BRCA1 Loss Preexisting in Small Subpopulations of Prostate Cancer Is Associated with Advanced Disease and Metastatic Spread to Lymph Nodes and Peripheral Blood

Natalia Bednarz1,3, Elke Eltze4, Axel Semjonow5, Michael Rink2, Antje Andreas1, Lennart Mulder6, Juliane Hannemann1, Margit Fisch2, Klaus Pantel1, Heinz-Ulrich G. Weier7, Krzysztof P. Bielawski3, and Burkhard Brandt1

Abstract

Purpose: A preliminary study performed on a small cohort of multifocal prostate cancer (PCa) detected BRCA1 allelic imbalances among circulating tumor cells (CTC). The present analysis was aimed to elucidate the biological and clinical roles of BRCA1 losses in metastatic spread and tumor progression in PCa patients.

Experimental Design: To map molecular progression in PCa outgrowth, we used fluorescence in situ hybridization analysis of primary tumors and lymph node sections, and CTCs from peripheral blood.

Results: We found that 14% of 133 tested patients carried monoallelic BRCA1 loss in at least one tumor focus. Extended molecular analysis of chr17q revealed that this aberration was often a part of larger cytogenetic rearrangement involving chr17q21 accompanied by allelic imbalance of the tumor suppressor gene PTEN and lack of BRCA1 promoter methylation. The BRCA1 losses correlated with advanced T stage (P < 0.05), invasion to pelvic lymph nodes (P < 0.05), as well as biochemical recurrence (P < 0.01). Their prevalence was twice as high within 62 lymph node metastases (LNM) as in primary tumors (27%, P < 0.01). The analysis of 11 matched primary PCa-LNM pairs confirmed the suspected transmission of genetic abnormalities between these two sites. In four of seven patients with metastatic disease, BRCA1 losses appeared in a minute fraction of cytokeratin- and vimentin-positive CTCs.

Conclusions: Small subpopulations of PCa cells bearing BRCA1 losses might be one confounding factor initiating tumor dissemination and might provide an early indicator of shortened disease-free survival.

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of CTCs in the patients’ peripheral blood as well as early biochemical recurrence of PCa and hence might account for metastasis formation.

The BRCA1 protein acts as a prominent tumor suppressor and, due to its “canonical” but yet largely unknown functions in cell cycle regulation and homologous recombination, is a key player in cellular control systems (4). The clinical relevance of BRCA1 alterations is well documented in both hereditary and sporadic breast and ovarian cancers (5). However, BRCA1 deficiency or AIs were also observed in some other types of tumors (6–8) and corresponded to a worsened survival (6, 7). The germline BRCA1 mutations were already reported to indicate a higher risk of PCa and its more aggressive phenotype (9–11). BRCA1 AIs were also detected in approximately 13% to 46% of tested cases of sporadic PCa (12–15). They coincided frequently with the AIs of other loci on chr17q21 and, to the best of our knowledge, only in one study were associated with the higher PCa stage (14). These AI analyses are conducted on a very low number of cases (23–25 samples) and did not exclude the possibility that alterations of another tumor suppressor gene located on 17q21 can play a pivotal role in prostate tumorigenesis. Therefore, the aim of this study was to investigate whether distinct BRCA1 alterations or larger rearrangements of chr17q12-21 may serve as a potential signature of progression in sporadic PCa. The BRCA1 gene locus, a gene-rich region located on 17q21 proximal of the BRCA1 gene (17q GRR), and ERBB-2/human epidermal growth factor receptor 2 (HER-2) were analyzed in primary tumors, lymph nodes, and CTCs and compared in clinical staging, grading, and outcome.

Materials and Methods

Patients

The patients with sporadic monofocal and multifocal PCa were treated by radical prostatectomy in the Department of Urology of University of Muenster during 1998-2000. They did not receive any hormone therapy and did not manifest any metastasis at the time point of diagnosis and surgery. The tumors were characterized by different clinical parameters (Table 1). Their pathologic stages ranged from T2N0M0 to T3N1M0 (TNM system UICC 2002) and the final Gleason sums from 2 to 10. The mean patient age at the moment of surgery was 64 years (range, 47-78 years). The concentration of serum prostate-specific antigen was measured after prostatectomy twice every year and occurrence of biochemical recurrence (PSA > 0.1 ng/ml followed by two increasing PSA concentration) was defined as the end point of disease-free survival. The follow-up was available for 186 patients with a median follow-up of 34 months (range, 1-114). Additionally, seven patients with metastatic PCa treated at the Department of Urology, University Medical Centre, Hamburg-Eppendorf, Germany, were selected for CTC analysis. Their short clinical description is given in Fig. 3B.

Clinical samples

Two tissue microarrays (TMA) containing 398 primary PCa and 196 LNM tissue cores (TC), corresponding to 199 and 98 patients, respectively, were prepared as described (16). Fifty-three (13%) TCs contained normal prostate tissue, 337 (85%) tumor TCs belonged to 191 (96%) patients, and 8 TCs were missing pathologic characteristics. All specimens were evaluated by a pathologist (E. Eltze) in the Gerhard-Domagk Institute of Pathology, University Clinic Münster, Germany. The patients with multifocal PCa were represented on the TMA by TCs originated from two different tumor foci. From this cohort, 31 cases of primary tumors were selected for additional AI analysis and 11 paired PCa-LNM formalin-fixed, paraffin-embedded tissues were chosen for both fluorescence in situ hybridization (FISH) and AI analyses.

Table 1. Clinical parameters of PCa patients

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Status</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>113 (57)</td>
<td></td>
</tr>
<tr>
<td>≥65</td>
<td>86 (43)</td>
<td></td>
</tr>
<tr>
<td>Focality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mono</td>
<td>39 (20)</td>
<td></td>
</tr>
<tr>
<td>Multi</td>
<td>160 (80)</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>18 (9)</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>12 (6)</td>
<td></td>
</tr>
<tr>
<td>2c</td>
<td>30 (15)</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>99 (50)</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>39 (20)</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>171 (86)</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>22 (11)</td>
<td></td>
</tr>
<tr>
<td>Gleason</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;7</td>
<td>76 (38)</td>
<td></td>
</tr>
<tr>
<td>≥7</td>
<td>118 (59)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Not all parameters are available for all 199 patients. n, number of cases.
At the Department of Urology, University Medical Centre, Hamburg-Eppendorf, Germany, 20 mL of peripheral blood from seven unrelated patients with metastatic PCa and three healthy volunteers were collected into EDTA tubes and processed for CTC enrichment within 3 hours after phlebotomy as described (17).

**Fluorescence in situ hybridization**

FISH probes were prepared from DNA isolated from BAC clones RP11-242D8 and RP11-506G7 using a Large DNA Construct Isolation Kit (Qiagen) and the BioPrime Total Genomic Labeling System (Gibco Invitrogen) according to the manufacturers’ protocols. The deparaffinized 4-μm-thick TMA or formalin-fixed, paraffin-embedded sections were fixed in 2% formaldehyde in methanol for 10 minutes at −20°C and pretreated in Spot-Light Tissue Heat Pretreatment Buffer (Zymed) for 10 minutes at 97°C. The cells were digested for 10 minutes in 100 μL of enzyme buffer (Zymed) at 37°C and subsequently dehydrated in a graded alcohol series. One microliter of COT1 Human DNA (Roche), 1 μL of CEP17 Spectrum Aqua (Abbott Molecular) as a reference probe, and 4 μL of Spectrum Orange–labeled probe (Abbott Molecular) for a target gene suspended in 4 μL of hybridization buffer (20% dextran sulfate/50% formamide/4× SSC buffer) were applied on the top of specimens. For ERBB-2/HER-2 detection, 10 μL of commercial ready-to-use probe (PathVysionTM Her-2 DNA Probe Kit, Abbott Molecular) were used. The specimens were denatured for 3 minutes at 95°C and hybridized for at least 16 hours at 37°C. Afterward, they were washed for 2 minutes each in 2× SSC/NP40 buffer at 72°C and at 21°C. The specimens were dehydrated in an alcohol series, counterstained with Vectashield Mounting Medium (Vector Laboratories, Inc.) containing 0.5 μg/mL 4′,6-diamidino-2-phenylindole, and analyzed under a fluorescence microscope (Zeiss). For each TC, target and reference probe signals were counted in at least 20 cells showing a minimum of two signals for the reference probe. The gene dosage, calculated as ratio of the number of target probe signal over reference probe signals, was determined according to experimental cutoffs established on 20 normal prostate tissues for every probe combination.

**FISH on CTCs**

Isolated CTCs on slides were fixed in ice-cold 75% ethanol for 2 minutes and then pretreated with 100 μg/mL RNase A for 40 minutes at 21°C before being immersed in 1× citrate buffer (pH 6.0; Dako) for 3 minutes at 120°C. The cells were postfixed in 1% formaldehyde in 1× PBS for 10 minutes and dehydrated in an alcohol series. Air-dried cells were denatured in denaturation buffer.
(70% formamide, 0.6× SSC, pH 7.4) for 5 minutes at 73°C and dehydrated in alcohol. Hybridization was conducted as described above with minor modifications (2 μL of Spectrum Orange–labeled RP11-242D8 probe, 0.5 μL of centromer 17 (CEP17), 6.5 μL of hybridization buffer; Abbott Molecular). The next day, the slides were washed three times for 10 minutes in 50% formamide/2× SSC buffer, two times for 10 minutes in 2× SSC buffer, and once for 5 minutes in 0.1× SSC/0.1% Tween 20 buffer at 46°C. The cells were permeabilized in three changes of 1× TBS/1% Tween 20 (TBST) for 3 minutes each. The unspecific binding was reduced by a 20-minute incubation in Blocking Serum (Dako). The primary mouse monoclonal antibodies against pan-cytokeratin (A45, Mikromet) or vimentin (RV202, DB Pharmingen), diluted 1:100 or 1:200, respectively, in DakoREAL Antibody Diluent (Dako), were applied to the cells for 45 minutes at 21°C. The specimens were washed three times for 3 minutes with 1× TBST and incubated with a secondary 1:200 diluted Alexa-488–labeled anti-mouse rabbit antibody (MiBioTech) for 45 minutes at 21°C. Finally, the slides were washed twice for 3 minutes with 1× TBST and once for 3 minutes with 1× PBS and counterstained with Vectashield Mounting Medium containing 4′,6-diamidino-2-phenylindole. Cytokeratin-positive [CK(+) or vimentin-positive [Vim(+)] cells, showing no apoptotic or necrotic morphology, were analyzed for their BRCA1 gene dosage. Additionally, a total of 500 cells per sample were scored for BRCA1 losses and compared with the scores obtained in three healthy volunteers (false hybridization index).

AI analysis
DNA isolation, microsatellite sequence–based PCR, and AI analysis of loci D17S855 (BRCA1) and D10S541 (PTEN) were done as described (3).

Methylation assay
To investigate the BRCA1 promoter methylation status, a multiplex ligation–dependent probe amplification (MLPA) assay, described in ref. 18, was done (Methylation Kit ME45, MRC-Holland) according to the manufacturer’s protocol with minor modifications (initial denaturation for 20 minutes, 40 PCR cycles). Data were normalized by methylation status analysis and further analyzed by the Coffalyser MLPA data analysis software.

Statistics
Kruskal-Wallis and Mann-Whitney tests were done to assess gene dosage associations with clinical outcome. To estimate the hazard risk, Cox proportional hazard regression analysis (95% confidence interval) was done. Disease-free survival curves were generated by Kaplan-Meier test with log-rank univariate analysis.

Results
Experimental strategy
To test the hypothesis that BRCA1 gene aberrations in PCa may be decisive events in primary PCa progression, our retrospective study investigated their frequency in primary PCa, LNM, and CTCs isolated from peripheral blood of metastatic PCa patients. The sample flow is presented in
Table 2. The BRCA1 gene dosage and PTEN AI in 11 PCa-LNM pairs assessed in TMA and formalin-fixed, paraffin-embedded sections (whole sections)

<table>
<thead>
<tr>
<th>No.</th>
<th>PTEN (AI)</th>
<th>TMA (FISH)</th>
<th>WS (FISH)</th>
<th>Final BRCA1 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Focus 1</td>
<td>○</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>2</td>
<td>Focus 2</td>
<td>-</td>
<td>-</td>
<td>L</td>
</tr>
<tr>
<td>3</td>
<td>Metastasis</td>
<td>e</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>4</td>
<td>Focus 1</td>
<td>e</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>5</td>
<td>Focus 2</td>
<td>e</td>
<td>N</td>
<td>L</td>
</tr>
<tr>
<td>6</td>
<td>Metastasis</td>
<td>-</td>
<td>-</td>
<td>L</td>
</tr>
<tr>
<td>7</td>
<td>Focus 1</td>
<td>-</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>8</td>
<td>Focus 2</td>
<td>-</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>9</td>
<td>Metastasis</td>
<td>e</td>
<td>L</td>
<td>N</td>
</tr>
<tr>
<td>10</td>
<td>Focus 1</td>
<td>e</td>
<td>N</td>
<td>L</td>
</tr>
<tr>
<td>11</td>
<td>Focus 2</td>
<td>e</td>
<td>N</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

NOTE: L, loss; N, normal gene copy number; G, gain; AI, allelic imbalance analysis; e, allelic imbalance; ○, normal heterozygote; WS, whole section.

Supplementary Table S1. To assess the specificity, efficiency, and accuracy of the selected FISH probes, 20 normal prostate tissues and different prostate and breast cancer cell lines were tested. The experimental cutoffs for normal gene dosage were calculated as 0.71-1.29, 0.72-1.28, and 0.80-1.20 for BRCA1, 17q GRR, and ERBB-2/HER-2, respectively.

**BRCA1 gene dosage in primary PCa**

Two hundred thirteen TCs from 133 PCa patients were evaluable for FISH analysis of the BRCA1 gene (Fig. 1A and B). BRCA1 losses and gains occurred in 18 (14%) and 5 (7%) patients, respectively. The BRCA1 gene dosage obtained by FISH agreed with BRCA1 AI status assessed in a subgroup of 31 selected tumors ($R^2 = 0.614; P < 0.001$; Supplementary Fig. S1). Eighty-one tested patients (62%) were investigated in two TCs, which enabled the assessment of heterogeneity of BRCA1 gene dosage within the analyzed PCa. Eighty-three percent of the BRCA1 aberrations were observed only in one TC, which suggested the unique character of these alterations confined to the small population of tumor cells.

**Characterization of BRCA1 aberrations and their clinical relevance**

All detected BRCA1 losses were monoallelic and appeared in majority of tumor cells localized in single TC (containing usually 20-1,000 tumor cells). Promoter analysis of BRCA1
showed lack of methylation in all 31 additionally analyzed PCa cases. Two other loci on chromosome 17q (17q GRR and ERBB-2/HER-2) were analyzed to investigate whether BRCA1 alterations are limited to BRCA1 or caused by larger alterations of chromosome 17q. The BRCA1 gains were found restricted to the gene locus. On the contrary, BRCA1 losses coexisted in 71% of the cases with the loss of 17q GRR containing genes already proposed to be involved in tumorigenesis (e.g., BECN1, EZH1, and VPS25; Fig. 1C; refs. 19–21). Losses of the entire region of chromosome 17 encompassing bands q12-21.1 were detected in 3% of all cases. Nevertheless, only BRCA1 alterations correlated significantly with clinical parameters (Supplementary Table S2). BRCA1 losses were associated with more advanced T status (P = 0.014) and higher Gleason score determined in TCs (P = 0.048). They occurred also more frequently in tumors with node-negative status (P = 0.032), whereas BRCA1 gains were observed only in node-negative patients.

**Prognostic value of BRCA1 aberrations**

When compared with the biochemical recurrence data, only BRCA1 losses classified according to the cutoff values ≤0.75 indicated the patients with shorter disease-free survival (P = 0.016; Fig. 2A). In addition, BRCA1 losses seemed to be prognostically significant in multivariate analysis (P = 0.048; Fig. 2B). However, the Cox regression model was done on a relatively small group of patients and needs to be confirmed on a larger cohort.

**BRCA1 aberrations in PCa metastases to pelvic lymph nodes and CTCs**

In LNM, losses were found in 27% of 62 evaluable cases and were significantly increased in comparison with primary tumors (P = 0.007). BRCA1 gains were detected at a frequency similar to primary PCa (8%). The comparison of corresponding pairs of primary tumors and their metastases revealed that 8 of 11 pairs exhibited the matching BRCA1 gene status in LNM and at least one tumor focus (Table 2). In this analysis, TCs were compared as well as the larger sections of tumor and metastasis specimens, which helped to determine the additional foci of tumor cells bearing BRCA1 aberrations within primary tumors. It confirmed the suspected transmission of genetic abnormalities between these two sites, suggesting that somatic BRCA1 mutations can predispose cells to dissemination. As a proof of principle, BRCA1 gene dosage was also assessed in tumor cells separated from peripheral blood of PCa patients suffering from metastasis. The BRCA1 losses were found in CK(+) and, of note, Vim(+) CTCs isolated from peripheral blood (Fig. 3A and B). Applying the strategy in which the frequency of cells with BRCA1 losses was counted in 500 randomly selected cells and compared with the indices of false hybridization obtained in the normal healthy volunteers, significantly elevated number of cells with BRCA1 loss were found in four of seven patients (P < 0.05). Of note is that both vimentin staining and scoring method helped to point out the cells, particularly when the pattern 2 BRCA1 copies:3 CEP17 copies was observed (Fig. 3B). Such a genotype was rarely detectable in the blood of healthy volunteers. The clinically established parameters such as T and N status showed that the cells bearing BRCA1 losses in PCa seem to be efficient to penetrate lymph nodes, possibly survive the stress conditions in bloodstream, and consequently might invade distant organs.
Determination of PTEN gene dosage
Another tumor suppressor gene, PTEN, is reported to be lost in both primary PCa (22) and a majority of prostate CTCs (3), which is also believed to be one of the initiating neoplastic events during prostate tumorigenesis in mice (23). In the current study, PTEN gene dosage was analyzed in a fraction of PCa and LNM and compared with the status of BRCA1 gene. The AI of PTEN paralleled BRCA1 losses and gains, but was also found in cases with normal BRCA1 gene dosage in 31 tested primary PCa and 11 corresponding PCa-LNM pairs (Table 2; Supplementary Table S3). This observation leads to the conclusion that PTEN losses always underlie BRCA1 losses but not vice versa.

Discussion
The high prevalence of PCa in the male population needs accurate diagnostic tools for early designating patients at risk of progression. The so far used prostate-specific antigen screening copes with this challenge only partially (1); hence, the characterization of new progression markers seems to be urgently required. In a previous study including nine loci, it was found that a fraction of CTCs delivered from one even very small focus bears BRCA1 AIs and may be associated with early biochemical recurrence after radical prostatectomy (3). To evaluate the biological and clinical effects of this phenomenon, we performed FISH for BRCA1 on a larger cohort of PCa patients. The present results confirm the BRCA1 aberrations, particularly losses, and define their heterogeneity within different foci of the single tumor. This shows the unique character of the cells carrying BRCA1 alterations, stays in agreement with the previous observations (3), and supports the hypothesis of monoclonal origin of PCa metastases (24).

Unlike in sporadic breast cancer where BRCA1 methylation is a frequent event (25), BRCA1 promoter was not methylated in any analyzed PCa sample, including the ones with the detected BRCA1 loss or gain. It suggests that BRCA1 can be haploinsufficient as it was observed already in breast and ovarian cancers (26, 27).

In more than 70% of cases, a simultaneous loss of BRCA1 and the 17q GRR locus was observed. At this locus, at least three genes can hypothetically amplify the effect of BRCA1 loss: BECN1, involved in autophagy (19); EZH1, responsible for epigenetic processes (20); and VPS25, identified as a potential tumor suppressor in D. melanogaster (21). Nevertheless, only the distinct loss of BRCA1 was found to be related to poor clinical-pathologic and early biochemical recurrence. Therefore, we applied BRCA1 FISH analysis on LNM and CTCs enriched from peripheral blood of metastatic PCa patients. The significantly increased frequency of BRCA1 loss in LNM and its high detection rate in LNM of the corresponding tissue pairs support the hypothesis of a critical role of BRCA1 loss in dissemination of distinct subclones of PCa cells.

The functional pathways of dissemination of PCa cells triggered by BRCA1 loss remain unclear and merit further investigation. The BRCA1 deficiency was already shown to mediate breast cancer cell migration (28, 29) and regulate some epithelial-mesenchymal transition (EMT)–associated proteins (28). It can be speculated that BRCA1 loss induces EMT processes in PCa. Intriguingly, in the current...
series, standard immunofluorescent CK staining applied for CTC detection underestimated the number of CTCs with BRCA1 loss. It could have been caused by the inability of the used antibody to recognize all types of CKs. However, contrary to CK staining, the use of the EMT marker vimentin increased the detection rate of these cells. The application of vimentin might be controversial, but indicated the cells with two copies of BRCA1 in the presence of three copies of CEP17, barely detected in blood of the healthy volunteers. It can supplement CK staining and, hence, potentially extend the spectrum and yield of CTCs. Of note is that increased vimentin expression had been reported in more aggressive types of PCa (30, 31).

BRCA1 aberrations coincided with AI of the coanalyzed PTEN gene, which stays in agreement with the previous observations in CTCs (3). The occurrence of PTEN AI without BRCA1 aberration supports the assumption that PTEN loss occurs as a “first hit” in prostate tumorigenesis, and a subsequent loss of BRCA1 can enhance its effect. It might be hypothesized that PTEN AI and other factors lead to an increased activation of the Akt pathway (32). As one option of explanation, it might result in impairment of BRCA1- and RAD51-driven homologous recombination by retention of these proteins in the cytoplasm (33). One may speculate that defects in homologous recombination generate different chromosomal aberrations, among others a loss of BRCA1, which promotes further tumorigenesis and triggers PCa dissemination (Fig. 4), for example, in an EMT-mimicking way.

Our current results show that even a small population of cells bearing BRCA1 losses can be associated with PCa tumor cell dissemination, potentially indicating those patients that are at high risk of metastasis. Further investigations should focus on the investigation of the clinical relevance of this phenomenon in a large cohort of patients and the definition of BRCA1-related functional proteins involved in this process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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