Promoter Hypermethylation of the PALB2 Susceptibility Gene in Inherited and Sporadic Breast and Ovarian Cancer

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Abstract

The partner and localizer of BRCA2 (PALB2) gene was recently identified as a BRCA2-interacting protein and subsequently shown to be a Fanconi anemia gene (FANCN). Disease-associated point mutations resulting in protein truncation have been found in BRCA1/2 mutation–negative breast cancer families identifying PALB2 as a susceptibility gene for breast cancer. Aberrant promoter hypermethylation is a mechanism of inactivation of many tumor suppressor genes, including BRCA1 and p16INK4a, in breast and ovarian cancer. We therefore investigated the methylation status of a 1512 bp typical CpG island located in the promoter and exon 1 region of the PALB2 gene in 130 sporadic and familial breast and ovarian primary tumors, 9 cell lines, and 10 normal cell specimens. We found two primary breast tumors from BRCA2 mutation carriers, four sporadic primary breast tumors, and four sporadic primary ovarian tumors showed hypermethylation of the core promoter region of PALB2. All 10 normal tissue DNA had an unmethylated PALB2 promoter region. Quantitative real-time reverse transcription–PCR showed PALB2 expression to be reduced 28-fold in primary breast tumor with PALB2 promoter hypermethylation compared with matched normal breast tissue RNA. Aberrant promoter hypermethylation of PALB2 is more frequent than the reported level of PALB2 point mutations in breast tumors from BRCA1/2-negative families and is similar to the frequency of BRCA1 hypermethylation in inherited and sporadic breast and ovarian cancers. [Cancer Res 2008;68(4):998–1002]

Introduction

Alterations in DNA methylation, an epigenetic process present in mammalian cells, are one of the hallmarks of human cancer. It has been shown that the silencing of tumor suppressor genes associated with promoter hypermethylation is a common feature in human cancer, and serves as an alternative mechanism for loss of tumor suppressor gene function (1). For example, the VHL gene is inactivated by hypermethylation in a subset of clear cell renal cancers (2), p16 hypermethylation associated with loss of expression is a common feature of many solid tumor types (3, 4), the mismatch repair gene hMLH1 can also be inactivated by hypermethylation (5) and BRCA1 hypermethylation is found in ~7% to 12% of inherited and sporadic breast and ovarian tumors (6, 7).

The PALB2 gene was recently identified as a protein interacting with BRCA2 (8) and as a Fanconi anemia gene (FANCN; refs. 9, 10). Disease-associated point mutations resulting in functional inactivation of an allele have been found in BRCA1/2 mutation–negative breast cancer families and identified PALB2 as a susceptibility gene for breast cancer (11–13). We therefore analyzed the methylation status of a typical CpG island in the promoter region of PALB2 in a large series of sporadic and inherited breast and ovarian tumors by bisulfite sequencing and quantitative real-time methylation-specific PCR (MSP).

Materials and Methods

Cell lines and tissue specimens. All breast, prostate, and other tumor cell lines were obtained from the American Type Culture Collection with the exception of the ovarian tumor cell lines that were a gift from Dr. T. Hamilton at Fox Chase Cancer Center (Philadelphia, PA). The breast, prostate, and other tumor cell lines were grown as described by the American Type Culture Collection. The ovarian tumor cell lines were grown in RPMI medium supplemented with 10% FCS. Primary breast (37 ductal, 11 lobular, and 12 papillary or other rare histologies) and ovarian (30 papillary serous, 12 clear cell, 7 endometrioid, and 4 mucinous) tumor and normal tissues were microdissected with the assistance of a pathologist (T. Al-Saleem). DNA was extracted using conventional techniques of digestion with proteinase K (Invitrogen) followed by phenol/chloroform extraction.

Bisulfite modification of DNA. Genomic DNA (1 µg) from tumor and normal specimens was denatured by NaOH (0.2 mol/L) for 10 min at 37°C and then modified by hydroquinone and sodium bisulfite treatment at 50°C for 17 h under a mineral oil layer. Modified DNA was purified using the Wizard DNA Clean-Up system (Promega). Modification was completed by bisulfite sequencing and quantitative real-time methylation-specific PCR (MSP).

PALB2 promoter CpG island analysis. The promoter sequence data were obtained from Ensembl Gene ID:00000083093. We selected the CpG island located between nucleotide position −1164 upstream and +348 bases downstream from the ATG codon. This region fulfilled the original CpG island definition criteria of Gardiner-Garden and Frommer (14) and the modifications suggested by Takai and Jones (13). No Alu repetitive elements were detected by REPEAT-MASKER mail server (University of Washington Genome Center, Seattle, WA).4

Bisulfite sequencing of the promoter CpG island. The 1512 bp CpG island was PCR-amplified in six overlapping fragments. PCR products were run on a 1.5% agarose gel and the gel slice purified by Qiaquick (Qiagen).
Direct sequencing was done on an ABI 3100A capillary genetic analyzer and data analyzed by Sequencer Version 4.2.2 software. Primer sequences used for PCR amplification and sequencing were as follows: fragment 1, sense 5'-TATTAGATTTGAGAAAGTAAA-3' (−1195 to −1172 nt); antisense 5'-TTGAGGTAGGAGAATRGTTTGA-3' (−93 to −72 nt); fragment 2, sense 5'-TTAAGAGTTAGGGATTTGTTTGG-3' (−1005 to −982 nt); antisense 5'-TATGAATAGGATGGGATTTGTTT-3' (−695 to −672 nt); fragment 3, sense 5'-TGATATATATAAAAGGTAGTGTAAAAG-3' (−744 to −720 nt); antisense 5'-GTATTTAGATTTGTTGTAGAGTA-3' (−425 to −401 nt); fragment 4, sense 5'-TAGGGTTGGAAAGAGGAGGA-3' (−474 to −454 nt); antisense 5'-TTTAATTGGTGGAGTTTAGGG-3' (−182 to −161 nt); fragment 5, sense 5'-TTTTAGGTGGTTTATTGGGATTTA-3' (−289 to −265 bp); antisense 5'-TTTTTAGTTGTGAGGAGAAGGA-3' (−22 to +44 nt); fragment 6, sense 5'-GAGTTTTTYGGGAAGTTTTTTAG-3' (−8 to +29 nt); antisense 5'-GTTGTATRGATATTTTTGTTTTTTTT-3' (−295 to +322 bp). Y represents degenerate C or T, R represents degenerate G or A base in the primer sequence.

**Quantitative real-time MSP.** Primer sequences to methylated DNA were designed together with an internal TaqMan probe labeled with FAM and MGB. In vitro methylated normal human genomic DNA, confirmed by bisulfite sequencing to show methylation for the gene to be analyzed, was used as a positive control. The concentration of this DNA was determined and a series of dilutions made for the standard curve. Unmethylated sequence of the ACTIN gene was used as a normalizing control. The percentage of methylated alleles was calculated for PALB2 based on the standard curve. An Applied Biosystems 7500 Real-time PCR machine was used for PCR and data analyzed with SDS 1.3.1 software. MSP Primer and probe sequences were as follows for the 3' portion of the CpG island sense 5'-ATTGACCGCGATTGAGG-3' (position 180 to 161 nt); antisense 5'-CCGCTTCGCCGCTGG-3' (position 95 to 80 nt), probe TTAGTTGATCGCGTATTGA (142 to 123 nt); and for the 5' portion of the CpG island sense 5'-AAAAATTTGGTTAGG-3' (position 84 to 69 nt); antisense 5'-TTTTGCTTTTATGCTGAGG-3' (position 906 to 883 nt). Tumor specimens positive for methylation by quantitative real-time MSP were re-amplified with sequencing primer set 5 or the 3' CpG island MSP primer set. The PCR product was run on a 1.5% agarose gel, purified from the gel slice and directly sequenced.

**Quantitative real-time reverse transcription-PCR.** RNA was isolated from 10 × 6 µm paraffin sections of both breast tumor and paired normal breast tissue using a RecoverALL Total Nucleic Acid Isolation kit as per the manufacturer's instructions (Ambion). Reverse transcription was performed using a SuperScript II Reverse Transcriptase kit as per the manufacturer's instructions (Invitrogen). The housekeeping gene GAPDH was used as a control for the template input and amplification efficiency. The primer sequences used for the GAPDH and PALB2 quantitative real-time
reverse transcription-PCR reactions were according to the TaqMan Gene Expression Assay (ABI) for each gene. Relative quantitation using the comparative Ct method was calculated.

Results and Discussion

We identified a 1512 bp typical CpG island according to standard criteria (14, 15) located between nucleotide positions −1164 and +348 relative to the ATG start codon extending 5’ from the promoter region upstream through exon 1 of PALB2. Direct bisulfite sequencing of the entire CpG island in 4 breast tumor cell lines MCF7, MDA231, T47D, and HS-578T; 10 primary breast tumors; 5 ovarian tumor cell lines OVCA3, OVCAR5, OVCAR10, A2780, and SKOV3; 10 primary ovarian tumors; 3 histologically normal breast tissue from age-matched women with no evidence of cancer; and 3 SV40-transformed normal human ovarian surface epithelium showed the PALB2 CpG island to be unmethylated in all cases. Representative bisulfite sequencing of the entire CpG island is shown in Supplementary Fig. S1.

The more sensitive and rapid quantitative real-time MSP assay was used to interrogate a portion of the CpG island (−180 to −40 nt), near the transcriptional start site in the core promoter region of PALB2, in a larger series of breast and ovarian tumors. MSP-based analysis can only interrogate the limited number of CG dinucleotide sites covered in the primer and probe sequences. However, analysis of tumor suppressor genes such as VHL (2), p16INK4a (3, 4), MLH1 (5), and BRCA1 (6) have invariably revealed hypermethylation near the transcriptional start site and across the core promoter region. By quantitative real-time MSP, we found PALB2 promoter hypermethylation in 2 of 8 inherited breast tumor, 4 of 60 sporadic breast tumor, 0 of 4 breast tumor cell line, 0 of 9 inherited ovarian tumor, 4 of 53 sporadic ovarian tumor, and 0 of 5 ovarian tumor cell line DNA. The four normal breast tissue, three normal ovarian surface epithelium, and three normal lymphocyte DNA were unmethylated. In total, 10 of 130 (8%) primary breast or ovarian tumors were positive for PALB2 hypermethylation. We repeated quantitative real-time MSP for these 10 tumor DNA and obtained identical results. The tumor DNA was then bisulfite-sequence to further confirm the methylation status. Dense hypermethylation of all CpG dinucleotides across the PALB2 core promoter region of breast tumor 6 from a BRCA2 mutation carrier is shown (Fig. 1A). RNA was isolated from the same tumor block as well as from normal breast tissue from breast cancer patient 6. Analysis of expression by quantitative real-time reverse transcription-PCR showed PALB2 expression to be 28-fold reduced in the microdissected tumor RNA (Fig. 2). In several of the sporadic tumors, the number of methylated alleles, indicated by quantitative real-time MSP, was <20% of the tumor DNA. These tumor DNA were PCR-amplified with methylated sequence-specific primers and the PCR product directly sequenced. All cases showed clear methylation (Fig. 1B). Although, the percentage of methylated alleles indicated that PALB2 hypermethylation was present in a subclone of the tumor cells, the level of methylation must be an underestimate because even after microdissection, a proportion of normal cells with unmethylated PALB2 alleles will be present in each tumor specimen DNA.

A second set of quantitative real-time MSP primers and probes were designed in order to analyze the methylation status of the upstream region of the CpG island (−946 to −809 nt). All 10 tumor DNA positive for methylation, and 10 tumor DNA negative for methylation, from the core promoter region were analyzed. There was no evidence of methylation in the upstream region of the PALB2 CpG island in the 20 tumor DNA. This finding suggests that the MSP-based analysis of several CpG dinucleotide sites in the core promoter region is a reliable estimate of the frequency of PALB2 hypermethylation in breast and ovarian cancer.

PALB2 promoter hypermethylation seems to be relatively infrequent in this series of breast and ovarian tumors representative of histologic cell type, grade, and stage. However, it is noteworthy that two of eight (25%) inherited breast cancers (breast tumors 5 and 6) showed PALB2 hypermethylation. Both tumors were from BRCA2 mutation carriers. Although the numbers we studied were small, examination of PALB2 methylation status in a larger number of inherited breast and ovarian tumors seems warranted. The clinicopathologic characteristics of the tumors with PALB2 hypermethylation are given in Table 1. It is also interesting that three of the four ovarian tumors with PALB2 hypermethylation are clear cell carcinomas and that the fourth has foci of clear cell carcinoma. Clear cell ovarian tumors have a poor prognosis and a lower response rate to standard chemotherapy compared with the more common papillary serous carcinoma. Three of the four sporadic breast tumors are ductal carcinomas whereas the fourth is a mucinous carcinoma. Hypermethylation of BRCA1 has been reported to be more frequent in the less common mucinous and medullary histologic subtypes of sporadic breast cancer (6). Nine of the 10 tumors positive for PALB2 hypermethylation were high grade. The hormone receptor status of the six breast tumors was estrogen receptor–negative (ER−), progesterone receptor–negative (PR−) in four cases, and ER+,PR− in two cases (Table 1).

Aberrant promoter hypermethylation of tumor suppressor and other cancer genes is clearly associated with loss of expression and thereby inactivation of gene function (1). What might be the potential significance of the frequency and density of hypermethylation we have identified in breast and ovarian cancer? The heterogeneity of genetic and epigenetic mutations in adult

Figure 2. Real-time quantitative reverse transcription-PCR of PALB2. TaqMan gene expression analysis of RNA from breast tumor 6 showed a 28-fold lower level of PALB2 expression compared with the paired normal breast RNA by relative quantification using the comparative Ct method. Bottom, cycle numbers.
epithelial cancers is such (16–18) that alterations as relatively infrequent as PALB2 hypermethylation could still represent an important biological subtype. Dense hypermethylation of PALB2 was present in breast tumor DNA from two BRCA2 mutation carriers, which suggests that an additional growth advantage is conferred to BRCA2-negative tumor cells by aberrant hypermethylation of PALB2 and a putative negative effect on the maintenance of genome integrity (8). In regard to the lower levels (<20% of tumor cells) of methylation observed in some tumors, the percentage of contaminating normal cells in the tumor specimen and that the second allele may be unmethylated in tumor cells could both be contributing factors. It is also possible that PALB2 methylation may be a late event in sporadic breast and ovarian tumorigenesis. The dynamics of clonal selection in tumors means that there will always be tumor cell clones with an additional growth advantage emerging. Within the heterogeneity of populations of cells in an individual tumor are some cells of greater tumorigenicity than other cells, described as cancer stem cells (19, 20). Another possibility is that PALB2 hypermethylation identifies such a subpopulation of cells. Tumors positive for PALB2 hypermethylation did not seem to have a methylator phenotype because we have profiled the methylation status of 10 to 15 other tumor suppressor and cancer genes, the majority of which were methylated when a methylator phenotype was present in SW48, SW480, HT29, and HCT116 colorectal; 786-0, 769P, A498, 1704, and ACHN renal cell; MG118/138 glioma; and H128 lung tumor cell lines (data not shown). Because breast and ovarian tumor cell lines were negative, but 8% of primary breast and ovarian tumors were positive for PALB2 hypermethylation in this study, it is possible that PALB2 hypermethylation is present in other types of primary epithelial tumors.

We conclude that aberrant promoter hypermethylation of PALB2 is relatively infrequent in breast and ovarian cancer yet is more frequent than the level of PALB2 point mutations in breast tumors from BRCA1/2-negative families reported to date (11–13), and is similar to the frequency of BRCA1 hypermethylation in inherited and sporadic breast and ovarian cancer (6, 7). The study of PALB2 methylation status in a larger number of inherited breast and ovarian cancers seems warranted. More studies are needed to determine the biological significance of the subclones with PALB2 methylation observed in several of the sporadic breast and ovarian tumors we analyzed, particularly with regard to clonal selection and outgrowth, chemoresistance, and cancer stem cell populations.

| Table 1. Clinicopathologic characteristics of PALB2 hypermethylation–positive tumors |
|-----------------------------|---------------------------|-----------------|-------|---|
| Age (y) | Cell type | Grade | Stage |
|-----------------------------|---------------------------|-----------------|-------|---|
| **Ovarian** |
| 1 | 48 | Clear cell | High | IIC |
| 2 | 56 | Mixed clear cell and papillary serous | High | IIC |
| 3 | 61 | Papillary serous with foci of clear cell | High | IIC |
| 4 | 58 | Clear cell | High | IIC |
| **Breast** |
| 1 | 49 | Ductal | III | ER+–/– | PR+– |
| 2 | 85 | Ductal | III | IIB | ER– | PR– |
| 3 | 50 | Ductal | III | IIB | ER+ | PR+ |
| 4 | 69 | Mucinous | I | IIB | ER– | PR– |
| 5 | 57 | Ductal (DCIS, LCIS) | III | I | ER– | PR– |
| 6 | 41 | Ductal (DCIS) | III | IIB | ER– | PR– |

NOTE: Staged according to American Joint Committee on Cancer stage grouping; graded according to American Joint Committee on Cancer criteria. Ovarian tumors and breast tumors 1 to 4 are sporadic cases. Breast tumors 5 and 6 are from BRCA2 mutation carriers. Abbreviations: DCIS, carcinoma in situ; LCIS, lobular carcinoma in situ.

Disease-associated PALB2 point mutation in a family with prostate cancer has been reported (11). Bisulfite sequencing of the LnCaP, DU145, PC3, and MDA2b prostate tumor cell lines showed PALB2 to be unmethylated. We also found PALB2 to be unmethylated by bisulfite sequencing in the SW48, SW480, HT29, and HCT116 colorectal; 786-0, 769P, A498, 1704, and ACHN renal cell; MG118/138 glioma; and H128 lung tumor cell lines (data not shown). Because breast and ovarian tumor cell lines were negative, but 8% of primary breast and ovarian tumors were positive for PALB2 hypermethylation in this study, it is possible that PALB2 hypermethylation is present in other types of primary epithelial tumors.

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