

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

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 mM each), 106 µl of H₂O, 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'-CCTGAGAGCCTGTATGA-3', 5'-AATCTC-TGCTTGTTGTT-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'-GCTGGAACCTCACTGGGTTGCC-3', 5'-CGGCCGAAGGAAGGAACCTGACCCAG-3', 72°C,

GAPDH: 5'-TGGTATCGTGAAGGACTCATGAC-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 µM) 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 trichostatin 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.

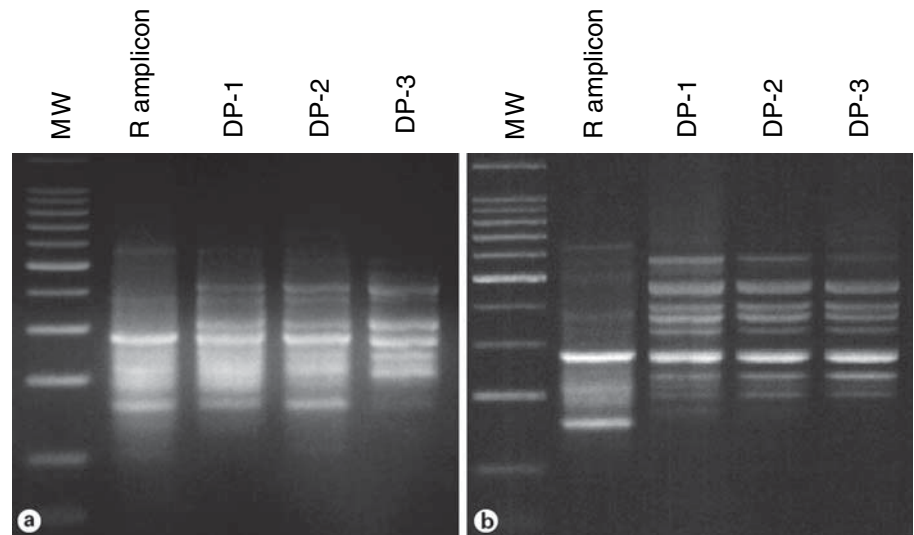


Table 1. Three K-H-DPs and six H-K-DPs are shown

	Gene name	Length	Homology	Accession No.
<i>K-H-DPs</i>				
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
<i>H-K-DPs</i>				
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-

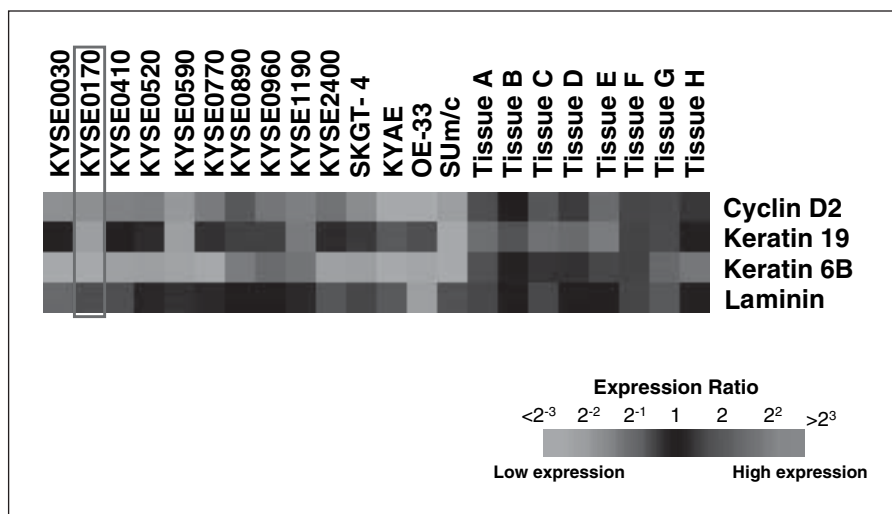


Fig. 2. cDNA microarray analysis for various esophageal cancer cell lines (squamous cell carcinomas; KYSE series and SUm/c, adenocarcinomas; SKGT-4, KYAE and OE-33) and esophageal cancer tissues (tissue A–H). The KYSE series includes KYSE 170, which was used as a driver in the current cDNA RDA (box).

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	Tumor samples (cancer cell lines)	LAGE-1	MAGE-A1	GAPDH										
Ovary	+	-	+	Prostatic adenocarcinoma	+	+	+										
Testis	+	+	+	Breast carcinoma	+	+	+										
Kidney	+	-	+	Colon adenocarcinoma 1	-	+	+										
Small intestine	-	-	+	Colon adenocarcinoma 2	+	-	+										
Heart	-	-	+	Ovarian carcinoma	+	-	+										
Prostate	-	-	+	Pancreatic adenocarcinoma	-	+	+										
Brain	-	-	+	Lung carcinoma (LX-1)	-	-	+										
Thymus	-	-	+	Lung carcinoma (GI-117)	-	-	+										
Placenta	-	-	+	Esophageal cancers (positive/total)													
Lung	-	-	+	Esophageal cancer cell lines	3/11	6/11											
Liver	-	-	+	Esophageal cancer tissues													
Colon	-	-	+	<table border="1"> <thead> <tr> <th rowspan="2">LAGE-1</th> <th colspan="2">MAGE-A1</th> </tr> <tr> <th>positive</th> <th>negative</th> </tr> </thead> <tbody> <tr> <td>Positive</td> <td>8</td> <td>2</td> </tr> <tr> <td>Negative</td> <td>6</td> <td>14</td> </tr> </tbody> </table>			LAGE-1	MAGE-A1		positive	negative	Positive	8	2	Negative	6	14
LAGE-1	MAGE-A1																
	positive	negative															
Positive	8	2															
Negative	6	14															
Muscle	-	-	+	p = 0.013 (Fisher's exact probability test)													
Pancreas	-	-	+														
Spleen	-	-	+														
Leukocyte	-	-	+														

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 μM 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	0 μ M	0.75 μ M	0.75 μ M	2 μ M	2 μ M	5 μ M	5 μ M	10 μ M	10 μ M	
Incubation time	-	2 days	5 days	2 days	5 days	2 days	5 days	2 days	5 days	
<i>Normal esophageal cell lines</i>										
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									
<i>Esophageal cancer cell lines</i>										
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	100 μ g/l	100 μ g/l	500 μ g/l	500 μ g/l	1 mg/l	1 mg/l	
Incubation time	2 days	5 days	2 days	5 days	2 days	5 days	
<i>Normal esophageal cell lines</i>							
HEEC-1	LAGE-1	-	-	-	-	-	
	GAPDH						
HEEC-2	LAGE-1	-	-	-	-	-	
	GAPDH						
HEEC-3	LAGE-1	-	-	-	-	-	
	GAPDH						
HEEC-4	LAGE-1	-	-	-	-	-	
	GAPDH						
<i>Esophageal cancer cell lines</i>							
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 transcrip-

tional 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 can-

cers. 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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