Amplification of the BRCA2 Pathway Gene EMSY in Sporadic Breast Cancer Is Related to Negative Outcome

Carmen Rodríguez,1 Luke Hughes-Davies,2 Hélène Vallès,3 Béatrice Orsetti,1 Marguerite Cuny,1 Lisa Ursule,1 Tony Kouzarides,2 and Charles Theillet4
1Génotype et Phénotypes Tumoraux E 229 INSERM, Centre Val d’Aurelle, Montpellier, France; 2Cancer Research UK/Wellcome Institute, Cambridge, United Kingdom

ABSTRACT

DNA amplification at band q13 of chromosome 11 is common in breast cancer, and CCND1 and EMS1 remain the strongest candidate genes. However, amplification patterns are consistent with the existence of four cores of amplification, suggesting the involvement of additional genes. Here we present evidence strongly suggesting the involvement of the recently characterized EMSY gene in the formation of the telomeric amplification. EMSY maps at 11q13.5, 100 kb centromeric to the GARP gene, which has been mapped within the core of the distal amplicon. The EMSY protein was shown to interact with BRCA2 and has a role in chromatin remodeling. This makes EMSY a strong candidate oncogene for the 11q13.5 amplicon. DNA amplification was studied in a total of 940 primary breast tumors and 39 breast cancer cell lines. Amplification profiles were consistent with the EMSY-GARP locus being amplified independently of CCND1 and/or EMS1. EMSY mRNA expression levels were studied along with those of five other genes located at 11q13.5 by real-time quantitative PCR in the 39 cell lines and a subset of 65 tumors. EMSY overexpression correlated strongly with DNA amplification in both primary tumors and cell lines. In a subset of 296 patients, EMSY amplification was found by both univariate and multivariate analyses to correlate with shortened disease-free survival. These data indicate that EMSY is a strong candidate oncogene for the 11q13.5 amplicon.

INTRODUCTION

DNA amplification is a common mechanism of oncogenic activation in human tumors, and band q13 of chromosome 11 is a frequent site of genetic aberration in a number of human malignancies, particularly breast and head and neck cancers (1). Several candidate oncogenes have been proposed, among which only CCND1 and EMS1 meet the criteria for genes activated by DNA amplification (2). Both genes map to chromosome 11q13.3, 0.8 Mb apart, with CCND1 occupying a more centromeric position than EMS1 (3). Because CCND1 is frequently rearranged by chromosomal translocations in hematologic malignancies and overexpressed in several human tumors, this gene has been considered the principal target for DNA amplification at 11q13 (4). However, some findings suggested that 11q13 amplification could be more complex. On the basis of the recently completed genome map, the 11q13 amplification domain spans up to 7 Mb and the existence of four distinct cores of amplification has been proposed (5). Although CCND1 and EMS1 occupy the centrally located cores, genes corresponding to both the proximal and distal amplicons remain to be identified. In a recent study we presented evidence strongly suggesting that the MYEOV gene, located 360 kb centromeric to CCND1, was a candidate oncogene for the proximal amplicon (6). In the present work we studied the involvement of the recently characterized EMSY gene in human breast cancer cell lines and primary tumors. Both its location (the gene maps at 11q13.5, adjacent to the GARP gene) and function (the protein interacts with BRCA2 and could possibly act as its antagonist) make EMSY an interesting candidate oncogene (7). We had previously shown that this region most probably corresponds to the core of an amplification unit that is independent of CCND1. According to genomic mapping work, GARP was located within the core of this distal amplicon; however, this gene could not be considered as a candidate because it was not expressed in breast tumors (8).

To verify the aberrant activation of the EMSY gene in breast cancer, we studied its DNA amplification status along with that of GARP, CCND1, and EMS1 in 875 primary breast tumors on Southern blots. Data were consistent with the existence of two distinct amplification cores: one proximal, represented by CCND1 and EMS1; the second distal, represented by EMSY and GARP. As a second step we undertook a real-time quantitative PCR analysis on an independent series of 65 breast tumors and 39 cell lines and studied both the DNA copy number and RNA expression status of eight genes located at 11q13, including EMSY, GARP, CCND1, and EMS1. Data clearly confirmed the existence of distinct amplification cores and were consistent with EMSY being a candidate gene for the distal amplicon. DNA amplification data in breast tumors were evaluated for clinical and prognostic significance and showed that EMSY amplification was indicative of poor outcome of the disease.
MATERIALS AND METHODS

Tumor Samples and Clinical Material. Collection and handling of tumor material and processing of clinical data were as described previously (9). The breast tumor series was composed of 79% invasive ductal, 14% invasive lobular, and 7% untyped or other invasive adenocarcinomas. Nodal invasion was stratified as follows: N−, 52%; N+, 48%. Scarff-Bloom and Richardson grading was as follows: grade 1, 8%; grade 2, 49%; grade 3, 43%. Steroid receptor status was as follows: estrogen receptor (ER)+ (>10 fmol/mg of protein), 67%; ER−, 33%; progesterone receptor (PR)+ (>10 fmol/mg of protein), 58%; PR−, 42%. Histologic types were determined according to WHO guidelines. Steroid receptor levels were determined by the radioligand binding assay. Normal breast tissue was obtained, after receipt of written consent, from three donors undergoing reductive mammoplasty.

Patients. Clinical follow-up data were collected retrospectively on a cohort of 296 breast cancer patients who had undergone surgery at the Cancer Center Val d’Aurelle-Paul Lamarque (Montpellier, France) between 1987 and 1992. All patients included in this study had primary cancer with unilateral breast tumors showing no macroscopic metastatic disease and no treatment before surgery. Minimum follow-up was 7 years. For 10 patients, contact was lost for more than 1 year. There are no other missing data (clinical or molecular) except tumor grading for the 296 cases included. Patients who died from causes other than breast cancer were censored at the time of death. At the time of analysis, 72 patients (24.3%) had relapsed: 2 with local recurrences, 8 with contralateral cancers, 5 with nodal metastases, 53 with distant metastases (among which 14 were at multiple sites), and 4 who developed a second cancer. Forty-one patients died from cancer.

Cell Lines. With the exception of CAL51 (3), all cell lines tested were from American Type Culture Collection (Manassas, VA). Culture conditions were as recommended by the supplier.

RNA Purification and Reverse Transcript Synthesis. Total RNA was purified according to the method described by Chomczynski and Sacchi (10). Reverse transcripts were prepared from 1 μg of total RNA treated with RNase-free DNase (Promega, Lyon, France), using 250 ng of random hexamer as a primer and the Moloney murine leukemia virus reverse transcriptase (Invitrogen, Cergy-Pontoise, France).

DNA Extraction and Southern Blotting. Southern blot preparation and hybridization were as described previously (11). The EMSY probe was a 478-bp cDNA (IMAGE clone W47611; obtained from HGMP, Hinxton, United Kingdom). DNA amplification analysis was performed as described previously (11). Hybridization signals were quantified in each lane for each probe by use of the HDG Analyser Visage software package (Genomic Solutions, Ann Arbor, MI). Test/control signal ratios were calculated. Control probes corresponded to genes (NMYC, ERBB3, and CDK2) that showed copy number variations in <1% of the tested tumors. All of the tumors tested were analyzed jointly with the four control and the test probes (EMSY, GARP, EMSY, and CCND1). Band intensities of the test probes were compared with the intensities for each control probe, and the relative intensities of the control probes were compared with each other. The final ratios correspond to those of the test probes relative to the mean of the controls.

Real-Time Quantitative PCR. Real-time PCR was carried out with an ABI Prism 7000 instrument (Applied Biosystems) under standard conditions using SYBR Green as detector. Briefly, PCR was done in final volume of 16 μL, including 8 μL of SYBR Green PCR master mixture, 0.5 μmol/L primers, and cDNA corresponding to either 6.5 ng of total RNA or 13 ng of genomic DNA. Primers were as follows: CCND1-f (5′-gggCAGTTTCTTCAATgGaAGTg-3′), CCND1-r (5′-CACCCAGTGCCCCACACT-3′), E2I4-f (5′-ACATAGCCCTTCTTGTTgCCAATg-3′), E2I4-r (5′-gggCCATgTgTTgTTgCAT-3′), EMS1-f (5′-TgggCCTgATgAgGTTAACC-3′), EMS1-r (5′-gggCTgTgCgTgTgACATgAgg-3′), EMSY-f (5′-AATgTTCCAAAGggCCTgTT-3′), EMSY-r (5′-gTggTAAGgAgTTgCgAAATgCT-3′), GARP-f (5′-gATCTgCCCTgCTCgCTCC-3′), GARP-r (5′-TgATgTTgTgCTCgCTCC-3′), PKAF1-f (5′-TgCTgCGATgTgCCCTgTgAgg-3′), PKAF1-r (5′-gggAgAACTACTgTggTgTg-3′), PRKRIR-f (5′-ACAATgActgTggCCAATATAT-3′), WNTI1-f (5′-gAgCgCgTgCCCTgTgAgg-3′), WNTI1-r (5′-gAgACTACgAgAggCCTgTT-3′), ALB-r (5′-ACTCATTggATgAgggTgTg-3′), DCK-f (5′-gCgCCACAAgACTAAgAT-3′), DCK-r (5′-CgATgCTgTCgCTCC-3′), 28S-f (5′-CgATgTCCTCATCCgCAAATg-3′), and 28S-r (5′-AggCCACAAgCTCgCAAC-3′). PCR products were 86, 103, 95, 106, 86, 96, 81, 139, 110 and 101 bp for, respectively, the CCND1, E2I4, EMS1, EMSY, GARP, PKAI, PRKRIR, WNTI1, ALB, DCK, and 28S genes. All PCRs were done at least twice. Results were considered reproducible when the threshold cycle (corresponding to the cycles number at which the amount of amplified target reached a fixed threshold) between duplicates did not differ by more than 0.5 (12). Standard curves were determined for each gene analyzed by use of serial dilutions of cDNA or genomic DNA. Relative quantities were calculated using these standard curves. Target gene quantities were normalized to endogenous references: 28S RNA for expression studies, and the ALB and DCK genes for DNA copy number analysis. For measurements of RNA level, variations were determined by calculating the ratio of the normalized level of each sample to the mean of normalized values for five normal breast samples. Ratio values exceeding 2.5 were considered indicative of hyperexpression, ratios between 2.0 and 2.5 were considered indicative of moderate overexpression. For DNA copy number determination, variations were determined by calculating the ratio of the normalized value of each sample to the median value observed for all tumors or cell lines. Values between 1.8 and 2.0 were considered indicative of moderate copy number increases (CNIs), values exceeding 2.0 were considered indicative of a clear copy number increase, and values below 0.5 were considered indicative of a DNA loss.

Comparative Genomic Hybridization and Fluorescence In situ Hybridization Analysis of Cell Lines. Comparative genomic hybridization (CGH) analysis was performed as previously described (13). Amplification levels for CCND1, EMS1, EMSY, and GARP were assessed in cell lines showing gains in the 11q13-q14 region on CGH. This was done by use of a bacterial artificial chromosome or cosmids clones that corresponded either to the genomic sequence of the tested genes or to
genomic sequences mapping nearby. Clones used were Cos9 (CCND1); RP11-21D20, or RP11-736L3 (EMSI); 855A11 (EMSY); and Cos105 (GARP). Fluorescent labeling and hybridization were as described previously (14). Copy numbers were scored by counting spots on metaphase spreads and dividing by 2.

**Statistics and Data Analysis.** Statistical analyses were performed with the Epilnfo 3.0 software package from the Centers for Disease Control and Prevention (Atlanta, GA), for classical $\chi^2$ and with Statview software (Abacus Concepts, Berkeley, CA) for survival analyses. Disease-free survival (DFS) was defined as the time from surgery to the first local or distant recurrence or last contact. Contralateral tumors and second cancers were not considered as recurrences for DFS determination. Breast cancer disease-specific survival was defined as the time from surgery to death, if the patient died from breast cancer, or to the last contact. Five-year survival rates were estimated, and survival curves were plotted according to the Kaplan–Meier method (15). Differences between groups were calculated by the log-rank test (16). In multivariate analysis, relative risks of recurrence or death from breast cancer, 95% confidence intervals, and $P$ values for censored survival data were calculated by use of Cox’s proportional hazards regression model (17). All $P$ values were two-sided and considered as significant if $<0.05$. The Cox model was built with the following clinical variables bearing prognostic significance: nodal status (pN), clinical tumor size (T), and ER status. Clinical variables were dichotomized as follows: nodal status (axillary enlarged lymph node), clinical tumor size ($T_1$-$T_3$ versus $T_4$), Scarff-Bloom and Richardson tumor grade (1 or 2 versus 3), and ER status [low ($\leq 10$) versus high (>10) fmol/mg of protein].

**RESULTS**

**EMSY Gene Amplification in Primary Breast Tumors and Cell Lines.** We analyzed DNA from 875 primary breast tumors by Southern blotting for CNI at the EMSY, GARP, EMS1, and CCND1 loci. These four loci map at 11q13 in an interval of 7 Mb. $^3$ CCND1 occupies the most centromeric position, EMSY has been mapped 0.8 Mb toward the telomere, whereas EMSY and GARP, localized approximately 100 Kb from each other, map 6 Mb telomeric of EMSY. EMSY amplification occurred independently of CCND1: Twenty-eight of 65 (44%) tumors with a CNI at EMSY had normal copy numbers at CCND1. Conversely, 65 of 102 tumors with a CNI at CCND1 showed normal copy numbers at EMSY (Fig. 1A). We also noted that EMSY was amplified independently of EMSY. Furthermore, 14 tumors had discontinuous CNIs involving both EMSY and CCND1 and leaving EMSY out (Fig. 1A). One tumor DNA showed a CNI at GARP and no apparent anomaly at EMSY (Fig. 1B, tumor 807). These data agree with the involvement of EMSY in the distal amplicon at 11q13.5.

Patterns of amplification in breast cancer cell lines supported this further. We studied eight cell lines by CGH and fluorescence in situ hybridization (FISH; not shown). Two cell lines, MDA-MB 134 and MDA-MB 175, had gains at 11q13 and increased copy numbers by FISH at all loci tested, thus corresponding to the coamplification of EMSY-GARP with EMSY and CCND1. Of the six remaining lines, one CGH analysis showed three gain gains at 11q13 (BT-474) or more distal gains at 11q13.5-q14 (MDA-MB-453 and T47D). FISH analysis showed slightly higher copy numbers at EMSY-GARP than at CCND1 (2-fold increase) in these cell lines. These observations in cancer cell lines further supported the idea of two independent amplification cores.

**Real-Time Quantitative PCR Analysis.** To ascertain the involvement of EMSY in the formation of the 11q13.5 amplification core, we studied jointly DNA copy numbers and RNA expression levels by real-time quantitative PCR of CCND1, EMSY, and GARP as well as of four genes (GARP, WNT11, PRRKR, E2F14, and PAK1) mapping close to the EMSY-GARP locus. In fact, WNT11 and PRRKR are immediate neighbors positioned centromeric to EMSY, whereas E2F14 is the closest gene telomeric to GARP (Fig. 2A). PAK1 is located at greater distance because it maps 0.9 Mb telomeric to EMSY and was included in this analysis because it had been reported to be amplified and overexpressed in ovarian cancer (18). We studied 65 breast tumors and 39 breast cancer cell lines by real-time quantitative PCR and assessed patterns of CNI relative to RNA expression levels.

Patterns of DNA copy numbers in both primary tumors and cell lines were consistent with Southern blot and FISH data showing that CCND1-EMS1 and EMSY-GARP represented sep-
Amplification of 11q13: CCND1, EMSY, WNT11, PRKRIR, and E2IG4, and PAK1, located at 11q13.5. (A) map of 11q13 showing respective locations of studied genes. Distances in Mb, as from http://genome.ucsc.edu/, are indicated in parentheses for each gene. (B) profiles of CNIs and hyperexpression in breast tumors. (C) profiles of CNIs and hyperexpression in cell lines. Rows, results for each sample (tumor or cell line); columns, results for genes. Genes are ordered according to their relative positions on the chromosome. For copy numbers (left-hand side), black-shaded areas indicate elevated copy number gain (values ≥ 2); gray-shaded areas indicate moderate gain (1.8 ≤ value < 2.0); nonshaded areas, no gain. Black-outlined boxes indicate reduced copy numbers. For expression data (right-hand side), black-shaded areas indicate hyperexpression (values ≥ 2.5); nonshaded areas, no hyperexpression. Moderately increased expression levels are indicated as gray-shaded areas, but were not considered for statistical analyses.

Clinical Correlations and Prognostic Significance of EMSY Gene Amplification. As a next step we were interested in determining whether DNA amplification of EMSY was related to a particular subgroup of breast tumors or to negative outcome of the disease. This was performed on the 875 tumors analyzed by Southern blotting.

Positive ER status was the only clinicopathologic variable showing correlation with EMSY amplification. In comparison, the presence of CCND1 or EM5 was found to correlate with positive ER status, positive PR status, histologic type, and tumor size (Table 2). CCND1 showed stronger correlation with positive ER status than EMSY; we therefore compared ER distributions in groups of tumors defined according to their patterns of CCND1 or EMSY amplification. It appeared that 59% of the CCND1+/EMSY+ tumors were ER+, whereas this number increased to 81% in CCND1+/EMSY− tumors. This difference was statistically significant (P = 0.03). Interestingly, the distribution of tumor grades differed in EMSY+ tumors. Indeed, CCND1+/EMSY+ tumors represented a larger proportion of grade 1 tumors (18.2%) than did CCND1+/EMSY− (4%) or even CCND1+/EMSY+ tumors (0%). These differences were close to significance.

Correlations with disease outcome were analyzed on a subset of 296 tumors for which we had complete follow-up information (see Materials and Methods). Minimum follow-up time was 7 years. Univariate analysis showed that CCND1, EMSY, and EM5 all showed significant association with shortened DFS. CCND1 was the only molecular marker that correlated with disease-specific survival as well (Table 3). Kaplan–Meier survival curves suggested that events associated with EMSY amplification were of later occurrence than with EM5.
Table 1  Incidence of CNI and coincidental hyperexpression of CCND1, EMS1, EMSY, GARP, and four neighboring genes located at 11q13.5

<table>
<thead>
<tr>
<th>Gene</th>
<th>Breast tumors</th>
<th>Cell lines</th>
<th>Mean RNA expression (copy numbers)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples with</td>
<td>Samples with</td>
<td>CNI/</td>
</tr>
<tr>
<td></td>
<td>n (%)/n (%)</td>
<td>n (%)</td>
<td>Hyperexpression, Hyperexpression, n (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hyperexpression, Hyperexpression, n (%)</td>
</tr>
<tr>
<td>CCND1</td>
<td>2/65 (18.5)</td>
<td>17/65 (26)</td>
<td>8/12 (66.6)</td>
</tr>
<tr>
<td>EMS1</td>
<td>8/65 (12.3)</td>
<td>7/65 (10)</td>
<td>5/8 (62.5)</td>
</tr>
<tr>
<td>WNT1</td>
<td>8/65 (12.3)</td>
<td>1/65 (1.5)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>PKRIR</td>
<td>2/65 (3)</td>
<td>1/65 (1.5)</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>EMSY</td>
<td>8/65 (12.3)</td>
<td>4/65 (6.1)</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>GARP</td>
<td>10/65 (15)</td>
<td>0/65 (0)</td>
<td>0/0 (0)</td>
</tr>
<tr>
<td>E2G4</td>
<td>4/65 (4.6)</td>
<td>1/65 (1.5)</td>
<td>1/4 (25)</td>
</tr>
<tr>
<td>PAK1</td>
<td>8/65 (12.3)</td>
<td>8/65 (12.3)</td>
<td>29/22 (80)</td>
</tr>
</tbody>
</table>

NOTE: The respective locations of the genes are shown in Fig. 2. Because primary breast tumors and cell lines did not always show similar incidences of either CNI or hyperexpression, data are presented separately. The $P$ values ($t$, Fisher exact test) indicate the statistical significance of the correlation observed between CNI and hyperexpression for each gene.

Abbreviations: NA, not analyzed.

Table 2  Clinicopathologic correlations for CCND1, EMS, GARP, and EMSY

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>CCND1 amplified</th>
<th>EMS1 amplified</th>
<th>EMSY amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N$</td>
<td>$n$</td>
<td>%</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>546</td>
<td>54</td>
<td>10.0</td>
</tr>
<tr>
<td>ILC</td>
<td>105</td>
<td>22</td>
<td>21.0</td>
</tr>
<tr>
<td>Other*</td>
<td>87</td>
<td>11</td>
<td>11.0</td>
</tr>
<tr>
<td>Total</td>
<td>738</td>
<td>86</td>
<td>11.0</td>
</tr>
<tr>
<td>$P$</td>
<td></td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Steroid receptor status†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER+</td>
<td>567</td>
<td>86</td>
<td>15.0</td>
</tr>
<tr>
<td>ER−</td>
<td>276</td>
<td>5</td>
<td>5.0</td>
</tr>
<tr>
<td>Total</td>
<td>843</td>
<td>101</td>
<td>12.0</td>
</tr>
<tr>
<td>$P$</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>PR+</td>
<td>482</td>
<td>70</td>
<td>15.0</td>
</tr>
<tr>
<td>PR−</td>
<td>358</td>
<td>30</td>
<td>8.0</td>
</tr>
<tr>
<td>Total</td>
<td>840</td>
<td>100</td>
<td>12.0</td>
</tr>
<tr>
<td>$P$</td>
<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Histologic size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 cm</td>
<td>123</td>
<td>13</td>
<td>11.0</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>235</td>
<td>31</td>
<td>13.0</td>
</tr>
<tr>
<td>Total</td>
<td>358</td>
<td>44</td>
<td>12.0</td>
</tr>
<tr>
<td>$P$</td>
<td></td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Statistical correlations were calculated using the standard $\chi^2$ computation. Correlations were considered statistically significant at $P \leq 0.05$. Above that threshold, association were considered nonsignificant ($P > 0.05$).

Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.

* Other, all other histologic types.
† Tumors were scored ER or PR positive for levels $\geq 10$ fmol/mg of protein.

DISCUSSION

An amplification unit at 11q13.5 occurring independently of CCND1 in breast cancer was proposed several years ago (5) and confirmed on independent tumor sets (19). Genome mapping work by Bekri et al. (8) showed that the GARP gene was located within the core of the amplification unit in breast tumors. However, the absence of increased GARP RNA expression in breast tumors disqualified it as a candidate gene. The recently discovered EMSY gene, mapping 100 Kb centromeric to GARP, brought some new and interesting insights. EMSY was isolated as part of a screening for protein partners binding to the transactivation domain of BRCA2 (7). Although it is not absolutely clear whether impairment of BRCA2 plays an important role in sporadic breast cancer, its roles in familial cancer and genetic stability make it an obvious candidate (20). The functions of the BRCA2 protein remain to be fully understood. It has been shown to interact with DNA repair proteins such as RAD51, but it has also been proposed to act as a transcription factor. The latter hypothesis is related to the presence in exon 3 of a conserved sequence that activates transcription. Noticeably, a deletion of this region in BRCA2 was found in a breast cancer family (21). This strongly suggests a role of the BRCA2 trans-

Table 3  Univariate analysis of gene alterations and clinical outcome

<table>
<thead>
<tr>
<th>Gene</th>
<th>N</th>
<th>5-year rate (%)</th>
<th>$P$</th>
<th>5-year rate (%)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCND1 Amplified</td>
<td>38</td>
<td>67</td>
<td>0.004</td>
<td>78.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Normal</td>
<td>258</td>
<td>82</td>
<td>89</td>
<td>84.1</td>
<td>1</td>
</tr>
<tr>
<td>EMS1 Amplified</td>
<td>31</td>
<td>61.3</td>
<td>&lt;0.001</td>
<td>79.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Normal</td>
<td>265</td>
<td>82.3</td>
<td>88.5</td>
<td>88.5</td>
<td>1</td>
</tr>
<tr>
<td>EMSY Amplified</td>
<td>35</td>
<td>64</td>
<td>0.02</td>
<td>83.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Normal</td>
<td>271</td>
<td>81.5</td>
<td>88</td>
<td>88.5</td>
<td>1</td>
</tr>
</tbody>
</table>
Amplification of 
pression status of

time quantitative PCR the DNA copy numbers and RNA ex-

To confirm these findings, we studied concomitantly by real-

blotting data strongly suggested that amplification of

EMSY and normal copy numbers at

EMS1 dependent of

EMS1 could occur in the absence of amplification of these other genes

can also be amplified independently. Patterns of coamplification

at 11q13.5 are not determined solely by physical proximity of

the genes, as shown by the 14 tumors with CNI at

CCND1-EMS1 in 55% of cases. This is important because

the EMS1 gene is localized 0.8 to 1 Mb telomeric to CCND1 and

can also be amplified independently. Patterns of coamplification at

11q13.5 are not determined solely by physical proximity of the genes, as shown by the 14 tumors with CNI at CCND1 and EMSY and normal copy numbers at EMS1. These Southern blotting data strongly suggested that amplification of EMSY could occur in the absence of amplification of other genes and was not just a consequence of CCND1-EMS1 amplification. To confirm these findings, we studied concomitantly by real-time quantitative PCR the DNA copy numbers and RNA expression status of CCND1, EMSY, EMT, and GARP, as well as four genes located in a close vicinity on either side of the EMSY-GARP locus. These data confirmed that CCND1-EMS1 and EMSY-GARP represented distinct amplification units and that EMSY overexpression correlated strongly with increased DNA copy numbers in both breast tumors and cell lines. These data are, thus, strong indications of EMSY being an active player in the formation of the 11q13.5 amplicon.

Interestingly, patterns of DNA copy changes and RNA expression supported the existence of a supplementary amplicon, represented by the PAK1 gene, located 1.2 Mb telomeric to EMSY. It was, noticeable, however, that PAK1 amplification/overexpression was less prevalent than that of EMSY in our set of breast tumors. This may be a difference from ovarian cancer because recent data showed that PAK1 was amplified in 30% of tumors, preferentially to the GARP locus. Gains in copy number were associated with increased expression (18). However, amplification at 11q13.5 may not conform to a universal pattern. Indeed, it has been shown that in oral squamous cell carcinoma cell lines, DNA amplification encompassed in its largest form a region starting ~400 kb centromeric to CCND1 and ending 300 kb telomeric to EMS1 (3). Thus, in oral squamous cell carcinoma, amplification more distally at 11q13.5 seems rare. It could be that amplification profiles at 11q13 vary according the tumor type. CCND1-EMS1 could constitute the common core, whereas amplicons at 11q13.5 occur differentially according to tumor type.

Once we had shown the involvement of the EMSY gene in 11q13.5 amplification, it was important to verify how it related to breast tumor phenotype. In agreement with previous studies (19, 22), the data presented here show that amplification of CCND1 strongly correlated with positive ER status and lobular carcinoma type. EMSY amplification correlated with ER positivity and tumor size but not tumor type, whereas EMSY correlated solely with ER positivity. It was noticeable that CCND1 was more tightly associated with ER than was EMSY, suggesting that the correlation between 11q13 amplification and positive ER status was driven by CCND1. In support of this, tumors with a EMSY+/CCND1− amplification pattern accounted for 59% of ER-positive tumors compared with 89% in the CCND1+/ EMSY− group. Moreover, we noted that EMSY+/CCND1− tumors were more commonly of grade 1 (18% versus 4% in the

activation domain in tumor suppression. The EMSY protein was shown to interact with this conserved region of BRCA2, and this led to reduced transactivation activity (7). Furthermore, EMSY relocates within the nucleus in the presence of DNA damage. Finally, the EMSY protein has been implicated in chromatin remodeling. These observations suggest a pivotal role for EMSY in conjunction with BRCA2, possibly counteracting it, making EMSY an interesting candidate oncogene for the 11q13.5 amplicon.

The data presented here show that the EMSY gene was amplified at the DNA level in primary breast tumors and cancer cell lines. This occurred independently of CCND1 in 44% of the tumors amplified for EMSY. EMSY amplification was also independent of EMS1 in 55% of cases. This is important because the EMS1 gene is localized 0.8 to 1 Mb telomeric to CCND1 and can also be amplified independently. Patterns of coamplification at 11q13.5 are not determined solely by physical proximity of the genes, as shown by the 14 tumors with CNI at CCND1 and EMSY and normal copy numbers at EMS1. These Southern blotting data strongly suggested that amplification of EMSY could occur in the absence of amplification of the other genes and was not just a consequence of CCND1-EMS1 amplification. To confirm these findings, we studied concomitantly by real-time quantitative PCR the DNA copy numbers and RNA expression status of CCND1, EMSY, EMT, and GARP, as well as four genes located in a close vicinity on either side of the EMSY-GARP locus. These data confirmed that CCND1-EMS1 and EMSY-GARP represented distinct amplification units and that EMSY overexpression correlated strongly with increased DNA copy numbers in both breast tumors and cell lines.
EMSY/CCND1+/+ group). This suggested that amplification of either gene may lead to different phenotypes. Amplification of CCND1, EMS1, or EMSY correlated with increased risk of relapse and was prognostically significant independently of nodal status, steroid receptors, or tumor size. Because EMSY amplification and grade 1 tumors showed a trend of association, we propose that EMSY amplification could be a marker of low-grade tumors at higher risk of relapse. It was of note that our data, on a collection of French breast cancers, were remarkably similar to the data for a set of breast tumors collected in western Canada (7). The only divergence between both data sets concerns the correlation with adverse disease outcome in node-negative patients, which we did not observe. This could possibly be due to the smaller numbers of patients in our cohort. Because the oncogenic mechanism associated with EMSY activation may be mediated by the perturbation of a BRCA2 pathway, it would be interesting to see whether breast tumors showing 11q13.5 amplification have genetic profiles similar to those shown by tumors that have developed on a constitutionally BRCA2-mutated background. Indeed, BRCA2 tumors have been shown to bear specific CGH patterns (23), distinct from those observed in sporadic tumors or tumors with a BRCA1 background. Along similar lines, BRCA2-mutated breast tumors were found to have a high prevalence of DNA amplification at 17q23, more specifically involving the TBX2 gene (24). It would be interesting to see whether EMSY-amplified tumors have genetic patterns similar to those of BRCA2 tumors, either globally or with regard to the TBX2 gene.

ACKNOWLEDGMENTS

We thank Profs. J. B. Dubois and P. Jeanteur for their constant help and support. Simon Thezenas from the Medical Statistics Unit is gratefully acknowledged for his help.

REFERENCES

Amplification of the BRCA2 Pathway Gene EMSY in Sporadic Breast Cancer Is Related to Negative Outcome


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/17/5785

Cited articles
This article cites 23 articles, 8 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/17/5785.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/10/17/5785.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.