Advances in Brief

Identification of Differentially Expressed Genes in Esophageal Squamous Cell Carcinoma (ESCC) by cDNA Expression Array: Overexpression of *Fra-1*, *Neogenin*, *Id-1*, and *CDC25B* Genes in ESCC[1]

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Abstract

Purpose: **This study aims to identify differentially expressed genes in esophageal squamous cell carcinoma (ESCC) through the use of a membrane-based cDNA array.**

Experimental Design: **Two newly established human ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding to a morphologically normal, esophageal epithelium tissue specimen, prospectively collected from the HKESC-2-related patient, were screened in parallel using a cDNA expression array containing gene-specific fragments for 588 human genes spotted onto nylon membranes.**

Results: **The results of cDNA expression array showed that 53 genes were up-regulated 2-fold or higher and 8 genes were down-regulated 2-fold or higher in both ESCC cell lines at the mRNA level. Semiquantitative RT-PCR analysis of a subset of these differentially expressed genes gave results consistent with cDNA array findings. Four of the differentially expressed genes that belong to the categories of oncogenes/tumor suppressor genes (*Fra-1* and *Neogenin*) and cell cycle-related genes (*Id-1* and *CDC25B*) were studied more extensively for their protein expression by immunohistochemistry. The two ESCC cell lines and their corresponding primary tissues, 61 primary ESCC resected specimens and 16 matching, morphologically normal, esophageal epithelium tissues were analyzed. The immunostaining results showed that *Fra-1*, *Neogenin*, *Id-1*, and *CDC25B* were overexpressed in both ESCC cell lines and their corresponding primary tumors at the protein level, validating the microarray findings. The results of the clinical specimens showed that the *Fra-1* gene was overexpressed in ESCC compared with normal esophageal epithelium in 53 of 61 cases (87%), *Neogenin* in 57 of 61 cases (93%), *Id-1* in 57 of 61 cases (93%), and *CDC25B* in 48 of 61 cases (79%). Furthermore, the expression of *Fra-1*, *Neogenin*, and *Id-1* in ESCC correlated with tumor differentiation.**

Conclusions: **Overall, this study demonstrates that multiple genes are differentially expressed in ESCC and provides the first evidence that oncogenes *Fra-1* and *Neogenin* and cell cycle-related genes *Id-1* and *CDC25B* are overexpressed in ESCC.**

Introduction

Esophageal carcinoma is the ninth most common human cancer in the world and the second most common cancer in China (1). In Hong Kong, ESCC[3] accounts for ~90% of esophageal malignant tumors and is the sixth most common cause of cancer death (2). Despite advances in multimodality therapy, the overall 5-year survival rates for ESCCs still remain poor (3). The development of new treatment modalities, diagnostic technologies, and preventive approaches will require a better understanding of the molecular mechanisms underlying esophageal carcinogenesis. Although recent reports have documented alterations of a few oncogenes and tumor suppressor genes in ESCC, the molecular and genetic basis of esophageal carcinogenesis still remains largely unknown (4, 5).

With the emerging technology of cDNA array hybridization, it is now possible to screen for alterations in the expression of many genes simultaneously (6–8). Because the development and progression of cancer are accompanied by complex changes in patterns of gene expression (9, 10), the cDNA array technology provides a very useful tool for studying these complex processes (6). In this study, we used cDNA expression array hybridization to examine the expression of 588 genes in two newly established ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding, morphologically normal, esophageal epithelium tissue specimen collected prospectively from the HKESC-2-related patient. By comparing gene expression levels between normal esophageal epithelium and the ESCC cell lines, we were able to identify the differentially expressed transcripts in ESCC. Subsequent semiquantitative RT-PCR analyses vali-

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[3] The abbreviations used are: ESCC, esophageal squamous cell carcinoma; *Fra-1*, fos-related antigen 1; *CDC25B*, cell division cycle 25B; *Id-1*, inhibitor of differentiation 1 (inhibitor of DNA binding 1); IH, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; *FPR1*, formyl peptide receptor 1; *RANTES*, regulated on activation, normal T expressed, and secreted; AP-1, activator protein-1; CDK, cyclin-dependent kinase; DAB, 3,3′-diaminobenzidine.

dated the cDNA array results. Expression of the protein products of four of these differentially expressed genes that belong to the categories of oncogenes/tumor suppressor genes (*Fra-1* and *Neogenin*) and cell cycle-related genes (*Id-1* and *CDC25B*) was further evaluated by IH in a large series of ESCC tumor specimens.

Materials and Methods

Cell Culture and Tissue Specimen. Two human ESCC cell lines, HKESC-1 and HKESC-2, have been established recently in our laboratory. HKESC-1 has been reported previously (11). Both cell lines were from Hong Kong Chinese patients with moderately differentiated ESCC; HKESC-1 was from a 47-year-old man, whereas HKESC-2 was from a 46-year-old woman. The squamous epithelial nature of HKESC-2 was confirmed by both electron microscopy (presence of tonofilaments and desmosomes) and immunohistochemical staining (positive for cytokeratins; data not shown). Both cell lines grew as adherent monolayers (11). HKESC-2 was maintained in the same conditions as HKESC-1 (11). Cells were harvested from passage 31 of HKESC-1 and passage 4 of HKESC-2 at 80–90% confluency, respectively.

One morphologically normal, esophageal epithelium tissue specimen, collected prospectively from the HKESC-2-related patient, was used as a control for the array experiment. For obtaining high-purity normal esophageal epithelium tissue specimen, the morphologically normal esophageal epithelium at least 5 cm away from the tumor margin was carefully dissected out from other tissues of the freshly resected esophagectomy specimen from the HKESC-2-related patient and evaluated microscopically. Unfortunately, the collected normal esophageal epithelium tissue from the HKESC-1-related patient could not be used as a control because the specimen was too small and only a small amount of RNA could be extracted from it.

cDNA Arrays, Probes, Hybridization, and Data Analysis. Atlas Human cDNA Expression Array membranes used in this study were purchased from Clontech (Palo Alto, CA). The membrane contained 10 ng of each gene-specific cDNA from 588 known genes and 9 housekeeping gene fragments (Fig. 1). Several plasmid and bacteriophage DNAs and blank spots are also included as negative and blank controls to confirm hybridization specificity.[4]

Total RNA was extracted using the Trizol reagent protocol (Life Technologies, Inc., Gaithersburg, MD) from the two ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding, morphologically normal esophageal epithelium collected prospectively from the HKESC-2-related patient. mRNA was then isolated from the total RNA using the Straight A's mRNA Isolation System (Novagen, Madison, WI). The ^{32}P-labeled cDNA probes were generated by reverse transcription of 1 μg of mRNA of each sample in the presence of [α-^{32}P]dATP. Equal amounts of cDNA probes (3×10^6 cpm/μl) from the ESCC cell lines and normal esophageal epithelium were then hybridized to separate Atlas Human cDNA array membranes for 24 h at 42°C and washed according to the supplier's instructions. The array membranes were then exposed to X-ray film at −70°C for 2–5 days. Autoradiographic intensity was analyzed using AtlasImage analysis software (version 1.01; Clontech). The signal intensities were normalized by comparing the expression of housekeeping genes *GAPDH* (G12) and *HLA-C* (G14):

$$\text{Intensity ratio} = \frac{\text{Adjusted intensity on array} - \text{HKESC-1 or HKESC} - 2}{\text{Adjusted intensity on array} - \text{normal}} \times \text{Normalization coefficient}$$

$$\text{Adjusted intensity} = \text{Intensity} - \text{Background}$$

$$\text{Normalization coefficient} = \left[\left(\frac{\text{Adjusted intensity } \textit{GAPDH} \text{ on array} - \text{normal}}{\text{Adjusted intensity } \textit{GAPDH} \text{ on array} - \text{HKESC-1 or HKESC-2}}\right)\left(\frac{\text{Adjusted intensity HLA} - \text{Con array} - \text{normal}}{\text{Adjusted intensity HLA} - \text{Con array} - \text{HKESC-1 or HKESC-2}}\right)\right] \div 2$$

Genes were considered to be up-regulated when the intensity ratio between expression in the ESCC cell lines compared with normal esophageal epithelium was 2-fold or greater. Genes were labeled as down-regulated when the ratio between normal and ESCC cell lines was 2-fold or higher.

RT-PCR. cDNA was generated using 1 μg of total RNA from the two ESCC cell lines (HKESC-1 and HKESC-2) and one corresponding, morphologically normal, esophageal epithelium collected prospectively from the HKESC-2-related patient as template and 2.5 mM Oligo $d(T)_{16}$ primers in a 20-μl reaction mixture, and the reverse transcription was carried out at 42°C for 1 h, followed by 95°C for 10 min using the GeneAmp RNA PCR Core kit (Perkin-Elmer, Branchburg, NJ). Two μl of cDNA were amplified in a 25-μl PCR reaction mixture containing 1× PCR buffer, 1.9 or 2.4 or 2.9 mM $MgCl_2$, 0.5 μM primers, 0.18 mM deoxynucleotides triphosphates, 1 unit of AmpliTaq Gold DNA polymerase with hot-start PCR as follows: 95°C for 10 min, followed by 25–35 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C (for primers of *cyclin D1*, *Id-1*, *CDC25B*, *FPR1*, *RANTES*, and *GAPDH*) or 62°C (for primers of *Fra-1*) or 65°C (for primers of *Neogenin*), 1 min extension at 72°C. Finally, PCR products were fully extended by incubating at 72°C for 10 min. The PCR reagents were purchased from Perkin-Elmer.

The sequences of gene specific primers for RT-PCR were the same as those of cDNA array (data not shown because of the copyright agreement by Clontech, Palo Alto, CA), except for the primers specific for *Fra-1*, which were the same as described before (12). All of the primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The cycle number was optimized for each gene-specific primer pair to ensure that amplification was in the linear range and the results were semiquantitative. Twelve μl of PCR product were visualized by

[4] A complete list of the 588 genes with array positions and GenBank accession numbers of the Atlas Human Expression Array used here can also be accessed through the web site http://www.clontech.com/clontech/APR97UPD/Atlaslist.html.

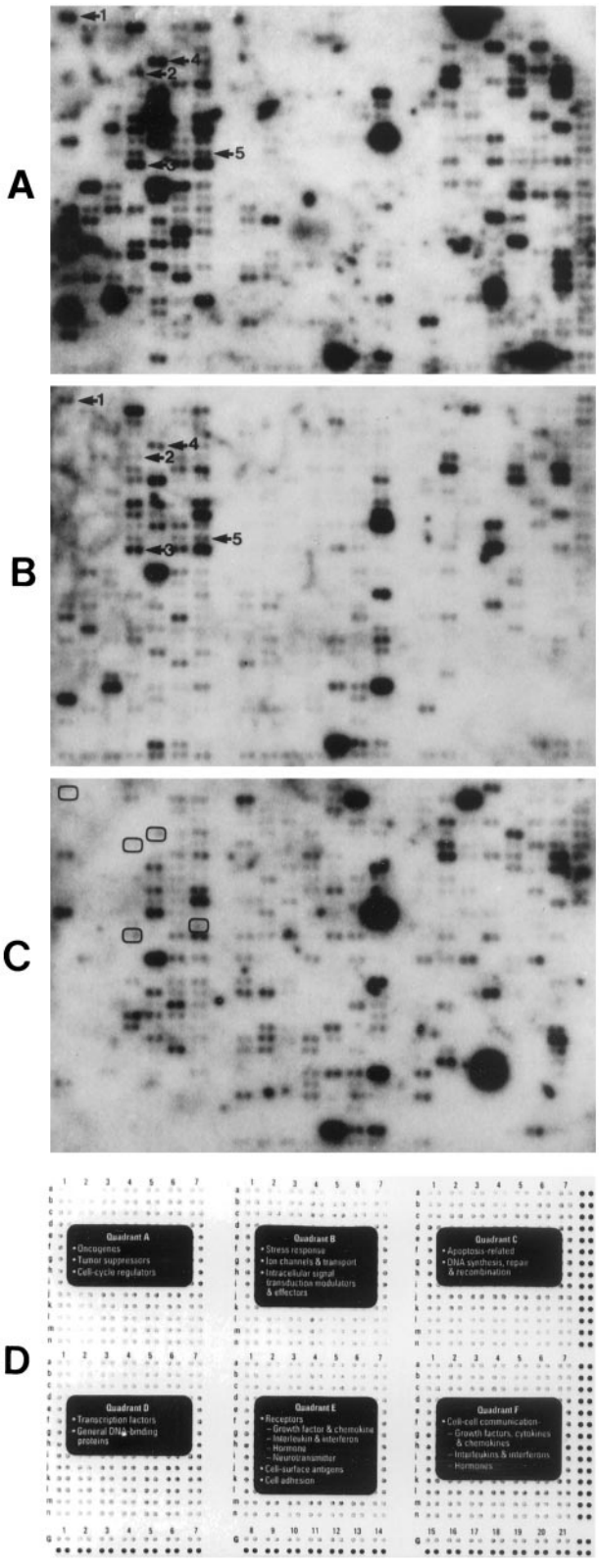

Fig. 1 *A–C*, gene expression profiles of two human ESCC cell lines HKESC-1 (*A*) and HKESC-2 (*B*) and one morphologically normal esophageal epithelium (*C*) from the HKESC-2-related patient using the Atlas Human cDNA Expression Array. Some of the differentially expressed genes are indicated: *1*, c-*myc* (A1a); *2*, *Fra-1* (A4f); *3*, *Neogenin* (A4n); *4*, *Id-1* (A5e); and 5, *CDC25B* (A7m). *D*, schematic diagram of Atlas Human cDNA Expression Array. The array contains 588 human genes spotted in duplicate and divided into six functional categories (*Quadrants A–F*). Three blank (G1, G8, and G15) and nine negative (G2–4, G9–11, and G16–18) controls are included to confirm hybridization specificity. Nine housekeeping genes (G5–7, G12–14, and G19–21) are also included in the array for normalizing mRNA abundance. Genomic DNA spots (*dark dots*) serve as orientation marks to facilitate in the determination of the coordinates of hybridization signals. A complete gene list with array coordinates and GenBank accession numbers is available at world wide web site http://www.clontech.com/clontech/APR97 UPD/Atlaslist.html.

electrophoresis on a 2% agarose gel stained with ethidium bromide and quantitated by densitometry using a dual-intensity transilluminator equipped with Gelworks 1D Intermediate software (version 2.51).

Collection of Tissues and Clinicopathological Data. The tissues were obtained from 61 (50 men and 11 women) patients with ESCC resected between 1996 and 1998 in Queen Mary Hospital, The University of Hong Kong. The patients' ages ranged from 41 to 83 years, with a mean age of 65 years. The specimens were dissected and examined in the fresh state. Representative tissue specimens from tumors and matching normal esophageal epithelium tissues were snap-frozen in liquid nitrogen and stored at −80°C. Other representative blocks were taken and processed in paraffin for histological examination. The carcinomas were found in the upper ($n = 10$; 16%), middle ($n = 35$; 57%), and lower ($n = 16$; 26%) third of the esophagus. The median length of the tumors was 5.5 cm (range, 1–11). The histology of the carcinomas was reviewed according to the criteria described previously (13). The ESCC tumors were well differentiated in 20 (33%) cases, moderately differentiated in 29 (48%), and poorly differentiated in 12 (20%). The carcinomas were staged according to the Tumor-Node-Metastasis classification (14). Many tumors were stage III ($n = 35$, 57%) or II ($n = 23$, 38%); of the remainder, one was stage I and two were stage IV.

Immunohistochemistry. Expression of *Fra-1*, *Neogenin*, *Id-1*, and *CDC25B* was investigated by the streptavidin-biotin-peroxidase complex method. Briefly, 6-μm frozen sections were cut from two pellets harvested from cultured cell lines HKESC-1 and HKESC-2, the cell lines' corresponding primary tissues, 61 primary ESCC tumors, and 16 matching, morphologically normal, esophageal epithelium specimens. After endogenous peroxidase activity was quenched and nonspecific binding was blocked, polyclonal rabbit anti-Fra-1, goat anti-Neogenin, rabbit anti-Id-1, and goat anti-CDC25B antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated at 4°C overnight at a dilution of 1:100 for *Fra-1*, 1:40 for *Neogenin* and *Id-1*, and 1:30 for *CDC25B*, respectively. The secondary antibody was biotinylated swine anti-rabbit (for *Fra-1* and *Id-1*) or rabbit anti-goat (for *Neogenin* and *CDC25B*) antibody (DAKO, Glostrup, Denmark) used at a dilution of 1:200 for 30 min at 37°C. After washing, sections were incubated with StreptABComplex/horseradish peroxidase (DAKO; 1:100 dilution) for 30 min at 37°C. Negative controls were performed by replacing the primary antibody by normal serum.

Each section was independently assessed by two histopathologists without prior knowledge of the patients' other data. Scoring was based on the percentage of positive cells. The staining was identified as: −, no expression; +, <10% of cells were stained; ++, 10–50% of cells stained; and +++, >50% of cells stained. From ++ to +++ was defined as overexpression.

Statistical Analysis. Comparisons between groups were performed using the χ^2 test and *t* test when appropriate. $P < 0.05$ was used to determine statistical significance. All statistical tests were performed with the GraphPad Prism software version 3.0 (GraphPad Software, Inc., San Diego, CA).

Results and Discussion

In this study, we first used cDNA expression array hybridization to identify genes that were differentially expressed in ESCC compared with normal esophageal epithelium. Two newly established ESCC cell lines in our laboratory were selected for cDNA array analysis to assure large quantities of high-purity tumor mRNA. The comparison of the autoradiographic intensities between ESCC cell lines and normal esophageal epithelium showed that 65 and 59 genes were up-regulated 2-fold or higher and 11 and 21 genes were down-regulated 2-fold or more in HKESC-1 and HKESC-2, respectively. Among these differentially expressed genes, 53 genes were up-regulated and 8 genes were down-regulated in both cell lines (Fig. 1 and Table 1). No signals were visible in the three blank spots (G1, G8, and G15) and nine negative control spots (G2–4, G9–11, and G16–18; Fig. 1), indicating that the cDNA array hybridization was highly specific. Among the 61 differentially expressed genes in both cell lines, 49 genes such as *Fra-1, Neogenin, Id-1,* and *CDC25B* genes were identified as differentially expressed in ESCC for the first time; 12 other differentially expressed genes have been described to be overexpressed in ESCC previously. The genes overexpressed in both of the ESCC cell lines belong to the categories of oncogenes/tumor suppressor genes, cell cycle-related genes, genes for DNA synthesis, DNA binding genes, or apoptosis-related genes (Table 1). The 8 genes that were down-regulated in both ESCC cell lines comprised genes for signal transduction (*guanine nucleotide regulatory protein NET1, protein kinase C-*β *II, cAMP-dependent protein kinase catalytic d-subunit and EPLG3*), genes for signaling proteins (*RANTES protein T-cell specific, Somatomedin A,* and *FPR1*), and the gene for MAL protein. These findings demonstrated that multiple genes are differentially expressed in ESCC at mRNA level.

To further validate the cDNA array approach, we performed semiquantitative RT-PCR to analyze the expression levels of 8 genes, *cyclin D1, Fra-1, Neogenin, Id-1, CDC25B, FPR1, RANTES,* and *GAPDH*. The results of RT-PCR analysis (Fig. 2) were consistent with the expression profiles obtained through cDNA array hybridization (Fig. 1).

Genes that belong to the categories of oncogenes/tumor suppressor genes and cell cycle-related genes are often implicated in the pathogenesis of various cancers (4, 5, 15). Significantly, a number of the differentially expressed genes identified by cDNA array hybridization in both ESCC cell lines belong to these categories. Four of these differentially expressed genes that were identified for the first time to be overexpressed in ESCC in this study, the oncogenes *Fra-1* and *Neogenin* and the cell cycle related genes *Id-1* and *CDC25B,* were selected for more detailed study for their protein expression in a large series of ESCC tumor specimens by IH. Moreover, these genes have been reported to be overexpressed in other tumor cell lines or primary tumors (16–24). The other consideration for selecting these particular genes for more extensive study was that the suitable antibodies of these genes were commercially available for the IH studies. The protein expression of these four genes was investigated in the two ESCC cell lines and their corresponding primary tissues, 61 primary ESCC tumors, and 16 matching, morphologically normal, esophageal epithelium specimens. The results of immunostaining are summarized in Tables 2 and 3 and are shown in Fig. 3. The protein products of *Fra-1, Neogenin, Id-1,* and *CDC25B* genes were found to be overexpressed in both the ESCC cell lines and their corresponding primary tumors (Table 2), validating the cDNA array results.

Fra-1 is one component of the AP-1 complex (25). The AP-1 components are considered to play key roles in signal transduction pathways involved in complex cellular growth, differentiation, and tumorigenesis (16). Previous studies indicated that increased AP-1 activity is a necessary event in the transformation of mouse epidermal cells (26, 27). *Fra-1* overexpression has been found in kidney and thyroid cancer (16, 17). These observations suggest that *Fra-1* overexpression might play an important role in malignant transformation of epithelial cells. In the present work, *Fra-1* mRNA overexpression was detected in both ESCC cell lines by cDNA array analysis (Fig. 1) and RT-PCR (Fig. 2*B*). Also, the majority of ESCC tumors (53 of 61, 87%; Table 3; Fig. 3*B*) had enhanced expression of *Fra-1*. Fra-1 protein expression was localized in the nuclei of ESCC tumor cells (Fig. 3*B*). In contrast, morphologically normal, esophageal epithelium tissues showed low expression of Fra-1. The expression of Fra-1 was often focal in morphologically normal esophageal epithelium and always restricted to the basal cell layer (Fig. 3*A*). The well or moderately differentiated ESCC showed more intense expression of Fra-1 than poorly differentiated ones ($P < 0.0001$; Table 3).

Neogenin encodes a 1461-amino acid protein with 50% amino acid identity to *DCC* (*deleted in colorectal cancer*; Ref. 18). It has been suggested to play an integral role in regulating differentiation and/or cell migration events within many embryonic and adult tissues (28). *Neogenin* expression has been detected at low levels in many adult tissues but not including esophagus (18). Overexpression of *Neogenin* has been observed in a wide variety of human cancer cell lines from cancers of breast, pancreas, brain, cervix, colon, and rectum (18). However, there is no information about the status of *Neogenin* expression in human primary cancers including esophageal cancer. In the current study, our cDNA array (Fig. 1) and RT-PCR (Fig. 2*B*) results showed that *Neogenin* mRNA was overexpressed in both ESCC cell lines. Neogenin protein overexpression was noted in 93% (57 of 61) of ESCCs (Table 3; Fig. 3*D*). The expression was localized in the cytoplasm of tumor cells (Fig. 3*D*). In contrast, the expression of Neogenin protein in morphologically normal esophageal epithelium was negative or negligible and was restricted to the highly proliferative basal cells (Fig. 3*C*). The well or moderately differentiated ESCC

Table 1 List of differentially expressed genes in the ESCC cell lines HKESC-1 and HKESC-2 when compared with one corresponding, morphologically normal, esophageal epithelium tissue specimen (N) from the HKESC-2-related patient using cDNA expression array

Location	Name of gene	Intensity Ratio HKESC-1/N	Intensity Ratio HKESC-2/N
Genes up-regulated in both ESCC cell lines			
Oncogene/Tumor suppressor genes			
A1a	c-*myc*	2.5	3.0
A2b	*IGFBP-2*	6.9	6.0
A3b	*Snon*	7.2	6.0
A3i	*rhoA* (MDR protein)	2.8	3.9
A3k	*DCC*	5.1	3
A4b	*APC*	2.6	3.9
A4c	*BRCA2*	30029/0	42730/0
A4f	*Fra-1*	10.8	7.8
A4g	*Ezrin*	21936/0	36475/0
A4h	*JUN-D*	35.4	60.6
A4j	*PEP1*	3.6	4.8
A4k	*EB1*	2.6	3.0
A4l	*C-CBL*	2.7	2.2
A4m	*SmadI*	3.2	3.0
A4n	*Neogenin*	2.4	3.2
Cell cycle-related genes			
A5e	*Id-1*	3.4	3.7
A5g	*P58/GTA1*	4.0	2.2
A6g	*Cyclin D1*	2.1	2.3
A6l	*Cyclin B1*	2.4	3.2
A6m	*Cyclin E*	3.5	3.3
A7b	*Cyclin G2*	2.4	3.1
A7d	*p35*	6.9	7.2
A7l	*C-1*	2.6	2.6
A7m	*CDC25B*	2.0	2.5
Apoptosis-associated genes			
C1i	*Adenosine A1 receptor*	22.2	5.9
C4l	*Apopain*	8.7	13.7
Genes for DNA synthesis/repair/recombination proteins			
C6d	*XRCC1*	3.2	2.9
C6l	*DNA Topoisomerase II*	14759/0	22201/0
C7n	*Dnase X*	5.1	4.4
Genes for DNA binding/transcription factors			
D1c	*CCAT-binding protein*	9.9	8.8
D1d	*Id-3*	6.5	6.6
D1e	*BTEB2*	7.4	9.0
D1g	*Id-2*	9.8	7.6
D1l	*TAX*	3.0	4.4
D1n	*CNBP*	4.3	2.1
D2a	*CCAAT displacement protein*	4.2	4.5
D2c	*APRF*	33987/0	29011/0
D2d	*hSNF2b*	42.2	40.7
D2f	*TAXREB67*	39440/0	49383/0
D2i	*TCF5*	37478/0	23080/0
D3a	*hSNF2a*	7.1	4.2
D3b	*DB1*	316.5	225.0
D3c	*D-binding protein*	37796/0	21621/0
D3g	*PAX-8*	13.6	7.2
D3j	*P15 subunit*	38725/0	40864/0
D3k	*Guanine nucleotide-binding protein G-S*	33.0	49.2
D4c	*AP-2*	7.1	4.9
D4j	*NF-E1*	34506/0	15924/0
D5k	*PAX3*	24.8	10.6
D7k	*TAFII31*	7.0	4.8
Genes for signal proteins			
F5a	*NGF-2*	16.7	4.6
F5b	*MIP2*α	39223/0	19827/0
F5f	*IL-8*	39239/0	11638/0
Genes down-regulated in both ESCC cell lines			
Gene for iron channel/transport protein			
B1b	*MAL protein*	1/3.3	0/11989
Genes for signal transduction			
B4g	*Guanine nucleotide regulatory protein NET1*	0/28564	1/23.7
B5j	*Protein kinase c-*β *II*	0/29588	0/29588
B6b	*cAMP-dependent protein kinase* α*-subunit*	0/41060	1/304
B6n	*EPLG3*	0/22304	1/2.5
Genes for cell signaling proteins			
F1a	*Somatomedin A*	0/19560	0/19560
F1k	*FMLP-related receptor I*	0/29220	0/29220
F2j	*RANTES protein T-cell specific*	0/36868	1/3.2

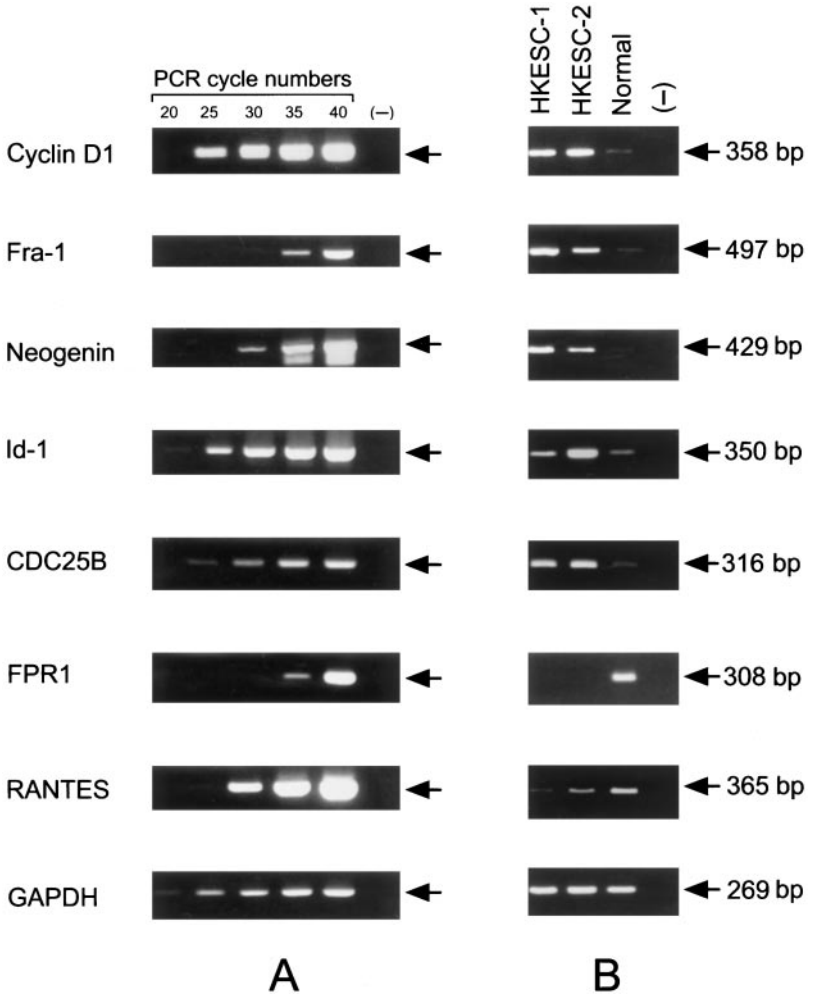

Fig. 2 RT-PCR analysis of *cyclin D1, Fra-1, Neogenin, Id-1, CDC25B, FPR1, RANTES,* and *GAPDH* genes in ESCC cell lines HKESC-1 and HKESC-2 and one corresponding, morphologically normal epithelium (*Normal*) from the HKESC-2-related patient. *A,* determination of optimal number of PCR cycles for different gene-specific primer pairs. mRNA from HKESC-1 was used to determine the optimal number of PCR cycles for genes *cyclin D1, Fra-1, Neogenin, Id-1, CDC25B,* and *GAPDH.* mRNA from the normal esophageal epithelium was used to determine the optimal number of PCR cycles for genes *FPR1* and *RANTES. B,* expression of *cyclin D1* (25 cycles), *Fra-1* (32 cycles), *Neogenin* (30 cycles), *Id-1* (25 cycles), *CDC25B* (28 cycles), *FPR1* (35 cycles), *RANTES* (28 cycles), and *GAPDH* (25 cycles) genes in two ESCC cell lines HKESC-1 and HKESC-2 and one corresponding, morphologically normal esophageal epithelium (*Normal*) from the HKESC-2-related patient.

Table 2 Summary of immunohistochemical staining results in ESCC cell lines and their corresponding primary tissue specimens

	Cell lines		Primary tissues[a]			
	HKESC-1	HKESC-2	T_1	N_1	T_2	N_2
Fra-1	+++[b]	+++	+++	+	++	+
Neogenin	+++	+++	+++	−	++	+
Id-1	+++	+++	+++	++	++	−
CDC25B	+++	+++	+++	−	++	+

[a] T, ESCC tumor; N, morphologically normal esophageal epithelium.
[b] Expression: −, no expression; +, <10% cells positive; ++, ≥10% and <50% cells positive; +++, ≥50% cells positive. ++ to +++ was considered as overexpression.

showed more intense expression of *Neogenin* than poorly differentiated ones ($P = 0.0047$; Table 3).

Id-1 is a cell cycle-related gene that encodes a helix-loop-helix protein. *Id-1* plays an important role not only in suppressing cellular differentiation but also in enhancing cellular proliferation (29, 30). Generally, Id-1 protein is highly expressed in growing cells, and its expression is down-regulated upon differentiation in many cell types. Although Id-1 is expressed in a variety of fetal tissues and overexpressed in tumors from brain and lung (19), its expression in ESCC is unknown. In this study, we observed that *Id-1* mRNA was overexpressed in both ESCC cell lines (Figs. 1 and 2*B*). Also, Id-1 protein overexpression was frequent in human primary ESCC tumors (57 of 61, 93%; Table 3; Fig. 3*F*). The Id-1 protein was localized in the cytoplasm of tumor cells (Fig. 3*F*). In contrast, the expression of Id-1 protein in morphologically normal esophageal epithelium was either negative or negligible and was restricted to the basal and parabasal cells (Fig. 3*E*). The well or moderately differentiated ESCC showed more intense expression of *Id-1* than poorly differentiated ones ($P = 0.0156$; Table 3).

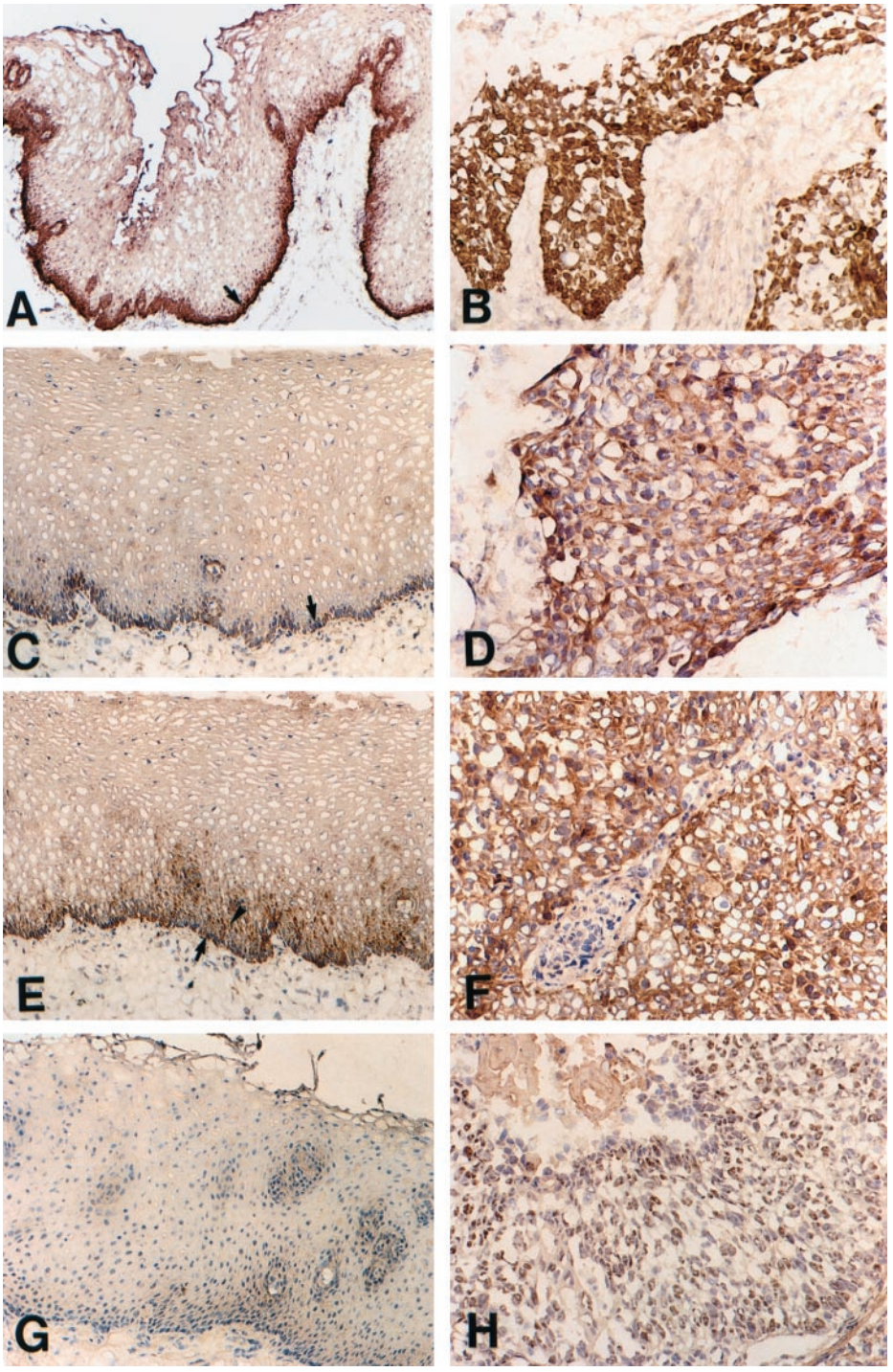

Fig. 3 Photomicrographs of *Fra-1, neogenin, Id-1,* and *CDC25B* expression by IH in morphologically normal esophageal epithelium and ESCC. *A, Fra-1* IH in morphologically normal esophageal epithelium showing that *Fra-1* expression was restricted to the basal cell layer (*arrow*); DAB ×160. *B, Fra-1* IH in ESCC showing that the nuclei of tumor cells are strongly positive for *Fra-1*; DAB × 400. *C, Neogenin* IH in morphologically normal esophageal epithelium showing that *Neogenin* expression was restricted to the basal cell layer (*arrow*); DAB ×330. *D, Neogenin* IH in ESCC showing *Neogenin* expression in the cytoplasm of tumor cells; DAB ×500. *E, Id-1* IH in morphologically normal esophageal epithelium showing *Id-1* expression was restricted to the basal (*arrow*) and parabasal (*arrowhead*) cell layers; DAB ×330. *F, Id-1* IH in ESCC showing Id-1 expression in the cytoplasm of tumor cells; DAB ×500. *G, CDC25B* IH in morphologically normal esophageal epithelium showing lack of immunoreactivity; DAB × 330. *H, CDC25B* IH in ESCC showing *CDC25B* expression in the nuclei of most tumor cells; DAB ×500.

CDC25B is a cell cycle-related gene. Its product is a phosphatase that catalyzes the removal of inhibitory phosphate from the CDK family of proteins (31). *CDC25B* can dephosphorylate threonine 14, tyrosine 15, or both on CDKs and activate cyclin/CDK complexes to stimulate cell proliferation (32). *In vitro* transforming experiments have demonstrated that *CDC25B* is also a potential oncogene (20). Overexpression of *CDC25B* has been found in cancers arising from breast (20), stomach (21), lung (22), and head and neck (23), and in non-Hodgkin's lymphoma (24). In this study, we demonstrated that the mRNA of *CDC25B* was highly expressed in both ESCC cell lines by cDNA array (Fig. 1) and RT-PCR (Fig. 2*B*). Furthermore, *CDC25B* was overexpressed in 79% (48 of 61) primary ESCC tumors by IH (Table 3; Fig. 3*H*). CDC25B protein

Table 3 Summary of IH staining results in clinical ESCC tumors and normal esophageal epithelium tissues

	Fra-1					Neogenin					Id-1					CDC25B				
Diagnosis	−[a]	+	++	+++	*P*	−	+	++	+++	*P*	−	+	++	+++	*P*	−	+	++	+++	*P*
Normal ($n = 16$)	1	15	0	0	<0.0001	6	10	0	0	<0.0001	3	12	1	0	<0.0001	9	7	0	0	<0.0001
Carcinoma ($n = 61$)	4	4	22	31		3	1	7	50		3	1	8	49		10	3	19	29	
Well ($n = 20$)	0	0	9	11		1	0	2	17		0	1	3	16		2	2	7	9	
Moderate ($n = 29$)	1	0	11	17	<0.0001	0	0	1	28	0.0047	0	0	3	26	0.0156	6	1	10	12	0.5720
Poor ($n = 12$)	3	4	2	3		2	1	4	5		3	0	2	7		2	0	2	8	

[a] Expression: −, no expression; +, <10% cells positive; ++, ≥10% and <50% cells positive; +++, ≥50% cells positive. ++ to +++ was considered as overexpression.

expression was localized mainly in the nuclei of tumor cells (Fig. 3*H*). On the other hand, the expression of *CDC25B* in morphologically normal esophageal epithelium tissues was either negative or very weak (Fig. 3*G*). In the case of *CDC25B*, there was no correlation between gene expression and ESCC differentiation ($P = 0.5720$; Table 3).

In summary, all four of the genes selected for further study demonstrated a significantly higher incidence of overexpression in primary ESCCs than morphologically normal esophageal epithelium tissues ($P < 0.0001$; Table 3). Furthermore, three of them, *Fra-1*, *Neogenin*, and *Id-1* were more highly expressed in tumors with greater differentiation. *CDC25B* did not demonstrate this correlation (Table 3). The expression of these genes did not correlate to age at presentation or gender of patients or tumor site, size, or stage. The differentiation of squamous cell carcinoma bears no relationship with the stage of the tumor (4). In this study, the expression of *Fra-1*, *Neogenin*, and *Id-1* was more often noted in the well/moderately differentiated squamous cell carcinoma. This is consistent with the theory that poorly differentiated squamous cell carcinoma arises at the early stage of carcinogenesis. In the later stages of tumor progression, the squamous cell carcinoma becomes more mature in appearance (well/moderately differentiated).

Unfortunately, the complete follow-up data were available only for some of these patients. Nevertheless, all these patients died within 2 years of resection of the primary tumors. Also, the *Fra-1*, *Neogenin*, *Id-1*, and *CDC25B* were highly expressed in squamous cell carcinomas. Thus, it is unlikely that the expression of these genes acts as an independent prognostic factor in these tumors.

Overall, our data demonstrate that multiple genes are differentially expressed in ESCC and show for the first time that oncogenes *Fra-1* and *Neogenin* and cell cycle-related genes *Id-1* and *CDC25B* are overexpressed in ESCC. Additional studies are required to determine the roles of these and other differentially expressed genes in the molecular pathogenesis of ESCC.

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