Cytogenetic characterization and gene expression profiling in the rat reflux-induced esophageal tumor model

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Objectives: The reasons for the increasing incidence of esophageal adenocarcinoma are not clear. A causal relation between gastroesophageal reflux disease and esophageal adenocarcinoma has been suggested. Support for this comes from the development of esophageal adenocarcinoma in the rat reflux model. However, to date, no systematic characterization of the tumors derived from this model has been reported.

Methods: We induced biliary reflux by creating esophagojejunal anastomoses in 12 Sprague–Dawley rats. The experiment was terminated at 9 months, and rat esophagi were harvested for histopathologic documentation of reflux-associated changes and evidence of tumor formation. Three cell lines were established from 2 of the reflux-associated tumors. We tested the ability of these cells to grow in vitro in tissue culture and in vivo as xenografts in an orthotopic location at the gastroesophageal junction. Furthermore, we performed a cytogenetic analysis and determined the array-based gene expression profiles of these 3 rodent carcinoma lines compared with normal esophageal mucosa.

Results: At 9 months, 12 of 12 rodents had histologic features of metaplastic columnar epithelium in the esophagus, with 7 having invasive carcinomas with glandular differentiation (either adenocarcinomas or adenosquamous carcinomas). The 3 cell lines established from 2 reflux-associated tumors were capable of sustained in vitro propagation and grew successfully as xenografts in both subcutaneous and orthotopic locations, confirming the tumorigenic nature of these lines. Despite their establishment from primary tumors with glandular features, the histology of the xenografts was that of well-differentiated squamous carcinomas. Karyotype analyses demonstrated cytogenetic heterogeneity and aneuploidy; furthermore, translocation (7:11) was present in all 3 lines. Array-based gene expression profiling confirmed upregulation of several cancer-related genes important in human esophageal cancer. Quantitative reverse transcription–polymerase chain reaction was used to confirm the differential expression of selected transcripts (vascular endothelial growth factor [*VEGF*], polo-like kinases [*PLK*], cyclin dependent kinase 4 [*CDK4*], hypoxia-inducible factor 1α [*HIF1α*], and insulin-like growth factor 1 [*IGF-1*]) in comparison with nonneoplastic esophageal mucosal scrapings.

Conclusions: The rodent reflux model is capable of inducing metaplastic epithelial changes simulating Barrett esophagus, as well as subsequent neoplastic transformation, at a high frequency. Cell lines have been established from these tumors that are capable of in vitro and in vivo passaging. The rodent reflux model should be a valuable model for studying therapy and chemoprevention efforts for Barrett esophagus, whereas the established cell lines provide a useful resource for drug discovery and other high-throughput studies.

The increasing incidence of esophageal adenocarcinoma has necessitated a search for the causes leading to this epidemic.¹ Epidemiologic studies have confirmed that this increase in the incidence of esophageal adenocarcinoma is strongly related to a similar increase in the incidence of gastroesophageal reflux disease.² How gastroesophageal reflux might be responsible for the development of

Abbreviations and Acronyms

<i>CDK-4</i>	= cyclin dependent kinase 4
<i>HIF1α</i>	= hypoxia-inducible factor 1 α
<i>IGF-1</i>	= insulin-like growth factor
<i>PLK</i>	= polo-like kinases
<i>RT-PCR</i>	= reverse transcription–polymerase chain reaction
<i>VEGF</i>	= vascular endothelial growth factor

esophageal cancer is still not fully understood. However, there are ample data from preclinical and clinical studies demonstrating that gastroesophageal reflux disease is injurious to the esophageal mucosa.^{3,4}

An animal model that is used for studying esophageal carcinogenesis is based on the rat reflux model originally proposed by Levrat and colleagues⁵ to study the effect of esophagitis induced after gastrectomy. This model has been extensively used, with and without carcinogens, to study various components of the refluxate.⁶⁻⁸ Others have used modifications of this original model to study carcinogenesis and chemoprevention.⁹⁻¹¹ For example, it has been shown that the reflux model does not need the carcinogen to induce esophageal tumors,¹² and the pure reflux model has been used in chemopreventive studies using inhibitors of cyclooxygenase 2.^{9,10} To date, there have been no attempts to confirm the malignant character of these tumors by growing them in vitro or in vivo. In addition, the exact genetic characteristics of these tumors are unclear and have not been systematically studied. The aim of the present experiment was to assess the malignant potential of cells from this model based on the ability of malignant, but not normal, cells to transform and grow in tissue culture and to examine chromosomal and genetic alterations in these tumors.

Materials and Methods

All procedures were prospectively approved by the Animal Care and Use Committee of Johns Hopkins University, and the animals received humane care in compliance with the "Guide for the care and use of laboratory animals," published by the National Research Council (National Academy Press, 1996). Animals were acclimatized in a central animal facility for 2 weeks before the experiment. Rats and mice were anesthetized with an intramuscular injection of ketamine and xylazine.

Surgical Model of Reflux-induced Esophageal Tumors

Biliary reflux was induced in 10-week-old male Sprague–Dawley rats (200–250 g, Harlan). The animals were allowed 2 weeks to acclimatize and were housed at a temperature of 20°C to 22°C and a humidity of 30% to 50% at 12-hour alternating light-dark cycle. The rats were fasted overnight but allowed water ad libitum until the operation. Briefly, the esophagus was mobilized, preserving the vagus nerves, and a loop of jejunum was then identified 4 cm from the ligament of Treitz. The gastroesophageal junction was divided

and ligated, and an end-to-side anastomosis was constructed between the distal esophagus and jejunum (modified Levrat's model⁵). Once the animals awoke, they were allowed water ad libitum; the feeds recommenced the following day. Animals received appropriate analgesia during the perioperative and postoperative periods. The experiment concluded 9 months postoperatively.

Harvesting of the Tumors

Abdominal and thoracic viscera were exposed through a midline incision, and the tumors were assessed for size and extent. The esophagus was mobilized from the neck down to and including the esophagojejunal anastomosis. The tumors were identified and carefully dissected away from the liver and adjoining structures. The esophagus was opened longitudinally to expose the lumen; a small representative tissue was then excised from the tumor mass and preserved in 10% formalin for histologic examination. The tumors were then harvested and washed immediately with phosphate-buffered saline solution to remove any food debris and contaminants and then stored in phosphate-buffered saline at 4°C and transported to the laboratory for tissue culture.

In Vitro Growth (Establishment of the Cell Lines)

Tumors were mechanically minced and enzymatically digested with collagenase. The disaggregated cells were then plated in appropriate growth media as a primary culture. The culture plates were checked for the presence of cancer cells everyday. After adequate growth, single clones were picked by using a cloning ring and transferred to 96-well plates. We froze and stored aliquots of the original lineage.

In Vivo Growth in Athymic Mice

Six- to 10-week-old female CD1 null/null mice (weight, 13–16 g) were used (Charles River Company). They were kept in wire cages in a low-pathogen environment (temperature, 20°–22°C; humidity, 30%–50%; 12-hour/12-hour light-dark cycle). Autoclaved chow pellets and water were available ad libitum.

Establishment of JA, JB, AMY cell line–derived tumor specimens. Approximately 5×10^6 cells (with 0.1 mL of Matrigel [BD Biosciences] and cells in 0.1 mL of Hank's buffered salt solution) were injected subcutaneously into a CD1 null/null mouse. Four weeks after implantation, the resulting tumors were explanted and cut into pieces of approximately 1 mm³. These cubes were then implanted subcutaneously in the CD1 null/null mice to obtain stable tumor growth. Tumors from these animals were examined by means of routine hematoxylin and eosin staining.

Surgical orthotopic implantation of JA, JB, AMY cell line–derived tumor specimens. After 1 passage, the tumor was harvested and cut into cubes of approximately 10 to 20 mm³. Tumor pieces were mixed to receive a homogeneous distribution of specimen from the center and the margin of the tumor. These pieces were subsequently implanted in 3 mice, respectively, by using microsurgical techniques. The stomach was exteriorized through a left-sided upper abdominal incision, and the serosa of the anterior wall of the forestomach (which has the similar histologic pattern as the esophagus) was removed with a scalpel. One tumor cube was sewn on the prepared gastric wall with 8-0 nonabsorbable sutures.

The laparotomy was closed in 2 layers with 6-0 non-absorbable sutures. Animals were killed 4 weeks later.

Cytogenetics

Cells at 75% confluency were arrested in metaphase with 0.05 $\mu\text{g/mL}$ Colcemid (Sigma, St Louis, Mo). Ethidium bromide was added at a final concentration of 0.05 mg/mL . Approximately 4 hours later, the monolayer was detached with trypsin, incubated at 37°C in 0.075 mol/L KCl for 10 minutes, and fixed in 3:1 methanol/acetic acid. Slides were prepared and G-banded by using standard techniques. At least 50 cells from each cell line were analyzed. Representative metaphases were photographed and karyotyped.

Microarray Analysis, Data Normalization, and Gene Ontology Analysis of Differentially Expressed Genes

Rat 8Kx2 cDNA microarrays were obtained from the Johns Hopkins Cancer Center Microarray Core. They contain 8000 genes and expressed sequence tags double printed on site from a cDNA set generated by the Institute for Genomic Research. The microarray gene list is available on request. Total cellular RNA was isolated with the RNeasy RNA extraction kit (Qiagen), according to the manufacturer's protocol. Parallel experiments were performed with a common reference design, in which 3 rat cancer cell lines (JA, JB, and AMY) were compared with reference normal rat esophageal mucosal cells (Normal_{pooled}; ie, a 1:1 mixture of Normal₁:Normal₂). The design included a dye swap for each comparison to minimize the effects of dye bias.¹³ The aims of the microarray study were to identify 2-fold or greater differentially expressed genes in each cell lines relative to normal values and to examine differentially expressed genes common to all 3 dye-swap pairs to identify cancer-related genes. Statistical analysis of the microarray data was performed with GeneTraffic software (Iobion Informatics). Problematic spots were flagged for exclusion from aggregate statistical calculations and normalization based on (1) raw signal intensity to a background intensity ratio of less than 1, (2) raw signal of less than 1 times average background, and (3) raw intensity of less than 150. Spot signals were normalized by using Lowess subgrid normalization. Differentially expressed transcripts were defined as spots with a mean log base 2 ratio absolute value of 2 or greater (ie, log base 2 ratio $\geq$ or $\leq$ 1), and gene lists were compiled for spots meeting this criterion for each of the 3 dye-swap pairs. Final gene lists identifying differentially expressed transcripts common to all 3 dye swaps were created from the raw lists by using an intersection set operator. Onto-Express¹⁴ analysis was performed on differentially regulated gene lists to construct functional profiles by using Gene Ontology¹⁵ terms for the following categories: biological process, cellular component, and molecular function. Onto-Express calculates statistical significance values (*P* values) for functional categories of genes within a profile by using a hypergeometric distribution in conjunction with a false-discovery rate probability correction method to compare the observed number of genes in a given category with the entire complement of genes within that category on the array. The *P* values distinguish between significantly overrepresented functional classes of genes and those representing random events. We focused our attention on functional categories containing at least 2 genes at a *P* value of less than .05.

Quantitative Reverse Transcription–Polymerase Chain Reaction for Detection of Genes of Interest

Total cellular RNA was isolated with the RNeasy RNA extraction kit (Qiagen), according to the manufacturer's protocol, and reverse transcription to cDNA was performed by using oligo dT synthesis (Invitrogen). All assays were carried out in 25- μL volumes for the Smart Cycler. Rat-specific reverse transcription–polymerase chain reaction (RT–PCR) primers were obtained from Applied Biosystems by using the Assay-by-Demand platform for prefabricated TaqMan assays and amplified by using ABI's Universal master mix. Thermal cycling for the Smart Cycler was performed as follows: 1 cycle at 95°C for 15 minutes, followed by 45 cycles of 94°C for 1 second and 56°C for 30 seconds and extension at 76°C for 30 seconds. Rat phosphoglycerate kinase 1 was used as a housekeeping gene for normalization of RT-PCR data, and the 2- $\Delta\Delta\text{Ct}$ method was used for calculating relative fold differential expression of candidate genes in the 3 cell lines compared with rat normal esophageal mucosal scrapings.

Results

Histology of Reflux-associated Metaplasia and Neoplasia

All 12 rodents had hyperplastic esophageal epithelium with several layers of esophageal epithelial cells (Figure E1, A). There was also evidence of mucosal ulceration and inflammation in the esophageal mucosa. All animals showed the presence of islands of columnar epithelium and tubular mucosal glands, with features both of mucous secretory and absorptive cells with interspersed intestinal-type goblet cells. In animals in which tumors developed, the islands of Barrett mucosa (Figure E1, B) were closely related to the lesions and sometimes formed the margin of the esophageal adenocarcinomas and adenosquamous cancers. The areas of columnar metaplasia extended upward from the anastomotic site for several millimeters. Some of the hyperplastic epithelium showed the presence of goblet cells, particularly in the basal layers. The morphological features of the jejunal mucosa were significantly distinct from those of the metaplastic epithelium. At 9 months postoperatively, 9 (75%) of 12 rodents had esophageal tumors. Four had esophageal adenocarcinomas (Figure E1, C), 2 had squamous cell carcinomas, and 3 had adenosquamous carcinomas (Figure E1, D). All of the tumors were located above the esophagojejunal anastomosis. Some of the tumors showed invasion of the submucosa (Figure E1, E). Mucin-secreting cells were obvious, even on hematoxylin and eosin stains, in the tumors with glandular differentiation (Figure E1, F).

In Vitro Characteristics of Esophageal Cancer Cell Lines

All 9 tumors were successful as primary cultures; however, only 5 could survive subsequent subculture at weekly interval. Two cell lines were found to be contaminated by *Candida* species and were abandoned. Finally, 3 cell lines could be established from 2 cancers, JA and JB from one cancer and AMY from another. The underlying histology of

the primary tumor from which JA and JB cell lines were established was adenosquamous, whereas the AMY cell line was established from an adenocarcinoma. These cell lines have replicated continuously for more than 50 population doublings. We froze aliquots of the original cells at regular intervals. There has been no morphological change noticed in all 3 cell lines over this period. The cytospin histology of JA and JB cell lines demonstrates that they consist of well-differentiated squamous cell cancer; cytospin preparations of the AMY cell line showed some presence of mucinous cells (Figure E2).

In Vivo Characteristics of Subcutaneous Implants

Three cell lines, JA, JB, and AMY, grew subcutaneously ($n = 4$ for each cell line) in either flank, and at 4 weeks after implantation, they grew to a diameter of 1.7 ± 0.5 cm (JA cell line), 1.9 ± 0.4 cm (JB cell line), and 1.7 ± 0.7 cm (AMY cell line), respectively. All the cell lines grew differentiated squamous cell cancers (Figure E3, A).

In Vivo Characteristics of Orthotopic Transplants

All the orthotopic implants at the gastroesophageal junction grew successfully ($n = 3$ per cell line) and reached greater than 1.5 cm in diameter in 4 weeks. The histology of these orthotopic xenografts also revealed well-differentiated squamous cell cancers for all 3 cell lines (Figure E3, B).

Karyotype Analyses

All 3 cell lines were very heterogeneous and aneuploid, with chromosome numbers in the near-tetraploid range. Chromosome gains and losses appeared to be somewhat random, although chromosomes 17 and 18 tended to be overrepresented, and chromosome 19 tended to be underrepresented. Occasional nonclonal structural changes in the chromosomes were also seen. All cell lines had lost the Y chromosome.

JA cell Line. This line had 2 major clones. The first had a modal chromosome number of 80, with a chromosome 4 that had a deletion of the long arm at band q22 (Figures E4, A.1, and E5) and a translocation consisting of what appeared to be an intact chromosome 8 and the whole short arm of chromosome 17 (Figures E4, A.1, and 5, C). The second clone had a modal number of 78 and 2 translocations, the 8:17 translocation seen in the first clone and a second translocation that appeared to be an intact chromosome 7 and most of the long arm of chromosome 11 with the breakpoint in band q11 (Figures E4, A.2, and E5, B). A representative karyotype is depicted in figure E4, A.3.

JB cell line. The JB cell line contained 2 major clones. The first had the same 8:17 translocation seen in the JA cell line (Figures E4, B.1, and E5, C), and the other had both 7:11 and 8:17 translocations observed in the JA cell line (Figures E4, B.2; E5, B; and E5, C). The modal chromosome number of both clones was 78 (Figure E4, B.3).

AMY cell line. The AMY cell line had a modal number of 80 (Figure 4, C.2) and the 7:11 translocation seen in both the JA and JB cell lines (Figures E4, C.1, and E5, B).

Gene Expression Profile by Means of cDNA Microarray

We observed that 1409 transcripts exhibited a 2-fold or greater differential expression in the JA cell line compared with the normal pooled sample (573 upregulated and 836 downregulated), the JB cell line had 1186 transcripts with 2-fold or greater differential expression (474 upregulated and 712 downregulated), and the AMY cell line had 1070 differentially expressed transcripts (390 upregulated and 680 downregulated). By using an intersection set operator, the expression of 676 transcripts was observed to be 2-fold or greater differentially expressed in all 3 cancer cell lines relative to pooled normal squamous mucosa, including 455 downregulated and 219 upregulated transcripts (representative subset in Table E1). Using Ontogene Express, we discovered that the overexpressed transcripts had a significant overrepresentation of genes related to regulation of cell cycle, cytoskeleton rearrangements, cytokine activity, apoptosis-related events, and protein serine threonine activity (Table E2). Similarly, we discovered that the transcripts with decreased expression in response to the condition under study had a significant overrepresentation of proteolysis, metalloendopeptidase activity, glutathione S-transferase activity, extracellular matrix-related activity, and hydrolase- and zinc-binding activity. Several of these genes have shown to be of importance in the human esophageal cancers, both squamous and Barrett-associated adenocarcinomas. In compliance with the recommendations of the Microarray Gene Expression Data Society (<http://www.mged.org/>), the microarray data for this study were deposited at the National Center for Biotechnology Information's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through the Gene Expression Omnibus series accession number GSE1707.

Quantitative RT-PCR of Genes of Interest

We further investigated the relative expression of a subset of 5 genes of interest by means of RT-PCR in the 3 esophageal cancer cell lines versus rat normal esophageal mucosal scrapings. These genes were selected based on known roles in human esophageal cancers.¹⁶⁻²⁰ The expression of vascular endothelial growth factor (*VEGF*), polo-like kinases (*PLK*), cyclin dependent kinase 4 (*CDK4*), hypoxia-inducible factor 1 α (*HIF1 α), and insulin-like growth factor (*IGF*) is depicted in Figure 1 and tabulated in Table E3 normalized to expression in the rat esophagus, confirming the overexpression of these transcripts in the cancer cells.*

Discussion

In this study we have extensively characterized 3 cell lines derived from a reflux-associated rat model of esophageal cancer. We have shown that the cell lines are capable of

Figure 1. Results of quantitative RT-PCR reaction for genes of interest: the relative expression level compared with that seen in normal tissues. **A**, Vascular endothelial growth factor (*VEGF*) level in the JA cell line is 541 times as much as the normal level and 230 and 23 times as much as the normal level in the JB and AMY cell lines. **B**, The level of polo-like kinases (PLK) is 1770, 621, and 40 times that of the normal level compared with the JA, JB, and AMY cell lines. **C**, Normalized values of cyclin dependent kinase 4 (CDK4) in the JA, JB, and AMY cell lines. The JB and AMY cell lines did not show overexpression of this particular gene compared with that seen in normal esophageal mucosal cells. **D**, Expression of hypoxia-inducible factor 1 α (*HIF1* α) in the 3 cell lines compared with normal levels. **E**, Overexpression of insulin-like growth factor (*IGF*) in the JA and JB cell lines compared with that seen in the AMY line, which did not have increased expression of IGF.

propagation in vitro, as well as in vivo, in both subcutaneous and orthotopic locations, confirming their malignant potential. Furthermore, karyotype analysis confirmed highly aneuploid cell populations, with common underlying clonal translocations (eg, translocation 7:11) that likely encode for oncogenes critical in esophageal carcinogenesis. Of note, all 3 cell lines demonstrated loss of the Y chromosome, a genetic aberration reported to be highly frequent in both Barrett esophagus and in esophageal adenocarcinomas.²¹⁻²³

Finally, the first global expression profiling of rodent esophageal cancer lines identified numerous genes that have been shown to be important in human esophageal carcinogenesis,^{24,25} corroborating the validity of this model.

In our study the 3 cell lines we have generated engrafted, as well differentiated, keratinizing squamous cell carcinomas, which was somewhat unexpected because the primary tumors from which these were derived had unequivocal adenocarcinomatous components. We postulate that this discrepancy is

either an artifact of sampling caused by intratumoral heterogeneity or a result of discrepant genetic differences in the adenocarcinoma and squamous components, with the latter having a growth or survival advantage in vitro. Nevertheless, the inherent squamous nature of the cell lines is a limitation, albeit their genesis in a Barrett-like background. Another limitation of this model includes the relatively high preponderance of tumors with an adenosquamous histology that is unusual in human Barrett esophagus-associated neoplasia. We believe that reflux-initiated chronic irritation might in part be responsible for the emergence of squamous components in the rodent tumors because constant irritation is a known risk factor for development of squamous cell cancer. In addition, the native rodent esophagus contains keratinized squamous epithelium, which becomes further pronounced in reflux, and this might be another contributing factor to the higher proportion of adenosquamous lesions. At the same time, this model has numerous advantages as a proved in vivo model of metaplasia-neoplasia in the rodent esophagus, wherein 100% of animals experience Barrett-like changes at 9 months, and 75% have neoplasia. This provides a unique opportunity to study the effects of cooperating carcinogens in the onset of malignancy, and conversely, this model can serve as a platform for attempts at chemoprevention and therapy.

Despite the squamous nature of the esophageal cancer lines, these can nevertheless serve as useful in vitro models for identifying new pathways involved in esophageal carcinogenesis and for testing novel therapies. For example, we recently demonstrated 2 “drugable” pathways, the Hedgehog-signaling pathway and the epidermal growth factor receptor pathway, in the JA, JB, and AMY cell lines.²⁶ We further investigated the efficacy of cyclopamine, a small-molecule inhibitor of the Hedgehog pathway,²⁷ and Iressa, an inhibitor of epidermal growth factor receptor tyrosine kinase, in the 3 lines and confirmed the efficacy of these agents in esophageal cancer. These cell lines are available from the investigators for drug discovery studies for esophageal cancer, such as high-throughput combinatorial testing, and will serve as a valuable resource. Similarly, by publicly depositing the gene expression data in the National Center for Biotechnology Information’s Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>), accessible through the GEO series accession number GSE1707, we hope to encourage researchers to explore additional pathways important in the pathogenesis of esophageal neoplasia, including molecular targets for therapy and prevention of this disease.

In summary, we describe a rodent model of reflux-induced metaplasia and neoplasia in the esophagus, which has generated 3 new cancer cell lines capable of continuous in vitro and in vivo propagation. We present extensive molecular characterization of these lines in anticipation of their use in the preclinical study of esophageal neoplasia.

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Figure E1. Reflux-induced tumor histology. A, Multilayered epithelium: a representative high-magnification photomicrograph of the esophageal mucosa subjected to surgically induced biliary reflux showing multilayered epithelium in the rat esophagus. B, Barrett esophagus: a high-magnification photomicrograph of the columnar metaplasia. C, Adenocarcinoma: remnants of squamous epithelium adjoining the adenocarcinomatous lesion in the rat esophagus. The *double arrow* shows squamous esophageal mucosa, and the *single arrow* shows adenocarcinoma. D, Adenosquamous cancer: a typical adenosquamous lesion is shown that is common in a surgically induced biliary rat reflux model. E, Invasive adenocarcinoma: submucosal extension of adenocarcinoma (*double arrow*) is depicted adjoining the normal squamous epithelium (*single arrow*). F, Adenocarcinoma (high magnification): a high-magnification photomicrograph showing mucin-producing cells (*arrows*) in the adenocarcinoma of the esophagus of reflux-induced tumor.

Figure E2. Cell line histology. A high-magnification cell cytospin preparation of the AMY cell line showing few interspersed mucin-producing cells.

Figure E3. Xenograft histology. A, A well-differentiated squamous cell cancer from the subcutaneous implant specimen. B, A similar high-magnification photomicrograph showing epithelial pearls and squamous cell cancer features of one of the orthotopic transplanted tumors.

Figure E4. A, The JA cell line had 2 major clones, one with a modal number of 80 and the other with a modal number of 78. *A.1*, Metaphase from first clone showing a deletion of chromosome 4 at band q22 (*large arrow*) and the unbalanced 8:17 translocation (*small arrow*). *A.2*, Metaphase from second clone showing an unbalanced 7:11 translocation (*long arrow*) and the unbalanced 8:17 translocation (*short arrow*). *A.3*, Typical karyotype, with chromosomes 17 and 18 being overrepresented. B, The JB cell line had 2 major clones with a modal chromosomal number of 78. *B.1*, Metaphase from first clone showing the unbalanced 8:17 translocation. *B.2*, Metaphase from the second clone showing the unbalanced 7:11 translocation (*long arrow*) and the unbalanced 8:17 translocation (*short arrow*). Two small marker chromosomes (*mar*) were often, but not always, seen in both clones. *B.3*, Representative karyotype from the JB cell line. C, The AMY cell line had the 7:11 translocation seen in JA and JB cell lines (*C.1, arrow*) and was a single clone with a modal chromosomal number of 80 (*C.2*).

Figure E5. A, Ideogram of chromosome 4 showing deletion at band q22. B, Ideogram showing the unbalanced 7:11 translocation. C, Ideogram showing the unbalanced 8:17 translocation.

TABLE E1. Representative differentially expressed genes common to all 3 cell lines compared with normal esophageal epithelium

Gene ID	Name	GenBank accession no.
RGICI01	2,3-Bisphosphoglycerate mutase	BC062240
RGIFD90	Actin-related protein 2/3 complex, subunit 1B	NM_019289
RGIEF73	Activated leukocyte cell adhesion molecule	AB008538
RGIBC32	Acyl-coenzyme A: cholesterol acyltransferase	D86373
RGIZB40	ADP-ribosylation-like 3	BQ204995
RGIAA10	Aldehyde reductase 1 (low Km aldose reductase) (5.8 kb <i>Pst</i> I fragment, probably the functional gene)	BC062034
RGIAM85	ATPase, Na ⁺ K ⁺ transporting, α 1	BC061968
UI-R-C2-ms-b-07-0-UI	Bone morphogenetic protein 6	AY184240
RGICC80	Cadherin 3, type 1, P-cadherin (placental)	XM_226426
RGICV80	Carboxyl ester lipase	X16054
RGICV07	Caspase 3	BF408215
RGIAAC47	Cyclin-dependent kinase 4	BC063158
RGIAS04	Cystatin C	BM391630
RGIAQ85	Cytochrome b5, outer mitochondrial membrane isoform	BE111603
RGICA13	Dihydro-orotate dehydrogenase	XM_341685
RGIAA27	Enolase 1, α	BC063174
RGIAM32	Follistatin	BF389575
RGIAD15	Glutathione reductase	U73174
RGIDR30	Glutathione synthetase	L38615
RGIAE95	Glyceraldehyde-3-phosphate dehydrogenase	AF106860
RGIAJ39	Growth factor-binding protein 1	AF142758
RGIAN82	H2A histone family, member Z	BQ201156
RGICS51	High-mobility group box 2	BG380210
RGIBD80	Hypoxia-inducible factor 1, α subunit	AF057308
RGICD34	Insulin-like growth factor 2 receptor	U59809
RGIAA38	Lactate dehydrogenase A	X01964
RGIEB96	Low-affinity Na-dependent glucose transporter (SGLT2)	U29881
RGIAAC38	Moesin	AF004811
RGIAV15	Natriuretic peptide precursor type B	BF395072
RGIDE33	Nuclear RNA helicase, DECD variant of DEAD box family	AF063447
RGIDS30	Nuclear ubiquitous casein kinase 2	CA510075
RGIBY81	Nucleosome assembly protein 1-like 1	BC061811
RGIAAC87	Ornithine decarboxylase 1	M16982
RGIAAG89	Ornithine decarboxylase antizyme 1	BG375293
RGIAK21	P21 (CDKN1A)-activated kinase 1	U49953
RGIAAC88	Polo-like kinase homolog (<i>Drosophila</i>)	U10188
RGIBW70	Polypyrimidine tract binding protein	BC061858
RGIFD46	Preproenkephalin, related sequence	BE111086
RGIAS16	Profilin	BI282907
RGIAV34	Programmed cell death 8 (apoptosis-inducing factor)	AB041723
RGIAAG94	RAN, member RAS oncogene family	BM384578
RGIFD16	S-adenosylhomocysteine hydrolase	BC061841
RGIBZ01	Sepiapterin reductase	XM_342714
RGIAE15	Serine/threonine kinase 16	D86220
RGIAW68	Similar to high-mobility group protein 1 (HMG-1) (Amphoterin) (heparin-binding protein p30) (LOC297927), mRNA	BQ198991
RGIBK38	Similar to Kruppel-like factor 7 (ubiquitous) (LOC363243), mRNA	BE112585
RGIBK44	Solute carrier family 1, member 3	NM_019225
RGIAH55	Solute carrier family 12, member 2	AF051561
RGIDZ81	Solute carrier family 2, member 5	D13871
RGIBC25	Thymic stromal-derived lymphopoietin, receptor	NM_134465
RGIAB90	Thymosin, β 10	BE117292
RGIDV78	Thyroid hormone-response protein 1	BE118252

TABLE E1. Continued

Gene ID	Name	GenBank accession no.
RGIEA66	Transketolase	BM384351
RGICK91	Vascular endothelial growth factor	BE098396
RGIBY29	Wingless-type MMTV integration site family, member 4	AF188608
RGIAH24	Zinc finger protein 36	NM_133290

ADP, Adenosine diphosphate; ATPase, adenosine triphosphatase.

TABLE E2. Functional categories significantly overrepresented in the differentially regulated gene sets**A. Functional categories significantly downregulated in all 3 cancer cell lines**

GO functional category	GO ID	Gene category	P value	Gene name	GenBank accession no.	UniGene ID
Molecular function	GO:0004289	Subtilase activity	3.52E-12	Subtilisin-like endoprotease	L31894	Rn. 950
				Proprotein convertase subtilisin/kexin type 7	U36580	Rn. 10653
Biological process	GO:0006508	Proteolysis and peptidolysis	.001873	Subtilisin-like endoprotease	L31894	Rn. 950
				Proprotein convertase subtilisin/kexin type 7	U36580	Rn. 10653
				Insulin-degrading enzyme	X67269	Rn. 45029
				Cathepsin K	NM_031560	Rn. 5598
				Cathepsin E	BC062002	Rn. 92738
				Cathepsin L	BC063175	Rn. 1294
				N-arginine dibasic convertase 1	NM_012993	Rn. 2081
				Matrix metalloproteinase 2 (72-kd type IV collagenase)	X71466	Rn. 6422
Molecular function	GO:0004222	Metalloendopeptidase activity	.002101	Insulin-degrading enzyme	X67269	Rn. 45029
				N-arginine dibasic convertase 1	NM_012993	Rn. 2081
				Matrix metalloproteinase 2 (72-kd type IV collagenase)	X71466	Rn. 6422
Molecular function	GO:0004364	Glutathione transferase activity	.002243	Glutathione-S-transferase, μ type 3 (Yb3)	BC059130	Rn. 6036
				Glutathione-S-transferase, μ 2	BI302435	Rn. 625
Molecular function	GO:0005201	Extracellular matrix structural constituent	.0023	Elastin	M60647	Rn. 54384
Biological process	GO:0006730	One-carbon compound metabolism	.003204	Procollagen, type I, α 2	AF121217	Rn. 107239
				Dihydrofolate reductase	BF406309	Rn. 24681
Molecular function	GO:0004252	Serine-type endopeptidase activity	.004305	Carbonic anhydrase 3	NM_019292	Rn. 1647
				Subtilisin-like endoprotease	L31894	Rn. 950
				Proprotein convertase subtilisin/kexin type 7	U36580	Rn. 10653
Cellular component	GO:0005615	Extracellular space	.004489	Reelin	NM_080394	Rn. 98353
				Insulin-like growth factor-binding protein 5	AF139830	Rn. 1593
				Glypican 3	M22400	Rn. 9717
				Cathepsin E	BC062002	Rn. 92738
				Insulin-like growth factor 2	X14834	Rn. 964
				Clusterin	M16975	Rn. 1780
				Cathepsin L	BC063175	Rn. 1294
				Cathepsin K	NM_031560	Rn. 5598
				ATP-binding cassette, subfamily D (ALD), member 3	D90038	Rn. 7024
				Albumin	V01222	Rn. 34353
				Secreted acidic cysteine-rich glycoprotein	BM384341	Rn. 98989
				Kinase insert domain protein receptor	U93306	Rn. 88869
				Superoxide dismutase 3, extracellular	Z24721	Rn. 10358
				Secretoglobulin, family 1A, member 1 (uteroglobin)	AI009524	Rn. 2206
				Aquaporin 1	X67948	Rn. 1618
				Branched chain aminotransferase 2, mitochondrial	BC061790	Rn. 981
				CD24 antigen	BC064439	Rn. 6007
				Matrix Gla protein	BM384331	Rn. 2379

TABLE E2. Continued

A. Functional categories significantly downregulated in all 3 cancer cell lines

GO functional category	GO ID	Gene category	P value	Gene name	GenBank accession no.	UniGene ID
Molecular function	GO:0004842	Ubiquitin-protein ligase activity	.008121	Progesterin-induced protein Ubiquitin-conjugating enzyme E2D 2	X64411 BE115845	Rn. 54812 Rn. 7390
Cellular component	GO:0005578	Extracellular matrix	.010148	Secreted acidic cysteine-rich glycoprotein	BM384341	Rn. 98989
				Elastin	M60647	Rn. 54384
				Extracellular matrix protein 1	XM_215645	Rn. 97792
				Procollagen, type I, α 2	AF121217	Rn. 107239
				Glypican 3	M22400	Rn. 9717
				Matrix metalloproteinase 2 (72-kd type IV collagenase)	X71466	Rn. 6422
Molecular function	GO:0008483	Transaminase activity	.024083	Branched chain aminotransferase 2, mitochondrial	BC061790	Rn. 981
Molecular function	GO:0019838	Growth factor binding	.024465	Aminolevulinic acid synthase 1	NM_024484	Rn. 97126
				Insulin-like growth factor-binding protein 5	AF139830	Rn. 1593
				Insulin-like growth factor-binding protein 6	BQ204169	Rn. 6431
Molecular function	GO:0008270	Zinc ion binding	.029383	Solute carrier family 11, member 2	AF008439	Rn. 11418
				Carbonic anhydrase 3	NM_019292	Rn. 1647
				Matrix metalloproteinase 2 (72-kd type IV collagenase)	X71466	Rn. 6422
Molecular function	GO:0016787	Hydrolase activity	.030582	Acid phosphatase, prostate	M32397	Rn. 40121
				Reelin	NM_080394	Rn. 98353
				N-arginine dibasic convertase 1	NM_012993	Rn. 2081
				Matrix metalloproteinase 2 (72-kd type IV collagenase)	X71466	Rn. 6422
				Cathepsin K	NM_031560	Rn. 5598
				Insulin-degrading enzyme	X67269	Rn. 45029
				Cathepsin E	BC062002	Rn. 92738
				Cathepsin L	BC063175	Rn. 1294
				Nudix (nucleoside diphosphate-linked moiety X)-type motif 6	U58289	Rn. 83600
				Proprotein convertase subtilisin/kexin type 7	U36580	Rn. 10653
				Dynamin 1	X54531	Rn. 10354
				Adenosine monophosphate deaminase 3	U90888	Rn. 11106
Molecular function	GO:0008237	Metalloproteinase activity	.031231	Subtilisin-like endoprotease	L31894	Rn. 950
				Insulin-degrading enzyme	X67269	Rn. 45029
				N-arginine dibasic convertase 1	NM_012993	Rn. 2081
				Matrix metalloproteinase 2 (72-kd type IV collagenase)	X71466	Rn. 6422
Molecular function	GO:0005520	Insulin-like growth factor binding	.038283	Insulin-like growth factor-binding protein 5	AF139830	Rn. 1593
				Insulin-like growth factor-binding protein 6	BQ204169	Rn. 6431
Cellular component	GO:0005576	Extracellular	.040352	Acid phosphatase, prostate	M32397	Rn. 40121
				Insulin-like growth factor 2	X14834	Rn. 964
				Insulin-like growth factor-binding protein 5	AF139830	Rn. 1593

Table E2. Continued

A. Functional categories significantly downregulated in all 3 cancer cell lines

GO functional category	GO ID	Gene category	P value	Gene name	GenBank accession no.	UniGene ID
Molecular function	GO:0004197	Cysteine-type endopeptidase activity	.050041	Extracellular matrix protein 1	XM_215645	Rn. 97792
				Matrix Gla protein	BM384331	Rn. 2379
				Insulin-like growth factor-binding protein 6	BQ204169	Rn. 6431
				Cathepsin K	NM_031560	Rn. 5598
				Cathepsin L	BC063175	Rn. 1294

B. Functional categories significantly upregulated in all 3 cancer cell lines

GO functional category	GO ID	Gene category	P value	Gene name	GenBank accession no.	UniGene ID
Cellular component	GO:0005694	Chromosome	2.67E-12	High-mobility group box 2	BG380210	Rn. 2874
Biological process	GO:0007286	Spermatid development	1.92E-04	H2A histone family, member Z	BQ201156	Rn. 112573
				Thymosin, β 10	BE117292	Rn. 5983
				RAN, member RAS oncogene family	BM384578	Rn. 107698
Molecular function	GO:0006330	Single-stranded DNA binding	.001618	Nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate	NM_022799	Rn. 21324
				Polypyrimidine tract-binding protein 1	BC061858	Rn. 64440
Biological process	GO:0030036	Actin cytoskeleton organization and biogenesis	.003425	Thymosin, β 10	BE117292	Rn. 5983
				Profilin 1	BI282907	Rn. 1152
Biological process	GO:0000074	Regulation of cell cycle	.004719	Cyclin-dependent kinase 4	BC063158	Rn. 6115
				RAN, member RAS oncogene family	BM384578	Rn. 107698
				Nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate	NM_022799	Rn. 21324
Molecular function	GO:0003690	Double-stranded DNA binding	.005205	Cell-cycle protein p55CDC	NM_171993	Rn. 9262
				Nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate	NM_022799	Rn. 21324
Molecular function	GO:0008415	Acyltransferase activity	.010213	High-mobility group box 2	BG380210	Rn. 2874
				Spermidine/spermine N1-acetyl transferase	BC060588	Rn. 107986
Biological process	GO:0006096	Glycolysis	.010961	Acyl-coenzyme A: cholesterol acyltransferase	D86373	Rn. 59
				Glyceraldehyde-3-phosphate dehydrogenase	AF106860	Rn. 91450
				Lactate dehydrogenase A	X01964	Rn. 107896
Molecular function	GO:0003676	Nucleic acid binding	.013416	Polypyrimidine tract-binding protein 1	BC061858	Rn. 64440
				Zinc finger protein 36	NM_133290	Rn. 82737
Biological process	GO:0007264	Small GTPase-mediated signal transduction	.017968	RAN, member RAS oncogene family	BM384578	Rn. 107698
				P21 (CDKN1A)-activated kinase 1	U49953	Rn. 9149
Molecular function	GO:0005125	Cytokine activity	.020391	ADP-ribosylation factor-like 3	BQ204995	Rn. 9538
				Thymic stromal-derived lymphopoietin, receptor	NM_134465	Rn. 22704
				Bone morphogenetic protein 6	AY184240	Rn. 40476

TABLE E2. Continued

B. Functional categories significantly upregulated in all 3 cancer cell lines

GO functional category	GO ID	Gene category	P value	Gene name	GenBank accession no.	UniGene ID
Cellular component	GO:0005634	Nucleus	.027878	Programmed cell death 8 (apoptosis-inducing factor)	AB041723	Rn. 8124
				H2A histone family, member Z	BQ201156	Rn. 112573
				Polypyrimidine tract-binding protein 1	BC061858	Rn. 64440
				Nuclear ubiquitous casein kinase and cyclin-dependent kinase substrate	NM_022799	Rn. 21324
				Polo-like kinase 1 (Drosophila)	U10188	Rn. 11034
				Zinc finger protein 36	NM_133290	Rn. 82737
				RAN, member RAS oncogene family	BM384578	Rn. 107698
				Hypoxia-inducible factor 1, α subunit	AF057308	Rn. 10852
				High-mobility group box 2	BG380210	Rn. 2874
Biological process	GO:0007049	Cell cycle	.032999	Cyclin-dependent kinase 4	BC063158	Rn. 6115
				Polo-like kinase 1 (Drosophila)	U10188	Rn. 11034
Molecular function	GO:0005516	Calmodulin binding	.034449	Brain-abundant, membrane-attached signal protein 1	BE110763	Rn. 37779
				Calponin 3, acidic	BC062020	Rn. 57635
Molecular function	GO:0004691	cAMP-dependent protein kinase activity	.035111	Cyclin-dependent kinase 4	BC063158	Rn. 6115
				Serine/threonine kinase 16	D86220	Rn. 13308
Molecular function	GO:0004682	Protein kinase CK2 activity	.039394	Cyclin-dependent kinase 4	BC063158	Rn. 6115
				Serine/threonine kinase 16	D86220	Rn. 13308
Cellular component	GO:0005856	Cytoskeleton	.04869	Thymosin, β 10	BE117292	Rn. 5983
				Profilin 1	BI282907	Rn. 1152
				Moesin	AF004811	Rn. 2762
Molecular function	GO:0004674	Protein serine/threonine kinase activity	.049573	Cyclin-dependent kinase 4	BC063158	Rn. 6115
				P21 (CDKN1A)-activated kinase 1	U49953	Rn. 9149
				Polo-like kinase 1 (Drosophila)	U10188	Rn. 11034
				Serine/threonine kinase 16	D86220	Rn. 13308

Onto-Express (OE) analysis of the differentially regulated gene lists uses Gene Ontology (GO) terms to identify functional categories significantly overrepresented within the downregulated (A) or upregulated (B) gene sets. OE also computes accompanying significance values (P values) by comparing the observed number of genes in a given category with the total number of genes within that category on the array. We focused our attention on those functional categories containing at least 2 genes at a P value of less than .05. Genes can be associated with more than 1 GO term. ATP, Adenosine triphosphate; GTPase, glutathione triphosphatase; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate.

TABLE E3. Results of quantitative real-time polymerase chain reaction analysis for 5 genes of interest in cell lines JA, JB, and AMY

	VEGF	CDK4	PLK	IGF	HIF
Normal	1	1	1	1	1
JA	541.1932	102.5369	1770.572	380.038	352.1387
JB	230.7201	0.570382	621.6678	7.260153	75.06144
AMY	23.91759	0.239816	40.22443	0.659754	4.257481

Changes in expression level relative to the normal esophageal mucosal control cell line are indicated by the values in the table. VEGF, Vascular endothelial growth factor; CDK4, cyclin dependent kinase 4; PLK, polo-like kinases; IGF, insulin-like growth factor; HIF, hypoxia-inducible factor.