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CpG island hypermethylation of *BRCA1* and loss of pRb as co-occurring events in basal/triple-negative breast cancer

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Abbreviations: TMA, tissue microarrays; TN, triple-negative; TNBC, triple-negative breast cancer; 5NP, five-negative phenotype; LumA, luminal A; LumB, luminal B; IHC, immunohistochemistry

Triple-negative breast cancer (TNBC) occurs in approximately 15% of all breast cancer patients, and the incidence of TNBC is greatly increased in *BRCA1* mutation carriers. This study aimed to assess the impact of *BRCA1* promoter methylation with respect to breast cancer subtypes in sporadic disease. Tissue microarrays (TMAs) were constructed representing tumors from 303 patients previously screened for *BRCA1* germline mutations, of which a subset of 111 sporadic tumors had previously been analyzed with respect to *BRCA1* methylation. Additionally, a set of eight tumors from *BRCA1* mutation carriers were included on the TMAs. Expression analysis was performed on TMAs by immunohistochemistry (IHC) for BRCA1, pRb, p16, p53, PTEN, ER, PR, HER2, CK5/6, CK8, CK18, EGFR, MUC1, and Ki-67. Data on *BRCA1* aberrations and IHC expression was examined with respect to breast cancer-specific survival. The results demonstrate that CpG island hypermethylation of *BRCA1* significantly associates with the basal/triple-negative subtype. Low expression of pRb, and high/intense p16, were associated with *BRCA1* promoter hypermethylation, and the same effects were seen in *BRCA1* mutated tumors. The expression patterns of BRCA1, pRb, p16 and PTEN were highly correlated, and define a subgroup of TNBCs characterized by *BRCA1* aberrations, high Ki-67 (≥ 40%) and favorable disease outcome. In conclusion, our findings demonstrate that epigenetic inactivation of the *BRCA1* gene associates with RB/p16 dysfunction in promoting TNBCs. The clinical implications relate to the potential use of targeted treatment based on PARP inhibitors in sporadic TNBCs, wherein CpG island hypermethylation of *BRCA1* represents a potential marker of therapeutic response.

Introduction

Triple-negative breast cancers (TNBCs) are defined as tumors negative for estrogen- (ER) and progesterone receptors (PR) without HER2 amplification/overexpression.[1] The clinical importance of this subgroup relates to poor prognosis and lack of treatment options. Currently, there are no established therapeutic targets for TNBCs leaving chemotherapy as the only effective treatment for these patients.[2] The biological importance of TNBCs as a distinct disease entity was established through microarray-based gene expression studies showing five distinct subtypes.[3] Of these, the basal-like subtype shows low or complete absence of ER and PR and lack overexpression of the HER2 gene. In concordance with this, most basal-like breast cancers classify as TNBCs.[4] More recently, a subtype of TNBCs characterized by features of mesenchymal and mammary stem cells was described and referred to as claudin-low in keeping with low expression of tight junction proteins including claudin-3, 4 and 7, and E-cadherin.[5] Importantly, residual tumor cells surviving chemotherapy are characterized by features associated with mesenchymal and tumor initiating cells, and are similar to claudin-low tumors.[6]

Inherited germline mutations in the *BRCA1* tumor suppressor gene confer greatly increased risk for developing breast cancer.[7] The incidence of TNBC in *BRCA1* mutation carriers is exceptionally high, or around 70%, and basal-like features are prominent among these tumors.[8,9] Additionally, high histological grade and expression of proliferation markers are common both in sporadic TNBCs and *BRCA1* mutated tumors.[10] The genomes of breast tumors derived from *BRCA1* carriers are highly rearranged, and this is also observed in sporadic TNBCs.[11] The shared characteristics with tumors arising in BRCA1 mutation carriers brought about the hypothesis that acquired defects in the *BRCA1* gene could lead to the development of TNBCs in sporadic disease.[12] Although somatic mutations in *BRCA1* have

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Table 1. BRCA1 gene aberrations with respect to breast cancer subtypes in sporadic and familial breast cancer

Breast Cancer Subtypes	LumA	LumB	HER2	Basal/TN
BRCA1 methylated (n = 18) [Sporadic]	5 (28%)	2 (11%)	1 (5%)	10 (56%)
Not BRCA1 methylated (n = 59) [Sporadic][a, b]	25 (42%)	21 (36%)	3 (5%)	10 (17%)
			Chi-Squared test; X2 = 12.9, P = 0.012	
BRCA1 mutated (n = 8) [Familial]	0	2 (26%)	1 (12%)	5 (62%)
Not BRCA1 mutated (n = 26) [Sporadic]a	14 (54%)	7 (27%)	2 (8%)	3 (11%)
			Chi-Squared test; X2 = 11.2, P = 0.011	
A->T BRCA1 polymorphism (n = 3)	2 (67%)	0	1 (33%)	0
BRCA1 Expression (IHC)		**Null/Weak (<10%)**	**Intermediate (10 - 50%)**	**Highly Expressed (>50%)**
BRCA1 methylated (n = 18) [Sporadic]		9 (50%)	8 (44%)	1 (6%)
No BRCA1 methylated (n = 61) [Sporadic][a]		6 (10%)	21 (34%)	34 (56%)
			Chi-Squared test; X2 = 20.1, P = 0.0002	
BRCA1 mutated (n = 8) [Familial]		6 (75%)	2 (25%)	0
No BRCA1 mutated (n = 25) [Sporadic][a,c]		5 (20%)	3 (12%)	17 (68%)
			Chi-Squared test; X2 = 11.6, P = 0.003	

a, matched against the BRCA1 affected group with respect to age- and year at diagnosis; b, two cases had missing information with respect to subtype-specific markers; c, one case had missing information with respect to BRCA1 expression.

not been found, other mechanisms could lead to inactivation of the *BRCA1* gene in sporadic disease.[13] In this relation, gene silencing through epigenetic modifications has been established as an important mechanism for inactivation of tumor suppressor genes.[14] Loss of *BRCA1* expression through CpG island hypermethylation of the promoter region has been described in breast cancer.[15,16] Importantly, epigenetic inactivation of the *BRCA1* gene is associated with pathological features that are also prevalent in tumors derived from *BRCA1* mutation carriers including ER negativity and the special histological types medullary and mucinous types.[15] We have previously demonstrated that CpG island hypermethylation of the *BRCA1* gene occurs in approximately 10% of all sporadic breast cancers, and that epigenetic silencing of *BRCA1* leads to similar patterns of genetic changes as those observed in *BRCA1* mutated tumors.[17,18] Collectively, these observations have established epigenetic inactivation of the *BRCA1* gene as an important event in sporadic breast cancer. It is, however, not yet clear whether epigenetic inactivation and transcriptional silencing of the *BRCA1* gene specifically relate to the development of TNBCs or basal-like breast cancers in sporadic cases, similar to that which occurs in *BRCA1* mutation carriers. There are contradictory data where some reports have described loss of *BRCA1* expression in association with the basal-like subtype,[19,20] but others have not found the same.[21,22] In this study, we used tissue microarrays to examine sufficiently large number of sporadic breast cancers to explore the relationship between subtype-specific markers and *BRCA1* promoter hypermethylation.

Results

Tissue microarrays (TMAs) were constructed representing 303 breast tumors derived from a well defined sample collection previously screened for the local 5193G→A *BRCA1* and 999del5 *BRCA2* germline mutations.[23,24] Out of the 303 tumors samples, 292 were derived from sporadic cases wherein a set of 111 tumors had previously been analyzed for CpG island hypermethylation of the *BRCA1* gene.[17,18] Additionally, we included a total of eight familial 5193G→A *BRCA1* mutated tumors, along with three tumors derived from carriers of a polymorphism 5142 ACT→TCT (Thr1675Ser) in exon 17 of the *BRCA1* gene.

***BRCA1* aberrations in sporadic- and familial breast cancers are associated with the basal/triple-negative phenotype.** The effects of genetic and epigenetic defects in the *BRCA1* gene were examined with respect to phenotype by looking at subtype-specific markers, i.e., ER, PR, HER2, CK5/6, EGFR and Ki-67 by IHC analysis on TMAs. Out of the 303 samples analyzed on TMAs, 286 were interpretable for subtype-specific markers analyzed on TMAs. In sporadic cases, the luminal-A (LumA) subtype was the most prevalent (119 of 275; 43%), followed by luminal-B (LumB) (84 of 275; 31%), triple-negative (TN) (53 of 275; 19%) and HER2 (19 of 275; 7%). To determine the effects of *BRCA1* aberrations on phenotype, we compared sporadic tumors displaying CpG island hypermethylation of the *BRCA1* gene (n = 18) and familial tumors obtained from 5193G→A *BRCA1* mutation carriers (n = 8) to those of non-*BRCA1*

Table 2. Characterization of breast cancer subtypes with respect to selected markers analyzed by immunohistochemistry (IHC)

Subtype		LumA*	LumB**	HER2	TN***
BRCA1	Null/Weak (<10%)	11 (10%)	8 (9%)	6 (29%)	20 (35%)
	Intermediate/High (>10%)	100 (90%)	77 (91%)	15 (71%)	37 (65%)
				Chi-Squared X2 = 23.2; P = 4 × 10-5	
p16 (CDKN2A)	Null/Weak <10%	50 (47%)	37 (43%)	8 (38%)	14 (25%)
	Intermediate 10 - 50%	52 (49%)	44 (51%)	7 (33%)	5 (9%)
	High/Intense >50%	5 (5%)	5 (6%)	6 (29%)	38 (67%)
				Chi-Squared X2 = 108.0; P < 1 × 10-10	
pRb (RB1)	Null/Weak <10%	4 (3%)	3 (3%)	1 (5%)	24 (42%)
	Intermediate 10 - 50%	28 (25%)	17 (20%)	6 (29%)	10 (17%)
	High >50%	82 (72%)	66 (77%)	14 (67%)	23 (40%)
				Chi-Squared X2 = 67.5; P < 1 × 10-10	
p53 (TP53)	Null/Weak <10%	36 (32%)	21 (25%)	5 (24%)	20 (34%)
	Intermediate 10 - 50%	73 (64%)	52 (61%)	4 (19%)	16 (28%)
	High/Intense >50%	5 (4%)	12 (14%)	12 (57%)	22 (38%)
				Chi-Squared X2 = 59.4; P < 1 × 10-10	
PTEN	Null (<1%)	10 (9%)	12 (14%)	3 (14%)	21 (37%)
	Positive (>1%)	105 (91%)	73 (86%)	18 (86%)	36 (63%)
				Chi-Squared X2 = 22.6; P = 5 × 10-5	
CK8 (KRT8)	Null/Weak <10%	1 (1%)	1 (1%)	2 (9%)	31 (54%)
	Intermediate (10 - 80%)	11 (9%)	5 (6%)	3 (14%)	20 (35%)
	High/Intense (>80%)	104 (90%)	80 (93%)	16 (76%)	6 (11%)
				Chi-Squared X2 = 164.9; P < 1 × 10-10	
CK18 (KRT18)	Null/Weak <10%	2 (2%)	1 (1%)	0	14 (25%)
	Intermediate (10 - 80%)	5 (4%)	3 (3%)	1 (5%)	24 (42%)
	High/Intense (>80%)	108 (94%)	82 (95%)	20 (95%)	19 (33%)
				Chi-Squared X2 = 116.5; P < 1 × 10-10	
MUC1	Null/Weak <10%	14 (12%)	6 (7%)	3 (14%)	17 (30%)
	Intermediate (10 - 80%)	55 (49%)	30 (35%)	3 (14%)	19 (34%)
	High >80%	44 (39%)	50 (58%)	15 (71%)	20 (36%)
				Chi-Squared X2 = 28.3; P = 8 × 10-5	
EGFR	Null <1%	112 (98%)	82 (96%)	11 (52%)	14 (25%)
	Weak (1 - 50%)	1 (1%)	2 (2%)	2 (10%)	10 (17%)
	High/Intense (>50%)	1 (1%)	1 (1%)	8 (38%)	33 (58%)
				Chi-Squared X2 = 153.6; P < 1 × 10-10	
CK5/6 (KRT5/6)	Null (<1%)	104 (90%)	78 (93%)	16 (76%)	20 (34%)
	Positive (>1%)	12 (10%)	6 (7%)	5 (24%)	38 (66%)
				Chi-Squared X2 = 84.4; P < 1 × 10-10	
Claudin-3 (CLDN3)	Null (<1%)	8 (7%)	3 (3%)	0	5 (9%)
	Positive (>1%)	108 (93%)	83 (97%)	21 (100%)	53 (91%)
				Chi-Squared X2 = 3.3; P = 0.35	

affected tumors matched for age-and year at diagnosis (n = 85) (**Table 1; Sup. Table 2 and Sup. Fig. 1**). This analysis demonstrates that CpG island hypermethylation of the BRCA1 gene significantly associates with the triple-negative (TN) phenotype in sporadic disease (**Table 1**). Similarly, the 5193G→A *BRCA1* mutated tumors were also enriched for the TN phenotype, whereas this phenotype was not identified in any of the three 5142 ACT→TCT polymorphic *BRCA1* tumors (**Table 1**). The effects of CpG island hypermethylation of the *BRCA1* promoter on expression were validated by IHC analysis for *BRCA1* gene products on tissue microarrays (TMAs) demonstrating a significant association (**Table 1**). Of interest in this relation is that *BRCA1* mutated tumors were also found to be associated with loss of *BRCA1* gene products (**Table 1**).

The assigned subtypes strongly associate with histological grade, with grade I mostly confined to LumA, and grade II being either of LumA or LumB, and grade III mostly of TN/Basal, LumB or HER2 subtypes (**Sup. Table 3**). Ductal carcinomas dominate all subtypes with other histological types being considerably less frequent wherein lobular carcinomas were mostly confined to the LumA subtype, and medullary features mostly of TN/basal or HER2 subtypes (**Sup. Table 3**). The large majority of sporadic tumors with CpG island hypermethylation of the *BRCA1* gene were of the ductal type, similar to that observed in tumors derived from *BRCA1* mutation carriers, and no significant associations were identified with respect to tumor morphology in this respect (**Sup. Table 4**).

Characterization of TN breast cancers with respect to *BRCA1* aberrations. To characterize the group of triple-negative tumors, a set of eight markers were selected based on published literature in relation to the basal-like phenotype, including markers of the DNA damage response, cell cycle progression and cellular phenotype, i.e., BRCA1, p53 (TP53), PTEN, p16 (CDKN2A), pRb (RB1), MUC1, CK8 (KRT8), CK18 (KRT18), CK5/6 (KRT5/6), EGFR and Claudin-3. This analysis shows that triple-negative breast cancers are characterized by null/weak BRCA1 expression, high/intense p16 (CDKN2A), null/weak pRb (RB1), negativity for PTEN, high/intense p53, weak/null MUC1, intermediate or low CK8/CK18, and positivity for CK5/6 and EGFR (**Table 2**). Cluster analysis based on these characteristics suggests that triple-negative breast cancers can be sub-categorized into two groups that differ with respect to pRb, p16, BRCA1 and PTEN, while similar with respect to CK8, CK18, CK5/6 and EGFR (**Fig. 1A–C**). Based on this model, loss of pRb expression coupled with high p16 levels, hereafter referred to as $pRb^{low}/p16^{high}$, and negativity for PTEN along with weak/null BRCA1 expression are highly coordinated expression patterns (**Fig. 1B**). Here, we find that sporadic tumors displaying CpG hypermethylation of the *BRCA1* gene mostly pertain to the same cluster of triple-negative tumors as the familial 5193G→A *BRCA1* mutated tumors (**Fig. 1C**). In line with this, the $pRb^{low}/p16^{high}$ pattern (indicating RB/p16 dysfunction) was found to be associated with sporadic tumors displaying CpG island hypermethylation of the *BRCA1* gene and, importantly, familial cases derived from 5193G→A *BRCA1* germline mutation carriers (**Table 3**). Notably, familial *BRCA1* mutated tumors, and sporadic tumors with acquired CpG island hypermethylation of the *BRCA1* gene, when coupled with RB/p16 dysfunction ($pRb^{low}/p16^{high}$), almost exclusively results in the TN subtype (**Sup. Table 5**).

Disparities in survival of patients with basal/triple-negative breast tumors. Consistent with previous studies, we find strong prognostic value associated with the assigned breast cancer subtypes wherein the basal-like, LumB and HER2 subtypes associate with poor prognosis (**Fig. 2**). The high fraction of basal/TN features in *BRCA1* affected tumors, whether sporadic or familial, is suggestive of prognostic effects. However, neither sporadic breast tumors displaying CpG island hypermethylation of the *BRCA1* gene, nor familial 5193G→A mutated tumors, were associated with patient survival (**Sup. Fig. 2A**). Nevertheless, looking exclusively at TN tumors, the $pRb^{low}/p16^{high}$ expression pattern was found to be related to more favorable disease outcome, where high/intense p16 was statistically significant (**Fig. 2B**). Additionally, the relatively few breast cancer patients with tumors of the TN/Claudin-Low phenotype, defined as TN breast tumors with complete loss of Claudin-3 expression, were associated with reduced survival (**Fig. 2B**). To account for the TN/Claudin-Low subtype, we classified triple-negative breast cancers into three prognostic subgroups based on the following scheme; Firstly, TN tumors that lack expression of Claudin-3 (null) are classified as TN/Claudin-Low; Secondly, the remaining tumors (TN) are classified based on the presence or absence of high/intense p16 levels referred to as $TN/Claudin^{+}/RB^{-}$ (loss) or $TN/Claudin^{+}/RB^{+}$, respectively. This classification scheme identifies TN subgroups with substantial differences in disease outcomes (**Fig. 2C**). Of particular importance in this respect is that the proportions of tumor cells positive for Ki-67, a marker of cellular proliferation, were significantly lower in the poor prognostic subgroups, i.e., $TN/Claudin^{-}$ and $TN/Claudin^{+}/RB^{+}$ (**Fig. 3A**). The optimal cutpoints for Ki-67 in predicting disease outcomes were different in TN- and luminal subtypes, whereas no survival-dependent effects for Ki-67 were identified within the HER2 subtype (**Fig. 3B**). Evidently, TN breast tumors displaying high levels of proliferation, i.e., ≥40%, associate with favorable disease outcomes with breast cancer-specific deaths observed in only 6% of these patients (2 of 34). This survival rate is similar to, and even better than that observed in luminal breast tumors displaying low levels of Ki-67 positive tumor cells, namely the LumA subtype (**Fig. 3C**).

Discussion

The results presented here demonstrate that *BRCA1* defects and RB/p16 dysfunction are co-occurring events in the development of triple-negative breast cancer (TNBC). Importantly, RB/p16 dysfunction was seen in association with acquired CpG island hypermethylation of the *BRCA1* gene, as well as in tumors derived from *BRCA1* mutation carriers. Additionally, TNBCs with RB/p16 dysfunction were characterized by high p53, loss of PTEN and high Ki-67 (highly proliferative). Given the link to *BRCA1* defects, we hypothesize that this phenotype i.e., RB/p16 dysfunctional TNBCs could be promoted by DNA repair defects, a feature associated with enhanced sensitivity to DNA damaging agents often used in treatment of hormone-receptor negative disease. This could explain the association with favorable disease outcomes for patients with TNBC of the RB/p16 dysfunctional type. The clinical significance of our findings relates to the potential use of less toxic and more targeted treatment based on PARP inhibitors in sporadic cases of TNBC, wherein CpG island hypermethylation of the *BRCA1* gene could be an important predictor of therapeutic response.

We have previously reported that CpG island hypermethylation of the *BRCA1* gene occurs in ~10% of sporadic breast cancers, and that epigenetic silencing of *BRCA1* in sporadic tumors leads to genomic changes similar to those observed in tumors derived from *BRCA1* mutation carriers.[17,18] Here, we have characterized the phenotype of sporadic tumors with acquired CpG island hypermethylation of the *BRCA1* gene on tissue microarrays

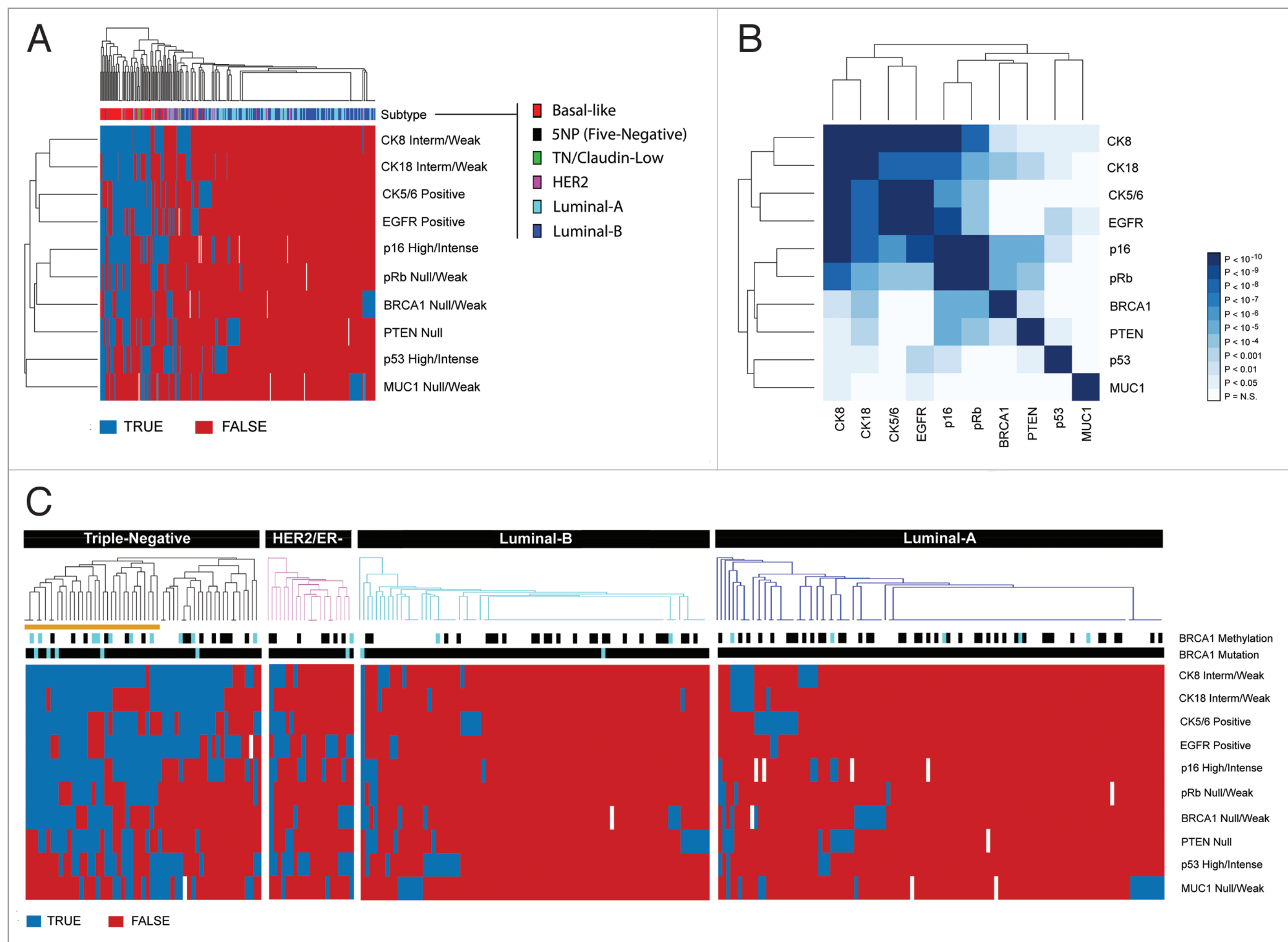


Figure 1. BRCA1, pRb, p16 and PTEN define a sub-class within triple-negative (TN) breast cancers. (A) Hierarchical cluster analysis for the IHC expression profiles suggesting sub-structure within the TN subtype with at least two branches enriched for the basal/triple-negative phenotype. (B) The association matrix for IHC markers representing the log-transform of adjusted p-values (Bonferroni adjustment for multiple testing) derived from Fishers exact hypothesis testing of pair-wise comparisons revealing two groups of strong correlative relationships, i.e., the expression profiles of BRCA1, PTEN, pRb and p16 more strongly correlate with each other than to CK8, CK18, CK5/6 and EGFR. The Bonferroni adjusted p-values are shown and represented in a spectrum of blue colors ranging from $<10^{-10}$ (deep blue) to <0.05 (light blue) and N.S = Not Significant (white). (C) Hierarchical cluster analysis performed separately on each of four main subtypes demonstrating that the TN subtype can be subdivided into two groups based on the co-ordinated expression of BRCA1, pRb, p16 and PTEN. Here, the familial tumors derived from BRCA1 mutation carriers along with sporadic tumors displaying CpG island hypermethylation predominantly cluster within the left branch (orange bar). The presence/absence of BRCA1 gene aberrations are indicated in cyan/black at the bottom of each dendrogram with white blanks representing unknown status.

(TMA) by comparing them to sporadic tumors, unmethylated at the BRCA1 gene promoter, matched for age- and year at diagnosis. This comparison shows that CpG island hypermethylation of the *BRCA1* gene is associated with TNBC and, importantly, that this same effect is observed in tumors derived from *BRCA1* mutation carriers. It has been proposed that the basal/TNBC phenotype can be useful in selecting patients for *BRCA1* mutation testing, and to classify variants of unknown significance in terms of disease predisposition.[8,29] We therefore included tumors derived from carriers of a *BRCA1* polymorphism, currently of unknown significance, that is 5142 ACT→TCT (Thr1675Ser). The results show that none of the *BRCA1* polymorphic tumors classify as TNBC, implying that this polymorphism is not pathogenic, or at least that it does not influence the course of tumor progression similar to that seen in *BRCA1* mutation carriers.

Currently, there is no internationally accepted definition of the basal-like phenotype, although it is widely accepted that most TNBCs are basal-like, and positivity for either CK5/6 or EGFR is often used to identify basal-like breast cancers.[4,30] In this respect, we found a strong association between TNBCs and positivity for the basal-like markers CK5/6 and EGFR, with either marker expressed in the large majority of all *BRCA1* mutated TNBCs (80%; 4 of 5) and similarly in sporadic TNBCs with acquired CpG hypermethylation of the *BRCA1* gene (80%; 8 of 10) (**Sup. Table 2**). Very few studies have analyzed basal markers in sporadic tumors with known CpG island hypermethylation of the *BRCA1* gene, although ER negativity has been consistently reported as an associated feature.[16,17,31] One study has reported similar frequencies of *BRCA1* methylation in sporadic tumors with basal-like phenotype and a control group

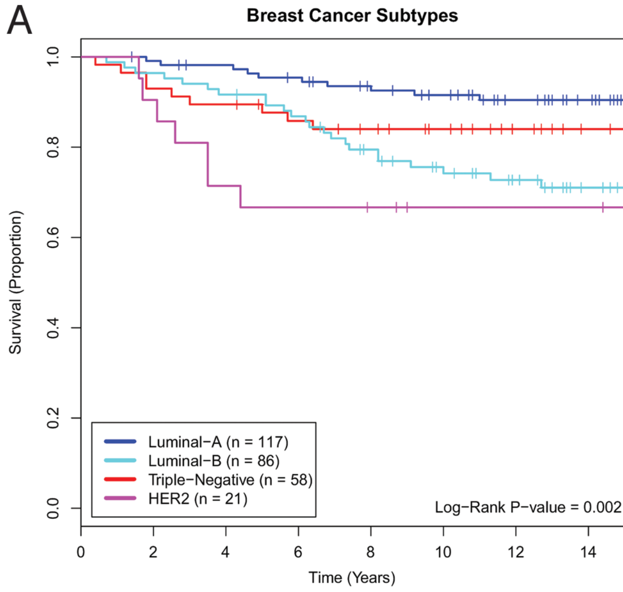
Table 3. Genetic and epigenetic defects in the BRCA1 gene with respect to expression of p16, pRb and PTEN by immunohistochemistry (IHC)

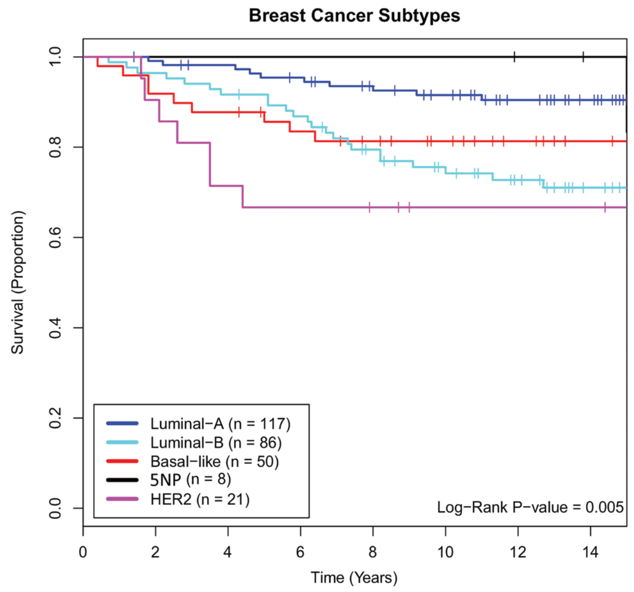
p16 (CDKN2A)	Null/Weak/Intermediate (≤50%)	High/Intense (≥50%)
BRCA1 methylated (n = 18) [Sporadic]	9 (50%)	9 (50%)
Not BRCA1 methylated (n = 60) [Sporadic][b,c]	48 (80%)	12 (20%)
		Chi-Squared χ2 = 4.9; p = 0.03
BRCA1 mutated (n = 8) [Familial]	3 (38%)	5 (62%)
Not BRCA1 mutated (n = 25) [Sporadic][b,c]	22 (88%)	3 (12%)
		Chi-Squared χ2 = 5.9; p = 0.01
BRCA1 Null/Weak (n = 38) (IHC < 10%) [Sporadic]	20 (53%)	18 (47%)
BRCA1 positive (n = 230) (IHC ≥ 10%) [Sporadic][a]	197 (86%)	33 (14%)
		Chi-Squared χ2 = 21.0; p = 5×10^{-6}
pRb (RB1)	**Null/Weak (<10%)**	**Intermediate/High (≥10%)**
BRCA1 methylated (n = 18) [Sporadic]	6 (33%)	12 (67%)
Not BRCA1 methylated (n = 60) [Sporadic][b,c]	7 (11%)	54 (89%)
		Chi-Squared χ2 = 3.4; p = 0.07
BRCA1 mutated (n = 8) [Familial]	5 (62%)	3 (38%)
Not BRCA1 mutated (n = 25) [Sporadic][b,c]	2 (8%)	23 (92%)
		Chi-Squared χ2 = 7.8; p = 0.005
BRCA1 Null/Weak (IHC < 10%) (n = 39) [Sporadic]	13 (33%)	26 (67%)
BRCA1 positive (IHC ≥ 10%) (n = 236) [Sporadic][a]	14 (6%)	222 (94%)
		Chi-Squared χ2 = 25.4; p = 5×10^{-7}
PTEN	**Negative (<1%)**	**Positive (≥1%)**
BRCA1 methylated (n = 18) [Sporadic]	7 (39%)	11 (61%)
Not BRCA1 methylated (n = 60) [Sporadic]b,c	12 (20%)	48 (80%)
		Chi-Squared χ2 = 1.7; p = 0.18
BRCA1 mutated (n = 8) [Familial]	2 (25%)	6 (75%)
Not BRCA1 mutated (n = 26) [Sporadic]b,c	7 (27%)	19 (73%)
		Chi-Squared χ2 = 0.1; p = 0.73
BRCA1 Null/Weak (IHC < 10%) (n = 40) [Sporadic]	17 (43%)	23 (57%)
BRCA1 positive (IHC ≥ 10%) (n = 237) [Sporadic]a	28 (12%)	209 (88%)
		Chi-Squared χ2 = 21.5; p = 3.6×10^{-6}

[a]All cases with available BRCA1 expression data, see also **Figure 1B**. [b]Matched against the corresponding BRCA1 affected group with respect to age- and year at diagnosis. [c]Information not available for all 61 matched against BRCA1 methylation, or 27 matched against BRCA1 mutated tumors.

matched for histological grade and age at diagnosis.[19] Our data, based on a larger number of samples, shows that the incidence of basal/TNBC in *BRCA1* methylated tumors is higher than can be expected by chance (50%; 9 of 18) and that this frequency is similar in *BRCA1* mutated tumors (62%; 5 of 8). Using the definition of basal-like as proposed by Nielsen et al. i.e., TNBCs positive for either CK5/6 or EGFR, leads to the same result (**Sup. Table 2**). Additionally, we find that CpG island hypermethylation of the *BRCA1* gene in sporadic cases of TNBC cannot be explained by special histological types, as the large majority of these tumors classify as ductal carcinomas not otherwise specified (89%; 8 of 9 TNBC w/*BRCA1* methylation) and, similarly, most of the *BRCA1* mutated TNBCs are also of the ductal type (80%; 4 of 5 TNBCs in *BRCA1* carriers) (**Sup. Table 4**). Taken together, our results are consistent with an important role for CpG island hypermethylation of the *BRCA1* gene in promoting the development of basal/TNBC in sporadic cases of the disease.

We demonstrate that the expression patterns of BRCA1, pRb, p16, PTEN and p53 are highly correlated with one another, and that these markers define a subgroup of TNBCs associated with genetic or epigenetic defects in the *BRCA1* gene. High/intense expression of p16 and loss of pRb are known to be associated events in breast cancer, and have been described as defining characteristics of basal-like breast cancer.[32] The retinoblastoma 1 gene (*RB1*) is a well known tumor suppressor gene with functions in regulating cell cycle progression, and in mediating response to DNA damage, and is a critical component of the cellular senescence program.[33] The $pRb^{low}/p16^{high}$ expression pattern is thought to reflect genetic mutations or epigenetic silencing of the retinoblastoma 1 gene (RB1) leading to loss of *RB1* gene products thereby alleviating transcriptional silencing of p16 (CDKN2A). Consistent with this, high expression of p16 is associated with loss of heterozygosity at the *RB1* gene locus and both are prominent characteristics of the basal-like phenotype.[34]


A
Breast Cancer Subtypes
Survival (Proportion)
Time (Years)
Luminal−A (n = 117)
Luminal−B (n = 86)
Triple−Negative (n = 58)
HER2 (n = 21)
Log−Rank P−value = 0.002


Breast Cancer Subtypes
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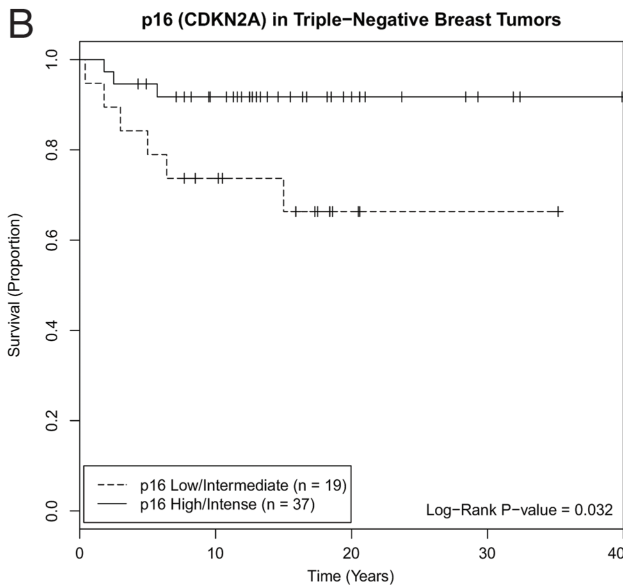
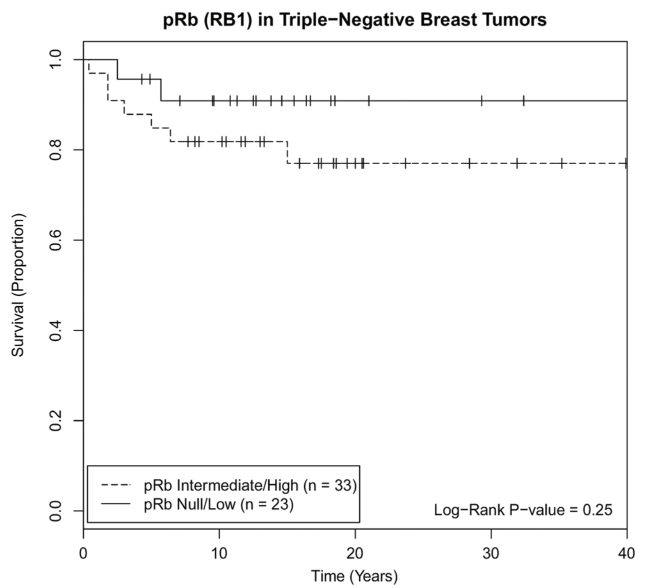
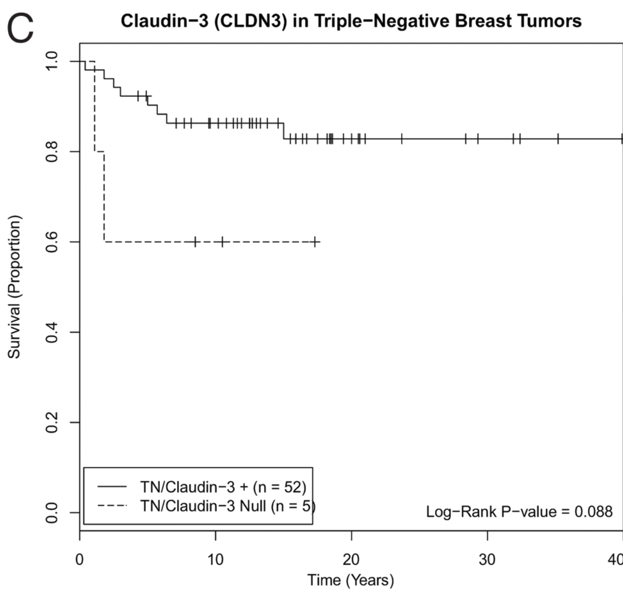
Luminal−B (n = 86)
Basal−like (n = 50)
5NP (n = 8)
HER2 (n = 21)
Log−Rank P−value = 0.005


B
p16 (CDKN2A) in Triple−Negative Breast Tumors
Survival (Proportion)
Time (Years)
p16 Low/Intermediate (n = 19)
p16 High/Intense (n = 37)
Log−Rank P−value = 0.032


pRb (RB1) in Triple−Negative Breast Tumors
Survival (Proportion)
Time (Years)
pRb Intermediate/High (n = 33)
pRb Null/Low (n = 23)
Log−Rank P−value = 0.25


C
Claudin−3 (CLDN3) in Triple−Negative Breast Tumors
Survival (Proportion)
Time (Years)
TN/Claudin−3 + (n = 52)
TN/Claudin−3 Null (n = 5)
Log−Rank P−value = 0.088

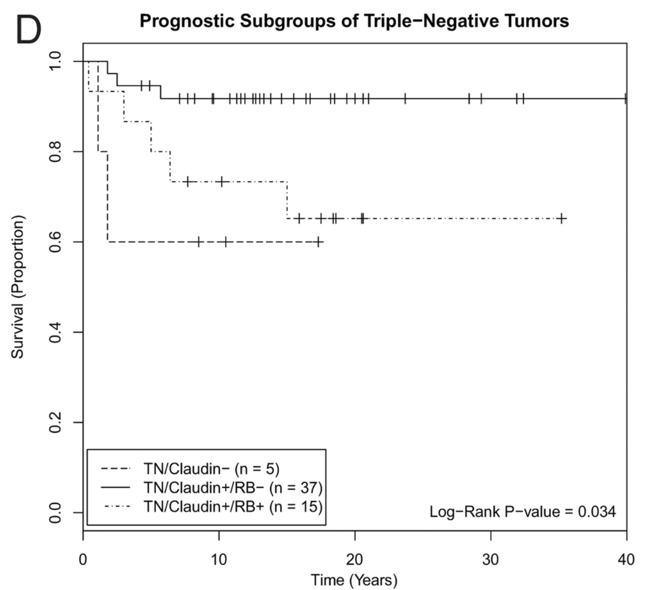

D
Prognostic Subgroups of Triple−Negative Tumors
Survival (Proportion)
Time (Years)
TN/Claudin− (n = 5)
TN/Claudin+/RB− (n = 37)
TN/Claudin+/RB+ (n = 15)
Log−Rank P−value = 0.034

Figure 2 (See opposite page). Survival with respect to phenotype and RB/p16 dysfunction in TN breast cancers. (A) Breast cancer-specific survival with respect to phenotype without (left) and with (right) positivity for either EGFR or CK5/6 to define the basal-like phenotype. (B) The high/intense p16 expression pattern significantly associates with differential survival outcomes in TN breast cancer. (C) TN tumors lacking of Claudin-3 expression relate to reduced short-term survival. (D) Prognostic stratification of triple-negative breast cancer. Here, TN tumors lacking Claudin-3 are sub-categorized as TN/Claudin-, with the remainder of TN tumors subdivided based on the prescence/absence of high p16 levels (IHC 3^+), referred to as TN/Claudin$^+$/RB$^-$ (loss), or TN/Claudin$^+$/RB$^+$, respectively.

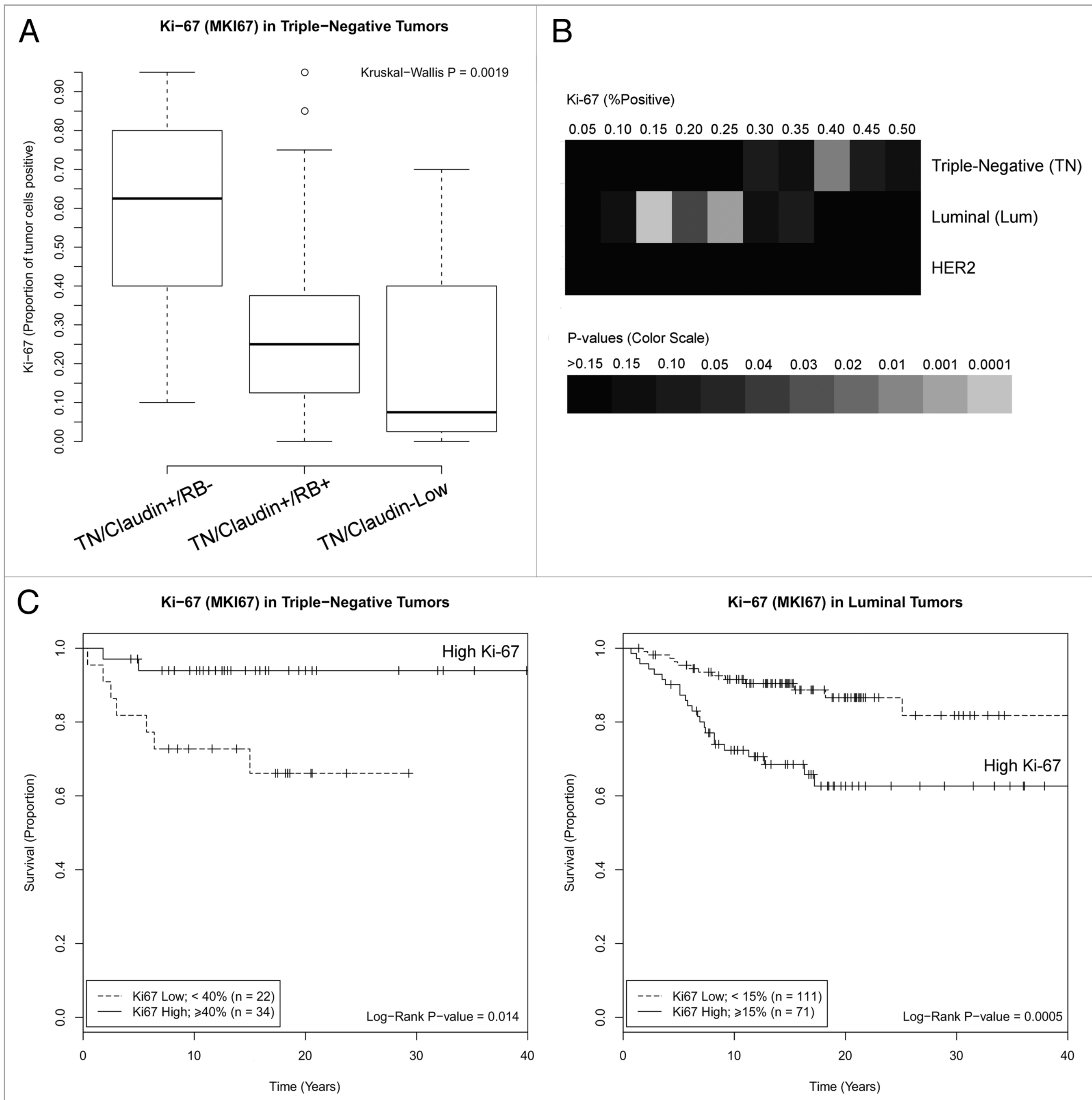


Figure 3. Ki-67 in triple-negative (TN) breast tumors. (A) The proportions of tumor cells undergoing proliferation, estimated by looking at Ki-67 on TMAs, were found to be significantly higher in TN tumors displaying loss of RB (TN/Claudin$^+$/RB$^-$) compared with the other two TN subroups (TN/Claudin$^+$/RB$^+$ and TN/Claudin-Low). (B) The thresholds of optimal seperation for Ki-67 with respect to breast cancer-specific survival differ between luminal- and triple-negative subtypes. Here, Ki-67 does not identify prognostic subgroups within the HER2 subtype. (C) Kaplan-Meier plots, coupled with the log-rank test, demonstrating significantly more favorable disease outcome for patients with TN breast cancer displaying high levels of Ki-67 positive tumor cells (left part). In contrast, highly proliferating tumors of the luminal phenotype are associated with significantly less favorable disease outcome (right part).

We demonstrate here, for the first time, a link between acquired CpG hypermethylation of the *BRCA1* gene and RB/p16 dysfunction, and that these events are co-dependent in the development of TNBCs. Importantly, we show that the development of TNBCs in *BRCA1* mutation carriers also depends on RB/p16 dysfunction. These results are consistent with our previous study, showing two very different progression paths of TNBCs, of which one was highly associated with *BRCA1* defects and extensive changes in DNA copy number indicative of genomic instability.[18] Given these observations, we propose here that RB/p16 dysfunction in TNBCs reflects a biologically meaningful subtype of TNBCs, and that a key event in promoting the development of this phenotype is inactivation of the *BRCA1* gene.

The functional importance of the relationship between *BRCA1* defects and RB/p16 dysfunction could reflect the need to suppress the cellular response to DNA damage due to defective DNA repair following inactivation of the *BRCA1* gene. It has been shown that acquired mutations in the *TP53* (p53) gene is a characteristic feature of tumors derived from *BRCA1* mutation carriers.[35] Our data, demonstrating a strong relationship between p53, p16 and pRb suggest cooperative effects for RB/p16 dysfunction and mutations in the *TP53* gene. Thus, both events could be required to fully suppress the DNA damage response in *BRCA1* defective cells. Inactivation of the *BRCA1* gene leads to defective DNA repair of double-stranded breaks by error-free homologous recombination, leading to error-prone non-homologous recombination (NHEJ).[36] This leads to accelerated accumulation of genetic lesions, from which advantageous changes can arise and contribute to progression of the disease. It has been shown that tumors derived from BRCA1 mutation carriers acquire gross mutations in *PTEN*, involving genetic lesions consistent with inappropriate DNA repair.[37] This is in line with results described here, showing loss of *PTEN* gene products in sporadic cases with acquired CpG island hypermethylation of the *BRCA1* gene. However, only two of the *BRCA1* mutated tumors were classified as PTEN null by IHC, although we note that in reviewing the arrays at least three additional cases were at borderline in this respect. The type of mutational mechanism affecting PTEN in *BRCA1* defective tumors could be at work in other genes as well, and it will therefore be of considerable interest to determine whether this will include the *RB1* gene.

Treatment options for patients with triple-negative breast cancers (TNBCs) are limited as they are negative for the expression of estrogen-receptors (ER), progesterone-receptors (PR) and the human epidermal growth factor receptor type 2 (HER2). Consequently, these patients are not responsive to tamoxifen for antagonizing the estrogen-receptor, or trastazumab for inhibiting the activity of HER2. However, chemotherapy is very effective in hormone-receptor negative disease, including TNBC, whereas the same does not apply for patients with hormone-receptor positive disease i.e., the luminal subtype.[38] It is important to note here that patients with TNBC are associated with poor prognosis, and this probably relates to the highly aggressive behavior seen following progression of TNBCs to metastatic disease.[39] Of considerable interest in this context is that we observed low mortality rates associated with RB/p16 dysfunction in the TNBC subtype. These tumors display features indicative of DNA repair deficiency, including CpG hypermethylation of the *BRCA1* gene. Defects in DNA repair pathways confer enhanced sensitivity to specific DNA damaging agents depending on the pathways affected. In this relation, breast cancer patients with hormone-receptor negative disease are commonly offered drug combinations that may include either of the two DNA damaging agent's cyclophosphamide or mitoxantrone. The mode of action for cyclophosphamide involves the formation of DNA adducts leading to crosslinks between and within DNA strands, and for mitoxantrone in inhibiting the activity of topoisomerase leading to DNA double-strand breaks at replication forks during DNA synthesis. Under normal circumstances, these types of DNA damage are repaired by error-free homologous recombination, a process that requires functional *BRCA1* gene products. The common use of these anti-cancer drugs in treatment of hormone-receptor negative disease could explain the relation to favorable disease outcome for TNBCs with RB/p16 dysfunction as this phenotype is linked to *BRCA1* defects. Interestingly and in support for this conclusion, platinum-based agents have been tested in patients with TNBC, and *BRCA1* methylation was found to be predictive of response.[40] It is noteworthy that the few claudin-low tumors identified here through IHC analysis of Claudin-3 (*CLDN3*) were related to poor disease outcome and showed low proliferative activity compared with the RB/p16 dysfunctional type of TNBCs. In this relation, chemotherapy-resistant tumors have been associated with features characteristic of tumor initiating cells (TICs) which, in turn, are naturally enriched in the claudin-low subtype.[6] The claudin-low phenotype has previously been associated with slower cell-cycling and poor prognosis.[41] This low proliferation activity likely relates to their mammary stem cell characteristics. Lim et al. derived a differentiation model to show that *BRCA1* mutated tumors are more similar to luminal progenitor cells rather than mammary stem cells.[42] This seems consistent with the hypothesis of differential TIC content in TNBCs, possibly explaining the differences in proliferative activity and disease outcome observed here between the three subclasses TN/Claudin^{-}, TN/Claudin^{+}/RB^{+} and TN/Claudin^{+}/RB^{loss}.

PARP inhibitors induce strong anti-tumor activity in patients with inherited *BRCA1* or *BRCA2* mutations.[43] Given the high clinical benefit for mutation carriers, it is of considerable interest to determine whether subsets of sporadic breast cancer patients could also derive benefit from PARP inhibitors. Recently, the addition of PARP inhibitors to a combination of gemcitabine and carboplatin showed significant improvements in patients with metastatic TNBCs.[44] The study of O'Shaughnessy et al. highlight the importance of PARP inhibitors in treatment of TNBC patients, although reports on PARP inhibitors as single agent treatment in TNBC have not shown clinical benefits.[2] Of potential importance in this respect is that PARP inhibitors can exert their anti-tumor effects in at least two independent ways; that is as single agents to induce synthetic lethality in tumors with HR defects (e.g., *BRCA1* silencing) or by potentiating the effects of

DNA damaging agents.[45] More comprehensive understanding of TNBCs is therefore essential to effectively guide the use of PARP inhibitors in clinical management of these patients. The potential importance of epigenetic inactivation of the *BRCA1* gene in this relation has been demonstrated in a pre-clinical study.[46] Thus, CpG island hypermethylation of the *BRCA1* gene could be an important marker in TNBCs for predicting response to PARP inhibitors, platinum-based drugs, or other agents leading to double-strand breaks.

In conclusion, our findings are consistent with a causative link between defects in the *BRCA1* gene by CpG island hypermethylation and the development of TNBCs characterized by RB/p16 dysfunction in sporadic cases of the disease.

Materials and Methods

Study group. The study group of 303 patients was derived from a well defined sample collection previously screened for the local 5193G→A *BRCA1* and 999del5 *BRCA2* germline founder mutations.[23,24] Extensive population and family studies have indicated that these two mutations account for all BRCA mutation related breast cancer risk in the population.[25,26] This is also supported by findings of the Cancer Genetic Counseling Clinic of the National University Hospital in Iceland. The patients were all diagnosed and treated at the same institution, the National University Hospital. Of the 303 cases, a total of 292 were derived from sporadic cases, of which 111 had previously been studied with respect to CpG island hypermethylation of the *BRCA1* gene.[17,18] Additionally, we included a selected set of breast tumors derived from 8 individuals carrying the same 5193G→A *BRCA1* germline mutation, as well as three from carriers of a polymorphism in the *BRCA1* gene were included in the study group. The *BRCA1* polymorphism is a base change from A to T affecting codon 1675 predicted to result in a single amino-acid change from Thr to Ser, and is currently of unknown clinical significance with respect to breast cancer risk. Sporadic cases were defined as those arising in non-*BRCA* carriers, i.e., individuals negative for the 5193G→A *BRCA1* and 999del5 *BRCA2* mutations and having negative family history for breast cancer.

Information on patient age and date of diagnosis were obtained from the Icelandic Cancer Registry. Matching for age- and year at diagnosis was carried out to compare *BRCA1* affected and non-affected tumors with respect to phenotype and breast cancer-specific survival. Age- and year at diagnosis within a range of ±3 years was achieved in all but two of non-*BRCA1* affected tumors matched for each of BRCA1 methylated- and BRCA1 mutated tumors (**Sup. Fig. 1A**). The number of selected tumors was targeted at four non-*BRCA1* affected cases for each one of *BRCA1* affected cases. This was achieved in 14 of 26 *BRCA1* affected tumors, with all but three of the remaining 12 *BRCA1* affected tumors having at least three matched non-*BRCA1* affected tumors. This yielded highly similar distribution of age- and year at diagnosis between *BRCA1* affected, and non-*BRCA1* affected cases (**Sup. Fig. 1B**).

Expression analysis on tissue microarrays (TMAs). Tissue microarrays (TMA) were constructed by selecting viable and representative region enriched for tumor cells from archived formalin fixed and paraffin-embedded (FFPE) tumor tissue as previously described in reference 18. TMAs representing three core samples from each case were studied using immunohistochemistry (IHC) for expression analysis of subtype-specific biomarkers, that is ER, PR, HER2, Ki-67, CK5/6 and EGFR, along with Claudin-3, coupled with additional selection of eight basal-linked markers based on published literature, i.e., BRCA1, p53 (TP53), PTEN, p16 (CDKN2A), pRb (RB1), MUC1, CK8 (KRT8), CK18 (KRT18). IHC expression analysis was performed as previously described in reference 18.

Breast cancer subtypes were assigned to using the five biomarker scheme based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), epidermal growth factor receptor (EGFR) and cytokeratin 5/6 (CK5/6).[27] Expression of ER and PR were scored positive (IHC score ≥1+) when IHC staining was observed in more than 1% of tumor cell nuclei. HER-2 positivity was scored for strong membranous staining (IHC score 3+) whereas EGFR was scored positive for any, weak or strong, membranous staining (IHC score ≥1+). Expression of CK5/6 was defined positive when cytoplasmic and/or membranous staining was observed and otherwise considered negative. Hormone-receptor positive tumors, those positive for either ER or PR, were assigned to the luminal subtype. Tumors negative for ER, PR and HER-2 were categorized as triple-negative (TN). Complete absence of Claudin-3 gene products in TN tumors were classified as TN/Claudin-Low, with the basal-like phenotype assigned to the remaining tumors (TN) where either EGFR or CK5/6 were expressed. Tumors negative for ER and PR negative tumors, but displaying HER-2 positivity (IHC 3+) were assigned to the HER2 subtype. Here, overexpression of HER2 (IHC 3+) was identified in 33 of 284 informative cases (33 of 284; 11.6%) of which 19 were hormone-receptor negative, thereby classified as HER2 subtype. Estimates for the expression of MKI67 (Ki-67) gene products represent counts for the proportion of positive tumor cells, based on nuclear expression, reported on a scale from 0.0–1.0 within intervals of 0.05. Luminal tumors showing Ki-67 expression ≥0.15 were assigned to the luminal B subtype whereas the remaining part was considered luminal A.[28]

The expression of BRCA1 was estimated on a discontinuous scale, based on counts for the proportion of tumor cells showing nuclear expression, Null/Weak (0; <10%), Intermediate (1+/2+; 10%–50%) and Strong (3+; >50%). Similarly, expression of p16 (CDKN2A), pRb (RB1) and p53 were scored by counting positively staining nuclei, i.e., Null/Weak 0 (<10%), Intermediate 1+ (10–25%), 2+ (25–50%) and Strong 3+ (>50%). In estimating p16, the highest score of 3+ almost invariably involved intense and diffuse cytoplasmic and nuclear staining, and therefore referred to as high/intense p16 (IHC Score 3+). Expression of PTEN was determined negative or positive based on the absence or presence of cytoplasmic IHC staining, respectively. MUC1, CK8 and CK18 were estimated by looking at cytoplasmic and/or membranous staining on the scale of Low (0; <10%), Intermediate (1+/2+; 10%–80%) and High (3+; >80%). The

antibodies and dilutions used in IHC expression analysis are listed in **Supplemental Table 1**.

Data analysis. Tabular data were analyzed by the chi-squared hypothesis test, using the chisq.test function in R 2.12.0. Hierarchical cluster analysis was performed using the heatmap function in R, with Euclidean distance as a measure of similarity and average linkage (cluster package in R 2.12.0). Differences in survival outcomes were compared by the log-rank statistic, and Cox proportional hazards model using R 2.12.0 (survival package). In all cases, the end-point was defined as breast cancer-specific survival from the time (days) of diagnosis. The underlying assumptions of proportionality for Cox hazards regression were assessed using the cox.zph function (R 2.12.0; survival package).

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Note

Supplemental materials can be found at:
www.landesbioscience.com/journals/epigenetics/article/15667

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