Silence of Chromosomal Amplifications in Colon Cancer

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ABSTRACT

Oncogene activation by gene amplification is a major pathogenetic mechanism in human cancer. Using comparative genomic hybridization, we determined that metastatic human colon cancers commonly acquire numerous extra copies of chromosome arms 7p, 8q, 13q, and 20q. We then examined the consequence of these amplifications on gene expression using DNA microarrays. Of 55,000 transcripts profiled, 2,146 were determined to map to one of the four common colon cancer amplions and to also be expressed in normal or malignant colon tissues. Of these, only 81 transcripts (3.8%) demonstrated a 2-fold increase over normal expression among cancers bearing the corresponding chromosomal amplification. Chromosomal amplifications are common in colon cancer metastasis, but increased expression of genes within these amplons is rare.

INTRODUCTION

Chromosomal aberrations may act as a fundamental pathophysiological event in human carcinogenesis (1). Common examples include inactivation of tumor suppressor genes by chromosome deletion, and creation of oncogenic fusion genes by chromosomal translocation (1). Additionally, as exemplified by the HER2 gene (ERBB2), chromosome amplification can activate a target gene to become an oncogene by inducing its expression to levels substantially greater than normal (2). However, cancer-associated chromosome amplifications may span entire chromosome arms, and it has been unresolved whether this class of chromosome aberration can act by altering expression of thousands of amplified genes or rather acts by deregulating only a select few of such amplified genes. In this study, we have combined comparative genomic hybridization and DNA microarray expression profiling to examine the expression of over 2000 genes that were identified as residing on chromosome arms that were amplified in metastatic colon cancers. We have found for nearly all these genes that chromosome amplification does not result in up-regulation of gene expression, or alternatively, that amplified genes that also demonstrated increased expression levels are quite rare.

MATERIALS AND METHODS

DNA Microarray Analysis. We designed two custom expression monitoring DNA microarrays using Affymetrix GeneChip technology (3) that contained essentially all expressed human genes in the public domain at the time of design. Briefly, we selected the sequences for inclusion on the arrays using genes predicted from the available human genome sequences and sequences derived from the expressed mRNAs and EST databases in GenBank (4). Consensus sequences representing human expressed sequences were generated using the Clustering and Alignment Tool software (DoubleTwist, Oakland, CA) using the mRNAs (nt) and EST (dbest) databases in GenBank. Prediction of the expressed genome from the human genome sequence was done using Ab initio exon prediction (5).

The arrays were hybridized with labeled cRNA derived from 10 µg of total RNA using standard protocols (6). The intensity data from the arrays were analyzed using a statistically based analysis methodology that allows for estimating expression levels and providing confidence intervals for these estimates. This method uses a gamma distribution model of the intensity data for normalization to control for the systematic variation attributable to nonbiological factors, such as array-to-array variability, and attributable to variation in sample quality. For each probe set, a single measure or average intensity was calculated using Tukey’s trimean of the intensity of the constituent probes (7).

Comparative Genomic Hybridization. Total genomic DNA from normal and tumor tissue was labeled with digoxigenin and biotin, respectively, using nick translation. Two µg each of digoxigenin-labeled normal DNA and biotin-labeled tumor DNA were ethanol precipitated together in the presence of 10 µg of salmon sperm DNA and 60 µg of Cot-1 fraction of human DNA (Life Technologies, Inc., Gaithersburg, MD). Hybridization conditions were as described previously (8). Briefly, probes were dried and resuspended in 10 µl of hybridization solution (50% formamide, 2 × SSC, and 10% dextran sulfate). DNA was denatured for 5 min at 80°C; repetitive sequences were allowed to preanneal for 1.5 h at 37°C and hybridized to normal human metaphase preparations. Normal metaphase slides were prepared from peripheral blood lymphocytes. Slides were treated with RNase (100 µg/ml) for 45 min, fixed, and dehydrated. DNA was denatured at 80°C for 1.5 min in 70% deionized formamide, 2 × SSC. The probe mixture was applied to the slide, covered with an 18-mm2 coverslip, sealed with rubber cement, and hybridized for 48 h at 37°C in a humidified chamber. Probe signals were detected using an amplification procedure and counterstained with DAPI, as described previously (9). Slides were mounted in antifade solution.

Microscopy and Image Analysis. Images were acquired with a cooled charge coupled device camera (Photometrics, Tuscon, Arizona) mounted on a Leica DMRBE epifluorescence microscope using filters specific for DAPI, fluorescein, and rhodamine (Chroma Technologies, Brattleboro, VT). CGH ratio profiles were calculated using Leica Q-CGH software (Leica Imaging Systems, Cambridge, United Kingdom) as described (9).

RESULTS

CGH of Metastatic Colon Cancer. To determine the relationship between chromosomal amplification and gene expression profiles, we characterized both processes in 23 independent metastatic colon cancers. This included 15 samples of metastatic tumor tissue resected from colon cancer liver metastases and an additional eight cell lines that were derived from biopsies of such colon cancer hepatic metastases. All metastatic tissue samples were dissected free of tissue contaminants and were confirmed by histology examination to be comprised of at least 70% malignant epithelial cells. We focused this study on colon cancer metastases, because they have had maximal opportunity in vivo to select for chromosome amplifications that could confer an aggressive cancer phenotype.

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2 These authors contributed equally to this work.
3 The abbreviation used are EST, expressed sequence tag; DAPI, 4,6-diamidino-2-phenylindole; CGH, comparative genomic hybridization; EGFR, epidermal growth factor receptor.

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metastases. Chromosomes 7p, 8q, 13q, and 20q are clearly amplified in substantial numbers of liver metastases, as shown in Fig. 1. CGH analysis of chromosome copy number changes in colon cancer liver metastases (n = 23). A, for each chromosome ideogram, every bar on the left side of ideogram indicates a sample with chromosomal loss (ratio, <0.5), and every bar on the right side indicates a sample with either chromosomal gains (ratios, >1 and <2; thin bar) or amplification (ratio, >2 thick bar). B, for each chromosome ideogram, every bar on the left side of ideogram indicates a sample with chromosomal loss (ratio, <0.5), and every bar on the right side indicates a sample with chromosomal amplification (ratio, >2). Chromosomes 7p, 8q, 13q, and 20q are clearly amplified in substantial numbers of liver metastases.

To identify chromosomal regions selected for amplification, these tumors were first characterized by comparative genomic hybridization (CGH) (9–11). Chromosomal regions demonstrating a ratio of 2 or greater compared with normal control were scored as amplified. Chromosomal regions demonstrating a ratio of between 1.5 and 2 were scored as gained (corresponding in a diploid genome to a chromosomal copy number of 3 and 4, respectively). Chromosomal regions showing a ratio of 0.5 or less were scored as lost. The CGH findings for all amplifications, gains, and losses detected in these metastatic samples are displayed in Fig. 1A, whereas only amplifications and losses are displayed in Fig. 1B. As shown in Fig. 1A, multiple different chromosomes frequently show chromosomal gains in colon cancer metastasis. In comparison with previous studies of lower stage colon cancers (8, 12), these metastatic samples more commonly showed genomic losses on chromosome 4 and uniquely showed gains without amplification of chromosome 2 (35% of samples). However, of the many chromosomal regions commonly gained in colon cancer metastasis, only four proved to also be recurrent sites of chromosomal amplification. These were chromosomes 7p, 8q, 13q, and 20q that proved amplified in from 26 to 43% of the cancer metastases (Fig. 1B; Table 1) in a pattern that was similarly observed both in primary metastases tumors and in metastases derived cell lines. Although detected in metastatic samples, these amplifications have also been identified by us and others in primary colon cancers (8, 12), thereby suggesting that chromosomal amplification likely mainly contributes to steps in colon carcinogenesis that are prior to metastases development.

Expression Profiling of Colon Cancer Amplicons. We next wanted to determine how each of the four major colon cancer amplicons affected expression of the genes that reside within the amplicons. Accordingly, cRNAs prepared from each of these samples were hybridized to DNA microarrays that measure gene expression of approximately 55,000 genes, EST clusters, and predicted exons. Analysis of the microarray data confirmed that the liver metastases samples expressed high levels of colon epithelial markers and, consistent with our histological determinations, were essentially free of hepatocyte-specific gene expression. The working draft genome assembly (13) was used to identify and order all transcription units present on the microarray that mapped to chromosomes 7p, 8q, 13q, and 20q. From this set, we selected for further analysis the 2146 transcription units that on the microarrays demonstrated either expression in control microdissected normal colon epithelial strips or expression in colon cancer liver metastases. This included all transcription units whose median expression in 9 normal colon epithelia exceeded a threshold of 100 average intensity units or whose expression in any of the 23 amplified liver metastases samples exceeded this threshold.

Fig. 2 displays the qualitative gene expression patterns for the 2146 transcription units that were mapped to and ordered across the four major colon cancer chromosomal amplicons. For each transcript, the median expression level was first calculated among the group of colon cancers determined by CGH to be amplified across the corresponding chromosomal region. Median gene expression in the colon cancer metastases with chromosome amplifications was then compared with the median level of gene expression among 9 control normal colon epithelial samples. Fig. 2 denotes in green the position of each transcript in which chromosome amplification was associated with a 2-fold or greater increase in average gene expression compared with normal colon epithelia. In contrast, red denotes the positions of transcripts in which chromosome amplification was associated with decreased average gene expression to <0.5 the level of normal controls. Brown regions contain transcripts for which chromosome amplification was associated with expression of between 0.5 and 2-fold the normal controls.

As is visually obvious from Fig. 2, chromosome amplification does not in general induce increased expression levels of the overwhelming majority of genes residing in the common colon cancer amplicons. The ubiquitous brown shading of the chromosomal ideograms reflects that 90% of the 2146 transcripts within these amplicons remain in the >0.5 and <2.0 range of expression relative to normal (coded brown), despite a greater than doubling of the corresponding gene copy number in each of the colon cancer samples represented (Table 2).

<table>
<thead>
<tr>
<th>Chr</th>
<th>7p</th>
<th>8q</th>
<th>13q</th>
<th>20q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of liver metastases</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>No. with amplifications</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>No. with gains</td>
<td>15</td>
<td>14</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>No. with no change</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. with losses</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1: CGH analysis summary
A summary is shown of the total number of liver metastases analyzed and of those, the number that displayed losses, gains, amplifications, or no change in chromosomes 7p, 8q, 13q, and 20q.
Indeed, a 2-fold or greater increase in gene expression was found in only 81 of 2146 (3.8%) genes subject to chromosome amplification (Table 2). In contrast, 164 genes (7.7%) residing within amplified regions actually demonstrated decreased expression to \(0.5\) the level of normal colon (Table 2). Moreover, 60 of the 81 upregulated genes showed only a 2–3-fold increased expression, suggesting they are not targets of high-level amplifications hidden within the broader regions of the chromosomal amplicons (Fig. 3). This observation is not attributable to any technical limitation in detection on the microarrays of increased gene expression. Indeed, the microarray analysis of genes residing on nonamplified chromosomes detected 14 transcripts whose median expression in the colon cancer metastases was increased from 4- to 12-fold over normal. Our first conclusion is thus that chromosomal amplification is associated with increased expression among only a small minority of genes residing within colon cancer chromosomal amplicons.

Additionally, this analysis reveals that the few genes residing in colon cancer amplicons that do show increased expression are scattered, and that none of the four amplicons demonstrates a “hotspot” in which a cluster of genes all show increased expression. This is illustrated in Fig. 2 by the magnified views of neighboring gene expression in the regions surrounding representative individual upregulated genes (coded in green). This observation contrasts with the finding in the MCF7 breast cancer cell line of a cluster of highly expressed genes mapping to a chromosome 17 amplicon, which thus may reflect events specific to MCF7 or to this specific breast cancer amplification (14).

The conclusion that genes within amplicons are rarely overexpressed remains valid even when applied to a single sample and to a segmental chromosomal amplicon delimiting a region smaller than the

<table>
<thead>
<tr>
<th>Amplifications of chromosome</th>
<th>7p</th>
<th>8q</th>
<th>13q</th>
<th>20q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total unique genes</td>
<td>530</td>
<td>542</td>
<td>501</td>
<td>573</td>
</tr>
<tr>
<td>No. of genes with increased expression</td>
<td>13</td>
<td>23</td>
<td>26</td>
<td>19</td>
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<tr>
<td>Highest fold expression increase</td>
<td>4.38</td>
<td>4.86</td>
<td>5.22</td>
<td>3.68</td>
</tr>
<tr>
<td>No. of genes with decreased expression</td>
<td>42</td>
<td>47</td>
<td>34</td>
<td>41</td>
</tr>
</tbody>
</table>
full chromosome arms we first characterized. For example, Fig. 4 shows CGH of an hepatic metastases-derived cell line (V394) in which a significant 13q amplification is restricted to only the proximal portion of the 13q arm. High-resolution CGH of this sample mapped this amplicon to three distinct chromosomal bands ranging from 13q11 to 13q21 (Fig. 4). However, only 8 of the 251 expressed genes lying within this amplicon showed a 2-fold or greater increase over normal (range 2.14–4.48-fold).

These data suggest that the selective advantage of chromosomal amplifications is most likely to lay in rare individual “target” genes that, similar to HER2 in some breast cancers, show both high copy number increases as well as an accompanying increase in gene expression. However, our analysis excludes some previously nominated candidate genes as being such specific targets of the major colon cancer-associated chromosomal amplifications. For example, genes posited as targets of chromosome 20q amplification in cancer that showed less than a 2-fold increased expression in colon cancer hepatic metastases ampliﬁed for 20q included: topoisomerase 1 (TOP1), the AIB1 transcription factor (NCOA3), the zinc ﬁnger transcription factor 217 (ZNF217), and the MYB transcription factor family member MYBL2 (Table 3) (15–18). [Our array did not measure expression levels for the BTAK mitotic kinase (STK15), which remains a potential candidate target gene (19, 20)]. Similarly, we found that the epidermal growth factor receptor gene (EGFR), residing on chromosome 7p, demonstrates on average a decreased expression to 0.49-fold of normal colon in cases in which chromosomes 7 is ampliﬁed (Table 3). Furthermore, consistent with published ﬁndings that increased MYC expression in colon cancer is not attributable to ampliﬁcation of this gene (21), which resides on chromosome 8q, we noted that the majority of the colon cancers demonstrating increased MYC expression derived from cases that did not bear chromosome 8q ampliﬁcation. Indeed, MYC expression was on average increased only 1.74-fold above normal in cases with ampliﬁcation of chromosome 8q (Table 3). Northern analysis also independently conﬁrmed the absence of induction of EGFR and MYC expression in representative hepatic metastases with high genomic ampliﬁcations of chromosomes 7p and 8q, respectively (data not shown). Thus, each of the four major colon cancer-associated chromosomal ampliﬁcations is presumptively based on a novel gene target.

**DISCUSSION**

Virtually all carcinomas reveal a tumor-speciﬁc distribution of genomic imbalances (22), and the acquisition of such chromosomal gains and losses is evidenced during early stages of tumorigenesis. The maintenance of these aberrations is strongly selected for even in the presence of gross aneuploidy and intratumor heterogeneity (23), and individual tumor-speciﬁc chromosomal imbalances are preserved even after years of cell culture (9, 11). Moreover, as illustrated by this study, chromosomes found to be frequently ampliﬁed in cancers also participate in an additional group of cases as chromosomes that are commonly gained. It is tempting to speculate that the functional consequence, and presumed selective advantage, of these chromosomal aneuploidies is exerted on the tumor cells via modiﬁcations in the expression status of genes on these chromosomes. The aim of this study was to determine whether chromosomal ampliﬁcation globally alters the expression of ampliﬁed genes or whether expression of only

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**Table 3 Gene expression by amplified colon cancers**

<table>
<thead>
<tr>
<th>Gene and GenBank accession</th>
<th>Chromosome</th>
<th>Fold increased expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>U07804</td>
<td>20</td>
<td>1.69</td>
</tr>
<tr>
<td>AA150333 W46488 N56493</td>
<td>20</td>
<td>0.71</td>
</tr>
<tr>
<td>ZNF217 N70546 MYBL2</td>
<td>20</td>
<td>0.71</td>
</tr>
<tr>
<td>X13293 MYC M13929 L00058</td>
<td>20</td>
<td>1.10</td>
</tr>
<tr>
<td>EGFR X00588</td>
<td>8</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.49</td>
</tr>
</tbody>
</table>
a select few genes is changed. Having examined the expression of
>2000 transcripts resident on amplified chromosomes present in
metastatic colon cancers, we found that chromosomal amplifications
do not result in global induction of gene expression, even when
examined in late-stage metastatic colon cancers. Thus, total relative
gene expression levels for the great majority of transcript units
must be tightly regulated. Genes that do demonstrate significantly
increased expression in association with chromosome amplification
are few in number and are not geographically clustered together. The
microarrays used in this study sampled 55,000 genes, ESTs, and
predicted exons and therefore provide a comprehensive analysis of the
human transcriptome that buttresses our conclusion that increased expression of amplified genes is a rare event. This conclusion lends
support to the likely importance in carcinogenesis of individual genes
that both are overexpressed in cancer and that map to sites of chro-
omosomal amplifications. We note for example in breast cancer the
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omosomal amplifications. We note for example in breast cancer the

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