

Targets of Gene Amplification and Overexpression at 17q in Gastric Cancer¹

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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 microarray revealed increased copy numbers of 11 known genes (*ERBB2*, *TOP2A*, *GRB7*, *ACLY*, *PIP5K2B*, *MPRL45*, *MKP-L*, *LHX1*, *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, cardia, gastroesophageal 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 CGH⁴ 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 (333–435 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, X57, 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 μ g of genomic DNA were digested for 14–18 h with *AluI* and *RsaI* restriction enzymes (Life Technologies, Inc., Rockville, MD) and purified by phenol/chloroform extraction. Digested gastric cancer test DNA (6 μ g) was labeled with Cy3-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ) and 6 μ g 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 scrapping, was used as a standard reference in all experiments. Reference RNA (100 μ g) was labeled with Cy5-dUTP and 80 μ g of test RNA with Cy3-dUTP by use of oligodeoxythymidylate-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 by 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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⁴ 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.

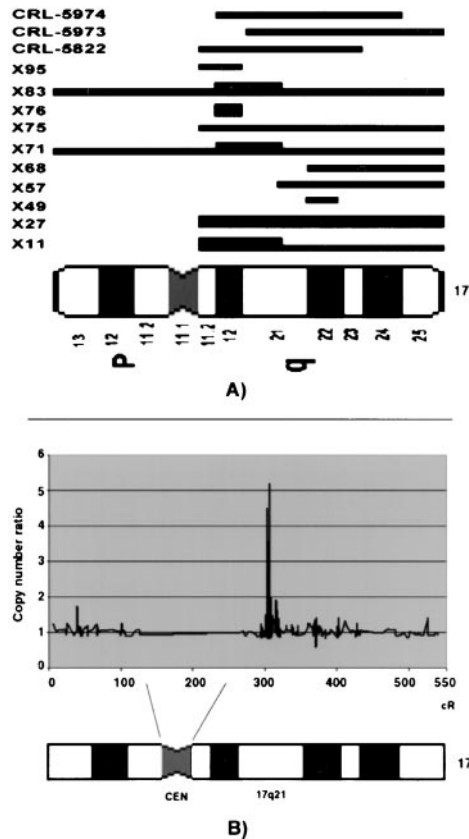


Fig. 1. DNA copy numbers in gastric cancer. A, summary of gains and high-level amplifications affecting chromosome 17 in gastric cancer xenografts and cell lines by chromosomal CGH. Horizontal bars, the extent of the copy number aberration in each sample. High-level amplifications are presented as wide bars. xenograft samples X43, X80, and cell line CRL-1739 had no detectable gains on chromosome 17. B, copy numbers survey of chromosome 17-specific genes in X83 gastric cancer xenograft by CGH microarray. The copy number ratios were plotted as a function of the position of the clones in the radiation hybrid map in cR scale. Individual data points were connected with a line. The chromosome 17 ideogram is shown below for visual comparison only.

copy number ratio ≥ 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 & Schuel, 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 $\mu\text{g}/\text{ml}$; Research Genetics, Huntsville, AL). Sequence-verified cDNA inserts were labeled with P^{32} 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 \times SCC/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 mRNeasy (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, X57, X71, X75, X79, X83, and X95) and three gastric cancer cell lines (CRL-5822, CRL-5973, and CRL-5974) that

Table 1 Summary of copy number ratios of 18 chromosome 17q12-q21 transcripts in eight xenografts and three cell lines of gastric cancer by CGH microarray^a

Gene	Unigene Id	Accession	Alignment ^b	Locus ^b	Samples											
					X11	X27	X57	X71	X75	X79	X83	X95	CRL-5822	CRL-5973	CRL-5974	
MRPL45 Mitochondrial ribosomal protein L45	Hs. 19347	AI277785	38274220/51922787	17q12/17q21.3	1.4	1.5	1.1	1	2.4	1.3	1.6	1.6	2	1.4	1.2	
MKP-1 like protein tyrosine phosphatase (MKP-L)	Hs. 91448	AA129677	38747279	17q12	1.9	1.6	0.8	1.4	1.1	1.1	1.2	2.8	1.4	1.4	1.7	
LIM homeobox protein 1 (LHX1)	Hs. 157449	AI375565	39307916	17q12	1	1.3	2.1	1.1	2.8	1.2	1	2.8	1.4	2.5	2.1	
Phosphatidylinositol-4-phosphate 5-kinase, type II, β (PIP5K2B)	Hs. 6335	H80263	40617731	17q12	1	0.9	1.6	1.7	1.3	1.3	0.9	1.7	1.4	2.8	0.8	
EST	Hs. 91668	H16094	41911205	17q21.1	1.2	0.8	1.2	1	1.4	3.8	4.7	0.9	3.1	1.2	0.8	
EST (FLJ20940 hypothetical protein)	Hs. 286192	AA552509	41868584	17q21.1	1.4	1.1	1.7	1.2	1.4	5.8	7.3	1.6	1.5	1.4	1.1	
H. sapiens MLN64 mRNA	Hs. 77628	AA504615	41877246	17q21.1	1.1	1.1	1.2	1.2	1.2	5.5	4.2	1.1	3.1	1.4	1.1	
V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2)	Hs. 323910	AA446928	41940229	17q21.1	1	1	1	1	1.1	2.6	2.1	1	1.7	1.1	1	
EST	Hs. 46645	AA283905	41972680	17q21.1	1.3	1.1	1	1.3	0.9	5.1	10.4	1.3	2	0.8	1.3	
EST	Hs. 318893	AA455291	41978415	17q21.1	1.1	0.9	1.1	1	0.9	7.8	2.5	0.8	3.2	1.4	0.9	
Growth factor receptor-bound protein 7 (GRB7)	Hs. 86859	H53703	41989210	17q21.1	1.4	1	1	1.1	1	5.7	8.9	0.9	2.7	1.4	1	
H. sapiens MLN51 mRNA	Hs. 83422	R52974	42331857	17q21.1	1.6	1.2	1.1	1.6	0.9	1.9	1.9	1.5	1.4	2.1	1.1	
Topoisomerase (DNA) II α (170kD) (TOP2A)	Hs. 156346	AA026682	42521254	17q21.2	1.3	1.1	1.2	1.6	1	1.7	1.6	1.4	1.6	1.4	1.1	
EST	Hs. 13268	AA514361	44056922	17q21.2	1.2	1.1	1	1.9	1.3	2.3	1.2	1.8	1.5	1.8	1.5	
ATP citrate lyase (ACLY)	Hs. 174140	R55974	44075311	17q21.2	1.2	1	1	1.6	1	1.6	1.1	1.6	1.4	1.6	2.3	
EST	Hs. 38039	H62271	44574937	17q21.2	5.7	2.9	2.3	6.7	3.1	1	1.7	2.5	3	4.3	2.7	
Ribosomal protein L27 (RPL27)	Hs. 111611	AA190881	45301136	17q21.2	4.6	2.5	2.2	6.1	1.7	0.9	2	2	2.1	1.4	2.9	
EST (DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 8, DDX8)	Hs. 171872	AI540663	46054957	17q21.3	1.4	1.7	0.5	1.4	0.9	2.9	1.6	1.2	1.9	3.7	1.1	

^a Copy number ratios above the 1.5 threshold are shown in bold.

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

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^a

Gene	Unigene Id	Accession	Alignment ^b	Locus ^b	Samples												
					X43	X49	X57	X68	X75	X76	X80	X95	CRL-5822	CRL-5973	CRL-5974		
Adaptor-related protein complex 2, β 1 subunit (AP2B1)	Hs. 74626	H29927	37327700	17q12	1.1	1	2.9	3.1	0.9	1.1	0.9	0.8	4.8	7.4	4.6		
EST (Hypothetical protein FLJ20940)	Hs. 286192	AA552509	41868584	17q21.1	21.9	4.5	6.4	7.6	10.2	10	17.3	0.6	12.1	11.2	0.6		
V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2)	Hs. 323910	AA446928	41940229	17q21.1	1	1	3	3.7	1.4	0.9	1.3	0.7	24.6	0.7	1		
Topoisomerase (DNA) II α (170kD) (TOP2A)	Hs. 156346	AA026682	42521254	17q21.2	4.1	6.1	16	4.5	2.8	6.6	3	1.4	5.6	7.6	6.8		
Keratin 14 (KRT14)	Hs. 117729	H44127	43757143	17q21.2	3.9	1.4	1.1	1.6	3.5	1	1.2	0.6	3.8	1.3	0.8		
Junction plakoglobin (JUP)	Hs. 2340	R06417	43994962	17q21.2	3.1	2.8	0.9	1.2	4.3	0.9	2.6	3.4	5	1.9	2.5		
Amine oxidase, copper containing 3 (AOC3)	Hs. 198241	T77398	45078066	17q21.2	4.6	1.9	4.5	2.6	2.1	2.6	4.2	1.6	3.1	1.8	5		
Integrin, α -3 (ITGA3)	Hs. 265829	AA424695	54688140	17q21.3	4.8	1.5	0.9	4.5	3.4	1.3	1.1	1.2	2.7	2.2	0.5		
EST	Hs. 56105	AA284262	65817334	17q23.2	1	1.7	5.2	3	15.6	2.8	0.6	1.9	1.3	0.6	0.4		
Growth factor receptor-bound protein 2 (GRB2)	Hs. 296381	AA449831	81840742	17q25.1	0.8	0.8	2.2	1.4	1.3	1.8	1.1	1.3	3.1	5.6	3.5		

^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.

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%), *ITGA3* (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

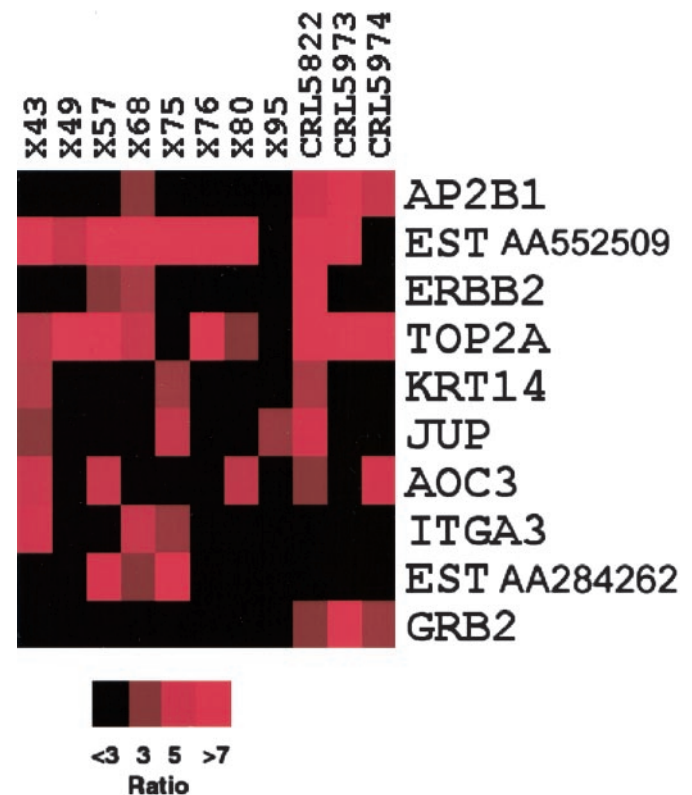
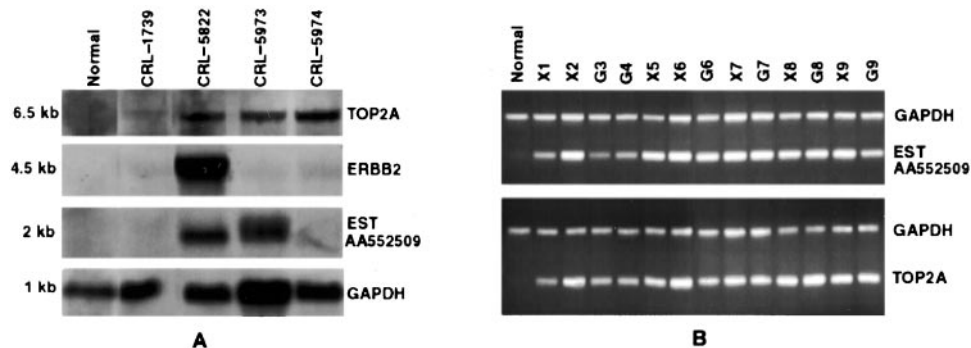


Fig. 2. Expression patterns of the most commonly overexpressed genes in gastric cancer xenografts and cell lines. Names of the genes are indicated on the right. Color coding for the expression ratios is shown below the graph. This image was created using Tree view software written by Michael Eisen, copyright 1998–1999, Stanford University.

⁵ Internet address: <http://www.ncbi.nlm.nih.gov/genemap>.

⁶ Internet address: <http://genome.ucsc.edu>.

Fig. 3. Validation of overexpressed genes in gastric cancer. A, Northern analysis of *TOP2A*, *ERBB2*, and EST AA552509 expression in normal gastric tissue and four gastric cancer cell lines. CRL-1739 had normal copy numbers by CGH. The size of each transcript is indicated on the right side of the corresponding picture. *GAPDH* was used as a loading control. B, expression analysis by multiplex RT-PCR in normal gastric tissue, seven xenografts (indicated by X-number), and six primary gastric cancers (indicated by G-number). Xenografts and their corresponding primary cancers have the same number. The names of the gene are shown on the right.



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 are 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 catabolism 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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