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Pattern of BRCA1 Expression is a Potential Prognostic Biomarker in Resectable Pancreatic Ductal Adenocarcinoma

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Abstract

Objectives—The tumor suppressor BRCA1 is a nuclear-cytoplasmic shuttling protein that when in the nucleus is required for DNA repair whereas when in the cytoplasm is important in activating cell death processes. Although BRCA1 mutations have been shown to be associated with an increased risk of pancreatic ductal adenocarcinoma (PDAC), its role in disease progression is yet to be determined. We hypothesized that BRCA1 expression pattern could be used as a prognostic biomarker.

Methods—67 patients who underwent resections for PDAC were included. A tissue microarray was constructed, stained with antibodies to BRCA1, and scored for intensity and subcellular location. Univariate and multivariate statistical analyses were performed.

Results—An increase in cytosolic BRCA1 distribution was associated with higher pathologic stage ($p=0.006$). Nuclear-cytoplasmic BRCA1 distribution was associated with a decrease in recurrence-free survival (RFS) with a hazard ratio of 1.4 ($p=0.059$). Decreased BRCA1 intensity was associated with higher pathologic stage ($p=0.027$), but BRCA1 intensity was not associated with overall survival or RFS.

Conclusions—Our results demonstrate a possible association of BRCA1 expression pattern with pathologic stage, implying a potential role of BRCA1 in PDAC development and progression.

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Keywords

BRCA1 localization; BRCA1 expression

Introduction

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in the United States and continues to have a dismal prognosis, with a 5-year overall survival rate less than 4% [1]. The only potential curative option is surgical resection, for which less than 20% of patients are eligible. Even in this subset of patients, the 5-year overall survival remains only 18-24% [2-6]. Given the poor survival with surgery alone, multiple attempts have been made to improve prognosis with adjuvant therapy. In multiple retrospective studies, chemoradiation therapy has been shown to confer a survival advantage compared with surgical resection alone [2, 7-8]. However, more recent randomized trials have shown a benefit to adjuvant chemotherapy but not to adjuvant chemoradiation therapy [9-11]. The role of adjuvant therapy in the management of localized pancreatic cancer remains controversial as many of the randomized clinical trials were statistically underpowered and used outdated radiation fractionation schema and techniques. Thus, the use of adjuvant therapy and the ideal treatment strategy remain controversial. Therefore, tumor biomarkers that could be used to predict which subset of patients is likely to benefit from adjuvant therapy would be useful for clinicians to tailor therapy based on that individual patient's tumor characteristics.

Many chemotherapeutic agents act by inducing DNA damage within rapidly dividing tumor cells. Radiation also causes DNA damage by inducing double-strand breaks. Molecular targets that regulate DNA damage and repair are hence likely to be good predictors of prognosis and response to treatment. The breast cancer susceptibility gene 1 (BRCA1) is a nuclear-cytoplasmic shuttling protein involved in DNA damage and repair pathways. In the nucleus, BRCA1 is involved in homologous recombination-mediated repair of DNA double-strand breaks through a signaling cascade [12-14]. Additionally, BRCA1 regulates cell cycle checkpoints through the BRCA1-BARD1 complex to facilitate entry into the S and G2 phases of the cell cycle, where homologous recombination is most efficient [15]. When located in the cytoplasm, BRCA1 enhances p53-independent cellular apoptosis [16]. One model proposes that BRCA1 is shuttled from the nucleus to the cytoplasm in the event of unsuccessful DNA damage repair, where it subsequently activates cell death pathways to remove these unrepaired cells [17]. Dysregulation of double-strand break repair function of BRCA1 and the induction of apoptosis will contribute to cancer cell resistance to DNA-damaging agents including chemotherapy and radiation.

BRCA1 has been well-studied particularly in breast and ovarian cancers and has been investigated as a molecular marker in various other cancers as well. Rosell *et al.* found that overexpression of BRCA1 mRNA was associated with poor survival in non-small cell lung cancer patients (Hazard ratio=2.4; p=0.04), demonstrating the prognostic utility of BRCA1 [18]. BRCA1 expression may also be useful to tailor treatment and predict treatment response. For instance, poly(ADP-ribose)polymerase (PARP) is a key nuclear enzyme in

DNA single-strand break repair whose inhibition induces 100-fold increased cell killing in BRCA1-deficient tumor cells, compared to BRCA1-proficient cells. PARP inhibition has also been shown to increase the cytotoxicity of DNA-damaging chemotherapy and radiation therapy in preclinical studies. PARP inhibitors have entered clinical testing both as single agents as well as in combination with chemotherapy and radiation [19,20]. It is possible that the pattern of BRCA1 expression may predict tumor cytotoxic response to PARP inhibition.

Wei *et al.* evaluated the mRNA expression of BRCA1 and survival after second-line docetaxel in advanced gastric cancer and found the risk of mortality was higher in patients with low BRCA1 levels (Hazard ratio for death=2.49; $p=0.037$), suggesting the potential of BRCA1 as a marker predictive of treatment response in cancers other than breast and ovarian [21]. BRCA1 mutation is associated with an increased risk of pancreatic cancer [22], but the role of BRCA1 in pancreatic cancer progression is yet to be determined. We hypothesized that BRCA1 expression pattern could be used as a prognostic biomarker in resectable pancreatic adenocarcinoma.

Materials and Methods

Patient Selection

This study was approved by the Vanderbilt University Medical Center Institutional Review Board. From 1984 to 2009, 67 patients were identified who had undergone curative resections for pancreatic adenocarcinoma and for whom both clinical data and tumor tissue were available. Only patients with histologically confirmed ductal adenocarcinomas were included. All tumors were restaged by a single pathologist (SCW) according to AJCC 7th edition criteria [23]. Data collected included patient demographics, operative details, treatment details and survival. Pathologic data obtained included tumor location, total number of nodes involved, total number of nodes resected, tumor size, differentiation and margin status. A positive margin was defined as tumor within 1 mm of the inked resection margin on microscopic examination. Tumor differentiation was recorded according to the guidelines outlined by the College of American Pathologists [24]. The lymph node ratio was defined as the number of positive lymph nodes as a fraction of the total number of lymph nodes examined/resected.

Construction of Tissue Microarray

Tissue microarrays were constructed using 1 mm cores of both tumor and background normal/reactive pancreas from 67 curative resection specimens, including pancreaticoduodenectomy/gastrojejunostomy procedures (Whipple procedures) and total or distal pancreatectomies. The microarrays were composed of single or duplicate cores from tumor and background pancreas. Due to the scarcity of clinical material, sometimes only one core was used. The microarrays were cut at 5 μ m-thickness and stained with hematoxylin and eosin.

Immunofluorescence study

5 μ m-thick sections of formalin-fixed, paraffin-embedded tissue microarrays were de-paraffinized and rehydrated. Samples were pretreated to promote antigen retrieval with

Target Retrieval Solution (DAKO, Carpinteria, CA, USA). Sections were blocked with 3% hydrogen peroxide, followed by blocking in 2% goat serum/0.1% Triton-X 100/PBS (1 hour). Slides were then incubated with primary antibody BRCA1 (1:50 dilution in blocking buffer, Calbiochem Cat. No. OP92) overnight at 4°C. Slides were washed in phosphate-buffered saline and incubated with secondary antibody (1:1000 goat anti-mouse Alexa594-conjugated antibody, Molecular Probes), stained with DAPI for 1–2 minutes, and analyzed by fluorescence microscopy (Carl Zeiss, Thornwood, NY). A total of 150–400 cells were counted. BRCA1 subcellular localization was determined to be nuclear-dominant (predominantly nuclear staining), nuclear-cytosolic (both nuclear and cytoplasmic staining) or cytoplasmic-dominant (predominantly cytoplasmic staining). Nuclei were stained with DAPI. The intensity of staining was scored as 1+ (weakly staining/least intense), 2+ (moderately staining), or 3+ (strongly staining/most intense) in tumor cells. Due to limited sample size, 1+ and 2+ staining intensity were grouped into “low” intensity, and 3+ staining intensity was referred to as “high” intensity. Quantitative and qualitative assessment of BRCA1 staining was performed by a single experienced pathologist blinded to patient outcomes. Examples of BRCA1 expression intensity and subcellular location staining are shown in Figures 1 and 2, respectively.

Statistical Analysis

The primary endpoint was correlation between BRCA1 expression and OS, and the secondary endpoint was correlation between BRCA1 expression and RFS. OS was defined as the time from surgery to the date of all-cause death or last follow-up. The secondary endpoint was defined as the time from surgery to the date of disease recurrence (RFS) or last follow-up in the absence of documented relapse. Patients' demographic and clinical variables were summarized using the median with the 25th and 75th percentiles (IQR) for continuous variables. For categorical variables, frequency and percentages were shown. The Wilcoxon rank sum test was used for continuous variables, and Pearson's χ^2 or Fisher's exact test was used to compare categorical variables between BRCA1 intensity groups (low or high). The Kaplan-Meier method, log-rank test, likelihood ratio test, and Cox proportional hazard models were used in univariate and multivariable analysis when appropriate to investigate the associations between the endpoints and the risk factors. The negative binomial model was used to investigate the association between BRCA1 intensity and the number of positive lymph nodes. For the multivariable Cox proportional hazard models, multiple imputations (MI) with 10 iterations were used to deal with the missing values [25]. The reliability of the final regression models was internally validated via bootstrap method [26] by measuring degree of overfitting quantified by optimism parameter in a calibration plot. One hundred fifty resamples were performed with bootstrap with replacement. All statistical inferences were assessed at a two-sided 5% significant level and all summary statistics, graphics, and survival models were generated using R version 2.13 statistical software [27].

Results

Patient Clinical and Pathologic Characteristics

From 1984-2009, 67 patients were identified who had undergone curative resections for pancreatic adenocarcinoma for whom tissue samples were also available for study. Table 1 summarizes the demographic and treatment details. Table 2 summarizes the clinicopathologic findings. Of the 67 patients undergoing surgical resection, 77.0% had microscopically negative surgical margins. The median overall survival for all patients was 20.5 (IQR: 8.4 - 42.1) months.

Univariate Analysis

Decreased BRCA1 intensity was associated with increasing TNM stage ($p=0.027$), but BRCA1 intensity was not associated with overall survival or recurrence-free survival ($p>0.05$). Despite a trend, the effects of higher BRCA1 intensity with lower numbers of positive lymph nodes was not statistically significant ($p=0.15$, negative binomial model, data not shown here). BRCA1 intensity was not associated with tumor grade, or the use of adjuvant chemotherapy ($p>0.05$). Findings are summarized in Table 2.

Nuclear-cytosolic BRCA1 distribution was associated with a decrease in RFS with a hazard ratio of 1.4, but statistical significance was not achieved ($p=0.059$). Increased cytosolic BRCA1 distribution was significantly associated with advanced disease stage ($p=0.006$). BRCA1 subcellular distribution was not associated with overall survival, tumor grade, lymph node ratio, or the use of adjuvant chemotherapy ($p>0.05$). Findings are summarized in Table 3.

Multivariable Analysis

When adjusted for known covariates as shown in Table 4, BRCA1 intensity and subcellular location were not associated with overall survival or recurrence-free survival ($p>0.05$). Due to our limited study sample size and to avoid model overfitting, we were unable to adjust some factors that are commonly adjusted in the literature such as stage and radiotherapy. The optimism for the final models were estimated as 0.167 and 0.106 for OS and RFS models respectively; i.e. the degree of overfitting was 16.7% and 10.6%, providing no evidence of overfitting.

Discussion

Loss of or reduced BRCA nuclear expression has been associated with high grade invasive breast carcinoma [28]. In this single institution retrospective study of 67 patients with PDAC, we found that decreased BRCA1 intensity and increased cytosolic BRCA1 distribution were significantly associated with advanced pathologic stage by univariate analysis. In supporting our observation, low mRNA expression of BRCA1 has been reported to be associated with higher risk of mortality in advanced gastric cancer after second-line docetaxel [21]. Taken together, these findings indicate that decreased BRCA nuclear function may be associated with worse clinical outcomes, including higher stage in

pancreatic cancer, higher grade in breast cancer, and decreased survival in advanced gastric cancer.

The DNA repair function of BRCA1 is well characterized. In the nucleus, BRCA1 is involved in homologous recombination-mediated repair of DNA double-strand breaks through a signaling cascade [12-14], and BRCA1 thus has a direct impact on genomic stability. Decreased BRCA protein level or mislocation to the cytosol will result in compromised function of BRCA1 in fidelity DNA repair and subsequent genomic instability in the cancer cell, which may contribute to high tumor grade. Interestingly, the most recent studies examining the role of BRCA found that BRCA1 deficiency is associated with cancer cell invasion and migration [29]. This new finding implies loss of BRCA1 may lead to increased metastatic capacity of the cancer cell. This supports our finding that decreased BRCA function is associated with higher pathologic stage in resectable pancreatic cancer.

Intriguingly, in our study we also found a trend supporting the recently discovered novel property of BRCA1 in suppressing cancer cell invasion and metastasis. While patients with higher BRCA1 intensity tended to have fewer positive lymph nodes, the effects of higher BRCA1 intensity on having less positive lymph nodes was not statistically significant ($p=0.15$). This data is also consistent with our results that lower BRCA1 intensity was associated with advanced disease stage. The lack of statistical significance is likely a result of the small sample size of this study. This hypothesis needs to be validated in a larger patient cohort.

We had anticipated that tumors with decreased BRCA1 function would be more sensitive to adjuvant therapy (chemotherapy with/without radiation). However, in our study, no association between BRCA expression pattern and treatment was found. Additionally, our study was limited by a small sample size, making it difficult to reach statistical significance. An interesting question to be addressed in future studies is the validity of BRCA1 as a predictive marker of chemosensitivity in pancreatic cancer given that the majority of pancreatic cancer patients suffer significant toxicity with little treatment benefit.

Since PARP inhibitors have been shown to increase the cytotoxicity of DNA-damaging chemotherapy and radiation therapy in breast and ovarian cancers expressing BRCA mutations and with relatively low toxicity, their potential utility in BRCA-mutation positive pancreatic cancer is of important clinical significance. One case report by Fogelman *et al.* describes a patient with a germline BRCA2 mutation and associated pancreatic cancer who demonstrated a complete pathologic response to the PARP inhibitor BSI-201 [30]. No pre-clinical studies have demonstrated whether BRCA expression or subcellular location can predict response to PARP inhibitors in pancreatic adenocarcinoma, though a study by Drew *et al.* found the pancreatic cancer cell line CAPAN-1 with mutated BRCA2 was sensitive to PARP inhibitor AG014699 monotherapy [31]. Clinical trials investigating the use of PARP inhibitors in pancreatic cancer are currently underway (clinicaltrials.gov: NCT01489865, NCT00515866, NCT01078662), one of which is targeting patients with known BRCA mutations.

In our study, we did not find the association between BRCA1 expression intensity and subcellular location with overall survival or recurrence-free survival. In addition, it is important to note that BRCA1 intensity and subcellular location were not associated with pathologic stage by multivariate analysis in the current study, and both of these negative findings may be due to the small sample size. Therefore, it is important to verify the findings of this study in a larger patient cohort. The other significant limitation of current study is the extended time period of time in collection of the surgical samples. Despite that we have restaged all patients at the time of this study according to AJCC 7th edition criteria, there could be bias in terms of treatment received by patients over this extended time frame. For example, over the time period included in this study gemcitabine emerged as the first-line chemotherapeutic agent for pancreatic cancer rather than 5-fluorouracil. Finally, it has been reported that the prognostic value of certain biomarkers are dependent on the stage of cancer. For example, high expression of ERCC1 and RRM1 are positive prognostic factors in early stage non-small cell lung cancer but are negative predictors in advanced disease [32,33]. Future studies are needed to further investigate whether BRCA1 expression pattern holds the same prognostic value in both early and advanced stage PDAC.

In summary, our results demonstrate an association between BRCA1 expression pattern and pancreatic cancer stage by univariate analysis, implying that BRCA1 may play a role in pancreatic cancer development and progression. These findings need to be confirmed in a larger cohort of patients.

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High BRCA1
intensity

Figure 1.
Representative immunohistochemical staining for BRCA1 expression in pancreatic adenocarcinoma. (A) and (B) are high intensity of BRCA1 expression. (C) and (D) are low intensity of BRCA1 expression.

Figure 2.

Representative immunohistochemical staining for BRCA1 subcellular location in pancreatic adenocarcinoma. BRCA1 subcellular localization was determined to be predominately (A) in nuclear, (B) in both nuclear-cytosolic, or (C) in cytoplasmic.

Table 1
Patient Clinical and Pathologic Characteristics

	N*	No. (%)
Age, years ^a	67	67 (59 - 73)
Gender	65	
Female		30 (45)
Male		37 (55)
Race	53	
African American		2 (3.8)
Caucasian		51 (96.2)
Tumor grade	64	
1		13 (20)
2		32 (50)
3		19 (30)
Tumor stage	66	
I		8 (12.1)
IIA		23 (34.9)
IIB		31 (47)
III		2 (3)
IV		2 (3)
Operation type	67	
Whipple		58 (86.6)
Distal pancreatectomy		4 (6)
Total pancreatectomy		2 (3)
En bloc resection		2 (3)
Non-definitive		1 (1.5)
Surgical margin status	66	
Negative		51 (77)
Positive		15 (23)
Adjuvant chemotherapy	61	
No		29 (48)
Yes		32 (52)
Adjuvant radiation therapy	61	
No		34 (56)
Yes		27 (44)
BRCA1 expression intensity	67	
Low		30 (45)
High		37 (55)
BRCA1 subcellular location	67	

	N*	No. (%)
Percentage cytosolic ^a		10 (4.8 – 22.5)
Percentage nuclear ^a		35 (25 – 45)
Percentage nuclear-cytosolic ^a		25 (19.2 – 30.4)

^a median (IQR)

* For N<67, data was missing for some patients.

Table 2
Univariate analysis of BRCA1 expression intensity

	Low Intensity, No.(%)	High Intensity, No.(%)	p-value
Tumor grade			0.425 ^a
1	7 (54%)	6 (46%)	
2	15 (47%)	17 (53%)	
3	6 (32%)	13 (68%)	
Tumor stage			0.027 ^{a*}
I-IIA	9 (29%)	22 (71%)	
IIB-IV	20 (57%)	15 (43%)	
Adjuvant chemotherapy			1 ^a
No	13 (45%)	16 (55%)	
Yes	15 (47%)	17 (53%)	
Lymph node ratio ^l	0.13 (0 - 0.3)	0 (0 - 0.1)	0.092 ^b
	Hazards Ratio (95% CI)		p-value
Recurrence-free survival	0.98 (0.57 - 1.68)	Ref	0.939 ^c
Overall survival	0.85 (0.47 - 1.53)	Ref	0.577 ^c

^a Fisher's Exact Test

^b Wilcoxon Rank Sum Test

^c Likelihood Ratio Test

^l Median (IQR)

* Statistically significant

Table 3

Univariate analysis of BRCA1 location

	Cytosolic (%)		Nuclear (%)		Nuclear-cytosolic (%)	
	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
Tumor grade		0.63 ^a		0.6 ^a		0.071 ^a
1	12.5 (7.5 – 22.9)		29 (25 - 38)		21.1 (17.1 - 30)	
2	10.5 (4.7 – 23.2)		33 (27 - 46)		26.7 (21.8 - 32)	
3	10 (5.1 – 16.1)		41 (26 - 46)		23.3 (16.1 – 28.6)	
Tumor stage		0.006 ^{b*}		0.28 ^b		0.75 ^b
I-IIA	6(3.5 – 16.8)		37 (28 - 46)		25 (19.6 – 30.4)	
IIB-IV	15.9 (7.9 – 24.1)		32 (24 - 45)		25 (19.2 – 30.6)	
Adjuvant chemotherapy		0.68 ^b		0.73 ^b		0.13 ^b
No	11 (6 – 17.9)		37 (25 - 46)		25 (22 – 30.3)	
Yes	9.1 (4.3 – 21.6)		34 (28 - 45)		22.9 (16.6 – 30.7)	
	Hazards ratio ¹ (95% CI)	p-value ^c	Hazards ratio ² (95% CI)	p-value ^c	Hazards ratio ³ (95% CI)	p-value ^c
Recurrence-free survival	0.81 (0.51 – 1.28)	0.362	1.15 (0.77 – 1.74)	0.495	1.41 (0.98 – 2.04)	0.059
Overall survival	0.84 (0.51 – 1.37)	0.475	1.16 (0.73 – 1.84)	0.527	1.29 (0.90 – 1.85)	0.158

^aKruskal-Wallis Test

^bWilcoxon Rank Sum Test

^cLikelihood Ratio Test

¹22.5% vs. 4.75% (upper quartile vs. lower quartile)

²30.4% vs. 19.3% (upper quartile vs. lower quartile)

³45.1% vs. 25.3% (upper quartile vs. lower quartile)

* Statistically significant

Table 4
Multivariable Cox proportional hazard analyses for OS and RFS

Variables	Overall Survival		Recurrence Free Survival	
	HR (95%CI)	P value	HR (95%CI)	P value
BRCA1 Intensity (Low vs. High)	0.70 (0.38 – 1.30)	0.264	0.76 (0.44 – 1.34)	0.344
Nuclear Cytosolic (30.45 vs. 19.25%) ^I	0.92 (0.62 – 1.37)	0.689	1.10 (0.74 – 1.62)	0.646
Lymph node ratio (24.63 vs. 0%) ^I	3.55 (1.79 – 7.05)	<0.001	3.84 (1.96 – 7.52)	<0.001
Adjuvant chemotherapy (No vs. Yes)	2.00 (1.07 – 3.72)	0.029	1.79 (1.02 – 3.14)	0.041

^I upper quartile vs. lower quartile