Combination of microarray profiling and protein-protein interaction databases delineates the minimal discriminators as a metastasis network for esophageal squamous cell carcinoma

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Abstract. Microarray profiling of 15 adjacent normal/tumor-matched esophageal squamous cell carcinoma (ESCC) specimens identified 40 up-regulated and 95 down-regulated genes. Verification of the microarray measurement by quantitative real-time reverse transcription PCR in the same set of samples as well as an additional 15 normal/tumor-matched samples revealed >95% consistency. These signatures can also be used to classify a recently reported ESCC microarray dataset. Moreover, these molecular signatures were used as templates to elucidate their corresponding protein-protein interaction (PPI) networks using the PPI databases, POINT and POINeT. As a result, 18 genes, of which six were not disclosed in the initial expression profile analysis, were found to be able to serve as the minimal discriminators for distinguishing ESCC tumors from normal specimens. Of these discriminators, ten (*BGN*, *COL1A1*, *COL1A2*, *MMP9*, *CD44*, *FN1*, *TGFBI*, *PXN*, *SPARC* and *VWF*) were associated with tumor metastasis and formed a highly interactive network with the first four molecules as 'hubs'. Our study not only reveals how novel insights can be obtained from gene expression profiling, but also highlights a group of highly interacting genes associated with metastasis in ESCC.

Introduction

Esophageal carcinoma is one of the most lethal cancers in the world, with a median survival of approximately 1 to 2 years among patients amenable to surgery (1,2). Adenocarcinoma is the major histological subtype in Western countries, yet esophageal squamous cell carcinoma (ESCC) is the most frequent histological subtype in Asia (2).

Microarray profiling has been used in the study of carcinogenesis, disease classification, as well as prediction of treatment response, lymph node metastasis and prognosis of ESCC (3-11). However, there has been a lack of systematic analysis of the molecular markers that characterize carcinogenesis of ESCC.

In this study, we used Affymetrix GeneChip technology to identify discriminators for ESCC, which can also be used to classify a recently reported ESCC microarray dataset. Furthermore, we employed the ESCC molecular discriminators as a template to deduce a protein-protein interaction (PPI) network by using two PPI databases, the Prediction Of INTeractome (POINT) and POINeT. This resulted in the uncovering of a group of genes that not only serves as the minimal discriminators for distinguishing ESCC tumors from normal specimens, but also forms a highly interactive network involved in the metastasis of ESCC.

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Table I. Demographic characteristics of 15 patients with esophageal squamous cell carcinoma (ESCC) enrolled for microarray analysis.

Patients	Gender	Age	Tumor location	TNM stage			AJCC stage	Differentiation
				T	N	M		
Y01	M	76	U/3	1	0	0	I	Moderate
Y02	M	75	M/3-L/3	4	1	0	III	Poor
Y03	M	65	M/3-L/3	4	1	0	III	Moderate
Y04	M	52	M/3-L/3	3	1	0	III	Moderate
Y05	F	88	M/3	1	0	0	I	Moderate
Yeso-05	M	69	L/3	3	1	0	III	Moderate
Yeso-06	M	47	L/3	3	1	0	III	Moderate
Yeso-07	M	50	M/3	2	1	0	IIB	Moderate
Yeso-08	M	45	L/3	3	1	0	III	Moderate
Yeso-10	M	77	M/3-L/3	3	0	1A	IV	Moderate
Yeso-11	M	68	M/3-L/3	3	1	1A	IV	Moderate
Yeso-19	M	46	U/3-M/3	3	0	0	IIA	Poor
Yeso-20	M	60	M/3	3	1	1A	IV	Moderate
Yeso-21	M	44	L/3	3	0	0	IIA	Moderate
Yeso-22	M	65	U/3	1	0	0	I	Moderate

Tumor location over esophagus: U/3, upper third; M/3, middle third; L/3, lower third. TNM stage: T, tumor size (depth); N, lymph node metastasis status; M, distal metastasis status. AJCC stage, tumor staging according to the American Joint Committee on Cancer.

Materials and methods

Patients and samples. This study was approved by the Institutional Review Board of Taipei Veterans General Hospital. Fifteen patients with newly diagnosed ESCC, who had received primary curative esophageal resection, were included in this expression profile study. None of them received neoadjuvant therapy prior to the resection (Table I). Fifteen additional adjacent normal/tumor-matched ESCC samples were obtained for quantitative real-time reverse transcription PCR (Q-RT-PCR). Informed consents were obtained from all patients. For tumor tissues, samples were obtained from the non-necrotic area of the tumor and cut into two equally sized pieces: one for storage and the other for pathological examination. Tumors were not microscopically dissected, but only those with cancer cellularity >80% under pathological examination were included for further study. For obtaining an adjacent normal counterpart, morphologically normal esophageal epithelium at least 5 cm from the tumor margin was carefully dissected from the freshly resected esophagectomy specimen and evaluated microscopically. All specimens were snap-frozen in liquid nitrogen immediately after resection and stored at -150˚C until needed for subsequent experiments. Tumor stage and grade were defined according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 6th edition.

RNA isolation, reverse transcription and microarray. Total RNA was isolated from the frozen esophageal tissues as previously described (12). The integrity of the RNA from the pooled samples was determined using a Spectra Max Plus (Molecular Devices), and the A260/A280 ratio was from 1.9 to 2.1. Reagents for hybridization and protocols for washing and staining were the same as previous methods (13) and the Affymetrix instructions (http://www.affymetrix.com/support/technical/manuals.affx).

Data analysis, clustering algorithm, gene ontology analysis and construction of the protein-protein interaction network. Following a quantitative scan of a chip, the images were transformed to text files containing intensity information using the GeneChip® Operating Software developed by Affymetrix. The microarray data were analyzed using the GeneSpring® GX 7.3.1 Software (Agilent Technologies, Santa Clara, CA, USA). A statistical comparison of gene expression between the matched normal/tumor specimens was performed using the Wilcoxon signed-rank nonparametric test, and this approach used false-discovery rate (FDR) to account for simultaneous testing on thousands of genes (multiple testing correction) (14,15). The difference in expression was expressed as fold change. To evaluate gene expression patterns, hierarchical clustering was carried out by the Pearson's correlation metric and average linkage. Differentially expressed genes were subjected to principal component analysis (PCA) to determine patterns in the variability of expression profiles (16).

Gene ontology (GO) analysis was performed using FatiGO (http://fatigo.bioinfo.cnio.es/) to distinguish differential distribution. Database POINT (http://point.bioinformatics.tw/) (17) and tool POINeT (http://poinet.bioinformatics.tw/) were used to study PPI and network construction.
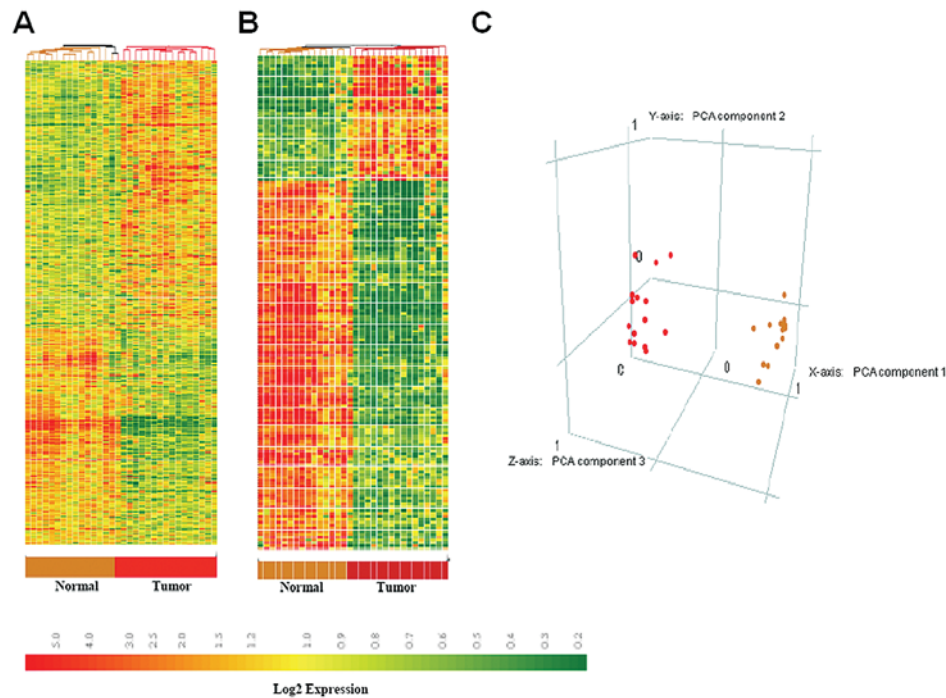
Figure 1. Gene expression profiles of 15 normal/tumor-matched ESCC samples. (A) Using the Wilcoxon signed-rank test, 4947 transcripts were found differentially expressed between adjacent normal and tumor-matched ESCC specimens (p<0.05). (B) With more stringent criteria, the expression profiles of 135 differentially expressed genes (166 transcripts), which displayed at least a 1.5-fold change (p<1x10^{-6}), were identified. (C) Principal component analysis (PCA) was used to further cluster the 166 transcripts into two distinct gene expression profiles of tumor (red)/adjacent normal (brown) specimens.

Q-RT-PCR. Q-RT-PCR analysis was used to confirm the microarray analysis data for the selected genes as described previously (12). Primer sequences (Table II) designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA) were used to perform Q-RT-PCR according to the manufacturer's instructions. To standardize the quantification of the selected target genes, DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (*DDX5*) served as an internal control for their smaller variance than glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin ß (*ACTB*) by using bootstrap re-sampling scheme (18), and was quantified on the same plate as the target genes. A melting-point (T$_m$) curve was used to identify the temperature at which only the amplicon and not the primer dimers, accounted for the SYBR Green-bound fluorescence. Assays were performed in triplicate using an Applied Biosystems Model 7700 instrument. Gene expression profiles of the Q-RT-PCR of adjacent normal/tumor-matched ESCC specimens were analyzed using the Wilcoxon signed-rank test.

Results

Distinct gene expression signatures characterize adjacent normal/tumor-matched ESCC specimens. Using the Wilcoxon signed-rank test, 4947 transcripts were found differentially expressed between adjacent normal and tumor-matched ESCC specimens (p<0.05). Supervised hierarchical clustering showed two main clusters, one representing the tumor specimens and the other representing the adjacent normal tissue specimens (Fig. 1A). The number of differentially expressed transcripts was narrowed down to 166 by using more stringent criteria (at least a 1.5-fold change with p<1x10^{-6}, Fig. 1B). The

discrimination ability of these 166 transcripts was further confirmed by PCA (Fig. 1C). The list of these 166 transcript (135 genes) discriminators, which is made up of 40 up-regulated and 95 down-regulated genes, is shown in Table III. Significantly, more than two-thirds of these discriminators were known to exhibit dysregulation profiles in many cancers as determined by PubMed search (Table III).

Differential distribution of gene-ontology (GO) terms in discriminators of ESCC. One hundred and thirty-five discriminative genes were classified with FatiGO to distinguish differential distribution in function through GO assignment. FatiGO implements 2 x 2 tables for comparison between up-regulated and down-regulated genes, extracting a list of GO-terms that are distributed among the groups with significant differences. In the cellular component, 32 out of 40 up-regulated genes and 57 out of 95 down-regulated genes had GO assignments. A comparison of the GO-term distribution between the up-regulated and down-regulated genes suggested that the significant GO-terms (p<0.05) were involved in the extracellular matrix (up vs. down, 31.25 vs. 1.75%), the membrane (up vs. down, 15.62 vs. 52.63%), and the cell fraction (up vs. down, 0 vs. 26.32%). In terms of molecular function, 33 out of the 40 up-regulated genes and 63 out of the 95 down-regulated had GO assignments. The only significant GO-term (p<0.05) involved was metallo-peptidase activity (up vs. down, 30.43 vs. 0%) (Table IV). In contrast, there was no significant GO-term for any biological process among these ESCC discriminators. Together, the GO assignments highlighted the functional categories of the discriminative genes involved in the tumorigenesis of ESCC.

Table II. Primers used for the Q-RT-PCR validation.

Gene symbol	RefSeq	Description		Primer sequence	Distance to 3' UTR (bp)
ASPM	NM_018136.3	Asp (abnormal spindle)-like, microcephaly associated	F	GTCTCTTCTGTAAAGATGCCGAATT	7722
			R	ATAAGCCAAGGTGACGGGAAA	
KPNA2	NM_002266.2	Karyopherin α2	F	GACTCCTGCCCTAAGAGCCATAG	865
			R	GCGAGTGCTCCTGCATCAAT	
SPP1	NM_000582.2	Secreted phosphoprotein 1	F	AATTGCAGTGATTTGCTTTTGC	1372
			R	AACTTCCAGAATCAGCCTGTTTAAC	
INHBA	NM_002192.2	Inhibin, ßA	F	AAGGCGGCGCTTCTGAA	1630
			R	CCCGTTCTCCCCGACTTT	
TPX2	NM_012112.4	TPX2, microtubule-associated	F	GATACCGCCCGGCAATG	651
			R	TCTCCATGCCCAATGACAAA	
AURKA	NM_003600.2	Aurora kinase A	F	TTCCAGGAGGACCACTCTCTGT	1139
			R	TGCATCCGACCTTCAATCATT	
CDC2	NM_001786.2	Cell division cycle 2, G1 to S and G2 to M (CDC2)	F	TTCAAAGCTGGCTCTTGGAAA	1154
			R	CGCAGCGGCAGCTACAA	
MMP3	NM_002422.3	Matrix metallopeptidase 3	F	CCTGGTACCCACGGAACCT	878
			R	GGACAAAGCAGGATCACAGTTG	
DPP3	NM_005700.3	Dipeptidylpeptidase 3	F	GGGCTTACCATCCTGTCTACCA	181
			R	CTGCGGGATCTAGACCAGTGA	
GPX3	NM_002084.3	Glutathione peroxidase 3 (plasma)	F	TCTCCCACTGCCTCCAAATATT	366
			R	GGGAGTGTGGTAGACCCAGAAA	
SERPINB3	NM_006919.1	Serpin peptidase inhibitor, clade B, member 3	F	GGCAGCAATACCACATTGGTT	1070
			R	TTCTCCCACTGCCCTTTGAA	
CCNG2	NM_004354.1	Cyclin G2	F	GCAGCTCTCCTCCCAGTGAT	884
			R	AAGCACAGTGTTTGTGCCACTTT	
MYH11	NM_002474.2	Myosin, heavy polypeptide 11, smooth muscle	F	CCACCTCATGGGAATTAATGTGA	5555
			R	TACCACATCTCGCCCAACCT	
FN1	NM_212482.1	Fibronectin 1	F	GAAAGTACACCTGTTGTCATTCAACA	5781
			R	ACCTTCACGTCTGTCACTTCCA	
BGN	NM_001711.3	Biglycan	F	GGAGGCGGTCCATAAGAATG	679
			R	ATGAGGAGGAGGAACAGAACATG	
MMP9	NM_004994.2	Matrix metallopeptidase 9	F	CCCGGAGTGAGTTGAACCA	231
			R	CAGGACGGGAGCCCTAGTC	
PXN	NM_002859.1	Paxillin	F	AGCGGCTCCCGATTCATC	3195
			R	GAGCACGGAGAGCCAACACT	
TGFBI	NM_000358.1	Transforming growth factor, ß-induced, 68 kDa	F	GGACTCCCTGGTCAGCAATGT	2106
			R	CTCGCCTGCCCACCATAT	
COL1A1	NM_000088.1	Collagen, type I, α1	F	GGCAAGACAGTGATTGAATACAAAA	1428
			R	ACGTCGAAGCCGAATTCCT	
COL1A2	NM_000089.3	Collagen, type I, α2	F	AACAACCAGATTGAGACCCTTCTT	1427
			R	TGGGTGGCTGAGTCTCAAGTC	
CD44	NM_000610.3	CD44 molecule	F	GCATTGCAGTCAACAGTCGAA	3213
			R	CGTTGAGTCCACTTGGCTTTC	
VWF	NM_000552.3	Von Willebrand factor	F	CAGTGTTCCCTATTGGAATTGGA	2980
			R	AGGAAGGAATTGCCCAAGGT	

Table II. Continued.

Gene symbol	RefSeq	Description	Primer sequence	Distance to 3' UTR (bp)
SPARC	NM_003118.2	Secreted protein, acidic, cysteine-rich (osteonectin)	F GGGAGCACGGACTGTCAGTT R CCCTGAGAAGAGCCCTGGTT	1380
CKS2	NM_001827.1	CDC28 protein kinase regulatory subunit 2 (CKS2)	F TTCGCGCTCTCGTTTCATTT R CTTGTCCGAGTAGTAGATCTGCTTGT	556
TJP1	NM_003257	Tight junction protein 1	F ACAAAGGAGAGGTGTTCCGTGTT R CGTTCTACCTCCTTATGATTTTTACCA	4921
TJP3	NM_014428.1	Tight junction protein 3	F GGAAGCAGGACATTTTCTGGAA R AGCTCAAAGTGAGTGCGGATGTA	1379
PPL	NM_002705.4	Periplakin	F GAGAGGGAGGTCAGCGATCTC R TCCAAGGCCCATATCTTTCG	2770
EVPL	NM_001988.1	Envoplakin	F GTTGGGCCAGGTAGGATACG R GAGCCCATCACCATGTTAGTAAAA	224
XLKD1	NM_006691.2	Extracellular link domain containing	F ATCCGGATGTCTCGGTTATGAA R AACTAGTCCGGATGGAGAGTTCTG	2367
NEFL	NM_006158.2	Neurofilament, light polypeptide 68 kDa	F CCGCTCCTTCCCGTCCTACT R GGTTTCCTCCACTTCGGTCTGC	260
IL1F9	NM_019618.2	Interleukin 1 family, member 9	F GGTTCCCAATGTGTTTTCGTCT R TGTCTCAGCACCAGCGTGAA	496
SLC1A4	NM_003038.2	Solute carrier family 1, member 4	F CAACCCCACCTTCCACCAT R GGGTCTGGGAGTCACAGCAA	1122
LOC441347	XM_496974.2	Similar to family with sequence similarity 9, member C	F TGTGCCATTAGGAGTCTGATAG R ATACTACCCAAAGCACTCTACA	
DUSP9	NM_001395.1	Dual specificity phosphatase 9	F CATCTGGTGGGCTGTTTTGTT R CCCCAGTGATCCCGTCAA	435
CD164	NM_006016.3	CD164 antigen, sialomucin	F ACCTGTGCGAAAGTCTACCTTTG R ACAGCCTGCACACCCAAGA	2409
OAS1	NM_016816.2	2',5'-oligoadenylate synthetase 1, 40/46 kDa	F CCAGGGATTTCGGACGGTCTTG R AGGCGTGGGTTTCGTGAGC	741
LOC285412		Similar to Epidermal Langerhans cell protein LCP1	F TTGGGTCACATAGTAGTAGAGT R TGCCATCTGTTCTTAGACT	
DDX5	NM_004396.2	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	F GAATTTCACTGAACCCACTGCTATT R TGCCACTCCAACCATATCCA	1741

Confirmation of the microarray measurement by Q-RT-PCR. Q-RT-PCR was performed to verify the microarray findings using 13 pairs of samples that were used in the microarray analysis as well as an additional 15 adjacent normal/tumor-matched ESCC samples. Two of the up-regulated discriminators, aurora kinase A (*AURKA*) and transforming growth factor, ß-induced, 68 kDa (*TGFBI*), were also found overexpressed in most of the tumors in comparison with the adjacent normal samples by Q-RT-PCR (Fig. 2A and B). Together, a total of 29 genes, including 19 up-regulated and 10 down-regulated ones, were selected from the genes listed in Table III. In 15 normal/tumor-matched samples, 26 of the 29 selected discriminators were shown differentially expressed between adjacent normal and tumor-matched specimens by Q-RT-PCR (p<0.05, asterisk) (Table V). The results were in good agreement with those from the microarray data.

Comparison of this expression profile with a published dataset. By comparing our expression profile with a recently published microarray dataset from 20 matched adjacent normal and tumor specimens of ESCC (8), 75 genes (25 up- and 50 down-regulated genes) (Table III) exhibited similar fold-changes without any contradictory result. This is a notable observation since there is usually a low percent overlap of differentially expressed genes observed when compared to the union of the different microarray platforms (19).

Table III. Differentially expressed genes of ESCC.

Discriminators revealed by microarray (135 genes, 166 transcripts)

Up-regulated[a] (40 genes)	Down-regulated[a] (95 genes)	
MMP1[b,c]	*P11*[b,c]	*RAGD*[b]
INHBA[b,c]	*ARS*[b,c]	*CEACAM1*[b,c]
MMP3[b,c]	*IL1RN*[b]	*ABLIM1*[b,c]
MMP10[b,c]	*SGP28*	*NMU*[b,c]
SPP1[b,c]	*SCEL*[b]	*PITX1*[b]
SERPINH1[b,c]	*CLIC3*	*KIAA0089*
CST1	*MYH11*[b]	*EHD3*[b,c]
IMP-2[b,c]	*CYP4B1*[b,c]	*TJP3*[d]
COL1A1[b,d]	*TMPRSS2*[b,c]	*IL8RB*[b,c]
COL1A2[b,c,d]	*KLK13*[b]	*CCNG2*[b,c]
OSF-2[b,c]	*CLCA4*	*KIAA0790*
MMP11[b,c]	*HPGD*[b,c]	*CEACAM7*[b,c]
APOL1	*RHCG*	*FLJ13841*[b,c]
APOE[b]	*FUT6*[b]	*MGLL*[b,c]
BGN[b,d]	*KIAA0227*	*NEBL*[b,c]
CDC2[b,c,d]	*XLKD1*	*NUCB2*[b,c]
FAP[b,c]	*DESC1*[b]	*CES2*[b]
PLAU[b,c]	*GPX3*[b,c]	*GALNT12*[b]
MMP9[b,d]	*CEACAM5*	*RAB25*[b]
IFI30[b,c]	*SERPINB4*	*MGC11335*
NETO2	*FLG*	*CAST*[b,c]
G1P3[b,c]	*SERPINB3*[b]	*MAPK3*
ECT2[b,c]	*EPS8R1*	*CYP2C9*[b]
PIR51[b,c]	*TGM1*[b,c]	*ZNF426*
DPP3[b]	*CYP3A5*[b,c]	*SORT1*
CKS2[b,c,d]	*PPL*[b,c,d]	*TM4SF6*[b,c]
LOXL2[b,c]	*HLF*[b,c]	*EPS8R2*
LOC146909	*PRSS3*[b,c]	*PIG3*
TPX2[b,d]	*EMP1*[b,c]	*ESPL1*
DKFZp762E1312	*EDN3*[b,c]	*FLJ10948*
MCM2[b]	*MGC4309*	*KIAA0165*
KIF14[b,c]	*HSHUR7SEQ*	*GSN*[b]
AURKA[b,c,d]	*SERPINB13*[b]	*LOC57228*
HMGB3[b,c]	*FLJ22408*	*TIAM1*[b,c]
PSMB9[b,c]	*PTN*[b,c]	*LPIN1*[b,c]
ANP32E	*BENE*[b,c]	*RANBP9*
CENPA[b,c]	*CRABP2*[b,c]	*TJP1*[b,c,d]
LAPTM4B[b,c]	*CYP2C18*	*FYCO1*
ASPM[b]	*FLJ21511*[b]	*FTS*
KPNA2[b,c]	*ATP1A2*	*NCOA1*[b,c]
	ZNF185[b]	
	CD24[b,c]	
	le1	
	SERPINB1[b,c]	
	CYP4F12	
	SULT2B1	
	UPK1A[b,c]	
	TRY6	
	SERPINB2[b,c]	
	FUT3[b,c]	
	ID4[b]	
	IL18[b,c]	
	EVPL[b,c,d]	
	PRSS2[b,c]	
	CYP3A5P2	

Table III. Continued.

Genes revealed by protein-protein interaction network analysis (6 genes, 10 transcripts)

Up-regulated (5 genes)	Down-regulated (1 gene)
FN1[b,d]	*VWF*[d]
CD44[b,d]	
SPARC[b,c,d]	
TGFBI[d]	
PXN[b,d]	

[a]Gene expression in tumor tissue <1.5-fold of the normal counterpart was defined as down-regulation and one with >1.5-fold of the normal counterpart was defined as up-regulation. [b]These genes have been reported in ESCC or others. [c]These genes showed similar fold-changes as reported by Greenawalt *et al.* [d]These genes served as the minimal discriminators based on components of the protein-protein interaction network of ESCC (Fig. 4).

To address whether the newly identified 135 ESCC (Table III) and 26 Q-RT-PCR validated (Table V) discriminators could be applied to classify other ESCC microarray datasets, we applied PCA to test their capability of discrimination in this recently published dataset (8). By using relative expression levels of 20 normal/tumor-matched ESCC specimens (http://www.ebi.ac.uk/microarray-as/aer/dataselection?expid=956697506), PCA correctly classified most of these 20 matched specimens by our ESCC signatures (Fig. 3).

From 135 molecular signatures to network construction. By using POINT and POINeT, 40 up-regulated genes (labeled in blue, yellow or red) were presented as queries to search for their interacting proteins (labeled in grey), and most of them interacted with other queries and formed a highly connective network (Fig. 4A). Five of them interacted with each other [matrix metallopeptidase-9 (*MMP9*), collagen, type I α2 (*COL1A2*), α1 (*COL1A1*), biglycan (*BGN*)] or themselves [CDC28 protein kinase regulatory subunit 2 (*CKS2*)]. These queries (red), which interact with more than one query, are referred to as hubs. Another four proteins [TPX2, microtubule-associated, homolog (*Xenopus laevis*) (*TPX2*)/*AURKA* and *CKS2*/cell division cycle 2 (*CDC2*), labeled in yellow/ red] also interacted with each other. It is believed that the increased number of interacting proteins associated with a query (or hub) is the most important feature that determines the essentialness of a given protein (20). We then added the interacting proteins (green) of these selected queries (Fig. 4B). In order to narrow down the network, these interacting proteins (green) must exhibit differentially expressed patterns (p<0.05) from the microarray data to remain in the network (Fig. 4C). In contrast, of the 95 down-regulated genes, only four of them [periplakin (*PPL*)/envoplakin (*EVPL*) and tight junction protein 3 (*TJP3*)/protein 1 (*TJP1*), labeled in yellow] interacted with each other or through additional interacting proteins (green) (Fig. 4D). However, these additional interacting proteins (green) (Fig. 4E) did not exhibit differential gene expression patterns between adjacent normal and tumor-matched specimens, and were therefore removed from the network (Fig. 4F). These analyses demonstrated that the
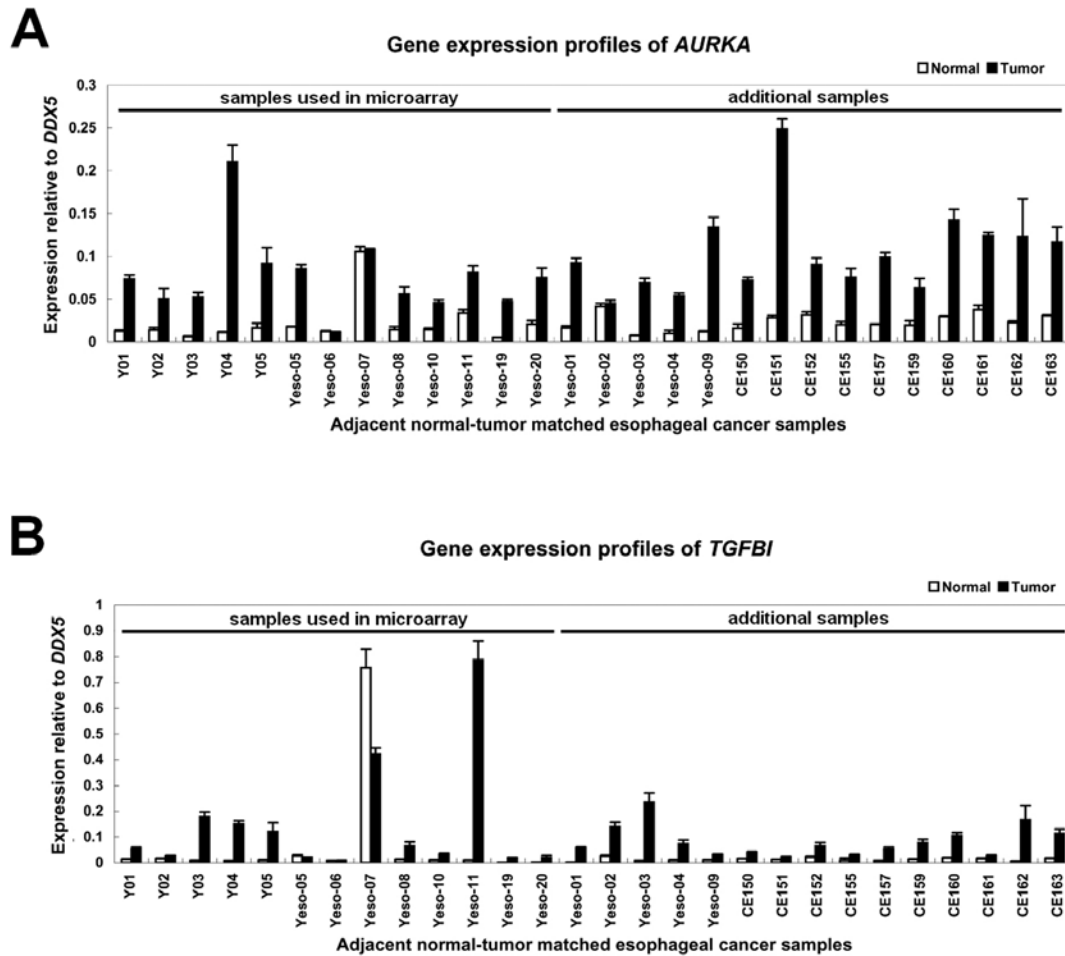
Figure 2. Q-RT-PCR confirmation of the microarray measurements. Gene expression of (A) *AURKA* and (B) *TGFBI* was found up-regulated in most of the ESCC specimens, except for one case.
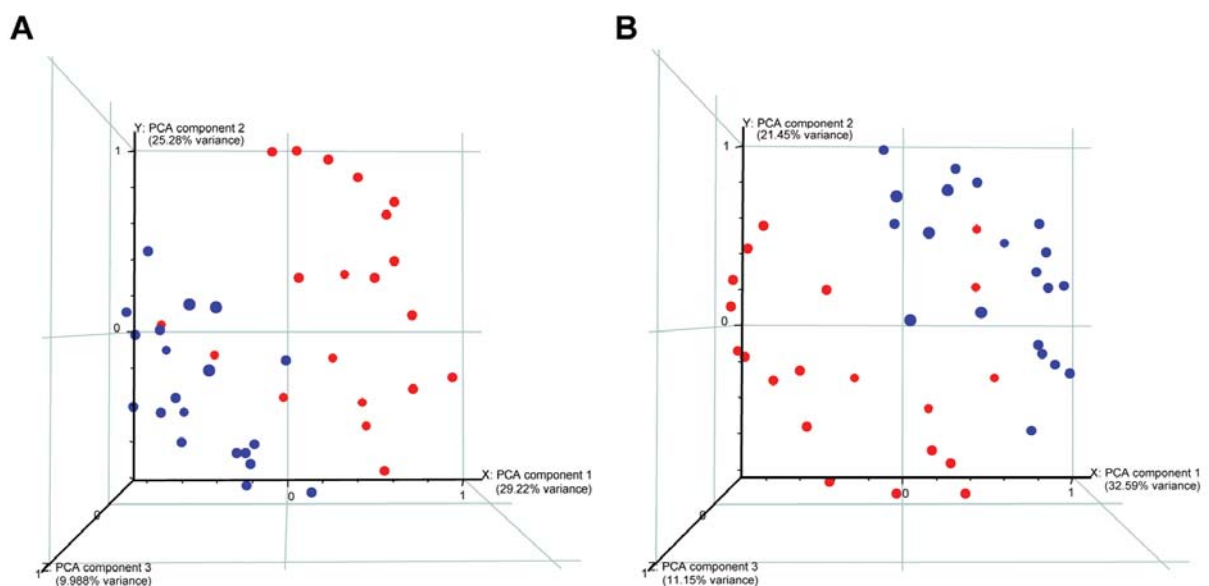

Figure 3. Classification capability of different matched ESCC specimens by our newly identified ESCC signatures. Twenty adjacent normal/tumor-matched ESCC specimens were reported by Greenawalt *et al*. Multidimensional scaling plot illustrates the ability of these 135 (A) and 26 Q-RT-PCR validated (B) genes to separate ESCC (red dots) from adjacent normal (blue dots) specimens. The proportion of variance explained by the first three principal components reached ~64 (A) and 65% (B), and thus justified a three-dimensional representation. The results indicated that the specimens were mostly correctly classified by these signatures.
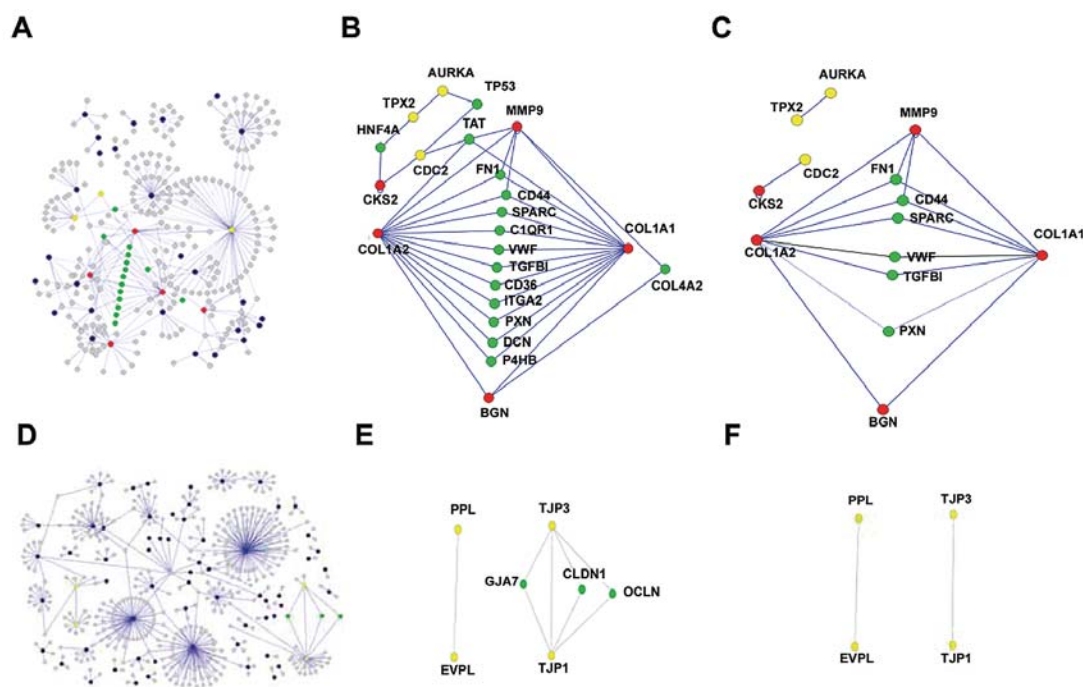
Figure 4. Construction of the protein-protein interaction (PPI) networks of the 166 ESCC molecular signatures by using POINT and POINeT. (A) The PPI network of the 40 up-regulated genes (referred to as queries and labeled in red, yellow or blue) and their interacting proteins (labeled in grey or green) are illustrated. Several queries can interact with >1 query or themselves (referred to as hubs and labeled in red). In contrast, some queries can interact with only one query (yellow) or none (blue). (B) In order to narrow down the network, we kept only those queries (red or yellow) that interacted with at least one query and their interacting proteins (green), and (C) eliminated those interacting proteins (green) that did not have differentially expressed patterns from the microarray data. (D) A similar network was constructed by using 95 down-regulated genes as a template. (E) Only *PPL/EVPL* and *TJP3/TJP1* (yellow) interacted with each other or through additional interacting proteins (green). (F) Since these interacting proteins did not exhibit differential gene expression patterns as determined by microarray profiling, they were removed from the network.
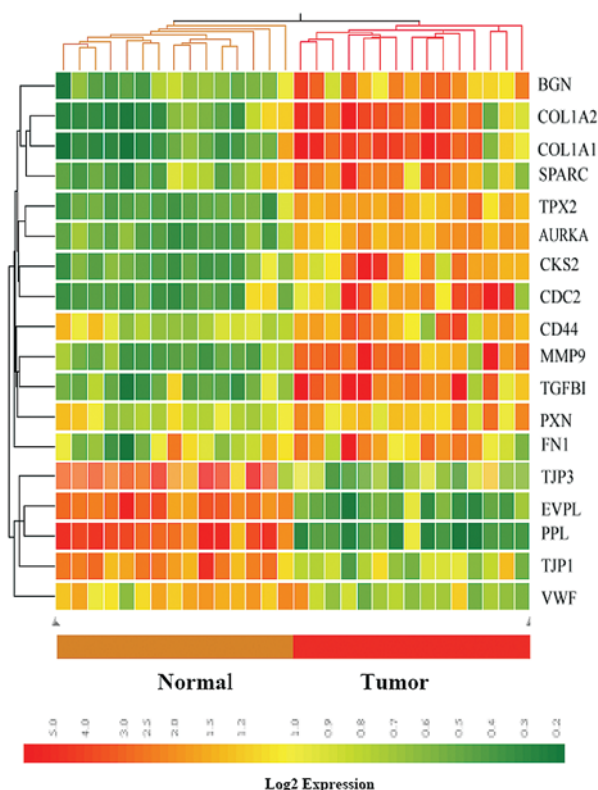

Figure 5. Eighteen genes (23 transcripts) derived from PPI analysis were used to illustrate the gene expression patterns of adjacent normal and tumor-matched ESCC specimens based on supervised hierarchical clustering to arrange the transcripts (y-axis) and samples (x-axis).

generation of a highly interactive network from the over-expressed ESCC gene signatures might not be a random process, because the chance to form an interactive network was not correlated with the size of the dataset (the larger down-regulated gene dataset was unable to form such a network).

To explore the existence of minimal discriminators to distinguish ESCC from matched adjacent normal specimens, those genes appearing in the final network, including 18 genes (23 transcripts) from Fig. 4C and F, consisting of 13 up-regulated and 5 down-regulated ones, were used. Six of them were not in the list of 135 discriminators (Table III). The gene expression patterns of 16 out of the 18 genes were confirmed by Q-RT-PCR (Table V). Using supervised hierar-chical clustering to arrange the transcripts (y-axis) and samples (x-axis), these 18 genes were able to serve as the smallest group of discriminators between tumor and adjacent normal-matched specimens in ESCC (Fig. 5). Finally, a literature review showed that 10 [*BGN*, *COL1A1*, *COL1A2*, *MMP9*, *CD44*, fibronectin 1 (*FN1*), *TGFBI*, paxillin (*PXN*), secreted protein, acidic, cysteine-rich (*SPARC*) and von Willebrand factor (*VWF*)] out of the 18 genes from the constructed networks of ESCC were associated with tumor metastasis (21-28).

Discussion

In this gene profiling study of ESCC, we were able to identify 135 normal/tumor discriminators, for which the ability of classification was confirmed in another dataset of microarray

Table IV. Comparison of Gene-Oncology (GO)-term distribution between up- and down-regulated genes.

GO-term	Up-regulated genes	No. of genes	Percentage	Down-regulated genes	No. of genes	Percentage	p-value
Cellular component							
Extracellular matrix	*MMP10*, *COL1A2*, *SPP1*, *MMP9*, *BGN*, *MMP11*, *OSF-2*, *MMP3*, *COL1A1*, *MMP1*	10	31.25	S*GP28*	1	1.75	9.17E-03
Membrane	*LAPTM4B*, *NETO2*, *LOXL2*, *FAP*, *G1P3*	5	15.62	*IL8RB*, *CEACAM5*, *TGM1*, *FUT3*, *CYP2C9*, *XLKD1*, *SORT1*, *GALNT12*, *RHCG*, *EMP1*, *FTS*, *FLG*, *NUCB2*, *TJP3*, *FYCO1*, *CYP3A5*, *TJP1*, *FUT6*, *CEACAM7*, *CEACAM1*, *UPK1A*, *EVPL*, *CD24*, *CYP2C18*, *CLCA4*, *CYP4F12*, *TMPRSS2*, *ATP1A2*, *CYP4B1*, *CLIC3*	30	52.63	2.25E-02
Cell fraction	No gene	0	0	*TGM1*, *FUT3*, *CYP2C9*, *XLKD1*, *EMP1*, *FLG*, *GPX3*, *CYP3A5*, *TJP1*, *CEACAM1*, *CYP2C18*, *EDN3*, *CYP4F12*, *CYP4B1*, *CLIC3*	15	26.32	2.25E-02
Molecular function							
Metallopeptidase activity	*MMP10*, *FAP*, *MMP9*, *MMP11*, *DPP3*, *MMP3*, *MMP1*	7	30.43	No gene	0	0	2.74E-02

Membrane, double layer of lipid molecule and its associated proteins in eukaryotes. Cell fraction, a generic term for parts of cells prepared by disruptive biochemical techniques which included soluble, insoluble, PME, and membrane fractions.

of ESCC (Fig. 3). Furthermore, in combination with a more functional-based PPI study, we identified 18 highly inter-acting genes, of which six were not disclosed in the initial expression profile analysis using stringent statistical criteria (Table III). Nonetheless, these 18 genes still served as the smallest group of normal/tumor discriminators (Fig. 5).

AURKA is an up-regulated gene from among the final 18 discriminators. *AURKA*, which encodes a serine/threonine kinase associated with chromosomal distribution, has been correlated with distant lymph node metastasis and poor prognosis in ESCC (29). *AURKA* can phosphorylate and interact with *TPX2*, and our study revealed the same gene expression patterns of both genes in ESCC specimens. Similar expression profiles were also observed between another two interacting genes, *CKS2* and *CDC2* (Table V and Fig. 5). Notably, these genes represented the 'proliferation signature' identified by Greenawalt *et al* (8). This result supports the view that common expression clusters could be functionally linked (30), and the conversion of gene expression profiling into functional features, such as protein-protein interactions, could reveal novel insights into the carcinogenesis of ESCC.

A set of 10 metastasis-associated genes, including *MMP9*, *BGN*, *COL1A1*, *COL1A2*, *SPARC*, *CD44*, *TGFBI*, *PXN*, *FN1*

and *VWF*, form a highly interactive network (Fig. 4). *MMP9*, *BGN*, *COL1A1* and *COL1A2*, which were identified as 'hubs' due to their interactions with more than one query in POINT, were identified as significantly up-regulated genes with GO-term 'extracellular matrix' (Table IV). *SPARC* and *COL1A2* were also revealed as genes in 'SPARC cluster' by Greenawalt *et al* (8). *COL1A1* has been shown to induce disruption of cell-cell contacts and promote proliferation of pancreatic carcinoma cells (24). Up-regulation of *TGFBI* has been reported in oral cancer (31), and *TGFBI*-induced expression of *BGN* has been implicated in cellular adhesion and migration (25). High *SPARC* and *FN1* expression was significantly associated with poor prognosis in ESCC (7,21,23). Overexpression of *PXN*, a multi-domain adaptor that recruits both structural and signaling molecules to focal adhesions, has been associated with enhanced metastatic potential of osteosarcoma (22). *MMP9* is a member of the metallopeptidases. *CD44* is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. Overexpression of *MMP9* and *CD44* has been correlated with metastasis and poor prognosis of ESCC (26,28). *VWF* is a glycoprotein functioning as both an antihemophilic factor carrier and a platelet-vessel wall mediator in the blood coagulation system, and in tumor cells it could play a protective role against dissemination (27).

Table V. Gene expression profiles of the Q-RT-PCR of 15 pair-wised ESCC specimens were analyzed using Wilcoxon signed-rank test.[a]

Gene name	Accession	N	Mean (normal)	Mean (tumor)	p-value
Up-regulated					
ASPM	NM_018136	15	0.010	0.037	0.001[b]
AURKA	NM_003600	15	0.026	0.086	0.001[b]
BGN	NM_001711	15	0.175	0.738	0.001[b]
CD44	NM_000610	15	0.957	1.598	0.015[b]
CDC2	NM_001786	15	0.009	0.037	0.001[b]
CKS2	NM_001827	15	0.353	0.987	0.002[b]
COL1A1	NM_000088	15	2.969	16.427	0.001[b]
COL1A2	NM_000089	15	2.080	8.563	0.003[b]
DPP3	NM_005700	15	0.171	0.256	0.017[b]
INHBA	NM_002192	15	0.012	0.056	0.005[b]
KPNA2	NM_002266	15	0.013	0.039	0.001[b]
MMP9	NM_004994	15	0.134	0.370	0.003[b]
PXN	NM_002859	15	0.056	0.121	0.001[b]
SPARC	NM_003118	15	0.859	3.529	0.005[b]
SPP1	NM_000582	15	0.308	3.181	0.006[b]
TGFBI	NM_000358	15	0.059	0.159	0.009[b]
TPX2	NM_012112	15	0.168	0.481	0.001[b]
FN1	NM_212482	15	0.034	0.040	0.307
MMP3	NM_002422	15	0.128	0.167	0.172
Down-regulated					
CCNG2	NM_004354	15	0.283	0.068	0.004[b]
EVPL	NM_001988	15	0.931	0.146	0.001[b]
GPX3	NM_002084	15	1.492	0.150	0.001[b]
MYH11	NM_002474	15	1.001	0.230	0.001[b]
PPL	NM_002705	15	0.422	0.035	0.001[b]
SERPINB3	NM_006919	15	4.901	0.504	0.009[b]
TJP3	NM_014428	15	0.080	0.011	0.001[b]
VWF	NM_000552	15	0.128	0.069	0.005[b]
XLKD1	NM_006691	15	0.023	0.012	0.011[b]
TJP1	NM_003257	15	0.101	0.069	0.211

[a]In total, 26 of 29 genes from 135 discriminators were differentially expressed between normal and tumor specimens. [b]$p<0.05$.
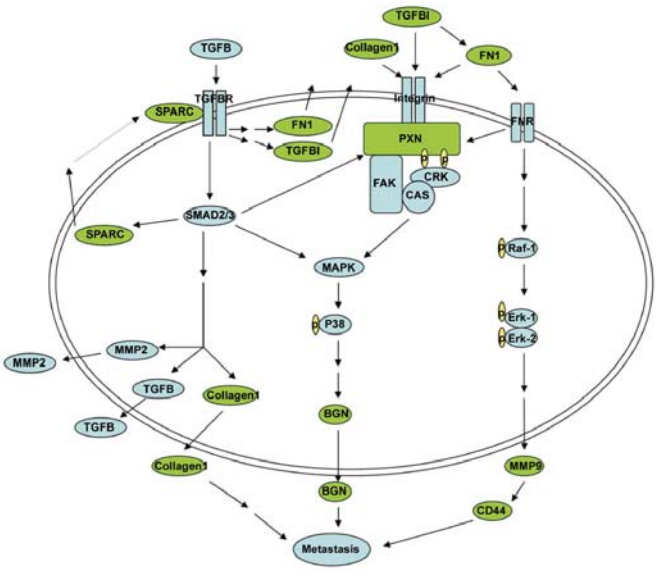

Figure 6. A proposed model for the putative relationships between tumor metastasis-associated genes in ESCC. Genes marked as green were those revealed by PPI analysis. *FAK*, focal-adhesion kinase; *CRK*, v-crk sarcoma virus CT10 oncogene homolog. For the other abbreviations and description of the model, please see Table II and text for details.

By using the 9 overexpressed genes as a basis and searching related genes through literature review, we were able to create an invasion and metastasis model for ESCC (Fig. 6). Transforming growth factor ß (*TGFB*) binds to its receptor (*TGFBR*) to modulate *SMAD2/3* signaling with subsequent induction of matrix metalloproteinase-2 (*MMP2*), collagen type 1, *SPARC*, and *TGFB* expression (32-34). *SPARC* could have positive feedback interaction with the *TGFBR* complex (34). The activated *TGFBR* complex could phosphorylate *SMAD3* and induce *BGN* expression through the *p38MAPK* cascade (25), as well as promote *FN1* (32) and *TGFBI* expression. *TGFBI*, *FN1* and collagen type 1 could

bind integrin and stimulate the expression of *PXN* protein (35), which then activates the *p38MAPK* pathway. In parallel, *TGFB1* induces the interaction between *FN1* and fibronectin receptor (FNR) (36), which increases the expression of *MMP9* through the v-raf-1 murine leukemia viral oncogene homolog (*RAF1*)/extracellular signal-regulated kinase (*ERK*) pathway (21,37). *CD44* serves as an anchor for *MMP9* on the cell surface (38), and the *CD44/MMP9* complex, *BGN* and collagen type 1 could then mediate tumor invasion and metastasis (24,25,38).

Among the 4 down-regulated genes, both *PPL* and *EVPL* encode components of desmosomes. *EVPL* is localized at the tylosis esophageal cancer (TOC) locus, which is commonly deleted in ESCC (39). *PPL* was found down-regulated in ESCC by proteomic analysis (40). *TJP1* and *TJP3* are membrane proteins located at intercellular tight junctions, and *TJP1* was found down-regulated in colon cancer cell with liver metastasis (41). Although the 4 down-regulated genes did not form a highly interacting network, their roles in the pathogenesis of ESCC still warrant further study.

In summary, our expression profiles provide disease-specific expression signatures for ESCC. Furthermore, by using PPI databases, we were able to identify additional important differentially expressed genes, and convert them into a functional network which is most likely involved in metastasis of ESCC. Based on the findings presented here, we postulate a model depicting the signaling pathways which induce cell invasion and metastasis of ESCC, and we offer a series of potential therapeutic targets for ESCC.

Acknowledgements

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