

## Amplification of *EMSY*, a novel oncogene on 11q13, in high grade ovarian surface epithelial carcinomas

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Dedicated to Dr. Desmond Robb, a great pathologist, teacher, diagnostician, and collaborator, who passed away after contributing to this work.

### Abstract

**Objectives.** Amplification of the 11q13 locus is commonly observed in a number of human cancers including both breast and ovarian cancer. *Cyclin D1* and *EMSI* have been implicated as candidate oncogenes involved in the emergence of amplification at this locus. Detailed analysis of the 11q13 amplicon in breast cancer led to the discovery of four regions of amplification suggesting the involvement of other genes. Here, we investigate the role of *EMSY*, a recently described BRCA2 interacting protein, as a key element of the 11q13 amplicon in ovarian cancer. *EMSY* maps to 11q13.5 and is amplified in 13% of breast and 17% of ovarian carcinomas.

**Methods.** *EMSY* amplification was assessed by fluorescent in-situ hybridization (FISH) in 674 ovarian cancers in a tissue microarray and correlated with histopathological subtype and tumor grade. A detailed map of the 11q13 amplicon in 51 cases of ovarian cancer was obtained using cDNA-array-based comparative genomic hybridization (aCGH). To further characterize the role of *EMSY* within this amplicon, we evaluated both the amplification profiles and RNA expression levels of *EMSY* and two other genes from the 11q13 amplicon in an additional series of 22 ovarian carcinomas.

**Results.** *EMSY* amplification was seen in 52/285 (18%) high grade papillary serous carcinomas, 4/27 (15%) high grade endometrioid carcinomas, 3/38 (8%) clear cell carcinomas, and 3/10 (30%) undifferentiated carcinomas. aCGH mapping of 11q13 in ovarian cancer showed that *EMSY* localized to the region with the highest frequency of copy number gain. *Cyclin D1* and *EMSI* showed a lower frequency of copy number gain. A highly significant correlation between *EMSY* gene amplification and RNA expression was also observed ( $P = 0.0001$ ). This was a stronger correlation than for other genes at 11q13 including *Cyclin D1* and *PAK1*.

**Conclusions.** These findings support the role of *EMSY* as a key oncogene within the 11q13 amplicon in ovarian cancer.

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### Introduction

DNA amplification has long been recognized as a means of oncogene activation during tumor development. Some genomic regions are frequently amplified in tumors; an example is chromosomal band 11q13. Amplification of this region is commonly seen in breast, head and neck, lung, and bladder cancer [1]. This region of 11q13 is gene dense, which has led to

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investigations into the potential genes that confer a growth advantage to tumor cells. Two genes, *Cyclin D1* and *EMSY*, have emerged as strong candidate genes for playing a causal role in the emergence of this amplification event on 11q13 in breast cancer [2,3]. However, detailed mapping of this region in breast cancer led to the discovery of two additional regions of amplification at 11q13, suggesting the presence of at least two other genes involved in breast carcinogenesis [4,5].

*Cyclin D1* and *EMSY* are located within 0.8 Mb of each other at 11q13.3. Both *Cyclin D1* and *EMSY* have been found to be commonly amplified and overexpressed in breast cancer [6,7]. A more telomeric region of amplification is found 6 Mb away and encompasses the gene *glycoprotein A repetitions predominant (GARP)* [4,5]. Amplification of this region is centered on the marker D11S533E, which was recently identified as the novel oncogene *EMSY* [8]. *EMSY* encodes a protein that binds and represses the activity of the transactivation domain of BRCA2. We have previously reported that *EMSY* gene amplification was found in 13% of sporadic breast cancers and in 17% of high grade ovarian cancers [8]. A recent publication demonstrated a strong correlation between overexpression of *EMSY* and gene amplification in several breast cancer cell lines and primary breast tumors [9]. These data support the role of *EMSY* as an oncogene in breast cancer.

To determine *EMSY*'s involvement in the emergence and maintenance of the 11q13 amplicon in ovarian cancer, a previously scored 674 ovarian tissue microarray was further analyzed to determine the histological features associated with *EMSY* amplification [8]. We then looked in more detail at the genes involved in the formation of the 11q13 amplicon, using array comparative genomic hybridization (aCGH) to map this region in 51 ovarian cancers. Lastly, we evaluated *EMSY* gene amplification and RNA expression along with that of other 11q13 genes, *Cyclin D1* and *p21/cdc42/Rad-activated kinase (PAK1)*, a novel oncogene [10], in 22 cases of ovarian cancer for which there were corresponding snap-frozen tumor samples.

## Materials and methods

### Ovarian cancer case series and tissue microarray construction (TMA)

Tissue microarrays were constructed from 674 archival formalin-fixed, paraffin-embedded ovarian tumor samples from Vancouver Hospital, Stanford Medical Center, and Memorial University. These included a spectrum of high and low grade tumors and cognate normal tissue. Representative areas of invasive carcinoma were selected and marked on the hematoxylin and eosin slide and its corresponding tissue block to be sampled for the tissue microarray (TMA). The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) as described previously [11]. Briefly, the instrument was used to create holes in a recipient block with defined array coordinates. A stylet was used to transfer the tissue cores into the recipient block. One 0.6 mm diameter core was taken from each case. Serial 6 µm sections were cut for FISH analysis.

### Fluorescent in-situ hybridization (FISH)

The tissue microarray sections were baked overnight at 60°C. Before hybridization, tissue array sections were deparaffinized in xylene and dehydrated in 100% ethanol. The slides were then subjected to pretreatment

washes, which included immersing the slides in 0.2 N HCl for 20 min, distilled water for 10 min, 2× SSC for 3 min, and 1 M NaSCN at 80°C for 30 min. Following protease treatment of the slides (protease solution at 37°C for 15 min), they were dehydrated in 100% ethanol and air-dried. The *EMSY* probes were created from DNA isolated from the PAC clones B4, dJ18D12, and dJ85A11 (all PCR verified for exon 2 or 8 of *EMSY*). *EMSY* probes were directly labeled, with Spectrum Green, by nick translation (Vysis, Downer's Grove, Illinois). The *EMSY* probe was combined with Spectrum Aqua labeled centromeric probe, CEP11 (Vysis), and Spectrum Orange labeled *Cyclin D1* (Vysis). The slides were co-denatured for 5 min at 73°C and hybridized for 18 h at 37°C on a HyBrite (Vysis, Downer's Grove, Illinois). Post-hybridization washes were done according to LSI procedure (Vysis). Slides were then counterstained with DAPI. FISH signals were enumerated in approximately 40 morphologically intact and non-overlapping nuclei. The average copy number for each probe was calculated and the amplification ratio determined. The amplification ratio was calculated as a ratio between the average copy number per cell for *EMSY* or *Cyclin D1* and the average copy number for centromere 11.

### Array comparative genomic hybridization (aCGH)

The cDNA microarrays used in this study included 28,000 unique characterized genes or ESTs represented by a total of 41,859 cDNAs, printed on glass slides by the Stanford Functional Genomics Facility (<http://www.microarray.org/sfgf/jsp/home.jsp>). The details of the construction of these arrays have been described previously [12]. Of the over 41,000 cDNA sequences represented on the microarrays used for this study, the chromosomal localization is known for 35,151 distinct mapped cDNAs, which represent 24,540 different Unigene clusters, and 3225 cDNAs not yet represented in Unigene clusters. Tumor DNA from formalin-fixed, paraffin-embedded tissue and reference DNA (normal gender-matched human leukocytes) was extracted, using protocols available at the following website (<http://genome-www.stanford.edu/DFSP/>). Reference DNA was digested with *DpnII* before further processing. Gel electrophoresis of digested and non-digested DNA isolated from formalin-fixed, paraffin-embedded tissue was run to determine DNA fragment size. Labeling of DNA isolated from tumor samples, after light-microscopic confirmation of the presence of non-necrotic tumor, was performed as described previously [13] (<http://cmgm.stanford.edu/pbrown/protocols/index.html>). Briefly, 4 µg of tumor DNA was fluorescently labeled (Cy5) in a volume of 50 µl, mixed with reference DNA labeled with Cy3, and hybridized overnight to the array. After washing, the array slides were scanned on a GenePix Scanner (Axon Instruments, Foster City CA), and fluorescence ratios (test/control) were calculated using GenePix software. Only cDNA spots with a ratio of signal over background of at least 1.4 in the Cy3 channel were included in further analysis. Chromosomal localization of the mapped genes was assigned as described previously [13] and is based on Goldenpath data from November 2002. For CGH data, the copy number for each locus was based on either single gene values or a moving average of the five nearest cDNA clones centered on that locus.

### Real-time quantitative PCR

Real-time PCR was carried out on an ABI 7900 Sequence Detection System (Applied Biosystems) under standard conditions. The *EMSY* TaqMan probe sequence was ctggatctcagcgggatgaatgcaaaagaa. The forward and reverse primers used for *EMSY* were 5'-tgcctgtgtgtgcccacaa-3' and 5'-ggacacggctcactgtg-3'. *Cyclin D1* and *PAK1* probes with primers included were obtained as a 20× target assay from Applied Biosystems (Foster, CA). Expression levels were first normalized to rRNA, and then the ratio of this expression level to the mean normalized expression level of all 22 samples was calculated. Expression levels were similarly compared to the expression of Stratagene's Universal Human Reference RNA mix consisting of 10 different human cell lines (La Jolla, CA).

### Statistical analysis

Statistical analysis was performed using SPSS 11.0 (Chicago, IL). A non-parametric Chi-squared test was used to determine the significance of *EMSY*

amplification events in high grade papillary serous versus grade 1 serous carcinoma and serous borderline tumors. Pearson correlation was used to assess the correlation between gene amplification and mRNA expression levels.

## Results

### *EMSY amplification in ovarian cancer*

A three-color FISH assay for *EMSY*, *Cyclin D1*, and centromere 11 copy number was successfully applied to 674 ovarian tumors or pseudoneoplastic lesions from three TMAs including cases from Vancouver Hospital, Stanford Medical Center, and Memorial University as previously described [8] (Fig. 1). The frequency of *EMSY* gene amplification in this series was, as reported previously, 17% [8]. The ovarian tissue arrays encompassed a wide range of ovarian pathologies, and, although the frequency of *EMSY* gene amplification was already determined, correlation with histological subtypes was not performed. The majority of cases were high grade serous carcinomas; however, all common subtypes of ovarian cancers were included on the arrays. FISH signals were successfully scored in 508 cases. The remaining 166 cases from this series were not scored as a result of core loss or lack of FISH signals. The histological subtypes associated with *EMSY* amplification can be seen in Table 1. No amplification was seen in normal ovary ( $n = 5$ ), germ cell tumors ( $n = 4$ ), sex cord stromal tumors ( $n = 12$ ), endometriosis ( $n = 11$ ), benign tumors, borderline tumors of any subtype ( $n = 89$ ), or in mucinous tumors of any grade ( $n = 27$ ). The frequency of *EMSY* amplification events in high grade (grade 2 or 3) serous carcinoma (52/285, 18%) was significantly greater than that seen in grade 1 serous carcinoma or serous borderline tumors (0/76, 0%) ( $P < 0.0001$ ).

The histological subtypes associated with *Cyclin D1* amplification are also shown in Table 1. As was the case for *EMSY* amplification, amplification of *Cyclin D1* was not seen in normal ovary, germ cell tumors, sex cord stromal tumors, endometriosis, benign tumors, borderline tumors of any subtype, or in mucinous tumors of any grade. Overall, *EMSY* amplification (37/360, 10%) was a significantly more frequent event than *Cyclin D1* amplification (14/360, 4%) in ovarian carcinomas ( $P = 0.001$ ).

Table 1

Evaluation of *EMSY* and *Cyclin D1* amplification in ovarian cancer

	<i>N</i>	<i>EMSY</i> amplified <i>n</i> (%)	<i>Cyclin D1</i> amplified <i>n</i> (%)	Co-amplified <i>n</i> (%)
<i>Histopathological diagnosis</i>				
Undifferentiated	10	3 (30)	2 (20)	2 (20)
Clear cell	38	3 (8)	1 (4)	1 (3)
Endometrioid	27	4 (15)	1 (4)	1 (4)
high grade				
Serous high grade	285	52 (18)	35 (12)	21 (7)

### *aCGH mapping of the 11q13 amplicon in ovarian cancer*

A detailed mapping of the 11q13 amplicon was obtained for 51 ovarian cancers (Fig. 2). Elevated copy numbers were observed across the 11q13 region. The highest frequency of copy number gain encompassed the genes *EMSY*, *GARP*, *PAK1*, and *GRB2-associated binding protein 2 (GAB2)* (Fig. 2). *Cyclin D1*, *ems1* sequence (*EMS1*), and *prolylcarboxypeptidase (PRCP)* exhibited lesser frequencies of copy number gain, suggesting that the minimal region encompassed by the 11q13 amplicon in ovarian cancer is bounded by *EMSY* at the centromeric end and *GAB2* at the telomeric end.

### *Correlation between EMSY gene amplification and RNA expression*

To determine whether *EMSY* gene amplification correlated with an increase in RNA expression, we studied *EMSY* gene amplification by FISH and RNA expression levels by quantitative real-time PCR in 22 ovarian tumors. To further establish *EMSY*'s involvement in the formation of the 11q13 amplicon, *Cyclin D1* and *PAK1* were also included in the FISH and real-time PCR analysis.

The resulting amplification profiles for each of the three genes along 11q13 can be seen in Table 2. Amplification of *Cyclin D1*, *EMSY*, and *PAK1* (amplification ratio  $\geq 1.5$ ) was commonly seen in the ovarian carcinomas analyzed. The majority of cases in which *EMSY* is amplified also showed co-amplification of *Cyclin D1* and *PAK1*. The expression profile for all three genes can also be seen in Table 2 (right-hand column, expression levels are based on the comparison to the

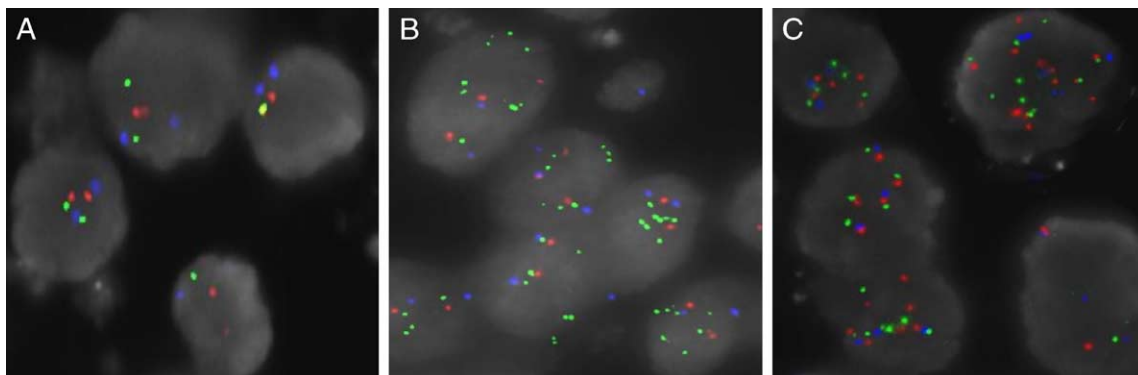


Fig. 1. *EMSY* gene amplification in ovarian carcinoma. (A) *EMSY* (green), *Cyclin D1* (orange), and centromere 11 (blue) are present in normal copy number. (B) *EMSY* gene amplification with normal copy number of *Cyclin D1* and centromere 11. (C) *EMSY* and *Cyclin D1* are present in higher copy number than centromere 11.

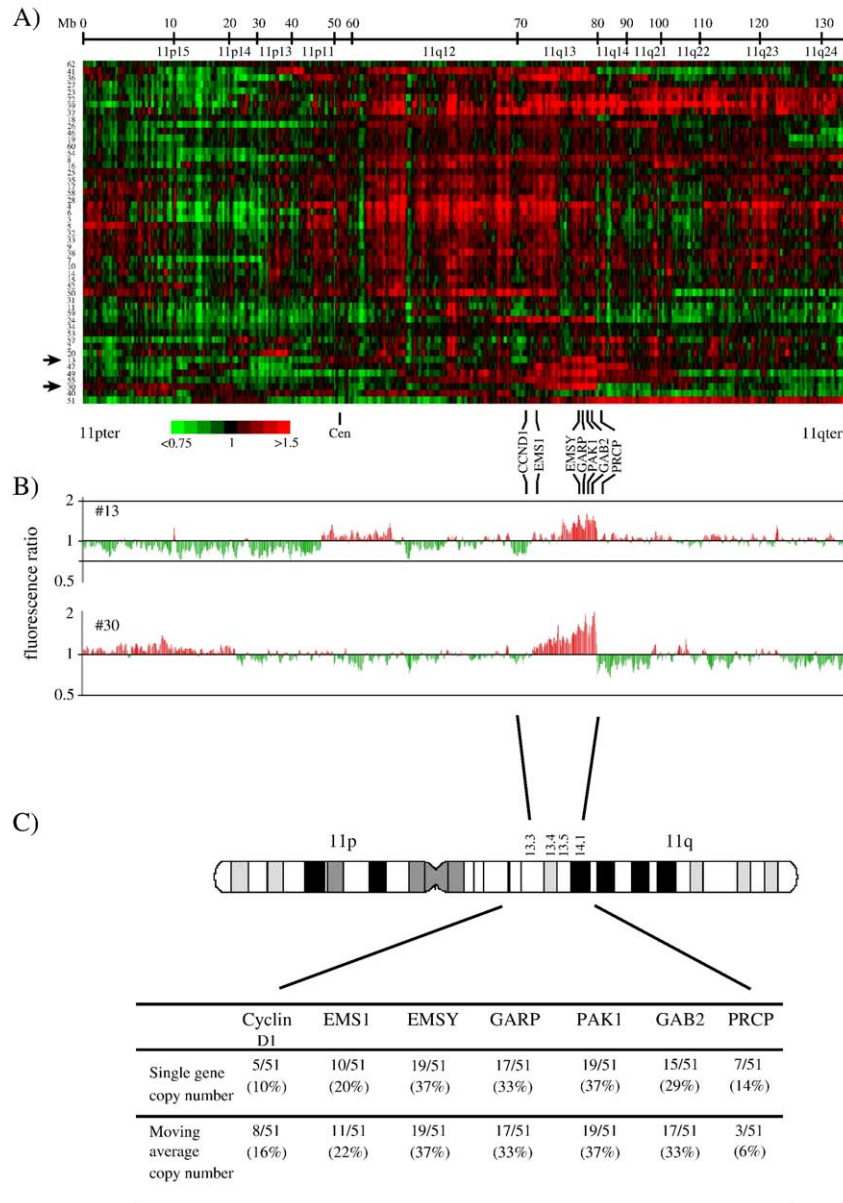


Fig. 2. Chromosome 11 array CGH data from 51 ovarian carcinomas based on a moving average of the five nearest genes centered on that locus. (A) Each row represents a single case of ovarian carcinoma, and each column is a cDNA mapped to the chromosomal locus depicted above the aCGH data. The cDNA copy number is depicted graphically, with red being copy number greater than normal and green less than normal, with the color intensity corresponding to copy number according to the scale shown. (B) Graphical representation of the 11q13 amplicon. Cases 13 and 30 both show that *Cyclin D1* and *EMS1* are not present in increased copy number, and the boundary of the amplicon is centromeric to *EMSY* and telomeric to *PAK1*. (C) Chromosome 11 ideogram showing the location of the 11q13 amplicon. The table below shows copy numbers expressed as single gene values and as a moving average of each gene and the nearest four flanking genes for 7 genes within the 11q13 amplicon. The criterion used for calling a gain at a particular gene or moving-average-5 locus was a ratio >1.2, which represents ratios above the 2 SD threshold in normal–normal hybridizations.

mean normalized expression of all 22 cases). Correlations between RNA expression and gene amplification were calculated for *Cyclin D1*, *EMSY*, and *PAK1*. *EMSY* gene amplification showed the strongest correlation with RNA expression ( $P < 0.0001$ ) (Table 3). *Cyclin D1* ( $P = 0.003$ ) and *PAK1* ( $P = 0.009$ ) also showed a significant correlation, although to a lesser degree. The above data were based on the comparison to the mean normalized expression of all 22 samples; when Stratagene’s Universal Human Reference RNA was used as the reference sample, the same correlations between gene amplification and overexpression were observed

(*EMSY* ( $P < 0.0001$ ), *Cyclin D1* ( $P = 0.003$ ), and *PAK1* ( $P = 0.011$ ); data not shown).

**Discussion**

Amplification of 11q13 is frequently seen in several tumor types, including head and neck, oral, lung, and breast cancer [14]. In breast cancer, amplification of this region has been reported in 15% of carcinomas [2] and is associated with a poor prognosis [15]. This region on chromosome 11 contains several candidate genes, including *Cyclin D1* and *EMS1* that could be

Table 2  
Gene amplification and overexpression profiles of *CCND1*, *EMSY*, and *PAK1* in ovarian tumors

Case #	Histology	Amplification ratios			RNA expression levels		
		<i>Cyclin D1</i>	<i>EMSY</i>	<i>PAK1</i>	<i>Cyclin D1</i>	<i>EMSY</i>	<i>PAK1</i>
1	undiffer grade 3	1.4	1.2	1.2	0.1	0.7	0.7
2	pap serous grade 3	2.1	2.1	1.4	2.0	1.2	2.3
3	pap seroushigh grade	1.0	0.8	0.9	1.4	1.2	1.0
4	pap serous grade 3	1.6	2.4	1.2	1.5	1.0	1.7
5	mixed carcinoma grade 3	1.9	1.9	1.8	5.3	3.1	2.9
6	pap serous grade 3	1.3	1.2	0.7	0.3	0.9	1.3
7	clear cell	1.1	0.9	0.8	0.3	0.5	0.5
8	undifferentiated	1.0	0.9	0.8	2.4	1.2	0.9
9	undifferentiated	2.5	2.4	2.4	0.8	1.3	1.0
10	pap serous grade 3	1.8	1.4	1.0	0.2	1.8	2.1
11	pap serous grade 3	2.1	1.8	1.4	2.3	2.6	1.0
12	pap serous grade 1	1.2	1.2	0.9	0.9	0.9	1.0
13	pap serous grade 3	1.2	1.0	1.0	1.8	0.4	0.5
14	pap serous grade 3	1.5	1.3	1.4	1.5	0.6	0.9
15	pap serous grade 3	1.1	0.8	1.2	6.0	2.9	2.1
16	pap serous grade 3	1.1	1.1	1.1	0.7	0.4	0.5
17	endometrioid grade 2	1.1	1.0	0.9	0.3	0.2	0.6
18	clear cell	0.9	0.9	1.0	0.3	0.2	0.4
19	undiffer grade 3	4.8	4.6	3.7	7.8	6.1	3.5
20	pap serous grade 3	1.2	1.5	2.6	2.5	2.5	0.8
21	pap serous grade 3	1.5	1.8	1.7	0.5	1.1	1.0
22	clear cell	0.9	0.9	0.9	0.8	0.6	0.3

Gene amplification ratios  $\geq 1.5$  are considered amplified, and those cases associated with amplification are shaded. For RNA expression, the shaded areas represent the top 6 expression levels for each gene. Genes are ordered according to their position on 11q13.

responsible for the emergence and maintenance of this amplification event. Cyclin D1 is a member of a family of proteins that regulate the activity of cyclin-dependent kinases and is an important regulator of the cell cycle [16]. Overexpression of Cyclin D1 shortens the G1 phase of the cell cycle, allowing cells to bypass critical checkpoints leading to the accumulation of mutations and genomic instability [17,18]. *Cyclin D1* is amplified in 15–20% of breast cancers [19–21] and overexpressed in up to 80% of tumors [1,2,22,23]. *Cyclin D1* amplification and overexpression have been associated with a poor prognosis in estrogen receptor positive breast tumors [24]. *EMSI*, located 0.8 Mb telomeric to *Cyclin D1*, encodes for the human homolog of cortactin, a cytoskeletal actin binding protein [25]. Several studies have shown that both *Cyclin D1* and *EMSI* are frequently co-amplified in breast cancer [15]. *EMSI* amplification can, however, occur independently of *Cyclin D1* amplification in breast cancer and is associated with an increased risk of relapse and death in estrogen negative tumors [3,6].

Detailed cytogenetic mapping of 11q13 in breast cancer has identified four distinct regions of amplification [2,4,21], suggesting the involvement of other genes in addition to *Cyclin D1* and *EMSI*. Other potential oncogenes telomeric to *Cyclin D1* and *EMSI* may exist within the 11q13 amplicon

Table 3  
Correlation between gene amplification and RNA expression

	Correlation coefficient	P value
<i>EMSY</i>	0.745	0.0001
<i>Cyclin D1</i>	0.609	0.003
<i>PAK1</i>	0.510	0.009

including *GARP*, *EMSY* [8], and *PAK1* [10]. *EMSY* is a recently cloned gene encoding a protein that binds and represses the activity of the transactivation domain of BRCA2. *EMSY* also localizes to sites of repair following DNA damage and could be a surrogate for BRCA2 loss in sporadic breast cancer. We have previously reported that *EMSY* is amplified in 13% of sporadic breast cancers and *EMSY* amplification was associated with a poor prognosis [8]. This finding has been confirmed in a recent study, which also showed a strong association between *EMSY* gene amplification and overexpression in primary breast tumors and cell lines [9]. These data suggest that the involvement of *EMSY* is an important element of the 11q13 amplicon in breast cancer; however, little is known about *EMSY* aberrations in ovarian cancer.

Here, we present additional evidence demonstrating that *EMSY* is frequently amplified in sporadic ovarian cancers. In particular, *EMSY* amplification is associated with specific histological subtypes of ovarian cancers, which include high grade papillary serous, clear cell, endometrioid, and undifferentiated carcinomas. Amplification of *Cyclin D1* was observed in the same histological subtypes associated with *EMSY* amplification, but less frequently. An association between Cyclin D1 protein overexpression and borderline or well differentiated, grade 1 ovarian tumors has been reported [7]. This dissimilarity in findings may be due to the smaller number ( $n = 43$ ) of sporadic ovarian carcinomas that were previously assessed for Cyclin D1 overexpression [7]. The data presented herein strongly demonstrate that *EMSY* amplification is associated with most subtypes of high grade carcinomas but not mucinous carcinomas, borderline tumors,

or non-epithelial tumors. Interestingly, this is the same profile of tumors associated with germline BRCA2 mutations [26–28]. *EMSY* gene abnormalities could be a surrogate for BRCA2 loss in ovarian cancer [8,29–31]. Therefore, *EMSY* abnormalities may denote a second and larger subset of ovarian cancers that could potentially respond to therapies targeting cells with BRCA1 and BRCA2 loss, such as PARP inhibitors.

aCGH mapping of 11q13 showed the most frequently amplified region in ovarian cancer to encompass *EMSY*, *GARP*, *GAB2*, and *PAK1*. We have previously shown that *EMSY* amplification was accompanied by *GARP* amplification in several cancer cell lines. In all of these cell lines, *EMSY* is overexpressed, as detected by real-time RT-PCR, and *GARP* expression is not detectable, providing evidence that *GARP* is not the oncogenic gene within this region [8]. *PAK1*, encoding a serine/threonine kinase involved in the regulation of anchorage-independent growth, invasiveness, and abnormal mitotic spindle organization [32,33], has recently been shown to be present in higher copy number in ovarian cancer [10], and array CGH confirms this. These results suggest that *EMSY* and *PAK1* may both be oncogenic elements in the 11q13 amplicon in ovarian cancer.

Gene amplification should lead to overexpression of genes playing a significant role in oncogenesis. Although *EMSY* gene amplification correlates with overexpression in breast cancer, it has not previously been studied in ovarian cancer. A strong significant correlation between *EMSY* gene amplification and mRNA expression was seen. A correlation, although to a lesser degree, was also seen between *Cyclin D1* and *PAK1* amplification and expression.

In summary, these results demonstrate that *EMSY* amplification is associated with high grade ovarian carcinomas, with the exception of mucinous carcinomas, and is a frequently amplified element of the 11q13 amplicon in ovarian cancer. Amplification of *EMSY* leads to overexpression, further supporting its role in oncogenesis. The complexity in the structure of the amplicons on 11q13 makes it unlikely that one gene is entirely responsible for the emergence of this amplification event. It is likely that co-amplification of several oncogenes on 11q13 is required to provide a selective advantage necessary for the development and progression of cancer.

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