Gene expression profiling of lymph node metastasis by oligomicroarray analysis using laser microdissection in esophageal squamous cell carcinoma

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Abstract. We applied oligomicroarray analysis of 17086 genes to identify the genes related to lymph node metastasis in esophageal squamous cell carcinoma (ESCC). The samples of cancer and non-cancerous paired tissue were taken from 16 patients with ESCC who underwent esophagectomy with lymph node dissection. Total ribonucleic acid was extracted from the cancer cells obtained by using laser microdissection and was amplified by T7 based-amplification for the application to the oligomicroarray. The oligomicroarray demonstrated 43 overexpressed genes, such as cell-cycle regulators, cell adhesion related genes, anti-apoptosis related genes, and 138 suppressed genes such as cell differentiation related and apoptosis related genes in ESCC cells with lymph node metastasis. Among them, 5 overexpressed genes (*SPP-1*, *CKS2*, *CCT5*, *STMN1*, *NDUFB9*) and one suppressed expression gene (*GJB2*) were selected in the gene profiles, and then the expressions of those genes were confirmed by real-time semi-quantitative reverse transcriptional polymerase chain reaction (RT-PCR) method for confirmation of the result not only in study cases but also in additional 21 cases. The gene expression by real-time semi-quantitative RT-PCR was in accordance with the microarray data. Although we were able to extract some genes related to nodal metastasis in ESCC, further examination is necessary in other genes as well as the interaction of stromal tissues.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive carcinomas of the gastrointestinal tract. Despite advances in multimodality therapy such as new surgical techniques and chemoradiotherapy, the overall 5-year survival rate in patients with ESCC still remains poor (1,2). One of the more critical factors affecting ESCC patient prognosis is lymph node metastasis. Recently, there have been studies of the biological factors associated with lymph node metastasis in ESCC such as overexpression of *cyclin D1* (3), *MMP-12* (4), *VEGF-C* (5) and *EF-1* (6), or reduced expression of *E-cadherin* (7) and *cystatin B* (8). However, there have been only a few analyses of multiple genes expressions for the exploration mechanisms of lymph node metastasis. The development of a large scale analysis of gene expression with a microarray allows the evaluation of the gene profiles at once (9). In practice, it is important for the molecular analysis of the gene expression profile to obtain high-quality ribonucleic acid (RNA) extracted from target cells. Therefore, we introduced the laser microdissection (LMD) technique that can separate and isolate populations of cancer cells from the tumor tissue of the same patient to clearly understand the molecular changes in a tumor (10), since the tumors consist of mixed populations of carcinoma cells and stromal cells such as fibroblasts, macro-phages and lymphocytes.

In this study, combining the methods of LMD, T7-based RNA amplification and oligomicroarray, we analyzed the differentially expressed genes between the cancer cells with and without lymph node metastasis in the patients with ESCC. Furthermore, we examined and confirmed our data by real-time semi-quantitative reverse transcriptional polymerase chain reaction (RT-PCR).

Patients and methods

Patients. The samples of cancer tissues and non-cancerous tissues were obtained from 32 patients with ESCC who underwent esophagectomy with lymph node dissection between 1999 and 2003 at Kagoshima University Hospital, Kogoshima, Japan. The Ethics Committee of Kagoshima University

Table I. The clinicopathological characteristics of the patients.

No.	Age	Gender	pT	pN	No.[a]	pM	pStage
1	55	Male	1	0	0	0	I
2	86	Male	2	0	0	0	IIA
3	73	Male	3	0	0	0	IIA
4	70	Male	3	0	0	0	IIA
5	73	Male	3	0	0	0	IIA
6	65	Male	1	1	5	1a	IVA
7	70	Male	2	1	2	0	IIB
8	76	Male	3	1	1	0	III
9	61	Male	3	1	2	0	III
10	74	Male	3	1	2	0	III
11	69	Male	3	1	4	0	III
12	69	Male	3	1	5	0	III
13	61	Female	3	1	9	1a	IVA
14	58	Male	3	1	11	0	III
15	69	Male	3	1	24	1a	IVA
16	68	Male	3	1	76	1b	IVB

[a]No., number of lymph node metastasis.

Hospital approved the study protocol and all patients in the study provided their written informed consent. We finally evaluated 16 of the 32 cases that passed a strict RNA quality check examination, as they were considered eligible for participation in the study. The patients ranged in age from 55 to 86 years (mean, 68.6 years). None of these patients underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy and/or radiotherapy, and none had synchronous or metachronous multiple cancer in other organs. Using the tumor node metastasis (TNM) classification of the International Union against Cancer (11), the 16 patients were classified as follows: two pT1, two pT2, twelve pT3 and none with pT4 tumors. Pathologically, all of the tumors were squamous cell carcinoma. Lymph node metastases were present in 11 of the 16 patients (68.8%). The number of lymph node metastasis ranged from 1 to 76 in the patients who had lymph node metastasis. The M1 tumors were all due to distant lymph node metastasis (Table I).

Tissue samples and preparation of the cancer cell population by laser microdissection. All samples were immediately cut from the resected esophagus, embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), and frozen in liquid nitrogen. The frozen tissues were sectioned by a cryostat (Leica Microsystems, Wetzlar, Germany) at 8 μm, mounted on glass slides and covered with PEN foil (2.5 μm thick; Leica Microsystems). The slice samples were quickly fixed using a mixture of 100% ethanol and acetic anhydride (19:1), and stored at -80˚C until use. Slides were stained with hematoxylin and eosin (H&E) at room temperature and dehydrated for 5 sec each with 70, 80, 95 and then 100% ethanol. After being air-dried, the sections were microdissected using the LMD system with a 337-nm nitrogen ultraviolet (UV) laser (Leica Laser Microdissection System, Leica Microsystems). The target cells dissected from a section were dropped immediately into a microcentrifuge tube cap filled with 30 μl of RLT lysis buffer (Qiagen, Hiden, Germany). At least 600 cancer cells were collected into a 0.5-ml tube, and then the total-RNA was extracted with an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The samples of paired non-cancerous tissues were from at least 5 cm away from cancer tissue, and pathologically composed of >75% normal esophageal squamous cells without cancerous and dysplastic cells. Total-RNA from the whole specimens of non-cancerous tissue was extracted with an RNeasy Mini Kit without laser microdissection. All total-RNAs extracted from cancer cells and non-cancerous tissue were assessed for quality by electrophoretic separation on an RNA Nano LabChip or RNA 6000 Pico Labchip (Agilent Technologies, Inc., USA) in Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) according to the manufacturer's instructions.

T7-based RNA amplification and labeling. The quantities of total-RNA consisted of integrated laser microdissected cancer cells (50 ng) and non-cancerous tissues (450 ng). We carried out T7-based RNA amplification using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Inc.). Cancer cell total-RNA was amplified for each of the 16 cases, while the non-cancerous tissues' total-RNA of the 16 cases was mixed and amplified for controllable variation in each sample. Total-RNA of all samples was reverse transcribed to first strand and second strand complementary DNA (cDNA) by MMLV-RT using an oligo(dT) primer that incorporated a T7 RNA polymerase promoter. These cDNAs were used as a template for *in vitro* transcription reaction in the presence of Cyanine labeled CTPs (NEW Life Science, Boston, MA) by T7 RNA polymerase. The cDNA of the non-cancerous tissue mixture was labeled with Cyanine 3-CTP (Cy3) and that of laser microdissected cancer cells was labeled with Cyanine 5-CTP (Cy5). Labeled complementary RNA (cRNA) samples were purified by using RNeasy mini kit (Qiagen). After purification, qualities of cRNA were re-evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Microarray hybridization and scanning. As shown in Fig. 1, the Cy3, Cy5 labeled cRNA targets and control targets were mixed and hybridized with the Human 1A Oligo Microarray (Agilent Technologies, Inc.) (12). The slides were placed into an Agilent hybridization chamber (Agilent Technologies, Inc.). Hybridization proceeded at 60˚C for 17 h. These were then removed from the chamber and washed and dried by using the *In situ* Hybridization kit-plus (Agilent Technologies, Inc.) according to the Agilent array protocol. Immediate scanning of slides was performed with the Agilent dual laser DNA microarray scanner (Agilent Technologies, Inc.).

Data analysis. The intensity of each hybridization signal was evaluated using Feature Extraction software (Agilent Technologies, Inc.). The common logarithm of Cy5/Cy3 ratio for each sample was calculated by averaging the spots. A cut-off value for expression level was automatically calculated according to the background fluctuation. Normalization of expression

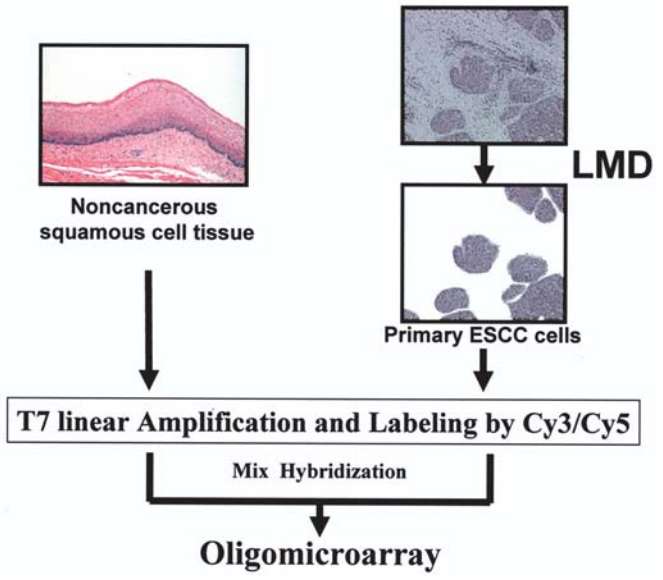

Figure 1. A schema of the LMD, T7 linear amplification and labeling by Cy3/Cy5, and oligomicroarray. The primary ESCC cells were obtained by using LMD. Whole tissue of a pair of non-cancerous squamous cells was used for the extraction of total-RNA without LMD. After the extraction of total-RNA, T7-based amplification was performed to obtain sufficient qualities of cRNA, and then the oligomicroarray analysis was performed on the non-cancerous tissue and the primary esophageal carcinoma cells.

Table II. The primer sequences used in the real-time semi-quantitative RT-PCR.

Gene name	Primer sequences (5'-3')	Size (bp)
SPP1	TGATGGCCGAGGTGATAGTG	125
	CTCGCTTTCCATGTGTGAGG	
CKS2	CTTCGACGAACACTACGAGTACC	112
	ACACCAAGTCTCCTCCACTCC	
CCT5	AGTTAGCCAAGAGGCGGATAAG	136
	GACTTCGGTCATAGTCTGGATGG	
STMN1	AGAATACACTGCCTGTCGCTTG	128
	TCTTTTGACCGAGGGCTGA	
NDUFB9	CACCTCCTATGAGAGATACGATTGC	133
	TCCCTCCGCAGTTTCTTCC	
GJB2	AGCGCAGAGACCCAAC	163
	GAGCCAGATCTTTCCAATGA	
GAPDH	TTGGTATCGTGGAAGGACTCA	249
	TGTCATCATATTTGGCAGGTTT	

was performed using LOWESS normalization (13). Differential expression between the groups of lymph node metastasis and the group of non-lymph node metastasis was considered significant, where $P<0.05$.

Real-time semi-quantitative RT-PCR. We selected 6 genes for the estimation of altered expression between lymph node positive cases and lymph node negative cases and examined their levels by applying the real-time semi-quantitative RT-PCR technique. Real-time semi-quantitative RT-PCR was carried out not only with the same samples used for array analysis but also with 21 additional samples that were not used. First-strand cDNA synthesis was performed in a total volume of 20 μl containing 300 units of M-MLV Reverse Transcriptase (Invitrogen Life Technologies, USA), 6.0 μl of 5X first-strand buffer, 3.0 μl of 0.1 M DTT, 6.0 μl of 10 mM dNTP mix, 100 ng of Random Primer (Takara Biotechnology, Japan), 20 units of RNasin Ribonucrease Inhibitor (Promega Corp., USA), 50 ng of total-RNA extracted from laser micro-dissected cancer cells or 450 ng of non-cancerous tissues. Next we incubated tubes at 25˚C for 10 min and 37˚C for 60 min, and then inactivated the enzyme by heating at 80˚C for 5 min (14). The *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene served as an internal control. The oligonucleotide primers designed for selected genes are shown in Table II. The reaction was performed in a LightCycler™ system (Roche Applied Science, Indianapolis, IN, USA) using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). Each reaction was carried out in a final volume of 20 μl containing 1 μl of the cDNA product sample, 3 mM $MgCl_2$, 0.5 μM of each primer and 1X reaction mix including FastStar DNA polymerase, reaction buffer, dNTPs and SYBR green. Thermal cycling for all genes was initiated with a denaturation step of 95˚C for 10 min and consisted of 45 cycles with denaturation at 95˚C for 10 sec, annealing at 60˚C (*SPP1*, *CKS2*, *GJB2*, *GAPDH*), 62˚C (*STMN1*) or 64˚C (*CCT5*, *NDUFB9*) for 10 sec (*SPP1*, *CKS2*, *GJB2*, *GAPDH*) or 5 sec (*STMN1*, *CCT5*, *NDUFB9*), and elongation at 72˚C for 5 sec. At the end of the PCR cycles, melting curve analyses and electrophoresis of the products on 2% agarose gels were done to ensure the generation of the expected PCR product. The data of selected gene expression analysis were calculated as a log T/N ratio: the value of target gene = $\log_{10}$ [(target gene expression of cancer cells/GAPDH expression of cancer cells)/(target gene expression of pair non-cancerous tissue/GAPDH expression of pair non-cancerous tissue)].

Statistical analysis. The associations of lymph node metastasis with expression levels were calculated with non-parametric Kruskal-Wallis and Mann-Whitney U tests. A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using the JMP 5 for Windows software (SAS Institute Inc., Cary, NC, USA).

Results

Quality check of total-RNA after LMD and labeled cRNA after T7-based amplification. In all samples, the target cells were cut out with at least several hundred cells per slide. Consequently, about 600-1000 cells were collected for total-RNA extraction. Total-RNA extracted after LMD had kept high quality without degradation in strict check by Agilent 2100 BioAnalyser (Fig. 2A and B). We selected 16 of 32 cases (50%) after RNA quality was thoroughly checked. Total-RNA extracted from primary cancer cells and non-cancerous squamous cell tissue was amplified and labeled using Cy3- or Cy5-conjugated dyes. As shown in Fig. 2C and D, total-RNA from all 16 patients was successfully amplified by an estimated 200-fold by using T7 RNA polymerase.

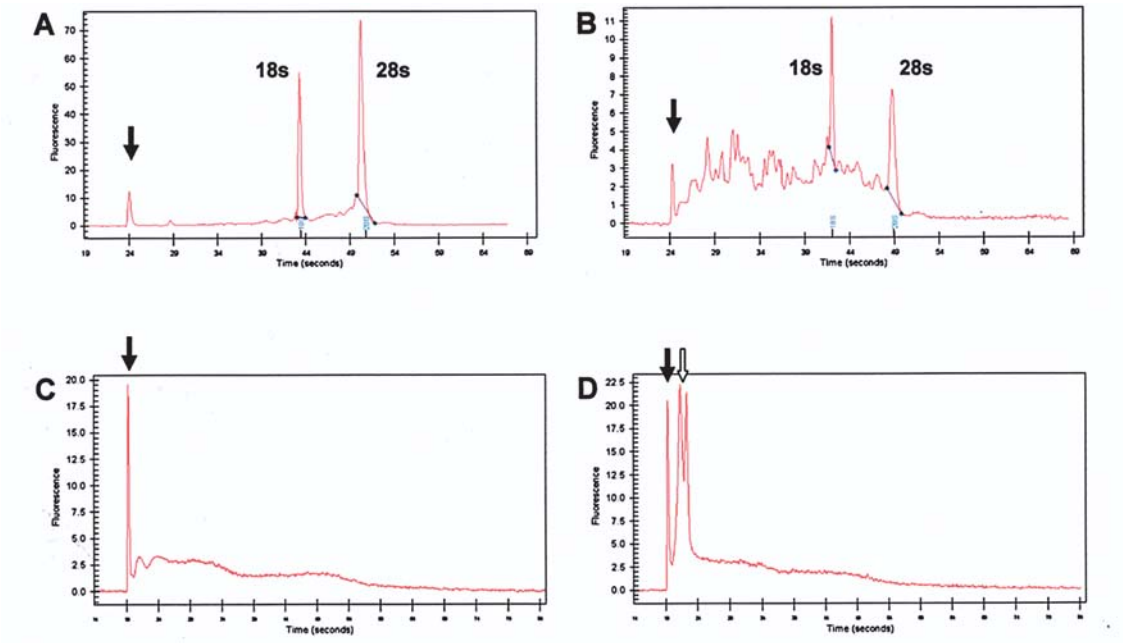

Figure 2. Quality check of total-RNA after LMD and labeled cRNA after T7-based amplification by Agilent 2100 BioAnalyser. (A), High quality total-RNA sample extracted from cancer cells with LMD. The distinct and intense features were the 18s and 28s ribosomal peaks, no peak between 18s and 28s, and no peak before 18s. (B), Degraded total-RNA sample extracted from cancer cells with LMD. There was a lower 28s ribosomal peak and smooth peak before 18s in comparison to the high quality RNA sample. (C), The amplification and Cy3-labeling of total-RNA (450 ng/μl) from non-cancerous tissue. The Cy3 labeled cRNA had the shape of a broad peak and high quality. (D), The amplification and Cy5-labeling of total-RNA (50 ng/μl) from primary carcinoma cells. Although the electopherograms of the Cy5 labeled cRNA had a peak of type M influenced by Cy5 (white arrow), it had the shape of a broad peak and high quality. The black arrow indicates a standard marker.

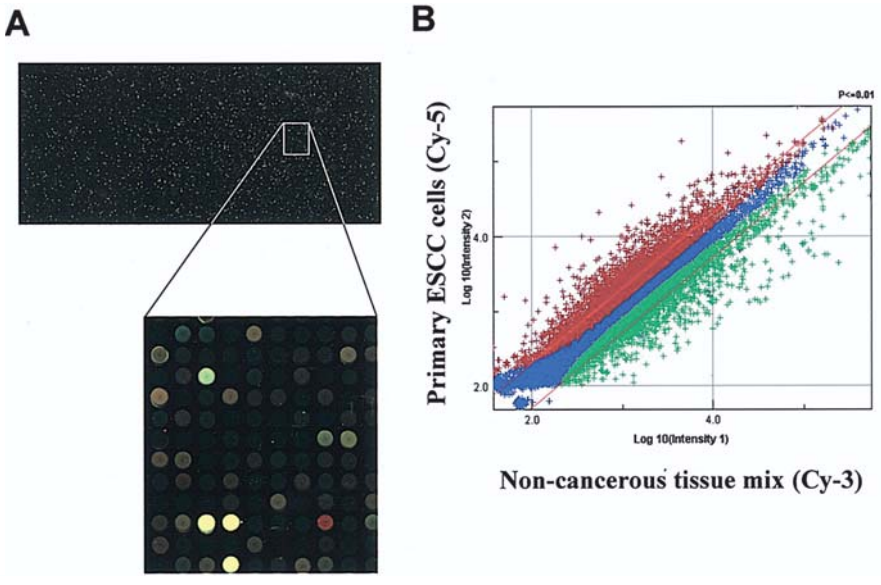

Figure 3. A representative finding of oligomicroarray expression patterns. (A), Example of the oligomicroarray data obtained from case 16. Total 17086 genes were studied. (B), Scatter plots of oligomicroarray analysis. Primary ESCC cells (Cy5) and non-cancerous tissue mix (Cy-3) from case no. 16 were labeled and hybridized to the oligomicroarray. The red spots illustrate overexpressed genes, while the green spots were suppressed genes, and blue spots were unchanged genes.

Identification of differentially expressed genes associated with lymph node metastasis by oligomicroarray. Each cRNA probe was hybridized to oligomicroarray with 17086 genes. We evaluated the expression profiles between the cancer cells and non-cancerous squamous cell tissue (Fig. 3). A representative scatter plot of microarray analysis between the cancer tissue and non-cancerous tissue in case 16 is shown in Fig. 3B. Overexpressed, suppressed and unchanged genes were indicated by red, green and blue spots, respectively. Each case was successfully analyzed as overexpressed, suppressed, and unchanged genes using microarray.

To identify the genes related to lymph node metastasis, 16 patients were divided into two groups: a metastatic group in which lymph node metastasis was positive in 11 patients

Table III. Overexpressed genes correlated with lymph node metastasis.

Gene name	Title	GenBank primate	Fold change
SPP1	Secreted phosphoprotein-1 (osteopontin)	M83248.1	10.04
KRT14	Keratin 14	BC042437.1	7.74
TACSTD1	Tumor-associated calcium signal transducer 1	AK026585.1	6.90
I_1152283	Protein containing a GP36 envelope protein domain	AK096414.1	6.00
STMN1	Stathmin (oncoprotein 18)	X53305.1	4.03
COL7A1	Collagen type VII alpha 1	NM_000094.2	3.98
COL1A1	Alpha 1 subunit of type I collagen	BC036531.1	3.78
BIRC5	Survivin	U75285.1	3.65
H2AFZ	H2A histone family Z	BC018002.1	3.63
CKS2	Protein that binds to CDC2/CDC28 protein kinase	BC006458.1	3.51
CCT5	Chaperonin containing T-complex 1 subunit 5 (epsilon)	BC035499.1	3.40
I_957781	Retired, primase polypeptide 2a	NM_000947.1	3.39
MLF1	Myelodysplasia/myeloid leukemia factor 1	L49054.1	3.36
CKS1B	CDC28 protein kinase 1	BC001425.1	3.34
STK31	*Homo sapiens* serine/threonine kinase 31	NM_031414.2	3.23
TK1	Thymidine kinase 1	NM_003258.1	3.22
KIFC1	Kinesin-like 2	BC000712.1	3.07
CTSZ	Cathepsin Z (cathepsin X)	BC042168.1	3.01
CENPF	Centromere protein F (350/400 kDa, mitosin)	U30872.1	3.00
BG1	Lipidosin	NM_015162.3	2.97
DEFB105	*Homo sapiens* defensin, beta 105 (DEFB105), mRNA	NM_152250.1	2.94
MCM7	Minichromosome maintenance deficient 7	NM_005916.2	2.94
ATP1B3	Na^+/K^+ transporting ATPase beta 3 subunit	BC011835.1	2.84
NM_144668.1	*Homo sapiens* hypothetical protein MGC33630	NM_144668.1	2.77
MYNN	Myoneurin	BC033620.1	2.77
ANLN	Aniline	AF273437.1	2.74
I_960942	Retired, member of the strictosidine synthase family	AB033767.1	2.74
KPNA2	Karyopherin alpha 2 (importin alpha 1)	U09559.1	2.68
H2AV	Protein with high similarity to S. cerevisiae Htz1p	BC000098.1	2.65
HMG4L	*Homo sapiens* high-mobility group protein 4-like	NM_178467.1	2.64
SPAG5	Sperm associated antigen 5	NM_006461.1	2.64
LOC152217	Protein of unknown function	AL832915.1	2.63
LIMS1	LIM and senescent cell antigen-like domains 1	BC005341.1	2.51
ENAH	Mammalian Ena (NPC derived proline rich protein 1)	NM_018212.1	2.47
CDC20	Cell division cycle 20	BC000624.1	2.46
MSH6	MutS homolog 6 (*E. coli*)	U28946.1	2.45
I_941570	Protein of unknown function	BC035925.1	2.45
HSPE1	Chaperonin 10	BC023518.1	2.44
NDUFB9	NADH dehydrogenase ubiquinone 1 beta subcomplex 9	AF044956.1	2.41
SNRPG	Sm core protein G	BC022432.1	2.40
RPA3	Replication protein A 3	L07493.1	2.34
ATR	Ataxia telangiectasia and Rad3 related	U49844.1	2.29
MGC10911	Protein of unknown function	BC004308.1	2.24

(nos. 6-11) and a non-metastatic group in which lymph node metastasis was negative in 5 patients (nos. 1-5) (Table I). When comparing gene expression profiles between two groups, 43 overexpressed genes and 138 suppressed genes were significantly correlated with lymph node metastasis (Tables III and IV). The overexpressed genes such as cell cycle regulation,

Table IV. Suppressed genes correlated with lymph node metastasis.

Gene name	Title	GenBank primate	Fold change
MAL	T-lymphocyte maturation associated protein	BC003006.1	-31.50
C1orf10	Chromosome 1 open reading frame 10	BC030807.1	-23.24
NICE-1	NICE-1 protein	AJ243662.1	-16.17
SPINK5	Serine protease inhibitor (Kazal type) 5	NM_006846.1	-15.50
HBB	Hemoglobin beta subunit	BC007075.1	-14.44
EMP1	Echinoderm microtubule-associated protein-like	U77085.1	-13.71
TGM1	Transglutaminase 1	BC034699.1	-13.47
SCEL	*Homo sapiens* sciellin (SCEL)	NM_003843.2	-13.16
KRT13	Keratin 13	BC002661.1	-12.22
CSTB	Cystatin B (stefin B)	L03558.1	-11.92
IL1RN	Interleukin 1 receptor antagonist	NM_173841.1	-11.83
I_1110078	Protein of unknown function	AF093250.1	-10.98
RUNX2	*Homo sapiens* runt-related transcription factor 2 (RUNX2)	NM_004348.1	-10.25
CSTA	Cystatin A (stefin A)	X05978.1	-9.55
PPL	Periplakin	AF013717.1	-9.31
SNTA1	Alpha 1 syntrophin	U40571.1	-8.95
CLIC3	Chloride intracellular channel 3	NM_004669.1	-8.94
LAGY	Protein containing a homeobox domain (homeodomain)	AF454763.1	-8.59
S100A9	S100 calcium-binding protein A9 (calgranulin B)	AF086362.1	-8.50
ANXA1	Annexin I	X05908.1	-8.45
SPRR3	Small proline-rich protein 3	NM_005416.1	-8.37
I_955703	Retired, cystatin A (stefin A)	NM_005213.2	-8.23
HAT	Airway trypsin-like protease	AB002134.1	-8.19
I_958335	Member of the trypsin family of serine proteases that contains a SEA domain	AL833167.1	-7.84
RHCG	Rhesus glycoprotein type C	AF081497.1	-7.57
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5	M30938.1	-6.90
K5B	*Homo sapiens* keratin 5b (K5B)	NM_173352.1	-6.74
SERPINB1	Monocyte neutrophil elastase inhibitor	BC009015.1	-6.70
SPRR1A	Small proline rich protein (cornifin) 1A	L05187.1	-6.65
I_959634	Protein containing eight leucine rich repeats	BC013767.1	-6.57
MYO1A	Myosin-IA (brush border myosin I)	AF105424.1	-6.33
I_966519	Protein of unknown function	I_966519	-6.26
BCE-1	Protein of unknown function	NM_007005.1	-6.24
I_1002369	Retired, Secreted lacrimal proline rich protein	NM_007244.1	-6.19
FLJ21511	Protein with low similarity to S	AK025164.1	-5.93
CLCA4	Chloride channel calcium-activated	AF127035.1	-5.90
PPP1R3C	Protein phosphatase 1 regulatory subunit 5	BC012625.1	-5.89
LOC84518	Member of the DUF614 protein of unknown function family	NM_032488.2	-5.85
TRAPPC1	*Homo sapiens* trafficking protein particle complex 1	NM_021210.	-5.63
I_1152130	Protein containing a calponin homology (CH) domain	AJ010306.2	-5.60
I_958949	Protein of unknown function	I_958949	-5.54
KRT4	Keratin 4	AK056254.1	-5.53
PSCA	Prostate stem cell antigen	AJ297436.1	-5.52
KIAA0790	Protein containing two SAM (sterile alpha motif) domains	NM_015278.1	-5.51
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	AK026603.1	-5.31
I_928865	Retired, involucrin	BC046391.1	-5.30
IBA2	Protein with high similarity to allograft inflammatory factor 1	AL136566.1	-5.29
FLJ20626	Protein containing three C2H2 type zinc finger domains	NM_017908.1	-5.26
MECP2	Methyl CpG binding protein 2	AF158180.1	-5.23

Table IV. Continued.

Gene name	Title	GenBank primate	Fold change
PCDH16	Protocadherin 16	NM_003737.1	-5.19
BRP17	Member of the metallo-beta-lactamase family	BC002937.1	-5.13
LOC146894	Protein containing an immunoglobulin (Ig) domain	NM_145273.1	-5.01
I_1109096	Protein containing three immunoglobulin (Ig) domains	BC016993.1	-4.92
S100A14	S100 calcium binding protein A14	AY007220.1	-4.92
EPS8R1	Protein containing an Src homology 3 (SH3) domain	NM_133180.1	-4.88
MGC4171	Member of the glycerophosphoryl diester phosphodiesterase family	NM_024307.1	-4.83
I_959851	Retired, lymphoid blast crisis oncogene	NM_006738.2	-4.68
FUT3	*Homo sapiens* fucosyltransferase 3	NM_000149.1	-4.61
DPM2	Dolichol phosphate mannose synthase regulatory subunit	AF061729.1	-4.56
HMOX1	Heme oxygenase (decycling) 1	X06985.1	-4.53
TGM3	Transglutaminase 3	L10386.1	-4.33
I_1109231	Retired, aplysia ras homolog I	AK096600.1	-4.28
FLJ25124	*Homo sapiens* hypothetical protein FLJ25124, mRNA	NM_144698.2	-4.26
SH2D2A	SH2 domain protein 2A	AJ000553.1	-4.22
MGC10540	Protein with low similarity to S. cerevisiae Vps25p	BC006282.1	-4.12
SCEL	Sciellin	NM_144777.1	-4.09
TCAP	Titin-cap (telethonin)	BC012628.1	-4.05
PIM1	*Homo sapiens* pim-1 oncogene	NM_002648.	-3.99
OR2A4	Member of the rhodopsin family of G protein-coupled receptors	AF399598.1	-3.97
ALOX12	Arachidonate 12-lipoxygenase	M62982.1	-3.94
C16orf7	Putative b subunit of ATP synthase	AB018551.1	-3.92
SPRR1A	*Homo sapiens* small proline-rich protein 1A, mRNA	NM_005987.	-3.91
KLK11	Kallikrein 11	AB041036.1	-3.80
EPS8R2	Protein containing an Src homology 3 (SH3) domain	AK025824.1	-3.78
I_963782	Retired, protein containing an Src homology 3 (SH3) domain	NM_022772.1	-3.71
ARS	*Homo sapiens* ARS component B (ARS), mRNA	NM_020427.2	-3.71
MGLL	Monoglyceride lipase	BC006230.1	-3.68
FLJ20261	Protein with high similarity to keratin 10 (human KRT10)	NM_019016.1	-3.65
FLJ35961	Protein containing an immunoglobulin (Ig) domain	NM_152372.1	-3.65
IVL	*Homo sapiens* involucrin (IVL), mRNA	NM_005547.1	-3.64
CCNG2	Cyclin G2	NM_004354.1	-3.61
CRABP2	*Homo sapiens* cellular retinoic acid binding protein 2	NM_001878.2	-3.58
KIAA1951	Protein containing 13 C2H2 type zinc finger domains	AB075831.1	-3.58
I_964796	Retired, cannabinoid receptor 2	X74328.1	-3.58
NUCB2	Nucleobindin 2	X76732.1	-3.57
NAGK	N-acetylglucosamine kinase	BC001029.1	-3.51
ITGB4BP	Integrin beta 4 binding protein	BC019305.1	-3.49
DNASE1L3	Deoxyribonuclease I-like 3 (DNase γ)	BC015831.1	-3.43
SULT2B1	Hydroxysteroid sulfotransferase cytosolic 2B1	U92315.1	-3.41
ZNF185	Zinc finger 185	Y09538.1	-3.41
GJB2	Gap junction protein beta 2 (connexin 26)	BC017048.1	-3.32
PDLIM1	PDZ and LIM domain 1	BC000915.2	-3.31
MGC22805	Protein containing two ankyrin (Ank) repeats	BC021671.1	-3.26
TIMP3	Tissue inhibitor of metalloproteinases 3	S78453.1	-3.20
DESC1	Differentially expressed in squamous cell carcinoma 1	AF064819.1	-3.17
DKFZp547I094	Protein of unknown function	NM_032155.1	-3.16
APM2	Protein of unknown function	BC004471.1	-3.15
FLJ14957	Protein of unknown function	NM_032866.1	-3.15

Table IV. Continued.

Gene name	Title	GenBank primate	Fold change
HPGD	Hydroxyprostaglandin dehydrogenase 15-NAD	AK058013.1	-3.07
PIG3	Member of the zinc-containing alcohol dehydrogenase family	BC000474.1	-3.05
SH3BP1	Protein with high similarity to mouse Sh3bp1	NM_018957.2	-3.02
SGP28	Specific granule protein 28 kDa	X94323.1	-2.99
TUBB2	Tubulin beta 2	BC019829.1	-2.97
HEBP2	Chromosome 6 open reading frame 34	AF117616.1	-2.94
SIM2	Single-minded (*Drosophila*) homolog 2	NM_005069.2	-2.93
EPHA1	Ephrin receptor A1	M18391.1	-2.92
PLOD3	Procollagen-lysine 2-oxoglutarate 5-dioxygenase 3	AF046889.1	-2.92
MASP1	Mannan-binding lectin serine protease 1	AF284421.1	-2.91
FLJ12168	Protein containing a TBC domain	NM_024682.1	-2.90
I_1860991.FL1	Protein of unknown function (104-aa form)	X96401.1	-2.86
I_959279	Protein containing two TPR (tetratrico peptide repeat) domains	D86980.1	-2.85
IFNA6	Interferon alpha family, gene 6	X02958.1	-2.85
I_1100026	Protein of unknown function	AF217403.1	-2.74
CTSG	Cathepsin G	M16117.1	-2.74
I_961562	Protein of unknown function	AB002354.2	-2.72
MGC13102	Protein of unknown function	NM_032323.1	-2.71
AIM1L	*Homo sapiens* absent in melanoma 1-like (AIM1L), mRNA	NM_017977.1	-2.69
SLC16A4	Solute carrier family 16 member 4	U59185.1	-2.66
RAGD	Protein with high similarity to S. cerevisiae Gtr2p	BC003088.1	-2.66
DHRS1	Member of the short-chain dehydrogenase-reductase family	AF418205.1	-2.64
SERPINB2	Plasminogen activator inhibitor 2	Y00630.1	-2.58
I_958627	Protein with high similarity to large form lysophospholipase	BC035836.1	-2.55
FUT5	Fucosyltransferase 5 [alpha (1,3) fucosyltransferase]	U27330.1	-2.53
FLJ32334	Protein of unknown function	NM_144565.1	-2.53
FLJ32859	*Homo sapiens* hypothetical protein FLJ32859	NM_152539.1	-2.49
FKSG2	Protein with high similarity to histamine-releasing factor	X16064.1	-2.47
SLC14A2	Solute carrier family 14 (urea transporter) member 2	NM_007163.2	-2.46
CNTFR	Ciliary neurotrophic factor receptor	BC001492.1	-2.45
ITGA9	Integrin alpha 9 subunit	D25303.1	-2.45
ALDH9A1	γ-aminobutyraldehyde dehydrogenase	NM_000696.2	-2.41
GPRC5D	G protein-coupled receptor family C group 5 member D	AF209923.1	-2.36
CARD6	Protein containing a caspase recruitment domain (CARD)	NM_032587.2	-2.35
OR2H3	Olfactory receptor family 2 subfamily H member 3	L35475.1	-2.35
DNAJC5G	*Homo sapiens* hypothetical protein FLJ40417	NM_173650.1	-2.27
RHO6	GTP-binding protein	BC026356.1	-2.24
IGSF8	Immunoglobulin superfamily member 8	AY044845.1	-2.23
CDH12	Cadherin 12 (Br-cadherin)	L34057.1	-2.22
PTX3	Pentaxin-related gene	BC039733.1	-2.21

cell adhesion and growth factor and/or proteinase were candidates related to lymph node metastasis. On the other hand, apoptosis and cell differentiation were included in the suppressed genes.

The relationship between lymph node metastasis and gene expression by real-time semi-quantitative RT-PCR. To confirm the reliability of the array data, real-time semi-quantitative RT-PCR was performed in 16 pair samples without amplification of total-RNA, which was used for array analysis, and 21 additional pairs, which were not used for the array analysis. Five genes in the overexpressed gene group and one gene in suppressed gene group were selected to measure the expression levels by real-time semi-quantitative RT-PCR. These selected

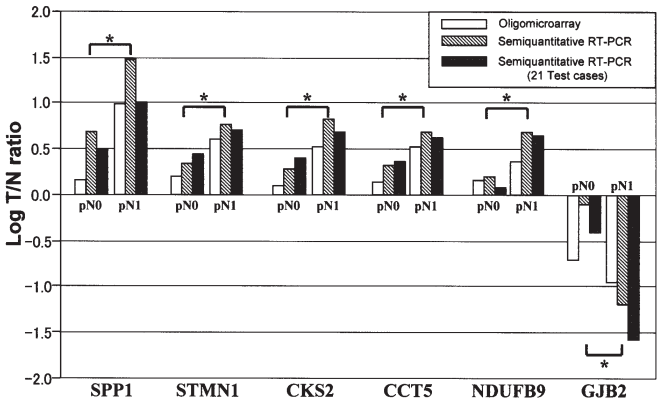

Figure 4. The relationship between lymph node metastasis and the expression of 6 genes in ESCC by real-time semi-quantitative RT-PCR. The expressions of each of the 6 genes (*SPP1*, *STMN1*, *CKS2*, *CCT5*, *NDUFB9*, *GJB2*) was analyzed using real-time semi-quantitative RT-PCR, including 21 test cases without microarray. Significant differences were observed between the gene expressions in the cancer cells of patients with (pN1) and without (pN0) lymph node metastasis. Note the similar results between oligomicroarray analysis and analysis of real-time semi-quantitative RT-PCR, where the asterisks represent $P<0.05$.

genes were as follows: the overexpressed genes, *SPP-1* (*osteopontin*), *CKS2* (*protein that binds to CDC2/CDC28 protein kinase*), *CCT5* (*chaperonin containing T-complex 1 subunit 5*), *STMN1* (*oncoprotein 18*), *NDUFB9* (*NADH dehydrogenase ubiquinone 1 beta subcomplex 9*), and suppressed gene, *GJB2* (*gap junction protein beta 2*). The *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) served as an internal control.

The expression of 6 genes extracted from the 16 array samples or from the 21 additional samples was similar to the microarray data. Thus, these results support the reliability and rationality of our methodology (Fig. 4).

Discussion

Many signaling cascades controlled by multiple genes have been related to the process of carcinogenesis, tumor progression, lymph node metastasis and poor prognosis. Owing to the development of microarray technology, the correlation between clinicopathological findings and gene expression in a single experiment has been elucidated by the analysis of many gene expression profiles (9,15). LMD is a powerful method to selectively extract only the desired cells from tissue specimens (10,16). In this study, the analysis of the gene expression profile was performed with RNA samples obtained from 600 cancer cells by using LMD, T7-amplification and oligomicroarray. It was possible to focus directly on the gene expression profile of the cancer cell population consisting of tumor tissue alone.

Lymph node metastasis is one of the most important prognostic factors in ESCC patients (17,18). However, the complicated mechanisms of metastasis is not sufficient to explain the alteration of a few genes and exercise considerable influence over interstitial interaction. With this point in mind, the LMD technique enables us to analyze differential gene expressions in only cancer cells without interstitial effects. In the present study we identified gene alterations correlated with lymph node metastasis using only cancer cells. Furthermore, we successfully confirmed our data by real-time semi-quantitative RT-PCR using an additional 21 test cases.

In this study, we showed that 43 overexpressed genes, including 3 unknown function genes, and 138 suppressed genes, including 12 unknown function genes, had different expression levels between the metastatic and non-metastatic groups. The overexpressed genes included cell-cycle regulators (*STMN1*, *CKS2*, *MLF1*, *CKS1B*, *CDC20*) (19-23), cell adhesion related genes (*SPP1*, *TACSTD1*, *ENAH*) (24-26), anti-apoptosis related gene (*BIRC5*) (27), DNA replication regulators (*TK1*, *MCM7*) (28,29), and a drug resistance related gene (*CCT5*) (30). The suppressed genes included cell differentiation related genes (*KRT4*, *KRT13*, *C1orf10*, *EMP1*, *CSTA*, *CSTB*, *S100A9*, *S100A14*, *ANXA1*, *SPRR1A*, *SPRR3*, *TGM3*, *TGM1*, *GJB2*) (8,31-39), epithelial transporter (*RHCG*) (40), and apoptosis related genes (*MAL*, *DNASE1L3*, *PIG3*) (41-43).

In these genes we selected 5 overexpressed genes and one suppressed gene, which were well documented in the literature regarding the malignant potential of some types of carcinomas. *SPP-1* (*osteopontin*) has a role of tumor progression in breast cancer and esophageal cancer (24) and was the most over-expressed gene in the lymph node metastasis group. *CKS2* (*protein that binds to CDC2/CDC28 protein kinase*) is known to interact with cyclin-dependent kinases and were expressed at significantly higher levels in colon cancer (21). *CCT5* (*chaperonin containing T-complex 1 subunit 5*) assists in the folding of actins and tubulins and showed drug resistance in gastric cancer (30), *STMN1* (*oncoprotein 18*) acts in the regulation of the cell cycle and increased expression in acute leukemia (20), and *NDUFB9* (*NADH dehydrogenase ubiquinone 1 beta subcomplex 9*) is a nuclear encoded mitochondrial protein with the respiratory electron transport chain (44,45). *GJB2* (*gap junction protein beta 2*) has a role in epidermal differentiation (39) and was suppressed in the lymph node metastasis group compared with the non-metastasis group.

These results might indicate that the important characteristics of cancer cells themselves with regards to metastatic potential to lymph nodes is the release of cell-cell adhesion, anti-apoptotic and proliferation ability. Since these genes are thought to affect each other, it is important to further analyze each gene in detail to elucidate the mechanism of lymph node metastasis in ESCC. Interestingly, we previously reported the precise function of the *MAL* gene, the most suppressive expression gene in our data, using TE3 cells derived from lymph node metastasis of ESCC (41). When we transfected *MAL* cDNA into TE3 cells, those cells lost the proliferation ability and acquired apoptosis function. Since *MAL* seemed to be one of the important candidate genes in relation to lymph node metastasis in ESCC, this gene expression should be examined by real-time semi-quantitative RT-PCR.

In conclusion, we were able to identify some genes related to lymph node metastasis in ESCC using microarray and demonstrated the close relationship between lymph node metastasis and a selection of genes extracted by gene expression profiling. In the present study, although we focused on the property of cancer cells alone, further study should be carried out on the interaction between cancer cells

and stromal tissues. When some of the important genes related to lymph node metastasis in ESCC are clarified the genes will be useful in predicting nodal metastasis.

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