Radiology

The mean tumor-to–normal esophageal wall attenuation difference was greatest in the second arterial phase.

Discussion

At conventional CT with contrast material enhancement, images are acquired in the venous phase, and esophageal cancer can be demonstrated in approximately 80% of lesions (14). This frequency of demonstration is nearly equivalent to that in the venous phase in our study (24 of 31 [77%]). Missed cancers at CT may result from poor delineation of early stage cancers without obvious wall thickening. Yoon et al reported that 69.2% of T1 lesions were not demonstrated at CT (14). At conventional CT, identification of esophageal cancers usually depends on wall thickening of the esophagus (15). Early stage cancers, however, may show minimal or no wall thickening. Wu et al (16) proposed a classification of tumor staging: Cancers with thickness greater than 5 mm and less than 15 mm are considered modified T2, those with thickness greater than 15 mm and with an irregular outer margin are considered T3, and tumor invasion of adjacent organs is considered T4. However, Wu et al had no criteria for T1 lesions because T1 lesions cannot be delineated by the thickness of the wall alone.

Our results showed that with dynamic CT, the second arterial phase is the optimal phase for visualization. Cancers in the second arterial phase can be identified as enhanced foci, even if the cancer does not cause wall thickening. As for advanced stage cancers with wall thickening, the second arterial phase most clearly depicts the tumors. These results are also supported by quantitative analysis of the difference in attenuation between the tumor and normal esophageal wall. Esophageal cancer shows a peak of enhancement around the second arterial phase, whereas normal esophageal wall shows gradual enhancement. Therefore, the best conspicuity of esophageal cancer against the esophageal wall can be obtained at this time.

Although the venous phase images seem inferior to the second arterial phase images with regard to the representation of esophageal cancer, venous phase images may be useful in evaluating mediastinal lymphadenopathy. In addition, by analyzing the venous phase images of the esophagus, multi-detector row CT can allow rapid imaging of the abdomen for the purpose of screening distant metastases and lymphadenopathy. In the evaluation of liver metastasis, liver parenchyma is not enhanced enough at the arterial phases and may not be adequate for depicting lesions other than hypervascular tumors, such as hepatocellular carcinoma (17). The first arterial phase images may have no clinical value in the evaluation of esophageal cancer and metastatic lesions. Ideally, both the second arterial and the venous phase images are required for the evaluation of esophageal cancers; however, increasing the number of scans causes increased radiation dose.

Improved delineation of esophageal cancer at multi-detector row CT can provide several potential clinical benefits. Precise localization of esophageal cancer is useful for planning radiation therapy or surgical resection. Once chemotherapy or radiation therapy is chosen, clear delineation of the tumor as an enhanced lesion may be useful for evaluating the therapeutic outcome. Moreover, clear delineation of esophageal cancer may improve recognition of relationships to the adjacent mediastinal organs and thus improve accuracy of local staging of the tumors.

There were some limitations in our study. First, the number of adenocarcinomas and T1a cancers was very limited in our population. The only case of adenocarcinoma in our study was a mucosal cancer (T1a cancer), and it was not demonstrated at CT. T1a cancer may not be delineated, even with dynamic CT. In such instances, the presence of enhanced lesions within nonthickened walls may become a useful radiologic feature that allows the differentiation of T1b cancers from T1a cancers, although further evaluation is required with early stage cases. Esophageal adenocarcinomas of advanced stages may show radiologic features and enhancing patterns that are different from those of squamous cell carcinomas. Although the prevalence of adenocarcinomas in Japan is increasing, the proportion of adenocarcinomas to squamous cell carcinomas is still low and differs from that in the United States

Figure 3

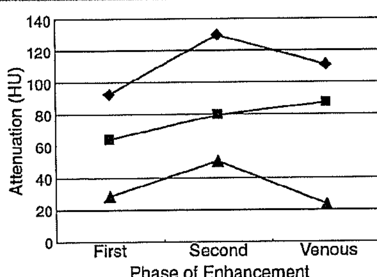

Figure 3: Graph of mean attenuation of normal esophageal wall (■) and tumor (♦). Note that attenuation of tumor and tumor-to–normal esophageal wall attenuation differences (▲) peak in second arterial phase, whereas attenuation of esophageal wall elevates gradually.

Table 3

Mean Attenuation of 28 Esophageal Tumors, Normal Esophageal Walls, and the Difference between Them

CT Imaging Phase	Mean Attenuation of Tumor (HU)	Mean Attenuation of Normal Esophageal Wall (HU)	Mean Tumor-to–Normal Esophageal Wall Attenuation Difference (HU)
First arterial	92.5 ± 14.7	64.2 ± 15.8	28.3 ± 17.1
Second arterial	130.0 ± 18.4	79.4 ± 20.6	50.6 ± 23.0
Venous	111.0 ± 13.7	87.2 ± 16.5	23.5 ± 19.0

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(18). Further evaluation is required to investigate whether this technique is applicable for patients with adenocarcinoma.

Second, our study population included only patients with esophageal cancer and did not consist of any healthy subjects because of radiation exposure. The readers were not blinded for the presence of the tumor. Hence, it is questionable whether this technique is useful in screening for esophageal cancer. In patients with inflammatory diseases, including gastroesophageal reflux disease or Barrett esophagus, CT appearances of these diseases may resemble early stage esophageal cancers (19). Distribution of inflammatory lesions, however, would be usually more superficial and diffuse than that of cancers.

Third, qualitative evaluation of CT images from all three phases was performed on the same day in our study, which may cause bias. We are convinced, however, that the results from quantitative analysis support reproducibility of qualitative analysis.

In conclusion, the results of our study show that the second arterial phase of dynamic CT is the optimal phase for visualization of esophageal cancer.

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A New Specific Gene Expression in Squamous Cell Carcinoma of the Esophagus Detected Using Representational Difference Analysis and cDNA Microarray

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Key Words
Representational difference analysis of cDNA · Esophageal squamous cell carcinoma · Human esophageal epithelial cell · LAGE-1 · HDAC inhibitor

Abstract
Objectives: To detect new specific gene expressions in squamous cell carcinoma of the esophagus. ***Methods:*** Representational difference analysis of cDNA (cDNA RDA) was applied to a human esophageal cancer cell line (KYSE170) and a human esophageal epithelial cell line (HEEC-1). ***Results:*** LAGE-1 was expressed specifically in KYSE170, but not in HEEC-1. It is also expressed in 27% of esophageal cancer cell lines (3/11) and 33% of esophageal cancer tissues (10/30), but not in other HEECs, normal esophageal epithelium, or other normal tissues except testis, ovary and kidney. The expression of LAGE-1 is strongly correlated with that of MAGE-A1 ($p = 0.013$, Fisher's exact probability test). Fibronectin, cytokeratin 6B, cytokeratin 19, cyclin D2 and Ten-m2 were detected as candidates for downregulated genes. Reduced expression profiles of them were also identified using cDNA microarrays. The expression of LAGE-1 was induced by 5′-aza-2′-deoxycytidine (5Aza-dC) and trichostatin A (TSA) in esophageal cancer cell lines, which did not express LAGE-1. In HEECs, 5Aza-dC induced LAGE-1 expression, but TSA did not. ***Conclusions:*** LAGE-1 expression was detected in esophageal cancer by cDNA RDA. LAGE-1 might have the potential to be a target antigen for anti-tumoral immunotherapy in esophageal cancers because of its tumor-specific expression similar to that of MAGE-A1.

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Introduction

Esophageal cancer is known to have one of the worst prognoses among all cancers. For improving the poor prognosis of esophageal cancer, elucidation of the mechanism of tumor progression and applications of tumor-specific treatment strategies are needed. We employed representational difference analysis of cDNA (cDNA RDA [1]) to identify overexpressed or repressed esophageal cancer-specific genes. This approach has been used

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to identify a number of new tumor-specific genes such as the MAGE [2], SAGE [3], BAGE [4], GAGE [5] and LAGE families [6], and CT10 [7]. These genes appear to be potential sources of antigens for cancer immunotherapy because they are expressed specifically in tumors of various histological types.

Recently, we established a protocol for culturing normal human esophageal epithelial cell lines (HEECs), and with those cell lines, we carried out gene expression profiling in human esophageal cancers using cDNA microarrays [8].

In the present study, we investigated the differences in gene expression between esophageal cancers and normal esophageal material using cDNA RDA and cDNA microarrays, and identified a candidate gene for cancer immunotherapy. Control mechanisms of genes that are specifically upregulated or downregulated in esophageal cancers were also examined.

Materials and Methods

Cell Lines and Tissues

Human esophageal cancer cell lines KYSE30, 150, 170, 410, 520, 590, 890, 960, 1190 and 2400, derived from squamous cell carcinoma [9], were grown in HAM/RPMI supplemented with 2% fetal calf serum, penicillin, and gentamicin. Other esophageal cancer cell lines, OE-33, KYAE and SKGT-4, derived from esophageal adenocarcinoma, were grown under the same conditions except in the case of OE-33, which was supplemented with 5% fetal calf serum. SUm/c that had been derived from squamous cell carcinoma of the esophagus floating in the thoracic duct was grown in culture medium with 5% fetal calf serum. HEEC-1, 2, 3 and 4, derived from the normal esophageal epithelium, were grown in Keratinocyte SFM (Invitrogen Corp., Carlsbad, Calif., USA)-containing supplements. The KYSE series, KYAE and HEECs were established in our laboratory, OE-33 was obtained from the European Collection of Cell Cultures (ECACC), and SKGT-4 and SUm/c were kindly donated by Dr. N. Altorki (Cornell University) and Dr. H. Watanabe (Tokyo, Japan), respectively. Specimens of normal and tumor tissues were obtained from our department under informed consent for all patients.

Preparation of cDNA

KYSE170 was derived from an esophageal cancer patient who had been alive for 12 years after surgery. Total RNA was extracted by the modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) method from KYSE170 and from HEEC-1. Poly(A)+ RNA was purified using an Oligotex dT-30 super [poly(A) purification kit; Takara Bio Inc., Japan], according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription from 2 μg of each poly (A) + RNA sample (First strand cDNA Synthesis kit; Amersham Pharmacia Biotech, Bucks., UK). For double-stranded cDNA synthesis, 20 μl (total volume) of the first strand cDNA solution was mixed with 15 μl of 10× second strand buffer, 3 μl of dNTPs (10 m*M* each), 106 μl of H_2O, 1 μl of *E. coli* ligase (Invitrogen), 4 μl of *E. coli* DNA polymerase I (Invitrogen), and 1 μl of RNase H (Invitrogen), incubated at 16°C for 2 h, and then supplemented with 2 μl of T4 DNA polymerase (Invitrogen) and incubated at 16°C for another 2 h. Synthesized cDNA was phenol-extracted, ethanol-precipitated and resuspended in 20 μl of TE.

Protocol of cDNA RDA

The protocol for RDA as described by Hubank and Schatz [1] was used with some modifications. Two micrograms of each cDNA were digested with *Dpn* II (New England Biolabs, Beverly, Mass., USA). Twelve microliters (~1.2 μg) of digested cDNAs were ligated to R-Bgl adapters and amplified by PCR to generate the KYSE170 and HEEC-1 representations. The R-Bgl adapters were removed with *Dpn* II digestion, and the products (driver amplicon) were purified using spin columns: MICROCON YM-100 (Millipore Corp., Mass., USA) to remove impurities such as residual adapters and dNTPs. For tester representations, 2 μg of each driver amplicon were ligated to the J-Bgl adapters. Sequences of oligonucleotides used in cDNA RDA were as described in Lisitsyn and Wigler [10].

Hybridization and Selective Amplification

All steps were performed as described in Hubank and Schatz [1] with the following modifications: the tester (200 ng) to driver (20 μg) ratio for the first hybridization was 1:100. The tester (50 ng) to driver (40 μg) ratio for the second and third rounds was kept at 1:800. Most of the purification steps for products were performed using Microcon YM-100 (Millipore). Mung bean nuclease digestion before the last PCR in each RDA round was omitted.

Subcloning and Sequencing of Different Products

Subcloning steps were performed according to published protocols [1]. Briefly, the difference products of the second and third rounds were digested with *Dpn* II and purified by electrophoresis using a gel purification kit (Invitrogen), cloned into the *Bam* HI site of pBluescript KS$^+$ II (STRATAGENE Cloning Systems, La Jolla, Calif., USA), and transformed into DH-5 alpha (Invitrogen). White colonies were picked up from solid LB medium containing penicillin and X-gal (blue-white selection). Inserted fragments were checked by PCR using T3 and T7 primers and sequenced using an ABI Prism 377 DNA Sequencer (Applied Biosystems, Calif., USA). Sequence homology searches were performed in the databases provided by the National Center for Biotechnology Information (Bethesda, Md., USA) using the BLAST program [11].

Reverse Transcription-Polymerase Chain Reaction

Difference products obtained respectively from KYSE170 and from HEEC-1 as the tester were investigated by reverse transcription-polymerase chain reaction (RT-PCR) in KYSE170 and HEEC-1, respectively. Their expression was also examined in other esophageal cancer cell lines, in 8 surgically resected esophageal cancers (squamous cell carcinoma), in normal esophageal epithelial tissues, and in various other cancer cell lines and normal tissues (human tumor MTC panel and human MTC panels I & II; BD Clontech, Palo Alto, Calif., USA). PCR protocols were as follows: 35 cycles of 30 s at 95°C, 30 s at each optimal annealing temperature, and 1 min at 72°C. GAPDH was used as a positive control, and RNA was used as a negative control for RT-PCR of all speci-

mens in this study. The primer sequences of oligonucleotides used in RT-PCR designed using OLIGO™ primer analysis software and the annealing temperatures were as follows:

ERT: 5′-AGAAGAGCTGGAAGTGAG-3′, 5′-ACCAAGTG-GAGAAGAACA-3′, 49°C,

PTI-1: 5′-GCTAAAAAGTGCCCGGAT-3′, 5′-ACATTCAG-TGCTCTACCC-3′, 49°C,

LAGE-1: 5′-CCAAACACAAGGTCTCAG-3′, 5′-ACAATGA-ACTGGCCACTC-3′, 55.5°C,

fibronectin: 5′-ACTGCCAACTCTTTTACT-3′, 5′-CTATTT-CCTCCTGTTTCT-3′, 51.9°C,

laminin: 5′-CCAAGACCCAGATCAACA-3′, 5′-GGGTATT-GTAGC AGCCTG-3′, 53.7°C,

keratin 6B: 5′-CCTGAGAGCCTTGTATGA-3′, 5′-AATCTC-CTGCTTGGTGTT-3′, 54°C,

keratin 19: 5′-GGCCTACCTGAAGAAGAA-3′, 5′-ATTCTG-CCGCTCACTATC-3′, 56°C,

cyclin D2: 5′-GTGGTGCTGGGGAAGTTG-3′, 5′-TCTGT-AGGGGTGCTGGCT-3′, 57°C,

Ten-m2: 5′-TGGGTG TGAATGTGTCTT-3′, 5′-GAAGAA-GGTGGACAGAGG-3′, 53.2°C,

MAGE-A1, 5′-GCTGGAACCCTCACTGGGTTGCC-3′, 5′-CGGCCGAAGGAAGGAACCTGACCCAG-3′, 72°C,

GAPDH: 5′-TGGTATCGTGGAAGGACTCATGAC-3′, 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′, 50°C.

cDNA Microarray Analysis

Fourteen esophageal cancer cell lines (10 KYSE series, SUm/c, SKGT-4, OE-33, and KYAE) and 8 surgically resected esophageal cancer tissue samples (squamous cell carcinoma) were used for cDNA microarray analysis. The reference probes used for cancer cell lines and cancer tissues were HEECs and pooled normal esophageal epithelial tissues which had been obtained from the same surgical specimens. Details of the procedure were as previously described [8]. Briefly, 1 μg of mRNA was extracted from each sample. Cy3-dUTP and Cy5-dUTP were used for fluorescent labeling, and each sample of labeled first-strand cDNA was mixed and hybridized with Human Cancer Chip Version 2.0 (Takara Bio Inc.). Fluorescent images were examined using an Array Scanner 428 (Affymetrix, Inc., Santa Clara, Calif., USA), and the signal intensities calculated using ImaGene 3.0 (BioDiscovery Inc., Marina Del Rey, Calif., USA). For data analysis, we used publicly available clustering analysis software 'Cluster' and visualization software 'Tree View' [11].

Treatment with 5′-Aza-2′-Deoxycytidine and Trichostatin A

Four esophageal cancer cell lines (KYSE150, KYSE520, KYSE960 and SKGT-4) and 4 HEECs (HEEC-1–4) were grown in medium with various and continuous concentrations (0, 0.75, 2, 5 or 10 μ*M*) of 5′-aza-2′-deoxycytidine (5Aza-dC, Sigma Mo., St. Louis, Mo., USA) and with 100 μg/l, 500 μg/l, or 1 mg/l of tricostatin A (TSA, Sigma). Each medium had been replaced every day by fresh medium with the same concentration of 5Aza-dC and TSA until total RNA was isolated at various time points (1, 2 days, or 5 days). The expression levels of LAGE-1 were then investigated by RT-PCR as described above.

Results

Genes Overexpressed in an Esophageal Cancer Cell Line and a Human Esophageal Epithelial Cell Line

For detecting tumor-specific genes, an esophageal cancer cell line (KYSE170) was used as the source of tester cDNA against driver cDNA from a human esophageal epithelial cell line (HEEC-1). For detecting the downregulated genes in cancer cells, HEEC-1 was processed as the source of tester cDNA against driver cDNA from KYSE170. Tester and driver cDNAs were digested with a restriction enzyme (*Dpn* II), ligated to a pair of adapters, and then subjected to PCR amplification. The amplified tester was hybridized to a large excess of driver. The hybridization product was amplified by PCR using the tester-specific adapters as primers. Under these conditions, only tester-tester homoduplexes, corresponding to KYSE170- or HEEC-1-specific sequences, were amplified exponentially. This first difference product was submitted to two additional rounds of subtraction, digestion, and amplification that produced the second and third difference products. We examined the quality of difference products from each round of cDNA RDA by agarose gel electrophoresis. As shown in figure 1a, a stepwise reduction of complexity was seen in the three successive difference products and clear bands with little background were visible with ethidium bromide staining in the third difference product. Cloning of the second and the third difference products produced inserts whose sizes ranged from 120 to 300 bp, and sequence analysis of the genes revealed that there were 9 different genes. For tumor-specific genes, three difference products were obtained from KYSE170 (K-H-DP1~DP3); they were identified as LAGE-1, ERT (*Homo sapiens* Ets-related transcription factor), and PTI-1 (*Homo sapiens* prostate carcinoma tumor-inducing gene 1). For downregulated genes, six difference products were detected from HEEC-1 (H-K-DP1~DP6). They were identified as fibronectin, laminin, cytokeratin 19, cytokeratin 6B, cyclin D2, and ten-m2 (odd Oz/ten-m homolog 2) (table 1).

Expression of Esophageal Cancer-Specific Genes by RT-PCR

The patterns of expression of ERT, PTI-1, and LAGE-1 were checked because these genes were considered to be potential candidate genes that might be tumor-specific. All of these genes were expressed in KYSE170 but not in HEEC-1, which suggested that RDA in the current study worked properly. However, the ERT and PTI-1 genes were expressed in many esophageal cancer

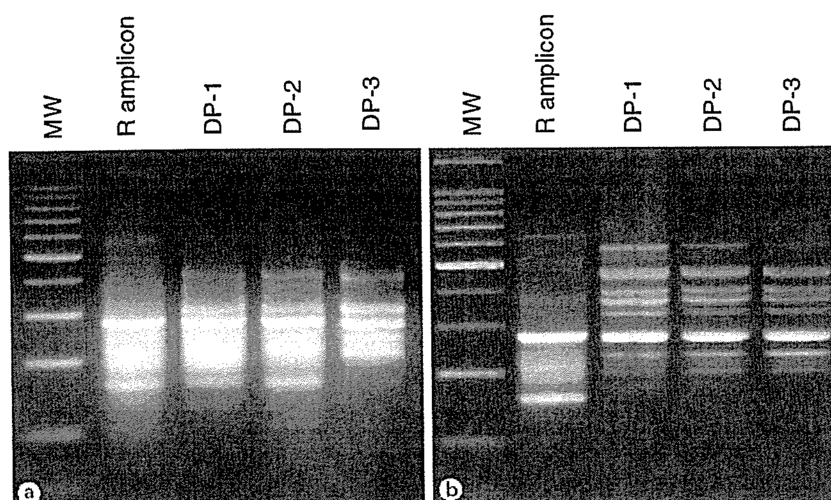

Fig. 1. Electrophoresis in 2% agarose gels with ethidium bromide staining of the final products from each round of cDNA RDA procedures. Molecular weight (MW), representative amplicon (R amplicon), difference product-1 (DP-1), DP-2 and DP-3 are shown from the left. Each band observed represents difference products. **a** KYSE170 vs. HEEC-1. KYSE170 was tester and HEEC-1 was driver. **b** HEEC-1 vs. KYSE170. HEEC-1 was tester and KYSE170 was driver.

Table 1. Three K-H-DPs and six H-K-DPs are shown

	Gene name	Length	Homology	Accession No.
K-H-DPs				
K-H-DP-1	LAGE-1	209 bp	100%	AJ223040
K-H-DP-2	ERT	189 bp	100%	AF017307
K-H-DP-3	PTI-1	301 bp	98%	L41498
H-K-DPs				
H-K-DP-1	fibronectin	181 bp	100%	X02761
H-K-DP-2	laminin	218 bp	99%	Z15008
H-K-DP-3	cytokeratin 19	278 bp	100%	Y00503
H-K-DP-4	cytokeratin 6B	262 bp	97%	BI335818
H-K-DP-5	cyclin D2	183 bp	99%	M90813
H-K-DP-6	odd Oz/ten-m homolog 2	153 bp	100%	NM_011856

All fragments turned out to have almost complete homology with known genes. Accession numbers represent genes in the GenBank database.

cell lines, all esophageal cancer tissues, and normal epithelial tissues of the esophagus (table 2). Both genes were also expressed in other normal tissues at a high rate (data not shown), which suggested that they were ubiquitously expressed. In contrast, LAGE-1 was expressed in 3/11 (27%) of esophageal cancer cell lines and 3/7 (43%) of esophageal cancer tissues, but was not expressed in normal epithelial tissues of the esophagus (table 2).

Expression of Genes Specifically Downregulated in Esophageal Cancer by RT-PCR

Nine genes identified from HEEC-1 when it was used as tester were examined as candidate genes representing the loss of normal genes in esophageal cancers. Fibronectin, laminin, cytokeratin 6B, and cytokeratin 19 were barely detected in KYSE170, but were distinctly detected in HEEC-1. In contrast, cyclin D2 and ten-m2 were detected in HEEC-1 but not in KYSE170. In additional examinations, laminin, cytokeratin 6B, and cytokeratin 19 were detected in most esophageal cancer cell lines and tissues, but cyclin D2 and ten-m2 were not detected in many esophageal cancer cell lines by RT-PCR (table 2).

cDNA Microarray Analysis

For comparison with the results from cDNA RDA, cDNA microarray analysis was performed. The DNA chips used in our microarray analysis did not have all genes that had been identified by cDNA RDA; neverthe-

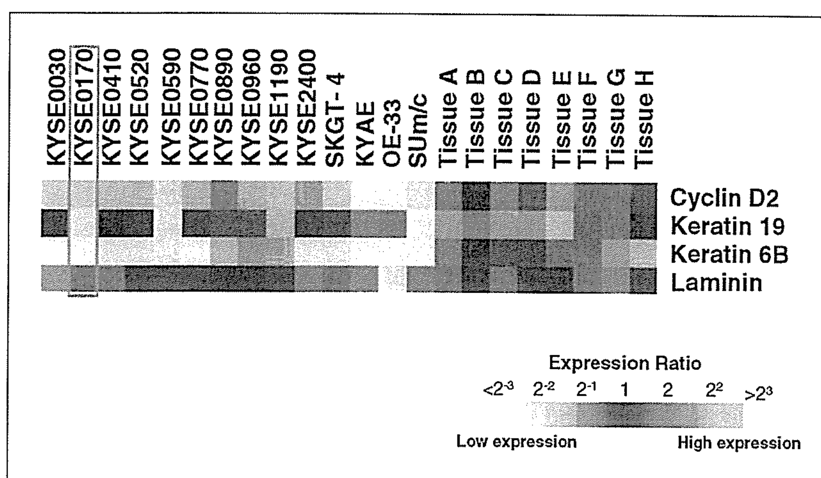

Fig. 2. cDNA microarray analysis for various esophageal cancer cell lines (squamous cell carcinomas; KYSE series and SUm/c, adenocarcinomas; SKGT-4, KYAE and OE-33) and esophageal cancer tissues (tissue A–H). The KYSE series includes KYSE 170, which was used as a driver in the current cDNA RDA (box).

Table 2. RT-PCR for various cell lines and tissue specimens

	KYSE170	HEEC-1	KYSE30	KYSE150	KYSE520	KYSE590	KYSE960	KYSE1190	SUm/c	SKGT-4	OE-33	KYAE	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5	Tissue 6	Tissue 7	*NT
LAGE-1	+	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	+	-
ERT	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
PTI-1	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Fibronectin	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+
Laminin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cytokeratin 19	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Cytokeratin 6B	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Cyclin D2	-	+	-	+	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+
ten-m2	-	+	-	-	-	-	+	+	-	-	-	-	+	-	+	+	+	+	+	+
GAPDH																				

All specimens were esophageal cancers except HEEC-1 and NT. * NT = Pooled sample of 10 normal tissues of esophagus. Tissue samples (tissue 1–7) were not identical to those in figure 2 (tissue A–H).

less, keratin 6B, keratin 19, laminin, and cyclin D2 were included. The expression of each of these genes obtained from cDNA microarrays was downregulated strongly in KYSE170 used as a driver (red box in fig. 2). These 4 genes were also found to be downregulated in most other esophageal cancer cell lines and esophageal cancer tissues (fig. 2).

Correlation between LAGE-1 and MAGE-A1

MAGE-A1 is known as one of the cancer/testis antigens and it has been reported that its expression was highly correlated with LAGE-1 [6]. For investigation of the correlation between these genes, the expression of LAGE-1 and MAGE-A1 in various specimens was examined by RT-PCR (table 3). The primers of MAGE-A1

Table 3. Comparison of LAGE-1 and MAGE-A1 by RT-PCR in various cancerous and normal specimens

Normal tissues	LAGE-1	MAGE-A1	GAPDH
Ovary	+	-	
Testis	+	+	
Kidney	+	-	
Small intestine	-	-	
Heart	-	-	
Prostate	-	-	
Brain	-	-	
Thymus	-	-	
Placenta	-	-	
Lung	-	-	
Liver	-	-	
Colon	-	-	
Muscle	-	-	
Pancreas	-	-	
Spleen	-	-	
Leukocyte	-	-	

Tumor samples (cancer cell lines)	LAGE-1	MAGE-A1	GAPDH
Prostatic adenocarcinoma	+	+	
Breast carcinoma	+	+	
Colon adenocarcinoma 1	-	+	
Colon adenocarcinoma 2	+	-	
Ovarian carcinoma	+	-	
Pancreatic adenocarcinoma	-	+	
Lung carcinoma (LX-1)	-	-	
Lung carcinoma (GI-117)	-	-	

Esophageal cancers	(positive/total)	
Esophageal cancer cell lines	3/11	6/11

Esophageal cancer tissues

LAGE-1	MAGE-A1	
	positive	negative
Positive	8	2
Negative	6	14

p = 0.013 (Fisher's exact probability test)

LAGE-1 was expressed in the kidney, ovary, and testis. MAGE-A1 was expressed in the testis. LAGE-1 and MAGE-A1 were also expressed in other cancer cell lines. Expression of LAGE-1 and MAGE-A1 was highly correlated in esophageal cancer cell lines and tissues.

used were the same as described in Hubank and Schatz [1]. LAGE-1 was not expressed in normal tissues except for testis, ovary, and kidney, and MAGE-A1 was expressed only in testis. LAGE-1 was expressed in 28% (3/11) of esophageal cancer cell lines and in 33% (10/30) of esophageal cancer tissues, while MAGE-A1 was expressed in 55% (6/11) and 47% (14/30), respectively. Moreover, the expression of LAGE-1 was strongly correlated with that of MAGE-A1 (p = 0.013, Fisher's exact probability test). The rate of expression of LAGE-1 among specimens in which MAGE-A1 was positive was 10/13 (77%).

Expression of LAGE-1 in Response to Treatment with 5Aza-dC or TSA

The expression of LAGE-1 induced by various continuous concentrations of 5Aza-dC and TSA for various incubation times is shown in table 4 A, B. 5Aza-dC was used at concentrations of 0.75, 2, 5 and 10 μ*M* for treatment times of either 2 days or 5 days. For all HEECs, LAGE-1 expression was not induced after 2 days at any concentration of 5Aza-dC tested, but it was induced after 5 days of 5Aza-dC treatment at every concentration tested. In all esophageal cancer cell lines, LAGE-1 was also induced under certain conditions (table 4A). TSA was examined at continuous concentrations of 100, 500 and

Table 4. Induction of LAGE-1 expression by 5Aza-dC or TSA

A Expression by 5-Aza-dC

Concentration Incubation time		0 µ*M* -	0.75 µ*M* 2 days	0.75 µ*M* 5 days	2 µ*M* 2 days	2 µ*M* 5 days	5 µ*M* 2 days	5 µ*M* 5 days	10 µ*M* 2 days	10 µ*M* 5 days
Normal esophageal cell lines										
HEEC-1	LAGE-1	-	-	+	-	+	-	+	NP	NP
	GAPDH									
HEEC-2	LAGE-1	-	-	+	-	+	-	+	NP	NP
	GAPDH									
HEEC-3	LAGE-1	-	-	+	-	+	-	+	NP	NP
	GAPDH									
HEEC-4	LAGE-1	-	-	+	-	+	-	+	NP	NP
	GAPDH									
Esophageal cancer cell lines										
KYSE150	LAGE-1	-	-		-	-	-	-	-	+
	GAPDH									
KYSE960	LAGE-1	-	-	-	+	+	-	-	+	+
	GAPDH									
SKGT-4	LAGE-1	-	-	-	+	+	NP	NP	NP	NP
	GAPDH									
KYSE520	LAGE-1	-	-	-	-	-	-	-	+	+
	GAPDH									

B Expression by TSA

Concentration Incubation time		100 µg/l 2 days	100 µg/l 5 days	500 µg/l 2 days	500 µg/l 5 days	1 mg/l 2 days	1 mg/l 5 days
Normal esophageal cell lines							
HEEC-1	LAGE-1	-	-	-	-	-	-
	GAPDH						
HEEC-2	LAGE-1	-	-	-	-	-	-
	GAPDH						
HEEC-3	LAGE-1	-	-	-	-	-	-
	GAPDH						
HEEC-4	LAGE-1	-	-	-	-	-	-
	GAPDH						
Esophageal cancer cell lines							
KYSE150	LAGE-1	-	-	+	NA	+	NA
	GAPDH						
KYSE960	LAGE-1	-	-	-	+	NA	NA
	GAPDH						
SKGT-4	LAGE-1	-	-	-	-	+	+
	GAPDH						
KYSE520	LAGE-1	-	-	-	-	-	-
	GAPDH						

5Aza-dC induced LAGE-1 in all esophageal cancer and normal cell lines. TSA induced LAGE-1 in KYSE150, KYSE960 and SKGT4, but not in KYSE520 or any HEECs. NP = Not performed; NA = not available.

1 μg/l for treatment times of 1, 2 or 5 days. TSA induced LAGE-1 expression in KYSE150, KYSE960 and SKGT4, but not in any of the HEECs (table 4B). KYSE150 and KYSE960 were so sensitive for TSA that RNA could not be obtained in some experiments (shown as NA in table 4B).

Discussion

The original RDA method established by Lisityn et al. [10] is a high through-put method that can detect genomic amplification or deletion between two genomes by subtractive PCR. Hubank and Schatz [1] applied this genomic RDA protocol to cDNA, employed four-cutter restriction enzyme *Dpn* II, which increased the number of restriction sites, and consequently succeeded in obtaining more genetic information. We have modified this cDNA RDA protocol as described in Materials and Methods. (A) We omitted mung bean nuclease, because our results were not affected by whether mung bean nuclease was used or not (data not shown). (B) The tester-to driver ratio was fixed at 1:800 in the second and third steps. (C) spin column was used in all purification steps of PCR products for removal of residual adapters or dNTPs.

Using our cDNA RDA protocol, we detected the expression of several specific genes (LAGE-1, ERT, PTI-1, cyclin D2 and ten-m2) from esophageal squamous cell carcinomas and normal esophageal epithelial cell lines. Furthermore, using cDNA microarray analysis, we confirmed that the same expression patterns for fibronectin, keratin 6B, keratin 19, laminin, and cyclin D2 genes were obtained between cancer and the normal epithelium from esophageal cell lines or esophageal tissues. This strong correlation between the results from cDNA RDA and cDNA microarray analyses implies that cDNA RDA is still a simple and useful tool either for detecting new specific genes or for selecting genes that should be spotted in original microarrays because there are still a number of unknown genes that are merely predicted or sequenced although the human genome project is nearly finished.

Among the genes thus detected, ERT has been suggested to be a transcriptional factor involved in the transcriptional regulation of the transforming growth factor-β (TGF-β) type II receptor (RII) gene. PTI-1 is a genetic element expressed in specific human carcinomas and implicated in mutagenic changes in elongation factor 1α (EF-1 α) as a potential contributor to the carcinogenic process. These genes have been reported to encode transcription factors, suggesting that alteration of transcriptional control may directly contribute to cancer development and evolution [13–15]. Our data imply that these transcriptional factors were also upregulated in squamous cell carcinoma of the esophagus, suggesting that similar mechanisms of cancer development would probably apply to the esophagus.

Ten-m2, also called Odz, is a novel gene involved in directing segmentation in *Drosophila melanogaster* [16, 17]. This gene encodes a protein involved in signal transduction on the cell surface, rather than in transcription, and its function is related to cellular adhesion, involving interactions with NCAM, laminin, proteo-glycans, and integrins. The loss of ten-m2 in squamous cell carcinoma of the esophagus suggests its moderate adhesive potential and might increase the tendency toward distant metastasis. Hence, our data from cDNA microarray analysis were compatible with these characteristics of esophageal cancer.

LAGE-1 is 3,245 bp long in genomic DNA and encodes three exons [6]. LAGE-1a, 1b, 1L, and 1S are collectively known as LAGE-1 family genes. The fragment we identified had 100% homology to LAGE-1b and LAGE-1L [18]. Although NY-ESO-1 [19] is a member of the LAGE family, it belongs to LAGE-2, which is quite distinct from the LAGE-1 family of genes. In the present study, NY-ESO-1 was not detected in cell lines or tumor samples of the esophagus by RT-PCR, despite our usage of published primers and protocols (data not shown). Gene LAGE-1 has been mapped to Xq28 and is located close to the MAGE-A1 gene of the MAGE family. In this study, coincidental expression of LAGE-1 and MAGE-A1 was shown. The functions of these genes are still unknown, but some reports have suggested that they might regulate the expression of other genes [18]. It has been reported that epigenetic changes are related to the regulatory mechanisms of many genes, including MAGE-A1 and LAGE-1 [6]. There is no direct evidence in the articles reported that LAGE-1 is methylated. When we refer to the public database 'UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway)' provided by the University of California, Santa Cruz, Calif., there are no reported CpG islands upstream of LAGE-1. LAGE expression was induced by 5-Aza-dC and TSA, suggesting that demethylation and histone acetylation may play important roles in the activation of this gene in tumors. The expression of LAGE-1 was more dependent on the duration of the incubation than the concentration of 5-Aza-dC in HEECs. In esophageal cancer cell lines, induction of the LAGE-1 gene by demethylation needed higher concentrations and longer durations. The involvement of his-

tone acetylation in the expression of LAGE-1 was also suggested to be quite different between HEECs and esophageal cancer cell lines. LAGE-1 was more inducible by TSA in some esophageal cancers than in HEECs. In each cell line, there may be variability in the ability of the components involved in acetylating mechanisms. Because of its tumor-specific expression similar to that of MAGE-A1, LAGE-1 has the potential to be a target antigen for anti-tumoral immunotherapy in esophageal cancers. In the near future, we should be able to improve the poor prognosis of esophageal cancer by vaccination utilizing such identified genes as antigens.

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Review

Clinical Aspects of Multimodality Therapy for Resectable Locoregional Esophageal Cancer

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Radical resection has been considered the only possible way to save the lives of patients with esophageal cancer. Therefore, tremendous efforts have been made in order to improve the surgical results for resectable locoregional esophageal cancer. Various surgical approaches have been developed. Combination therapies such as neoadjuvant, adjuvant chemotherapy, and neoadjuvant chemoradiation have been extensively investigated in numerous randomized clinical trials. Due to insufficient surgical results and high postoperative mortality rates, definitive chemoradiation has been studied as alternative treatment in selected patients, based on the concept that combined-modality therapy allows simultaneous treatment of locoregional disease and systemic micrometastases. Chemoradiation has shown survival rates equivalent to surgery in some non-randomized comparative studies. Presently, concerns appear to be shifting to the question of whether definitive chemoradiation could be an alternative to surgery in the primary treatment of resectable locoregional esophageal cancer. Recently, 2 randomized trials, comparing definitive chemoradiation with chemoradiation and surgery were published. These trials seem to show at first glance that definitive chemoradiation can achieve results comparable to surgery with neoadjuvant chemoradiation. More sophisticated trials should be conducted as treatment modalities used in these trials are far from routine. (Ann Thorac Cardiovasc Surg 2006; 12: 234–41)

Key words: esophageal cancer, neoadjuvant therapy, definitive chemoradiation, salvage esophagectomy

Introduction

Esophageal cancer is an increasingly common malignancy. This neoplasm is devastating because of its aggressive clinical course and high mortality rate. The long-term survival rates of under 10% are disappointing in part, due to the high incidence of advanced and/or metastatic disease at the time of diagnosis. Over 2 decades, several treatment modalities have been developed to improve the survival of patients with esophageal cancer. Among these, surgical resection remains the preferred choice. It currently provides the best palliation for dysphagia and local control and the best chance for cure when compared with other therapeutic options. However, as definitive chemoradiation has been gradually improving, the boundaries of treatment strategies have become blurred in the patients with resectable locoregional esophageal cancer.

This overview will examine the available data on current treatment modalities and discuss the future direction clinical research and treatment.

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Preoperative Chemotherapy

Since surgery alone cannot achieve a good survival rate, trials evaluating the efficacy of preoperative chemotherapy followed by surgery for resectable esophageal cancer have been conducted since the 1980s. The rationale of preoperative chemotherapy includes down-staging of the tu-

Table 1. Phase III trials to investigate the impact of neoadjuvant chemotherapy in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	R0 (%)	Mortality (%)	Median survival (mos)	2-year survival (%)	*P*
Kelsen et al.[1]	1998	Surgery	SCC/AC	227	59	6	16.1	35	ns
		CDDP/5-FU		213	62	7	14.9	37	
MRCOCWP[2]	2002	Surgery	SCC/AC	400	54	10	13.3	34	0.004
		CDDP/5-FU		402	60	10	16.8	43	

R0, microscopically complete resection; mos, months; CDDP, cisplatin; 5-FU, 5-fluorouracil; SCC, squamous cell carcinoma; AC, adenocarcinoma; MRCOCWP, Medical Research Council Oesophageal Cancer Working Party; ns, not significant.

mor and elimination of micrometastatic disease.

Two recent randomized trials evaluating the utility of preoperative chemotherapy for esophageal cancer are summarized in Table 1. They are reported from the U.S. Intergroup trial 0113[1] and the Medical Research Council Oesophageal Cancer Working Party (MRCOCWP).[2]

In the U.S. Intergroup trial, 440 patients with operable esophageal cancer, histologically squamous cell carcinoma (SCC) or adenocarcinoma (AC), stage I to III, were eligible and follow-up data was adequate. A total of 213 patients were assigned to receive chemotherapy consisting of 3 cycles of 5-fluorouracil (5-FU: 1,000 mg/m^2/24 hours for 5 days) and cisplatin (CDDP: 100 mg/m^2/bolus day 1) followed by surgery. Two additional cycles of the same chemotherapy were administered to these patients after curative resection. The remaining 227 patients were assigned to undergo immediate surgery. There were no significant differences between the 2 groups in the median survival of 14.9 months for the patients who received preoperative chemotherapy and 16.1 months for those who underwent immediate surgery (p=0.53). Among the patients whose resection was curative, median survival in each group was over 2 years. At 2 years, the survival rate was 35% and 37% respectively and at 3 years, the survival rate was 23% and 26% respectively. Furthermore, no statistically significant differences were observed between the 2 groups in terms of curative resection rate (59% vs. 62%) and treatment mortality rate (6% vs. 7%). The pattern of failure was almost identical for the 2 groups. The histology of the tumor did not have an effect on response to treatment. Complete responses as assessed by pathological examination were achieved in 2.5% of patients who had received at least 1 cycle of chemotherapy. Preoperative chemotherapy with a combination of 5-FU and CDDP did not demonstrate a survival benefit in the patients with SCC and AC of the esophagus. This trial concluded that surgery alone remains the standard treatment for patients with localized esophageal cancer.

In contrast, in the MRCOCWP trial, 802 previously untreated patients with resectable esophageal cancer were accrued regardless of histologic cell type, 31% with SCC and 69% with AC or undifferentiated carcinoma. Eligible patients were randomly allocated to receive chemotherapy consisting of 2 cycles of 5-FU (1,000 mg/m^2/24 hours for 4 days) and CDDP (80 mg/m^2/bolus day 1) with an interval of 3 weeks between the first day of each cycle followed by surgery 3 weeks apart or to undergo immediate surgery. In this trial, the curative resection rate was 59% in the preoperative chemotherapy group versus 62% in the resection alone group. The treatment effect was similar for the patients with SCC and those with AC. There were no differences in the postoperative mortality rate of 10% in both groups. The median survival of 16.8 months in the preoperative chemotherapy group was better than 13.3 months in the resection group, and the survival rate at 3 years was 43% and 34% respectively. The MRCOCWP trial suggested that the preoperative chemotherapy regimen used in the study should be considered for patients with resectable cancer of the oesophagus.

It is difficult to explain the difference in survival benefits between these 2 trials. This conflicting difference might be dependent upon the resectability rate of only 80% in the Intergroup and 92% in the MRCOCWP. Although the MRCOCWP trial is not included in this analysis, recently published meta-analysis of 11 randomized controlled trials that compared neoadjuvant chemotherapy and surgery with surgery alone for esophageal cancer did not demonstrate a survival benefit for the treated patients.[3] In the papers on randomized trials including esophagectomy, the type of procedures performed and there distribution into groups should be clearly mentioned. The efficacy of neoadjuvant chemotherapy remains controversial but, in any case, seems to be limited.

Table 2. Phase III trials to investigate the impact of adjuvant chemotherapy in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	5-year survival (%)	*P*
Ando et al.[4]	1997	Surgery	SCC	100	45.1	ns
		CDDP/VDS		105	48.3	
Ando et al.[5]	2003	Surgery	SCC	122	52	ns
		CDDP/5-FU		120	61	

CDDP, cisplatin; VDS, vindesine; 5-FU, 5-fluorouracil; SCC, squamous cell carcinoma; ns, not significant.

Postoperative Chemotherapy

The use of chemotherapy in the adjuvant setting could prevent undesired delays in definitive surgery. However, due to the high postoperative mortality and morbidity that accompany complicated surgical procedures, there has been little impetus to promote clinical trials with adjuvant chemotherapy in esophageal cancer, postoperative chemotherapy has not been widely studied in randomized trials.

Postoperative chemotherapy has not been widely studied in randomized trials. However, 2 Japanese randomized trials, involving patients with SCC compared it with surgery alone without preoperative therapy[4,5](Table 2). In the first trial, chemotherapy consisted of 2 cycles of vindesine (VDS: 3 mg/m^2/bolus day 1) and CDDP (70 mg/m^2/2 hours day 1). In the second trial, chemotherapy consisted of 5-FU (800 mg/m^2/24 hours for 5 days) and CDDP (80 mg/m^2/2 hours day 1) with an interval of 3 weeks between the first day of each cycle. Chemotherapy was well tolerated in the postoperative setting. Though the first trial did not demonstrate the benefit of adjuvant chemotherapy, the second trial showed a trend toward improved 5-year disease-free survival.

In the second trial with 5-FU and cisplatin, both groups showed no remarkable difference in overall survival. The 5-year survival rate of the surgery alone group was 52% while that for the postoperative chemotherapy group was 61% (p=0.13). In contrast, the 5-year disease-free survival rate of 55% in patients in the surgery plus chemotherapy group was significantly better than the 45% achieved by patients in the surgery alone group (p=0.037). Furthermore in subgroup analyses, the subgroup with lymph node metastasis showed risk reduction of 52% in the adjuvant group versus 38% in the surgery alone group which was also statistically significant.

The efficacy of postoperative chemotherapy is currently unclear because of the small number of trials. However, a potential benefit might exist for certain patient subgroups. As long as postoperative mortality and morbidity rates are not decreased, postoperative chemotherapy cannot be easily adapted to the context of clinical trials.

Preoperative Chemoradiation

Another common treatment strategy is preoperative chemoradiation. Neoadjuvant chemoradiation followed by surgery has been extensively studied over the past decade as a result of the pattern of both local and distant failure associated with surgery alone. The rationale of preoperative chemoradiation includes down-staging of the tumor and eradication of micrometastases. Moreover, most chemotherapeutic agents that have an effect on esophageal cancer simultaneously act as radiosensitizers. Although 8 randomized controlled trials have been reported that evaluate the benefit of preoperative chemoradiotherapy in patients with esophageal cancer,[6–13] a sufficient number of patients to allow statistically meaningful results have been accrued in only 2 of these trials[10,13](Table 3). Bosset et al.[10] reported the results of a randomized trial of preoperative combined modality therapy from the European Organization for Research and Treatment of Cancer (EORTC). A total of 282 patients with clinically resectable esophageal cancer, histologically SCC and stage I to II, were randomized to receive either preoperative combined modality therapy or surgery alone. The preoperative radiotherapy was unconventional in design and consisted of 5 daily fractions of 3.7 Gy each followed by a 2-week rest and another 3.7 Gy for 5 days. Also unusual was the chemotherapy which consisted of a single use of CDDP given at a dose of 80 mg/m^2 on day 0–2 before starting radiotherapy. Though the 3-year disease-free survival rate of 40% in patients who received preoperative combined modality therapy was a signifi-

Table 3. Phase III trials to investigate the impact of neoadjuvant chemoradiation in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	R0 (%)	Mortality (%)	Median survival (mos)	3-year survival (%)	*P*
Nygaard et al.[6]	1992	Surgery CDDP/BLM+35 Gy	SCC	41 47	37 55	13 24	7.5 7.5	9 17	ns
Le Prise et al.[7]	1994	Surgery CDDP/5-FU+20 Gy	SCC	45 41	84 85	7 8.5	10 10	14 19	ns
Apinop et al.[8]	1994	Surgery CDDP/5-FU+40 Gy	SCC	34 35	na	15 14	7.4 9.7	20 26	ns
Walsh et al.[9]	1996	Surgery CDDP/5-FU+40 Gy	AC	55 58	na	8 4	11 16	6* 32	0.01
Bosset et al.[10]	1997	Surgery CDDP+37 Gy	SCC	139 143	na	3.6 12.3	18.6 18.6	37 39	ns
Law et al.[11]	1998	Surgery CDDP/5-FU+40 Gy	SCC	30 30	42 80	na	27 26	na	ns
Urba et al.[12]	2001	Surgery CDDP/VBL/5-FU+45 Gy	SCC/AC	50 50	na	2 16.9	17.6 30	16 30	ns
Burmeister et al.[13]	2002	Surgery CDDP/5-FU+35 Gy	SCC/AC	128 128	na	4.6*	18.5 21.7	na	ns

R0, microscopically complete resection; mos, months; CDDP, cisplatin; BLM, bleomycin; 5-FU, 5-fluorouracil; VBL, vinblastine; SCC, squamous cell carcinoma; AC, adenocarcinoma; na, not available; ns, not significant; *, overall treatment related mortality.

cantly better than the 28% achieved by patients treated with surgery alone, there was no significant difference in the 3-year overall survival rate (37% and 39% respectively) and median survival of 18.6 months in both groups.

A recent randomized trial of 256 patients who received either neoadjuvant chemoradiation consisting of 1 cycle of 5-FU and CDDP given with radiotherapy at a dose of 35 Gy in 15 fractions or surgery alone was reported in abstract form by Burmeister et al.[13] The results did not show a survival benefit that could be attributed to the added chemoradiation. In a subgroup analysis, patients with SCC had an increased disease-free survival, but not overall survival. The administration of single agent chemotherapy is not effective enough to eradicate micrometastatic disease.

Given the above data and, despite the widespread use of preoperative chemoradiation, randomized trials have raised significant methodological concerns and have yielded conflicting outcomes. There are several unanswered questions, i.e., optimal radiation dose, adequate radiation field, chemotherapeutic agents and administration schedule, histological distribution, etc. Moreover, preoperative chemoradiation tends to increase postoperative mortality; this may be most important factor swaying the results of trials.[6,10,12]

Chemoradiation without Surgery

Treatment with chemotherapy and radiotherapy, has been shown to be superior to radiation alone.

In 1985, the Radiation Therapy Oncology Group (RTOG) started RTOG 85-01, a prospective randomized controlled trial. They evaluated the hypothesis that concurrent chemoradiation could achieve a higher overall survival rate in patients who had localized carcinoma of the thoracic esophagus than was possible with radiation alone.[14] In this study 121 patients were randomly assigned to receive either combined-modality therapy or radiation therapy alone. The chemoradiation therapy consisted of protracted infusion of 5-FU (1,000 mg/m^2/24 hours for 4 days) and CDDP (75 mg/m^2/bolus day 1) on weeks 1, 5, 8 and 11 with 50 Gy of radiation therapy, while the radiation alone comprised 64 Gy. In this study, patients who received chemoradiation showed a significant improvement in median survival of 12.5 months compared with 8.9 months for patients who received radiation alone. With a 2-year survival rate of 38% and 10% respectively. Histologic type did not have a significant affect on the outcomes. The protocol was closed early because of positive results at the interim analysis. An additional 69 patients treated with the same combined-modality therapy after the closure of the randomization confirmed the results of

Table 4. Phase III trials to investigate the impact of neoadjuvant chemoradiation vs definitive chemoradiation in patients with resectable esophageal carcinoma

Author/ ref. no.	Year	Protocol	Histology	No. of patients	Mortality (%)	Median survival (mos)	2-year survival (%)	*P*
Bedenne et al.[18]	2002	I-CRT → Surgery	SCC/AC	259	9*	17.7	34	0.56
		I-CRT → CRTx				1*	19.3	40
Stahl et al.[19]	2005	I-CT+CRT → Surgery	SCC	86	12.8	16.4	39.9	ns
		I-CT+CRT → CRTx		86	3.5	14.9	35.4	

mos, months; I-CRT, induction chemoradiotherapy; I-CT+CRT, induction chemotherapy + chemoradiotherapy; SCC, squamous cell carcinoma; AC, adenocarcinoma; ns, not significant; *, the death rate within 3 months after starting induction treatment.

chemoradiation with a median survival of 17.2 months and 3-year survival rate of 30%.[15,16] Based on this positive result, concurrent chemoradiotherapy has become the standard therapy for patients with localized carcinoma of the thoracic esophagus selected for nonsurgical treatment. Although chemoradiation was associated with a higher incidence of toxicity, this problem has led to studies aimed at improving the efficacy of radiotherapy, especially in determining the appropriate radiation dosage a part of combined modality therapy.

In the intergroup INT 0123 (RTOG 94-05),[17] 236 patients with esophageal cancer, histologically SCC or AC, clinical stage T1 to 4, N0/1, M0 were allocated to receive either combined-modality therapy consisting of 4 monthly cycles of 5-FU (1,000 mg/m^2/24 hours for 4 days) and CDDP (100 mg/m^2/bolus day 1) with concurrent 64.8 Gy radiation or the same chemotherapy dose and schedule but with 50.4 Gy. Since the data revealed that it was highly unlikely there would be any advantages to using the high dose radiation compared with the standard dose, this trial was closed at the time of the planned interim analysis. For the 218 eligible patients, there was actually no significant difference in median survival of 13.0 months for the high dose arm and 18.1 months for the standard dose arm. The survival rate at 2 years for the 2 arms was 31% and 40%, and local persistence or failure, respectively. Although 11 treatment related deaths occurred in the high dose arm compared with 2 in the standard dose arm, 7 of the 11 deaths occurred in patients who had received 50.4 Gy or less. It is unlikely that the increase in treatment related deaths in the high dose arm was related to a higher dose of radiation. Thus the standard radiation dose for patients treated with 5-FU and CDDP chemotherapy is 50.4 Gy.

This randomized trial data suggest the clear superiority of chemoradiation over radiotherapy alone. Definitive chemoradiation is thus widely recognized as the standard of nonsurgical treatment for locoregional operable esophageal cancer.

Necessity for Surgery after Chemoradiation

Chemotherapy and radiotherapy without surgery has not been compared with surgery alone in prospective clinical trials. Recently 2 trials have been reported from the Federation Francaise de Cancerologie Digestive (FFCD) and the German Oesophageal Cancer Study Group (GOCSG)[18,19] that indicate the necessity of surgery after radiation (Table 4).

In the FFCD trial (FFCD 9102), 445 patients with operable thoracic esophageal cancer, histologically SCC, stage T3-4N0-1M0 were eligible. Induction therapy consists of 2 cycles of 5-FU and CDDP (days 1–5 and 22–26) plus concurrent protracted (46 Gy in 4.5 weeks) or split-course (15 Gy in days 1–5 and 22–26) radiation. The 259 patients who had at least a partial response were randomly allocated to receive additional chemoradiation or perform surgery. The additional chemoradiation consisted of 3 cycles of the same chemotherapy and concurrent protracted (20 Gy) or split-course (15 Gy) radiation. The 2-year survival rate was 34% in the surgery group versus 40% in the chemoradiation group, which was not significant (p=0.56). Furthermore, median survival was 17.7 months versus 19.3 months respectively. Thus, the FFCD 9102 trial concluded that additional chemoradiation is an alternative to surgery in patients with locally advanced resectable esophageal cancer who respond to initial chemoradiation.

The GOCSG compared preoperative chemoradiation followed by surgery with chemoradiation alone. In this trial, 172 patients with locally advanced SCC of the esophagus, stage T3-4N0-1M0 were randomized to ei-

ther induction chemotherapy followed by chemoradiation (40 Gy) followed by surgery, or the same induction chemotherapy followed by definitive chemoradiation (at least 65 Gy) without surgery. Induction chemotherapy consists of 3 cycles of bolus 5-FU, leucovorin, etoposide, and CDDP on days 1–3 every 3 weeks. This was followed by concomitant chemoradiation with CDDP and etoposide. In the surgery arm, tansthoracic esophagectomy was performed 3–4 weeks after the end of irradiation. In the definite chemoradiation arm, the same combined chemoradiation was administered with a radiation dose of 40 Gy. Afterwards, the radiation dose was increased to at least 65 Gy with hyperfractionated external-beam radiotherapy or high dose-rate afterloading brachytherapy. Although the local progression-free survival rate at 2 years (64.3% in the surgery group and 40.7% in the chemoradiation group) was significantly different, (p=0.003), overall survival in both treatment groups, was roughly equal (39.9% at 2 years and 35.4%, 31.3% and 24.4% at 3 years). Median survival of each group also showed no difference. As a result, the GOCSG concluded that chemoradiation followed by surgery can no longer be recommended as routine treatment in patients with good tumor response to induction therapy. However, surgery is recommended in limited cases to provide survival benefit in patients defined as nonresponders.

In these 2 trials, definite chemoradiation appears to be an alternative to surgery in the initial treatment of locoregional advanced esophageal cancer. However, several issues should be raised, e.g., 1) since each trials include clinical T4 which could lead to non-curative resection (R1–R2), the patients with this stage of tumor should be excluded because prognoses were extremely poor in patients whose resection was incomplete; 2) treatment schedule in radiotherapy should be integrated into 1 regimen, and there are significant methodological concerns, including radiation field, dose, fraction and split; 3) even now, a combination of 5-FU and cisplatin remains the standard regimen for esophageal cancer. Since the protocol used for chemotherapy in the German study is not the common protocol, the validity of the study's conclusion is not easily acceptable; and 4) of the randomized trials comparing concurrent chemoradiation followed by surgery with surgery alone, only one trial reported by Walsh et al.,[9] in which only adenocarcinoma patients were eligible, demonstrated a significantly better median survival and 3-year survival rate. This result means histology could have an important influence on outcome. Thus randomized controlled trials of non-surgical approaches ought to be planned for patients with different histologies.

Salvage Esophagectomy after Definitive Chemoradiation Therapy

Isolated persistence or local failure of the disease to respond is not uncommon after definitive chemoradiation. Although salvage esophagectomy is one of the strategies for selected patients, it is a far riskier operation from the standpoint of mortality and morbidity than planned esophagectomy with or without neoadjuvant therapy. In general, anastomotic leakage and pulmonary complications are more common when esophagectomy is performed after definitive chemoradiation. Swisher et al.[20] reported anastomotic leak rates of 39% in patients who underwent salvage esophagectomy. This was significantly higher than the 7% leak rate experienced by those who received planned esophagectomy (p=0.005) and the average hospital stay (29.4 days) for the former was significantly longer than that for the latter (18.4 days) (p=0.03). Postoperative mortality rates for salvage esophagectomy patients trended upward (15% vs. 6%, p=0.2). Swisher et al.[20] also described how salvage esophagectomy increased the complexity or difficulty of resecting the relapsed tumors. Meunier et al.[21] also stressed the difficulty of surgery.

Thus surgeons who perform salvage esophagectomy face a difficult challenge in trying to reduce concomitant postoperative mortality and morbidity.

Conclusion

The view that surgery might not be essential and that the apparent advantage of chemoradiation alone arises from the avoidance of perioperative mortality. The treatment modality of resectable locoregional esophageal cancer seems to be evolving from surgery alone to definitive chemoradiation and preoperative chemoradiation. Though the survival rates for definitive chemoradiation and surgery appear similar, surgery-related death rates might be most important factors swaying the results of studies. On the other hand, local failure occurs more frequently in the patients treated with definitive chemoradiation alone. Patients with local recurrence or residual disease after chemoradiation should be referred for surgery by a medical oncologist. Considering the high postoperative mortality and morbidity rates, salvage esophagectomy could be considered a difficult challenge for a surgeon. If de-

finitive chemoradiation is carried out on the assumption that local failure can be salvaged by surgery, it is difficult to regard it as a valid treatment strategy. Moreover, if a method of predicting results before and during treatment can be developed, definitive chemoradiation could become acceptable as a separate treatment option. There are no widely accepted clinical trials contrasting definitive chemoradiation with surgery using standard and appropriate protocols. These issues can only be resolved by carefully designed, randomized, controlled trials. At present, such trials have yet to be carried out. It should be noted that neoadjuvant therapy which is mainly performed in Western countries might increase the postoperative mortality rate. The possibility cannot be denied that surgical treatment might prove to be superior as long as concomitant mortality can be reduced.

The survival advantage over surgery in all neoadjuvant and adjuvant settings remains unclear. A breakthrough seems impossible unless more promising chemotherapeutic agents are developed, as the efficacy of both definitive chemoradiation and surgical results have reached their limit. Thus one must conclude that surgery remains the gold standard for resectable locoregional esophageal cancer with which other treatment options must be compared.

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Ultrasensitive DNA Chip: Gene Expression Profile Analysis without RNA Amplification

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We have developed a new DNA chip whose substrate has a unique minute columnar array structure made of plastic. The DNA chip exhibits ultrahigh sensitivity, up to 100-fold higher than that of reference DNA chips, which makes it possible to monitor gene expression profiles even with very small amounts of RNA (0.1–0.01 μg of total RNA) without amplification. Differential expression ratios obtained with the new DNA chip were validated against those obtained with quantitative real-time PCR assays. This novel microarray technology would be a powerful tool for monitoring gene expression profiles, especially for clinical diagnosis.

Key words: agitation, columnar array structure, DNA chip, microarray, sensitivity.

The Completion of the Human Genome project has significantly accelerated functional genomic studies. Nowadays, DNA microarrays, such as a DNA chip, are being used for high-throughput analysis. Microarray technology has recently been shown to be the most useful among many functional genomic approaches (*1–5*). Moreover, the DNA chip is promising to be a powerful tool not only for genetic diagnosis (*6–10*) but also for personalized therapy (*3*).

Generally speaking, there are two dominant methods for immobilizing oligonucleotides on a substrate for DNA chips; one is the direct synthesis of nucleic acids step by step on the solid substrate, and the other is the immobilization of synthesized oligonucleotides on the solid substrate using a high speed robot. The advantage of the photolithography method is the mass production of high-density DNA chips on which high-density oligonucleotides are immobilized. On the other hand, it requires special facilities and many photomasks to prepare the DNA chips, resulting in high cost. Taking genetic diagnosis with DNA chips into account, made-to-order DNA chips containing selected DNA probes for individual patients will be required for personalized therapy. From the viewpoint of personalized therapy, the DNA chips prepared by the second method are preferable.

However, most current DNA chips are not applicable for clinical use because of their low sensitivity. Assersohn *et al.* (*11*) reported that the mean recovery of breast fine needle aspirate was 202,500 cells, which corresponds to approximately 0.1 μg of total RNA. In general, a large amount of total RNA (1–100 μg) is required for a DNA chip (*12*). For example, the Affymetrix GeneChip prepared by the photolithography method operates with 1–15 μg of total RNA with a single round cDNA synthesis. Analysis of the gene expression profile in a small amount of sample using current DNA chip technology requires RNA amplification (*15–17*), which may lead to biased results (*18, 19*). If only a small amount of sample, such as a biopsy one, is available for the assay, gene expression analysis of the sample is difficult using the existing DNA chips.

Here, we succeeded in highly sensitive detection of hybridization signals using our newly developed DNA chip having a unique structure made of plastic. The level of nonspecific adsorption of target DNA was reduced and the signal intensity of hybridization was increased on the DNA chip, hence the signal/noise (S/N) ratio was remarkably improved. Moreover, our DNA chip makes it possible to perform gene expression profiling with 0.1 μg of total RNA without any amplification. This performance strongly suggested that our new DNA chip would be useful for genetic diagnosis.

MATERIALS AND METHODS

Oligonucleotides—In most experiments, 64 different 70 bases oligonucleotides selected from a commercially available oligonucleotide set (QIAGEN, Human sample set, ver. 3.0) were used as DNA probes. In some experiments (Table 1 and Fig. 8), a 60 bases oligonucleotide (sequence, 5′-ACATTTTGAGGCATTTCAGTCAGTTGC-TCAATGTACCTATAACCAGATCGTTCATCTGGA) complementary to plasmid pkF3 (Takara Bio) was used. A 20 bases oligonucleotide (sequence, 5′-TGGAGAACTT-GATCGACAAG) was also used (Fig. 7). All oligonucleotides were chemically modified with an amino group at the 5′-terminal.

New DNA Chip—Substrate: A poly (methyl methacrylate) (PMMA) substrate, 76 × 26 × 1 mm, was used as the substrate for our newly developed DNA chip. This substrate has a unique structure, as shown in Fig. 1. A depressed structure with 256 arrayed pillars is located at the center of the substrate. The diameter of the top of the pillars is 0.15 mm, and their height is 0.2 mm. The DNA

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