Array-based comparative genomic hybridization of circulating esophageal tumor cells

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Abstract. Esophageal squamous cell carcinoma (ESCC) shows a high frequency of lymphatic and/or systemic metastasis, even when the tumor invades only the submucosa. To investigate the genetic alterations in circulating esophageal tumor cells, we performed array-based comparative genomic hybridization (CGH) analysis of 8 DNA samples of xenografts, which were previously established from the thoracic duct lymph of 13 ESCC patients. A total of 5 loci (or genes), 10q21.3 (*EGR2*), 11q13.3 (*CCND1/CyclinD1*, *FGF4*, and *EMS1*), 11q14 (*PAK1*), and 22qtel (*ARSA*) were found to be candidate amplified loci in the xenograft. In contrast, a total of 24 loci including 9p21 (*p16* and *MTAP*) were found to be homozygously deleted candidates in the xenograft. Both *p16* homozygous deletion and *CCND1* amplification were detected in 6 (75%) and 5 (62.5%) of the 8 xenografts. Furthermore, by quantitative Southern blot analysis, we found *p16* homozygous deletion in 30.8% (8/26) of the primary tumors and in 50% (4/8) of the metastasized lymph nodes. The frequency of *CCND1* amplification and *p16* homozygous deletion is suggested to be associated with ESCC progression. Matrigel invasion assays of *p16*-deleted ESCC cells showed that restoring wild-type *p16* activity into the cells significantly inhibits tumor-cell invasion, suggesting that *p16* inactivation could be involved in ESCC invasion. This is the first report showing the genetic alteration of concealed tumor cells in the thoracic duct lymph. The present gene list should be helpful for identifying new amplified and deleted genes in primary ESCCs as well as in metastasized lymph nodes.

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Introduction

In East Asian countries including Japan and China, and in some parts of Europe, esophageal carcinoma consists mainly of squamous cell carcinomas located mostly in the thoracic esophagus, while adenocarcinoma in the distal part of the esophagus has increasingly become the major pathological type found in Europe and North America. Esophageal squamous cell carcinoma (ESCC) is a cancer with one of the poorest prognoses. ESCC shows lymphatic and/or systemic metastasis, even when the tumor invades only the submucosa (1). Therefore, identification of the genetic alterations associated with ESCC progression is thought to be important. However, a comparative study between distantly metastasized tumors and primary tumors is rarely found compared with that between metastasized lymph nodes and primary tumors, because distantly metastasized tumor samples themselves are difficult to obtain. Furthermore, it is quite difficult to identify genetic or epigenetic alterations in circulating tumor cells, since only rare tumor cells exist in the lymphatic duct or blood vessels (2).

Here we performed array-based comparative genomic hybridization (CGH) analysis of DNA samples of the xenografts, which were previously established from the thoracic duct lymph (3), and report that the accumulation of *CCND1* amplification and *p16* homozygous deletion is associated with ESCC progression. Furthermore, matrigel invasion assays of *p16*-deleted ESCC cells showed that restoring wild-type *p16* activity into the cells significantly inhibits tumor-cell invasion.

Materials and methods

Xenografts from thoracic duct lymphs in esophageal cancer. A thoracic duct lymph was collected independently from 13 patients with ESCC during surgery by cannulation into the thoracic duct. The collected volume varied from 20 to 30 ml.

The collected lymph was centrifuged and the pellets were subcutaneously injected into the abdomen of BALB/c-nude mice. Eight established xenografts were previously reported from 8 out of the 13 patients (3). Here we named the 8 xenografts as Xeno-TDL1-8.

Genomic DNA purification from surgical specimens of ESCC patients and xenografts. ESCC tissues were obtained from patients at the National Cancer Center Hospital (Tokyo). Written informed consent was obtained from all the patients. All of the surgical specimens and the 8 xenografts were frozen immediately in liquid nitrogen and stored at -80˚C until use. Genomic DNA was extracted from the frozen materials by the conventional phenol-chloroform procedure.

Array-based CGH. The gene copy number was assessed using a commercial array (Genosensor™ Array 300 v1.0, Vysis, IL, USA) according to the manufacturer's protocol. The array contains 287 BAC clones corresponding to various chromosome loci which have been reported to be altered in various human cancers (list available from the manufacturer's web site, http://www.vysis.com/). Briefly, DNA samples isolated from normal human lymphocytes (reference DNA) and tumor samples (test DNA) were labeled by random priming with Cy3- or Cy5-labeled dCTP. The DNA probes (0.1 μg) were mixed with 20 μg of unlabeled Cot-1 DNA and were hybridized to the genomic array, which was then counter-stained with DAPI and analyzed by the fluorescent image capturing system, GenoSensor.

Southern blot analysis. Five micrograms of *Eco*RI-digested DNA per lane was loaded onto 1% agarose gel, and blotted onto a nylon membrane filter, Hybond N^+ (Amersham). The probes for the full-length of the *p16* cDNA and the *CCND1* cDNA were labeled with [^{32}P]dCTP using Random Primed DNA labeling kits (Boehringer Mannheim), and hybridized at 42˚C in 5X SSC/0.1% sodium dodecyl sulfate (SDS)/50% Dextran for 12 h. The filters were washed three times in 0.1% SSC/0.1%SDS at 65˚C, and were exposed to X-ray film at -80˚C. To control the contamination of the tumor samples by normal cells, we performed quantitative Southern blot analysis. Hybridization and washing were done under the same stringent conditions as the above procedure. Using a Bio-image-analyzer (BAS2000; Fujix, Kanagawa, Japan), the ratio of the signal intensity of the *p16* gene/a control gene (*PAX-5*) was calculated. Homozygous deletion was defined by the signal intensity of the *p16* gene being <20% of the internal control gene, *PAX-5*, located at chromosome 9p13. For the *PAX-5* probe, a 298-bp DNA fragment was amplified by PCR with the primers (see below) from genomic DNAs.

Genomic PCR amplification of the p16 gene. Sequences of the primers were as follows: A forward primer, 5'-GGTGTT TCTTTAAATGGCTC -3', and a reverse primer, 5'-AGCCT TCATCGAATTAGGTG-3' for *p16*; a forward primer, 5'-GCGGTGCTTCTCCTATGTGAC -3', and a reverse primer, 5'-TTTAAAGTGCTCTGCGTGATG-3' for *PAX-5*. PCR was performed using Takara ExTaq (Takara Corp., Shiga, Japan) in a total volume of 50 μl containing 100 μM of each primer and 50 ng of template DNA. The thermal cycling conditions were 35 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, and extension at 68˚C for 1 min. The last cycle had an additional extension at 68˚C for 10 min. The sizes (437 bp of *p16* exon2 and 298 bp of *PAX-5*) and sequences of the PCR products were confirmed by agarose gel electrophoresis and direct sequencing.

Matrigel invasion assay. Two esophageal cancer cell lines, TE1 and TE3, and a mouse fibroblast cell line, STO were used in this study. TE1 has been reported previously to show no alteration of *CCND1* or *p16*, whereas TE3 has shown *p16* homozygous deletion but no *CCND1* amplification (4,5). To assess the infective ability of the adenoviral vectors, the cells were infected with an adenovirus carrying the *E coli* ß-galactosidase gene under the control of the human cyto-megalovirus promoter (Ad-lacZ), and 24 h later they were stained with X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactppyranoside). Increased doses of the adenovirus, from 0 to 200 MOI, were used to ascertain the MOI necessary to infect 80% or more of each cell line. The invasion of the esophageal tumor cells *in vitro* was measured by the BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences) according to the manufacturer's protocol. After infection of Ad-lacZ and an adenovirus carrying *p16* (Ad-*p16*) at 100 MOI, the cells were trypsinized and 500 μm of cell suspension ($1x10^6$ cells/ml) was added in triplicate wells. After 24-h incubation, the cells that passed through the filter into the lower wells were fixed and stained with 100% methanol and 1% Toluidine blue, respectively. The number of cells invaded was counted by photographing the membrane through a microscope.

Results

Array-based CGH analysis of xenografts derived from thoracic duct lymph of ESCC patients. We previously reported that xenografts were established from the thoracic duct lymph in 8 (61.5%) of the 13 advanced ESCC patients, whereas only 4 (30.8%) patients showed tumor cells in the thoracic duct lymph as revealed by skillful cytologists (3). These facts suggest that circulating tumor cells in the thoracic duct lymph are very few, but have tumor forming activity in nude mice. To conclude this, however, we have to provide more evidence, such as the presence of ESCC-type genetic alterations in the xenograft. The xenografts are composed of mouse mesenchymal cells and human tumor cells. This composition of no contamination of human mesenchymal cells provides an advantage in identifying homozygously deleted loci, which are very difficult to detect by many molecular biological techniques such as genomic subtraction or differential display. To investigate the genetic alterations in this unique material derived from circulating esophageal tumor cells, we performed array-based CGH analysis, which has a great potential for comprehensive analysis of a relative gene-copy number in tumors (6,7) and subjectively enables us to identify new amplified and homozygously deleted genes. To investigate the genetic alterations in the xenografts, we used bacterial artificial chromosome (BAC) clone-arrays containing the 287 amplified or lost loci reported previously in each type of tumor (see Materials and

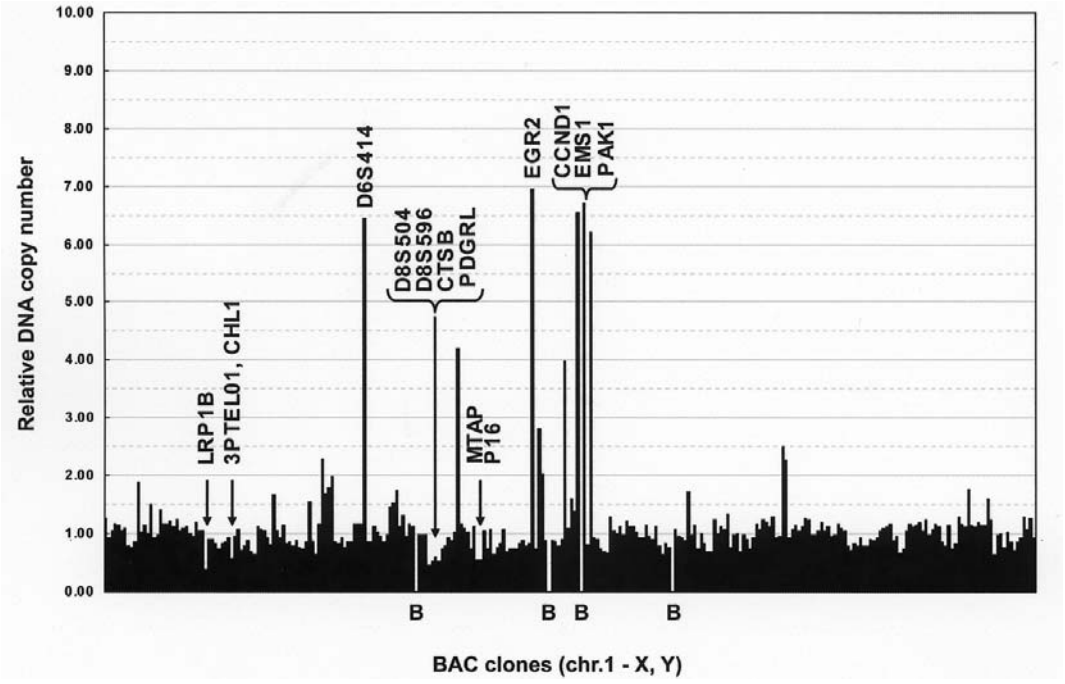

Figure 1. Representative results of array-based CGH on xenograft DNA derived from the thoracic duct lymph of ESCC patients. Fluorescence ratios on all the 287 chromosome loci between a xenograft DNA (Xeno-TDL2 in Table I) from an ESCC patient and a normal male DNA. Amplified or homozygously deleted gene candidates and their chromosome loci, whose ratios between the two samples were changed >5-fold or <0.6-fold (arrows), are also indicated. B, no DNA spot on the array used.

methods). The array-based CGH in the Xeno-TDL2 DNA sample was shown as a representative result (Fig. 1). A >5-fold increased gene (or marker) and its chromosomal locus, which was found in at least one xenograft, are summarized in Table I. A total of 5 loci (or genes), 10q21.3 (*EGR2*), 11q13.3 (*CCND1*, *FGF4*, and *EMS1*), 11q14 (*PAK1*), and 22qtel (*ARSA*) were found to be the candidates amplified in the xenograft. In the same way, a <0.6-fold decreased gene (or marker) and its chromosomal locus are also summarized in Table I. A total of 24 loci were found to be homozygously deleted candidates in the xenograft. Nine telomeric regions, 1qtel (*1QTEL10*), 3ptel (*CHL1*), 4ptel (*SHGC4-207*), 5qtel (*NIB1408*), 8ptel (*D8S504* and *D8S596*), 8q24-qtel (*PTK2*), 12qtel (*stSG8935*), 19ptel (*129F16/SP6* and *stSG42796*), and 19qtel (*D19S238E*) were found to be decreased. The other 15 homozygously deleted candidate loci were 1p12 (*D1S2465* and *D1S3402*), 2q21.2 (*LRP1B*), 3p24.3 (*THRB*), 3p14.2 (*FHIT*), 3q21 (*RBP1*, *2*), 3q26.2 (*EIF5A2*), 6q25.1 (*ESR1*), 7q32-34 (*TIF1*), 8p22 (*CTSB*, *PDGRL*, and *LPL*), 9p21 (*p16* and *MTAP*), 9p11.2 (*AFM137XA11*), 10p13 (*BMI1*), 16q24.2 (*CDH13*), 18q11.2 (*LAMA3*), and 18q21.3 (*DCC*), respectively.

CCND1 amplification and p16 homozygous deletion in the xenografts. Among oncogene amplifications in primary ESCCs, *CCND1* amplification has been reported to be most frequent (8). Consistent with these data, *CCND1* amplification was also revealed by array-based CGH analysis of the xenografts (Table I). To confirm the CGH results, we first investigated this gene amplification by a classical but faithful method, Southern blot hybridization, in the 8 xenograft DNA samples. Of them, 5 xenografts (Xeno-TDL1-5) showed *CCND1* amplification (Fig. 2A), thereby providing evidence that the xenograft was derived from the circulating tumor cells in the thoracic duct lymph.

As shown in Table 1, the array-based CGH analysis also showed frequent deletion of the 9p21 locus containing *MTAP* and *p16*. In the 8 xenografts, we next checked for *p16* homozygous deletion by genomic PCR using human specific primers. Six (75%) out of the 8 xenografts showed *p16* homozygous deletion (Fig. 2B). No change in the *p16* copy number in 2 xenografts (Xeno-TDL6 and Xeno-TDL8) shown by genomic PCR was also demonstrated by the array-based CGH (Xeno-TDL6: 1.13 and Xeno-TDL8: 1.17 in Table I). In the xenograft DNA samples, any homozygously deleted genes are detectable by PCR only using human specific primers.

Of the 8 xenografts, only one (Xeno-TDL8) showed no alteration in both *CCND1* and *p16*. Southern blot and genomic PCR analyses of these two genes suggest that most xenografts were derived from the circulating tumor cells in the thoracic duct lymph.

Quantitative Southern blot analysis of p16 in metastasized lymph nodes of ESCCs. We previously reported *p16* mutations in 4 (16%) of 25 primary ESCCs (5). Other investigators successfully detected *p16* homozygous deletion in metastasized lymph nodes (16%, 5/31) by comparative multiplex PCR, and found a decreased amount of *p16* PCR product in 2 out of 5 primary tumors exhibiting *p16* homozygous deletion in metastasized lymph nodes (9). Quantitative PCR analysis provides a quick method for determining the copy number of specific DNA sequences in a large number of clinical samples including paraffin-embedded tissues and biopsy samples.

Table I. Homozygous deleted or amplified candidate loci identified by array-based CGH.

	Gene or marker	Chromosomal loci	Xeno-TDL[a] 1	2	3	4	5	6	7	8
>5-fold	*D6S414*	6p12.1-p21.1	2.27	6.45	1.60	2.58	2.30	1.17	1.27	1.09
	EGR2	10q21.3	6.28	6.94	4.38	6.91	6.45	0.90	1.1	0.78
	CCND1	11q13	5.51	6.53	4.83	6.09	5.88	1.00	1.18	1.07
	EMS1	11q13	5.91	6.71	4.87	6.35	4.58	1.09	1.62	1.3
	PAK1	11q13-q14	5.43	6.20	4.13	6.03	4.55	0.89	1.73	0.93
	9ARSA	22q tel	1.33	1.59	1.01	1.63	1.79	1.10	7.32	1.32
<0.6-fold	*D1S2465*, *D1S3402*	1p12	0.97	0.94	0.92	0.87	1.07	0.98	0.57	0.87
	1QTEL10	1q tel	0.91	1.25	0.72	1.14	0.89	0.82	0.53	0.82
	LRP1B	2q21.2	0.54	0.39	0.66	0.48	0.56	0.66	0.94	0.5
	3PTEL01, *CHL1*	3p tel	0.66	0.58	0.70	0.64	0.68	0.39	0.91	0.48
	THRB	3p24.3	0.85	0.71	0.91	0.77	0.84	0.64	0.51	0.66
	FHIT	3p14.2	0.76	0.70	0.83	0.71	0.69	0.47	0.78	0.54
	RBP1, *RBP2*	3q21-q22	1.17	1.07	1.13	1.04	0.95	1.48	0.54	1.59
	EIF5A2	3q26.2	0.96	0.92	0.92	0.95	0.83	1.46	0.57	1.38
	SHGC4-207	4p tel	0.93	0.83	0.96	0.89	0.81	0.92	0.45	0.88
	NIB1408	5q tel	0.73	0.85	0.71	0.90	0.77	1.04	0.52	1.08
	ESR1	6q25.1	0.84	0.85	0.82	0.87	0.85	0.57	0.85	0.64
	TIF1	7q32-q34	1.15	0.99	1.09	1.00	0.96	1.04	0.57	1.01
	D8S504	8p tel	0.57	0.46	0.59	0.54	0.56	0.53	0.75	0.57
	D8S596	8p tel	0.60	0.52	0.80	0.47	0.75	0.58	0.7	0.54
	CTSB	8p22	0.66	0.60	0.69	0.53	0.62	0.66	1.78	0.47
	PDGRL	8p22-p21.3	0.51	0.52	0.66	0.00	0.76	0.62	1.99	0.8
	LPL	8p22	0.74	0.75	0.81	0.80	0.73	0.65	0.51	0.66
	PTK2	8q24-qter	0.97	1.16	0.96	1.07	1.04	1.47	0.55	1.43
	MTAP	9p21	0.62	0.55	0.79	0.58	0.69	1.08	0.65	1.11
	CDKN2A (*p16*)	9p21	0.65	0.54	0.80	0.62	0.76	1.13	0.71	1.17
	AFM137XA11	9p11.2	1.15	1.04	1.11	0.95	1.04	0.79	0.38	0.74
	BMI1	10p13	0.89	0.79	1.04	0.81	0.77	1.05	0.51	0.87
	stSG8935	12q tel	1.16	1.15	1.04	1.12	1.11	1.13	0.55	1.13
	CDH13	16q24.2-q24.3	0.74	0.70	0.84	0.77	0.81	0.98	0.54	0.9
	LAMA3	18q11.2	0.69	0.71	0.85	0.72	0.70	0.55	2.16	0.51
	DCC	18q21.3	0.67	0.79	1.12	0.60	0.64	0.81	1.44	0.99
	stSG42796	19p tel	0.99	1.18	0.86	1.20	0.75	1.11	0.44	1.27
	2D19S238E	19q tel	0.67	0.66	0.78	0.77	0.60	0.70	0.61	0.72

[a]Xenografts established from the thoracic duct lymph of ESCC patients.

However, the PCR method is so unstable that we often suffer low reproducibility, and an experiment requires several repetitions (10).

In this study, to examine the frequency of *p16* homozygous deletion in metastasized lymph nodes and primary ESCCs, quantitative Southern blot analysis was performed. Each blot contains 1, 3 and 9 μg of *Eco*RI-digested DNA of normal portions, primary tumors and metastasized lymph nodes to control for possible contamination of the tumor samples by various amounts of normal cells. Representative results of the quantitative Southern blot analysis are shown in Fig. 3. Consistent with previous reports (10,11), a homozygous deletion was defined if the *p16* signal was <20% of the signal from a control gene, *PAX-5*, located on chromosome 9q. We found that *p16* homozygous deletion in primary ESCC and metastatic lymph nodes was detected in 30.8% (8/26) and 50% (4/8) of the cases, respectively (Fig. 3). In summary, *p16* homozygous deletion frequency is likely found to

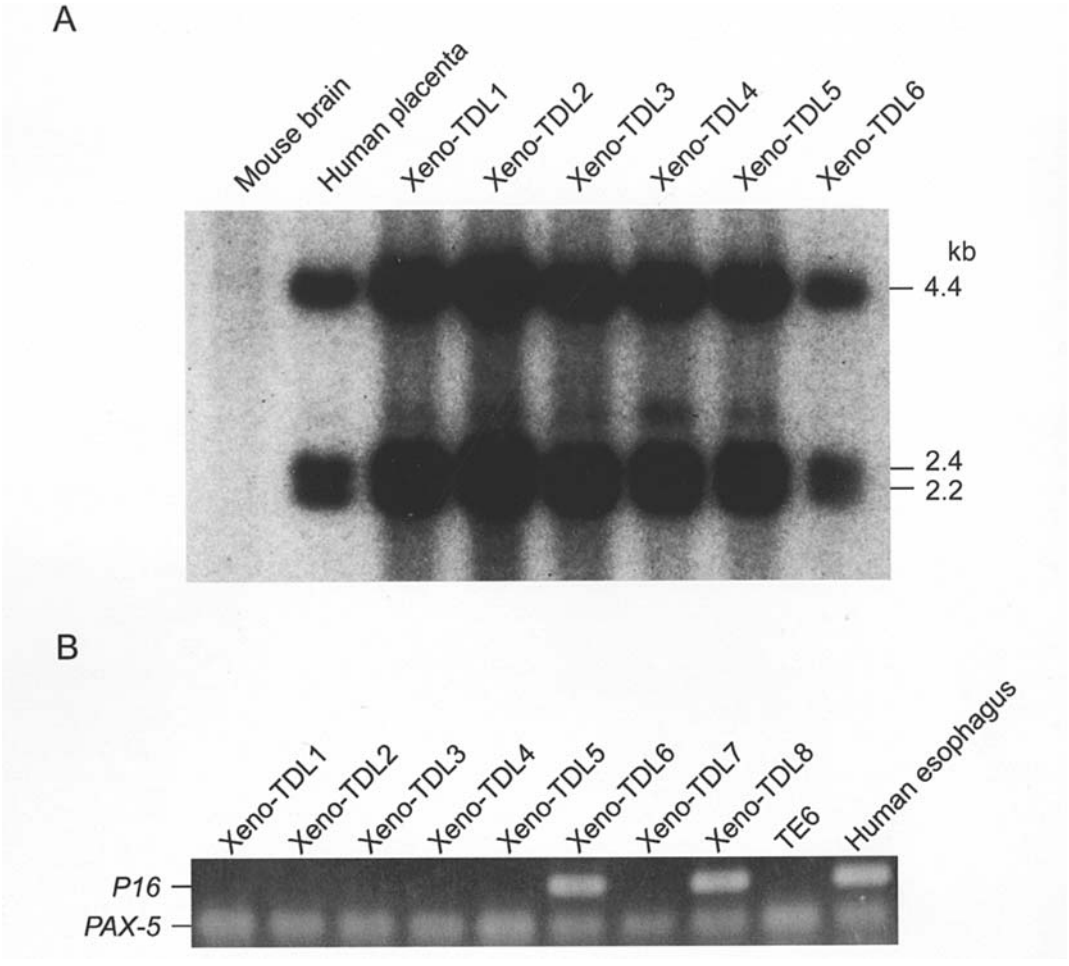

Figure 2. *CCND1* amplification and *p16* homozygous deletion in the xenografts. (A) Southern blot analysis with *CCND1* of 6 xenografts, Xeno-TDL1-6, mouse genome DNA, and human genome DNA. *CCND1* amplification was found in the Xeno-TDL1-5. (B) Genomic PCR of *p16* exon2 and *PAX-5* in 8 xenografts, Xeno-TDL1-8. Two DNA fragments (437 bp of *p16* exon2 and 298 bp of *PAX-5*) amplified by PCR from 50 ng xenograft DNA was analyzed by ethidium bromide-stained 2% agarose gels. An esophageal cancer cell line TE6, in which *p16* has been reported to be deleted, is used as a negative control, and human normal esophagus DNA as a positive control. *p16* homozygous deletion was found in 6 (75%) of the 8 xenografts.

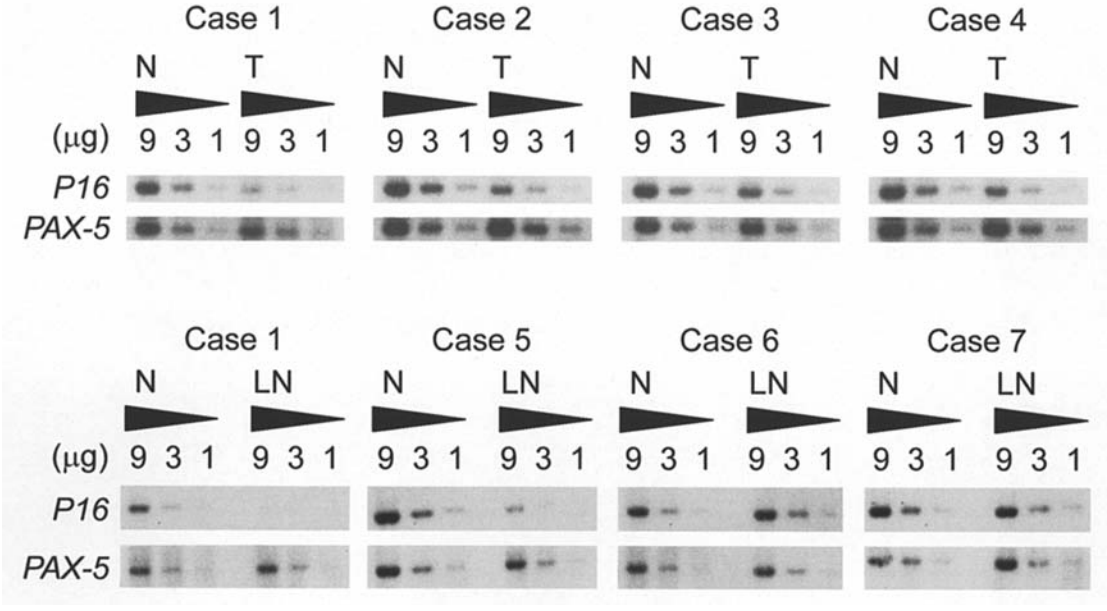

Figure 3. Quantitative Southern blot analysis of the *p16* gene in both primary ESCCs and metastasized lymph nodes. Various amounts of *Eco*RI-digested genomic DNA (9, 3, 1 μg) are loaded to compare the intensity among primary tumor (T), metastasized lymph node (LN) and normal tissue (N). In cases 1, 2, 4, and 5, DNA from the primary tumor or the metastasized lymph node show a remarkable decrease in the signal intensity of *p16* compared to normal tissues, whereas the internal control gene *PAX-5* demonstrated the same intensity in each volume of the genomic DNA between tumor and normal tissues.

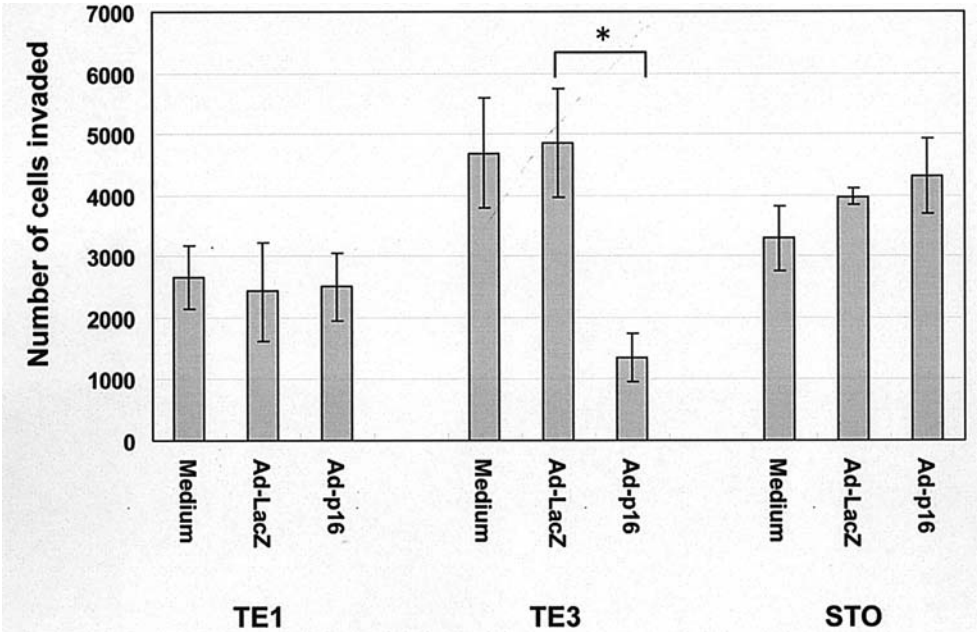

Figure 4. Matrigel invasion assays of esophageal cancer cells infected with Ad-*p16*. The invasion of *p16*-transfected TE3 cells was reduced compared to that of the Ad-lacZ adenovirus control and mock-infected cells; however, no difference of invasion was observed between *p16*-transfected TE1 cells and these two controls. TE1 and TE3 (*p16*-null), esophageal cancer cell lines, and STO, a mouse fibroblast cell line, which is used for a control of invasion assay. *p <0.005.

increase in association with ESCC progression (primary tumors, 30.8%; metastatic lymph nodes, 50%; and circulating tumor cells, 75%).

Adenovirus-mediated p16 gene transfer suppresses invasion of p16-deleted esophageal tumor cells in vitro. The increment of *p16* deletion frequency associated with ESCC progression suggested different functions for *p16* aside from its control of the cell cycle. Therefore, we performed matrigel invasion assays to understand the biological consequences of the *p16* inactivation in ESCC progression. In this assay, we used two esophageal tumor cell lines, TE1 and TE3. TE1 has been reported previously to show no alteration of both *CCND1* and *p16*, whereas TE3 has shown *p16* homozygous deletion but no *CCND1* amplification (4,5). After infection of Ad-lacZ and an adenovirus carrying *p16* (Ad-*p16*) at 100 MOI, which were necessary to infect 80% or more of each cell line (data not shown), the cells were trypsinized and used for the matrigel assay. The invasion of the *p16*-transfected TE3 cells was reduced compared to that of the Ad-lacZ adenovirus control and mock-infected cells; however, no difference of invasion was observed between *p16*-transfected TE1 cells and these two controls (Fig. 4). These results suggest that *p16* inactivation could be involved in ESCC invasion.

Discussion

The amplification frequency (62.5%) of the *CCND1* gene in the xenografts was much higher than that reported previously (28% and 38%) in both 32 primary ESCCs and 13 ESCC cell lines reported previously (8). In regard to primary ESCCs, we previously reported that the 1p34 locus containing *MYCL1*, 2p24 (*MYCN*), 7p12 (*EGFR*), 8p11 (*FGFR1*), and 12q14 (*MDM2*) were amplified in one of the 32 cases (3%), and the 17q12 locus (*ERBB2*) in 2 of the 32 cases (6%), while only the 11q13 locus (*CCND1*, *FGF4*, and *EMS1*) was frequently amplified (28%, 9/32) (8). Another group reported that the 11q22 locus containing *cIAP1* and *MMPs* has been reported to be amplified in 4 of 42 primary ESCCs (9.5%) (12). Therefore, it has been concluded that the 11q13 locus is the most frequently amplified and a major target in ESCC development. *EMS1* in the same amplified locus is known to be involved in invasion and metastasis (13), a function that may account for a report that amplification of the 11q13 locus is useful for predicting outcome and distant organ metastasis in ESCC patients (14).

We found that the *p16* deletion frequency increases in association with ESCC progression (primary tumors, 30.8%; metastatic lymph nodes, 50%; and circulating tumor cells, 75%). Matrigel invasion assays of *p16*-deleted ESCC cells showed that restoring wild-type *p16* activity into the cells significantly inhibits tumor-cell invasion, suggesting that *p16* inactivation could be involved in ESCC invasion. Recently, there is accumulating evidence showing different functions including migration, angiogenesis, and skeletogenesis for *p16* aside from its control of the cell cycle (15,16). It has been reported that adenovirus-mediated *p16* gene transfer suppresses glioma invasion (17). This report also showed that exogenous *p16* expression significantly reduced the expression of matrix metalloproteinase-2 (*MMP-2*), an enzyme involved in tumor-cell invasion. Recently, it has also been reported that *p16* inhibits *MMP-2* expression through the attenuation of Sp1 binding to the *MMP-2* promoter (18). In ESCCs also, the targets for a transcription factor Sp1 should be identified for understanding the detailed mechanism of *p16* in invasion inhibition and for developing new anti-tumor drugs.

Our established xenografts can provide highly sensitive results in detecting gene amplification and deletion by array-based CGH. Many genetic alterations in ESCCs have also been found in other squamous cell carcinomas, especially in

head and neck SCCs. Therefore, the present gene list should be helpful for identifying new amplified and deleted genes in primary tumors as well as in metastasized lymph nodes not only in ESCCs but also in head and neck SCCs.

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