Down-Regulation of BRCA1 in Chronic Pancreatitis and Sporadic Pancreatic Adenocarcinoma

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INTRODUCTION

Purpose: BRCA1 and BRCA2 are considered to be breast cancer susceptibility genes that may also contribute to pancreatic cancer development because family studies revealed mutation carriers to have an increased risk of developing pancreatic cancer. However, as demonstrated for breast and ovarian cancer, inactivation of BRCA in sporadic diseases is based on alteration in gene expression or functional alteration.

Experimental Design: To study a potential correlation of BRCA1 and BRCA2 to chronic pancreatitis and development of sporadic pancreatic adenocarcinoma, we have analyzed the expression of these genes by quantitative PCR and performed immunohistochemical analyses in normal pancreatic tissues, chronic pancreatitis, and pancreatic cancer specimens.

Results: BRCA1 expression was down-regulated in chronic alcoholic pancreatitis, in particular on the RNA level. Furthermore, our data indicate suppressed BRCA1 expression in pancreatic cancer on both the RNA and protein levels. Quantitative analysis of BRCA1 protein expression demonstrated regular staining in 50% of tumor specimens tested and reduced staining in 50% of tumor specimens tested. Correlation with the clinical outcome revealed a significantly better 1-year overall survival for patients with BRCA1-regular as compared with BRCA1-reduced or BRCA1-absent tumors. In contrast, no substantial differences in BRCA2 expression were found in chronic pancreatitis and pancreatic cancer samples.

Conclusions: Our data demonstrate alteration of BRCA1 expression in chronic pancreatitis and sporadic pancreatic adenocarcinoma. We, for the first time, provide evidence for a role of BRCA1 in pancreatic carcinogenesis of noninherited tumors and for clinical outcome.

References

1. Beger C. BRCA1 and BRCA2 are cancer susceptibility genes that have been associated with breast and ovarian cancer (1, 2). Although no BRCA1 mutations can be detected in sporadic breast or ovarian cancers, down-regulation of BRCA1 expression has been attributed to involvement in pathogenesis of the majority of noninherited breast and ovarian cancers (3–5). In contrast, BRCA2 expression has been found diverse in sporadic breast and ovarian cancers: some cases overexpressed BRCA2; whereas others showed loss of BRCA2 expression (6, 7). As hypothesized for BRCA1, inactivation of BRCA2 due to its altered expression or functional inactivation is thought to also contribute to cancer development of sporadic tumors (8).

BRCA protein function has been specifically attributed to cellular regulation of DNA damage repair and gene transcription events (9). Recent data suggest that BRCA1 serves as a scaffold in a large protein ensemble that functions in multiple biological processes including (a) DNA double-strand break repair, (b) DNA mismatch repair, (c) gene transcription, (d) transcription-coupled DNA damage repair, and (e) chromatin remodeling (10, 11). Similar functions have been described for BRCA2, although regulation pathways and interacting proteins may differ between both genes (9).

Besides breast and ovarian cancer, mutations in BRCA1 and BRCA2 have been identified to correlate with an increased risk for some other cancer types such as prostate cancer (BRCA1 and BRCA2), pancreatic cancer (BRCA1 and BRCA2), cancer of the uterus (BRCA1), gallbladder and bile duct cancer (BRCA2), stomach cancer (BRCA2), and malignant melanoma (BRCA2 (10, 11)). Therefore, it may be of particular interest to analyze whether BRCA1 and BRCA2 inactivation may also contribute to tumor development of sporadic forms in cancer types other than breast and ovarian cancer. However, to date, only one study has been undertaken to analyze the expression levels of BRCA1 in lung and colon cancer samples in comparison with corresponding normal control tissues using semiquantitative reverse transcription (RT)-PCR (12). The analysis of pancreatic cancer is of particular interest because family studies have demonstrated that both BRCA1 and BRCA2 mutation carriers have an increased risk of developing pancreatic cancer (10, 11, 13–15).

In an attempt to test whether BRCA1 and BRCA2 may be important for development of noninherited forms of pancreatic adenocarcinoma, we have analyzed their expressions by quantitative real-time RT-PCR and immunohistochemical analyses in pancreatic cancer specimens as compared with normal pancreatic controls and chronic pancreatitis samples. Our data sug-
gest that expression of BRCA1 is down-regulated in pancreatic cancer on both the RNA and protein levels. Furthermore, patients with down-regulation of BRCA1 protein expression in pancreatic cancer cells had a significantly poorer outcome than patients with BRCA1-positive cancers. Interestingly, BRCA1 expression was significantly down-regulated in chronic pancreatitis. In contrast, we did not observe significant changes in BRCA2 protein expression in chronic pancreatitis or pancreatic cancer samples. BRCA2 RNA expression was slightly up-regulated in pancreatic cancer as compared with normal pancreatic tissue or chronic pancreatitis specimens.

MATERIALS AND METHODS

Tissue Specimens. Pancreatic tissue specimens were obtained from 53 patients with pancreatic adenocarcinoma and 30 patients suffering from chronic pancreatitis who underwent resection at the Department of General Surgery, University of Ulm (Ulm, Germany). Tissues were collected after obtaining informed consent and according to the guidelines of the local ethic committee. The group of pancreatic cancer patients included 22 female and 31 male patients. The median age was 64.2 years (range, 42.2–82.9 years). The group of chronic pancreatitis patients included 4 female and 26 male patients, with a median age of 51.3 years (range, 35.3–69.7 years). Thirteen normal pancreatic tissue samples served as controls. Tissues were collected after surgical removal, rinsed in normal saline solution, snap-frozen immediately in liquid nitrogen, and stored at −80°C, or they were fixed in 4% formalin for 1 day at room temperature, processed, and embedded in paraffin. Immunohistochemistry was performed on 50 pancreatic cancer tissues, 28 specimens from patients suffering from chronic pancreatitis, and 10 normal control samples. RNA expression analysis was performed on 16 pancreatic cancer tissues, 13 samples from...
patients with chronic pancreatitis, and 6 (BRCA1) or 3 (BRCA2) normal pancreatic tissues.

RNA Preparation and Quantitative RT-PCR. For quantitative real-time RT-PCR analysis, the TaqMan technology (7700 Sequence Detector; Applied Biosystems, Foster City, CA) was applied according to the manufacturer’s instructions using the standard curve method. Primers (MWG Biotech, Ebersberg, Germany) and probes (Eurogentec Inc., Seraing, Belgium) were chosen with the help of Primer Express Software (Applied Biosystems); sequences and cycling conditions are available on request. Probes were labeled with 6-carboxyfluorescein (5’/H11032-FAM; reporter) and 6-carboxytetramethylrhodamine (3’/H11032-TAMRA; quencher). Total RNA was extracted from shredded frozen tissue samples using RNeasy kit (Qiagen, Hilden, Germany). RNAs were quantified photometrically, and input amounts were optimized for each amplicon, resulting in threshold values between 20 and 35 cycles. Before cDNA synthesis, RNA samples were DNase-digested to avoid potential contamination with genomic DNA. cDNA was prepared by reverse transcription of total RNA using random hexamer oligonucleotides (MWG Biotech) and Superscript II (Life Technologies, Inc., Karlsruhe, Germany). After reverse transcription, cDNA was diluted with distilled water (1:4 to 1:100), and real-time PCR was performed using TaqMan PCR Core Reagents (Eurogentec Inc.) according to the manufacturer’s instructions. Each sample was analyzed in at least two independent assays with duplicate samples. Mean values of the gene of interest were normalized to GAPDH levels.

Immunohistochemistry. Paraffin-embedded tissues were cut in 5-µm-thick sections and adhered to silanized slides, deparaffinized, and hydrated by passing through xylene (3 × 5 min), a graded series of isopropanol (1 × 100%, 80%, 70%, and 50%; 5 min each), and distilled water (10 min). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (30 min) between the first two steps of washing with isopropanol. After each of the following steps, sections were washed with PBS (pH 7.45) three times for 10 min, respectively. Tissue sections were covered with 2% normal rabbit (BRCA1) or swine (BRCA2) serum (DAKO, Glostrup, Denmark) in PBS for 60 min, followed by incubation overnight with the primary antibodies detecting BRCA1 (mouse IgG1, clone MS13, diluted 1:20 in PBS; DPC Biemann, Bad Nauheim, Germany) or BRCA2 (clone Ab-2, rabbit polyclonal antibody, diluted 1:50 in PBS; Dianova, Hamburg, Germany). These antibodies have been demonstrated to specifically detect the corresponding proteins (4, 16). For each case, a corresponding section was incubated in PBS without the primary antibody as a control for nonspecific staining. Antibody specificity was further validated by staining sections with nonimmune isotype serum. The dilution of the antibodies was optimized using pancreatic sections. To ensure comparability, all samples were stained in parallel for
BRCA1 or BRCA2, respectively. Biotinylated rabbit antimouse secondary antibody (BRCA1) or biotinylated swine antirabbit secondary antibody (BRCA2) was added for 45 min, followed by the avidin-biotinylated peroxidase complex for an additional 45 min. After washing with PBS for 10 min, staining was achieved by using 3,3'-diaminobenzidine. The sections were counterstained with Mayer's hemalaun and mounted.

**Semiquantitative Analysis of Immunohistochemical Findings.** For grading of immunohistochemical findings, the percentage of positively stained nuclei of acinar and ductal cells in at least 10 high-power fields was counted. Stromal cells that showed positive immunoreaction in all tissue types (normal pancreas, chronic pancreatitis, and carcinomas) were excluded. This semiquantitative grading was used to distinguish tissues with a high number of positively stained cells from tissues with a low number of positively stained cells or undetectable staining. For the correlation with clinical and pathological data, the specimens were scored as positive with a labeling index (LI) of \( \geq 50\% \) (\( \geq 50\% \) of the nuclei stained positively) and scored as reduced or absent in case of a LI of \(< 50\% \). All sections were graded independently by two experienced investigators without knowledge about patients’ clinicopathological data and outcome.

**Statistical Analysis.** Significance was defined as \( P < 0.05 \). Student's \( t \) test, Mann-Whitney \( U \) test, Kaplan-Meier estimates, and \( \chi^2 \) test were computed using the MedCalc or Prism software packages.

**RESULTS**

**Reduced Protein Expression of BRCA1 in Pancreatic Adenocarcinoma.** Positive immunohistochemical staining of BRCA1 could be detected in all normal pancreatic tissue samples analyzed (Fig. 1A). This staining was characterized by a clear nuclear immunoreactivity in \( \geq 50\% \) of the cells (acinar cells as well as cells of ductal origin). In tissue specimens from
patients suffering from chronic pancreatitis, the LI was found to be ≥50% in all sections examined (Fig. 1B). In clear contrast, only 25 of 50 (50%) pancreatic cancer samples reached the cutoff of 50% positively stained nuclei (Fig. 1C). In the other 25 cancer sections, only <50% of all nuclei showed an immunoreactivity using the anti-BRCA1 antibody, with some cancers showing almost no cells positive for BRCA1 (Fig. 1D). Direct comparison of the LIs of the three groups revealed a highly significant reduction of BRCA1 immunostaining in pancreatic cancer when compared with normal pancreatic or chronic pancreatitis tissues (Fig. 2). Furthermore, we found a significant down-regulation of the overall LIs of chronic pancreatitis tissue samples (n = 28) in comparison with normal control samples (Fig. 2).

**Reduced RNA Expression of BRCA1 in Chronic Pancreatitis and Pancreatic Adenocarcinoma.** In parallel, expression analysis of BRCA1 mRNA was performed using quantitative real-time RT-PCR on the following samples: 6 normal pancreatic specimens; and 13 or 16 samples from patients suffering from chronic pancreatitis or pancreatic cancer, respectively. Quantitative RT-PCR using primers that detect all BRCA1 splice variants revealed a significant reduction of BRCA1 RNA expression in both chronic pancreatitis and pancreatic cancer when compared with normal samples (Fig. 3A).

Because immunohistochemical analysis particularly demonstrated reduction of nuclear staining in pancreatic cancer tissues, we analyzed expression of the BRCA1 splice variant Δ11b, which lacks nuclear localization signals and is known to be expressed predominantly in the cytoplasm (12, 17). Our data suggest an overall reduction of BRCA1-Δ11 in pancreatic cancer samples, which parallels expression level of total BRCA1 mRNA (data not shown). However, on analyzing the ratio of total BRCA1/BRCA1-Δ11, we found a slight increase of the total BRCA1/BRCA1-Δ11 ratio in pancreatic adenocarcinoma, whereas no clear change in the ratio was detectable in chronic pancreatitis when compared with normal tissue controls (Fig. 3B).

**No Reduction of BRCA2 Protein Expression in Pancreatic Adenocarcinoma.** As already shown for BRCA1, immunohistochemical detection of BRCA2 proteins demonstrated positive staining in ≥50% of all pancreatic cells (again, acinar and ductal cells) of normal pancreatic tissue samples (Fig. 4A). Furthermore, all chronic pancreatitis samples showed a clear nuclear immunoreactivity in ≥50% of the cells (Fig. 4B). Forty-two of the 50 adenocarcinomas (84%) scored positive for BRCA2, with a LI of ≥50% stained nuclei (Fig. 4C), and only 8 of 50 carcinomas (16%) showed a LI of <50% (Fig. 4D). However, the overall comparison of LIs of the three groups revealed a marked down-regulation of BRCA2 immunostaining in pancreatic cancer when compared with normal pancreatic or chronic pancreatitis tissues (Fig. 5).

**No Reduction of BRCA2 RNA Expression in Pancreatic Adenocarcinoma.** Again, we paralleled our immunohistochemical analysis by quantitative determination of BRCA2 mRNA in specimens. As already suggested by immunohistochemical data, no reduction in expression was observed during cancer development (Fig. 6). In contrast, expression was slightly increased in pancreatic cancer when compared with tissue samples from normal control or chronic pancreatitis.

**No Coexpression Pattern of BRCA1 and BRCA2 in Pancreatic Adenocarcinoma.** Because 42 of 50 adenocarcinomas stained positive with the antiserum directed against human BRCA2, but only 25 of 50 were positive for BRCA1 immunostaining, we found no statistically significant correlation between both proteins (data not shown). **Vice versa,** of the 42 carcinomas categorized as positive for BRCA2, half (21 samples) stained positive, and half (21 samples) showed reduced or undetectable immunoreactivity for BRCA1. In the case of the eight carcinomas with reduced BRCA2 expression, four were...
negative for BRCA1, and 4 were positive for BRCA1 ($\chi^2$ test, $P = 0.70$).

Correlation of BRCA1 and BRCA2 Immunohistochemistry Results with Clinicopathological Features. To elucidate the clinical importance of the immunohistochemical findings, we correlated the immunohistochemical analyses with parameters of the Union Internationale Contre le Cancer classification (tumor extent, lymph node metastases, and distant metastases), tumor grading, sex, or age. No correlation was found for any of the parameters listed above when compared with BRCA1 and BRCA2 LIs, respectively (Table 1). However, on analysis of the 1-year overall survival rate in the different groups, a significant difference was found between pancreatic carcinoma patients categorized as BRCA1 protein positive and patients whose tumors showed reduced or undetectable expression of BRCA1 protein (Table 1; Fig. 7). Of the patients with tumors showing a BRCA1 LI of $\geq 50\%$ (defined as BRCA1 positive), 76% were alive after 1 year, whereas among the patients with a BRCA1 LI of $< 50\%$ (defined as BRCA1 negative), only 40% survived at least 1 year ($\chi^2$ test, $P = 0.02$). In contrast, no differences in 1-year overall survival were found between patients with BRCA2-positive and BRCA2-reduced or BRCA2-absent tumors (Table 1; Fig. 7).

**DISCUSSION**

Pancreatic cancer is the human cancer of the gastrointestinal tract with the poorest prognosis, and most patients will die within 1 year after diagnosis (18). The identification of molecular mechanisms associated with pancreatic carcinogenesis is of major importance for understanding the fundamental nature of pancreatic cancer. Recent data suggest that mutation carriers of the breast cancer susceptibility genes *BRCA1* and *BRCA2* not only develop breast and ovarian cancer but also have an increased risk of developing pancreatic cancer (10, 11, 13–15). However, inactivation of BRCA function in sporadic breast and ovarian cancers is not based on mutations, rather these tumors reveal an alteration in gene expression or functional inactivation (3–7). To study a potential correlation of BRCA1 and BRCA2 in chronic pancreatitis and sporadic pancreatic cancer, BRCA1 and BRCA2 expression analyses were performed in samples from patients suffering from chronic pancreatitis and sporadic pancreatic cancer and compared with normal tissue specimens.

Our data clearly indicate down-regulation of BRCA1 expression on both the RNA and protein levels in sporadic pancreatic adenocarcinoma when compared with normal pancreatic tissue controls. To our knowledge, this is the first study demonstrating a correlation between cancer development and BRCA1 expression status in sporadic pancreatic cancer. Interestingly, BRCA1 expression was also found to be down-regulated in chronic pancreatitis samples, in particular on the RNA level. Because chronic pancreatitis is considered a precancerous lesion on an epidemiological (19, 20) as well as a molecular biological (21) level, *BRCA1* RNA down-regulation may be an initial step during carcinogenesis. Furthermore, because down-regulation of BRCA1 expression in chronic pancreatitis samples was detectable mainly on the RNA level, whereas BRCA1 protein expression remained unaltered, these cells may have preserved their capacity to compensate for altered BRCA1 expression on protein level. Later, during carcinogenesis, these pathways may be disrupted, as indicated by decreased RNA and protein expression found in cancer specimens. There are multiple reasons, such as transcriptional or posttranscriptional regulation, reduced protein stability, or increased degradation, that could account for the reduced BRCA1 protein levels observed in 50% of the pancreatic cancer specimens. However, because we also found reduced *BRCA1* RNA levels in pancreatic cancer,
BRCA1 and BRCA2 in Pancreatic Cancer

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rived from pancreatic cancer or chronic pancreatitis. Analyzing significant down-regulation of expression in tissue samples de-
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drawn from clinical data because down-regulation of BRCA1
mechanisms may contribute in a subset of samples with unde-
for the reduced protein levels observed, although additional
transcriptional regulation might be at least one potential cause
for the reduced protein levels observed, although additional
mechanisms may contribute in a subset of samples with unde-
tectable BRCA1 protein expression.

Further evidence of clinical importance of BRCA1 can be
drawn from clinical data because down-regulation of BRCA1
expression was associated with a poorer prognosis among pa-
tients suffering from pancreatic cancer. Surprisingly, after sur-
gery, 1-year overall survival was 76% in patients with BRCA1-
positive cancers as compared with 40% in patients with
BRCA1-negative cancers. This observation might be explained
by a more aggressive and uncontrolled growth of tumor cells in
BRCA1-reduced or BRCA1-absent cancers due to loss of
BRCA1-dependent regulation of DNA repair (9). These data
will be confirmed on a larger number of patients allowing a multivariate analysis.

Studying expression of BRCA2, we did not observe a
significant down-regulation of expression in tissue samples de-
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chronic pancreatitis, a precancerous lesion for cancer of the pancreas.

Future studies will have to analyze the chromosomal structure of the BRCA1 loci in pancreatic cancer samples, i.e., detect mutations and loss of heterozygosity to gain insight into the mechanism of BRCA1 inactivation in sporadic pancreatic cancer development. Furthermore, the biological importance of BRCA1 inactivation should be studied in detail using pancreatic cancer cell culture or animal models. Finally, it would be of major interest to perform expression analyses on a larger group of patients to evaluate the potential diagnostic value of BRCA1 alterations in chronic pancreatitis and pancreatic cancer.

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