Esophageal squamous cell carcinomas with distinct invasive depth show different gene expression profiles associated with lymph node metastasis

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Abstract. We examined the gene expression profiles of esophageal squamous cell carcinomas (ESCCs) with respect to degree of invasive depth and lymph node (LN) involvement in a large cohort. We used high-density oligonucleotide microarrays to examine the expression of 22,115 genes in 54 ESCCs and 11 non-cancerous esophageal tissues. We found that 4,155 genes were biologically significant in both ESCC and non-cancerous esophageal tissue by analysis of Present Call (hybridization quality by Affymetrix) throughout all samples. From these genes, we used a supervised learning method to select genes responsible for the development of ESCC. We found that 999 genes were expressed differentially in pT1/pN0 tumors vs. non-cancerous esophageal tissue. In the same manner, 48, 66 and 30 genes were expressed differentially in pT1/pN0 tumors vs. pT1/pN1 tumors, pT1/pN0 tumors vs. pT2-4/ pN0 tumors and pT2-4/pN0 tumors vs. pT2-4/pN1 tumors, respectively. Intriguingly, there were no overlaps between the 48 LN metastasis-related genes of pT1 tumors and the 30 LN metastasis-related genes of pT2-4 tumors, suggesting that ESCCs with distinct invasive depths express different genes linked to LN metastasis. Our present results suggest that the degree of invasive depth must be considered when predicting LN metastasis of ESCC from gene expression profiles.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most fatal malignancies in the world because progression occurs without symptoms and many patients are malnourished at the time of diagnosis (1,2). It is well known that lymph node (LN) metastasis correlates with a poor outcome for ESCC patients (3). In treatment of advanced ESCC with LN metastasis, surgery remains the best palliation for patients; however, the 5-year survival rate is ~20% (4). This poor outcome may be explained in part by the fact that it remains unclear how LN metastasis develops and which genes are involved in the process. Tumorigenesis is a complex, multi-stage process that involves many genes. Therefore, analysis of gene expression patterns in ESCC is necessary in order to develop diagnostic, prognostic and therapeutic tools to improve outcome.

DNA microarrays have been used to comprehensively analyze many genes involved in the pathogenesis of various malignancies (5). This technology has made it possible to predict the cancer outcome accurately (6,7).

With respect to ESCC, there have been many DNA microarray studies identifying genes related to pathogenesis or therapy of ESCC (8-16); however, only two studies considered and analyzed LN metastasis (17,18). Kan *et al* (17) reported that molecular profiling of 60 genes accurately predicted LN metastasis in 10 of 13 (77%) ESCCs. Molecular profiling by Tamoto *et al* (18) correctly predicted LN metastasis in 16 of 18 (89%) ESCCs. However, in these studies (17,18), the number of samples tested was too small to validate the predictive performance or to clarify the molecular basis of LN metastasis. To resolve these issues, molecular profiling of a larger number of samples is needed.

In the present study, we used high-density oligonucleotide microarray to identify LN metastasis-related genes in a large number of ESCC (n=54) and non-cancerous esophageal tissues (n=11). We focused in particular on the relation between invasion depth and LN metastasis and then classified the ESCC

Table I. Clinicopathological features.

Factors	Non-cancerous tissue	pT1/pN0	pT1/pN1	pT2-4/pN0	pT2-4/pN1	P-value
Gender (male/female)	11/0	9/0	9/1	6/1	24/4	NS
Age (mean ± SD)	62.0±9.1	64.1±9.0	59.9±7.5	60.1±7.8	63.4±12.1	NS
Histological grading (G1/G2/G3)[a]		1/6/2	3/5/2	4/2/1	8/12/8	NS
No. of involved lymph nodes (mean ± SD)		-	3.2±2.7	-	4.68±2.9	NS

[a]G1, well-differentiated tumor; G2, moderately-differentiated tumor; G3, poorly-differentiated tumor by TNM classification (24). NS, not significant.

samples into four different tumor classes according to degree of invasive depth (pT1 vs. pT2-4) and the presence or absence of LN metastasis (pN0 vs. pN1). We used our original supervised learning method (19-23) to compare genes whose expression differed significantly between the four tumor classes and the non-cancerous tissue. Our gene expression profiles provide new insights into the mechanisms underlying carcinogenesis and progression of ESCC, particularly LN metastasis.

Materials and methods

Patients. Fifty-four patients underwent surgical treatment for ESCC at Yamaguchi University Hospital between September 1997 and March 2003; none had undergone prior chemotherapy or radiotherapy. Preoperative imaging analysis revealed that no patient had distant metastases at the time of surgery. Written informed consent was obtained from all patients prior to surgery. The study protocol was approved by the Institutional Review Board for the Use of Human Subjects at the Yamaguchi University School of Medicine. All ESCC was diagnosed histopathologically by at least two trained pathologists. Tumor stage and grade were classified according to the pTNM Classification of the International Union Against Cancer (24). Eleven non-tumorous esophageal samples from 11 patients who underwent esophageal resection and who had histologically normal esophageal mucosa were included as control.

DNA microarray analysis. Each cancer tissue sample was divided into two specimens; one part was frozen in liquid nitrogen immediately after surgical resection, and the other part was used for histopathological examination. The clinical and pathologic data for these patients are summarized in Table I. RNA extractions, cDNA and cRNA syntheses and oligonucleotide microarray screening were performed as described previously with minor modifications (6,19). In the present study, we used the Human Genome U133 A® DNA microarray chip (Affymetrix, Santa Clara, CA), which contains 22,115 genes.

Digitized image data from the microarray chip were processed using Affymetrix® Microarray Suite 5.0 software, and initial absolute analysis was performed without scaling. For comparative analyses using Roche Affymetrix Chip Experiment-Analysis (RACE-A) software, the average fluorescence intensity of all the genes on each chip was set to 1000. We then used the average difference of the hybridization signal, which measures the mean difference in fluorescence intensity between a perfect match and centrally mismatched oligonucleotides of a probe set.

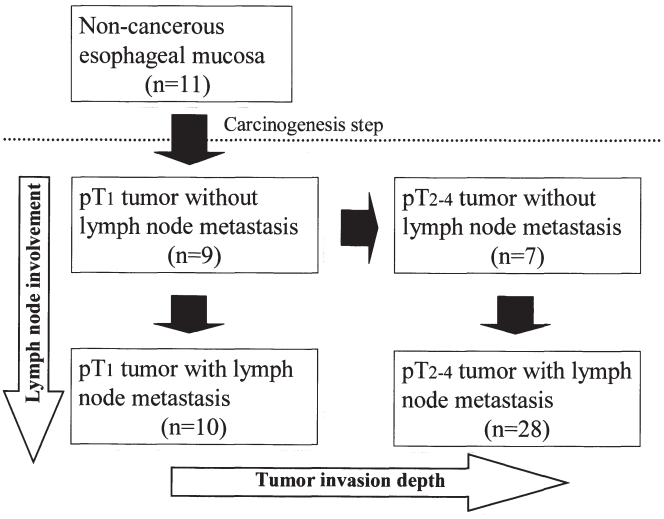

Figure 1. Schematic carcinogenesis and progression of ESCC. We classified the 65 esophageal tissues into five groups on the basis of tumor invasive depth and LN involvement, and then compared gene expression variations between groups using our original supervised learning theory (see Materials and methods).

Gene selection. We first selected genes that had all present call (arbitrary units by Affymetrix) in all 54 ESCC samples and 11 non-tumorous esophageal mucosa samples. This filtering resulted in the identification of 4,155 genes (18.8%) of the 22,115 genes. We then profiled genes among non-cancerous esophageal tissues and the four classes of ESCC (Fig. 1). We used the Fisher ratio (6,19-23) to evaluate the abilities of the selected genes to discriminate non-cancerous esophageal tissue (n=11) and pT1/pN0 tumors (n=9), pT1/pN0 tumors and pT1/pN1 tumors (n=10), pT1/pN0 tumors and pT2-4/pN0 tumors (n=7), and pT2-4/pN0 tumors and pT2-4/pN1 tumors (n=28).

Results

To examine changes in gene expression in ESCC related to different clinicopathological characteristics, we performed

Table II. Top 30 genes whose expression levels differed between non-cancerous tissue and pT1 tumors without lymph node metastasis.

Fisher ratio	Fold change	Accession no.	Abbreviation	Locus	Function
Thirteen genes up-regulated in pT1 tumors without lymph node metastasis					
12.874	2.393	D26600	PSMB4	1q21	Ubiquitination
12.346	3.577	D26361	KIF14	1pter-q31.3	Microtubule associated complex
11.010	2.617	D38551	RAD21	8q24	Apoptosis, cell cycle, cell motility
10.899	1.867	U75503	ADAR	1q21.1-q21.2	Double-stranded RNA adenosine deaminase activity
9.945	1.959	BC022432	SNRPG	2p13.3	Small nucleolar ribonucleoprotein complex
9.188	2.003	D26598	PSMB3	17q12	Ubiquitination
9.034	2.005	G54029	PSMB4	1q21	Ubiquitination
8.889	2.340	D78151	PSMD2	3q27.3	Ubiquitination
8.818	2.563	AF001212	PSMD11	17q12	Ubiquitination
8.674	1.993	X78669	RCN2	15q23	Calcium-binding protein
8.345	1.782	J03827	NSEP1	1p34	DNA-binding protein B
8.222	2.162	L77701	COX17	3q13.33	Intracellular copper ion transporter
8.135	2.166	X65867	ADSL	22q13.2	Purine ribonucleotide biosynthesis
Seventeen genes down-regulated in pT1 tumor without lymph node metastasis					
25.798	0.299	BC003370	CSTB	21q22.3	Cysteine protease inhibitor activity
21.042	0.199	Y07909	EMP1	12p12.3	Cell proliferation
14.871	0.554	BF683426			Unknown
13.199	0.362	X05908	ANXA1	9q12-q21.2	Cell motility
11.827	0.338	AB067506	KIAA1919	6q21	Unknown
11.748	0.487	U10248	RPL29	3p21.3-p21.2	Structural protein of ribosome
10.950	0.336	L33930	CD24	6q21	Cell adhesion
10.816	0.523	U70063	ASAH1	8p22-p21.3	Fatty acid metabolism
9.898	0.228	L05187	SPRR1A	1q21-q22	Structural molecule activity
9.885	0.186	M18216	CEACAM6	19q13.2	Cell-cell signaling
9.585	0.294	AY007220	S100A14	1q21.1	Calcium ion binding
9.488	0.385	N92498			Unknown
9.372	0.242	M93056	SERPINB1	6p25	Serine protease inhibitor activity
8.982	0.389	L33930	CD24	6q21	Cell adhesion
8.940	0.415	X05978	CSTA	3q21	Cysteine protease inhibitor activity
8.275	0.373	M58664	CD24	6q21	Cell adhesion
8.025	0.603	BC047024	SUCLG2	3p14.3	Succinyl-CoA synthetase, ß-G chain

Fold change, average of pT1 tumors without lymph node metastasis/non-cancerous tissue. Accession nos. and abbreviations were obtained from the URL website (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene).

microarray analysis of 22,115 and found that 4,155 were differentially expressed in ESCC. For each comparison, we ranked the identified 4,155 genes in order of decreasing magnitude of the Fisher ratio. To determine the number of genes that should be considered, we performed a random permutation test as described previously (19-23). From the distribution of the Fisher ratios based on the randomized data, we selected all genes that passed the random permutation test ($P<0.01$). We found that the levels of expression of 999 genes with Fisher ratios greater than 1.54 differed significantly between pT1/pN0 tumors and non-cancerous esophageal tissue. Information for the top 100 genes listed here is available in the supplementary Tables. In the same manner, 48 (Fisher ratio >1.65), 66 (Fisher ratio >2.10) and 30 (Fisher ratio >1.34) genes showed differential expression in pT1/pN0 tumors vs. pT1/pN1 tumors, pT1/pN0 tumors vs. pT2-4/pN0 tumors and pT2-4/pN0 tumors vs. pT2-4/pN1 tumors, respectively (supplementary Tables). Tables II-V and Fig. 2 show the 30 genes with the highest Fisher ratios in each comparison. These genes had various biological functions.

Interestingly, there were no overlaps between the 48 LN metastasis-related genes of pT1 tumors and the 30 LN meta-

Table III. Top 30 genes whose expression levels differed between pT tumors without lymph node metastasis and pT1 tumors with lymph node metastasis.

Fisher ratio	Fold change	Accession no.	Abbreviation	Locus	Function
Five genes up-regulated in pT1 tumors with lymph node metastasis					
2.783	1.495	AA789278		Unknown	Unknown
2.457	1.425	AF077043	RPL36	19p13.3	Structural constituent of ribosome
2.313	1.307	X64707	RPL13	16q24.3	Structural constituent of ribosome
2.257	1.376	AW574664		Unknown	Unknown
1.992	1.359	Z98200		Unknown	Unknown
Twenty-five genes down-regulated in pT1 tumors with lymph node metastasis					
4.473	0.561	AF054174	H2AFY	5q31.3-q32	Chromosome organization and biogenesis
3.954	0.648	AB023229	KIAA1012	18q12.1	Intracellular transporter activity
3.701	0.612	L09604	PLP2	Xp11.23	A4 differentiation-dependent protein
3.637	0.558	BC015178	C18orf10	18q12.2	Unknown, DKFZP586M1523 protein
3.154	0.695	BC002939	MCLC	1p13.3	Unknown, Mid-1-related chloride channel 1
2.911	0.682	AF380179	SON	21q22.11	Anti-apoptosis
2.863	0.646	BC014840	MADH2	18q21.1	Regulation of transcription
2.724	0.673	AF351618	FBXO11	2p21	Ubiquitin conjugating enzyme activity
2.706	0.595	AJ007590	RP2	Xp11.4-p11.21	ß-tubulin folding
2.674	0.614	P56211	ARPP-19	15q21.1	Positive regulation of glucose import
2.666	0.638	AK001152	FLJ10290	5q33.1	Nucleic acid binding
2.607	0.727	U39317	UBE2D2	5q31.3	Invasive growth, ubiquitin-conjugating enzyme
2.481	0.727	L23805	CTNNA1	5q31	Cell adhesion molecule activity
2.444	0.708	U83117	UBL1	2q33	Ubiquitin conjugating enzyme activity
2.318	0.557	D64110	BTG3	21q21.1-q21.2	Negative regulation of cell proliferation
2.271	0.715	AF063015	FTSJ1	Xp11.23	Cell division protein
2.267	0.629	AK000101	ZCCHC10	5q31.1	Unknown, nucleic acid binding
2.241	0.734	AF100615	MORF4L1	15q24	Regulation of cell growth
2.214	0.680	AL122045	CNOT8	5q31-q33	Regulation of transcription
2.206	0.734	J04977	XRCC5	2q35	Unknown, DNA recombination
2.106	0.649	M16827	ACADM	1p31	Fatty acid metabolism
2.046	0.747	AB030181	ARID4B	1q42.1-q43	Retinoblastoma-binding protein 1-like 1
2.013	0.691	M74525	UBE2B	5q23-q31	Ubiquitin conjugating enzyme activity
1.983	0.630	AL136810	TIP120A	12q14	TIP120 protein
1.960	0.709	O43189	PHF1	6p21.3	Regulation of transcription

Fold change, average of pT1 tumors with lymph node metastasis/pT1 tumors without lymph node metastasis. Accession nos. and abbreviations were obtained from the URL website (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene).

stasis-related genes of pT2-4 tumors, suggesting that ESCCs with distinct invasive depths have different genetic pathways linked to LN metastasis.

Discussion

Despite recent surgical improvements, the prognosis of ESCC patients remains poor, with a 5-year survival rate of ~20% (4). LN metastasis is one factor that strongly affects the outcome of ESCC (3). It was also reported that the degree of invasive depth of a tumor is closely related to LN metastasis in ESCC (25). In the present study, to elucidate the molecular basis of LN metastasis of ESCC, we examined gene expression patterns from two viewpoints: invasion depth of tumor and LN metastasis. We found that pT1 ESCCs and pT2-4 ESCCs do not commonly express any genes linked to LN metastasis (supplementary Tables). This result suggests that prediction of LN metastasis of ESCC on the basis of expression profiles is limited unless we consider the depth of tumor invasion. This concept is supported by the results of previous studies in which molecular profiling misclassified the presence or absence of LN metastasis in 3 (23%) of 13 ESCCs (17) and 2 (11%) of 18

Table IV. Top 30 genes whose expression levels differed between pT1 tumors without lymph node metastasis and pT2-4 tumors without lymph node metastasis.

Fisher ratio	Fold change	Accession no.	Abbreviation	Locus	Function
Fourteen genes up-regulated in pT2-4 tumors without lymph node metastasis					
6.575	2.007	G09498	MYO1B	2q12-q34	Unknown, myosin-I α
5.600	1.602	U61734	TMP21	14q24.3	Intracellular protein transport
4.782	1.659	AF151893	TTC11	7q22.1	Tetratricopeptide repeat domain 11
4.515	1.607	U84371	AK2	1p34	Adenylate kinase, mitochondrial
3.907	1.350	AK000120	OTUB1	11q13.1	Ubiquitin-specific protease otubain 1
3.559	2.561	M14328	ENO1	1p36.3-p36.2	Negative regulation of transcription
3.443	1.535	BC002475	PFN1	17p13.3	Cytoskeleton organization and biogenesis
3.387	2.268	L42584	KRT6B	12q12-q13	Structural constituent of cytoskeleton
3.111	1.681	K03515	GPI	19q13.1	Humoral immune response, cytokine activity
2.906	1.777	BC010273	PAICS	4pter-q21	Purine nucleotide biosynthesis
2.827	1.367	BC008926	RPL29	3p21.3-p21.2	Structural protein of ribosome
2.812	1.864	M86400	YWHAZ	8q23.1	Protein domain specific binding
2.764	1.621	AL117616	SRI	7q21.1	Calcium channel regulator
2.730	1.612	BC010430	NSEP1	1p34	Major histocompatibility complex, class II
Sixteen genes down-regulated in pT2-4 tumors without lymph node metastasis					
9.543	0.639	Q13618	CUL3	2q36.3	Induction of apoptosis by intracellular signals
5.079	0.412	AA486366	RGS5	1q23.1	Peripheral plasma membrane protein
4.799	0.561	AL021366	PHF1	6p21.3	Regulation of transcription, DNA-dependent
4.113	0.581	AF351618	FBXO11	2p21	Ubiquitin conjugating enzyme activity
4.078	0.572	M16827	ACADM	1p31	Fatty acid ß-oxidation
3.540	0.435	X76732	NUCB2	11p15.1-p14	Nucleobindin 2
3.389	0.719	AF251063	P17.3	Xp11.3	Neuronal protein 17.3
3.346	0.396	D16360	GPX3	5q23	Glutathione peroxidase activity
3.323	0.707	AF044321	COX11	17q22	Cytochrome c oxidase biogenesis
3.198	0.738	AA443738	HBXIP	1p13.2	Viral replication, invasive growth
2.978	0.686	AF315687	AHCYL1	1p12	Dendritic cell expressed AHCY-like protein
2.930	0.572	AF092135	CRI1	15q21.1-q21.2	Chromosome 15 open reading frame 3
2.830	0.512	J03600	ALOX5	10q11.2	Lipoxygenase activity
2.780	0.617	M15182	GUSB	7q21.11	Glycosaminoglycan catabolism
2.765	0.541	AF176085	PTBP2	1p22.1-p21.3	Neural polypyrimidine tract binding protein
2.710	0.602	AF251038	C5orf5	5q31	Unknown, chromosome 5 open reading frame 5

Fold change, average of pT2-4 tumors without lymph node metastasis/pT2-4 tumors with lymph node metastasis. Accession nos. and abbreviations were obtained from the URL website (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene).

ESCCs (18). Thus, in conjunction with our present finding, we propose that predictors for LN metastasis in ESCC should be established separately according to tumor invasion depth (pT1 and pT2-4) to obtain high predictive ability in a large cohort.

It is clinically important to accurately predict LN metastasis in pT1 ESCC to avoid unnecessary treatment of patients who have been cured by resection alone. In addition, elucidating the molecular basis of LN metastasis in this type of ESCC may identify new therapeutic targets. We found that the levels of 25 of the top 30 genes were lower in pT1/N1 ESCC than in pT1/N0 ESCC and that these 25 down-regulated genes were involved in various biological function activities (Table III). The five up-regulated genes included *RPL36* and *RPL13*, both of which are structural components of ribosomes. It was reported that the levels of expression of many *RPL* family genes were altered in ESCC cells showing acquired resistance to the anti-cancer agent cisplatin (12). Thus, it is possible that both *RPL36* and *RPL13* may be involved in LN metastasis of pT1 ESCC.

No angiogenesis-related genes showed differential expression when we compared pT1/pN0 ESCC and pT1/pN1 ESCC. In contrast, expression levels of *VEGF* were

Table V. Top 30 genes whose expression levels differed between pT2-4 tumors without lymph node metastasis and pT2-4 tumors with lymph node metastasis.

Fisher ratio	Fold change	Accession no.	Abbreviation	Locus	Function
Seventeen genes upregulated in pT2-4 tumors with lymph node metastasis					
2.654	1.616	AF389880	RC3	15q15.3	Rabconnectin-3
2.620	1.530	BC034418	FLJ20719	1p31	Unknown, hypothetical protein FLJ20719
2.534	1.964	BC059385	UBL3	13q12-q13	Ubiquitin-like 3
2.392	1.523	X80115	NOTCH2	1p13-p11	Notch 2 preproprotein
2.039	1.448	O94806	PRKCN	2p21	Protein amino acid phosphorylation
1.929	1.829	AW516297	FLJ11946	Unknown	Unknown, hypothetical protein FLJ20719
1.686	1.563	M15182	GUSB	7q21.11	ß-glucuronidase activity
1.682	1.347	AF047442	SEC22L1	1q21.2-q21.3	Unknown, hypothetical protein DJ328E19.C1.1
1.660	1.392	AF113124	FEZ2	2p21	Fasciculation and elongation protein ζ 2
1.636	1.504	U77493	NOTCH2	1p13-p11	Unknown, notch 2 preproprotein
1.622	1.482	AK001016	FLJ10154	13q33.2	Unknown, hypothetical protein FLJ10154
1.543	1.647	M63978	VEGF	6p12	Vascular endothelial growth factor receptor binding
1.463	1.688	G28996	FLJ20719	Unknown	Unknown, hypothetical protein FLJ20719
1.442	1.385	AK024685	SCD4	4q21.3	Stearoyl-CoA desaturase 4
1.435	1.260	AK025605	ZDHHC6	10q25.3	DHHC domain containing 6, integral to membrane
1.408	1.471	W72053	FLJ21904	Unknown	Unknown
1.394	1.967	AB018298	SEC24D	4q27	Protein transport protein Sec24D
Thirteen genes downregulated in pT2-4 tumors with lymph node metastasis					
2.611	0.743	BC017218	PLA2G12A	4q25	Lipid catabolism
2.268	0.648	BF215996	MYO1B	2q12-q34	Myosin-I α
2.188	0.715	U84371	AK2	1p34	Adenylate kinase activity
1.971	0.638	L26318	MAPK8	10q11.23	Microtubule-based movement
1.945	0.673	AF047434	NDUFS5	1p34.2-p33	Mitochondrial electron transport
1.844	0.741	BC002475	PFN1	17p13.3	Cytoskeleton organization and biogenesis
1.719	0.597	U90906	HSPB1	7q11.23	Heat shock protein
1.603	0.745	M27132	ATP5B	12p13-qter	ATP synthesis coupled proton transport
1.517	0.768	D14878	C10orf7	10p13	Positive regulation of cell proliferation
1.448	0.715	U66669	HIBCH	2q32.3	Unknown, 3-hydroxyisobutyryl-CoA hydrolase activity
1.409	0.766	AK074302	FLJ21901	2q31	Unknown, hypothetical protein FLJ21901
1.395	0.721	P05218	OK/SW-cl.56	6p21.32	Natural killer cell mediated cytolysis, ß5-tubulin
1.368	0.749	U63743	KIF2C	1p34.1	Microtubule motor activity

Fold change, average of pT2-4 tumors with lymph node metastasis/pT2-4 tumors without lymph node metastasis. Accession nos. and abbreviations were obtained from the URL website(http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene).

significantly higher in pT2-4/pN0 ESCC than in pT2-4/pN1 ESCC (Table V). It was reported that VEGF expression correlates with outcome and LN metastasis in ESCC (26). Interestingly, the percentage of VEGF-positive cells appears to increase with the invasive depth of ESCC (27). These reports support our finding that *VEGF* is related to LN metastasis in pT2-4 ESCC but not in pT1 ESCC. In the present study, we also identified *NOTCH2* as a gene responsible for LN metastasis of pT2-4 ESCC. Recent evidence shows that Notch signaling from tumor cells can activate endothelial cells and trigger tumor angiogenesis both *in vitro* and in a xenograft mouse tumor model and that selective interruption of Notch signaling within tumors may have anti-angiogenic effects (28). Collectively, these findings suggest that both VEGF and Notch play important roles in LN metastasis of pT2-4 ESCC via altered angiogenesis.

We found that 999 genes were expressed differentially between pT1/pN0 ESCC and non-cancerous esophageal tissue, this number of genes was much larger than that from other comparisons between tumor classes, suggesting that the levels of many genes (999/4,155, 24%) change drastically during carcinogenesis. Several studies have examined the expression

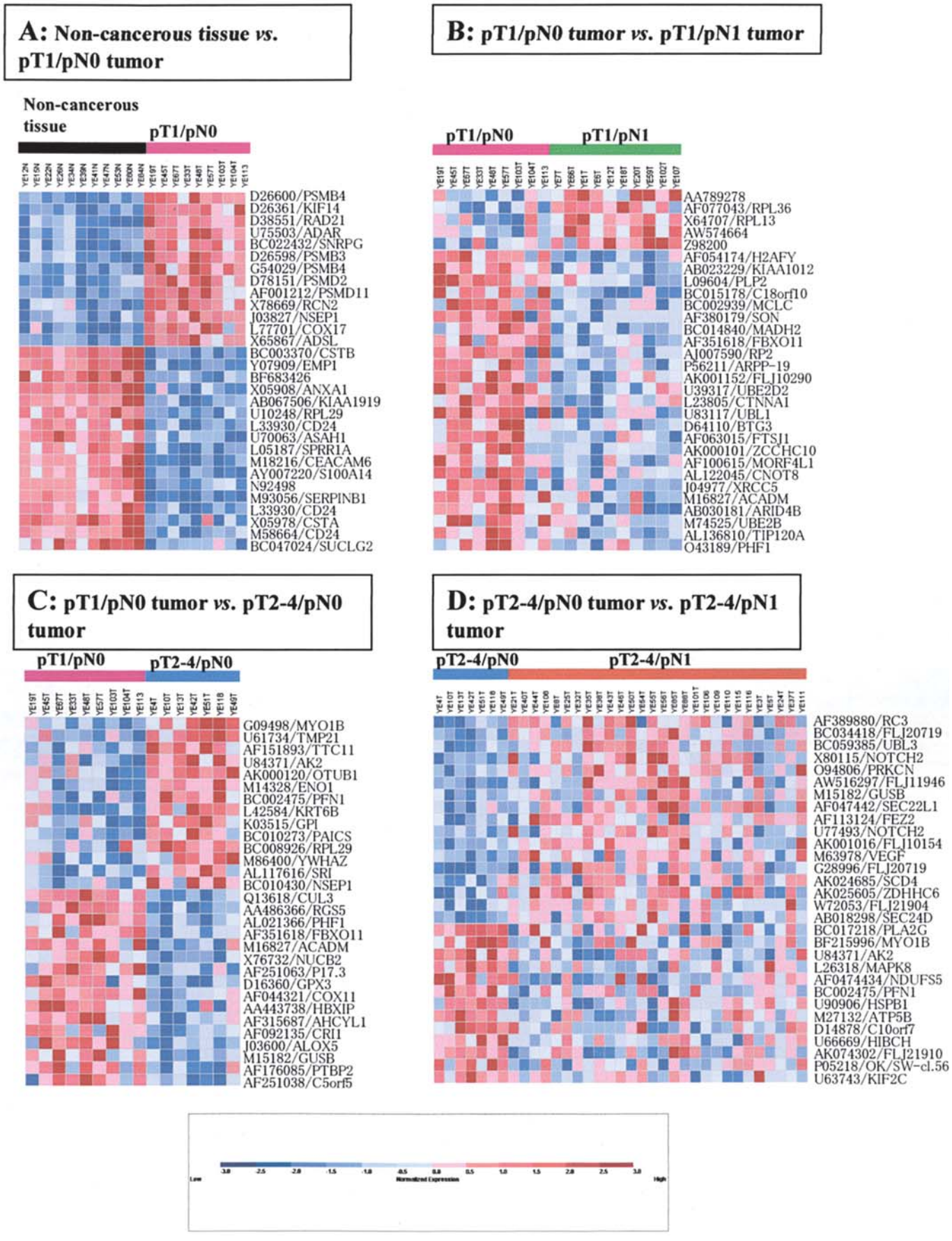

Figure 2. Genes linked to carcinogenesis and progression of ESCC. Color displays show the expression of the top 30 genes in pT1/pN0 tumors vs. non-cancerous esophageal tissue (A), pT1/pN0 tumors vs. pT1/pN1 tumors (B), pT1/pN0 tumors vs. pT2-4/pN0 tumors (C) and pT2-4/pN0 tumors vs. pT2-4/pN1 tumors (D). Each gene was ranked in decreasing order of the Fisher ratio (see Materials and methods) and listed by accession no. and symbol, which were obtained from the Entrez Gene (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene) or TIGR database (http://www.tigr.org/tdb/hgi/searching/reports.html).

of carcinogenesis-related genes in ESCC (8-10); however, all of these studies compared non-cancerous esophageal samples with heterogeneous ESCC samples consisting of pT1 to pT4 tumors with or without LN metastasis, resulting in a failure to identify key genes. Recently, it was reported that the number of differentially expressed genes dramatically increased in a step-by-step fashion from normal esophageal epithelium to papilloma, dysplasia and invasive carcinoma in a rat ESCC model (15). Because our present cohort did not include pre-neoplastic lesions such as papilloma and dysplasia, further studies are needed to identify genes with expression that differs between pre-neoplastic and non-cancerous esophageal tissues.

Among the 30 genes with differential expression between pT1/pN0 ESCC and non-cancerous esophageal tissues, increased expression of ubiquitination-related genes (*PSMB3, PSMB4, PSMD2* and *PSMD11*) was the most characteristic gene signature (Table II). It was reported that levels of *PSMB3* are increased in breast cancer (29). However, to our knowledge, there are no reports of up-regulation of expression of *PSMB4, PSMD2*, or *PSMD11* in cancer. The ubiquitin pathway plays a central role in the regulation of cell growth and cell proliferation by degrading tumor suppressor proteins (30) and controlling the levels of key cell cycle proteins (31), suggesting that increased levels of ubiquitination-related genes may account for some growth advantage to cancer cells. Thus, the four genes described here may be molecular targets for prevention or treatment of ESCC.

In the present study, DNA microarray analysis revealed that many genes are involved in carcinogenesis and progression of ESCC. We also found that pT1 ESCC and pT2-4 ESCC express no common genes responsible for LN metastasis, raising the difficulty of accurate prediction of LN metastasis in ESCC without also considering tumor invasion depth. Further studies are needed to clarify whether this strategy can be used to predict LN metastasis in other types of cancer in the alimentary tract.

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Supplementary Table I. Top 100 genes whose expression levels differed between non-cancerous tissue and pT1 tumors without lymph node metastasis.

Order	Gene Accession no.			Symbol	Function
1	201201_at	\	BC003370	CSTB	Cysteine protease inhibitor activity
2	201324_at	\	Y07909	EMP1	Cell proliferation
3	213969_x_at	\	BF683426		Unknown
4	201012_at	\	X05908	ANXA1	Cell motility
5	202244_at	/	D26600	PSMB4	Ubiquitin-dependent protein catabolism
6	206364_at	/	D26361	KIF14	Microtubule associated complex
7	216379_x_at	\	AB067506	KIAA1919	Unknown, KIAA1919 protein
8	200823_x_at	\	U10248	RPL29	Structural protein of ribosome
9	200608_s_at	/	D38551	RAD21	Apoptosis, cell cycle, cell motility
10	266_s_at	\	L33930	CD24	Cell adhesion
11	201786_s_at	/	U75503	ADAR	Double-stranded RNA adenosine deaminase activity
12	210980_s_at	\	U70063	ASAH1	Fatty acid metabolism
13	205644_s_at	/	BC022432	SNRPG	Small nucleolar ribonucleoprotein complex
14	214549_x_at	\	L05187	SPRR1A	Structural molecule activity
15	211657_at	\	M18216	CEACAM6	Cell-cell signaling
16	218677_at	\	AY007220	S100A14	Calcium ion binding
17	212593_s_at	\	N92498		Unknown
18	213572_s_at	\	M93056	SERPINB1	Serine protease inhibitor activity
19	201400_at	/	D26598	PSMB3	Ubiquitin-dependent protein catabolism
20	202243_s_at	/	G54029	PSMB4	Endopeptidase activity
21	208651_x_at	\	L33930	CD24	Humoral immune response
22	204971_at	\	X05978	CSTA	Cysteine protease inhibitor activity
23	200830_at	/	D78151	PSMD2	Tumor necrosis factor receptor-associated protein 2
24	208777_s_at	/	AF001212	PSMD11	26S proteasome regulatory subunit 9
25	201486_at	/	X78669	RCN2	Calcium-binding protein
26	208628_s_at	/	J03827	NSEP1	DNA-binding protein B
27	209771_x_at	\	M58664	CD24	Humoral immune response
28	203880_at	/	L77701	COX17	Intracellular copper ion transporter
29	202144_s_at	/	X65867	ADSL	Purine ribonucleotide biosynthesis
30	215772_x_at	\	BC047024	SUCLG2	Succinyl-CoA synthetase, ß-G chain
31	210999_s_at	/	U66065	Grb10	Cell-cell signaling
32	211939_x_at	\	X74070	HSBTF3	Regulation of transcription, DNA-dependent
33	201612_at	\	U34252	ALDH9	Aldehyde dehydrogenase (NAD+) activity
34	205394_at	/	AF016582	CHK1	Negative regulation of cell proliferation
35	202770_s_at	\	U47414	CCNG2	Cyclin G2, cell cycle, cytokinesis
36	202794_at	\	L08488	INPP1	Signal transduction
37	39248_at	\	N74607	1NFLS	Transport of nonionic small solutes
38	209369_at	\	M63310	ANX3	Phospholipase A2 inhibitor activity
39	202096_s_at	\	M36035	BZRP	Mitochondrial translocation
40	201348_at	\	D00632	GPX3	Glutathione peroxidase activity
41	213699_s_at	/	X56468		Regulation of cell cycle
42	201637_s_at	/	U25165	FXR1	Fragile X mental retardation-related protein 1
43	200703_at	\	U32944	PIN	Microtubule motor activity
44	210142_x_at	/	AF117234	FLOT1	Integral to membrane
45	214091_s_at	\	M24613	PHO2	Unknown
46	201853_s_at	/	M25515	CDC25B	Cell division cycle, regulation of cell cycle
47	214737_x_at	/	AV725195		Unknown
48	202537_s_at	\	AL080122	HTC	Unknown, DKFZP564O123 protein
49	214328_s_at	/	R01140		Unknown
50	204751_x_at	\	Z15207	DSC2	Cell adhesion molecule activity
51	208517_x_at	\	DQ131479	BTF3	Regulation of transcription, DNA-dependent

Supplementary Table I. Continued.

Order			Gene Accession no.	Symbol	Function
52	209932_s_at	/	U90223		dUTP pyrophosphatase activity
53	212586_at	\	AA195244		Proteolysis and peptidolysis
54	201528_at	/	M63488		DNA repair
55	221702_s_at	/	AF353992	BLP2	BBP-like protein 2
56	201477_s_at	/	X59543	RRM1	DNA replication
57	202731_at	\	U96628	PDCD4	Programmed cell death 4
58	210927_x_at	/	BC004239	MGC	Oncogenesis
59	211761_s_at	/	BC005975	MGC	Calcyclin binding protein
60	222229_x_at	\	AL121871	RPL26	Unknown, Pseudogene
61	202546_at	\	AF053233	VAMP8	Vesicle-associated membrane protein
62	204068_at	/	U60206	STK3	Protein amino acid phosphorylation, apoptosis
63	211026_s_at	\	BC006230	MGC	Lipid metabolism
64	212371_at	/	AL049397	DKFZ	Unknown
65	200633_at	/	BC006230	UBB	Protein modification
66	212725_s_at	/	N37081		Unknown
67	210027_s_at	/	M80261	APE	Multifunctional DNA repair enzyme
68	204219_s_at	/	L02426	PSMC1	26S proteasome
69	213929_at	\	AL050204		Unknown
70	209484_s_at	/	AF201941		Unknown, DKFZP566O1646 protein
71	203396_at	/	BC005361	PSMA4	Ubiquitin-dependent protein catabolism
72	212372_at	/	AK026977	MYH10	Motor activity
73	212597_s_at	/	AL079310	HS510H16B	Endosome to lysosome transport
74	212058_at	/	X54649	DMFRIZZ2	Ser/Arg-rich domain protein, RNA processing
75	208627_s_at	/	BE966374		Major histocompatibility complex, class II
76	208749_x_at	/	M33375	HUMCCDR	Flotillin complex
77	201584_s_at	/	U90426	DDXL	Nuclear RNA helicase
78	202605_at	/	M15182	GUSB	Glycosaminoglycan catabolism
79	203432_at	/	U09086		Unknown, lamin/chromatin binding
80	201178_at	/	AF129537	FBXO7	Ubiquitin-dependent protein catabolism
81	202754_at	/	BC041093	KIAA0029	Unknown, binds single-stranded nucleic acids
82	201433_s_at	/	D14694	PTDSS	Unknown, phosphatidylserine synthase 1
83	202983_at	/	AI760760		Regulation of transcription, DNA-dependent
84	201534_s_at	\	AF044221	HCG-1	Ubiquitin-like 3
85	208091_s_at	/	BC016650	DKFZp564K0822	Unknown, hypothetical protein DKFZp564K0822
86	210574_s_at	/	AF241788	NPD011	Cell proliferation, regulation of cell cycle
87	208616_s_at	/	U48297	PTPCAAX2	Protein tyrosine phosphatase
88	218350_s_at	/	AL133264	CLAPS3	Negative regulation of DNA replication
89	212788_x_at	/	BG537190		Unknown
90	211208_s_at	/	AB039327	CASK	Actin cytoskeleton, cell adhesion
91	211069_s_at	/	BC006462	SUMO1	Ubiquitin conjugating enzyme activity
92	208771_s_at	\	J02959.1	LTA4H	Proteolysis and peptidolysis
93	202246_s_at	/	U79269	CDK4	Cell proliferation, oncogenesis, regulation of cell cycle
94	219787_s_at	/	AK001323	FLJ10461	Cell growth and/or maintenance
95	211762_s_at	/	BC005978	RAG	Protein transporter activity
96	200965_s_at	\	D31883	ABLIM	Cytoskeleton organization and biogenesis
97	200681_at	/	L07837	GLO1	Lactoylglutathione lyase
98	202666_s_at	/	AB015907	BAF53A	Structural constituent of cytoskeleton
99	203255_at	/	AF264714	VIT1	Protein modification
100	200744_s_at	/	AI741124		RAS protein signal transduction

/, up-regulated genes in pT1 tumors without lymph node metastasis; \, down-regulated genes in pT1 tumors without lymph node metastasis.

Supplementary Table II. Top 48 genes whose expression levels differed between pT tumors without lymph node metastasis and pT1 tumors with lymph node metastasis.

Order			Gene Accession no.	Symbol	Function
1	207168_s_at	\	AF054174	H2AFY	Chromosome organization and biogenesis
2	207305_s_at	\	AB023229	KIAA1012	Intracellular transporter activity
3	201136_at	\	L09604	PLP2	A4 differentiation-dependent protein
4	212055_at	\	BC015178	C18orf10	Unknown, DKFZP586M1523 protein
5	213628_at	\	BC002939	MCLC	Unknown, Mid-1-related chloride channel 1
6	214988_s_at	\	AF380179	SON	Anti-apoptosis
7	203075_at	\	BC014840	MADH2	Regulation of transcription, DNA-dependent
8	214351_x_at	/	AA789278		Unknown
9	203255_at	\	AF351618	FBXO11	Ubiquitin conjugating enzyme activity
10	205191_at	\	AJ007590	RP2	ß-tubulin folding
11	221482_s_at	\	P56211	ARPP-19	Positive regulation of glucose import
12	218134_s_at	\	AK001152	FLJ10290	Nucleic acid binding
13	201345_s_at	\	U39317	UBE2D2	Invasive growth
14	210844_x_at	\	L23805	CTNNA1	Cell adhesion molecule activity
15	219762_s_at	/	AF077043	RPL36	Structural constituent of ribosome
16	211069_s_at	\	U83117	UBL1	Ubiquitin conjugating enzyme activity
17	213134_x_at	\	D64110	BTG3	Negative regulation of cell proliferation
18	208929_x_at	/	X64707	RPL13	Structural constituent of ribosome
19	205324_s_at	\	AF063015	FTSJ1	Cell division protein
20	221193_s_at	\	AK000101	ZCCHC10	Unknown, nucleic acid binding
21	212191_x_at	/	AW574664		Unknown
22	217982_s_at	\	AF100615	MORF4L1	Regulation of cell growth
23	202163_s_at	\	AL122045	CNOT8	Regulation of transcription, DNA-dependent
24	208642_s_at	\	J04977	XRCC5	Unknown, DNA recombination
25	202502_at	\	M16827	ACADM	Fatty acid metabolism
26	212591_at	\	AB030181	ARID4B	Retinoblastoma-binding protein 1-like 1
27	202334_s_at	\	M74525	UBE2B	Ubiquitin conjugating enzyme activity
28	215963_x_at	/	Z98200		Unknown
29	208839_s_at	\	AL136810	TIP120A	TIP120 protein
30	40446_at	\	O43189	PHF1	Regulation of transcription, DNA-dependent
31	219489_s_at	\	AL139260	RHBDL2	Serine-type endopeptidase activity
32	209224_s_at	\	BC003674	NDUFA2	NADH dehydrogenase (ubiquinone) activity
33	202557_at	\	AI718418		Unknown, Stress 70 protein chaperone
34	206272_at	\	X82554	SPHAR	DNA replication
35	219454_at	\	AF186084	EGFL6	Cell cycle, oncogenesis
36	209090_s_at	\	AF263293	RPL17	Unknown, endophilin B1
37	201390_s_at	\	X57152	CSNK2B	Protein kinase CK2, intrinsic regulator
38	218738_s_at	\	AF162680	STRIN	Ring finger protein 138
39	205335_s_at	\	X12791	SRP19	Signal recognition particle
40	212372_at	\	AK026977	MYH10	Cytokinesis
41	201546_at	\	D28476	TRIP12	Thyroid receptor interacting protein 12
42	218350_s_at	\	AF067855		Cell cycle arrest
43	201441_at	\	BC001015	COX6B	Cytochrome c oxidase activity
44	209787_s_at	\	BC001282	HMGN4	High mobility group protein N4
45	203689_s_at	\	AI743037		Unknown, fragile X mental retardation 1
46	213857_s_at	\	BG230614		Unknown
47	215245_x_at	\	AA830884		Unknown, nucleoplasm
48	203077_s_at	\	U59911	MADH2	Regulation of transcription, DNA-dependent

/, up-regulated genes in pT1 tumors with lymph node metastasis; \, down-regulated genes in pT1 tumors with lymph node metastasis.

Supplementary Table III. Top 66 genes whose expression levels differed between pT1 tumors without lymph node metastasis and pT2-4 tumors without lymph node metastasis.

Order			Gene Accession no.	Symbol	Function
1	201371_s_at	\	Q13618	CUL3	Induction of apoptosis by intracellular signals
2	212365_at	/	G09498	MYO1B	Unknown, myosin-I α
3	200929_at	/	U61734	TMP21	Intracellular protein transport
4	218353_at	\	AA486366	RGS5	Peripheral plasma membrane protein
5	40446_at	\	AL021366	PHF1	Regulation of transcription, DNA-dependent
6	218034_at	/	AF151893	TTC11	Tetratricopeptide repeat domain 11
7	208967_s_at	/	U84371	AK2	Adenylate kinase, mitochondrial
8	203255_at	\	AF351618	FBXO11	Ubiquitin conjugating enzyme activity
9	202502_at	\	M16827	ACADM	Fatty acid ß-oxidation
10	201245_s_at	/	AK000120	OTUB1	Ubiquitin-specific protease otubain 1
11	217294_s_at	/	M14328	ENO1	Negative regulation of transcription
12	203675_at	\	X76732	NUCB2	Nucleobindin 2
13	200634_at	/	BC002475	PFN1	Cytoskeleton organization and biogenesis
14	218320_s_at	\	AF251063	P17.3	Neuronal protein 17.3
15	209126_x_at	/	L42584	KRT6B	Structural constituent of cytoskeleton
16	201348_at	\	D16360	GPX3	Glutathione peroxidase activity
17	211727_s_at	\	AF044321	COX11	Cytochrome c oxidase biogenesis
18	202299_s_at	\	AA443738	HBXIP	Viral replication, invasive growth
19	208308_s_at	/	K03515	GPI	Humoral immune response, cytokine activity
20	200849_s_at	\	AF315687	AHCYL1	Dendritic cell expressed AHCY-like protein
21	211698_at	\	AF092135	CRI1	Chromosome 15 open reading frame 3
22	201014_s_at	/	BC010273	PAICS	Purine nucleotide biosynthesis
23	204446_s_at	\	J03600	ALOX5	Lipoxygenase activity
24	200823_x_at	/	BC008926	RPL29	Structural protein of ribosome
25	200641_s_at	/	M86400	YWHAZ	Protein domain specific binding
26	202605_at	\	M15182	GUSB	Glycosaminoglycan catabolism
27	218683_at	\	AF176085	PTBP2	Neural polypyrimidine tract binding protein
28	208921_s_at	/	AL117616	SRI	Calcium channel regulator
29	208627_s_at	/	BC010430	NSEP1	Major histocompatibility complex, class II
30	218518_at	\	AF251038	C5orf5	Unknown, chromosome 5 open reading frame 5
31	212195_at	\	AL049265		Unknown
32	202850_at	\	M81182	ABCD3	ATP-binding cassette
33	213969_x_at	/	BF683426		Unknown
34	200703_at	/	U32944	PIN	Microtubule motor activity
35	202797_at	\	AB020658	KIAA0851	Suppressor of actin 1
36	203053_at	\	AF081788	BCAS2	Pre-mRNA splicing factor activity
37	213251_at	\	AV712064	SWISNF	Chromatin modeling
38	201259_s_at	/	Q16563	SYPL	Pantophysin
39	214988_s_at	\	X63071	DBP-5	Apoptosis inhibitor activity
40	201841_s_at	/	U90906	HSPB1	Heat shock protein activity
41	201897_s_at	/	BC001425	CKS1	Cytokinesis
42	222294_s_at	\	AW971415		Unknown
43	217772_s_at	/	BC000875	MTCH2	Mitochondrial carrier homolog 2
44	203455_s_at	\	M77693	SAT	Diamine N-acetyltransferase activity
45	202429_s_at	\	L14778	DKFZp761L0516	Protein phosphatase-3
46	205401_at	/	Y09443	AGPS	Alkylglycerone-phosphate synthase activity
47	208767_s_at	/	AW149681		Putative integral membrane transporter
48	209363_s_at	/	U46837	SRB7	Regulation of transcription from PolII promoter
49	217975_at	\	AF125535	LOC51186	pp21 homolog
50	207168_s_at	\	AF054174	H2AFY	Chromosome organization and biogenesis
51	201408_at	\	W67887		Unknown, protein phosphatase 1
52	208675_s_at	/	D29643	KIAA0115	Glycosyltransferase
53	209022_at	\	BC001765	ARS	Unknown, cell cycle

Supplementary Table III. Continued.

Order			Gene Accession no.	Symbol	Function
54	201892_s_at	/	J04208	IMPDH2	Purine nucleotide biosynthesis
55	201063_at	\	D42073	RCN1	Endoplasmic reticulum lumen
56	202961_s_at	/	AF047436	ATP5J2	Hydrogen ion transporter
57	209112_at	\	U10906	Kip1	TGFß receptor, cytoplasmic mediator activity
58	212287_at	\	BF382924		Unknown, joined to JAZF1
59	201231_s_at	/	M14328	ENO1	Transcription co-repressor
60	203310_at	\	D63506	STXBP3	Intracellular protein transport
61	210338_s_at	/	AB034951	HSC54	Heat shock protein activity
62	202371_at	\	AF271783	FLJ21174	Unknown, hypothetical protein FLJ21174
63	202362_at	\	M22995	RAP1A	Small GTPase mediated signal transduction
64	218622_at	/	BC000861	MGC5585	Protein transporter activity
65	213347_x_at	\	AW132023		Unknown
66	215245_x_at	\	AA830884		Fragile X mental retardation 1

/, up-regulated genes in pT2-4 tumors without lymph node metastasis; \, down-regulated genes in pT2-4 tumors without lymph node metastasis.

Supplementary Table IV. Top 30 genes whose expression levels differed between pT2-4 tumors without lymph node metastasis and pT2-4 tumors with lymph node metastasis.

Order			Gene Accession no.	Symbol	Function
1	212820_at	/	AF389880	RC3	Rabconnectin-3
2	212854_x_at	/	BC034418	FLJ20719	Unknown, hypothetical protein FLJ20719
3	221027_s_at	\	BC017218	PLA2G12A	Lipid catabolism
4	201534_s_at	/	BC059385	UBL3	Ubiquitin-like 3
5	202443_x_at	/	X80115	NOTCH2	Notch 2 preproprotein
6	212365_at	\	BF215996	MYO1B	Myosin-I α
7	208967_s_at	\	U84371	AK2	Adenylate kinase activity
8	218236_s_at	/	O94806	PRKCN	Protein amino acid phosphorylation
9	213476_x_at	\	L26318	MAPK8	Microtubule-based movement
10	201757_at	\	AF047434	NDUFS5	Mitochondrial electron transport
11	214722_at	/	AW516297	FLJ11946	Unknown
12	200634_at	\	BC002475	PFN1	Cytoskeleton organization and biogenesis
13	201841_s_at	\	U90906	HSPB1	Heat shock protein
14	202605_at	/	M15182	GUSB	ß-glucuronidase activity
15	201103_x_at	/	AF047442	SEC22L1	Unknown, hypothetical protein DJ328E19.C1.1
16	215000_s_at	/	AF113124	FEZ2	Fasciculation and elongation protein ζ 2
17	212377_s_at	/	U77493	NOTCH2	Unknown, notch 2 preproprotein
18	218067_s_at	/	AK001016	FLJ10154	Unknown, hypothetical protein FLJ10154
19	201322_at	\	M27132	ATP5B	ATP synthesis coupled proton transport
20	210512_s_at	/	M63978	VEGF	Vascular endothelial growth factor receptor binding
21	201725_at	\	D14878	C10orf7	Positive regulation of cell proliferation
22	218330_s_at	/	G28996	FLJ20719	Role in colorectal carcinogenesis
23	213374_x_at	\	U66669	HIBCH	Unknown, 3-hydroxyisobutyryl-CoA hydrolase activity
24	220232_at	/	AK024685	SCD4	Stearoyl-CoA desaturase 4
25	218249_at	/	AK025605	ZDHHC6	DHHC domain containing 6, integral to membrane
26	219002_at	\	AK074302	FLJ21901	Unknown, hypothetical protein FLJ21901
27	212043_at	/	W72053	FLJ21904	Unknown
28	209026_x_at	\	P05218	OK/SW-cl.56	Natural killer cell mediated cytolysis
29	202375_at	/	AB018298	SEC24D	Protein transport protein Sec24D
30	211519_s_at	\	U63743	KIF2C	Microtubule motor activity

/, up-regulated genes in pT2-4 tumors with lymph node metastasis; \, down-regulated genes in pT2-4 tumors with lymph node metastasis.