

## Original Article

# Amplification of *EMSY* Gene in a Subset of Sporadic Pancreatic Adenocarcinomas

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**Abstract:** Mutations in the breast cancer susceptibility gene 2 (*BRCA2*) are commonly found in familial pancreatic cancer. Recently, *EMSY* (11q13.5) has been described as a *BRCA2* interacting protein capable of binding and inactivating the protein domain encoded by exon 3 of the *BRCA2* gene. Amplification of *EMSY* occurs in 13% of sporadic breast cancers and is directly linked to increased expression. Here we investigate the amplification status of this new potential oncogene in 59 sporadic pancreatic cancers using fluorescence in situ hybridization (FISH) and tissue microarray (TMA). Real-time quantitative RT-PCR was performed on 20 pancreatic cancer cell lines and overexpression was calculated using the delta-delta-Ct-method. Amplification of *EMSY* was found in 8/59 cases (13.6%). 9/20 (45%) cell line samples showed overexpression of *EMSY*. In conclusion, sporadic pancreatic cancer shows amplification of *EMSY* at prevalence similar to that found in other cancers.

**Key Words:** Pancreatic cancer, *EMSY*, *BRCA2*, FISH, amplification

## Introduction

Pancreatic cancer is the deadliest of the common solid cancers and the fourth leading cause of cancer-related death in the United States [1]. Due to the late development of clinical symptoms, 80% of patients with pancreatic cancer have metastasis at the time of initial diagnosis [2] such that 1-year and 5-year survival is 19% and 4%, respectively [3]. Surgical resection remains the best option for cure, but only about 20% of patients are eligible for surgery [4]. Numerous genetic (*K-ras*, *p16*, *p53*, *SMAD4* [5-7], telomere shortening) and epigenetic (e.g. *SPARC*, *RELN*, *TFPI-2* [8-11] and others [12, 13]) alterations occur during the development of pancreatic adenocarcinoma. In addition, germline mutations of the *BRCA2* gene occur in 5-15% of individuals with familial pancreatic cancer [14-16] and in about 5% of patients with clinically sporadic pancreatic cancer [17]. A polymorphic stop codon near the 3' end of *BRCA2* is more common in patients with familial pancreatic cancer than in controls [18].

*BRCA2* is a cancer-susceptibility gene that functions in DNA repair pathways which are essential for the maintenance of genetic integrity [19, 20]. *BRCA2* promotes highly accurate homologous recombination (HR) mediated double strand break (DSB) repair through direct interaction with *RAD51* [21]. Cancer in *BRCA2* mutation carriers is associated with loss of heterozygosity (LOH) of the wild-type allele, resulting in a functional deficiency [22]. Even though functional deficiency of *BRCA2* strongly reduces HR-mediated DSB repair, other error-prone DSB repair systems such as nonhomologous end joining (NHEJ) and single-strand annealing (SSA) remain intact [21, 23]. Rerouting of DSB processing through these repair systems may cause the accumulation of structural chromosomal aberrations as seen in *BRCA2*-deficient human cancer cells [24]. *BRCA2*-deficient cells exhibit an impaired DSB repair mechanism resulting in chromosomal instability and carcinogenesis [25]. Their inability to successfully repair DSB renders *BRCA2*-null cells especially vulnerable to DNA damaging agents such as mitomycin C (MMC)

[26, 27] and poly(ADP-Ribose) polymerase (PARP1) [28-35]. These findings have led to a clinical trial testing the utility of using MMC and gemcitabine in patients with *BRCA2* germline mutations [36].

Although inherited mutations of *BRCA2* are well known to predispose to breast and ovarian cancer, somatic *BRCA2* mutations are rarely seen in sporadic cancer cases [37, 38]. In addition, in response to DNA damage the Fanconi anemia (FA) pathway functioning upstream of *BRCA1* and *BRCA2* forms a Fanconi protein complex that mono-ubiquitinates *FANCD2* which then interacts with *BRCA1/BRCA2*. Mostly somatic mutations in the Fanconi anemia genes, *FANCC* and *FANCG* occur in 5-10% of pancreatic cancers [39-42]. Fanconi anemia gene-deficient cells are also hypersensitive to DNA damaging agents such as MMC [27, 43, 44].

Other proteins that interact with the *BRCA2* pathway are genetically altered in certain cancers. Recently, a novel gene, *EMSY*, has been described providing a new mechanism possibly linking *BRCA2* to sporadic breast and ovarian cancer [45]. *EMSY* is amplified in 13% of sporadic breast cancer and 17% of high grade ovarian cancer [46]. *EMSY* (11q13.5) maps to the same amplicon as *GARP*, glycoprotein A repetitions predominant, a gene not expressed in breast cancer [47], presenting *EMSY* as the gene of interest within this amplicon. Amplification of the 11q13 locus is common in several tumor types. Investigation of this gene dense region has led to several candidate targets of amplification [46, 48]. Cyclin D1 (*CCD1*) and *EMS1* (cortactin) – located on separate amplicons at 11q13.3 (*CCD1* mapping 0.8 Mb proximal to *EMS1*) – are considered strong candidate oncogenes within this region and are frequently co-amplified in breast cancer [49]. Other genes in this region include *RSF-1*, a gene that was recently described as amplified in a subset of ovarian carcinomas [50]. Amplification of *EMSY* occurs independently of adjacent *CCD1*, and correlates directly with increased levels of mRNA [45, 51-53]. The amino terminal of *EMSY* (ENT-domain) binds to the independent activation domain of *BRCA2* encoded by exon 3 of the *BRCA2* gene. Deletion of this specific region is known to be the sole mutation in a Swedish breast/ovarian cancer family [54]. Overexpression of *EMSY* interferes with the activation potential domain

of *BRCA2* encoded by exon 3 resulting in decreased *BRCA2* activity, mimicking the genomic instability phenotype as seen in *BRCA2* deficient cells [45]. The importance of *EMSY* in tumorigenesis remains uncertain although *EMSY* amplification was associated with a poor outcome in one study [53].

This study examines the prevalence of *EMSY* amplification in pancreatic adenocarcinomas using fluorescence in situ hybridization (FISH) and *EMSY* mRNA quantification in pancreatic cancer cell lines. We also determined if pancreatic cancers overexpressed the nearby gene, *RSF-1*.

## Materials and Methods

### *Patients and Samples*

The pancreatic cancer samples were chosen from tissue microarrays (TMAs) developed on patients with resectable infiltrating adenocarcinoma of the pancreas that underwent pancreatico-duodenectomy at Johns Hopkins Hospital, Baltimore, MD, between 1/2/1998 and 7/25/2003 [55]. Because the natural history for variant pancreatic neoplasms differs from usual pancreatic ductal adenocarcinoma, patients with IPMN's, mucinous cystic adeno-carcinomas and medullary adenocarcinomas were excluded. Patients were also excluded if gross metastatic or unresectable disease beyond the Whipple margins was found at the time of surgery.

All clinical and pathologic patient information is maintained in a regularly updated clinical database. The primary outcome of the study was overall survival as determined from date of Whipple resection to the time of death or last follow-up. Patients, their family or their primary physicians are contacted by postcard at least annually to confirm patient status with the last observation recorded in February 2007. This study was conducted as part of a Johns Hopkins Hospital IRB approved protocol.

### *Fluorescence In Situ Hybridization*

DNA from *EMSY* PAC clones DJ824N10, DJ855A11 and DJ180012 provided by Dr. Carlos Caldas, University of Cambridge Department of Oncology, was extracted and purified and directly labeled with Spectrum Green by NICK translation. A Spectrum aqua-labeled centromeric probe, CEP11 was used to

stain centromere-11. FISH was performed on 4 TMAs of formalin fixed, paraffin embedded tissues [55]. The slides were heat treated overnight at 56°C, deparaffinized (Xylene, 100%, 95% and 75% ethanol), demasked using 50TE2 pH 9.0 for 30min at 95°C, washed in 2XSSC and RNase treated for 1 hr at 37°C (0.1mg/μL in 2 x SSC), after which they were washed again in 2XSSC, digested in a pepsin solution (0.1% in dH<sub>2</sub>O, pH 2.0) for 10min at 37°C, washed in PBS and dehydrated in ethanol (75%, 95% and 100%). Probes and target DNA were denatured simultaneously for 12 min at 80°C. Hybridization was done for 72 hrs under coverslips at 37°C. Posthybridization washes were performed using NP-40 rapid wash procedure. Nuclei were counterstained with 4,6-diamidino 2-phenylindole (DAPI, 1μg/mL) diluted 1:5 in antifade solution. Slides were analyzed with a Leica DMRA2 fluorescence microscope at a 100X magnification using FITC, DAPI and Aqua filters. Signals were counted manually only in non-overlapping, intact nuclei. A total of 150 nuclei were counted per case, divided over two TMA cores, unless fewer representative nuclei were available. The average number of probe signals per nucleus was calculated for both *EMSY* and CEP11. The amplification ratio was determined as the ratio between the average *EMSY* copy number and the average centromere-11 copy number. *EMSY* amplification was found when the amplification ratio exceeded 1.5 [45]. If the average copy number of *EMSY* per nucleus was higher than 2.5 but the amplification ratio did not exceed 1.5 due to a concomitant increase in the centromere-11 signal these cases were labeled as having an increased *EMSY* copy number (ICN) without gene amplification. Documentation of the images was done using the Leica DC350 FX camera and Leica CW4000 FISH software.

#### *Real-Time RT-PCR Expression Analysis*

Quantitative SYBRGreen RT-PCR was performed on 20 cell line RNA samples to screen for over expression of the *EMSY* gene. Results were normalized to the housekeeping gene *GAPDH* and expression was compared to normal levels in the diploid pancreatic cell lines HPDE and HPNE using the delta-delta-Ct-method. Cell-lines with a ratio higher than 2.5 were considered as having *EMSY* overexpression [53]. Primers detecting the

*GAPDH* gene were run in a separate reaction tube from the *EMSY* primers (*EMSY*-HF1 5'CCA CCC CAC ATG TCT CCT GTA 3', *EMSY*-HR1 5' TGA GCT TGG TGA TGT GGT GAC 3'). The PCR reaction mix was prepared according to the standard protocol with a final primer concentration of 0.3 μM. The samples were run using the Cepheid SmartCycler System. The protocol consisted of a 30 min, 50°C reverse transcriptase step followed by 15 min at 95°C for the initial activation. 45 cycles of denaturation (15 sec at 94°C), annealing (30 sec at 58°C) and extension (30 sec at 72°C) were performed after which a melting curve was used to confirm the specificity of the PCR products. Experiments were performed in triplicate.

#### *Immunohistochemistry*

Immunohistochemistry (IHC) for RSF-1 was performed as previously described [50].

#### *Statistics*

Simple descriptive statistics were used to determine the prevalence of clinico-pathological differences between pancreatic cancer with *EMSY* amplification and those without.

## **Results**

#### *Fluorescence In Situ Hybridization*

In total 4 TMAs were stained with 18 cases per TMA using a FISH-array for *EMSY* and centromere 11. A total of 59 cases of pancreatic cancer were successfully scored. Each case consisted of four cores on the TMA, two of which tumor tissue and two controls. Approximately 150 random intact nuclei were scored per case. No gain in copy number was found in any of the control tissue (**Figure 1**).

As can be seen in **Table 1**, low-level amplification of *EMSY* (*EMSY*/CEP11 copy number ratio >1.5) was found in eight of 59 cases (13.6%). An increased copy number (ICN) of *EMSY* (>2.5 *EMSY* probe signals on average per nucleus) was found in 12 of 59 cases (20.3%) including the 8 with *EMSY* amplification. But in 4 of these cases, the centromeric probe signal was also increased suggesting that in these cases the increased *EMSY* signal was due to aneuploidy. There were no obvious clinicopathologic differences

**Table 1** Summary of FISH analysis of 59 cases of sporadic pancreatic cancer

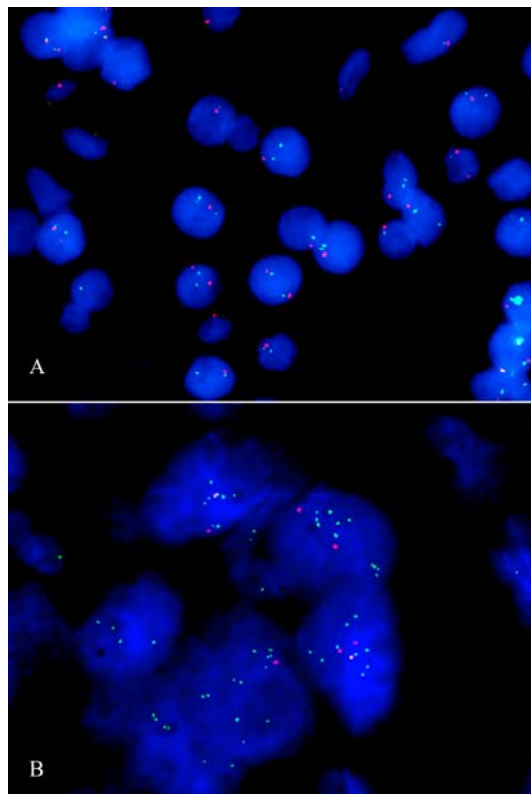
case #	# of nuclei scored	Average # of signals per nucleus		EMSY/C11 ratio
		EMSY	C11	
1	133	1.86	1.56	1.19
2	128	1.69	1.67	1.01
3	123	1.51	1.49	1.01
4	115	1.46	1.42	1.03
5	135	2.55	2.02	1.26
6	129	2.58	2.35	1.1
7	79	1.72	1.78	0.97
8	122	2.02	1.91	1.06
9	103	1.75	1.63	1.07
10	118	1.52	1.47	1.03
11	122	1.83	1.73	1.06
12	169	3.93	2.39	1.64
13	126	1.57	1.45	1.08
14	109	2.99	1.87	1.6
15	121	2.11	1.75	1.21
16	86	1.76	1.55	1.14
17	152	3.31	1.42	2.33
18	131	1.81	1.54	1.18
19	135	1.44	1.37	1.05
20	104	1.55	1.5	1.03
21	45	1.66	1.44	1.15
22	153	2.2	1.39	1.58
23	126	1.54	1.58	0.97
24	73	2.47	2.44	1.01
25	135	1.62	1.51	1.07
26	138	2.47	1.49	1.66
27	67	1.49	1.43	1.04
28	99	1.59	1.58	1.01
29	128	1.94	1.68	1.15
30	111	1.62	1.48	1.09
31	123	1.57	1.49	1.05
32	110	2	1.66	1.2
33	171	2.61	1.49	1.75
34	62	2.69	2.36	1.14
35	117	1.83	1.69	1.08
36	54	2.98	2.11	1.41
37	127	2.02	1.91	1.06
38	134	2.19	2.06	1.06
39	140	3.42	2.26	1.51
40	145	4.01	2.99	1.34
41	137	1.47	1.53	0.96
42	160	1.67	1.54	1.08
43	198	2.96	2.27	1.3
44	141	2.27	1.91	1.19
45	123	1.56	1.53	1.02
46	158	2.16	1.95	1.11
47	84	1.49	1.57	0.95
48	146	1.64	1.62	1.01
49	166	1.8	1.53	1.18
50	119	1.88	1.67	1.13

51	72	2.27	1.99	1.14
52	164	2.68	1.74	1.54
53	132	1.99	1.65	1.21
54	102	1.59	1.55	1.03
55	74	1.54	1.47	1.05
56	170	1.63	1.67	0.98
57	168	1.58	1.56	1.01
58	166	1.93	1.71	1.13
59	191	1.58	1.54	1.03

37.3% had an increased copy number (>2.0 signals on average per nucleus) 13.6% had EMSY amplification (ratio >1.5)

EMSY amplification

Increased EMSY copy number



**Figure 1** FISH analysis of EMSY amplification, conducted on paraffin embedded pancreatic tissue. The EMSY probe is colored green, while the control signal, the centromere-11 probe is red. **A.** No EMSY amplification in normal pancreatic tissue. **B.** EMSY amplification in a cluster of pancreatic tumor cells.

**Table 2** Pancreatic cancer cell lines and corresponding EMSY expression level

Cell lines	EMSY mRNA expression ratio	BRCA2 mutation status [17]	Fancd2 mono-ubiquitination [44]	Hypersensitivity to MMC [44]
Colo357	2.1		pos	NA
PL14	2.2		NA	NA
Hs766T	1		neg	pos
CFPAC1	2	LOH	pos	NA
BxPC3	1.5	LOH	pos	neg
PL4	3.1		NA	NA
PaCa2	0.9		NA	NA
PL11	1.2		neg	pos
Su86.86	2.7	LOH	pos	neg
CAPAN2	2.3		pos	NA
PL3	3		pos	NA
PL9	2.5		NA	NA
PL6	2		pos	NA
PL1	1.1		NA	NA
PL12	2.6		NA	NA
Aspe1	2		NA	NA
CAPAN1	2.6	6174delT/LOH	pos	pos
MiaPaCa	3.4		NA	NA
AsPC1	3.1	LOH	pos	NA
Panc-1	4.1	LOH	pos	NA

LOH, loss of heterozygosity; del, deletion; pos, positive; neg, negative; NA, not applicable

(age, gender, tumor size, differentiation, stage) between the eight cases with *EMSY* amplification and the remaining cases without such amplification, although our sample size was not powered to identify such differences.

#### Real-Time RT-PCR Expression Analysis

*EMSY* amplification is associated with an increase in RNA levels. We determined the RNA expression levels in 20 pancreatic cell lines. Nine of 20 cell lines tested (45%) had an *EMSY* expression ratio >2.5 relative to the mean expression ratio in 2 non-neoplastic pancreas cell lines, but none of the pancreatic cancer cell lines showed high levels (>5-fold above reference normal levels) of *EMSY* overexpression. A subset of cell lines have been previously studied with regard to BRCA2 mutation status [17], defects in FANCD2 monoubiquitination and hypersensitivity to MMC due to FA or BRCA2 deficiency [44]. Results have been incorporated in **Table 2**.

#### Immunohistochemistry

Expression of the nearby gene *RSF-1* recently identified as overexpressed in ovarian cancers was evaluated in pancreatic ductal adenocarcinomas by immunohistochemistry of pancreatic cancer TMAs. *RSF-1* protein was similarly expressed in pancreatic cancer and normal epithelium of the pancreatic duct.

#### Discussion

Given the poor survival of patients with pancreatic adenocarcinoma, there is a tremendous need to characterize the molecular profiles of these cancers in the hopes that molecular profiles can guide early diagnosis and the optimal use of novel therapies. *EMSY* has recently been proposed as a potential oncogene in breast and ovarian cancer through its interaction with BRCA2. The involvement of the BRCA2 pathway in pancreatic cancer led us to believe *EMSY* may

also play a role in pancreatic carcinogenesis. *EMSY* is exclusively nuclear and, like *BRCA2*, re-localizes to DSB repair sites following DNA damage. Furthermore, the ENT-domain of *EMSY* interacts with a number of chromatin-regulator proteins, including *HP1 $\beta$*  and *BS69*, suggesting a role in chromatin remodeling [45, 56-58].

Here we looked at *EMSY* copy number aberrations in 59 cases of sporadic pancreatic carcinoma and expression of *EMSY* in 20 pancreatic cancer cell lines. We identified eight of 59 (13.6%) cases of low-level *EMSY* amplification (*EMSY*/*CEP11* copy number ratio >1.5) in sporadic pancreatic carcinomas. *EMSY* overexpression was found in 9 of 20 pancreatic cancer cell lines, but the level of overexpression was modest with no cell lines showing expression more than five-fold above the reference level.

Twelve (20.3%) cases demonstrated increased copy number of *EMSY* (>2.5 *EMSY* probe signals on average per nucleus), with most cells appearing aneuploid containing also aberrant copy numbers of centromere 11. Nonetheless, six out of 12 cases do not show *EMSY* amplification due to an increase in centromere-11 copies. In all 12 cases increase in *EMSY* copy number was greater than that of centromere 11.

Cells containing increased copy numbers of *EMSY* appeared to be relatively large and clustered, surrounded by tumor cells expressing normal copy numbers, suggesting a late onset amplification with disease progression, rather than a mutation early in carcinogenesis. This may explain an earlier report that found a negative association of this mutation on disease outcome.

The inability to effectively repair DSB caused by DNA damaging agents results in the accumulation of chromatid breaks and aberrations leading to cell cycle arrest and eventually apoptosis. *EMSY*'s inhibiting effect on the *BRCA2* pathway suggests cells overexpressing *EMSY* may also express an increased sensitivity to DNA damaging agents. A subset of pancreatic cancer cell lines had been studied prior with regard to *BRCA2* mutation status [17], defects in *FANCD2* monoubiquitination and hypersensitivity to MMC due to FA or *BRCA2* deficiency [44]. One cell line overexpressing *EMSY* was found not

to be hypersensitive to MMC treatment. However, the level of *EMSY* amplification and RNA overexpression ratio of this particular cell line was not very high and it maybe that a higher level of *EMSY* expression is needed to be functionally relevant.

In summary, we find that pancreatic cancers display low-level amplification of *EMSY* at prevalence similar to what has been reported in other cancers. Further investigation is needed to determine if such low-level amplification of *EMSY* is sufficient to affect the pancreatic cancer sensitivity to DNA damaging agents such as MMC and PARP1.

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