Targets of Gene Amplification and Overexpression at 17q in Gastric Cancer

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ABSTRACT

DNA copy number gains and amplifications at 17q are frequent in gastric cancer, yet systematic analyses of the 17q amplicon have not been performed. In this study, we carried out a comprehensive analysis of copy number and expression levels of 636 chromosome 17-specific genes in gastric cancer by using a custom-made chromosome 17-specific cDNA microarray. Analysis of DNA copy number changes by comparative genomic hybridization on cDNA microarrays revealed increased copy numbers of 11 known genes (ERBB2, TOP2A, GRB7, ACLY, PIP5K2B, MPRL45, MKP-1, LIHX1, MLN51, MLN64, and RPL27) and seven expressed sequence tags (ESTs) that mapped to 17q12-q21 region. To investigate the genes transcribed at the 17q, we performed gene expression analyses on an identical cDNA microarray. Our expression analysis showed overexpression of 8 genes (ERBB2, TOP2A, GRB2, AOC3, AP2B1, KRT14, JUP, and ITGA3) and two ESTs. Of the commonly amplified transcripts, an uncharacterized EST AA552509 and the TOP2A gene were most frequently overexpressed in 82% of the samples. Additional studies will be initiated to understand the possible biological and clinical significance of these genes in gastric cancer development and progression.

INTRODUCTION

Gastric carcinoma is one of the most common malignancies worldwide and is the second most frequent cause of cancer-related death (1). Moreover, cardiomyosporophagic junction and esophageal adenocarcinomas have the most rapidly rising incidence of all visceral malignancies in the United States and Western world for reasons that are unclear (2). Previous studies have documented the importance of genetic alterations affecting known oncogenes, tumor suppressor genes, and mismatch repair genes in the development of gastric cancer (3, 4). Several genes, such as cMET, ERBB2, MYC, and MDM2, are amplified in 10–25% of tumors, and their amplification is associated with advanced disease (3, 5). Comprehensive DNA copy number analyses of gastric cancers using CGH have demonstrated recurrent DNA copy number changes on several chromosomal regions. Gains at 17q have been shown to be frequent in gastric cancers (6). However, the critical regions of genetic alterations are large, and the target genes for amplification at 17q remain unknown.

Characterization of the chromosomal regions involved in DNA copy number changes is likely to reveal genes important for the development of gastric cancer. In the present study, we used a custom-made chromosome 17-specific cDNA microarray to systematically evaluate copy numbers and expression levels of genes at 17q in gastric carcinomas.

MATERIALS AND METHODS

Samples. Sixteen gastric cancer xenografts, four gastric cancer cell lines (CRL-5822, CRL-5974, CRL-5973, and CRL-1739) from the American Type Culture Collection (Manassas, VA), and five primary gastric cancers were used in this study. The cell line (CRL-1739) with normal DNA copy number of chromosome 17 was included as a control in Northern blot hybridizations. The cell lines were cultured under recommended conditions. Xenografting of gastric cancers was performed as described earlier (7). All tumors included in this study were dissected and verified histologically to be composed predominantly of neoplastic tissues. We have earlier characterized the DNA copy numbers of the cell lines and xenografts using “chromosomal” CGH. The details of the DNA copy numbers of the xenografts have been reported elsewhere (7). Fig. 1A summarizes the chromosomal CGH results for chromosome 17.

Chromosome 17-specific cDNA Microarray. The construction of the chromosome 17-specific cDNA microarray has been described previously (8). Briefly, the cDNA microarray contained a total of 636 clones, including 88 house keeping genes, 201 known genes from chromosome 17, and 435 EST clones from radiation hybrid map intervals D17S933-D17S930 (293–325 cR), the 17q12-q21 region, and D17S791-D17S795 (333–343 cR, the 17q23-q24 region). The preparation and printing of the cDNA clones on glass slides were performed as described elsewhere (9).

Copy Number and Expression Analyses by cDNA Microarrays. Genomic DNA was extracted from eight xenografts (X11, X27, X71, X75, X79, X83, and X95) and three cell lines (CRL-5822, CRL-5973, and CRL-5974). All cases had gains or high-level amplification at 17q by chromosomal CGH (Fig. 1). Normal genomic DNA was used as a reference in all experiments. Copy number analysis using CGH microarray was performed as described previously (8, 10). Briefly, 20 μg of genomic DNA were digested for 14–18 h with Alul and RsaI restriction enzymes (Life Technologies, Inc., Rockville, MD) and purified by phenol/chloroform extraction. Digested gastric cancer test DNA (6 μg) was labeled with Cy3-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ) and 8 μg of reference DNA with Cy5-dUTP using Bioprime Labeling kit (Life Technologies, Inc.). Hybridization was done according to the protocol by Pollack et al. (10) and posthybridization washes as described previously (11).

Total RNA was extracted from eight xenografts (X43, X49, X57, X68, X75, X76, X80, and X95) and three gastric cancer cell lines (CRL-5822, CRL-5973, and CRL-5974) by using RNeasy kit (Qiagen, GmbH, Hilden, Germany). A pool of four normal gastric epithelial tissue samples, enriched for the epithelial layer of the stomach through dissection and mucosal scraping, was used as a standard reference in all experiments. Reference RNA (100 μg) was labeled with Cy5-dUTP and 80 μg of test RNA with Cy3-dUTP by use of oligodeoxynucleotide-primed polymerization by SuperScript II reverse transcriptase (Life Technologies, Inc.). The labeled cDNAs were hybridized on microarrays as described previously (11, 12).

For both the copy number and expression analyses, the fluorescence intensities at the cDNA targets were measured using a laser confocal scanner (Agilent Technologies, Palo Alto, CA). The fluorescent images from the test and control hybridizations were scanned separately, and the data were analyzed using the DEARRAY software (13). After the subtraction of background intensities, the average intensities of each spot in the test hybridization were divided by the average intensity of the corresponding spot in the control hybridization. On the basis of our earlier reports (8, 14), clones that showed a

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The abbreviations used are: CGH, comparative genomic hybridization; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; AP, adapter protein.
Copy number ratios ≥ 1.5 were considered as amplified, and clones that showed an expression ratio ≥ 3 were considered as overexpressed. Clones that showed such increased ratios in the self versus self control experiment were excluded from the analysis.

Northern Hybridization. Total RNA was extracted from four gastric cancer cell lines and two normal stomach specimens using the RNeasy kit (Qiagen, GmbH). The Northern hybridization was performed using standard methods. Briefly, 10 μg of total RNA were size-fractionated on a 1% agarose gel containing formaldehyde and transferred on a Nytran membrane (Schleicher & Schuell, Keene, NH). The membrane was prehybridized for 1 h at 65°C in Express hybridization solution (Clontech, Palo Alto, CA) together with sheared Herring sperm DNA (10 μg/ml; Research Genetics, Huntsville, AL). Sequence-verified cDNA inserts were labeled with P32 by random priming (Prime-It; Stratagene, La Jolla, CA). Hybridization was performed in the Express hybridization solution (Clontech) at 65°C overnight followed by washes in 2 × SSC/SDS solutions. Signals were detected by autoradiography. The normal gastric tissues and CRL-1739 cell line (normal chromosome 17 on CGH) were used as control samples. A GAPDH cDNA was used as a control probe.

Multiplex RT-PCR. Multiplex RT-PCR was used to validate the cDNA array results for the two most overexpressed genes (ESTAA552509 and TOP2A) using seven xenografted and six primary gastric cancer samples. For reference expression, a pool of normal gastric epithelial tissues obtained from different individuals was used. Primary tumors of four xenografts were included in the analyses. mRNA was purified from the tissues using RNeasy (Qiagen), and cDNA synthesis was performed using Advantage RT-for-PCR Kit (Clontech). In each PCR reaction, primers for the human GAPDH gene were used as an internal reference. The PCR reactions were done using standard protocol for 28 cycles. We confirmed the reproducibility of the method by repeating the RT-PCR twice, and the results were consistent. The primers used for the RT-PCR were obtained from GeneLink (Hawthorne, NY), and their sequences are available on request.

RESULTS

Detailed Characterization of the 17q Amplification Using Chromosome-specific Microarray. Copy number levels of 636 chromosome 17-specific genes were evaluated by CGH microarray in eight xenografts (X11, X27, X37, X71, X75, X79, X83, and X95) and three gastric cancer cell lines (CRL-5822, CRL-5973, and CRL-5974) that

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1 Copy number ratios above the 1.5 threshold are shown in bold.
2 Alignment (bp position) and locus are shown according to Santa Cruz August freeze 2001 assembly.
showed gain or high-level amplification affecting chromosome 17 by chromosomal CGH (Fig. 1). CGH microarray analysis revealed increased DNA copy numbers ratio (≥1.5) in three or more cases for 11 genes and seven ESTs that map to 17q12 (4 clones) and 17q21 (14 clones; Table 1). The amplified genes/ESTs were localized at 302–321 cR in the radiation hybrid map5 (Fig. 1B) and between 38274220–46054957 bp at 17q, according to the University of California Santa Cruz’s August freeze 2001 assembly of the human genome sequence.6 The two most consistently amplified clones were EST (H62271) and ribosomal protein L27 (82%). Other frequently amplified genes included TOP2A, EST AA552509, and ERBB2. The details of the copy numbers and location of these genes/ESTs are listed in Table 1.

**Gene Expression Profiling of 17q Using cDNA Microarrays.**

Parallel expression survey in eight xenografts (X43, X49, X57, X68, X75, X76, X80, and X95) and the three cell lines identified 10 transcripts at 17q whose expression was elevated (ratio ≥3) in at least three specimens, as compared with the normal gastric epithelial cells (Table 2; Fig. 2). Three of the commonly amplified sequences (TOP2A, ERBB2, and EST AA552509) that map to 17q21 were also overexpressed frequently in our cDNA expression analyses. The two most consistently affected transcripts were EST AA552509 (82%) and the TOP2A (82%).

Other frequently overexpressed genes included AOC3 (45%), JUP (36%), ERBB2 (27%), ITAG3 (27%), and KRT14 (27%) at 17q21 region, as well as AP2B1 at 17q12, EST AA284262 at 17q23, and GRB2 at 17q25 (Table 2; Fig. 2).

**Northern Blotting.** Northern analysis was used as an independent expression assay to validate the cDNA microarray results. Because of the limited availability of RNA from the xenografted tumors, only cell lines were analyzed. Three genes, EST AA552509, TOP2A, and ERBB2, that showed overexpression in one or more cell lines by cDNA microarray were selected for analysis. Results from the Northern analysis confirmed the cDNA microarray data. ERBB2 was highly overexpressed in CRL-5822 cell line, TOP2A in all three cell lines, and EST AA552509 in CRL-5822 and CRL-5973 (Fig. 3). These genes were not expressed in the normal gastric epithelial sample or the gastric cell line (CRL-1739) that had normal chromosome 17 DNA copy numbers by chromosomal CGH (Fig. 3).

**Multiplex RT-PCR.** Expression analyses with RT-PCR showed elevated expression of TOP2A and EST AA552509 in all tested tumor samples, whereas no expression was seen in the pool of normal gastric epithelial tissues (Fig. 3). The xenografts and their corresponding primaries showed similar levels of expression.

**DISCUSSION**

Studies by chromosomal CGH have indicated that 17q is amplified frequently in gastric cancer. Here we used a custom-made cDNA

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**Table 2** Summary of expression levels of 10 chromosome 17q12-q21 transcripts in eight xenografts and three cell lines of gastric cancer by cDNA microarray:

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<td>3.8</td>
<td>1.3</td>
<td>0.8</td>
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<td>Junction plakoglobin (JUP)</td>
<td>Hs. 2340</td>
<td>R06417</td>
<td>43994962</td>
<td>17q21.2</td>
<td>3.1</td>
<td>2.8</td>
<td>0.9</td>
<td>1.2</td>
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<td>2.6</td>
<td>3.4</td>
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<td>1</td>
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<tr>
<td>Amine oxidase, copper containing 3 (AOC3)</td>
<td>Hs. 198241</td>
<td>T77398</td>
<td>45078066</td>
<td>17q21.2</td>
<td>4.6</td>
<td>1.9</td>
<td>4.5</td>
<td>2.6</td>
<td>2.1</td>
<td>2.6</td>
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<td>1.6</td>
<td>3.1</td>
<td>1</td>
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<tr>
<td>Integrin, α-3 (ITGA3)</td>
<td>Hs. 265829</td>
<td>AA424695</td>
<td>54688140</td>
<td>17q21.3</td>
<td>4.8</td>
<td>1.5</td>
<td>0.9</td>
<td>4.5</td>
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<td>EST</td>
<td>Hs. 56105</td>
<td>AA284262</td>
<td>65817334</td>
<td>17q23.2</td>
<td>1</td>
<td>1</td>
<td>5.2</td>
<td>3</td>
<td>15.6</td>
<td>2.8</td>
<td>0.6</td>
<td>1.9</td>
<td>1.3</td>
<td>0.6</td>
<td>0.4</td>
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<tr>
<td>Growth factor receptor-bound protein 2 (GRB2)</td>
<td>Hs. 296381</td>
<td>AA449831</td>
<td>81840742</td>
<td>17q25.1</td>
<td>0.8</td>
<td>0.8</td>
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<td>1.4</td>
<td>1.3</td>
<td>1.8</td>
<td>1.1</td>
<td>1.3</td>
<td>3</td>
<td>5.6</td>
<td>3.5</td>
</tr>
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</table>

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a Expression ratios above the 3 threshold are shown in bold.

b Alignment (bp position) and locus are shown according to Santa Cruz August freeze 2001 assembly.

* Internet address: http://genome.ucsc.edu.
microarray that contained 636 cDNA clones from chromosome 17 to systematically analyze the copy number changes at 17q in eight gastric cancer xenografts and three cell lines. The CGH microarray analyses showed increased copy number ratios for 18 clones that were localized to the 17q12-q21 region. To identify those genes that are activated through increased copy number, we performed a comprehensive gene expression profiling using the same chromosome 17-specific cDNA microarray. Three of the commonly amplified transcripts (TOP2A, ERBB2, and EST AA552509) that map to 17q21 were overexpressed frequently in our analyses and might, therefore, represent putative amplification target genes in gastric cancer. The cDNA microarray results were validated using Northern and RT-PCR analyses.

The two most frequently overexpressed genes in our samples were the EST AA552509 and TOP2A. In addition, ERBB2 was also amplified and overexpressed in >30% of tumors. Our data show that these genes have been overexpressed in gastric cancers with no indication of their expression in normal gastric epithelial tissues. The overexpression of EST AA552509 has not been reported before and might be important for gastric carcinogenesis or have a possible value as a tumor marker or therapeutic target. On the other hand, the importance of TOP2A, and ERBB2 in cancer, especially breast cancer, is well known (15, 16). TOP2A is an enzyme that catalyzes ATP-dependent strand-passing reactions and functions in DNA replication and chromosome condensation and segregation (17). TOP2A is a molecular target for many anticancer drugs (topo2 inhibitors). ERBB2 is amplified frequently in breast cancer and has been shown to be an independent prognostic factor (18, 19). In breast cancer, TOP2A is often coamplified with ERBB2 (20, 21). In our gastric adenocarcinomas, amplification and overexpression of TOP2A were independent of and also more frequent than ERBB2. Previous studies of ERBB2 in gastric cancer have shown that the frequency of its overexpression varies from 9 to 38% (22, 23), which is in agreement with our findings. Our results provide additional evidence that clinical studies are required to determine the possibility that TOP2A and ERBB2 are useful targets for cancer therapy in gastric cancer patients with these molecular alterations.

The up-regulation of GRB2, JUP, and ITAG3 genes in the present study supports our earlier results that show these genes to be overexpressed in gastric cancer (7). Interestingly, studies in breast cancer suggest that GRB2 may mediate transmission of ERBB2 oncogenic signals, which in turn activate mitogen-activated protein kinase pathway (24, 25). GRB2 is a widely expressed protein, which plays a crucial role in activation of several other growth factors (26).

KRT14, AOC3, and AP2B1 were overexpressed in ≥3 of 11 of our gastric cancers. Copper-containing amino oxidases, such as AOC3, are involved in the catalolysis of putrescine and histamine and are also involved in the regulation of growth and apoptosis (27). The AP2B1 is a member of AP complexes that function as vesicle coat components in different membrane traffic pathways. AP-2 complex associates with the plasma membrane and directs the internalization of trafficking cell surface protein (28). However, there is no information about the possible role of these genes in cancer.

Our study has identified genes that are coamplified at 17q12 and 17q21 amplicons that are not altered transcriptionally in comparison of tumors to normal reference samples. The lack of correlation between some amplified genes and their expression profile suggests that these genes are not critical targets at the 17q amplicon but might be coamplified together with critical genes within the amplicon structure. We also found genes that were overexpressed but not amplified by CGH microarrays. These results in CGH microarray may be attributed to the resolution of CGH-based technologies. On the other hand, upstream gene regulation and/or mutations are known as important biological mechanisms in transcriptional regulation irrespective of gene copy number.

Comparison of this gastric cancer study with our earlier data from breast cancer using the same cDNA microarray revealed a different pattern of alterations affecting chromosome 17 (8, 14). In breast cancer, two common regions of increased copy number and expression, 17q12-q21 and 17q23, were observed. In addition, the genes influenced by the 17q12-q21 amplification in gastric cancer differed from those in breast cancer where ERBB2 was among the most strongly affected (8, 14). These results indicate that although 17q is involved frequently in copy number alterations in several cancers, the target loci and genes might be different from one tumor type to another.

In summary, the present study demonstrates that although the 17q region contains hundreds of genes, only three genes were frequently amplified and overexpressed in gastric cancers, as compared with normal gastric epithelial tissues. The consistent overexpression of TOP2A in our gastric cancers suggests that this gene may be a potential target for topo2 inhibitors in gastric cancer patients. The overexpression of EST AA552509, in the majority of our samples, suggests that this novel gene may play a critical role in gastric tumorigenesis. We have initiated additional studies to explore the possible biological and clinical significance of these genes in gastric cancer development and progression.

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Targets of Gene Amplification and Overexpression at 17q in Gastric Cancer

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