

Detection of Differentially Expressed Genes in Esophageal Carcinoma Using Non-R1 Differential Display with High Specificity

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Differentially expressed genes in human esophageal carcinoma/normal tissue pairs were identified by means of a modified differential display technique to overcome the limitations of the conventional technique. Out of the eight cDNAs isolated, three were novel. The other five clones consisted of cDNA encoding cytokeratin 13, complement 7, KIAA1160, expression sequence tag (EST)-Hs110855 and EST-Hs13662. Quantitative reverse transcription-PCR (RT-PCR) for 10 carcinoma/normal tissue pairs confirmed down-regulation of cytokeratin 13, complement 7 and KIAA1160 mRNAs in carcinoma tissue and up-regulation of EST-Hs110855 mRNA. EST-Hs13662 mRNA also seemed to increase in carcinoma tissues but not to a statistically significant extent. In situ hybridization confirmed that cytokeratin 13 mRNAs localized in differentiating keratinocytes of normal epithelia and had disappeared in carcinomas, suggesting that this down-regulation reflects de-differentiation during carcinogenesis. The functions of complement 7 and KIAA1160 mRNAs are unclear in normal tissue but their down-regulation in carcinoma tissue may help the development of esophageal carcinoma. The expression of EST-Hs110855 mRNA reportedly observed in carcinomas of different origins suggests that this EST is a carcinogenesis-related gene. Our modified technique, which eliminates the source of false-positives, reduces the screening time, and dispense with the radioisotope, was found to be useful for isolating differentially expressed genes from clinical specimens with apparently genetically distant cellular populations and a very limited mass.

Key words — differential display, esophageal carcinoma, cytokeratin 13, EST

INTRODUCTION

The prognosis for patients with esophageal carcinoma is poor, because carcinomas frequently metastasize when they invade the sub-mucosal layer of the esophagus. Although the survival of patients undergoing surgery for advanced esophageal carcinoma has improved mainly due to advances in surgical techniques and perioperative management, the 5-year survival rate is no more than 10–40%.¹⁾ To overcome difficulties in the treatment of esophageal carcinomas, understanding the biological characteristics of esophageal carcinomas and identification of their etiology are important. Epidemiological studies found that environmental exposures such as cigarette smoking, alcohol consumption, and dietary habits are risk factors for esophageal carcinomas.²⁾

However, it is still largely unknown how these factors can cause carcinogenesis of esophageal mucosa.

Accumulation of multiple genetic changes in both oncogenes and tumor suppressor genes during tumor development has been shown in a model of colon adenocarcinoma.³⁾ In esophageal carcinoma, the mutation of the p53 gene (50–70%),^{4,5)} and allelic deletions of 5q21-22 implicating the *apc* genes (30–70%),⁶⁾ are frequently observed. Moreover, frequent loss of heterozygosity (LOH) has been detected at several chromosome locations, particularly in chromosomes 4, 5, 9, 13, 17, and 18.⁷⁾ However, except for chromosome 17, where p53 gene is supposed to be the primary target of allelic deletion, neither candidate tumor suppressor gene has been mapped on the LOH sites nor has it been established what kind of genetic products are encoded. Along with such genomic changes, alterations in mRNA expression have recently been reported. For example, cyclinD1,⁸⁾ alpha6 integrin,⁹⁾ polo-like kinase (PLK),¹⁰⁾ and ErbB-2¹¹⁾ have been identified as genes

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showing the over-expression in esophageal carcinoma tissues compared with their normal counterparts and on the other hand, cystatin B¹²⁾ and manganese superoxide dismutase (Mn-SOD)¹³⁾ as genes showing reduction in carcinomas. Most of such mRNAs have been detected by means of differential displays using human clinical specimens. Isolation of these mRNAs could provide not only a better understanding of the biological features of tumor development but also new diagnostic markers and targets for cancer therapy.

Differential display is a sensitive technique for detecting variations in gene expression between two cellular populations.¹⁴⁾ However, the conventional radio-labeling differential display is plagued by false-positives. The identification of genes that are truly differentially expressed, therefore becomes a labor- and cost-intensive task. We made some modifications in the conventional technique, which was then evaluated for its ability to identify differentially expressed genes in ten carcinoma/normal tissue pairs. With this modified differential display, eight differentially expressed genes, comprising three known genes, two expression sequence tags (ESTs), and three novel genes were obtained.

MATERIALS AND METHODS

Clinical Samples and RNA Extraction — Histologically confirmed squamous cell carcinomas of the esophagus from ten patients were included in this study. All of the patients were treated by curative surgical resection with their informed consent at Kanazawa University Hospital during 1997–1998. The carcinoma and the corresponding normal specimens were immediately frozen in liquid nitrogen after resection, and kept at -80°C until use. From these specimens, total RNA was prepared with the guanidium-isothiocyanated and cesium chloride method,¹⁵⁾ and stored at -80°C .

Differential Display — To circumvent individual differences of clinical specimens, pairs of normal and carcinoma mRNAs from four patients were simultaneously analyzed using differential displays. Total RNA (2.5 μg) was reverse transcribed in the RT buffer containing 20 μl of 0.2 μM anchored poly dT primer 5'-AAGCT11V-3' (GeneHunter Corp., MA, U.S.A.), 100 μM dNTPs, and 1 U MMuLV reverse transcriptase (GeneHunter Corp.). After reverse transcription, the aliquot was diluted with 80 μl of 10 mM Tris/HCl–1 mM EDTA (pH 8.0) and stored

in 10 μl each at -80°C until use. Two μl of cDNA solution was subjected to PCR amplification with 10 μM of one of the arbitrary primer (GeneHunter Corp.) in 50 μl of 100 μM dNTPs, PCR buffer, and 1U of *Taq* polymerase (Takara, Shiga, Japan). PCR was processed at 94°C for 2 min, 40°C for 5 min, and 72°C for 5 min for 2 cycles, and at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min for 28 cycles. The obtained products were separated on non-denaturing 8% polyacrylamide gels made in 16×14 cm gel slabs. The gel was stained by 10,000-times diluted SYBR Green I (Molecular Probes, OR, U.S.A.) and cDNA was visualized by an imaging analyzer (Digital image analyze, Molecular Dynamics, U.S.A.). The bands showing reproducible differential expression were excised and the precise excision was confirmed again by imaging analyzer. The cDNA fragments was extracted by boiling, PCR re-amplified, subcloned into pGEM-T vector (Promega, Madison, WI, U.S.A.). The obtained clones were sequenced with a dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Norwalk, CT, U.S.A.) and analyzed using the GenBank BLAST and FAST homology search programs. The cloned P4 cDNA was also utilized to generate probes for in situ hybridization.

Quantitative Reverse Transcription (RT)-PCR

— RT-PCR was utilized for quantitation of mRNAs as described elsewhere.¹⁶⁾ Briefly, oligodT-primed cDNA from 0.2 μg of total RNA was subjected to PCR processed at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. The sequences of the primers used were as follows: P2, upstream (5'-TGGGCCCTCAGGATGTTTCAA-3') and downstream (5'-GCCGGCTTTGTTTTGTCTCAC-3); P4, upstream (5'-GAGGAATGGTTCCA-CGCCAAG-3') and downstream (5'-GCGACCAG-AGGCATTAGAGGT-3); P5, upstream (5'-CAGTCCTGCCACTTTCTACTAAGGTACCCAA-AAGCCCTA-3') and downstream (5'-CTAGAA-GGTGAGTGTCAGTTC-3); P7, upstream (5'-ATGTCAGCGCTGGGAGAACT-3') and downstream (5'-CAAGGACTTATGCTGGTGACA-3); P8, upstream (5'-GCAGTCACTCTCAGAATCT-TG-3') and downstream (5'-GCTGTGATTCCAC-ATCAAACC-3); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), upstream (5'-CATGGGG-AAGGTGAAGGTCGGA-3'), and downstream (5'-TTGGCTCCCCCTGCAAATGAG-3'). The PCR products were separated by electrophoresis on a 2% agarose-ethidium bromide gel and visualized under UV light. Camera images were converted to PICT

files and analyzed using the NIH image 1.55 program. The mRNA expression of P2, P4, P5, and P8 was estimated by means of standardization with that of GAPDH as the internal control for each sample.

In Situ Hybridization — The P4 cDNA clone obtained by differential display was linealized by restriction enzyme digestion using *Nco*I or *Sall*I. From the linealized clone, the bidirectional cRNA probes were obtained using either T7 or SP6 RNA polymerase (Takara) and the DIG Labeling Kit (Boehringer, Mannheim, Germany). Frozen samples were cut into 4 μ m sections, mounted on coated glass slides, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min. The sections were then treated with 0.2 N HCl, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min, dehydrated with ethanol series, and air dried. After prehybridization with the hybridization solution containing 50% formamide, 10% dextran sulfate, 1 \times Denhardt's solution, 600 mM NaCl, 0.25% SDS, 150 μ g/ml yeast tRNA at 50°C for 2 hr, each slide was allowed to hybridize with approximately 0.5 μ g/ml of the cRNA probe for 16 hr at 50°C. The slides were washed briefly in 5 \times SSC¹⁵ at ambient temperature and in 50% formamide and 2 \times SSC at 50°C for 30 min, treated with 10 μ g/ml RNase A at 37°C for 30 min and again washed once with 2 \times SSC and twice with 0.2 \times SSC for 20 min each at 50°C. After hybridized digoxigenin-labeled probes were detected with the Nucleic Acid Detection Kit (Boehringer), the slides were rinsed with 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, and fixed.

Statistical Analysis — The statistical significance of differences in the mRNA expression between carcinoma/normal tissue pairs was calculated using Wilcoxon's signed rank test.

RESULTS

Isolation of Differentially Expressed Gene

When the PCR products of differential display were electrophoresed, each lane consisted of about 20–30 bands (Fig. 1). Most bands showed the same intensities between carcinoma and normal samples in all four cases. However, bands seen in all normal samples were substantially reduced or had disappeared in all carcinoma samples (Fig. 1, F1 & F3) and vice versa (Fig. 1, F2). Differential display using 20 arbitrary primers could isolate 14 cDNAs which showed differential expression in all of the four tissue pairs, and eight of these cDNAs could be

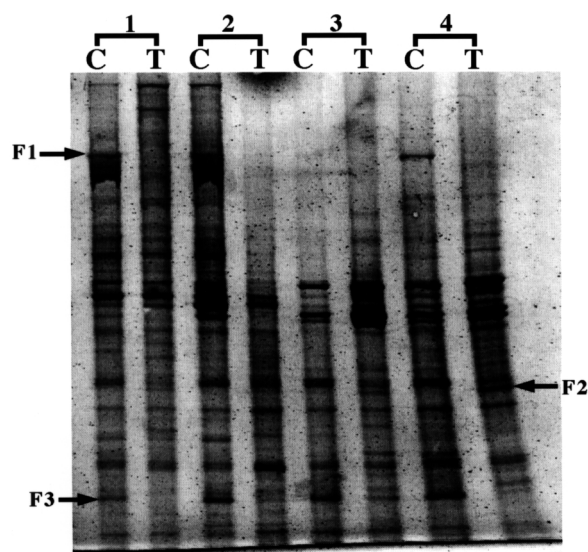


Fig. 1. Differential Display of mRNAs from Esophageal Carcinoma and Normal Tissues

mRNAs of carcinoma/normal pairs from four patients were analyzed by differential display using a set of primers; AAGCT11C (5'-AAGCTTTTTTTTTTC-3') and AP61 (5'-AAGCTTGACCTT-3'). F1, F3, fragments appeared in normal tissues but decreased or almost absent in carcinoma tissues; F2, fragments appeared in carcinoma tissues but not in their normal counterparts; N, Normal tissue; C, carcinoma tissue.

reamplified, cloned, and subjected to sequence analysis (Table 1). A homology search showed that two cDNAs matched those encoding cytokeratin 13 for P4 and complement 7 for P7 (Table 2). Three cDNAs, P2, P5, and P7, were EST or KIAA with unknown function. In addition, three novel cDNAs were isolated, which were registered as ESTs.

Quantitation of Differentially Expressed mRNAs Using Quantitative RT-PCR

The amounts of mRNAs, except for those of the novel ones in ten carcinoma/normal tissue pairs were estimated by means of quantitative RT-PCR. A preliminary study disclosed that the intensity of PCR products for GAPDH increased linearly from 20 to 28 cycles in both normal and carcinoma tissues (Fig. 2, A and B). The 447-bp-products for P4 cDNA appeared at 22 cycles and increased linearly from 24 to 30 cycles in normal tissue, while those in carcinoma tissue were faint even at 32 cycles. When the intensities of bands were plotted against cycles, 28 cycles showed exponential amplification both in carcinoma and normal tissues (Fig. 2, C). The expression of P4 cDNA was therefore assessed as the intensity of products at 28 cycles over that of GAPDH at 24 cycles (Fig. 2, D). This procedure was

Table 1. Partial Sequences of ddPCR Products Differentially Displayed between Esophageal Carcinoma and Its Normal Counterparts

Product	sequence ^{a)}	Accession no.
P1	acctaagtcccatgacatcccatgggatcagatactgaatttagcttctgtttgtccagttcagaaaatgagttgtc attgttctaaacttattagatgacttacatgtgacngtttacaggctgttcttatgggaacatattaaggcattgaggag	BF219668
P2	gccagccgagcctctctctctctcttccacttttccactaacctggctctgagtttctttatattctgttccaaaggcaca ataacaccatcatcttcatctaggtaacatagctactaaaatcgattg	BF219669
P3	ctcattaatafatatcgcgacactcttttctaatacatctgacatattcacctcacagtgctcattaccgtatatagcggtcatt gattttctctgagcagtttaattctttagaagaatttcacgatttttactttcaacagtaccattcatataatggtctataa gccccaaaataaattgattaacggggcacagaagtttctgtttttacgctctctctctcagctcatttcaagcatat agatggactctcagcttcccagttccaaggcttctcagttcacgtttacattgagtagccctccagcgtaccataaa ccggaagccattctaacaactctctctgtataaccgtttgtcgacttttagtaacgagc	BF219670
P4	cctgagagcagagggactgagcctgggtccagcagccctctctctctcagggactgccgctctctctctct ctgggtctgaagacagaggggagggcagccagcagattaaagcctacggacatcagaagtgcggcgaccag aggcattagagtggtgtaacagagcactagaagtcgtgtaacagagtgctacggggctgacgcttctctgg gaaacaagagacaagacatgctcagcgtgagaagaccacctcagcagggcagggccccctggaggggct acaaactcctcattagtcaggtggtgctgggat	BF219671
P5	cttttaataacaagactaactgtaccagtactgataacatcctgaataaagactgcaataccacattct	BF219672
P6	taaaancttggcctnccatcaaaaantaatgtaaaacaatgtttccatgggtcagatttaatttttacaccaagccca aaggcgaentggtaantttgagcacttttatgcatttttgatttngcagatgaagcaagtgtagaattaatgcntaac ttgattgagcactcnncaagcgcctgatagaaaaatcaagcaataccgtattactatgacgtgcaggatcc gcttttttgcattataagctaaataataaccggcatcaagcttccacttttcataatagatgctttagttatcataattaagcc atatntcaccggctggaactcctcaattcattctttacatccgtattagttacattacgccg	BF219673
P7	gaagaatagcatcctaagtggttctactctgtcatctgaagttttgaaatataattaccagggtcagaacaatacaga gatagattttgataatagcttattttaaattcattaaagccctctcatttctctcccacagctgctgtttgtttgatgaaa gagaaataagggaataaata	BF219674
P8	aaaggacctngtttgggtggagtagcagggtaggaggtgaggaaaacaagaaggagctggagattggccaag gagcaactcagaaggacagagagcagcagcattaggaggagcc	BF219675

a) Recognition sequences are given using single-letter International Union of Pure and Applied Chemistry codes and standard abbreviations to represent ambiguity ($n = a, c, g, \text{ or } t$).

Table 2. Identification of the ddPCR Amplification Products Differentially Displayed between Tumor and Normal Tissues of the Esophagus

Product	ddPCR primers	Amplification extent			Accession number
		Normal	Tumor	Homology search	
P1	dT11 C/AP-53	-	+	No match	
P2	dT11 C/AP-56	++	-	KIAA1160	Hs.33122
P3	dT11 A/AP-61	-	+	No match	
P4	dT11 C/AP-61	++	-	cytokeratin 13	X14640
P5	dT11 G/AP-62	-	++	EST	Hs.110855
P6	dT11 A/AP-54	+	++	No match	
P7	dT11 G/AP-52	++	-	complement 7	Hs.78065
P8	AP-62/AP-62	+	++	EST	Hs.13662

A selection of 8 ddPCR products showing differentially expressed pattern between tumor and normal tissues were isolated from non-denaturing acrylamide gels, reamplified, and sequenced in both directions using the corresponding primers. The sequences were confronted to EMBL/GenBank databases using the Blast algorithms to search for homology with existing sequences.

also found to be suitable for estimation of other mRNAs. Expression of P4 mRNA, namely cytokeratin 13 mRNA, in normal tissue was 0.18 ± 0.10 times as much as that of GAPDH mRNA

and that in carcinoma tissue was significantly reduced to 0.02 ± 0.01 (Fig. 3). The carcinoma/normal ratio (T/N ratio) was 0.10 ± 0.03 on average. Complement 7 mRNA, *i.e.* P7 mRNA, was expressed 0.08 ± 0.01 times as much as that of GAPDH mRNA in normal tissue. Although the T/N ratio was higher than 1 in one case, its reduction in carcinoma tissue was statistically significant, at 0.43 ± 0.05 . Constitutive expression of P2 mRNA, KIAA1160 mRNA, was also observed in normal tissue, 0.12 ± 0.01 times more than that of GAPDH mRNA and its reduction in carcinoma tissue was also significant even though two cases had a T/N ratio of more than 1. The increase in P5 mRNA, EST-Hs110855, in carcinoma tissue was statistically significant because seven out of ten normal tissues displayed a very low expression, while the expression in the remaining three tissues was almost the same as that in carcinoma tissue. Although P8, EST-Hs13662, was selected as being enriched in carcinoma tissues, the increase was not always statistically significant. For the novel cDNAs, primers suitable for quantification were unfortunately not avail-

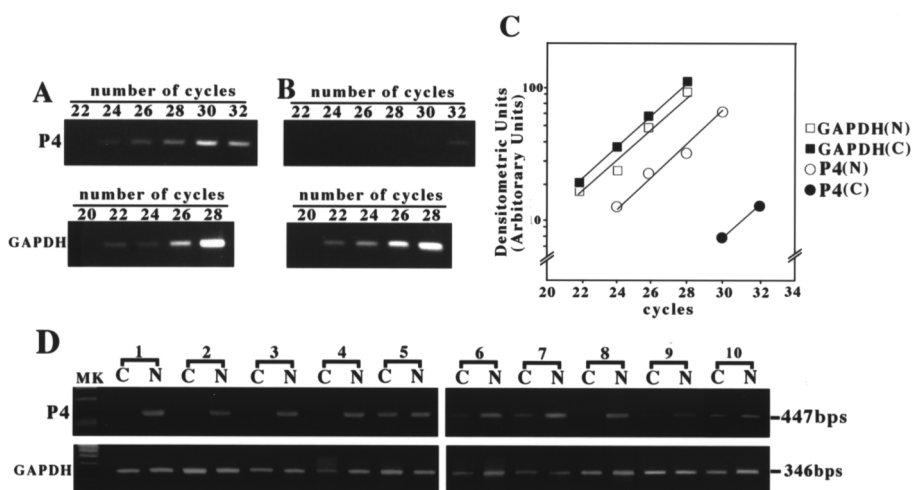


Fig. 2. Semi-Quantitative RT-PCR Analysis of mRNA Expression of P4 in Paired Normal/Carcinoma Tissue Pairs
 Increases in RT-PCR products for P4 and GAPDH according to PCR cycles in the normal (A) and the carcinoma (B) tissue from case1. C: The intensities of the bands based on the PCR cycle number were plotted. D: mRNA expression was measured at of 28 cycles of PCR amplification for P4 and at 24 cycles for GAPDH.

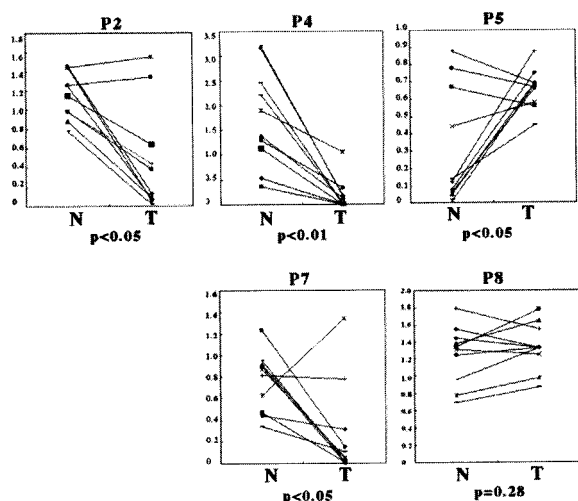


Fig. 3. Confirmation of Differential Expression of the Genes Obtained by Differential Display
 Expression is given as $\times 10^{-1}$ of GAPDH mRNA expression. Statistical differences between normal (N) and carcinoma (C) tissues were calculated using Wilcoxon's signed rank test.

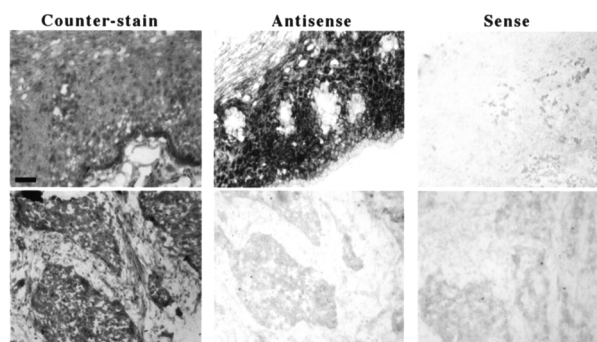


Fig. 4. Localization of P4 mRNAs in the Normal Esophageal Tissue and in the Carcinoma Tissue Determined by in Situ Hybridization
 Upper panels show the normal esophageal mucosa and lower panels the carcinoma tissue. Serial sections were stained by hematoxylin, antisense probe, and sense probe. The bar indicates 150 μ m.

able.

In Situ Hybridization Analysis for P4 mRNA

In situ hybridization demonstrated that the antisense cRNA probe for P4 mRNA was hybridized to the cells in the suprabasal layers of normal esophageal epithelia (Fig. 4). The signal intensity was especially strong in the parabasal regions and became weaker toward the surface. No positivity was observed in the basal cells of epithelia and sub-epithelial cells, nor was antisense or sense cRNA posi-

tive for carcinoma tissue.

DISCUSSION

By means of differential display using carcinoma/normal tissue pairs of esophagus, a total of eight cDNAs were cloned as differentially expressed genes. Further quantitative RT-PCR confirmed that cytokeratin 13, complement 7 and KIAA1160 mRNAs were constitutively expressed in normal esophagus and their expression reduced in carcinoma tissue. EST-Hs13662 expression was not statistically significant but seemed to increase more in carcinoma

tissue than in normal tissue. On the other hand, the elevation on EST-Hs110855 mRNA in carcinoma tissue was statistically significant, while its expression was high in three normal tissues.

Differential display is useful for isolating differentially expressed genes in more than two cellular populations,¹⁴⁾ and helps the isolation of not only known mRNAs with specific functions for their originating cells, but also novel mRNAs whose function is not yet clear. Specifically, the number of genes related to cell cycles could be isolated by means of differential display.¹⁷⁾ The conventional radiolabeling differential display is extremely sensitive but includes many false-positives, lack of 3' end representation and redundancy between cDNA subsets. These problems are the result of low annealing temperature of primers, inhibition of polymerase activity by radioisotopes, pseudo-amplification during extraction/cloning procedure from denaturing acrylamide gels, and other characteristics. Such difficulties are especially noticeable when the populations are genetically distant as in carcinoma/normal tissue pairs. In the study presented here, we made three modifications to the conventional differential display. First, amplified products underwent further analysis only when their differential expression was confirmed in multiple carcinoma/normal tissue pairs. Although Northern blot analysis, reverse Northern blot analysis and other procedures so on are used to confirm reproducibility in the conventional differential display, these methods are not only time consuming but also sometimes less sensitive than the differential display itself. Moreover, they can be performed only when a great deal of RNA is available. Second, the products were not labeled by radioisotopes during amplification but stained with SYBR green after electrophoresis. Finally, the products were separated on non-denaturing acrylamide gels, which were easy to handle compared with denaturing gels. SYBR green staining was not always as sensitive as radioisotope labeling but not using radioisotopes avoids impairment of polymerase activity so that longer cDNAs can be obtained with high fidelity. Moreover, cDNA destruction caused by radioisotopic decay during storage could be avoided. In fact, only 14 bands in our study were of interest although 20 arbitrary primers were used. Out of these 14, eight cDNAs (57%) could be isolated. In a previous study at our department, it was found that conventional radio-labeling differential display using ten arbitrary primers could amplify 280 candidate bands, but only ten of these were confirmed

to be truly differentially expressed.¹⁸⁾ In addition, eight out of the ten were mitochondrial or ribosomal so that only two cDNAs seemed to be functionally significant.

Among cDNAs enriched in normal tissue but down-regulated in carcinoma tissue, cytokeratin 13 belongs to the family of intermediate filament protein which is expressed mostly in epithelial cells and associated with cytoskeleton organization. Cytokeratins are consisted of acidic type I (K9-20) and basic type II (K1-8).¹⁹⁾ Specific combinations of type I and II are expressed depending on their developmental and differentiation status.²⁰⁾ The finding that cytokeratin 13 mRNA originated from the suprabasal layers of normal esophageal epithelia but decreased toward the surface agrees with the result of a immunohistochemical analysis demonstrating that K5/K14 are expressed in proliferating basal cells but that once they detach from the basal layer, they are replaced with K4/K13 in the internal non-cornified epithelia.²¹⁾ Moreover, its down-regulation in carcinoma tissue indicates that the loss of cytokeratin 13 immunoreactivity²²⁾ occurred transcriptionally and that its expression regulation was associated with de-differentiation during carcinogenesis.

Although complements have been considered to be serum proteins of hepatic origin, the allotypic changes occurring from transplant recipients to its donors quantifies that approximately 30% of systemic complement 7 is derived from non-marrow/non-hepatic organs.²³⁾ Although the contribution of esophageal complement 7 mRNA to this non-marrow/non-hepatic pool is unknown, the normal esophagus is, in any case, one of the local origins of complement 7. In addition to the esophagus, preferential expression of complement 7 mRNA was reportedly observed in colon, heart, brain *etc.*²⁴⁾ although the function of locally expressed complement 7 is still unclear. The esophagus is continuously exposed to external stimulation and the expression of complement components is known to be induced by cytokines.²⁵⁾ It is important to clarify whether the preferential expression in normal tissue is truly constitutive or is induced by such stimulation. It is also considered important to explore whether the down-regulation in carcinoma tissue is associated with carcinogenesis itself or is the result of changes in microenvironments including cytokines.

KIAA1160, supposedly encoding a protein consisting of 350 amino acids, has been isolated from the human brain library to target the coding sequence of large proteins from long mRNAs. Although

KIAA1160 mRNA was found to localize ubiquitously among tissues, its function remains unknown. EST-Hs13662 was also found to localize ubiquitously, while EST-Hs110855 is reportedly characterized by relatively limited localization in germ cells, renal cell carcinoma, colon adenocarcinoma, etc. Hence, the transcriptional regulation of EST-Hs110855 seems to be more closely related to carcinogenesis than that of EST-Hs13662 and KIAA1160. Isolation of the full length cDNA sequence as well as the promoter sequence of EST-Hs110855 should provide essential information about carcinogenesis-associated alteration in gene expression regulation and the resulting phenotypic changes in carcinoma tissue.

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