Molecular Dissection of 17q12 Amplicon in Upper Gastrointestinal Adenocarcinomas

Nazif Maqani,1 Abbes Belkhiri,1 Christopher Moskaluk,2 Sakari Knuutila,3 Ailtaf A. Dar,1 and Wael El-Rifai1

1Department of Surgery and Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, Tennessee; 2Department of Pathology, University of Virginia, Charlottesville, Virginia; and 3Molecular Cytogenetics Laboratories, University of Helsinki Central Hospital, Helsinki, Finland

Abstract

DNA amplification at 17q is frequently detected in upper gastrointestinal adenocarcinomas (UGC; stomach and esophagus). In this study, we did fluorescence in situ hybridization on a tissue microarray that contained 304 UGCs and 89 normal stomach samples using a ~168-kb BAC clone (CTD-2019C10) that maps to 17q12-q21.1. This 168-kb region contains the following genes: PPP1R1B/DARPP-32, STARD3, TCAP, PNMT, PERLD1, ERBB2, C17orf37, and GRB7 as well as the first two exons of ZNFN1A3. DNA amplification (≥5 signals) was detected in 85 of 282 (30%) of UGCs, and high-level amplification (≥10 signals) was seen in 28 of 282 (10%) of all tumors. Adenocarcinomas of gastrointestinal junction and lower esophagus had the highest frequency of amplification (45%) compared with stomach tumors (27%; P = 0.04). On the other hand, 38% of tumors with intestinal-type morphology had amplification compared with 26% of diffuse-type tumors (P = 0.02). We further did quantitative real-time reverse transcription-PCR on 74 frozen tissue samples from UGCs for 11 genes located within or adjacent to the boundaries of this ~168-kb genomic region. These genes include all 9 genes that are fully or partially located inside the CTD-2019C10 clone as well as 2 additional adjacent genes (NEUROD and TOP2A). Overexpression of PPP1R1B/DARPP-32, TCAP, and TOP2A was seen in approximately half of the tumors, whereas STARD3 and ZNFN1A3 were rarely overexpressed (12%). Interestingly, there was a statistical correlation between expression of all 8 genes that map between PPP1R1B/DARPP-32 and GRB7, whereas expression of NEUROD, ZNFN1A3, and TOP2A that are partially inside or adjacent to the boundaries of the CTD-2019C10 clone did not correlate with the expression of any of these 8 genes. These data show a transcriptionally active oncogenic region bounded by PPP1R1B/DARPP-32 and GRB7 in UGCs and provide further insight into expression levels of several critical genes. (Mol Cancer Res 2006;4(7):449–55)

Introduction

Upper gastrointestinal adenocarcinomas (UGC; i.e., adenocarcinomas of the stomach and esophagus) are the second most common cause of death from cancer in the world (1, 2). UGCs are poorly responsive to therapy (3) and are characterized by complex genetic changes and frequent genomic amplification at 17q (4). Today, esophageal and gastroesophageal junction (GEJ) adenocarcinomas have the fastest rising incidence of all cancers in the western world (5-7), most notably in the last three decades. Still, the critically important genes involved in the development of these tumors remain largely undefined. Using comparative genomic hybridization and microarrays, the critical region of 17q amplification has been defined around 17q12-q21 (8). Amplification at 17q is associated with several malignancies, such as breast, gastric, pancreatic, and neuroblastomas (9-15). The 17q12-q21 region is a gene-rich area that contains several candidate cancer genes, including PPP1R1B/DARPP-32, ERBB2, TOP2A, and GRB7. The 17q12-q21 region has been heavily implicated in breast and UGC. The amplification of a 280-kb core region at 17q12 leads to activation of two hypothetical proteins in breast cancer (16). However, detailed analysis of this region in UGC is missing. PPP1R1B/DARPP-32 has also been recently mapped close to the ERBB2 (17), ERBB2 and TOP2A are known to have prognostic implications in breast cancer (18-21). Hereceptin, an ERBB2 antagonist, and several TOP2A antagonists, such as epirubicin and doxorubicin, have been used for patients who are positive for the gene amplification and/or overexpression of ERBB2 and TOP2A in breast cancer. A multicenter phase II study of trastuzumab in combination with epirubicin and docetaxel was recently reported as first-line treatment for HER-2-overexpressing metastatic breast cancer (22-24). In UGCs, the rate of amplification and overexpression of ERBB2 usually varies between 8% and 28%, whereas amplification and expression of TOP2A seems to be more frequent (8, 25-28).

Our previous work identified a common region of amplification at 17q12-q21.1 (8). In this study, we did fluorescence in situ hybridization (FISH) on a tumor tissue...
array to evaluate the amplification of a ~168-kb region in primary tumor samples. We next explored the expression level of 11 genes/transcripts that map to this 168-kb region and its adjacent boundaries to determine the transcriptional effects of this amplification.

Results

DNA Copy Number Amplification

Using the 168-kb BAC clone for FISH, 22 tumors and 13 normal tissues did not show satisfactory FISH signals and were excluded from analysis. None of the normal samples showed amplification of the 168-kb BAC clone. DNA amplification (≥5 signals) was observed in 85 of 282 (30%) of analyzable tumors and high-level amplification (≥10 signals) was observed in 28 of 282 (10%) of tumors. Barrett’s adenocarcinomas of the lower esophagus and GEJ had the highest frequency of DNA amplification (45%) compared with stomach tumors (antrum, body, and cardia; 27%; P = 0.04). On the other hand, 38% of intestinal type had amplification with 26% of diffuse-type tumors (P = 0.02). The details of the results are shown in Tables 1 and 2 and Fig. 1.

Quantitative mRNA Expression

Quantitative real-time reverse transcription-PCR (RT-PCR) analysis of tumor samples, compared with normal samples, showed overexpression of PPP1R1B/DARPP-32, TCAP, and TOP2A in approximately half of the tumors, whereas GRB7 was overexpressed in 32%, ERBB2 in 22%, and PERLD1 in 20% of tumors. On the other hand, STARD3, PNMT, C17orf37, and ZNFN1A3 were rarely overexpressed (≤14%; Fig. 2). The expression of these genes was more frequently observed in intestinal type versus diffuse type and more in the tumor invasion group T1 + T2 versus the T1 + T3 group. The details of the results, and the observed frequencies compared with clinicopathologic variables, are shown in Table 3. Interestingly, there was a significant statistical correlation of expression of all 8 genes that map between PPP1R1B/DARPP-32 and TOP2A (Fig. 3). On the other hand, the expression of genes that are partially inside (ZNFN1A3) or adjacent (NEUROD and TOP2A) to the borders of the CTD-2019C10 clone did not correlate with the expression of any of the other 8 genes that are fully inside the amplicon area (Fig. 3). This may indicate that genes located between PPP1R1B/DARPP-32 and TOP2A follow a distinct pattern that is different from other overexpressed genes adjacent to them.

Discussion

This study has characterized a 168-kb amplicon at 17q12-q21 in UGCs. The 168-kb amplified region contains 8 known genes as well as the first two exons of ZNFN1A3 (as shown in Fig. 4). Analysis of the mRNA expression levels of 11 genes in genomic order (NEUROD, PPP1R1B/DARPP-32, STARD3, TACP, PNMT, PERLD1, ERBB2, C17orf37, GRB7, ZNFN1A3, and TOP2A) showed a significant statistical correlation between the mRNA levels in genes bounded by PPP1R1B/DARPP-32 and GRB7 but not with NEUROD, ZNFN1A3, and TOP2A mRNA expression levels. Therefore, the results may suggest a transcriptionally active oncogenic region bounded by PPP1R1B/DARPP-32 and GRB7 that is uniquely amplified and overexpressed in UGCs. A recent study suggested an evolutionary recombination hotspot around the GSML-GSDM that is adjacent to ZNFN1A3 (29). Although we have not investigated recombination, the findings of Katoh and Katoh (29) may suggest an underlying genetic rearrangement of this region that could explain the correlation of expression pattern of the aforementioned genes in our study. DNA amplification was statistically more frequent in proximal adenocarcinomas of the GEJ and lower esophagus than in tumors of the stomach (cardia, body, and antrum; P = 0.04).

Adenocarcinomas of the GEJ and lower esophagus are often associated with gastroesophageal reflux disease that leads to a premalignant Barrett’s esophagus condition. The incidence of gastroesophageal reflux disease is increasing at alarming rates (30, 31); today, esophageal and GEJ adenocarcinomas have the fastest rising incidence of all cancers in the western world (5-7). We observed a correlation between DNA amplification and intestinal-type adenocarcinomas. Furthermore, mRNA expression levels of most of the genes that we tested showed a similar association with intestinal-type adenocarcinomas as shown in Table 3. We have also observed that amplification of the 168-kb region was more often observed in T1-T2 invasive tumors and in patients with a poor survival rate at 5 years. These findings agree with reports in breast cancer (16, 32).

The amplification of the 168-kb region was seen on average in 30% of tumors. Interestingly, our study has shown that

<table>
<thead>
<tr>
<th>Table 1. Summary of FISH Results on 393 Tissue Samples</th>
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<tbody>
<tr>
<td>Samples*</td>
</tr>
<tr>
<td>FISH score, n (%)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>NR</td>
</tr>
<tr>
<td>Tumor (n = 304)</td>
</tr>
<tr>
<td>Normal (n = 89)</td>
</tr>
</tbody>
</table>

*The tissue microarray contained adenocarcinomas of the stomach (antrum, body, and cardia), GEJ, and lower esophagus.

†NR, not reported; 0, 2 signals; 1, 3-4 signals; 2, 5-9 signals; 3, 10-15 signals; 4, ≥15 signals.

<table>
<thead>
<tr>
<th>Table 2. Histopathologic and Clinical Variables of FISH Results</th>
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<tbody>
<tr>
<td>Site</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Stomach (n = 125)</td>
</tr>
<tr>
<td>GEJ and esophagus (Barrett’s; n = 47)</td>
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<tr>
<td>Histology</td>
</tr>
<tr>
<td>Diffuse (n = 39)</td>
</tr>
<tr>
<td>Intestinal (n = 94)</td>
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<tr>
<td>Tumor-node-metastasis stage</td>
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<tr>
<td>T1 (n = 12)</td>
</tr>
<tr>
<td>T2-T4 (n = 156)</td>
</tr>
<tr>
<td>5-y Survival</td>
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<tr>
<td>Alive (n = 41)</td>
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<tr>
<td>Dead (n = 78)</td>
</tr>
</tbody>
</table>

NOTE: 0, 2 signals; 1, 3-4 signals; 2, 5-9 signals; 3, 10-14 signals; 4, ≥15 signals per cell; stomach, antrum, body, and cardia. P ≤ 0.05 were considered significant and are indicated in bold.
overexpression for several genes was more frequent than the amplicon frequency. \textit{PPP1R1B/DARPP-32} was most frequently overexpressed (55%) followed by \textit{TCAP} (54%) and \textit{GRB7} (32%). Therefore, other transcriptional or post-transcriptional mechanisms may also play a role for expression of genes within this region. We previously identified \textit{PPP1R1B/DARPP-32} as a cancer gene (29). \textit{PPP1R1B/DARPP-32} is now known to be overexpressed in several tumors, such as adenocarcinomas of the breast, colon, esophagus, and stomach (33-36). We recently showed that \textit{PPP1R1B/DARPP-32} is a novel cancer gene with a potent antiapoptotic potential that may contribute to the drug resistance and poor outcome phenotype in UGCs (37). Amplification and expression of \textit{ERBB2} and \textit{GRB7} was recently reported in Barrett’s adenocarcinoma (38-40). However, these studies lacked data related to the expression of other genes that are between or at the boundaries of \textit{ERBB2} and \textit{GRB7}. On the other hand, we detected a rare overexpression of \textit{STARD3} and \textit{C17orf37} in UGCs. Therefore, these differences

**FIGURE 1.** FISH on UGCs. FISH using BAC clone CTD-2019C10 as a probe. FISH was done using CTD-2019C10 FISH using the CTD-2019C10 and FITC for signal detection. Arrows, green FITC hybridization signals. Amplification seen as several signals in each cell from the intestinal-type GEJ adenocarcinoma sample.

**FIGURE 2.** mRNA expression levels of 11 genes. A. Quantification of mRNA levels of 11 genes (\textit{NEUROD, PPP1R1B/DARPP-32, STARD3, TCAP, PNMT, PERLD1, ERBB2, C17orf37, GRB7, ZNFN1A3, and TOP2A}) in 74 UGCs using an iCycler. The expression of test genes was compared with \textit{HPRT1} gene, which had minimal variation in all normal and neoplastic UGC samples that we tested. Tumor samples were compared with the average expression of normal gastric mucosa samples (SE was <5%). We used an arbitrary cutoff value of 3-fold as a minimum for any overexpression, which is represented by a horizontal line crossing at this threshold. B. Standard ethidium bromide staining of 1.2% agarose gel electrophoresis of PCR products of the 11 genes and \textit{HPRT1} in six (S1-S6) matched tumor (T) and nontumor (N) samples.
Table 3. Summary of Quantitative Real-time RT-PCR Analysis of 11 Genes on 74 Adenocarcinomas of the Stomach, GEJ, and Lower Esophagus

<table>
<thead>
<tr>
<th>Gene</th>
<th>n (%) tumors showing mRNA overexpression compared with normal</th>
<th>NEUROD</th>
<th>PPP1R1B</th>
<th>STARD3</th>
<th>TCAP</th>
<th>PNMT</th>
<th>PERLD1</th>
<th>ERBB2</th>
<th>C17orf37</th>
<th>GRB7</th>
</tr>
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<tbody>
<tr>
<td>Total (n = 74)</td>
<td></td>
<td>24 (32)</td>
<td>41 (55)</td>
<td>6 (8)</td>
<td>40 (54)</td>
<td>9 (12)</td>
<td>14 (19)</td>
<td>17 (23)</td>
<td>8 (11)</td>
<td>24 (32)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Male (n = 55)</td>
<td></td>
<td>16 (29)</td>
<td>31 (56)</td>
<td>5 (9)</td>
<td>5 (9)</td>
<td>27 (49)</td>
<td>6 (11)</td>
<td>10 (18)</td>
<td>4 (7)</td>
<td>17 (31)</td>
</tr>
<tr>
<td>Female (n = 12)</td>
<td></td>
<td>6 (50)</td>
<td>8 (67)</td>
<td>0 (0)</td>
<td>10 (83)</td>
<td>3 (25)</td>
<td>25 (32)</td>
<td>4 (33)</td>
<td>3 (25)</td>
<td>6 (50)</td>
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<tr>
<td>Site</td>
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<td></td>
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</tr>
<tr>
<td>Stomach (n = 33)</td>
<td></td>
<td>8 (24)</td>
<td>17 (52)</td>
<td>1 (3)</td>
<td>16 (48)</td>
<td>5 (15)</td>
<td>4 (12)</td>
<td>6 (18)</td>
<td>1 (3)</td>
<td>12 (36)</td>
</tr>
<tr>
<td>GEJ and esophageal (n = 41)</td>
<td></td>
<td>16 (39)</td>
<td>24 (59)</td>
<td>5 (12)</td>
<td>24 (59)</td>
<td>4 (10)</td>
<td>10 (24)</td>
<td>11 (27)</td>
<td>7 (17)</td>
<td>12 (29)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Diffuse (n = 9)</td>
<td></td>
<td>2 (22)</td>
<td>3 (33)</td>
<td>0 (0)</td>
<td>3 (33)</td>
<td>1 (11)</td>
<td>1 (11)</td>
<td>0 (0)</td>
<td>1 (11)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>Intestinal (n = 20)</td>
<td></td>
<td>12 (60)</td>
<td>10 (50)</td>
<td>1 (5)</td>
<td>15 (75)</td>
<td>4 (20)</td>
<td>5 (25)</td>
<td>7 (35)</td>
<td>1 (5)</td>
<td>9 (45)</td>
</tr>
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<td>Tumor-node-metastasis stage</td>
<td></td>
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<tr>
<td>T1 + T2 (n = 22)</td>
<td></td>
<td>8 (36)</td>
<td>11 (50)</td>
<td>1 (5)</td>
<td>10 (45)</td>
<td>4 (18)</td>
<td>4 (18)</td>
<td>4 (18)</td>
<td>2 (9)</td>
<td>7 (32)</td>
</tr>
<tr>
<td>T1 + T4 (n = 42)</td>
<td></td>
<td>12 (29)</td>
<td>25 (60)</td>
<td>4 (10)</td>
<td>25 (60)</td>
<td>4 (10)</td>
<td>8 (19)</td>
<td>11 (26)</td>
<td>7 (35)</td>
<td>15 (36)</td>
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<td>Grade</td>
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<td></td>
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<tr>
<td>Well differentiated to moderately differentiated (n = 17)</td>
<td></td>
<td>10 (59)</td>
<td>14 (82)</td>
<td>2 (12)</td>
<td>11 (65)</td>
<td>3 (18)</td>
<td>6 (35)</td>
<td>7 (41)</td>
<td>2 (12)</td>
<td>8 (47)</td>
</tr>
<tr>
<td>Poorly differentiated (n = 29)</td>
<td></td>
<td>9 (31)</td>
<td>11 (38)</td>
<td>14 (48)</td>
<td>14 (48)</td>
<td>2 (7)</td>
<td>2 (7)</td>
<td>4 (14)</td>
<td>1 (3)</td>
<td>8 (28)</td>
</tr>
<tr>
<td>Node</td>
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<td></td>
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<tr>
<td>N0 (n = 19)</td>
<td></td>
<td>10 (53)</td>
<td>11 (58)</td>
<td>2 (11)</td>
<td>11 (58)</td>
<td>3 (16)</td>
<td>4 (21)</td>
<td>6 (32)</td>
<td>3 (16)</td>
<td>7 (37)</td>
</tr>
<tr>
<td>N1-N4 (n = 35)</td>
<td></td>
<td>10 (22)</td>
<td>25 (56)</td>
<td>2 (4)</td>
<td>24 (53)</td>
<td>5 (11)</td>
<td>8 (18)</td>
<td>9 (20)</td>
<td>2 (4)</td>
<td>15 (33)</td>
</tr>
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</table>

NOTE: Eight genes are fully located inside the boundaries of clone CTD-2019C10 that was used for FISH. Exon 1 and intron 1 of ZNFN1A3 are located inside the CTD2019C. NEUROD and TOP2A are adjacent to either ends of the CTD-2019C10 (see Fig. 1 for details). Numbers in bold indicate statistical significance (P ≤ 0.05).

in gene expression may suggest that STARD3 and C17orf37 are transcriptionally more tightly regulated than other adjacent genes. Overexpression of ERBB2 was seen in 23% of our tumors. This finding agrees with several earlier reports showing ERBB2 amplification and/or expression in gastric cancers (25, 41-43). TOP2A, which is located outside the boundaries of the 168-kb amplicon region, was more frequently overexpressed than ERBB2. Interestingly, there was a lack of correlation of expression of TOP2A with ERBB2 or with any of the 8 genes located inside the 168-kb amplicon. This finding may indicate that overexpression of TOP2A is an unrelated event to the formation of the 168-kb amplicon or expression of genes inside it. This observation confirms our earlier results in gastric cancer where expression of ERBB2 and TOP2A was in many cases independent even when coamplification of both genes was present (43). There are reports in breast and lung cancer that indicate concordant amplification of ERBB2 and TOP2A in some cases; however, expression levels were not provided (20, 44). Both ERBB2 and TOP2A are therapeutic targets for approved chemotherapeutic agents (hereceptin and epirubicin, respectively) and are used in clinical trials for breast cancer patients as well as few other malignancies (22-24). Expression of ERBB2 and/or TOP2A could be a decision-making step for treatment of several cancers because these genes provide resistance to standard chemotherapeutic options (20, 45). Tumors that express these targets are likely better responders to the targeted treatment as shown in breast cancer (20, 45-47). However, expression of other molecular targets, such as expression of estrogen receptors and ERBB2, can significantly influence the response to therapy of TOP2A inhibitors (48). These factors should be considered collectively when selecting therapeutic options for patients. The role of targeted therapy for ERBB2 and TOP2A in adenocarcinomas of the stomach and esophagus has not been fully investigated.

In summary, we have characterized a 168-kb amplicon at 17q12-q21 and the expression levels of 11 genes in UGCs. The expression level of genes bounded by PPP1R1B/DARPP-32 and GRB7 correlated with each other but not with other genes adjacent to their boundaries. We suggest that this may be a transcriptionally active oncogenic region that plays an important role in tumorigenesis. Further studies are required to understand the complexity, biological significance, and interaction of these genes in tumorigenesis.

Materials and Methods

Tissue Samples

A total of 393 paraffin-embedded tissue blocks from adenocarcinomas of the stomach and lower esophagus (n = 304) and normal gastric mucosa (n = 89) were available for the construction of a tissue microarray and FISH analysis. In addition, frozen samples from 74 tumors and 21 normal gastric epithelial samples were collected. All frozen tumors were dissected following a H&E slide examination to ensure at least 70% of neoplastic cells per sample and used for RNA extraction, cDNA synthesis, and subsequent quantitative real-time RT-PCR assays. All tissue samples were collected in accordance with institutional review board–approved protocols. Tissues were stained with H&E, and representative tissue microarray, tissue cores were prepared with a diameter of at least 70% of neoplastic cells per sample and used for RNA extraction, cDNA synthesis, and subsequent quantitative real-time RT-PCR assays. All tissue samples were collected in accordance with institutional review board–approved protocols. Tissues were stained with H&E, and representative regions were selected for inclusion in a tissue array. For the tissue microarray, tissue cores were prepared with a diameter of 0.6 mm and were retrieved from the selected regions of the

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donor blocks and punched to the recipient block using a manual tissue array instrument (Beecher Instruments, Silver Spring, MD). All samples were punched in duplicates. Sections of 5 μm were transferred to polylysine-coated slides (SuperFrostPlus, Menzel-Gläser, Braunschweig, Germany) and baked in an oven at 37°C for 2 hours. We used this tumor tissue array for FISH analysis. The collected adenocarcinomas ranged from well differentiated to poorly differentiated, from stages I to IV, with a mix of intestinal-type and diffuse-type tumors.

Fluorescence In situ Hybridization

Before hybridization, tumor tissue array sections were deparaffinized and pretreated in a microwave oven (10 minutes at 92°C in 0.01 mol/L citrate acid). After rinsing in PBS, slides were digested with pepsin solution (Digest-All 3, Zymed Laboratories, Inc., South San Francisco, CA) for 10 minutes at 37°C, rinsed in PBS, dehydrated in graded ethanol series, and air dried. The BAC clone CTD-2019C10 (Research Genetics, Huntsville, AL) for 168-kb amplicon region was obtained for in situ hybridization. This BAC clone was sequence verified and contains the following genes: PPP1R1B/DARPP-32, STAR3D, TCAP, PNMT, PERLD1, ERBB2, C17orf37, and GRB7 as well as the first two exons of ZNFN1A3. We did FISH using this clone as a measure of amplification of all genes within this region. The CTD-2019C10 probe was labeled with biotin-14-ATP (Life Technologies, Invitrogen Corp, San Diego, CA) by nick translation, precipitated with herring sperm DNA (0.62 μg/μL, Sigma, St. Louis, MO), and human Cot-1 DNA (0.62 μg/μL; Life Technologies), and dissolved in hybridization buffer (50% formamide, 20% dextran sulfate, 2× SSC). The slides were denatured in 70% formamide/2× SSC (pH 7) at 75°C for 5 minutes and the probes were denatured at 75°C for 5 minutes and applied onto the slides.

**FIGURE 3.** Frequency and correlation of mRNA overexpression levels. A. Frequency of overexpression of each of the 11 genes as seen in stomach tumors (n = 33) and GEJ and lower esophageal tumors (n = 41). X axis, gene name; Y axis, frequency percent value. B. Magnitude of statistically significant correlations of expression of the 11 genes. Gray levels indicate the correlation values (bottom right). The expression of NEUROD, ZNFN1A3, and TOP2A does not correlate with any other gene. Genes bounded by PPP1R1B/DARPP-32 and GRB7 showed significant correlation with each other with a variable magnitude.
Hybridization was done at 37°C for 2 days. Posthybridization washes were done at 45°C using a series of the following solutions (pH 7): 50% formamide, 2× SSC, twice in 0.1× SSC and 4× SSC/0.2% Tween 20. Signal detection was achieved using fluorescein avidin and fluorescein anti-avidin D (Vector Laboratories, Inc., Burlingame, CA). The slides were mounted with an antifading medium that contained 4',6-diamidino-2-phenylindole counterstain (Vector Laboratories). The tumor array preparations were surveyed with a Zeiss (Oberkochen, Germany) Axiophot fluorescence microscope (for the fluorescence-labeled arrays) and an Olympus (Tokyo, Japan) BH-2 light microscope. A minimum of 50 nonoverlapping nuclei were scored from each case. FISH results were scored based on the number of signals per cell as follows: FISH score of 0-1 signals; 2, 5-9 signals; 3, 10-15 signals; 4, >15 signals. Amplification was defined as follows: FISH score of 0-1, 2-4 signals; 2, 5-9 signals; 3, 10-15 signals; 4, >15 signals. Amplification was defined as ≥5 signals (score ≥2) in ≥50% of cancer cells.

Quantitative Real-time RT-PCR

For quantitative real-time RT-PCR, 74 primary UGCs were analyzed and compared with 21 normal gastric mucosa samples. The mRNA was isolated using RNaseasy kit (Qiagen GmbH, Hilden, Germany). Single-stranded cDNA was synthesized using Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). Quantitative PCR was done using iCycler (Biol-Rad, Hercules, CA). The threshold cycle number was determined using iCycler software version 3.0. Reactions were done in triplicate and threshold cycle numbers were averaged. Gene specific primers for 11 genes (NEUROD, PPP1R1B/DARPP-32, STARD3, TCAP, PNMT, PERLD1, ERBB2, GRB7, ZNFN1A3, and TOP2A) were designed. NEUROD and TOP2A were adjacent to the CTD-2019C10 borders (Fig. 4). The primers were obtained from GeneLink (Hawthorne, NY) and their sequences are available on request. The results were normalized to HPRT1, which had minimal variation in all normal and neoplastic samples that were tested and is a reliable and stable reference gene for real-time RT-PCR. Fold overexpression was calculated according to the formula: 2(ΔΔCt) = 2(Rt − En) / 2(Rn − En), as earlier described (33), where 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 Rt is the threshold cycle number for the reference gene observed in the tumor sample. Rn and En values were an average for the 21 normal samples that were analyzed.

Statistical Analysis

Frequencies and other summary statistics were calculated. χ² tests of association were done to examine potential relationships between amplification and expression levels via PCR and variables of a demographic, clinical, or pathologic nature.

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References


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Nazif Maqani, Abbes Belkhiri, Christopher Moskaluk, et al.


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