staining and found that Aurora-A immunoreactivity in established stable subclone 510 A and 1440 A was homogeneously depleted (Fig. 2B). We investigated the effect of suppressing the expression of Aurora-A on proliferation in ESCC cell lines. With suppression of Aurora-A expression, cell growth was inhibited by 38.7 ± 10.5% in 510 A and by 24.3 ± 10.0% in 1440 A compared with empty vector–transfected cells (510 m and 1440 m; $P < 0.01$ and 0.01, respectively; Fig. 2C). To investigate the growth suppression caused by inhibition of Aurora-A expression, cell cycle changes in 510 A and 1440 A as well as in empty vector–transfected cells were examined by flow cytometry. The population of cells in G_2-M phase was significantly larger in 510 A (41.13 ± 6.94%) and 1440 A (38.35 ± 1.06%) than that in the empty vector–transfected cells (510 m: 24.12 ± 2.33%, $P < 0.01$; 1440 m: 22.55 ± 2.25%, $P < 0.01$; Fig. 2D).

The effect of suppressing Aurora-A on chemosensitivity to docetaxel in vitro. Chemosensitivity to docetaxel was investigated by WST assay and cell number determinations. In the WST assay, we found that IC_{50} of stable transfectants of shRNA for Aurora-A was lower than that of the empty vector transfectant (510 A, $2.7 \times 10^{-7} \pm 0.4 \times 10^{-7}$ mol/L; 510 m, $4.8 \times 10^{-7} \pm 0.7 \times 10^{-7}$ mol/L; 1440 A, $2.6 \times 10^{-7} \pm 0.8 \times 10^{-7}$ mol/L; 1440 m, $4.9 \times 10^{-7} \pm 0.4 \times 10^{-7}$ mol/L; Fig. 3A and B). We also confirmed the effect of suppressing Aurora-A expression on chemosensitivity to docetaxel by cell number determinations. In the cell number determinations, the suppression of the expression of Aurora-A allowed even 0.3 nmol/L docetaxel to be effective (Fig. 3C and D). Discrepancy in effective concentration of docetaxel between WST assay and cell number determinations could be explained by the difference in the initial numbers of cells, cell concentration at the beginning of each experiment, as well as the difference in size of culture plates and observation period. We repeated the cell number determinations with the same cell concentration and observation period as WST assay and confirmed that growth-inhibitory effect of docetaxel in these two different experiments was similar (Supplementary Data 2). Importantly, in respective experiments, we found that the suppression of Aurora-A expression enhanced chemosensitivity to docetaxel. To investigate the effect of chemosensitivity to docetaxel by the suppression of Aurora-A expression, apoptotic cells in the 510 A and 1440 A clones as well as empty vector–transfected clones were examined by flow cytometry and the type of cell death induced by docetaxel was assessed. In the Aurora-A shRNA-transfected cells, 0.3 nmol/L docetaxel induced a notable level of apoptosis compared with the empty vector–transfected cells (510 A, 71.3%; 510 m, 36.12%; 1440 A, 78.1%; 1440 m, 26.28%; Fig. 3E and F). These findings suggested that the suppression of Aurora-A expression augmented the apoptosis induced by docetaxel. To confirm the validity of the experiment, cells treated with 3% formalin for 30 min and stained with both Annexin V and propidium iodide served as a positive control for double staining (Supplementary Data 3).

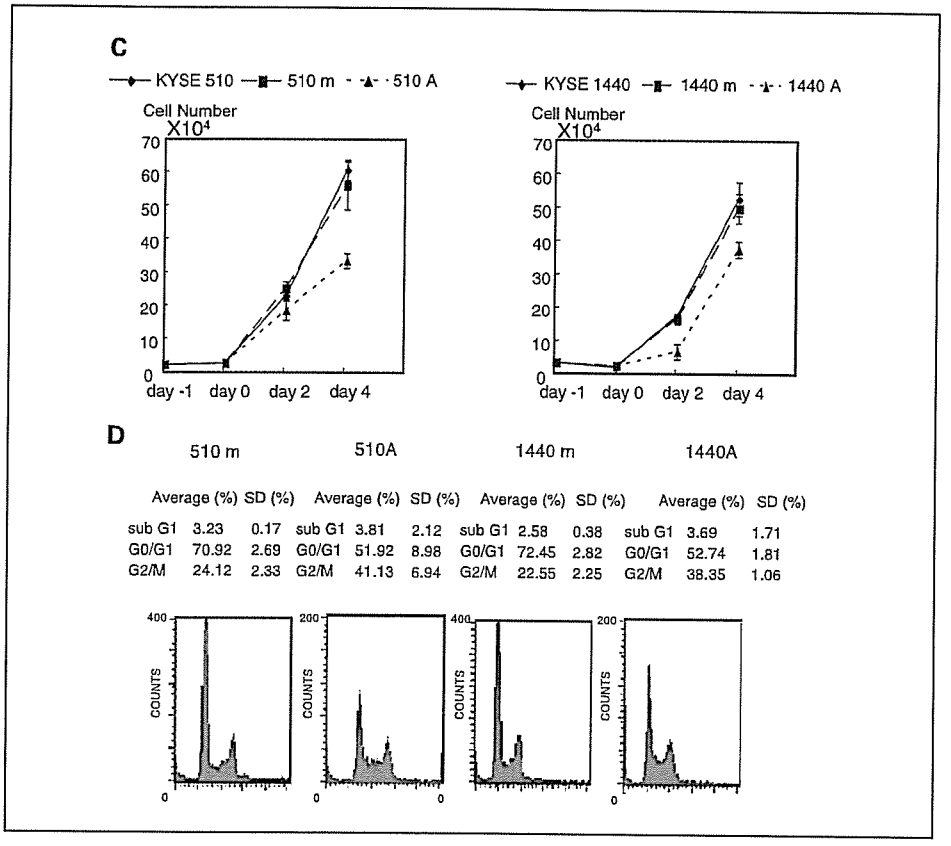

Fig. 2 *Continued. C, in vitro* growth assay in cells transfected with the Aurora-A shRNA vector (510 A or 1440 A) or an empty vector (510 m or 1440 m). The number of cells was counted every 2 d. The experiments were repeated thrice. *D*, cell cycle profiles obtained by flow cytometry. Ratios of cell populations in G_1 and G_2-M were the average and SD. These experiments were done thrice, and representative results are shown. The experiments were repeated thrice, and the representative results are shown.

The effect of Aurora-A suppression on tumor growth in vivo. To examine the possible effect of Aurora-A shRNA on tumor growth *in vivo*, s.c. tumor formation assay in nude mice was done. As shown in Fig. 4A, the tumors formed from Aurora-A shRNA-transfected cells (510 A) were clearly smaller than that formed from empty vector–transfected cells (510 m). Then, we examined tumor volume and weight at 46 days after inoculation. The average tumor volume of the mice at day 46 was reduced by 36.1% in 510 A compared with 510 m (P = 0.03; Fig. 4C), and the average tumor weight was also decreased by 31.5% in 510 A compared with 510 m (P = 0.03; Fig. 4C). All of the tumors were stained with H&E and for Aurora-A (Fig. 4D), and Aurora-A immunoreactivity was confirmed to be reduced in tumors grown from Aurora-A shRNA-transfected cells but not in tumors grown from empty vector–transfected cells.

The effect of Aurora-A suppression on chemosensitivity to docetaxel in vivo. To examine the possible effect of Aurora-A shRNA on chemosensitivity to docetaxel *in vivo*, s.c. tumors were generated in nude mice followed by treatment with docetaxel or PBS. The tumors formed from Aurora-A shRNA-transfected cells (510 A) were apparently smaller than those formed from empty vector–transfected cells (510 m) after the treatment with docetaxel at day 39 (Fig. 5A, *b* and *d*). To confirm this, we measured tumor volume at 39 days after inoculation. As shown in Fig. 5C, following the treatment with docetaxel, the average tumor volume of 510 A and 510 m was 175.3 ± 70.4 mm^3 and 312.8 ± 28.0 mm^3, respectively, and thus, the suppression of Aurora-A expression resulted in a 44.0% inhibition of tumor growth (P = 0.03).

Discussion

We previously reported that the expression of Aurora-A was frequently up-regulated in ESCC tissues and cell lines and contributed to a poor prognosis (14). In an attempt to determine the potential of Aurora-A as a therapeutic target, we used a vector-based shRNA technique to knock out its expression and analyzed its phenotypes.

In the current study, we were able to suppress the expression of Aurora-A using vector-based shRNA for two different target sequences in two different ESCC cell lines and obtained very similar results at each examination *in vitro*. That is, a reduction of Aurora-A protein expression was clearly related to cell growth inhibition and increased sensitivity to docetaxel.

Recently, a relationship between Aurora-A activity and G_2-M transition was reported in cancer cells (28). In the current study, we showed that the suppression of Aurora-A expression caused an accumulation of the cells in the G_2-M phase *in vitro*, resulting in the inhibition of proliferation of ESCC cell lines. Moreover, in recent report, Hata et al. (17) showed that the suppression of Aurora-A expression had an antitumorigenic effect *in vivo* in pancreatic cancer, and we similarly showed that the suppression of Aurora-A expression in ESCC cell lines inhibited tumor growth *in vivo* using our vector-mediated shRNA strategy. Our results were consistent with Hata et al. in

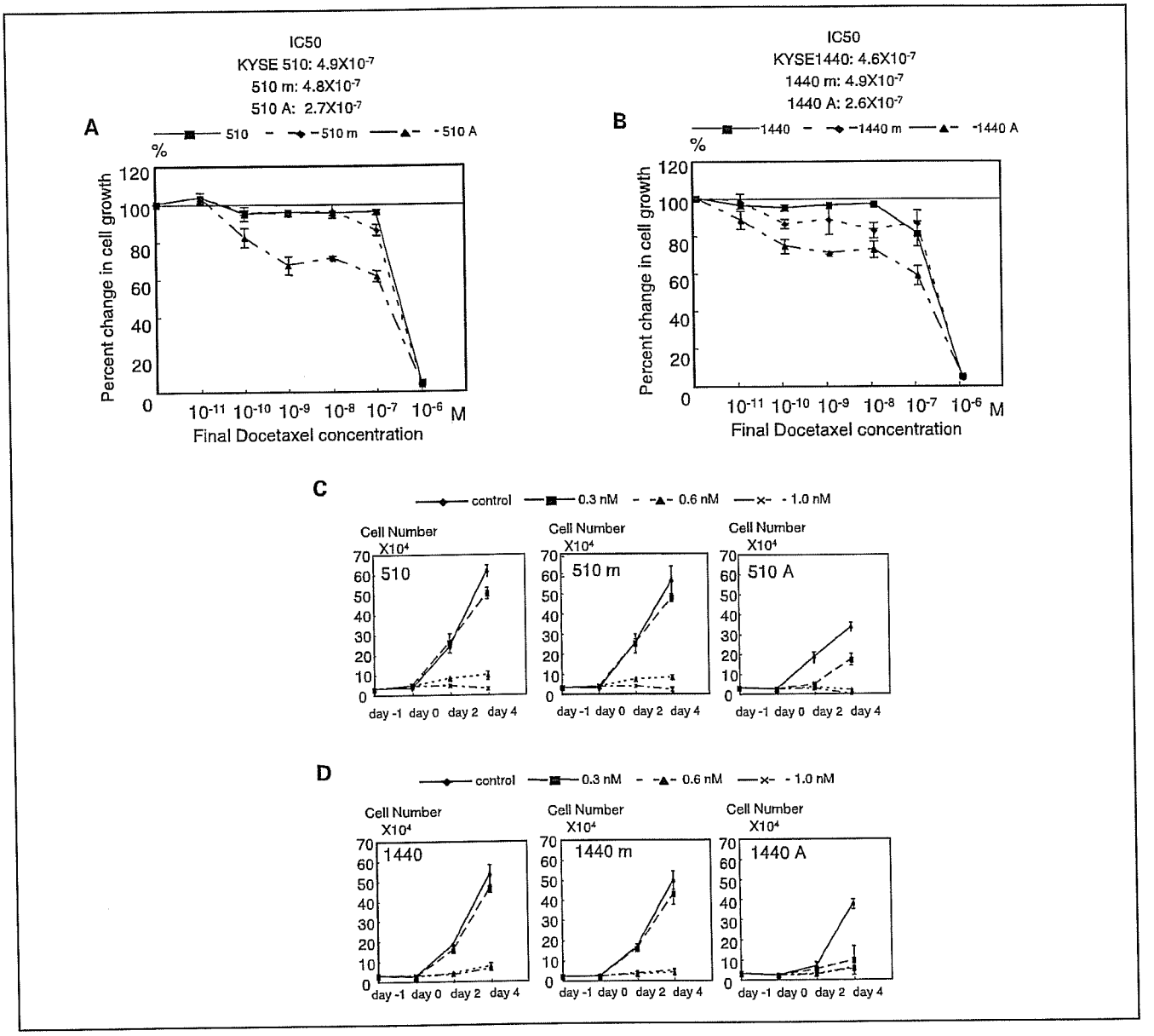

Fig. 3. Effect of the suppression of Aurora-A expression on sensitivity to docetaxel. *A* and *B*, cytotoxic activities of docetaxel were measured by WST-8 assay. Graded concentrations of docetaxel (10^{-5} to 10^{-10} mol/L) were added to the wells, and the cells were cultured for 48 h. Control cultures received normal growth medium only. Cell viability was measured as absorbance at 450 nm, and values obtained were compared with that of the control. The IC_{50} value was defined as the concentration that reduced the absorbance in each test by 50%. X axis, final docetaxel concentration (10^{-6} to 10^{-11} mol/L). The experiments were repeated thrice. *C* and *D*, cytotoxic activities of docetaxel were confirmed by cell counting. Graded concentrations of docetaxel (0, 0.3, 0.6, and 1.0 nmol/L) were added for the first 2 d, and normal growth medium was added for the next 2 d. The number of cells was counted every 2 d. These experiments were repeated thrice.

terms of antitumor activity that the suppression of Aurora-A expression caused in ESCC. Consequently, Aurora-A seemed to be a critical factor for the proliferation of cancer cells, and therefore, it should be a good therapeutic target for halting proliferation of ESCC.

Furthermore, we found that the suppression of Aurora-A expression enhanced the sensitivity to docetaxel-induced apoptosis both *in vitro* and *in vivo*. Taxanes bind to free tubulin and promote the assembly of tubulin into stable microtubules. Hence, they stop cell cycle progression, causing cells in the M phase to accumulate at the metaphase-anaphase transition and subsequently leading them to apoptosis, which is consistent with our findings that the suppression of Aurora-A expression resulted in the accumulation of cells in the G_2-M phase. As previously discussed, Aurora-A is essential for the proper arrangement of centrosomes and microtubules, and Hata et al. showed that a combination of the suppression of Aurora-A expression and use of taxanes resulted in an augmented induction of apoptosis in pancreas cancer *in vitro* (17). Additionally, Anand et al. (15) showed that the overexpression of Aurora-A induced increased resistance to taxanes via a decrease in spindle checkpoint activity *in vitro*. Our results

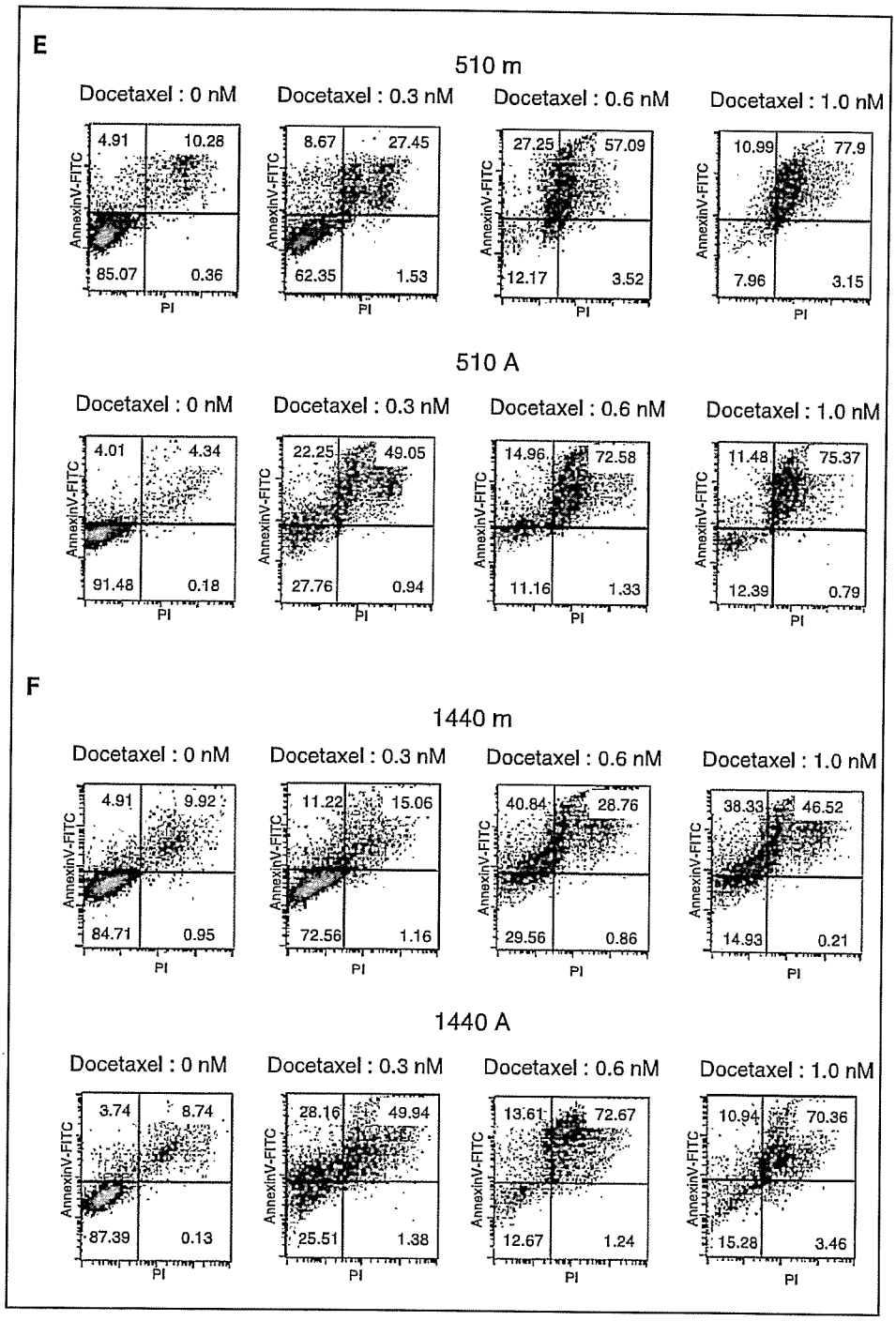

Fig. 3 *Continued.* *E* and *F*, to assess the type of cell death induced by docetaxel, flow cytometry was done. Cells in bottom left quadrant (unstained), top left quadrant (stained only with Annexin V), and top right quadrant [stained with both Annexin V and propidium iodide (*PI*)] represent viable cells, cells in early apoptosis, and cells in late apoptosis, respectively. The experiments were repeated thrice, and the representative results are shown.

are consistent with this study and were able to show that the suppression of Aurora-A expression enhanced chemosensitivity to docetaxel not only *in vitro* but also *in vivo*. These results suggest that, by suppressing Aurora-A expression, spindle checkpoint activity might have recovered and, thus, increased the sensitivity to taxanes. In the meantime, the mechanism that triggers apoptosis after inhibition of Aurora-A expression, as well as the complicated biological activity of Aurora-A, remains to be clarified.

RNA interference has become conventional applications for *in vivo* cancer therapy (29, 30), and an efficient way of delivering small interfering RNA into solid tumors has been developed (31). In the current study, we explored the possibility that the RNA interference–mediated suppression of Aurora-A could be used as a specific gene-targeting therapy to suppress the progression of ESCC. Moreover, the function of Aurora kinase inhibitors (including the patent literature) has been studied recently, revealing potentially promising anticancer

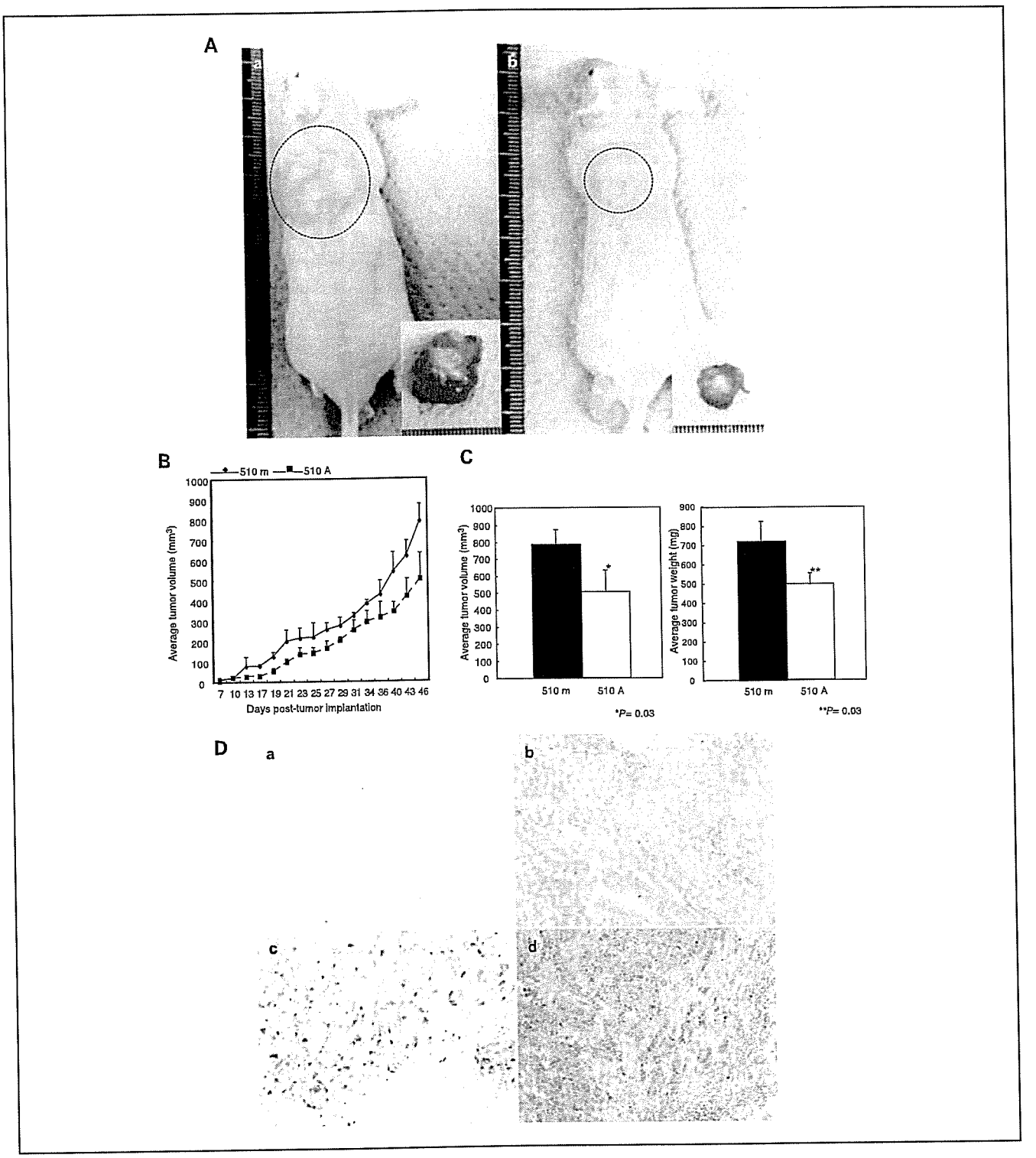

Fig. 4. Effect of the down-regulation of Aurora-A expression on the formation of tumors *in vivo*. *A*, representative features of tumors in a mouse 46 d after the inoculation. a, tumors formed from empty vector – transfected cells (510 m) in the left flank; b, tumors formed from Aurora-A shRNA-transfected cells (510 A) in the left flank. *B*, growth of tumors in the mice injected with Aurora-A shRNA-transfected cells (510 A) or empty vector – transfected cells (510 m). The inoculation was done in five mice. *C*, tumor volume and weight at day 46 after inoculation. Left, black column, average tumor volume at day 46 after the inoculation of empty vector – transfected cells (510 m); white column, average tumor volume at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A; $n = 5$). *, $P = 0.03$. Right, black column, average tumor weight at day 46 after the inoculation of empty vector – transfected cells (510 m); white column, average tumor weight at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A; $n = 5$). **, $P = 0.03$. *D*, a, immunohistochemical staining of Aurora-A in s.c. tumors at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A). Magnification, ×200. b, H&E staining of s.c. tumors at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A). Magnification, ×200. c, immunohistochemical staining of Aurora-A in s.c. tumors at day 46 after the inoculation of empty vector – transfected cells (510 m). Magnification, ×200. d, H&E staining of s.c. tumors at day 46 after the inoculation of empty vector – transfected cells (510 m). Magnification, ×200.

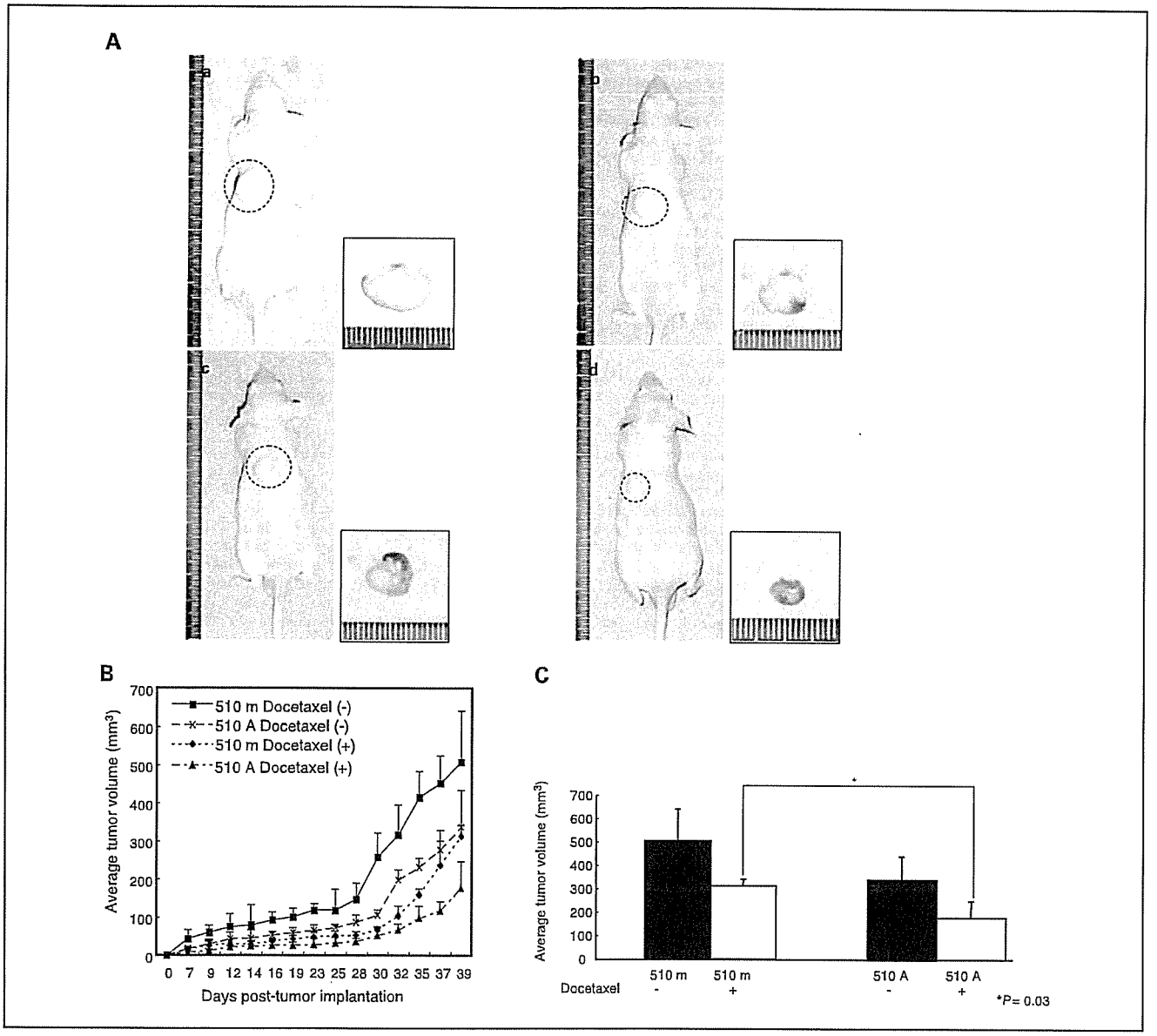

Fig. 5. Effect of the suppression of Aurora-A expression on sensitivity to docetaxel *in vivo*. Mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly) or with 0.1 mL PBS (pH 7.4; i.p., thrice weekly). *A*, representative features of tumors 39 d after inoculation. a, empty vector – transfected cells (510 m) were injected s.c. into the left flank and the mice were treated with PBS (i.p., thrice weekly). b, empty vector – transfected cells (510 m) were injected s.c. into the left flank and the mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly). c, Aurora-A shRNA-transfected cells (510 A) were injected s.c. into the left flank and the mice were treated with PBS (i.p., thrice weekly). d, Aurora-A shRNA-transfected cells (510 A) were injected s.c. into the left flank and the mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly). *B*, growth of tumors in the mice injected with Aurora-A shRNA-transfected cells (510 A) or empty vector – transfected cells (510 m) with or without docetaxel. The inoculation was done in five mice. *C*, tumor volume at day 39 after the inoculation. Left, black column, average tumor volume at day 39 after the inoculation of empty vector – transfected cells (510 m) in mice treated with PBS; white column, average tumor volume at day 39 after the inoculation of empty vector – transfected cells (510 m) in mice treated with docetaxel ($n = 5$). Right, black column, average tumor volume at day 39 after the inoculation of Aurora-A shRNA-transfected cells (510 A) in mice treated with PBS; white column, average tumor volume at day 39 after the inoculation of Aurora-A shRNA-transfected cells (510 A) in mice treated with docetaxel ($n = 5$). *, $P = 0.03$.

effects (32, 33). Therefore, our results in combination with these findings suggest that taxane-mediated chemotherapy could be more effective in combination with these anti-Aurora agents in ESCC.

In summary, the suppression of Aurora-A expression is shown to inhibit tumor growth of ESCC and enhanced chemosensitivity to docetaxel both *in vitro* and *in vivo*. Consequently, the therapeutic regimen to suppress the Aurora-A expression is a feasible candidate to become a novel therapeutic strategy for the treatment of ESCC.

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分子生物学を考慮した食道癌3領域郭清の適応

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臨床

分子生物学を考慮した食道癌3領域郭清の適応

Indication of three fields lymph node dissection in esophageal cancer based on molecular biology

嶋田　裕
SHIMADA Yutaka

食道癌の3領域郭清患者の選別について術中微小転移の検討から，以下の適応が考えられる．

①MtLt患者の微小転移を含めた上縦隔LN転移陽性例は頸部郭清を行うべきである．②MtLt患者の術中上縦隔LN転移陰性例では頸部郭清を省略できる．③Ut患者は上縦隔LN転移の有無にかかわらず頸部郭清は必須である．微小転移検出をテクニシャンのルーチン業務とすることにより臨床導入に至った．今後は，複数の検出マーカーとより迅速な微小転移の検出機器の導入で腹膜播種検出とともに一般化されることが必要である．

はじめに

本邦では食道癌の3領域郭清はT1b以深の患者では標準術式とされてきた[1)-3)]．しかしながら，一律に3領域郭清をすべての患者に適応することに対しては批判があり，2領域郭清を標準とする施設も多く存在する．その原因としては，3領域郭清適応患者の選別のための適切な指標がないことがあげられてきた．われわれは3領域郭清適応患者の選別に微小転移の検出を応用し，臨床導入を図ってきたので，その中期成績を含めて概説する．

表1　反回神経周囲リンパ節転移と頸部転移の関係

	頸部LN転移(+)	頸部LN転移(−)	文献
106Rec(+)	10	13	10
106Rec(−)	7	56	
106Rec(+)	16	15	11
106Rec(−)	5	38	
106rec(+)	15(2*)	−	12
106rec(−)	1	−	
106rec(+)	21(5*)	30(4*)	9
106rec(−)	17	87	

*微小転移

I. 頸部転移と縦隔リンパ節転移

胸部食道癌における頸部転移頻度は16〜43%であり[1)4)-8)]，われわれの微小転移を含めた検討でも32.6%(38/117)[9)]に認められている．従来から頸部リンパ節転移と106Recリンパ節の相関が報告されてきたが，微小転移を含めた検討からさらに有意な相関が確認されている[9)-12)]（表1）．われわれは以前からMtLt食道癌において106Recの術中迅速HE検査が陽性の場合は頸部郭清を行い，陰性の場合は頸部郭清を省く試みを行ってきた．その結果，頸部郭清の省略では5/69(7.2%)に頸

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Key words：食道癌／3領域郭清／分子生物学

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部再発をきたした．そこでretrospectiveな免疫染色を行ったところ，頸部再発の80%(4/5)に106Rec微小転移が認められた[13]．したがって，微小転移を含めた解析により予測精度が向上すると考えられ，さらには免疫染色より感度が高い手法の導入により，微小転移検出の感度の向上を目指した．

II. 分子生物学的手法による微小転移検出

われわれは微小転移の検出においてはSCCmRNAを標的としたnested RT-PCRがサイトケラチン染色に比し感度が高く，有用であることを示してきたが[14]，術中迅速検査には検査時間が長時間かかりそのままでは活用できなかった．そこで，Light cyclerによるreal time RT-PCRの導入を行い，標本採取から結果判定まで平均2.5時間で行える体制を整えた[15]．この術中診断法では胸部操作の始めに上縦隔郭清を行えば，胃管作成および腹部郭清中に結果が判明することになる．

III. 微小転移検出による頸部郭清の検討

Yoshiokaらの検討では106 Rec微小転移をRT-PCRレベルで検出し，遺伝子診断のみが陽性で頸部郭清を行った患者の2/6(33.3%)に病理検査で頸部リンパ節転移陽性であり，頸部郭清を省略した患者の2/9(22%)に頸部再発をきたしたと報告している[16]．したがって106Rec転移陽性例では頸部郭清が望ましいが，106rec微小転移陰性患者での頸部郭清の必要性の判定には臨床試験が必要と考えられた．ターゲットを106Recだけに絞るかどうかについては，①106Recは頸部転移のsentinel nodeであるかどうかについては検証がなされていない，②われわれのretrospectiveな検討では106Recの微小転移検出のみでは，予測できない患者も存在する[13]，を考慮し106Recだけにターゲットを絞り込まないこととした．

そこで，上縦隔LN微小転移陰性に対して頸部郭清の省略の是非を検討するrandomized studyを倫理委員会の承認を得て開始した．本試験の目的は，上縦隔LN転移(微小転移を含む)と頸部転移・再発の関係を検討することであり，3領域郭清の意義について検討する試験ではない．したがって，頸部転移を疑う患者に対しての頸部郭清のrendomized studyは倫理上問題があり，上縦隔LN転移陰性患者についてのみrandomizationを行った．

IV. 頸部郭清選択の臨床試験

上記のごとく，臨床試験ではMtLtにおける106Recのみならず，105，106pre，106tbLを含めた上縦隔LNを頸部転移予測の対象とした．Ut患者では42%に頸部転移が認められることと[9]，食道を直接上行する経路や他のリンパ経路を経由する可能性も否定できないことから原則的に頸部郭清を行った．すなわち，①Ut患者や術前画像診断で頸部転移が疑われる患者には頸部郭清を行う，②MtLtにて上縦隔LN(106Rec, 106pre，105)に通常迅速HEが陽性であるか，またはreal time RT-PCR陽性患者には頸部郭清を行う，③MtLtにて上縦隔LNに通常迅速HE陰性かつreal time RT-PCR陰性患者に対して頸部郭清の有無のrandomized studyを行うこととした．リンパ節はそれぞれ2分割し，1個は術中迅速病理検査，1個はRT-PCR検査を行い，適格患者をrandomizeした(図1)．

2001年から2004年の8月まで58人の胸部食道癌に対して臨床試験を行った．Follow upは17～57ヵ月．Ut患者では上縦隔LN転移が術中迅速HE検査で7/13，RT-PCRで8/13に転移を認めた．全例に頸部郭清を行い9/13(69.2%)に微小転移を含めた頸部転移が認められた．また頸部転移の認められた2人は術中迅速HEおよびRT-PCRともに陰性であった．Ut患者では食道壁を長軸方向に転移していく経路や106，105を経由しない頸部転移経路が示唆された．

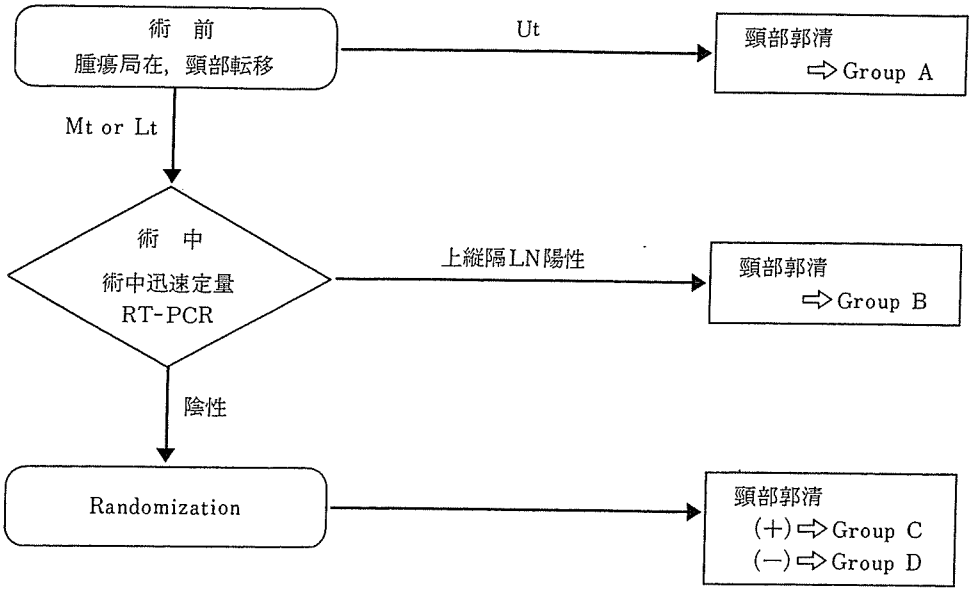

図1　頸部郭清選択の臨床試験プロトコール

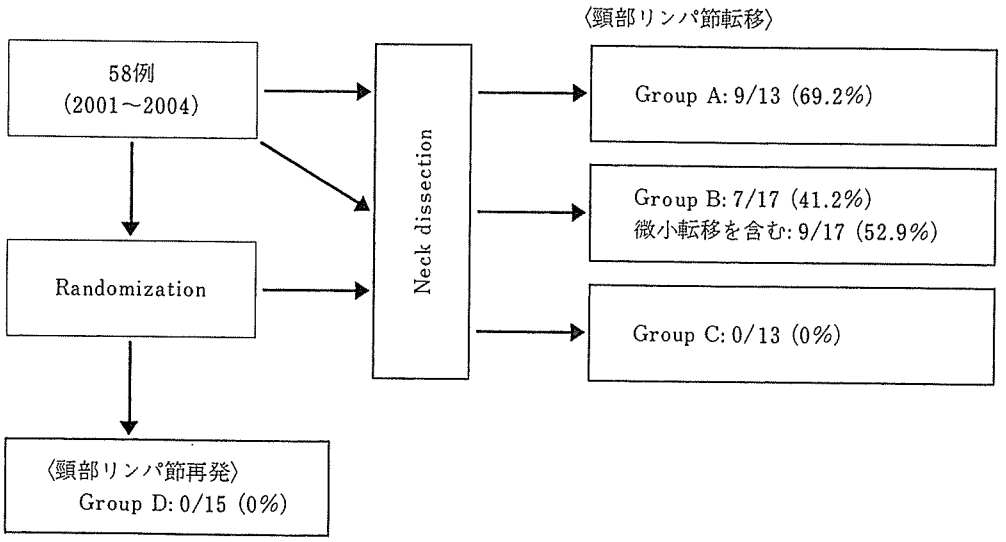

図2　頸部郭清臨床試験の結果
Ut症例とMtLtで上縦隔リンパ節転移陽性患者は頸部郭清をすべきで，MtLtで上縦隔リンパ節転移が陰性である患者は頸部郭清が省略可能である．

MtLtの術中迅速陽性患者17人に頸部郭清を行い9/17(52.9%)に微小転移を含む頸部転移を認めた．頸部転移のうち5人は術中RT-PCRのみが陽性であった．術中検査陰性症例(迅速検査およびRT-PCR)28人にrandomized studyを行い，15人が頸部非郭清，13人が郭清群に振り分けられた．頸部郭清群での頸部転移および頸部非郭清群での頸部再発は全例認められず，頸部非郭清の不利益は認められなかった[17]．

以上の結果をまとめると，Ut患者およびMtLt患者の術中上縦隔LN転移陽性例では頸部郭清をすべきで，MtLt患者の術中上縦隔LN転移陰性例では頸部郭清を省略できることが示唆された(図2)．

V. 微小転移による頸部郭清患者選択の妥当性の検証

頸部郭清臨床試験の短期(3年以内)予後では予後向上が認められ，頸部郭清効果によるものと推測された[17]．さらにテクニシャンによる微小転移検出頸部郭清患者選択による9患者(2004年9月～4月)，および除外項目で上縦隔LN微小転移陽性またはUtでありながら頸部郭清が行われなかった4患者(2001～2004年)について妥当性を検討した．

2004年9月以降の頸部郭清の選択基準で頸部郭清を行わなかった患者の頸部再発は認められていない(図3)．しかしながら，2004年以前の肝硬変により頸部郭清を控えた患者とRT-PCR陽性でありながら再建胃管虚血のために頸部郭清を控えた患者で頸部再発をきたした．腹部リンパ節転移優位で頸部郭清を控えた患者は腹部リンパ節再発をきたし，頸部郭清省略の不利益は認められなかった[19](表2)．

したがって，われわれの微小転移検出に基づく頸部郭清患者の選択は頸部転移の観点からは妥当であると考えられる．さらに患者背景により安易に頸部郭清を控えると頸部再発をきたすことから，安易な頸部郭清の手控えはすべきではない．

VI. Sentinel node navigation surgeryの可能性

近年，sentinel node navigation surgeryが消化器癌でも導入されており，早期胃癌では有用な

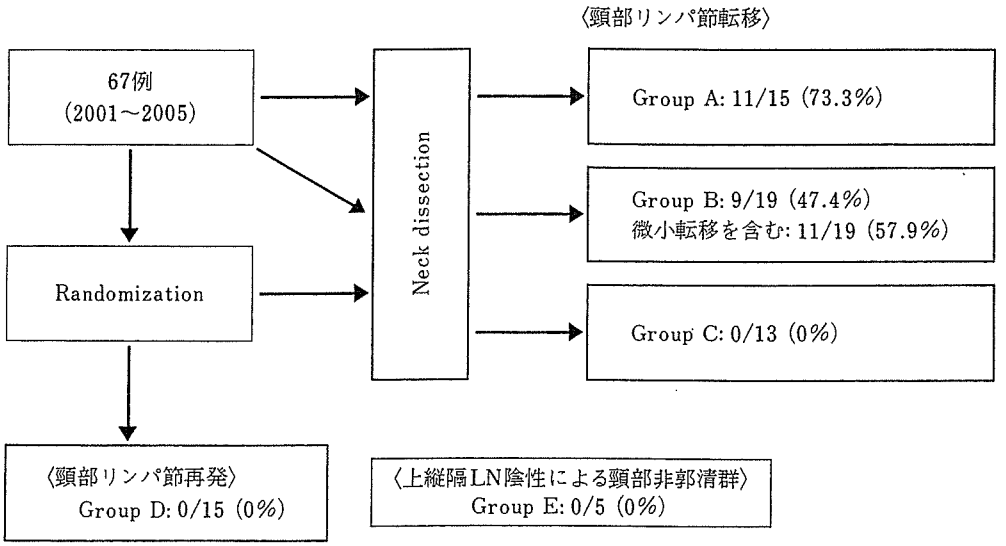

図3 頸部郭清臨床試験中期結果
継続した患者集積でも上縦隔リンパ節転移が陰性である患者では頸部再発は認められていない．

表2 頸部非郭清だが術中RT-PCRを行い得た患者の経過

患者	局在	pT	pN	pM	pTNMStage	迅速PCR	頸部郭清除外理由	再発	再発後治療	予後
1	Ut	1a*	0	0	1	陰性	肝硬変	1Y後右104	頸部郭清，照射	2Y死
2	Mt	1b	1	0	2b	陽性	胸腔内吻合	なし		4Y4M生
3	Lt	3	0	0	2a	陽性	胃管血流不良 右102，左102	3Y4M後	頸部郭清，照射	4Y2M生 (担癌)
4	Mt	4	1	0	3	陽性	腹部リンパ節優位	No8，血行性転移	化学療法	1Y3M死

＊術前1b診断