Transcriptional Oncogenomic Hot Spots in Barrett's Adenocarcinomas: Serial Analysis of Gene Expression

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Serial analysis of gene expression (SAGE) provides quantitative and comprehensive expression profiling in a given cell population. In our efforts to define gene expression alterations in Barrett’s-related adenocarcinomas (BA), we produced eight SAGE libraries and obtained a total of 457,894 expressed tags with 32,035 (6.9%) accounting for singleton tags. The tumor samples produced an average of 71,804 tags per library, whereas normal samples produced an average of 42,669 tags per library. Our libraries contained 67,200 unique tags representing 16,040 known gene symbols. Five hundred and sixty-eight unique tags were differentially expressed between BAs and normal tissue samples (at least twofold; $P < 0.05$), 395 of these matched to known genes. Interestingly, the distribution of altered genes was not uniform across the human genome. Overexpressed genes tended to cluster in well-defined hot spots located in certain chromosomes. For example, chromosome 19 had 26 overexpressed genes, of which 18 mapped to 19q13. Using the gene ontology approach for functional classification of genes, we identified several groups that are relevant to carcinogenesis. We validated the SAGE results of five representative genes (ANPEP, ECGF1, PP1201, EIF5A1, and GKN1) using quantitative real-time reverse-transcription PCR on 31 BA samples and 26 normal samples. In addition, we performed an immunohistochemistry analysis for ANPEP, which demonstrated overexpression of ANPEP in 67/86 (78%) Barrett’s dysplasias and 35/65 (54%) BAs. ANPEP is a secreted protein that may have diagnostic and/or prognostic significance for Barrett’s progression. The use of genomic approaches in this study provided useful information about the molecular pathobiology of BAs.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a major health problem in the United States with a prevalence of 5–7% in the general population and an increasing incidence rate (Serag, 2006). Approximately 10% of patients with chronic GERD develop a metaplastic condition known as Barrett’s esophagus (BE) in which the normal squamous epithelium of the esophagus is replaced by a columnar epithelium with goblet cells. BE is a serious premalignant lesion that can ultimately progress from metaplasia to dysplasia and subsequently to Barrett’s adenocarcinoma (BA) (Ferraris et al., 1997; O’Connor et al., 1999; Rana and Johnston, 2000). The incidence of BA has rapidly increased in the Western world over the past three decades (Hamilton et al., 1988; Phillips et al., 1991; Blot et al., 1993), and is comprised of aneuploid tumors characterized by complex molecular alterations (El-Rifai et al., 2001; El-Rifai and Powell, 2002). Several genetic abnormalities have been associated with Barrett’s tumorigenesis, including microsatellite instability (Meltzer et al., 1994), loss of heterozygosity (Dolan et al., 1999), gene-promoter hypermethylation (Sato and Meltzer, 2006), as well as up- and down-regulation of various genes (Wu et al., 1993; Swami et al., 1995; Regalado et al., 1998; Brabender et al., 2002). Comprehensive molecular analyses of DNA amplifications and gene expression have revealed complex genetic alterations in gastroesophageal and lower esophageal adenocarcinomas (El-Rifai et al., 1998; Varis et al., 2002; van Dekken et al., 2004; Kuwano et al., 2005).

The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute, University of Virginia, or Vanderbilt University.

Supported by: National Cancer Institute; Grant numbers: R01CA106176 (WER), GI SPORE CA 95103.

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Received 10 April 2007; Accepted 27 June 2007
DOI 10.1002/gcc.20479
Published online 17 July 2007 in Wiley InterScience (www.interscience.wiley.com).
Analyses of the human transcriptome map of normal tissues have shown clustering of highly expressed genes in chromosomal domains (Caron et al., 2001). Chromosomal arms and bands are known to occupy specific locations within the nucleus known as chromosome territories (CTs). The positioning of a gene(s) can influence its access to the machinery responsible for specific nuclear functions such as transcription and splicing (Cremer and Cremer, 2001). Recently, a few reports have suggested the presence of transcriptional hot spots in the cancer genome, (Wu et al., 2006) where overexpressed genes tend to cluster in defined chromosomal domains; however, similar information remains lacking for most cancer types. Serial analysis of gene expression (SAGE) provides unlimited, comprehensive, genome-wide analysis of gene expression in a given cell population (Velculescu et al., 1995, 2000). The major advantage in using SAGE is the quantitative ability to accurately evaluate transcript numbers without prior sequencing information. This method has proven invaluable in studies of several tumor types, including adenocarcinomas of the colon (Parle-McDermott et al., 2000; St Croix et al., 2000), prostate (Culp et al., 2001), pancreas (Argani et al., 2001), ovary (Hough et al., 2000), and breast (Seth et al., 2002). In this study, we explored the BA transcriptome using SAGE and mapped gene-expression changes to chromosomal positions, thereby generating a map of transcriptional oncogenic hot spots of this deadly cancer.

**MATERIALS AND METHODS**

**Serial Analyses of Gene Expression**

High-quality total RNA (500 μg) was extracted from four intestinal-type, moderately to poorly differentiated, BA cases (three gastroesophageal junctional [GEJ] and one lower esophageal) using an RNeasy kit (QIAGEN, Hilden, Germany). In addition, four normal gastric mucosa pools were used as reference samples. Each of these pools consisted of four normal gastric mucosal biopsy samples from four different individuals. The tumors selected for SAGE analysis were estimated to consist of more than 70% tumor cells. All normal samples had histologically normal mucosa confirmed on review of hematoxylin- and eosin-stained sections. Importantly, histopathological examination confirmed that none of the normal samples had any areas of inflammation or necrosis. All samples were collected with consent in accordance with approved Institutional Review Board protocols. SAGE libraries were constructed using NlaIII as the anchoring enzyme and BsmFI as the tagging enzyme as described in SAGE protocol version 1.0e, June 23, 2000, which includes a few modifications of the standard protocol (Velculescu et al., 1995). A detailed protocol and schematic of the method is available at (http://www.sagenet.org/protocol/index.htm). We sequenced 20,000 clones with an average of 2,500 clones per library, using the Cancer Genome Anatomy Project (CGAP). eSAGE 1.2a software was used to extract SAGE tags, remove duplicate ditags, tabulate tag contents, and link SAGE tags in the database to UniGene clusters using the recently reported ehm-Tag-Mapping method (Margulies and Innis, 2000; Margulies et al., 2001). The resulting libraries’ tags were compared with UniGene clusters and the SAGE tag “reliable” mapping database (http://www.sagenet.org/resources/genemaps.htm).Statistical analyses of these tags were then performed using eSAGE software.

**Quantitative Real-Time Reverse-Transcription PCR**

Quantitative real-time reverse-transcription PCR (qRT-PCR) was performed on 31 adenocarcinomas of Barrett’s-related origin, 26 normal gastric epithelial tissues, and 6 Barrett’s metaplasia tissue samples. All tissues were dissected to obtain ≥70% cell purity. All of the adenocarcinoma samples were collected from the GEJ or lower esophagus and ranged from well differentiated (WD) to poorly differentiated (PD), Stages I–IV, with a mix of intestinal- and diffuse-type tumors. RNA was purified from all samples using an RNeasy Kit. Single-stranded cDNA was generated using an Advantage™ RT-for-PCR Kit (Clontech, Palo Alto, CA). qRT-PCR was performed using an iCycler (BioRad, Hercules, CA) with SYBR Green technology, and the threshold cycle numbers were calculated using iCycler software v3.0. Reactions were performed in triplicate and threshold cycle numbers were averaged. For validation of SAGE results, we designed gene-specific primers for human ANPEP, ECGF1, PP1201, EIF5A1, GKN1, and HPR1. These primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and their sequences are available upon request. A single-melt curve peak was observed for each product, thus confirming the purity of all amplified cDNA products. The qRT-PCR results were normalized to HPR1, which had minimal variation in all normal and neoplastic samples tested. Fold overexpression was calculated according to the formula, \(2^{(R_t - E_t)}/2^{(R_i - E_i)}\), as described earlier (Buck-
### TABLE 1. The Top 93 Deregulated Genes in Barrett’s Adenocarcinomas

| Tag sequence | UniGene cluster ID | Gene symbol | Title | Location | T4 tag count | N4 tag count | Ratio, T4/N4 | P value |
|--------------|--------------------|-------------|-------|----------|--------------|--------------|-------------|---------|
| T4 tag count | N4 tag count       |             |       |          |              |              |             |         |
| T4 tag count | N4 tag count       |             |       |          |              |              |             |         |

**Upregulated genes**

| Tag sequence | UniGene cluster ID | Gene symbol | Title | Location | T4 tag count | N4 tag count | Ratio, T4/N4 | P value |
|--------------|--------------------|-------------|-------|----------|--------------|--------------|-------------|---------|
| GTGGCCACGG   | Hs.112405          | S100A9      | S100 calcium binding protein A9 | 1q21 | 355          | 0             | 418         | <0.001  |
| GAGCAGGGCC   | Hs.112408          | S100A7      | S100 calcium binding protein A7 | 1q21 | 95           | 0             | 112         | <0.001  |
| AAGATGGGTG   | Hs.114286          | CD9         | CD9 antigen (p24) | 12p13.3 | 112          | 7             | 10          | <0.001  |
| GCACCTGTCG   | Hs.11239           | ANPEP       | Aminopeptidase | 15q25-q26 | 76           | 0             | 89          | <0.001  |
| GTGACAGAGA   | Hs.1125673         | EIF4A1      | EIF4A translation initiation factor 4A, isoform 1 | 17p13 | 92           | 4             | 14          | <0.001  |
| TTTCTCTGTG   | Hs.1139222         | SPPR3       | Small proline-rich protein 3 | 1q21-q22 | 308          | 0             | 362         | <0.001  |
| GTTCAAGTGA   | Hs.1186810         | REPS2       | RALBP1 associated eps domain containing 2 | Xp22.2 | 107          | 2             | 32          | <0.001  |
| ACTGTATTTT   | Hs.1194691         | G protein   | G protein-coupled receptor, family C, group 5, member A | 12p13-p12.3 | 103          | 6             | 10          | <0.001  |
| TGGATCCTGA   | Hs.302145          | HBG2        | Hemoglobin, gamma G | 11p15.5 | 75           | 0             | 88          | <0.001  |
| CAGGGAGAAG   | Hs.308709          | GRP58       | Protein disulfide isomerase family A, member 3 | 15q15 | 79           | 2             | 32          | <0.001  |
| CTAGCTTTTT   | Hs.335175          | AGPAT4      | I-acetylglucosamine 3-phosphate O-acetyltransferase 4 | 1q21-q22 | 308          | 0             | 362         | <0.001  |
| TCACCCAGGGG  | Hs.391464          | ABCC1       | ATP-binding cassette, subfamily C member 1 | 16p13.1 | 52           | 0             | 61          | <0.001  |
| CCTCGGCTCA   | Hs.414510          | KRT7        | Keratin 7 | 12q12-q13 | 179          | 1             | 106         | <0.001  |
| TTCTTCTTAA   | Hs.419125          | TMEM38B     | Transmembrane protein 38B | 9q31.2 | 58           | 1             | 34          | <0.001  |
| TACCTGCAGA   | Hs.416073          | S100A8      | S100 calcium binding protein A8 | 1q21 | 343          | 1             | 204         | <0.001  |
| CAGCAGAGAC   | Hs.412416          | SERF2       | Small EDRK-rich factor 2 | 15q15 | 79           | 4             | 12          | <0.001  |
| GGCAGGCGATG  | Hs.445351          | LGALS1      | Lectin, galactoside-binding, soluble, 1 | 22q11.3 | 89           | 0             | 105         | <0.001  |
| GACATGCTGA   | Hs.447579          | LOC339290   | Hypothetical protein LOC339290 | 18p11.2 | 95           | 0             | 112         | <0.001  |
| GTTGGGATTG   | Hs.459927          | PTMA        | Prothymosin, alpha (gene sequence 28) | 2q35-q36 | 162          | 9             | 11          | <0.001  |
| TCACCCACAC   | Hs.462859          | SCFD2       | Short-chain dehydrogenase/reductase | 17q12 | 337          | 31            | 6           | <0.001  |
| CCCCCCGGGA   | Hs.466507          | LISCH7      | Liver-specific bHLH-Zip transcription factor | 19q13.3 | 48           | 0             | 56          | <0.001  |
| CCGGACAC    | Hs.473583          | NSEPI       | Y box binding protein 1 | 1p34 | 76           | 2             | 33          | <0.001  |
| CCGCGGTTG    | Hs.501293          | BSG         | Basigin (OK blood group) | 19q13.3 | 77           | 4             | 11          | <0.001  |
| GATACCTGGA   | Hs.505911          | GALNTL4     | Casein kinase 2, alpha 1 polypeptide | 11p15.3 | 94           | 0             | 111         | <0.001  |
| ACAGGCTACG   | Hs.503998          | TAGLN       | Transgelin | 11q23.2 | 71           | 3             | 14          | <0.001  |
| GTGGCTACA    | Hs.504820          | MGC14817    | Hypothetical protein MGC14817 | 12q14.3 | 242          | 16            | 9           | <0.001  |
| TAATTTTGCA   | Hs.508113          | OLPH4       | Olfactomedin 4 | 13q14.3 | 228          | 1             | 136         | <0.001  |
| GTGACAGGATG  | Hs.509736          | HSPCB       | Heat shock 90 kDa protein 1, beta | 6p12 | 149          | 13            | 7           | <0.001  |
| TGTCACTCTG   | Hs.512350          | LOC440676   | Heat shock 90 kDa protein 1, beta | 1q21.1 | 108          | 1             | 64          | <0.001  |
| AGTGGTGGAGC  | Hs.512488          | HSPB1       | Heat shock 27 kDa protein 1 | 12q12 | 98           | 1             | 58          | <0.001  |
| GCGGACGTCA   | Hs.513490          | ALDOA       | Aldolase A, fructose-bisphosphate | 16q22-q24 | 206          | 4             | 31          | <0.001  |
| ACCGCGTTG    | Hs.513803          | CYBA        | Cytochrome b-245, alpha polypeptide | 16q24 | 77           | 0             | 91          | <0.001  |
| AGCAGGACCA   | Hs.515714          | S100A16     | S100 calcium binding protein A16 | 1q21 | 61           | 0             | 72          | <0.001  |
| GATCTGTTTTG  | Hs.516488          | S100A2      | S100 calcium binding protein A2 | 1q21 | 61           | 0             | 72          | <0.001  |
| ATCGTGGGCG   | Hs.520942          | CLDN4       | Claudin 4 | 7q11.23 | 62           | 0             | 73          | <0.001  |
| CCACAGGCTAG  | Hs.520973          | HSPB1       | Heat shock 27 kDa protein 1 | 7q11.23 | 175          | 7             | 15          | <0.001  |
| AACCGTGCCA   | Hs.523302          | PRDX3       | Peroxiredoxin 3 | 10q25-q26 | 46           | 0             | 54          | <0.001  |
| CTACTCATCT   | Hs.531719          | ADCYAP1     | Adenylate cyclase activating polypeptide 1 | 18p11 | 85           | 1             | 51          | <0.001  |
| AACCTAGGGG   | Hs.5333            | KIAA0711    | Kelch repeat and BTB (PO2) domain containing 11 | 8p23.3 | 94           | 0             | 111         | <0.001  |

(Continued)
| Tag sequence | UniGene cluster ID | Gene symbol | Title | Location | T4 tag count | N4 tag count | Ratio, T4/N4 | P value |
|--------------|--------------------|-------------|-------|----------|--------------|--------------|--------------|---------|
| GACTTTCCAG   | Hs.534293          | SERPINA3    | Serpin peptidase inhibitor, clade A member 3 | 1q32.1    | 125          | 1            | 74          | <0.001  |
| CATTCCAGT    | Hs.54483           | NMI         | N-myc (and STAT) interacting                | 2p24.3-q21.3 | 285 | 0  | 335       | <0.001  |
| GACCGGCCAG    | Hs.546251          | ECGF1       | Endothelial cell growth factor 1            | 22q13     | 46        | 0            | 54          | <0.001  |
| TAGCTTAA      | Hs.554202          | SVIL        | Supervillin                                   | 1p11.2  | 210 | 0  | 247       | <0.001  |
| TGCCATCTG     | Hs.555971          | PPI 20I     | Transmembrane BAX inhibitor motif containing 1 | 2p24.3-p24.1 | 90 | 1  | 54        | <0.001  |
| CTATCTCTC     | Hs.75227           | NDUPA9      | NADH dehydrogenase (ubiquinone) I alpha subcomplex, 9, 39 kDa | 1p23.3 | 51 | 0  | 60        | <0.001  |
| ACTGCCTG      | Hs.81071           | ECM1        | Extracellular matrix protein I                | 1q21     | 77        | 1            | 46        | <0.001  |
| TACTTTTTG     | Hs.110401          | GIF         | Gastric intrinsic factor (vitamin B synthesis) | 1p13.1-p21.3 | 7  | 185 | 0.020   | <0.001  |
| ACAGAGCAAG    | Hs.131603          | EMI domain containing 2 | 2p24.3-p24.1 | 90 | 1  | 54        | <0.001  |
| ACCCTCCCCCA   | Hs.132087          | FLJ6299     | Kelch domain containing 6                     | 3q21.3    | 36 | 595 | 0.026   | <0.001  |
| AACCTCCCCA    | Hs.133539          | MAST4       | Microtubule associated serine/threonine kinase family member 4 | 5q12.3  | 1  | 51        | 0.010   | <0.001  |
| AACCTCCCCC    | Hs.134074          | ARL2BP      | Solute carrier family 35, member E1           | 19p13.11 | 1 | 42  | 0.010   | <0.001  |
| CTGCCAGCTC    | Hs.162071          | TFF1        | Trefoil factor 1                              | 2q21.3    | 95 | 174 | 0.3      | <0.001  |
| TTGAGATAGA    | Hs.16757           | GDDDR       | Down-regulated in gastric cancer GDDR         | 2p13.3    | 5 | 474 | 0.010   | <0.001  |
| CACCTCTGAT    | Hs.17324           | CKB         | Creatine kinase, brain                       | 1q32.2    | 9 | 74  | 0.070   | <0.001  |
| GACCTCCCCA    | Hs.178728          | MB3D4       | Methyl-CpG binding domain protein 3           | 1q33.3    | 6 | 64  | 0.020   | <0.001  |
| AGTGGCTCTC    | Hs.1867            | PGC2        | Progastricin (pepsinogen C)                   | 6p21.3-p21.1 | 36 | 595 | 0.040   | <0.001  |
| CGCTCCTGAA    | Hs.209217          | ASTN2       | Astrotactin 2                                 | 9q11      | 0 | 24  | 0.035   | <0.001  |
| CAGGTCTTC     | Hs.220864          | CHD2        | Chromodomain helicase DNA binding protein 2   | 15q21     | 1 | 42  | 0.010   | <0.001  |
| CGGGGAGGGA    | Hs.2681            | GAS         | Gastrin                                      | 1q21      | 0 | 100 | 0.009   | <0.001  |
| CACCTCCCCA    | Hs.283739          | BEB1       | Ubiquitin 4                                   | 1q21      | 4 | 76  | 0.030   | <0.001  |
| AGGCTTCTGA    | Hs.2859            | OPRL1       | Opiate receptor-like 1                       | 2q21.3    | 62 | 1086 | 0.030   | <0.001  |
| AACATCTGGG    | Hs.2979            | TFF2        | Trefoil factor 2 ( spasmytic protein 1)       | 1q21.2    | 5 | 76  | 0.030   | <0.001  |
| GCAGGCTCCA    | Hs.30131           | GHR1        | Ghrelin precursor                            | 3p26-2p25 | 5 | 50  | 0.060   | <0.001  |
| TGCAATTTA     | Hs.307835          | PGMS        | Phosphoglucomutase 5                         | 9p12-q12 | 6 | 40  | 0.090   | <0.001  |
| CCCTGGAAGC    | Hs.309228          | CUGBP2      | CUG triplet repeat, RNA binding protein 2     | 1p13      | 1 | 33  | 0.020   | <0.001  |
| CTGAGTCTGC    | Hs.36992           | ATP4A       | ATPase, H+/K+ exchanging, alpha polypeptide   | 1q21.2    | 10 | 384 | 0.020   | <0.001  |
| GATCTGCTGC    | Hs.370480          | ABCB7       | ATP-binding cassette, sub-family B (MDR/TAP), member 7 | Xq12-q13 | 1 | 26  | 0.020   | <0.001  |
| AACCTCTCCA    | Hs.38698           | C10orf27    | Chromosome 10 open reading frame 27           | 1q22.1    | 0 | 29  | 0.029   | <0.001  |
| TATCTAGTG     | Hs.393854          | ATP6V1G1    | ATPase, H+ transporting, lysosomal 13 kDa, V1 subunit G isoform 1 | 9q32 | 3 | 48  | 0.040   | <0.001  |
| AACCTCTCCA    | Hs.432854          | PGAS4       | Porin, putative                              | 1q21.3    | 365 | 6637 | 0.030   | <0.001  |
| GGAAGCGAAG    | Hs.434202          | ATP4B       | ATPase, H+/K+ exchanging, beta polypeptide    | 1q21.2    | 4 | 138 | 0.020   | <0.001  |
| TCTATCACTC    | Hs.438454          | FBXO25      | F-box protein 25                              | 8p23.3    | 12 | 376 | 0.020   | <0.001  |
| TCCCTTTAAG    | Hs.438824          | CK1P-1      | CK2 interacting protein 1                     | 1q21.2    | 3 | 49  | 0.040   | <0.001  |
| TTTTTAAGA     | Hs.445586          | UNQ473      | DMIC                                             | 1q12      | 2 | 35  | 0.030   | <0.001  |
| CAGTCTCTG     | Hs.445680          | H.445680    | Similar to anaphase promoting complex subunit 1 | 2q12.3 | 1 | 42  | 0.010   | <0.001  |
| ACTGATCTG     | Hs.447547          | VPS35       | Hypothetical protein MGC34800                | 16q12     | 5 | 34  | 0.090   | <0.001  |

(Continued)
RT is the threshold cycle number for the reference gene observed in the tumor, ET is the threshold cycle number for the experimental gene observed in the tumor, RN is the threshold cycle number for the reference gene observed in the normal sample, and EN is the threshold cycle number for the experimental gene observed in the normal sample. RN and EN values were averages of the corresponding normal analyzed samples. The ratio was calculated after normalization to total tag numbers.

Immunohistochemistry

Immunohistochemical (IHC) analysis of ANPEP protein expression was performed on a tumor tissue microarray (TMA) that contained 65 adenocarcinomas. Samples from adjacent normal and dysplastic tissues were included when available. All tissue samples were histologically verified, and representative regions were selected for inclusion in the TMA. All of the adenocarcinoma samples were collected from either the GEJ or lower esophagus and ranged from WD to PD, Stages I–IV, with a mix of intestinal- and diffuse-type tumors. Tissue cores with a diameter of 0.5 mm were retrieved from the TMA and mounted on a microscope slide. The slides were counterstained with hematoxylin.

TABLE 1. The Top 93 Deregulated Genes in Barrett's Adenocarcinomas (Continued)

| Tag sequence | UniGene cluster ID | Gene symbol | Title | Location | T4 tag count | N4 tag count | Ratio, T4/N4 | P value |
|--------------|--------------------|-------------|-------|----------|--------------|--------------|-------------|---------|
| TCATTTGAA    | Hs.464472          | MRLC3       | Myosin regulatory light chain MRLC2 | 18p11.2       | 0             | 27              | 0.031     | <0.001  |
| CAATGCCCTTCT | Hs.474571          | MYH9        | Myosin, heavy polypeptide 9, nonmuscle | 22q13.1       | 2             | 70              | 0.020     | <0.001  |
| TGCGAGACCA   | Hs.490038          | CPA2        | Carboxypeptidase A2 (pancreatic) | 7q32          | 0             | 24              | 0.035     | <0.001  |
| CATGTTCTCC   | Hs.516297          | TCF7L1      | Transcription factor 7-like 1 (T-cell specific, HMG-box) | 2p11.2       | 0             | 82              | 0.010     | <0.001  |
| CAGTTCTTTT   | Hs.518611          | TBC1D14     | TBC1 domain family, member 14 | 4p16.1       | 2             | 29              | 0.040     | <0.001  |
| AAGTACCAAA   | Hs.523130          | LIPF        | Lipase, gastric | 10q23.31      | 1             | 51              | 0.010     | <0.001  |
| CAGTGCTCC    | Hs.527922          | DLEU1       | Deleted in lymphocytic leukemia, 1 | 13q14.3       | 349           | 8046            | 0.020     | <0.001  |
| ACCTCACCAC   | Hs.529117          | CYP2B7P1    | Cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1 | 19q13.2       | 1             | 41              | 0.010     | <0.001  |
| CAGTGCTTCTT  | Hs.551178          | Hs.551178   | CDNA EF5627 fs, clone TRACHD010272 | 1             | 60              | 60              | 0.001     | <0.001  |
| GAGATTAGTG   | Hs.551521          | KCNE2       | Potassium voltage-gated channel, iso-related family, member 2 | 21q22.12      | 5             | 55              | 0.050     | <0.001  |
| TGACCTCAG    | Hs.558365          | ORM2        | Orosomucoid 2 | 9q32          | 1             | 25              | 0.020     | <0.001  |
| TCATTCTGAA   | Hs.69319           | GKN1        | Gastrokine I | 2p13.3        | 51             | 3592            | 0.010     | <0.001  |
| AAGTGCCCATA  | Hs.76253           | ATXN2       | Ataxin 2 | 12q24.1       | 2             | 37              | 0.030     | <0.001  |
| TTAACCCCTCTC | Hs.78224           | RNASEI      | Ribonuclease, RNase A family, 1 (pancreatic) | 14q11.2       | 26            | 219             | 0.070     | <0.001  |

T4, tag number in all tumor samples tested; N4, tag number in all normal samples. The expression of all genes was significantly altered in at least three tumor samples (P < 0.05), as compared to all normal samples. At least two tumors showed more than fivefold change (P ≤ 0.01). Tags with "0" value were replaced with arbitrary 0.5 values for relative calculation of fold expression. The ratio was calculated after normalization to total tag numbers.

Chromosomal localization of deregulated genes. Chromosomal regions that contain up-regulated genes are shown in red, whereas those that contain down-regulated genes are shown in green. Regions which contain both up- and down-regulated genes are colored in yellow. The distribution of these genes did not follow a random distribution pattern and several genomic regions contain clusters of deregulated genes. Some of the more significant "hot spots" can be seen here on chromosomes 1 (P < 0.01), 3 (P < 0.02), 12 (P < 0.01), 15 (P < 0.01), and 19 (P < 0.01).
from the selected regions of the donor blocks and punched to the recipient block using a manual tissue array instrument (Beecher Instruments, Silver Spring, MD). Each tissue sample was represented by four tissue cores on the TMA. Sections (5 μm) were transferred to polylysine-coated slides (SuperFrostPlus, Menzel-Gläser, Braunschweig, Germany) and incubated at 37°C for 2 hr. The resulting TMA was used for IHC analysis utilizing a 1:50 dilution of ANPEP antibody (CD13/aminopeptidase-M, clone 1A5, Serotec, England).

### TABLE 2. Chromosomal Minimal Common Overlapping Regions of Transcription Hot Spots

| Overexpressed genes | Number of genes | Gene symbols |
|---------------------|-----------------|-------------|
| 1q21                | 13              | S100A16, S100A2, S100A7, S100A9, S100A8, ECM1, S100A10, S100A6, LMNA, SPRR3, HDGF, HIST2H2BE, TAGLN2 |
| 6p21                | 6               | HSPA1A, HLA-A, HSPA1B, HLA-C, RPL10A, CLIC1 |
| 8q24-qter           | 4               | AW103531L, LY6D, LY6E, FLJ32440 |
| 11q13               | 4               | FTH1, CCND1, DKFZP61E198, TNCRNA |
| 12p13               | 9               | GAPD, C1R, C15, PHB2, MLF2, PTMS, FLJ22662, NDUFA9, CD9 |
| 14q32.3             | 4               | CRIP2, C14orf173, CRIP1, IGHG1 |
| 17q21               | 4               | KRT17, PPP1R1B, GRN, COL1A1 |
| 17q25               | 4               | LGALS3BP, MRPL12, ACTG1, NT5C |
| 19q13.4             | 5               | RPS9, RPS5, LENGB, CDC42EP5, Hs.534672 |
| 20q13               | 5               | P13, PGBP, TMEPA1, C20orf149, GATA5 |
| 22q13               | 7               | RPL3, Hs.102336, CDC42EP1, LGALS1, ATXN10, PLXNB2, ECGF1 |

| Downregulated genes | Number of genes | Gene symbols |
|---------------------|-----------------|-------------|
| 4q21                | 4               | IGj, CCNI, SEC31L1, CDS1 |
| 19q13.1             | 4               | UNQ473, CYP2B7P1, FCGBP, ATP4A |
| 21q22               | 4               | KCN2E, CLIC6, TFF1, TFF2 |

### TABLE 3. Chromosomal Location of Frequent Gene Alterations in Barrett’s Adenocarcinomas

| Chromosome | Upregulated transcripts = 242 | Downregulated transcripts = 153 | Grand total |
|------------|-------------------------------|-------------------------------|-------------|
| p arm | q arm | Total | p arm | q arm | Total |
| 1 | 15 | 20 | 35 (0.01) | 10 | 11 | 21 (0.35) | 56 |
| 2 | 7 | 10 | 17 (0.2) | 4 | 8 | 12 (0.39) | 29 |
| 3 | 3 | 4 | 7 (0.13) | 1 | 2 | 3 (0.06) | 10 |
| 4 | 1 | 4 | 5 (0.11) | 3 | 8 | 11 (0.02) | 16 |
| 5 | 0 | 8 | 8 (0.26) | 2 | 4 | 6 (0.4) | 14 |
| 6 | 8 | 2 | 10 (0.38) | 3 | 1 | 4 (0.2) | 14 |
| 7 | 3 | 3 | 6 (0.08) | 3 | 5 | 8 (0.12) | 14 |
| 8 | 2 | 6 | 8 (0.27) | 2 | 3 | 5 (0.37) | 13 |
| 9 | 1 | 7 | 8 (0.46) | 0 | 8 | 8 (0.29) | 16 |
| 10 | 5 | 7 | 12 (0.27) | 3 | 6 | 9 (0.28) | 21 |
| 11 | 5 | 9 | 14 (0.3) | 1 | 5 | 6 (0.11) | 20 |
| 12 | 10 | 11 | 21 (0.01) | 1 | 8 | 9 (0.04) | 30 |
| 13 | NA | 3 | 3 (0.36) | NA | 2 | 2 (0.24) | 5 |
| 14 | NA | 10 | 10 (0.27) | NA | 4 | 4 (0.17) | 14 |
| 15 | NA | 8 | 8 (0.01) | NA | 5 | 5 (0.19) | 13 |
| 16 | 3 | 3 | 6 (0.11) | 2 | 4 | 6 (0.07) | 12 |
| 17 | 4 | 8 | 12 (0.3) | 1 | 5 | 6 (0.22) | 18 |
| 18 | 4 | 0 | 4 (0.3) | 1 | 0 | 1 (0.44) | 5 |
| 19 | 8 | 18 | 26 (0.01) | 3 | 4 | 7 (0.37) | 33 |
| 20 | 1 | 8 | 9 (0.26) | 2 | 3 | 5 (0.41) | 14 |
| 21 | NA | 2 | 2 (0.23) | NA | 4 | 4 (0.05) | 6 |
| 22 | NA | 8 | 8 (0.45) | NA | 2 | 2 (0.02) | 10 |
| X | 2 | 1 | 3 (0.07) | 4 | 5 | 9 (0.08) | 12 |
| Y | 0 | 0 | NA | NA | 0 | NA | 0 |

A total of 568 transcripts were up- or down-regulated with statistical significance in which 395 known gene symbols were identified. In order to investigate and find statistically significant hot spots, the location of altered genes was compared with the list of all genes that are transcribed in both tumor and normal samples. The analysis was performed using Onto-Express online software (http://vortex.cs.wayne.edu/index.htm).

*Values in parentheses are P values.
dase-N Ab-3 mouse monoclonal antibody; Lab Vision Corporation, Fremont, CA). Sections were deparaffinized and rehydrated. TMA slides were treated in a microwave with citrate buffer for 20 min and incubated with the antibody at room temperature. Detection was performed using an avidin–biotin immunoperoxidase assay. Cores with no evidence of staining, or only rare scattered positive cells less than 3%, were recorded as negative. The overall intensity of staining was recorded as that for the core with the strongest intensity. IHC results were evaluated for intensity and frequency of staining. The intensity of staining was graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The frequency was graded from 0 to 4 by percentage of positive cells as follows: Grade 0, <3%; Grade 1, 3–25%; Grade 2, 25–50%; Grade 3, 50–75%; Grade 4, >75%. The index score was the product of multiplication of the intensity and frequency grades, which was then classified into a 4-point scale: index score 0 = product of 0, index score 1 = products 1 and 2, index score 2 = products 3 and 4, index score 3 = products 6 through 12.

RESULTS

Sequence Analyses of SAGE Libraries

Sequence analyses of 20,000 clones from eight SAGE libraries produced 457,894 expressed tags, with 32,035 tags (6.9%) accounting for singleton tags. The four tumor SAGE libraries (GSM758, GSM757, HG7, and HS29) produced 287,219 tags with an average of 71,804 tags per library. The normal samples (GSM14780, GSM784, 13S, and 14S) produced 170,675 tags with an average of 42,669 tags per library. The comparison of expressed tags to the UniGene cluster release of May 2005 identified 67,200 unique SAGE tags. These tags represented 16,040 known gene symbols according to UniGene information. Of these, 568 unique tags were differentially expressed between BAs and normal tissue samples (at least twofolds and \( P \leq 0.05 \)). These unique tags matched 395 known genes (242 upregulated and 153 downregulated) that regulate diverse cellular functions and signaling pathways, which may prove to be quite significant in the detection and prevention of cancer. Ninety-three genes were significantly altered, showing a greater than fivefold expression change in at least two tumor libraries as compared to all four normal libraries (\( P \leq 0.01 \)) (Table 1). Forty-eight genes showed up-regulation, whereas 45 were down-regulated. The group of over-expressed genes contained several with known cancer-related functions, including members of S100A calcium-binding proteins, heat-shock protein 27 kDa (HSPB1), heat-shock 90 kDa protein beta (HSPCB), prothymosin (PTMA), transmembrane bax inhibitor motif containing-1 (PP1201), peroxiredoxin-3 (PRDX3), and endothelial growth factor-1 (ECGF1). Down-regulated transcripts included genes such as gastronine (GKN1), down-regulated in gastric cancer (GDDR), gastrin intrinsic factor (GIF), methyl-CpG binding domain protein 3 (MBD3), and trefoil factor 2 (TFF2). CGAP maintains the public SAGE database for gene expression in human cancer (Lal et al., 1999), and sequence data are publicly available at http://www.ncbi.nih.gov/geo and http://cgap.nci.nih.gov/SAGE/.

Transcriptional Oncogenomic Hot Spots and Functional Classification of Genes

Onto-Express online software (http://vortex.cs.wayne.edu/index.htm) (Khatri et al., 2002; Draghici et al., 2003) was used to identify potential transcriptional oncogenomic hot spots in the genome and obtain the functional classification of the deregulated genes. We mapped all SAGE unique transcripts (16,040 gene symbols) to their corresponding cytogenetic locations. The altered transcripts (395 known gene symbols) were analyzed against all transcripts to generate an expression ideogram and identify transcription hotspots (Fig. 1). Interestingly, the distribution of altered genes was not uniform along the human chromosomes. Overexpressed genes tended to cluster in well-defined hot spots across the human genome (Table 2). For example, 26 overexpressed genes mapped to chromosome 19, of which 18 mapped to the single chromosome band 19q13. Similarly, 35 genes mapped to chromosome 1, of which 13 mapped to the chromosome band 1q21. Table 3 and Figure 1 summarize these data and map the genes to their corresponding cytogenetic locations.

Gene ontology (GO) terms are organized in three general categories: biological process, cellular role, and molecular function; terms within each GO category are linked in defined parent–child relationships that reflect current biological knowledge (Ashburner et al., 2000). Among the 395 differentially expressed genes, the number corresponding to each category was tallied and compared with the number expected for each GO category based on its representation in the reference gene list, which contained all of the unique 16,040 known gene symbols detected by analysis of the eight SAGE libraries. Significant differences
### TABLE 4. Functional Classification of Deregulated Genes in Barrett’s Related Adenocarcinomas Using Gene Ontology (GO)

| Gene symbol | Ratio | Gene symbol | Ratio | Gene symbol | Ratio | Gene symbol | Ratio |
|-------------|-------|-------------|-------|-------------|-------|-------------|-------|
| ALS2CR19    | 0.13  | DUSP6       | 27.38 | IGFBP7      | 3.14  | PTMA        | 10.71 |
| AURKAIP1    | 27.38 | EMP1        | 10.27 | ILK         | 27.38 | PTMS        | 6.19  |
| CRIP1       | 4.17  | GKN1        | 0.01  | LGALS1      | 105.95| S100A6      | 3.83  |
| BTG1        | 0.31  | GRN         | 4.63  | MACF1       | 6.07  | SFN         | 42.86 |
| CCND1       | 32.14 | HDGF        | 33.33 | MDK         | 10.12 | TIMP1       | 9.97  |
| CDKN2A      | 27.38 | HIF3A       | 5.21  | MTSS1       | 0.17  | TM4SF4      | 11.31 |
| CHEK1       | 4.03  | IFITM1      | 23.21 | PPP2R1B     | 23.21 | TSPAN1      | 0.01  |
| DNA binding and replication |       |             |       |             |       |             |       |
| ABCB7       | 0.02  | CTGF        | 22.62 | HIST2HBE    | 28.57 | PTMS        | 6.19  |
| ABCC1       | 61.9  | CUGBP2      | 0.02  | HSPA1B      | 11.61 | RAB40C      | 71.43 |
| ACTA1       | 20.24 | DUT         | 0.04  | ILK         | 27.38 | RBM17       | 0.09  |
| ACTB        | 4.5   | EGFR        | 54.76 | MAST4       | 0.01  | RHOD        | 26.19 |
| ACTG1       | 3.06  | EEF2K       | 0.03  | MBD3        | 0.02  | ROD1        | 28.57 |
| ARF1        | 28.57 | EIF5A       | 8.52  | MYH9        | 0.02  | SERPINA3    | 74.4  |
| ATP1A1      | 14.05 | ELF3        | 38.1  | NCL         | 25    | SET         | 0.29  |
| ATRIA4      | 0.02  | ENO1        | 9.23  | NTSC        | 2.52  | VNK1        | 0.02  |
| PTBP1       | 0.23  | EPHA4       | 0.03  | OBFC2A      | 0.23  | YBX1        | 22.62 |
| CDKN2A      | 27.38 | GNA12       | 15.18 | PKF1        | 8.23  | ZFHXB1      | 0.26  |
| CHD2        | 0.07  | GNAS        | 0.02  | PPP2R1B     | 23.21 | ZNF480      | 30.95 |
| CHEK1       | 4.03  | HDLBP       | 28.57 |            |       |             |       |
| RNA binding |       |             |       |             |       |             |       |
| CUGBP2      | 0.02  | NCL         | 25    | RNASE1      | 0.07  | RPS5        | 3.07  |
| EIF1AX      | 0.16  | PTBP1       | 0.23  | ROD1        | 28.57 | SERBP1      | 4.32  |
| HDLBP       | 28.57 | RBM17       | 0.09  | RPL18       | 5.7   | SNRPB       | 9.33  |
| MRPL12      | 15.48 | RBM19       | 0.03  | RPL3        | 21.73 | YBX1        | 22.62 |
| Transcription |     |             |       |             |       |             |       |
| ZFHXB1      | 0.26  | FOXA2       | 0.11  | NTSC        | 2.52  | RPLP0       | 19.05 |
| ZF36L1      | 41.67 | FOXD4L1     | 32.14 | CDKN2A      | 27.38 | EIFS1       | 28.57 |
| ELF3        | 38.1  | LASS6       | 0.16  | NMI         | 339.29| HSPB1       | 14.88 |
| EEF1B2      | 0.37  | RA17        | 25    | PTBP1       | 0.23  | BTG1        | 0.31  |
| AES         | 3.79  | TC7L1       | 0     | ROD1        | 28.57 | PPP2R1B     | 23.21 |
| ENO1        | 9.23  | TIMELESS    | 0.36  | SNRPB       | 9.33  | ESRG        | 0.05  |
| HIF3A       | 5.21  | YBX1        | 22.62 | HSPA1B      | 11.61 | PCBD2       | 0.36  |
| MBD3        | 0.02  | ZNF480      | 30.95 | EIFIAX      | 0.16  | GATAs       | 48.81 |
| PHB2        | 9.33  | CHD2        | 0.07  | EIF5A       | 8.52  |             |       |
| PTMA        | 10.71 | JUND        | 12.2  | EEF2K       | 0.03  |             |       |
| Receptor related |   |             |       |             |       |             |       |
| ANPEP       | 90.48 | F3          | 19.05 | INTS6       | 13.67 | PHB2        | 9.33  |
| ANXA1       | 4.6   | GNB2L1      | 34.52 | ITGB1       | 4.84  | PLXNB2      | 8.81  |
| ARF1        | 28.57 | GPR68       | 0.16  | LGALS3BP    | 47.62 | SLAMF7      | 46.43 |
| OPRL1       | 0.02  | HSPIA1A     | 55.95 | LRGP1       | 38.1  |             |       |
| DRD5        | 0.02  | IFITM1      | 23.21 | MTSS1       | 0.17  |             |       |
| EPHA4       | 0.03  | IL6ST       | 4.06  |             |       |             |       |
| Calcium ion binding | |             |       |             |       |             |       |
| ACTN4       | 10    | EEF2K       | 0.03  | MRLC2       | 3.71  | S100A7      | 113.1 |
| ANXA1       | 4.6   | EFHD2       | 11.31 | PADI1       | 42.86 | S100A8      | 204.17|
| ANXAI0      | 0.24  | ITGB1       | 4.84  | PRKCSH      | 29.76 | S100A9      | 422.62|
| ANXAI1      | 16.67 | ITPR3       | 0.22  | REP52       | 3.18  | SPARC       | 4.31  |
| C1R         | 24.4  | LRPB1       | 38.1  | S100A10     | 4.16  | SVIL        | 250   |
| C1S         | 19.05 | MACF1       | 6.07  | S100A16     | 72.62 | TKT         | 35.71 |
| CLTB        | 10.32 | MMP11       | 14.58 | S100A2      | 72.62 | VMD2L3      | 27.38 |
| CSP2G2      | 27.38 | MRCL3       | 4.76  | S100A6      | 3.83  |             |       |
| Zinc ion binding | |             |       |             |       |             |       |
| ALPPL2      | 34.52 | CRIP2       | 25    | MMP11       | 14.58 | S100A7      | 113.1 |
| ANPEP       | 90.48 | ESRG        | 0.05  | MT1F        | 0.17  | TRM2        | 0.18  |
| RA117       | 25    | GATA5       | 48.81 | PARK2       | 0.02  | ZFHXB1      | 0.26  |
| CA2         | 0.26  | GIT2        | 27.38 | PDLIM1      | 15.48 | ZF36L1      | 41.67 |
| CPA2        | 0.01  | HERC2       | 36.9  | PDLIM7      | 46.43 | ZNF480      | 30.95 |
| CRIP1       | 4.17  | HINT1       | 24.4  |             |       |             |       |

(Continued)
from the expected were calculated with a two-sided binomial distribution. False discovery rates (Benjamini et al., 2001) and Bonferroni adjustments were also calculated. The biological meaning of the \( P \) values obtained depends upon the list of genes that are submitted; as our gene list is from a comparison of BA samples, it can be inferred that this cancer stimulates the processes involved within the functional groups that were most highly represented in the results of the GO classification.

In our set of differentially expressed genes, the functional groups demonstrating the most significant representation appear under the biological-process ontology and map to the cell-cycle regulation, DNA binding and regulation, cell–environment interaction, and cell-signaling categories.

Table 4 summarizes several important GO functional classes.

**Validation of Transcriptional Targets**

To evaluate further the SAGE data, we selected five novel genes (ANPEP, ECGF1, PP1201, EIF5A1, and GKN1, all of which have important cellular or biological features) for validation with qRT-PCR. We confirmed over-expression of ANPEP, ECGF1, PP1201, and EIF5A1 and down-regulation of GKN1 in primary GEJ and lower esophageal adenocarcinoma samples (Table 5, Fig. 2). Interestingly, GKN1 was not expressed in normal esophageal mucosa samples but showed a transient expression in BE samples where 4/6 of these samples demonstrated expression levels com-

| Gene symbol | Ratio | Gene symbol | Ratio | Gene symbol | Ratio | Gene symbol | Ratio |
|-------------|-------|-------------|-------|-------------|-------|-------------|-------|
| ADCYAPI     | 50.6  | EPFA4       | 0.03  | IL6ST       | 4.06  | PDLIM1      | 15.48 |
| ANXA1       | 4.6   | FKBP8       | 41.67 | ILK         | 27.38 | PRMT1       | 30.95 |
| ARFI        | 28.57 | FMOD        | 0.17  | ITGB1       | 4.84  | PRKCSH      | 29.76 |
| WNT4        | 0.03  | GAST        | 0     | ITPR3       | 0.22  | PRMT1       | 30.95 |
| BSG         | 11.46 | GHRH        | 0.06  | LGALS3BP    | 47.62 | PDCR       | 47.62 |
| BTRC        | 7.54  | GNAS        | 0.02  | LY6E        | 7.29  | RAB40C      | 71.43 |
| C15         | 19.05 | GN2B2LI      | 34.52 | MDK         | 10.12 | REP52       | 31.85 |
| C9orf86     | 25    | GPR68       | 0.164 | MKLN1       | 6.45  | RHOD        | 26.19 |
| CDS1        | 0.01  | GRN         | 4.63  | MTSS1       | 0.17  | SFN         | 42.86 |
| CEACAM6     | 8.57  | HDGF        | 33.33 | MYH9        | 0.02  | SNX6        | 34.52 |
| DRD5        | 0.02  | HINT1       | 24.4  | NMI         | 339.29 | SPARC      | 4.31 |
| ECGF1       | 54.76 | IFIT1       | 23.21 | OPRL1       | 0.02  |            |       |
| Inflammation |     |             |       |             |       |             |       |
| ANXA1       | 4.6   | LGALS3BP    | 47.62 | PDLIM1      | 15.48 | SERPINA3    | 74.4  |
| CYBB        | 0.018 | LY6E        | 7.29  | PRMT1       | 30.95 | TFF1        | 0.32  |
| GPR68       | 0.164 | ML2F        | 6.94  | PTMS        | 6.19  | TFF2        | 0.03  |
| GPX1        | 9.92  | NMI         | 339.29 | S100A8     | 204.17 |            |       |
| ILIRN       | 7.94  | ORM2        | 0.024 | S100A9      | 422.62 |            |       |
| Cell environment interaction | | | | | | | |
| ACTN4       | 10    | ECGF1       | 54.76 | LY6D        | 45.83 | S100A6      | 3.83  |
| ADCYAPI     | 50.6  | EMLIN1      | 26.19 | MDK         | 10.12 | S100A9      | 422.62 |
| ANPEP       | 90.48 | ENAH        | 0.01  | MKLN1       | 6.45  | SLAMF7      | 46.43 |
| ANXA1       | 4.6   | FCGGBP      | 0.18  | MTSS1       | 0.17  | SPON2       | 6.67  |
| BTG1        | 0.31  | GRN         | 4.63  | PGMS        | 0.09  | TSPAN1      | 0.01  |
| CD9         | 9.52  | IL32        | 17.86 | PPIB2       | 0.05  | WNT4        | 0.03  |
| CEACAM6     | 8.57  | KLK6        | 35.71 | PPP2R1B     | 23.21 |            |       |
| CTGF        | 22.62 | LGALS3BP    | 47.62 | PDCR        | 47.62 |            |       |

The average ratio is shown. This ratio was calculated by comparing the total number of tags in tumor samples and normal samples.

*Examples: GO: 0007049 cell cycle, GO: 0008283 cell proliferation, and GO: 0006915 apoptosis.
*Examples: GO: 0000166 nucleotide binding, GO: 0003677 DNA binding, and GO: 0006260 DNA replication.
*Examples: GO: 0003723 RNA binding and GO: 0003730 mRNA 3′-UTR binding.
*Examples: GO: 0003700 transcription factor activity, GO: 0006350 transcription, and GO: 0006355 DNA dependent regulation of transcription.
*Examples: GO: 0004872 receptor activity, GO: 0005102 receptor binding, and GO: 0005057 receptor signaling protein activity.
*Examples: GO: 0005509 calcium ion binding.
*Examples: GO: 0002702 zinc ion binding.
*Examples: GO: 0007165 signal transduction, GO: 0007166 cell surface receptor linked signal transduction, and GO: 0007186 G-protein coupled receptor protein signaling pathway.
*Examples: GO: 0006952 defense response and GO: 0006954 inflammatory response.
*Examples: GO: 0006928 cell motility, GO: 0007155 cell adhesion, and GO: 0007267 cell–cell signaling.

Table 4. Functional Classification of Deregulated Genes in Barrett’s Related Adenocarcinomas Using Gene Ontology (GO) (Continued)
parable to those observed in normal gastric mucosa. We did not have samples with Barrett’s dysplasia for qRT-PCR. The GKN1 expression was lost in almost all adenocarcinoma samples (Fig. 2). The qRT-PCR products were run on 1.2% agarose gels for visual confirmation of these results (Fig. 3). RT-PCR results for all five genes were also compared in each individual primary tissue sample to determine any correlations in combined gene expression levels; however, we were unable to find any correlations of statistical significance.

Expression of ANPEP in Tumor TMA

The IHC analysis demonstrated a lack of immunostaining for ANPEP in normal esophageal and gastric epithelial tissues. On the other hand, BAs showed overexpression of ANPEP (Score +1 to +3) in 35/65 (54%) tumors. A weak to moderate expression of ANPEP (Score +1 to +2) was observed in 6/7 (86%) high-grade Barrett’s dysplasia samples. The immunostaining pattern of ANPEP was cytoplasmic with strong extracellular and luminal expression (Fig. 4). The immunostaining for ANPEP was observed in tumors with intestinal and diffuse histological subtypes and in all stages (Table 6). However, the relatively small sample size did not provide a sufficient statistical power to detect significant correlations between the IHC staining patterns and clinicopathological factors such as tumor histology, grade, or stage.

DISCUSSION

In this study, we performed a comprehensive analysis of the transcriptome of BAs using SAGE. The major advantage to using SAGE is the quantitative ability to evaluate accurately transcript numbers without prior sequence information. The SAGE analysis produced a great deal of information about transcripts and candidate cancer genes, and we have interpreted these data in terms of possible genomic and functional organization of candidate cancer genes. SAGE analysis requires laborious and extensive sequencing that often limits the number of samples that are subjected to analysis. We obtained a total of 457,894 expressed tags from eight SAGE libraries with minimal singleton tags (32,035; 6.9%). The qRT-PCR analysis on a larger sample size confirmed the SAGE results and validated the overexpression of ANPEP, ECGF1, PP1201, and EIF51 and downregulation of GKN1. ECGF1 (thymidine phosphorylase) expression has been shown to correlate with the angiogenic activity of some tumors (Mazurek et al., 2006). ECGF1 expression may be a sign of tumor-stromal interac-

### TABLE 5. Summary of qRT-PCR Results

| Overexpressed genes | Downregulated gene |
|---------------------|-------------------|
| EIF51 | ECGF1 | ANPEP | PP1201 | GKN1 |
| All cases | 9/31 (29)* | 15/31 (48) | 14/31 (45) | 15/31 (48) | 30/31 (97) |
| Gender | | | | | |
| Male | 4/19 (21) | 8/19 (42) | 10/19 (53) | 14/19 (74) | 19/19 (100) |
| Female | 2/4 (50) | 3/4 (75) | 1/4 (25) | 1/4 (25) | 0/4 (0) |
| Site | | | | | |
| GEJ | 4/10 (40) | 7/10 (70) | 7/10 (70) | 9/10 (90) | 16/10 (160) |
| ESO | 3/10 (30) | 4/10 (40) | 4/10 (40) | 5/10 (50) | 10/10 (100) |
| NA | 2/5 (40) | 4/5 (80) | 3/5 (60) | 0/5 (0) | 4/5 (80) |
| Stage | | | | | |
| T1–T2 | 2/8 (25) | 3/8 (37) | 5/8 (62) | 6/8 (75) | 8/8 (100) |
| T3–T4 | 5/14 (36) | 7/14 (50) | 5/14 (36) | 8/14 (56) | 14/14 (100) |
| NA | 3/9 (33) | 5/9 (55) | 4/9 (44) | 1/9 (11) | 8/9 (88) |
| Grade | | | | | |
| WD-MD | 3/10 (30) | 5/10 (50) | 5/10 (50) | 8/10 (80) | 10/10 (100) |
| PD | 2/9 (22) | 4/9 (44) | 5/9 (56) | 6/9 (67) | 9/9 (100) |
| NA | 4/12 (33) | 6/12 (50) | 4/12 (33) | 1/12 (8) | 11/12 (92) |
| Node | | | | | |
| N0 | 2/8 (25) | 2/8 (25) | 5/8 (63) | 6/8 (75) | 8/8 (100) |
| N1–N2 | 4/13 (31) | 7/13 (54) | 4/13 (31) | 7/13 (54) | 13/13 (100) |
| N3–N4 | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) |
| NA | 3/10 (30) | 6/10 (60) | 5/10 (50) | 2/10 (20) | 9/10 (90) |

*Values in parentheses are percentages.

NA, information not available; GEJ, gastroesophageal junction; ESO, esophageal; WD, well-differentiated; MD, moderately-differentiated; PD, poorly differentiated. We did not observe statistical significance with any of the correlates due to small sample size.
Figure 2. Quantitative real-time reverse-transcription PCR showing fold expression changes at the mRNA level of five representative genes. qRT-PCR analysis was performed using iCycler on 31 lower esophageal and GEJ adenocarcinoma samples (Tu) and 6 Barrett’s esophagus (BE) samples in comparison with 26 normal glandular mucosa samples (N). The horizontal axis shows sample numbers, whereas the fold expression in tumor samples compared with that in normal samples is shown on the vertical axis. The fold expression was calculated according to the formula: $2^{\Delta C_T} = 2^{(C_T^{normal} - C_T^{tumor})}$ as detailed in the “Materials and Methods” section. Each bar represents one sample. The displayed mean fold expression for each sample is calculated in comparison with the expression average of the 26 normal samples. The expression of each gene was normalized to the expression of HPRT1, which showed minimal variation in all normal and neoplastic samples tested. GKN1 shows downregulation (<0.4-fold expression) whereas ANPEP, PP1201, EIF5A1, and ECGF1 demonstrate overexpression (>2.5 fold expression) in primary tumors as compared to normal tissue samples.
tion promoting greater vascularization around the cancer lesion and has also been found to protect cells from DNA-damaging agents and related apoptosis (Jeung et al., 2006). EIF5A1 (eukaryotic translation factor 1) has been shown to be involved in cell proliferation through the action of polyamines (Nishimura et al., 2002, 2005), and plays a role in the regulation of TP53-related apoptosis (Li et al., 2004). PP1201, also known as transmembrane Bax inhibitor motif-containing 1 (TMBIM1), is a novel gene of cancer cells. Although very little is known regarding GKN1, it has been previously reported as highly expressed in normal gastric epithelium (Martin et al., 2003) and down-regulated in gastric carcinomas (Oien et al., 2004). We have detected strong expression of GKN1 in BE that was followed with loss of its expression in adenocarcinomas. This transient expression of GKN1 may be a protective response to acid-induced reflux-disease injury that is the lost with cellular progression to cancer. ANPEP, also known as CD13, is of a particular clinical interest since it is a secreted protein that may be used as a potential biomarker. Using IHC, analysis of ANPEP expression demonstrated protein expression at the outer cell membrane layers with significant secretion into the lumen of 6/7 Barrett’s high-grade dysplasia samples and generally greater expression in 35/65 adenocarcinomas, suggesting that ANPEP overexpression may be an early event in carcinogenesis. ANPEP expression plays a role in angiogenesis where a reduction in expression has been shown to cause reduced capillary formation (Fukasawa et al., 2006), cell motility (Chang et al., 2005), and adhesion (Fukasawa et al., 2006). Inhibition of ANPEP decreases the invasive potential of metastatic tumor cells in vitro (Saiki et al., 1993). Interestingly, ANPEP is also a cell-surface metalloproteinase that acts as a receptor for human coronavirus (Yeager et al., 1992) and is considered to be a marker for epithelial–mesenchymal interaction (Sorrell et al., 2003).

The combination of transcriptional analysis together with cytogenetic information provided a powerful tool to align altered transcripts across the human genome. Interestingly, the distribution of deregulated genes did not follow a uniform pattern across the genome. Instead, we found a remarkable pattern of distribution with the presence of transcriptional hot spots along chromosomal domains. From this pattern, we were able to identify novel, transcriptionally active, and oncogenic hot spots. One of our surprising findings was the clustering of 26 overexpressed genes in one of the smallest human chromosomes, 19. We also identified a number of other hot spots, such as 1q21 (13 genes), 12p13 (9 genes), and 6p21.2 (6 genes) (Table 2) in a recent analysis of amplification-based clustering demonstrated that cancers with similar etiology, cell-of-origin, or topographical location have a tendency to obtain convergent amplification profiles (Myllykangas et al., 2006). In line with this observation, Vogel et al. (2005) reported that genes expressed in concert are organized in a linear arrangement for coordinated regulation. The present evidence suggests organization of a large proportion of the human transcriptome into gene clusters throughout the genome, which are partly regulated by the same transcription factors, share biological functions, and are characterized by non-housekeeping genes (Vogel et al., 2005). Taken together, our results further highlight the complex organization of the cancer genome and suggest that integrated analysis of the transcriptome may reveal similar findings in other tumors as well.

Each cancer candidate gene was assigned to a functional group based on GO information (Table 4).
Using this approach, several groups that are highly interesting and relevant to carcinogenesis were identified including transcriptional regulators (38 genes) and zinc finger transcription factors (23 genes). Similarly, several candidate genes were found to be involved in the notable functional groups of cell-environment interaction and signal transduction. Subsets of these groups were of interest and included metalloproteinases and G proteins and their regulators. Among the interesting groups, we also observed deregulation of 31 genes that regulate cell calcium homeostasis. The role of calcium-binding proteins in carcinogenesis has drawn a complex picture showing downregulation or overexpression depending upon the tumor type and location (Kao et al., 1990; Mueller et al., 1999).

Figure 4. Immunohistochemical staining for ANPEP. (A, B) Normal gastric tissue glands (A) and normal esophageal squamous tissues (B) are negative for ANPEP immunostaining (Score 0). (C) Barrett’s dysplastic tissue demonstrates immunostaining for ANPEP that is secreted in the lumen (Score +2). (D) Barrett’s metaplasia tissue shows glandular staining (Score +2). (E) Diffuse-type esophageal adenocarcinoma tissue shows staining for ANPEP in the cell cytoplasm with significant localization along the cell membranes (Score +3). (F) Intestinal-type esophageal adenocarcinoma tissue showing high levels of ANPEP along the cell membranes as well as luminal secretion (Score +3). All photos (insets at upper-right quadrant) are taken at 200× and 400× magnification.
TABLE 6. Summary of Immunohistochemistry Analysis of ANPEP on Tissue Microarrays

|                | IHC score |          |          |          |          |
|----------------|-----------|-----------|-----------|-----------|-----------|
|                | 0         | 1         | 2         | 3         | Total     |
| All cases      | 30 (46)   | 21 (32)   | 6 (9)     | 8 (12)    | 65 (100)  |
| Gender         |           |           |           |           |           |
| Male           | 22 (73)   | 16 (76)   | 6 (100)   | 7 (88)    | 51 (78)   |
| Female         | 2 (7)     | 2 (10)    | 0 (0)     | 1 (13)    | 5 (8)     |
| NA             | 5 (17)    | 3 (14)    | 0 (0)     | 0 (0)     | 8 (13)    |
| Site           |           |           |           |           |           |
| GEJ            | 11 (37)   | 8 (38)    | 3 (50)    | 6 (75)    | 28 (43)   |
| ESO            | 15 (50)   | 11 (52)   | 3 (50)    | 2 (25)    | 31 (48)   |
| NA             | 3 (10)    | 2 (10)    | 0 (0)     | 0 (0)     | 5 (8)     |
| Histology      |           |           |           |           |           |
| Diffuse        | 10 (33)   | 7 (33)    | 0 (0)     | 2 (25)    | 19 (29)   |
| Intestinal     | 19 (63)   | 14 (67)   | 6 (100)   | 6 (75)    | 45 (69)   |
| Stage          |           |           |           |           |           |
| T1–T2          | 6 (20)    | 10 (48)   | 2 (33)    | 1 (13)    | 19 (29)   |
| T3–T4          | 15 (50)   | 6 (29)    | 3 (50)    | 4 (50)    | 28 (43)   |
| NA             | 8 (27)    | 5 (24)    | 1 (17)    | 3 (38)    | 17 (26)   |
| Grade          |           |           |           |           |           |
| WD             | 3 (10)    | 3 (14)    | 1 (17)    | 0 (0)     | 7 (11)    |
| MD             | 4 (13)    | 5 (24)    | 2 (33)    | 2 (25)    | 13 (20)   |
| PD             | 19 (63)   | 13 (62)   | 3 (50)    | 6 (75)    | 41 (63)   |
| Node           |           |           |           |           |           |
| N0             | 18 (60)   | 10 (48)   | 4 (67)    | 2 (25)    | 34 (52)   |
| N1–N2          | 3 (10)    | 8 (38)    | 1 (17)    | 4 (50)    | 16 (25)   |
| N3–N4          | 1 (3)     | 0 (0)     | 0 (0)     | 0 (0)     | 1 (2)     |
| NA             | 7 (23)    | 3 (14)    | 1 (17)    | 2 (25)    | 13 (20)   |

NA, information not available; GEJ, gastrointestinal; ESO, esophageal; WD, well-differentiated; MD, moderately-differentiated; PD, poorly-differentiated.

*Values in parentheses are percentages.

We thank Mr. Frank Revetta for his technical assistance and Mrs. Sheryl Mroz for editing this manuscript.

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