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An Ultrasensitive New DNA Microarray Chip Provides Gene Expression Profiles for Preoperative Esophageal Cancer Biopsies without RNA Amplification

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Key Words
Microarray · Esophageal cancer · Preoperative · Biopsy · Diagnosis

Abstract
Objectives: Gene expression profiling using pretreatment biopsies has been limited due to their small sample sizes. This study evaluated the usefulness of an ultrasensitive new DNA microarray chip, which has a unique array structure, for the clinical diagnosis of esophageal cancer using preoperative biopsies. ***Methods:*** Paired cancer and normal esophageal epithelial tissues from 56 patients who underwent esophagectomy and from 48 patients who underwent preoperative endoscopy were studied. Among 2 feature gene sets selected by a reference DNA chip discriminating malignant status of samples, 20 feature genes were selected for the development of the new DNA chip. The new DNA chip was hybridized with 0.1 μg of total RNA per slide without RNA amplification. ***Results:*** Twenty feature genes, including *RRM-2* and *XRCC-3*, for the new DNA chip could discriminate cancer from noncancer at a 95.2% rate of accuracy in 42 biopsies (sensitivity 95.7%, specificity 94.7%). A receiver operating characteristic (ROC) curve analysis showed that the area under ROC curve for the prediction was 0.966. ***Conclusions:*** The gene expression profiles from the preoperative biopsies could diagnose esophageal cancer accurately, using the ultrasensitive DNA chip without RNA amplification. This new DNA chip technology might contribute further to the development of customized therapeutic strategies for various cancer patients.

Introduction

Esophageal cancer has the sixth highest mortality rate of all cancers [1]. Overall survival is less than 10% and 5-year survival for resected patients is 20–40% despite the recent advances of several therapeutic strategies [1, 2]. Selection for suitable therapeutic strategies before the treatment is, however, sometimes difficult in current diagnos-

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tic approaches. Therefore, a new pretreatment diagnostic method is truly needed.

Microarray-based gene expression profiles can provide fundamental insights into cancer biology [3] and have the potential to predict patient outcome [4, 5] and response to therapy [6]. Application of this technology into pretreatment diagnosis using biopsy samples seems to be one of most promising approaches for improving outcomes of patients with esophageal cancer. The first consideration for pretreatment diagnosis using biopsy samples is the requirement of a large amount of high-quality RNA. Although an average microarray experiment requires 10–100 μg of total RNA, most biopsies do not yield this amount due to their small sizes. Despite the progress of recent RNA amplification technology [7], it was reported that some noises or biases can be introduced and alter the gene expression profiles [8, 9]. The second consideration is the clinical utility of the platform in terms of its ease of use. The process of laser capture microdissection or RNA amplification takes considerable time and expense for the clinical setting. To solve these problems, we applied a newly developed high-performance DNA chip to the preoperative diagnosis. The new DNA chip exhibits ultrahigh sensitivity, up to 100-fold higher than that of a reference DNA chip, and requires only 0.01–0.1 μg of total RNA without any amplification [10].

We describe here the application of a new valuable diagnostic DNA chip with accurate feature genes for clinical diagnosis of esophageal cancer using preoperative biopsy samples. This new DNA chip technology might contribute further to customized therapeutic strategies for patients with various cancers.

Material and Methods

Tumor Samples

Frozen paired cancer and normal esophageal epithelial tissues were obtained for the microarray analysis from 56 patients with primary esophageal cancer who underwent surgery and from 48 patients who underwent endoscopic examination at the Kyoto University Hospital from 1992 to 2006. Tissue samples of resected specimen after surgery were obtained by experienced surgeons. Tissue samples of endoscopic examination were obtained from 4 sites of cancer tissues and 2 sites of normal epithelial tissues by expert endoscopists. A pair of biopsy samples of each esophageal primary tumor or each normal tissue of each patient was acquired from the same location. One of the pair was studied by microarray analyses, while the other aliquot was examined histologically by experienced pathologists. All tumors were confirmed as esophageal cancer by the clinicopathological department of the hospital. All cases were classified according to the sixth edition of the pathological tumor node metastasis (TNM) classification of 2002 [11]. Information on gender, age, stage of disease and histopathological factors were abstracted from the medical records. Written informed consent was obtained from all patients for surgery and the use of samples for research. The study protocol was approved by the Institutional Review Board of Kyoto University (approval No. G-116).

Total RNA Extraction

Tissue samples were frozen in liquid nitrogen immediately after resection and stored before use. Frozen samples were treated with RNAlater-ICE (Ambion, Austin, Tex., USA) at –20°C for 2 h, crushed into pieces, and extracted by a phenol-chloroform RNA extraction method (Trizol; Invitrogen, Carlsbad, Calif., USA). Qualities of the purified total RNAs were analyzed by a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, Calif., USA). The criterion for the use of a sample was that the 18s and 28s ribosomal RNA peaks were twice or more as high as other peaks, as described previously [12].

The Reference DNA Chip

The reference DNA chip produced by Toray Industries Inc. (Kanagawa, Japan) [10] was used to identify the feature genes for the new DNA chip. The 8,068 DNA probes which comprise 70-base oligonucleotides were spotted on a commercially available glass slide (Matsunami Glass Industries, Kishiwada, Japan). An Affymetrix GeneChip U133A array (Affymetrix, Santa Clara, Calif., USA) with 14,500 human transcripts was used for the preliminary experiments to pick up the candidate genes for the reference DNA chip. For this preliminary experiment using the Affymetrix array, the surgically resected 5 matched pairs of esophageal normal epithelial tissues and primary esophageal cancer tissues (a total of 10 samples) from 5 patients were used.

The New DNA Chip '3D-Gene'

The new DNA chip was developed by Toray Industries Inc. as described previously [10]. In brief, the new DNA chip has a unique depressed structure with 256 arrayed pillars, which enable a low level of nonspecific absorption of target DNA and high signal intensity. On the surface of the substrate made by polymethyl methacrylate, 5'-amino-modified 70-base oligonucleotides were spotted. For the new DNA chip, 20 feature genes which can discriminate esophageal cancer from esophageal normal epithelial tissues and 107 genes which have the same expression levels for esophageal cancer and esophageal normal epithelial tissues were selected through analysis of the reference DNA chip.

Hybridizations

For the reference DNA chip or the new DNA chip, 1 or 0.1 μg of total RNA, respectively, was reverse transcribed to cDNA and labeled using the CyScribe First-Strand cDNA Labeling Kit (GE Healthcare, Piscataway, N.J., USA) as described previously [10]. A commercially available reference RNA (Human Universal Reference RNA; Stratagene, La Jolla, Calif., USA) was used for cohybridization with the sample total RNA. Each sample cDNA labeled with Cy3 was cohybridized in duplicate with the universal reference cDNA labeled with Cy5 at 42°C for 16 h. After the hybridization, each DNA chip was washed and dried. Hybridization signals were scanned using a DNA microarray scanner (Agilent Technologies) for the reference DNA chip, and GenePix4000B (Molecular Devices, Sunnyvale, Calif., USA) for the new DNA

chip, and processed by the Feature Extraction Software, version 7.1 (Agilent Technologies) or GenePixPro version 4.0 (Molecular Devices). Each DNA chip data set was normalized by a pin-tip LOWESS method using the R software, which is available from the Comprehensive R Archive Network site (http: //cran.r-project.org).

Feature Gene Selection and Support Vector Machine

All Pearson linear correlation coefficients were calculated in multiple experiments, and data which have correlation coefficients less than 0.7 were excluded from the following analyses. Feature gene selection was done by a bootstrap method as described previously [13]. A temporary feature gene list was selected by a random sampling from each data set and the selection was repeated for the number of filtered genes. Among these temporary feature gene lists, an average ranking of each gene was calculated and a permanent feature gene list was determined. Within the feature genes selected, a leave-one-out cross-validation analysis was done with a support vector machine algorism [13]. Then, the number of feature genes was determined to optimize the accuracy of prediction.

Quantitative Real-Time Reverse Transcription PCR

Quantitative real-time reverse transcription PCR was carried out using a Light Cycler (Roche Diagnostics, Basel, Switzerland) with a Light Cycler DNA master SYBR-Green I kit (Roche Diagnostics) according to the manufacturer's instruction. *XRCC-3* and *RRM-2* were used for this assay, because these genes were expressed differentially among samples in the microarray analysis. The expressions of these genes were normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase. The primer sequences for *XRCC-3*, *RRM-2* and *glyceraldehyde-3-phosphate dehydrogenase* were as follows. The forward primers were 5′-AGTCCTTACTCCCCCACCAC-3′, 5′-GGCTTTAAAGTGAGGGGTGA-3′ and 5′-TGGTATCGTGGAAGGACTCATGAC-3′, and the reverse primers were 5′-TGGCACAGGCCACACTACTA-3′, 5′-GCTCCCATTAGTGGTCAGGA-3′ and 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′, respectively.

Receiver Operating Characteristic Curve Analysis

A receiver operating characteristic (ROC) curve analysis was done by Analyze-it, version 1.73 (Analyze-it Software, Leeds, UK), to evaluate the accuracies of the prediction models according to the manufacturer's instruction.

Results

Preparation of Total RNA

We extracted total RNAs from 112 pairs of surgically resected samples from 56 patients and 96 pairs of preoperative biopsy samples from 48 patients for this study. Among these 112 surgically resected and 96 biopsy samples, total RNAs of 12 surgically resected samples (12/112; 10.7%) and 15 biopsy samples (15/96; 15.6%) were severely degraded on the RNA quality assay. Therefore, these degraded samples were excluded and the remaining 100 surgically resected and 81 biopsy samples from 102 cases were subjected to the further steps. The average total RNA yield from the biopsy samples was 37.9 μg (range 4.3–103 μg). Since 0.1 or 1 μg of total RNA was used for the new DNA chip or the reference DNA chip, respectively, these amounts were sufficient for the hybridizations and the following analyses. Clinicopathological characteristics of the tumors, which were used in our analyses, are provided in table 1. There are no significant differences in the clinicopathological factors between the 3 groups of patients: (1) surgery patients for the reference DNA chip (n = 56), which included 46 cancer samples and 54 noncancer samples; (2) biopsy patients for the reference DNA chip (n = 20), which included 20 cancer samples and 19 noncancer samples; (3) biopsy patients for the

Table 1. Clinicopathological characteristics of the tumors

Variables	Surgery ref. chip (n = 56)	Biopsy ref. chip (n = 20)	Biopsy new chip (n = 26)
Age, years			
Mean	66.2	61.2	63.1
SD	6.9	5.6	6.5
Gender			
Male	48	19	25
Female	8	1	1
Primary tumor			
pT1	15	4	6
pT2	18	5	5
pT3	20	9	10
pT4	3	2	5
Lymph node metastasis			
pN0	20	9	10
pN1	36	11	16
Distant metastasis			
pM0	45	18	24
pM1	11	2	2
pTNM stage			
I	8	3	5
IIa	12	6	4
IIb	13	3	4
III	12	1	9
IV	11	2	3
Histology			
SCC G1	10	5	9
SCC G2	29	10	13
SCC G3	11	5	2
AC	2	0	1
Other	4	0	1

Ref. chip = Reference DNA chip; SCC = squamous cell carcinoma; AC = adenocarcinoma; G1–G3 = grade 1–3.

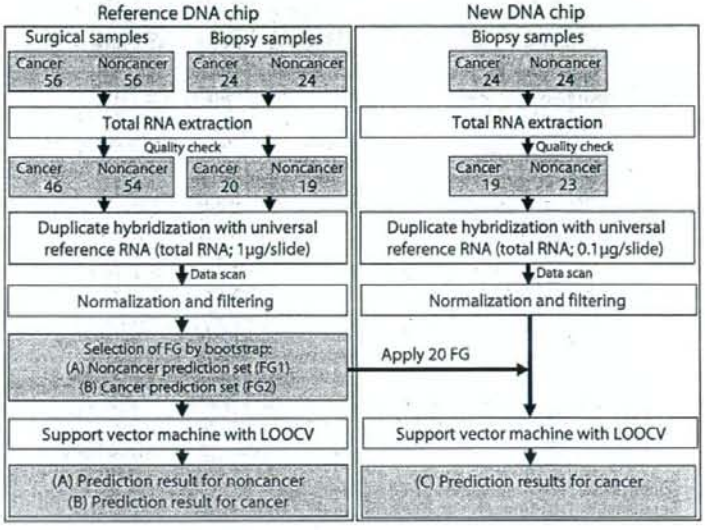

Fig. 1. Outline of this study. One hundred surgically resected and 81 biopsy samples were included in this study after the RNA quality assay. The reference DNA chip was used to select the feature genes for the new DNA chip. Two types of feature genes (FG1 and FG2) were selected for the noncancer and the cancer prediction model using the reference DNA chip through the analysis by a support vector machine and a leave-one-out cross-validation analysis. The 20 feature genes were applied to and validated by the new DNA chip. The numbers shown in the boxes are the numbers of clinical samples. FG = Feature genes; LOOCV = leave-one-out cross-validation.

new DNA chip (n = 26), which included 19 cancer samples and 23 noncancer samples. Of the 102 cases, 94 (92.2%) had squamous cell carcinoma, 3 (2.9%) had adenocarcinoma and 5 (4.9%) had other histological tumors. In the surgery group, biopsy (reference chip) group and biopsy (new chip) group, there are 12, 6 and 4 patients who received neoadjuvant chemotherapy, respectively, and 3, 1 and 0 patients who received preoperative radiotherapy, respectively.

Feature Gene Selection by the Reference DNA Chips

For the reference DNA chip, an Affymetrix GeneChip U133A array with 14,500 human transcripts was used for the preliminary experiments to pick up the candidate genes for the reference chip. Among the 14,500 genes, 5,923 had differential expression patterns between normal esophageal epithelium and esophageal cancer, and 2,407 genes had the same expression patterns for both.

The outline of the analyses is shown in figure 1. To identify feature genes for the new DNA chip, we analyzed the expression profiles of the reference DNA chips. Total RNAs from surgically resected samples with 46 cancer and 54 noncancer (normal epithelial) tissues and biopsy samples with 20 cancer and 19 noncancer tissues were included in this analysis. After duplicate hybridization with the commercially available reference RNA, the raw data sets were normalized and subjected to a data filtering. We selected 2 different feature genes from the data sets to discriminate the malignant status of the biopsy samples, which comprise 18 feature genes for a noncancer prediction (FG1) and 20 feature genes for a cancer prediction (FG2). Among them, 20 feature genes were selected from a combination of FG1 and FG2 for the development of the new DNA chip according to the optimization for the prediction accuracies. Ten feature genes overlap in the feature genes for both noncancer (FG1) and cancer prediction (FG2). A list of the FG1, FG2 and optimized 20 feature genes selected by the reference DNA chip is shown in table 2. Among the optimized 20 feature genes, only 1 gene *(SPRR3)* was known to associate with esophageal cancer [14–16].

Prediction Using the Reference DNA Chips

We divided the samples to the following 4 groups as shown in table 3a: (I) surgically resected noncancer tissues (n = 54); (II) surgically resected cancer tissues (n = 46); (III) noncancer tissues in biopsy samples (n = 19); (IV) cancer tissues in biopsy samples (n = 20). For the prediction for noncancer, the training data set included groups I, II and IV. Eighteen feature genes (FG1) were used to discriminate all 139 samples and gave a predictive accuracy of 95.0% (sensitivity 93.2%, specificity 97.0%; table 3b). For the prediction for cancer, the training data set included groups I–III. Twenty feature genes (FG2)

Table 2. Feature genes selected by the reference DNA chip

No.	Gene name, description	Symbol[1]	Gene annotation[2]	RefSeq	Map
	FG1 for noncancer prediction				
1	fibroblast growth factor 3	**FGF3**	growth factor	NM_005247	11q13
2	galactosamine (N-acetyl)-6-sulfate sulfatase	**GALNS**	polysaccharide metabolism, sulfur metabolism	NM_000512	16q24.3
3	calcium/calmodulin-dependent protein kinase II inhibitor 1	**CAMK2N1**	not classified	NM_018584	1p36.12
4	X-ray repair cross-complementing protein 3	XRCC3	DNA repair	NM_005432	14q32.3
5	calcium/calmodulin-dependent protein kinase II-beta	**CAMK2B**	serine/threonine protein kinase	NM_001220	7p14
6	small praline-rich protein 3	**SPRR3**	not classified	NM_005416	1q21-q22
7	ribonucleotide reductase, M2 subunit	**RRM2**	not classified	NM_001034	2p25-p24
8	chromosome 14 open reading frame 162	**C14orf162**	not classified	NM_020181	14q24.1
9	septin 6	SEPT6	small GTPase, cytokinesis	NM_145799	Xq24
10	eukaryotic translation initiation factor 4E-binding protein 2	EIF4EBP2	translation factor, tumor suppressor	NM_004096	10q21-q22
11	trophinin-associated protein (tastin)	**TROAP**	not classified	NM_005480	12q13.12
12	presenilin-associated rhomboid-like protein	**PSARL**	serine protease	NM_018622	3q27.3
13	growth arrest-specific 1	GAS1	not classified	NM_002048	9q21-q22
14	cystatin B	CSTB	cysteine protease inhibitor	NM_000100	21q22.3
15	apolipoprotein M	APOM	not classified	NM_019101	6p21.33
16	cytochrome b-561	CYB561	electron transport processes	NM_001915	17q11
17	ret finger protein	RFP	ubiquitin-protein ligase	NM_006510	6p22
18	solute carrier family 2 (facilitated glucose transporter), member 14	**SLC2A14**	carbohydrate transporter	NM_153449	12p13.31
	FG2 for cancer prediction				
1	galactosamine (N-acetyl)-6-sulfate sulfatase	**GALNS**	polysaccharide metabolism, sulfur metabolism	NM_000512	16q24.3
2	fibroblast growth factor 3	**FGF3**	growth factor	NM_005247	11q13
3	presenilin associated rhomboid-like protein	**PSARL**	serine protease	NM_018622	3q27.3
4	calcium/calmodulin-dependent protein kinase II-β	**CAMK2B**	serine/threonine protein kinase	NM_001220	7p14
5	special AT-rich sequence-binding protein 2	SATB2	mRNA transcription regulation	NM_015265	2q32-q33
6	ribonucleotide reductase, M2 subunit	**RRM2**	not classified	NM_001034	2p25-p24
7	calcium/calmodulin-dependent protein kinase II inhibitor 1	**CAMK2N1**	not classified	NM_018584	1p36.12
8	glyoxylate reductase/hydroxypyrvate reductase	GRHPR	amino acid biosynthesis	NM_012203	9q12
9	trophinin-associated protein (tastin)	**TROAP**	not classified	NM_005480	12q13.12
10	capping protein (actin filament), gelsolin-like	CAPG	cell structure	NM_001747	2cen-q24
11	mannose-6-phosphate receptor	M6PR	not classified	NM_002355	12p13
12	echinoderm microtubule-associated protein-like 1	EML1	not classified	NM_004434	14q32
13	yippee-like 5	YPEL5	not classified	NM_016061	2p23
14	slit homolog 2	SLIT2	cell adhesion, motility	NM_004787	4p15.2
15	solute carrier family 2 (facilitated glucose transporter), member 14	**SLC2A14**	carbohydrate transporter	NM_153449	12p13.31
16	small praline-rich protein 3	**SPRR3**	not classified	NM_005416	1q21-q22
17	acetyl-coenzyme A acyltransferase 2	ACAA2	transferase	NM_006111	18q21.1
18	dolichyl-phosphate mannosyltransferase polypeptide 3	DPM3	glycosyltransferase	NM_153741	1q22
19	chromosome 14 open reading frame 162	**C14orf162**	not classified	NM_020181	14q24.1
20	calmodulin 2	CALM2	phosphorylase kinase	NM_001743	2p21

Table 2 (continued)

No.	Gene name, description	Symbol[1]	Gene annotation[2]	RefSeq	Map
	FG for new DNA chip cancer prediction				
1	galactosamine (N-acetyl)-6-sulfate sulfatase	**GALNS**	polysaccharide metabolism, sulfur metabolism	NM_000512	16q24.3
2	fibroblast growth factor 3	**FGF3**	growth factor	NM_005247	11q13
3	calcium/calmodulin-dependent protein kinase II-β	**CAMK2B**	serine/threonine protein kinase	NM_001220	7p14
4	calcium/calmodulin-dependent protein kinase II inhibitor 1	**CAMK2N1**	not classified	NM_018584	1p36.12
5	presenilin-associated rhomboid-like protein	**PSARL**	serine protease	NM_018622	3q27.3
6	X-ray repair cross-complementing protein 3	XRCC3	DNA repair	NM_005432	14q32.3
7	capping protein (actin filament), gelsolin-like	CAPG	cell structure	NM_001747	2cen-q24
8	glyoxylate reductase/hydroxypyrvate reductase	GRHPR	amino acid biosynthesis	NM_012203	9q12
9	trophinin-associated protein (tastin)	**TROAP**	not classified	NM_005480	12q13.12
10	ribonucleotide reductase, M2 subunit	**RRM2**	not classified	NM_001034	2p25-p24
11	special AT-rich sequence-binding protein 2	SATB2	mRNA transcription regulation	NM_015265	2q32-q33
12	chromosome 14 open reading frame 162	**C14orf162**	not classified	NM_020181	14q24.1
13	septin 6	SEPT6	small GTPase, cytokinesis	NM_145799	Xq24
14	mannose-6-phosphate receptor	M6PR	not classified	NM_002355	12p13
15	small praline-rich protein 3	**SPRR3**	not classified	NM_005416	1q21-q22
16	echinoderm microtubule-associated protein-like 1	EML1	not classified	NM_004434	14q32
17	yippee-like 5	YPEL5	not classified	NM_016061	2p23
18	eukaryotic translation initiation factor 4E-binding protein 2	EIF4EBP2	translation factor, tumor suppressor	NM_004096	10q21-q22
19	solute carrier family 2 (facilitated glucose transporter), member 14	**SLC2A14**	carbohydrate transporter	NM_153449	12p13.31
20	slit homolog 2	SLIT2	cell adhesion, motility	NM_004787	4p15.2

[1] Bold symbol represents overlapped 10 feature genes between FG1 and FG2.
[2] Molecular function or biological process according to the PANTHER classification system (http://panther.appliedbiosystems.com).

were used to discriminate all 139 samples and gave a predictive accuracy of 95.0% (sensitivity 97.3%, specificity 92.4%; table 3c).

Prediction Using the New DNA Chips and ROC Curve Analysis

To diagnose esophageal cancer by a handy and accurate platform, we developed the new DNA chip with the 20 feature genes which can discriminate between cancer and noncancer tissues. The predictive accuracy using these 20 feature genes on the new DNA chip was 95.2% (sensitivity 95.7%, specificity 94.7%; table 3d).

A photograph of the new DNA chip and a magnified image of pillars at the center of the substrate are shown in figure 2. An average value of feature gene expression in each subset of sample group in the new DNA chips is shown in figure 2c. The expressions of these feature genes in normal tissues were higher than those in cancer tissues in biopsy samples, which were all compatible with the finding in the analysis using the reference DNA chip. Real-time reverse transcription PCR confirmed the differential expression patterns of *XRCC-3* and *RRM-2*, validating these findings (fig. 3). *XRCC-3* and *PRM-2* had lower expressions in cancer biopsy tissues compared with corresponding normal epithelial biopsy tissues in the validation study, which were consistent with microarray expression findings of the new DNA chip.

As shown in figure 4, a ROC curve analysis was done to evaluate the accuracies of the predictions. For the training set by the reference DNA chip, the area under ROC curve (AUROC) was 0.970 and 0.962 for noncancer and cancer prediction models, respectively. For the validation set by the new DNA chip, the AUROC was 0.966. This analysis showed that the prediction models for both the reference DNA chip and the new DNA chip could diagnose the malignant status of the samples accurately.

All processes for obtaining a diagnostic result from each biopsy sample using the new DNA chip took approximately 24 h, including 3 h of RNA preparation and 16 h of hybridization.

Discussion

Microarray analysis has been shown to be a powerful tool for molecular diagnosis as well as for personalized therapy in a variety of malignancies. In our previous study, the microarray analysis can discriminate cancer subtypes and predict patient outcomes of esophageal cancer [17]. Some of the genes in the expression profiles presented to date seem to be derived from nonepithelial components of the tumor [18], suggesting that stromal elements represent an important contributing factor to the cancer phenotypes. Laser capture microdissection is now widely used to obtain a pure expression profile for cancer cells; however, the process is thought to be too expensive and time consuming for clinical application so far. We have also shown previously that a microarray analysis of surgically resected samples by artificial neural networks can predict lymph node metastasis of esophageal cancer with high accuracy [19], suggesting that the metastatic state can be deciphered from the gene expression pattern of primary tumor and that treatment can be substantially improved. In that study, however, we could not use preoperative biopsy samples because the cDNA microarray required 120 μg of total RNA per slide. Now the new ultrasensitive DNA chip enables us to use preoperative biopsy samples and to proceed to the next step to predict the status of lymph node metastasis in esophageal cancer.

We selected 20 feature genes to discriminate esophageal cancer from normal epithelial tissues. The predictive accuracy using these 20 feature genes on the new DNA chip was 95.2% (sensitivity 94.7%, specificity 94.7%) and the AUROC was 0.966, demonstrating that the prediction models for the new DNA chip could diagnose the malignant status of the samples accurately. Thus, these 20 feature genes were thought to have a potential to diagnose esophageal cancer before the treatment. Among 5,923 differentially expressed genes in the Affymetrix chip analysis, 3,108 genes were upregulated and 2,815 genes were downregulated in cancers. Among FG1, all 18 genes were downregulated in cancers. Among FG2, 19 genes were downregulated in cancers and only 1 gene *(CALM2)* was upregulated. This finding may suggest that downregulated genes in cancers primarily contribute to these prediction models in the reference DNA chip and the new DNA chip. In the diagnosis on the new chip for 42 biopsy samples, 1 normal sample from a 73-year-old male (tumor status: pT4N1M1, stage IV, SCC G1) was classified as malignant and 1 cancer sample from a 64-year-old male (tumor status: pT-3N0M0, stage I, SCC G1) was classified as benign. However, there were no related characteristics between these 2 cases.

Table 3. Prediction results

a Sample grouping for prediction

	Noncancer	Cancer	Total
Surgery	I (n = 54)	II (n = 46)	100
Biopsy	III (n = 19)	IV (n = 20)	39
Total	73	66	139

b Prediction for noncancer

Reference chip prediction	Histology		Chip characteristics
	non-cancer	cancer	
Noncancer	68	2	feature genes: 18; learning sets: I + II + IV; n = 120; accuracy: 0.950; sensitivity: 0.932; specificity: 0.970
Cancer	5	64	
Total	73	66	

c Prediction for cancer

Reference chip prediction	Histology		Chip characteristics
	non-cancer	cancer	
Noncancer	71	5	feature genes: 20; learning sets: I + II + III; n = 119; accuracy: 0.950; sensitivity: 0.973; specificity: 0.924
Cancer	2	61	
Total	73	66	

d Prediction for cancer

New chip prediction	Histology		Chip characteristics
	non-cancer	cancer	
Noncancer	22	1	feature genes: 20; biopsy; n = 42; accuracy: 0.952; sensitivity: 0.957; specificity: 0.947
Cancer	1	18	
Total	23	19	

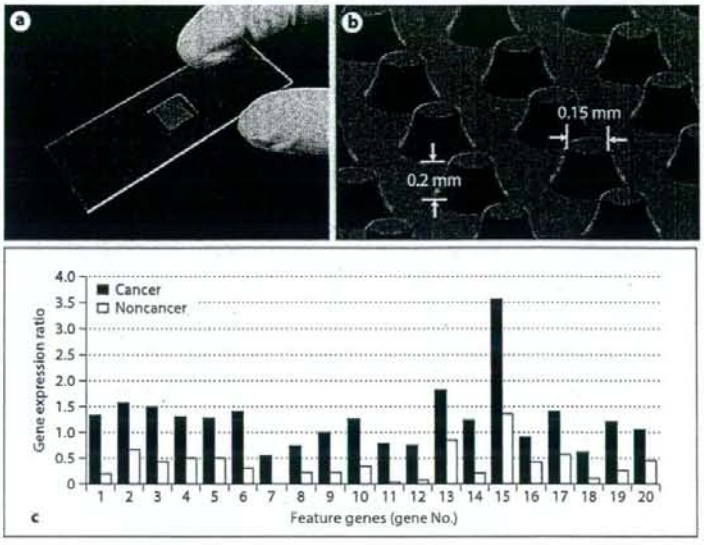

Fig. 2. a Photograph of the new DNA chip. **b** Magnified image of pillars at the center of the substrate. **c** Average values of feature gene expression in each subset of biopsy sample groups in the new DNA chips. The y-axis stands for the expression ratio of each feature gene in noncancer or cancer to the commercially available reference RNA. The gene numbers in this figure are identical to those in table 2.

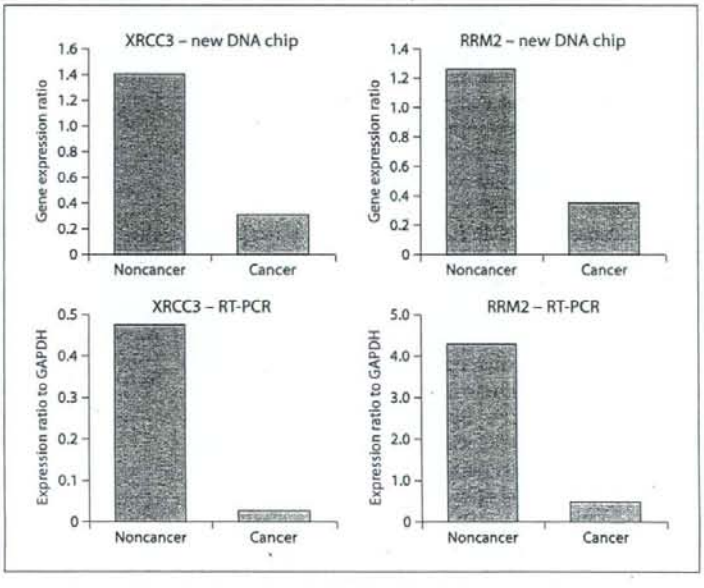

Fig. 3. Validation study by real-time reverse transcription PCR using cancer biopsy tissues and corresponding normal epithelial biopsy tissues. The y-axis for the new DNA chip and the RT-PCR stands for the expression ratio of each gene to the commercially available reference RNA and the normalized expression by the expression of *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, respectively.

A remarkable feature of the expression profiles identified in previous studies is that they usually involve fewer than 100 genes [19, 20], in one instance only 17 genes [18]. Less than hundreds rather than thousands of genes are therefore likely to be sufficient, indicating that a classification can be achieved using less expensive, faster and more robust platforms for examining gene expression. Therefore, this 256-array platform is thought to be suit-

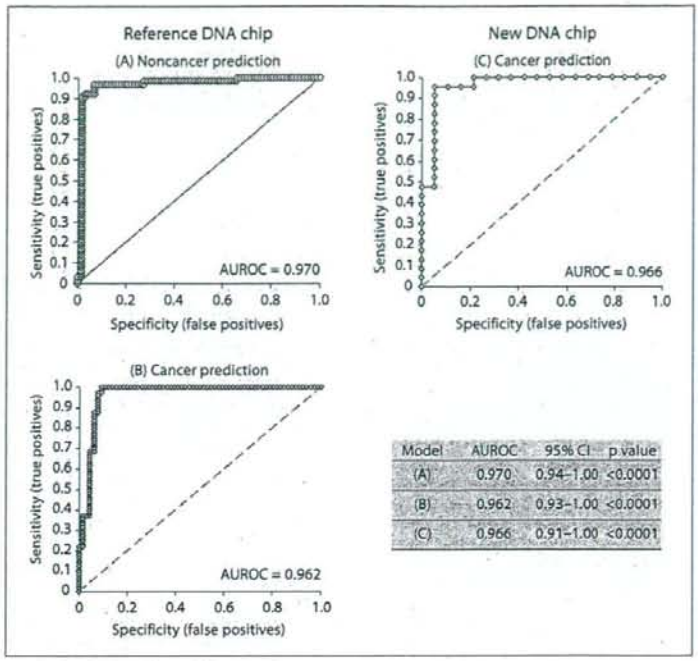

Fig. 4. ROC curve analysis. The result of the analysis is summarized in a table on the right below. (A) Noncancer prediction by the reference DNA chip. (B) Cancer prediction by the reference DNA chip. (C) Cancer prediction by the new DNA chip. AUROC = Area under ROC curve; CI = confidence interval.

able for the diagnostic purpose. Among the 20 feature genes we selected, only 1 esophageal cancer-related gene *(SPRR3)* was included. *SPRR3*, which is also known as *esophagin*, a possible tumor suppressor gene for esophageal cancer, was previously cloned by our colleagues and is thought to promote esophageal epithelial cell differentiation [14, 15]. However, 8 were other cancer-related genes and 11 genes have not been implied in human carcinogenesis, and these genes may increase the pool of potentially useful molecules for diagnosis and therapy of esophageal cancer.

A number of studies for pretreatment diagnosis using microarray analysis have been testing in other malignancies such as breast, prostate or colon cancer [12, 21, 22]. According to Centeno et al. [12], an average total RNA yield from fine needle aspiration biopsies in 18 tumor samples of various cancers was 3.63 μg (range 0.72–12.5 μg). Thus, the new DNA chip can also be applicable to the analysis of samples smaller than those from tissue biopsy, such as from fine needle aspiration biopsies.

In this study, the gene expression profiles from a very small amount of RNA (0.1 μg of total RNA) can achieve an accurate prediction for the diagnosis of esophageal cancer using the new ultrasensitive DNA chip without any RNA amplification. Further projects for customized therapy using our new DNA chip technology are currently under way to achieve accurate pretreatment diagnoses for effective lymph node resection, radiotherapy and/or chemotherapy.

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ORIGINAL ARTICLE

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Retrospective analysis of 27 consecutive patients treated with docetaxel/nedaplatin combination therapy as a second-line regimen for advanced esophageal cancer

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Abstract
Background. The aim of this study was to evaluate the efficacy and safety of combination therapy with docetaxel and nedaplatin in advanced esophageal cancer as a second-line regimen in an outpatient setting.
Methods. Twenty-seven consecutive patients with advanced esophageal cancer who received docetaxel/nedaplatin combination therapy as a second-line regimen were retrospectively evaluated. The combination therapy consisted of intravenous administration of docetaxel 30 mg/m^2 and nedaplatin 40 mg/m^2 every 2 weeks.
Results. The patients received a median of 7.4 cycles of treatment (range, 2–25 cycles). No complete response was observed, and 3 of the 27 patients (11%) achieved partial responses. The disease control rate (partial response + stable disease) was 52%. The median survival time (MST) was 11.4 months. Severe hematological adverse events (grade 3–4) were: neutropenia (n = 10; 37%) and anemia (n = 5; 19%); there was neither febrile neutropenia nor grade 3-4 thrombocytopenia. Furthermore, no severe nonhematological adverse events or treatment-related deaths were observed.
Conclusion. Combination therapy of docetaxel with nedaplatin was safe and well tolerated; however, the development of more effective therapy is warranted to improve the prognosis of esophageal cancer.

Key words Docetaxel · Nedaplatin · Esophageal cancer · Chemotherapy

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Introduction

The incidence of esophageal cancer is increasing in Western countries as well as in Japan. Interestingly, squamous cell carcinoma accounts for more than 90% of esophageal cancers in Japan, while adenocarcinoma has rapidly increased over the past decade and has now become the most common histological type in Western countries.[1,2] In both histological subtypes, early lymphogenic and hematogenous spread is a common feature, resulting in the poor prognosis of this disease.[3] Even patients with the earliest stage disease have a significant risk of recurrence. Thus, locoregional treatment is not sufficient to treat this highly lethal disease, and the development of effective systemic chemotherapy is urgently needed. A variety of chemotherapeutic regimens has been tested until now;[4–6] however, no gold standard chemotherapy regimen has been established for advanced esophageal cancer. At present, a combination of cisplatin and continuous-infusion 5-fluorouracil (5-FU) is one of the most commonly used regimens, with a 25%–35% response rate in metastatic disease.[3,7] The major problem of cisplatin-based chemotherapy lies in its nephrotoxity. The use of this chemotherapy mandates large-volume hydration, which limits the management of this agent in an outpatient setting, and badly hampers the patient's quality of life.

Docetaxel has been proven to show antitumor activity against various cancers, including esophageal cancer.[8–10] Clinical trials of single-agent docetaxel have demonstrated 20%–28% response rates for its use as a second-line regimen in advanced esophageal cancer.[9,10]

Nedaplatin (*cis*-diammine-glycolate platinum) is a second-generation platinum derivative developed in Japan, and several in vitro studies have demonstrated that nedaplatin has equivalent antitumor activity to cisplatin, with less nephrotoxity.[11,12] Consistent with the results of the in vitro studies, nedaplatin in combination with other agents has shown modest antitumor activity for several human tumors, with less nephrotoxicity and gastrointestinal toxicity.[13,14] These reports prompted us to use a combination of docetaxel with nedaplatin as a second-line regimen in patients with advanced esophageal cancer, because pretreated patients have poorer tolerance to second-line chemotherapy, and a less toxic treatment is desirable. Supporting our idea, at the time we started this combination therapy, several Japanese groups were also using docetaxel/nedaplatin combination regimens for treating advanced esophageal cancer, as well as for head and neck cancer. We carried out the present retrospective study to evaluate docetaxel/nedaplatin combination therapy as a second-line regimen in patients with esophageal cancer, and we report our findings here.

Patients and methods

Patients

Twenty-seven consecutive patients who received docetaxel/nedaplatin combination therapy as a second-line regimen at Kyoto University Hospital, between January 2004 and June 2006, were retrospectively analyzed. All the patients had histologically proven esophageal cancer with metastasis. Docetaxel/nedaplatin combination therapy was given if the patient met the following criteria: an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; adequate baseline bone marrow function (hemoglobin level, ≥8 g/dl; neutrophil count, ≥1000/mm^3; and platelet count, ≥75 000/mm^3); adequate hepatic function (total bilirubin level, ≤1.5 mg/dl; and aspartate aminotransferase and alanine aminotransferase levels, ≤3.0 × the upper limit of normal); adequate renal function (serum creatinine level, ≤1.5 mg/dl); and written informed consent to receive chemotherapy at our hospital. Toxicities were graded according to the Common Terminology Criteria for Adverse Events v 3.0 (CTCAE v 3.0). Measurable target lesions were assessed by the Response Evaluation Criteria in Solid Tumors (RECIST).[15]

Treatment plan

Docetaxel 30 mg/m^2 (Taxotere; Aventis Pharma, Tokyo, Japan) was infused in 40 min, followed by nedaplatin 40 mg/m^2 (Aqupla; Shionogi Pharma, Tokyo, Japan) over 1.5 h, every 2 weeks. These doses were based on a phase I trial of chemotherapy using docetaxel and nedaplatin in chemotherapy-naïve patients with oral squamous cell carcinoma in Japan.[15] That phase I trial recommended 60 mg/m^2 docetaxel and 100 mg/m^2 nedaplatin every 4 weeks in chemotherapy-naïve patients. Because our treatment was to be repeated every 2 weeks in patients who had had prior chemotherapy, we adjusted the doses of docetaxel and nedaplatin to 30 mg/m^2 and 40 mg/m^2, respectively. Premedication with an H_2-blocker and dexamethasone, as well as post-therapy hydration with 500 ml saline was given for every administration. 5-HT_3 antagonists were added when the oncologist judged it necessary. If the neutrophil count was less than 1000/μl or the platelet count was less than 75 000/μl on the day of administration, treatment was adjourned until the count recovered. The dose of docetaxel and nedaplatin was reduced by 20% when the oncologist judged it necessary, according to hematological and other toxicities observed in the previous course. Treatment was continued until disease progression, unacceptable adverse events, patient withdrawal, or death.

Follow-up evaluation

Standard radiological examinations, using computed tomography (CT) scans, were applied for response every 1 to 3 months. Tumor response was assessed based on RECIST.[14] Metastatic lymph nodes within a previous radiation field and the primary lesion were also evaluated. Complete response (CR) was defined as the disappearance of all evidence of cancer, and partial response (PR) as a more than 30% reduction in the sum of the longest diameters of target lesions. Stable disease (SD) was defined as either a less than 30% reduction in the sum of the longest diameters of target lesions, or less than a 20% increase without any new lesions. Progressive disease (PD) was defined as more than a 20% increase in the sum of the longest diameters of target lesions or the appearance of new lesions. The best response achieved during the treatment course was reported.

Statistical methods

Overall survival was measured from the start of the treatment to the date of death. If the patients had not died, overall survival was censored on the last confirmed date of survival. Time to treatment failure (TTF) was measured from the start of the treatment to the date when the treatment was discontinued. The Kaplan–Meier method was used to estimate the overall survival curve. Survival time was censored at the last confirmation date if the patients were alive.

Results

Patient characteristics

Twenty-seven consecutive patients who received docetaxel/nedaplatin combination therapy as a second-line regimen at Kyoto University Hospital were evaluated for response, survival, and toxicity. Patient baseline characteristics are summarized in Table 1. The most common histological type was squamous cell carcinoma ($n = 26$; 96%), and there

Table 1. Patients' baseline characteristics

Characteristics	*n* (%)
Age (years)	
Median	62.5
Range	45–84
Sex	
Male	25 (93)
Female	2 (7)
ECOG performance status	
0	25 (93)
1	2 (7)
2	0
Histology	
Squamous cell carcinoma	26 (96)
Adenocarcinoma	1 (4)
Previous treatment	
Chemoradiotherapy	14 (52)
Chemoradiotherapy → surgery	5 (19)
Surgery → chemoradiotherapy	3 (11)
Surgery → chemotherapy	5 (19)
Evaluated lesions	
Lung	7 (23)
Liver	4 (13)
Lymph nodes	15 (50)
Others	4 (13)

Table 2. Tumor response

No. of patients	Response				Disease control rate (%)
	CR	PR	SD	PD	
27	0	3	11	13	52

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; disease control rate, CR + PR + SD

was one adenocarcinoma case. All patients had a history of prior treatment, consisting of chemoradiotherapy (n = 14), chemoradiotherapy/surgery (n = 5), surgery/chemotherapy (n = 5), and surgery/chemoradiotherapy (n = 3). A combination of cisplatin (70 mg/m^2) on day 1 and continuous-infusion 5-FU (700 mg/m^2 per day on days 1 through 5) had been repeated at 4-week intervals, with or without radiotherapy in 24 patients (89%). No patients had a history of prior chemotherapy containing docetaxel or nedaplatin.

Response and survival

Patients received a median of 7.4 cycles of treatment (range, 2–25 cycles). Response rates are summarized in Table 2. There was no CR, and 3 of the 27 patients (11%) achieved a PR. The disease control rate (PR + SD) was 52%. Twelve patients had measurable lymph nodes within the previous radiation field. Among these 12 patients, only one patient had another target size of lung. The response rates in these 12 patients were as follows; 1PR (8%), 95D (75%) and 2PD (17%) field, PR, SD, and PD were observed in 1/12 (8%), 9/12 (75%), 2/12 (17%), respectively. When this article was submitted, 8 of the 27 patients were still alive. The overall survival of the 27 patients is shown in Fig. 1. The median survival time was 11.4 months (95% confidence interval [CI], 6.8–18.7), the 1-year survival rate was 46.5% (95% CI, 25.0–68.0), and the median time to treatment failure (TTF) was 4.3 months (range, 1.0–20.2 months).

Table 3. Worst grade of toxicity per patient during all cycles: CTCAE v 3.0 grade [No. (%)]

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Anemia	10 (37)	8 (30)	4 (15)	1 (4)
Neutropenia	0	2 (7)	7 (26)	3 (11)
Febrile neutropenia	–	–	0	0
Leukopenia	0	9 (33)	7 (26)	0
Thrombocytopenia	7 (26)	1 (4)	0	0
Anorexia	14 (51)	3 (11)	0	0
Fatigue	14 (51)	2 (7)	0	0
Diarrhea	9 (33)	0	0	0
Nausea	5 (19)	2 (7)	0	0
Vomiting	3 (11)	1 (4)	0	0
Neuropathy-sensory	5 (19)	0	0	0

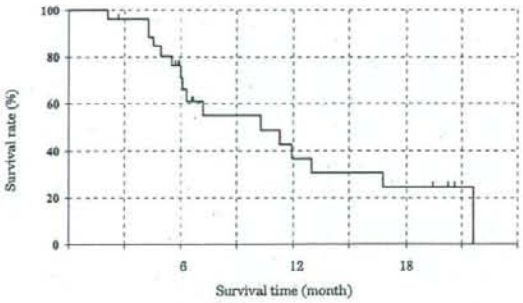

Fig. 1. Overall Survival for 27 patients

Toxicity

The docetaxel/nedaplatin combination therapy was generally safe and well tolerated. Dose reduction was required in 5 of the 27 patients (18.5%). Hematological and nonhematological toxicities are summarized in Table 3. The most common and severe, but reversible, toxicity of this combination therapy was neutropenia. Grade 3 and grade 4 neutropenia were observed in 7 (26%) and 3 (11%) patients, respectively. Febrile neutropenia was not observed in this study. Grade 3–4 anemia was observed in 5 (19%) patients. Anorexia and fatigue were the most common nonhematological toxicities (grade 1–2 anorexia, 62%; grade 1–2 fatigue, 58%). However, neither severe nonhematological adverse events (grade 3–4) nor treatment-related deaths were observed.

Discussion

A number of studies have reported on chemotherapy for advanced esophageal cancer; however, most of these re-

ports evaluated a first-line regimen for chemonaïve patients and only a limited number of studies focused on second-line chemotherapy for advanced esophageal cancer. The low incidence and poor prognosis of esophageal cancer also limit therapeutic trials for this disease. Among the limited number of studies focusing on second-line chemotherapy, several groups evaluated the efficacy of single-agent docetaxel for advanced esophageal cancer. Two independent studies, from France and Japan, reported overall response rates of 28% and 20%, respectively, with single-agent docetaxel at 70–100 mg/m^2 every 3 weeks.[9,10] Because pretreated patients have poorer tolerance to second-line chemotherapy, lower toxicity, as well as efficacy, is an important factor when considering the second-line chemotherapy. By combining nedaplatin at 40 mg/m^2 with docetaxel at 30 mg/m^2 every 2 weeks, we expected less toxicity, with preserved efficacy. In our current study, we observed a partial response (PR) in 3 of the 27 patients (11%), a disease control rate (PR + SD) of 52%, a 1-year survival rate of 46.5% and the MST of 11.4 months.

On the other hand, this combination regimen demonstrated less nephrotoxicity and gastrointestinal toxicity, as we expected. Only 37% of the patients developed grade 3–4 neutropenia and neither febrile neutropenia nor treatment-related deaths were observed in this study. Interestingly, while we were revising this manuscript, Osaka et al.[16] reported the feasibility of docetaxel/nedaplatin combination therapy for patients with recurrent esophageal cancer. Their regimen consisted of docetaxel 30 mg/m^2 and nedaplatin 40 mg/m^2 every 2 weeks, which was completely consistent with the regimen in our study. They evaluated 28 patients with recurrent esophageal cancer who had had prior chemotherapy, and they reported a response rate of 39.3%, with one complete response and an MST of 8.5 months. They also noted that the toxicity was relatively low and that this regimen could be safely managed in an outpatient setting. Table 4 summarizes the results of second-line chemotherapy using docetaxel/nedaplatin combination therapy for advanced esophageal cancer. The docetaxel/nedaplatin combination appears to have a modest effect on advanced esophageal cancer, with less toxicity however, we must be cautious in interpreting these results, because both our study and that of Osaka et al.[16] are retrospective, with a small sample size. Furthermore, because of the lack of phase I studies, the dose-finding method was not scientific in either of the studies, and the lower toxicity observed with this regimen may be attributable to the low dose intensity. However, a recent phase I study from another group (Abe et al.[17]) reported a recommended dose of 30 mg/m^2 docetaxel and 30 mg/m^2 nedaplatin every 2 weeks for advanced esophageal cancer. That report scientifically supports the dose used in our regimen.

Table 4. Results of second-line chemotherapy with a combination of docetaxel/nedaplatin for advanced esophageal cancer

	Our report	Osaka et al.[16]
No. of patients	27	28
Response rate	11%	39%
MST (months)	11.4	8.5
Grade 3–4 leukopenia	7/27 (26%)	2/28 (7.1%)
Grade 3–4 anemia	5/27 (19%)	1/28 (3.6%)
Grade 3–4 thrombocytopenia	0/27 (0%)	0/28 (0%)

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REVIEW ARTICLE

Yutaka Shimada · Fumiaki Sato

Molecular factors related to metastasis of esophageal squamous cell carcinoma

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Abstract The aggressive behavior of esophageal cancer leads to a low survival rate for patients with this disease. Isolated esophageal cancer cells seem to have the potential for regrowth and metastasis. To control the metastasis of esophageal carcinoma, detailed analysis of various molecular and biological factors should be done in each patient. Recent progress in molecular biology has revealed that oncogenes, suppressor genes, growth factors and their receptors, adhesion molecules, angiogenic factors, cytokines, and apoptotic factors are related to the growth and metastasis of esophageal carcinoma. Among them, several genes have been identified as having a crucial role in establishing the metastasis of esophageal carcinoma, and multiple genetic alterations may underlie the metastatic process. Microarray analysis has also revealed several genetic networks that are involved in the metastasis of esophageal cancer. These recent findings regarding the metastasis of esophageal cancer are summarized in the present review.

Key words Esophageal cancer · Lymph node metastasis · Hematogenous metastasis · Isolated tumor cell · Metastatic potential

Introduction

Esophageal cancer is the sixth most frequent cause of cancer death worldwide, which is a higher rank than its incidence (eighth) because of the low survival rate [1,2]. According to the Surveillance Epidemiology and End Results (SEER) program, the stage distribution of patients with esophageal cancer diagnosed at the U.S. National Cancer Institute was as follows: one-third of the patients had localized disease (no sign of progression), one-third of them had regional disease (usually with lymph node involvement), and the remaining one-third had distant metastasis [3]. Thus, understanding the mechanism of metastasis for esophageal cancer may help us to treat this deadly disease more effectively.

There are several steps in the process of establishment of metastasis by cancer [3,4]. The initial phase is an invasive process and the second phase is a metastatic process. The invasive process has several steps, which are (1) loss of contact inhibition, (2) tumor vascularization, (3) detachment of cells from the primary tumor, (4) penetration of the basement membrane, and (5) migration through the stroma. The metastatic process also has several steps, comprising (1) penetration of lymphatics and/vessels, (2) escape from immune surveillance and survival in the circulation, (3) entrapment in the capillary bed of a distant organ, (4) penetration of the lymphatic or vessel wall to enter the tissue, (5) growth in the new location, and (6) tumor vascularization. Thus, invasion and metastasis are both dynamic multistep processes [4].

With continued advances in the field of molecular biology, our understanding of the behavior of esophageal cancer continues to evolve. The available evidence suggests that multiple genetic alterations at the nucleotide and chromosomal levels may be involved in the metastasis of esophageal cancer. These changes may include alterations of oncogene or tumor suppressor genes, as well as genes for growth factors, angiogenic factors, adhesion molecules, and cytokines, and those involved in signal transduction and apoptosis. Recent findings regarding the metastasis of esophageal cancer are summarized in this review.

Invasion

Invasion initiates the metastatic process and requires changes of tumor cell adhesion to other cells and to the extracel-

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lular matrix (ECM), as well as proteolytic degradation of the tissue matrix and increased motility of tumor cells. Then, it is necessary for tumor cells to penetrate into the lymphatics or blood vessels.

Cadherins

Cell-to-cell adhesion is mediated by cadherins, and E-cadherin is the main regulator in epithelial tissues [5]. Loss or dysfunction of E-cadherin decreases intercellular adhesion and results in the acquisition of invasive capacity by esophageal cancer cell lines [6]. Epidermal growth factor (EGF) counteracts E-cadherin-mediated junctional assembly through phosphorylation of beta-catenin and allows tumor cell behavior to become more aggressive [7]. Also, hematogenous recurrence of esophageal squamous cell carcinoma (ESCC) is reported to show a correlation with E-cadherin expression [8].

Alpha-catenin (alpha-cat) is a cytoplasmic protein that forms a link to the cytoskeleton and thus regulates the activity of E-cadherin (E-cad) [9]. The frequency of lymph node metastasis by E-cad(+/–)/alpha-cat(–) tumors is significantly higher than by E-cad(+)/alpha-cat(+) tumors or E-cad(+/–)/alpha-cat(+/–) tumors. Thus, not only E-cad but also alpha-cat are important regulators of intercellular adhesion. In particular, reduction of alpha-cat expression in human esophageal cancer is more strongly correlated with an invasive phenotype and lymph node metastasis than increased E-cad expression [10].

Although such data exist, two multicenter studies have failed to confirm any relation between E-cadherin expression and lymph node metastasis or hematogenous metastasis in patients with either advanced or superficial esophageal cancer [11,12]. Thus, other mechanisms are suspected to contribute to cadherin-related regulation of cell-to-cell attachment in patients with esophageal cancer. The *Snail* gene product is a transcriptional repressor of E-cadherin and an inducer of the epithelial-mesenchymal transition in several epithelial tumor cell lines [13,14]. Deletion of E-cadherin is regulated by hypermethylation and Snail expression, but overexpression of Snail is unrelated to clinicopathological factors [15]. On the other hand, *Slug*, a member of the *Snail* family of transcriptional factors, is a newly identified suppressor of E-cadherin transcription [16]. Slug expression is significantly correlated with reduced E-cadherin expression, and tumors with low E-cadherin expression or positive Slug expression are more likely to show lymph node metastasis than tumors with preserved E-cadherin expression or negative Slug expression [17].

Adhesion molecules

TSLC1/IGSF4 has been identified on chromosome 11q23.2 as a tumor suppressor gene in non-small cell lung cancer by functional complementation of a lung adenocarcinoma cell line [18]. In a clinicopathological study, loss of TSLC1 expression by ESCC was significantly correlated with the depth of invasion and the metastatic status. The in vivo motility and invasiveness of ESCC cells were significantly suppressed by TSLC1 transfection in vivo [19].

Thrombomodulin (TM) is a thrombin receptor that was originally identified on the endothelium and acts as a natural anticoagulant [20]. However, TM is also expressed by squamous epithelial cells, mainly at intercellular bridges. Tumors usually express TM at cell–cell boundaries and in the cytoplasm, and a decrease of TM expression is associated with lymph node metastasis. This action is very similar to the influence of E-cadherin, although the structure of the two molecules is quite different [21].

Desmoglein 1 (DG1) is a major component of the desmosomal membrane core that plays an important role in epithelial cell adhesion [22]. A strong inverse correlation has been found between DG1 expression and tumor invasion, lymph node metastasis, lymphatic invasion, and vascular invasion [23].

Focal adhesion kinase (FAK) is a tyrosine kinase that is localized to focal cellular adhesions and is associated with a number of other proteins, such as integrin adhesion receptors [24]. A significant correlation has been observed between FAK overexpression and the presence of regional lymph node metastasis, as well as the number of involved lymph nodes [25].

Thus, not only the cadherin cell–cell adhesion system, but also various other adhesion molecules, including TSLC1, thrombomodulin, desmoglein 1, and FAK, play a role in the metastasis of esophageal squamous cell carcinoma.

Matrix metalloproteinases

Degradation of the extracellular matrix (ECM) is mediated by matrix metalloproteinases (MMPs), and this is one of the key steps during tumor invasion and metastasis [26]. MMPs are known to be involved in the cleavage of cell-surface receptors, the release of apoptotic ligands, and inactivation of chemokines. MMPs are also thought to play a major role in various aspects of cell behavior, such as proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defenses.

Among the many MMPs, the expression of MMP-2, -3, -7, -9, -12, -13, -14, and -26 appears to be closely related to lymph node metastasis of ESCC [27–32]. In addition, MMP-26 (matrilysin-2) expression is significantly correlated with distant metastasis [32].

Tissue inhibitors of metalloproteinases (TIMPs) inhibit the activity of MMPs and may also play an important role in tumor invasion/metastasis [26]. TIMP-1 and -3 have been associated with lymph node metastasis of ESCC [33,34].

Growth potential and motility

Cell proliferation

Cyclin D1 has been mapped to the 11q13 region of chromosome 11, and its expression has been detected in SCC cell lines and several primary esophageal cancers [35,36]. Overexpression of *cyclin D1* is correlated with distant metastasis after curative resection, but there is no significant difference in lymph node recurrence between patients with or without overexpression [37,38]. This relationship of cyclin D1 to hematogenous metastasis has been confirmed by logistic regression analysis based on 11 different molecular and cellular factors [39]. However, the reason why cyclin D1 is related to hematogenous metastasis, but not to lymph node metastasis, is unclear.

The *p16/MTS1/CDKN2* gene regulates cyclin-dependent kinase 4-mediated phosphorylation of pRB in normal cells. The mean number of metastatic lymph nodes was significantly higher in patients who had ESCC without p16 expression than in patients whose tumors showed p16 expression [38].

Ornithine decarboxylase (ODC) is a key enzyme involved in the biosynthesis of polyamines, which are essential for cell proliferation [40]. ODC-overproducing transfectants show enhanced MAP kinase activity that parallels the extent of their invasiveness [41]. In patients with esophageal cancer, overexpression of ODC mRNA shows a significant correlation with lymph node metastasis [42].

Aurora-A/STK15/BTAK (Aurora-A) encodes a serine/threonine kinase associated with the distribution of chromosomes, and its upregulation induces chromosomal instability that leads to aneuploidy and cell transformation [43]. A correlation between the upregulation of Aurora A and tumor aggressiveness has also been detected for several cancers [44], and upregulation of Aurora-A mRNA and protein is correlated with distant lymph node metastasis of ESCC [45].

Thus, various genes related to cell proliferation may have a role in the metastasis of esophageal cancer.

Motility

Growth factors

Epidermal growth factor (EGF) is one of the best known growth factors expressed by malignant tumors, and upregulation of the *EGF receptor (EGFR)* gene is associated with a poor prognosis of ESCC [46,47]. Although the EGFR is thought to have a role in metastasis of ESCC, there has only been one study that demonstrated an association between lymph node metastasis of ESCC and EGFR expression [48].

Nerve growth factor (NGF) is overexpressed not only in the nervous system but also by several types of cancer [49,50]. An immunohistochemical study has revealed that NGF overexpression is associated with lymph node metastasis and distant metastasis of ESCC [51]. NGF overexpression is also associated with strong TrkA expression and with the lack of low-affinity neurotrophin receptor (p75NTR) expression. Cultured ESCC cell lines secrete detectable amounts of NGF and also express both TrkA and p75NTR. A neutralizing anti-NGF antibody, a TrkA inhibitor, and a small interfering RNA for NGF have all been shown to significantly decrease the motility of an ESCC cell line overexpressing NGF [51].

Signal transduction

Growth factor receptor-bound protein 7 (Grb7) has an Src homology 2 (SH2) domain and shares structural homology with a molecule related to cell migration in *Caenorhabditis elegans* (designated as Mig-10) [52,53]. In esophageal carcinoma cells, Grb7 protein undergoes tyrosine phosphorylation by epidermal growth factor and becomes attached to various extracellular matrix proteins, including fibronectin. This fibronectin-dependent phosphorylation of Grb7 is regulated by integrin signaling that leads to an interaction with focal adhesion kinase protein. Ectopic expression of a Grb7-SH2 dominant-negative fragment has been found to inhibit fibronectin-dependent phosphorylation of endogenous Grb7 and reduce the migration of esophageal carcinoma cells. Grb7 protein overexpression in resected ESCC tumors is significantly correlated with the presence of lymph node metastasis [54].

Members of the *Smad* family play a key role in regulating gene expression in the transforming growth factor (TGF)-beta-1 signaling pathway [55]. Activation of Smads leads to their translocation from the cytoplasm to the nucleus, where these molecules act as transcription factors. Esophageal cancer patients with preserved expression of Smad4 have fewer lymph node metastases than those with reduced Smad4 expression. However, tumor cell expression of TGF-beta-1 is not associated with any clinicopathological factors [56].

Activin A is a member of the transforming growth factor-beta (TGF-beta) superfamily and is a strong differentiation factor for embryonic stem (ES) cells [57]. *Activin-betaA (Act-betaA)* is a subunit of activin A that shows significant overexpression in cancer tissue. Although expression of Act-beta A mRNA is not associated with the differentiation of ESCC, it is associated with lymph node metastasis [58].

The Ras homologue (Rho) family of small guanosine triphosphatases (GTPases) comprise a large branch within the Ras family of low molecular weight guanine nucleotide-binding proteins. Rho GTPases are thought to be involved in regulating diverse physiological responses, such as cell proliferation and motility, as well as pathological processes such as cell transformation and tumor metastasis [59]. Despite the marked similarity of the different Rho isoforms (Rho A, Rho B, and Rho C), their physiological roles are distinct. In transfected ESCC cells, transfection of RhoC promoted migration more effectively compared to RhoA [60]. In a nude mouse experimental metastasis model,

RhoA was shown to stimulate tumor growth more than RhoC, whereas RhoC induced lung metastasis more often than RhoA [60].

Recent studies have demonstrated that the Sonic Hedgehog (SHh) signaling pathway plays an important role in the development of various organs, including the neural tube, lungs, gastrointestinal tract, pancreas, and prostate [61]. SHh proteins act via the transmembrane proteins Patched (Ptch) and Smoothened (Smo), which regulate the transcriptional activity of three Gli zinc-finger transcription factors [61,62]. Increased Hh signaling can lead to the development of tumors (including gastrointestinal cancer), and activation of the Hh signaling pathway is associated with tumor metastasis [63,64].

Gli-1 was expressed by 31 of 34 esophageal cancer cell lines (91%), whereas SHh, Ptch, and Smo were expressed by all 34 cell lines [65]. Cyclopamine (a selective inhibitor of the Hh signaling pathway) significantly inhibited the proliferation and migration of ESCC cells expressing Gli-1. Also, SiRNA targeting Gli-1 inhibited the growth of ESCC cells. Furthermore, the detection of Gli-1 expression in resected tumors was associated with deeper tumor invasion, lymph node metastasis, and a poor prognosis [65].

Migration factors

Caveolin-1 is the principal structural component of the caveolar membrane domain in nonmuscle cells, including the ductal epithelium of the breast. There is now clear evidence that caveolin-1 influences the development of cancer in humans [66]. In cultured cells, caveolin-1 is known to act as a negative regulator of the Rasp42/44 MAP kinase cascade and as a repressor of *cyclin D1* gene transcription, possibly explaining its in vitro suppressive effect on cell transformation. However, overexpression of caveolin-1 is associated with lymph node metastasis of ESCC and with a worse prognosis after surgery [67].

Fascin is an actin-binding protein that induces membrane protrusions and increases the motility of various transformed cells [68]. An immunohistochemical study has revealed that fascin expression is usually stronger in tumors compared with normal epithelium. The fascin-immunopositive rate was associated with the extent of tumor invasion and with lymph node metastasis in ESCC patients [69], and an in vitro study revealed that all the 33 ESCC cell lines tested were positive for fascin protein expression. One of the fascin-overexpressing cell lines showed a decrease of motility and invasiveness after downregulation of fascin expression by a vector-based small interfering RNA [69].

Autocrine motility factor (AMP) and its receptor (gp78) have been shown to play an important role in tumor cell migration, invasion, and metastasis [70]. In ESCC, gp78 expression shows a significant association with tumor size, infiltrative growth, depth of invasion, and lymph node metastasis [71].

Laminin-5 gamma-2 chain (LN-5 gamma-2) is an extracellular matrix protein that plays an important role in cell migration and tumor invasion [72]. LN-5 gamma-2 expression in the invasive edge of the tumor was correlated with the depth of invasion and lymph node metastasis [48].

Although the mechanism of action of the transmembrane superfamilies, motility-related protein-1 (MRP-1/CD9), and KAI1/CD82 is not well known, these molecules have been reported to suppress the metastasis of several cancers [73,74]. Expression of both MRP-1/CD9 and KAI1/CD82 is positive in the membranes of normal esophageal epithelial cells but is reduced or negative in the case of cancer cells. Decreased expression of MRP-1/CD9 and KAI/CD-82 is significantly correlated with lymph node metastasis of ESCC, but not with distant metastasis [75].

Thus, growth factors, signal transduction players, and migration-related factors also contribute to the metastasis of esophageal cancer.

Metastasis

Some tumor cells are able to escape from the host immune system and survive in the blood vessels or lymphatics until they become trapped in the capillary bed of a distant organ. Then, these tumor cells penetrate the lymphatic or vessel wall and enter the tissues, after which metastatic deposits grow in the new location along with neovascularization. To carry out these steps, tumor cells always employ the same system, which is part of the invasion process.

Migration to specific organs

Chemokines

Tumor cell migration and metastasis share many similarities with leukocyte migration, which is regulated by various chemokines and their receptors. Among them, CC chemokine receptor 7 (CCR7) plays a critical role in the migration of activated dendritic cells into regional lymph nodes [76]. CCR7 mRNA was detected in 9 of 20 ESCC cell lines. In vitro studies have demonstrated that CCL21 (the ligand of CCR7) significantly increases the migration of ESCC cell lines, with pseudopodia being induced by CCL21 stimulation. Furthermore, CCL21 markedly enhances the motility of esophageal carcinoma cell lines in a phagocytosis assay. An immunohistochemical study has shown that high CCR7 expression is significantly correlated with lymphatic involved by ESCC and subsequent lymph node metastasis [77].

Bone marrow-homing receptor CXCR4 has also been suggested to have a role in the metastasis of various cancers [76]. CXCR4 expression has a significant association with increased lymph node micrometastasis and with increased bone marrow micrometastasis. Thus, CXCR4 may have a role in early metastatic spread because its expression is associated with micrometastasis to both the lymph nodes and bone marrow [78].

Isolated tumor cells

Lymph node micrometastasis

Although lymph node micrometastasis is thought to be a poor prognostic factor for ESCC, the clinical impact is still controversial [79,80]. Scheunemann et al. showed that there is a tumorigenic potential of apparently tumor-free lymph nodes in esophageal cancer patients [81]. Thus, prediction of micrometastasis may be useful for deciding on esophageal cancer treatment. However, the expression of E-cadherin, cyclin D1, MMP 9, and vascular endothelial growth factor by primary ESCC does not predict lymph node micrometastasis [82].

Circulating tumor cells

To detect circulating esophageal tumor cells, immunostaining for carcinoembryonic antigen (CEA) mRNA and SCC mRNA is usually employed [83–85]. The overall incidence of recurrence and that of hematogenous recurrence are significantly higher in patients who are positive for CEA mRNA than in those who are negative [84]. Detection of SCCA mRNA in the peripheral blood on admission is correlated with the depth of tumor invasion and with venous invasion. The recurrence rate is correlated with the SCC mRNA status on admission and during manipulation of the tumor [85]. Thus, the existence of circulating tumor cells is a risk factor for metastasis. Furthermore, a higher colony formation rate in cultures of blood samples is associated with the number of circulating tumor cells (Fig. 1). The establishment of metastasis thus may be associated with the number of circulating tumor cells and the ability to survive in culture.

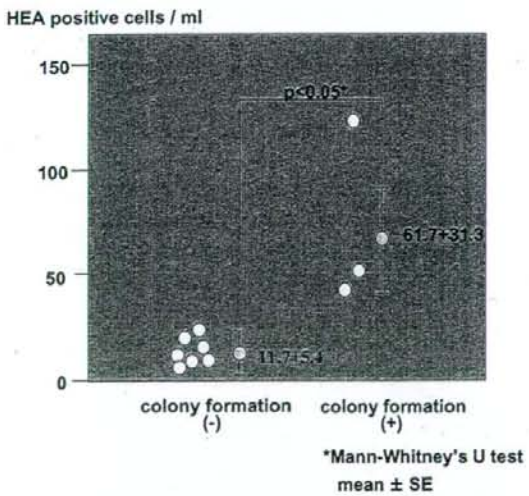

Fig. 1. Association between the number of isolated tumor cells (*ITC*) obtained with a magnetic cell sorter (*MACS*) and colony formation. A larger number of colonies in cultures from blood samples was associated with the number of circulating tumor cells. *HEA*, human epithelial antigen–125/EpCAM antigen

Free tumor cells in the thoracic duct

Watanabe et al. showed that thoracic duct fluid from ESCC patients had a high rate (61%: 8/13) of tumorigenicity in nude mice [86]. This very high rate of tumorigenicity suggests that esophageal cancer is already a systemic disease at an early stage. Homozygous deletion of the *p16* gene and amplification of the *cyclin D1* gene were observed in xenografts derived from thoracic duct lymph fluid of the ESCC patients [86].

The HSA and Sum cell lines were established from ESCC cells floating in the thoracic duct. These two cell lines had a high migratory ability in vitro and a high lymph node metastatic potential in vivo (Figs. 2, 3; see Table 1) These cells expressed NGF, TIB929 (GTPase superfamily), and osteopontin [51,87,88]. These findings suggest that tumor cells floating in the thoracic duct have been selected from the primary tumor and have adapted to the environment, and may thus have a high metastatic potential.

Only certain cancer cells (so-called cancer stem cells) show the potential for growth at sites of metastasis. Thus, it is necessary to find markers for cancer stem cells or isolate such stem cells [89,90].

Attachment

SLea, SLex, and E-selectin

The carbohydrate determinants sialyl Lewis A and sialyl Lewis X are frequently expressed by human cancer cells and serve as ligands for E-selectin, a cell adhesion molecule from the selectin family that is expressed by vascular endothelial cells. These carbohydrate determinants are involved in the adhesion of cancer cells to the vascular endothelium and thus contribute to hematogenous metastasis of cancer [91].

The expression of sLeA and sLex is correlated with hematogenous recurrence of ESCC and sLeA expression is also correlated with pM (lymph) status. Although neither sLeA nor sLeX expression has a significant influence on the survival of patients with ESCC, logistic regression analysis has demonstrated that increased tumor expression of sLeA and sLeX is a risk factor for hematogenous recurrence of ESCC [92].

Serum levels of soluble E-selectin were significantly higher in a hematogenous recurrence group than in a non-hematogenous recurrence group. Logistic regression analysis showed that high serum soluble E-selectin levels, lymph node metastasis, and intraepithelial spread were associated with postoperative hematogenous recurrence of ESCC [93].

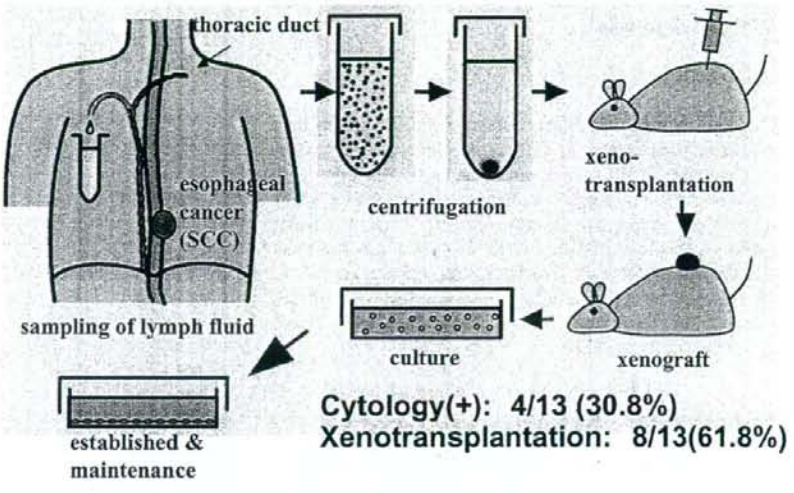

Fig. 2. Establishment of cancer cell lines derived from thoracic duct lymph

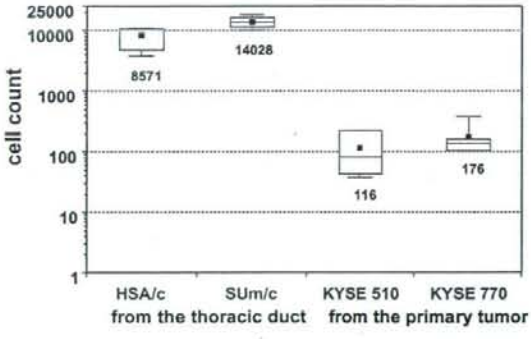

Fig. 3. Migration assay. The HSA and Sum cell lines were established from esophageal squamous cell carcinoma (ESCC) cells floating in the thoracic duct. These two cell lines had a higher migration ability compared with cells from the primary tumor

Angiogenesis

VEGF

Vascular endothelial growth factor (VEGF) has an influence on the growth of malignant tumors by promoting angiogenesis [94]. A significant correlation has been found between VEGF expression and lymph node metastasis in patients with ESCC. The average microvessel density (MVD) shows a correlation with VEGF expression [95], whereas the expression of VEGF, TGF-alpha, platelet-derived endothelial cell growth factor (PD-ECGF), and fibroblast growth factor (FGF) is significantly correlated with lymph node metastasis [96,97].

VEGF-C plays a key role in the process of lymphangiogenesis [94]. Four of five human esophageal carcinoma cell lines were found to constitutively express VEGF-C mRNA [98]. When resected specimens were examined, VEGF-C mRNA was detected in tumor tissues, but not in normal mucosa, by reverse transcriptase-polymerase chain reaction (RT-PCR). There was a significant relationship between VEGF-C and flt-4 mRNA expression. However, VEGF-C was detected in the cytoplasm of many cancer cells and stromal cells whereas Flt-4 was mainly expressed by the endothelial cells of lymphatics. Normal and dysplastic esophageal squamous epithelium shows little or no cytoplasmic staining for VEGF-C, whereas VEGF-C expression is correlated with venous invasion, lymphatic invasion, and lymph node metastasis. The vessel count was found to be significantly higher in VEGF-C-positive tumors than in VEGF-C-negative tumors [98]. Furthermore, VEGF-C overexpression by ESCC with submucosal invasion is a high risk factor for lymph node metastasis (including micrometastasis) [99].

HIF

Hypoxia-inducible factor (HIF)-1-alpha is a transcription factor that regulates the transcription of genes associated with cell proliferation and angiogenesis [100]. HIF-1-alpha was expressed by all five ESCC cell lines that were tested, and HIF-1-alpha expression was found to show a correlation with lymphatic invasion and VEGF-C expression [101]. Furthermore, HIF-1-alpha expression was correlated with both lymph node metastasis and distant metastasis [102].

Other factors

Osteopontin (OPN) is a secreted integrin-binding glycophosphoprotein that is thought to have a role in the metastasis of malignant tumors [103]. cDNA microarray analysis has revealed that high levels of osteopontin mRNA expression are associated with poor survival in ESCC patients. In an immunohistochemical study, osteopontin protein ex-