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BRCA1-related gene signature in breast cancer: the role of ER status and molecular type

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1. ABSTRACT

There is an ongoing debate whether hereditary breast cancer is a clinical entity distinct from sporadic breast cancer. We tried to shed some light on this issue by comparing the molecular profiles of these two types of cancer using DNA microarrays. Our results show that a previously reported marked difference between *BRCA1*-mutation linked and sporadic breast cancer was probably due to uneven stratification of samples with different ER status and basal-like versus luminal-like subtype. We observed that apparent difference between *BRCA1*-linked and other types of breast cancer found in univariate analysis was diminished when data were corrected for ER status and molecular subtype in multivariate analyses. In fact, the difference in gene expression pattern of *BRCA1*-mutated and sporadic cancer is very discrete. These conclusions were supported by the results of Q-PCR validation. We also found that *BRCA1* promoter hypermethylation had similar effect on global gene expression as mutation-induced protein truncation. Thus, in the molecular studies of hereditary breast cancer, *BRCA1* promoter methylation should be recognized and considered together with gene mutation.

2. INTRODUCTION

Since wide implementation of mutation screening and genetic counseling, breast cancer has been frequently regarded either as a sporadic or a hereditary disease (hereditary breast cancer, HBC). Hereditary cancers may arise due to germline mutation in *BRCA1* or *BRCA2* genes, and rarely also due to other known mutations (e.g. in *TP53*, *ATM*, *PTEN* or *CHEK2*), or they may be of unknown etiology (called non-BRCA1/2 or BRCAx cases). There is an ongoing debate whether pathology and clinical behavior of HBC is distinct from those of sporadic breast cancer. Undoubtedly, patients with HBC develop the disease at a younger age, but it is not clear whether they have worse prognosis, as suggested in some studies. When assessing pathology and immunophenotype, *BRCA1* mutation-linked breast cancer is regarded as the most distinct category of HBC. Among its characteristics are: high tumor grade (these cases are often characterized by high proliferative activity, resulting in tumors with pushing margins and high mitotic index), elevated lymphocyte infiltration and low estrogen receptor expression (1-4).

The question of putative molecular differences between hereditary *BRCA1* or *BRCA2* mutation-linked and

sporadic tumors was first analyzed by Hedenfalk *et al.* who used early generation cDNA microarrays (5). The authors claimed that these three categories of breast cancer could be easily distinguished on the basis of distinct gene expression pattern. This issue was also indirectly addressed in two further microarray studies, one concerning the multi-gene signature correlated with clinical outcome (6) and the other describing molecular subtypes of breast cancer (7). Van't Veer *et al.* observed that tumor samples from patients with *BRCA1* mutation fall within ER-negative cluster while Sorlie *et al.* found that *BRCA1*-mutated tumors associated with basal-like subtype. In our opinion, the results of these two studies give a clear indication that molecular differences between mutation-induced and sporadic breast cancers are less pronounced than originally proposed in (5). However, this discrepancy was not discussed therein and its causes have not been systematically investigated so far.

The aim of our study was to verify the magnitude of differences in gene expression profile between *BRCA1*-associated and sporadic breast cancers. In our analyses we took into account the estrogen receptor status and molecular subtype of the tumor, the two most significant features affecting global gene expression pattern in breast cancer. We also checked for *BRCA1* promoter methylation in tumor samples and analyzed gene expression profile in tumors with *BRCA1* gene inactivated either by mutation or epigenetic silencing..

3. MATERIALS AND METHODS

3.1. Clinical samples

We analyzed 35 breast cancer specimens (Table 1). Surgical samples obtained during mastectomy were flash-frozen in liquid nitrogen and stored at -80°C. Only samples from patients without neoadjuvant chemotherapy were used in this study as chemotherapy may seriously affect gene expression profile. All tissue samples were collected at the Pomeranian Medical University in Szczecin.

Seventeen tumor samples were collected from patients with hereditary breast cancer: 12 were derived from tumors affecting women with hereditary *BRCA1* mutation, the only one from a woman with *BRCA2* mutation, while another eight cases had familial history of breast/ovarian cancer, but were negative for the *BRCA1/2* mutations (so called BRCAx cases). Proportion of BRCA1 and BRCA2 mutated tumors was typical for the Polish population (8-10). Ten samples were derived from patients with apparently sporadic disease (no familial history of cancer) while 4 patients had a history of familial cancer aggregation (FCA) but without prevalence of breast/ovarian cancers. Thus, these samples were merged with sporadic samples in most of the analyses.

All *BRCA1* mutation-linked tumors in our study were negative for estrogen receptor (by immunohistochemistry, standard procedures for ER, PGR and HER2 staining were applied), while the only *BRCA2*-mutated tumor was ER-positive. As we considered hormone receptor status being a very important source of variability in gene expression profile, we carefully matched the control group of sporadic cancers to the group of hereditary breast tumors. Thus, 10 sporadic cases were selected with respect to the estrogen receptor status: 9 tumors were ER-negative, while only one was ER-positive. The larger proportion of ER-positive tumors was observed only in BRCAx group and FCA group (3 and 2 ER-positive samples, respectively). Average age of the patients was 51.8 years. There were 26 ductal and 5 medullary carcinomas within the study group, which is consistent with the distribution of histopathological types in *BRCA1* mutation carriers. Patients were diagnosed at stage T1-2, N0-1 and M0.

3.2. *BRCA* mutation-testing

All patients with familial history of breast/ovarian cancer or familial cancer aggregation (FCA) were diagnosed and tested at the International Hereditary Cancer Center of the Pomeranian Medical University in Szczecin. The most common founder mutations that account for over 90% of all BRCA mutations in the Polish population (5382insC, 300T/G and 4153delA in *BRCA1* gene) were checked by multiplex PCR (patent no. P-335917, Poland). Patients who were negative for these three mutations were further tested for 185delAG in *BRCA1* and 6174delT in *BRCA2* gene by allele specific PCR, according to (11). Tumor samples from patients with strong familial history of breast or breast/ovarian cancers, but who tested mutation-negative, were assigned to the BRCAx group for the purpose of microarray data analysis.

3.3. RNA isolation

Total RNA was isolated according to Chomczynski and Sacchi (12), as it worked best in our hands for fat-rich breast tissue. Frozen tissue (20-40 mg) was homogenized in 600 microliters of ice-cold Solution D with Lysing Matrix D ceramic spheres using a FastPrep instrument (MP Biomedicals, Irvine, CA, USA). After extraction RNA was cleaned up with simultaneous on-column digestion of DNA traces using RNeasy Mini Kit and DNAse I (Qiagen), according to the manufacturer's recommendations. RNA quantity was estimated with ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed using Agilent platform: RNA 6000 Nano LabChip Kit, RNA Integrity Number software and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

3.4. Detection of *BRCA1* promoter methylation

DNA was extracted from frozen tissue with Genomic Mini kit (AA Biotechnology). Sodium bisulfite modification of DNA was performed according to (13). Methylation-specific PCR was done with primers specific for methylated sequence and, separately, with primers specific for unmethylated sequence (14). PCR conditions for the unmethylated sequence were as follows: initial denaturation: 10 min at 95°C, then 40 cycles at 95°C (for 30 s), at 60°C (for 55 s) and at 72°C (for 30 s); final elongation at 72°C for 7 min. For methylated sequence: initial denaturation at 95°C for 10 min, then 2 cycles at 95°C (for 30 s), at 64°C (for 55 s) and at 72°C (for 30 s), 2 cycles at 95°C (for 30 s), at 62°C (for 55 s), and at 72°C

Table 1. Characteristics of tumor samples used in microarray experiment

No	Sample name	Germ-line *BRCA1/2* mutation	Group	ER status, est. by IHC[1]	Molecular subtype, acc. to (7)	Histology	*BRCA1* promoter methylation
1	t01	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Medullary	-
2	t02	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Ductal	-
3	t09A	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Ductal	-
4	t09B	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Ductal	-
5	t11	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Medullary	-
6	t12	*BRCA1* 4153delA Ex11	BRCA1	Negative	Basal	Ductal	-
7	t14	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Medullary	-
8	t17	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Ductal	-
9	t21	*BRCA1* C61G Ex5	BRCA1	Negative	Basal	Ductal	-
10	t26	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Ductal	-
11	t28	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Ductal	-
12	t33	*BRCA1* 5382insC Ex20	BRCA1	Negative	Basal	Ductal	-
13	t10	*BRCA2* 9631delC Ex25	BRCA2	Positive	Luminal	Ductal	-
14	t04	No mutation	BRCAx	Negative	Basal	Ductal	-
15	t19	No mutation	BRCAx	Negative	Basal	Ductal	Methylated
16	t24	No mutation	BRCAx	Negative	Basal	Ductal	-
17	t32	No mutation	BRCAx	Negative	Luminal	Ductal	-
18	t35	No mutation	BRCAx	Negative	Luminal	Ductal	-
19	t08	No mutation	BRCAx	Positive	Luminal	Ductal	-
20	t36	No mutation	BRCAx	Positive	Luminal	Ductal	-
21	t37	No mutation	BRCAx	Positive	Luminal	Ductal	-
22	t06	No mutation	Sporadic	Negative	Basal	Medullary	Methylated
23	t07	No mutation	Sporadic	Negative	Basal	Ductal	Methylated
24	t13	No mutation	Sporadic	Negative	Basal	Medullary	-
25	t20	No mutation	Sporadic	Negative	Luminal	Ductal	-
26	t22	No mutation	Sporadic	Negative	Luminal	Ductal	-
27	t27	No mutation	Sporadic	Negative	Luminal	Ductal	-
28	t29	No mutation	Sporadic	Negative	Luminal	Ductal	-
29	t31	No mutation	Sporadic	Negative	Basal	Ductal	Methylated
30	t34	No mutation	Sporadic	Negative	Basal	Ductal	-
31	t05	No mutation	Sporadic	Positive	Luminal	Ductal	-
32	t15	No mutation	Sporadic/FCA	Negative	Luminal	Ductal	-
33	t18	No mutation	Sporadic/FCA	Positive	Luminal	Ductal	-
34	t25	No mutation	Sporadic/FCA	Negative	Basal	Ductal	Methylated
35	t30	No mutation	Sporadic/FCA	Negative	Basal	Ductal	Methylated

[1] - ER status estimated by immunohistochemistry

(for 30 s), 36 cycles at 95°C (for 30 s), at 60°C (for 55 s) and at 72°C (for 30 s), then final elongation at 72°C for 7 min. For each sample PCR was repeated three times.

3.5. Oligonucleotide microarrays

We used HG U133 Plus 2.0 Gene Chip oligonucleotide arrays (Affymetrix) allowing detection of 47 000 human gene transcripts. The hybridization target was prepared according to the recommendations from microarrays' manufacturer. Total RNA (8 micrograms) was used for synthesis of double stranded cDNA. Half of the cDNA volume was used for synthesis of biotinylated cRNA with the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics). Both cDNA and cRNA were purified with Gene Chip Sample Cleanup Module (Affymetrix). cRNA (16 micrograms) was fragmented and hybridized to the microarray for 16 h at 45°C. After washing and staining the microarrays were scanned with GeneChip Scanner 3000 (Affymetrix). Data were acquired using GCOS 1.2 software (Affymetrix). The preprocessing was performed by Robust Multi-array Analysis (RMA, Bioconductor). Raw pre-processed dataset is available at www.genomika.org/publications/hereditarybreastcancer, together with descriptions of the samples.

3.6. Quantitative RT-PCR

Quantitative RT-PCR analysis was done using the ABI 7700 Sequence Detection System and dedicated software (Applied Biosystems, Foster City, CA, USA). The reactions were performed using MasterAmp Real-Time RT-PCR Kit (Epicentre) according to the manufacturer's recommendations. Primers for the SYBR Green system were designed using Primer3 online software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of the PCR primer pairs are shown in Table 2. As a reference gene to normalize RNA quantity we used the Eukaryotic Translation Initiation Factor 4 gamma 2 (EIF4G2), that appears to be equally transcribed in all tissues analyzed by microarrays. Gene expression values were obtained by a standard delta-delta Ct method. Primers specificity was verified by sequencing of selected RT-PCR products for each gene.

3.7. Methods of data analysis

Gene expression comparisons by Welch t-test were performed using GeneSpring 7.2 software (Agilent), with non-corrected threshold of p-value less than 0.001. False Discovery Rate (FDR) was estimated by Benjamini-Hochberg algorithm. Two-way analysis of variance (ANOVA), with random variance assumption and global testing were carried out by procedures implemented in BRB Array (developed by Richard Simon and Amy Peng Lam and available on the National Cancer Institute website). Principal Component Analysis of microarray data was carried out by prcomp package of R environment. The reanalysis of the data of Hedenfalk *et al.* was done on raw microarray data for 3226 clones and 22 tumor samples

Table 2. Oligonucleotide primers used in real-time RT-PCR

Gene	Sequence	Product size (bp)
EIF4G2	F 5`- GCAAGGCTTTGTTCCAGGTGA -3` R 5`- AGGCTTTGGCTGGTTCTTTAGTCA -3`	100
FANCA	F 5`- TCCCCACCTGATTCTCTGTCATGT -3` R 5`- GAGGCTCCGTCAACTAAGTGAGA -3`	218
HIP2	F 5`- GCAATGACTCTCCGCACGGTA -3` R 5`- GCCCAAAGTCGAGCTGTCTG -3`	140
TOB1	F 5`- ATTGTTTCTACGACATGGTATTGCATTTA -3` R 5`- CAAGTATTCGTACATTTTAATTCCACCACT -3`	182

Table 3. Summary of the microarray data analyses

No	Subject of analysis	Samples compared	No of genes	See results	
A. Summary of the univariate comparisons					
1.	*BRCA1* mutation status	*BRCA1*-mutated *versus* all other breast ca.	**234 (p less than 0.001)** **GT: p=0.001**	Supplementary Tab. 1	
2.	*BRCA1* mutation status	*BRCA1*-mutated *versus* all sporadic breast ca.	41 (p less than 0.001) GT: p=0.065	-	
3.	*BRCA1* mutation status	*BRCA1*-mutated *versus* ER (-), sporadic breast ca.	27 (p less than 0.001) GT: p=0.16	-	
4.	Comparison of *BRCA1* inactivation pathways	*BRCA1*-mutated *versus* *BRCA1*-methylated	43 (p less than 0.001) GT: p=0.24	-	
5.	*BRCA1* inactivation status	*BRCA1*-mutated or *BRCA1*-methylated *versus* all other breast ca.	**609 (p less than 0.001)** GT: p=0.001	Supplementary Tab. 3	
B. Summary of the results of two-way ANOVA comparisons					
No	**First variable**	**Second variable**	**No of genes**	**No of genes**	**See results**
1.	*BRCA1* mutation	ER status	*BRCA1* mutation: 0 (FDR less than 20%) **101 (p-value less than 0.001)**	ER status: 1380 (FDR less than 20%)	Supplementary Table 2
2.	*BRCA1* mutation	molecular subtype	*BRCA1* mutation: 0 (FDR 20%) **37 (p-value less than 0.001)**	Molecular subtype: 5705 (FDR less than 20%) 1222 (p-value less than 0.001)	Supplementary Table 5
3.	*BRCA1* inactivation	ER status	*BRCA1* inactive: 259 (FDR less than 10%) 250 (p-value less than 0.001)	ER status: 101 (FDR less than 10%) 185 (p-value less than 0.001)	Supplementary Table 4
4.	*BRCA1* inactivation	molecular subtype	*BRCA1* inactive: 0 (FDR less than 20%) 57 (p-value less than 0.001)	Molecular subtype: 2441 (FDR less than 20%) 526 (p-value less than 0.001)	Supplementary Table 6

(among them 7 sporadic and 7 *BRCA1*-mutated tumors were analyzed), provided by BRB Array Tools repository. Data were analyzed by the same approach and software as our dataset. Class prediction procedure was carried out using Support Vector Machines (SVM) class prediction engine with leave-one-out cross-validation (BRB Array Tools). Real-time PCR gene expression values were compared by non-parametric Kolmogorov-Smirnov test by SPSS 13 software (SPSS), with two-sided p-value threshold of less than 0.05.

3.8. Data analysis workflow

Searching for the *BRCA1*-mutation signature we performed several supervised analyses (Table 3). The results of each univariate analysis were further verified by the global test. This allowed to estimate the probability of getting that number of genes by chance, despite lack of real differences between the classes. In the multivariate comparisons, the analysis carried out with respect to *BRCA1* truncation was corrected for other features that were suspected to influence the molecular profile of breast cancer. Full gene lists obtained in all comparisons are freely available as a supplementary data at www.genomika.org/publications/hereditarybreastcancer
Not all gene lists are discussed here, due to the large number of comparisons and low statistical significance of some lists. However, we show the numbers of genes obtained in consecutive analyses (Table 3), as in our opinion, that numbers illustrate which features are truly related to significant changes in molecular profile and which analyses are biased due to samples stratification.

Class prediction was applied in order to estimate whether the *BRCA1*-mutation linked breast cancers can be distinguished on the basis of their gene expression profile. Finally, we applied Principal Component Analysis (PCA) and searched for the features which correlate with most distinct gene expression profiles.

4. RESULTS

4.1. Search for the BRCA1-mutation signature in breast cancer

First, we compared gene expression profile of *BRCA1*-mutation linked breast cancer samples to that of all other breast cancer samples without *BRCA1* mutation (sporadic, BRCAx, FCA and the only one BRCA2-mutated). Univariate analysis revealed 234 differentially expressed probesets (selected with non-corrected p less than 0.001). This result proved significant also in the global test (p=0.001). However, when *BRCA1*-mutated samples were compared exclusively to sporadic cases, we obtained only 41 probesets (univariate non-corrected p less than 0.001) and this result turned out non-significant in the global test (p=0.065).

Than we reanalyzed of the data of Hedenfalk *et al.* (5) using the same approach and software as for our

dataset. Raw microarray data for 3226 clones and 22 tumor samples, among them 7 sporadic and 7 *BRCA1*-mutated tumors were retrieved from BRB Array Tools repository. Comparison of *BRCA1*-linked tumors vs. sporadic ones revealed only 11 differentially expressed cDNA clones (non-corrected p-value less than 0.001, non-significant in the global test: p=0.065). Thus, it appeared that the difference in gene expression pattern between *BRCA1*-mutated and sporadic tumors was similarly weak in both datasets.

To further analyze the magnitude of difference between *BRCA1*-mutated samples and other tumors, class prediction was carried out using 423 probesets with non-corrected p-value p less than 0.001 and Support Vector Machine engine with leave-one-out cross-validation. The maximum achieved accuracy of discrimination of two classes ("*BRCA1*-mutated tumors" and "other tumors") was only 69%. The same method was applied to the dataset from (5). Maximal possible accuracy (64%) in discrimination of two classes was obtained with 96 probesets (p-value less than 0.001). In both cases the classification accuracy was not much higher than could be achieved by chance, suggesting that there is no significant difference between the tumor classes defined by presence or absence of *BRCA1* mutation. There was also almost no overlap between the two gene lists, what may indicate that majority of selected genes were false positives. The only two genes occurring on both lists were *TOB1* and *ALCAM*. One can speculate that these genes may be of functional significance for *BRCA1* mutation-linked breast cancer.

4.2. *BRCA1*-related expression signature is strongly influenced by ER status of the tumor

ER status is well known factor affecting both, clinical course of breast cancer and gene expression pattern in breast tumor samples. Thus, we were aware that a difference in the frequency of ER-positive tumors between groups of "*BRCA1*-mutated" and "non-mutated" cancers (Table 1) may influence the results of analysis. Indeed, the univariate comparison revealed 589 probesets differentially expressed in ER (+) and ER (-) tumors (non-corrected p-value less than 0.001, global test p-value less than 0.001; data not shown).

To avoid the impact of ER-positive samples on gene selection procedure we excluded from further analysis all ER-positive samples. When we repeated a comparison of "*BRCA1*-mutated" and "sporadic" tumors in the homogenous ER-negative group of tumors, the difference between both classes was not significant (27 genes at p-value less than 0.001, global test probability p=0.16). A similar conclusion was reached after two-way ANOVA that included both variables: *BRCA1* mutation and ER status. In this analysis, ER status was associated with significant changes in gene expression (1380 probesets) while no genes showed altered expression in the context of *BRCA1* status at FDR less than 20%. Also at FDR less than 10% none of genes passed these criteria in the comparison of *BRCA1*-mutated and non-mutated tumors. When the less stringent criteria were applied (non-corrected p-value less than 0.001, *nota bene* the threshold similar to the that used in (5).), we obtained 375 probesets related to ER status and 101 probesets potentially associated with *BRCA1* mutation status.

We found also that the majority of genes selected in our first comparison ("*BRCA1*-mutated" versus "all other" tumors) were related to ER status. This suggests that in fact we analyzed the difference between ER-negative and ER-positive tumors in this first comparison, what could account for statistical significance of the result. In our opinion, the results described in this chapter suggest that the majority of variance attributed previously to the presence of hereditary *BRCA1* mutation was dependent on ER-positive sample imbalance between *BRCA1*-mutated and remaining tumors.

4.3. The biological context of the gene expression differences between *BRCA1*-mutated and sporadic tumors

Although the diversity in molecular profile of *BRCA1*-mutated and non-mutated breast tumors appeared rather discrete and nonsignificant, we tried to analyze its biological background. Thus, we carried out gene set analysis based on two repositories: Biocarta (gene sets related to cellular signaling pathways) and BROAD (different gene sets). Taking into account the results of previous analyses, we used the data exclusively from ER-negative samples. Ten gene sets from Biocarta repository showed significantly changed expression (p-value less than 0.005) between *BRCA1*-mutated and sporadic tumors, in at least one of four statistic tests used. One gene set (called Msp/Ron receptor signaling), with up-regulation of immunity-related genes *TNF, IL1B, CCL2* and *CSF1*, was found to be significant in all 4 tests. Two further gene sets showed differences in 3 tests; this were Antigen Processing and Presentation, with over-expression of *TAP2/1* and *HLA-A/DRA* genes, and interestingly, BRCA1-dependent Ub-ligase activity, relying on the differences in *FANCA/FANCE* and *BRCA1/BARD* genes.

In analysis of curated gene sets from BROAD repository, only two gene sets were significant in all 4 tests (gene set described by Kang in (15) and Msp/Ron pathway). The "Kang gene set" (17 genes in total) was described as downregulated in gastric cancer cell lines resistant to doxorubicin, comparing to parent chemosensitive line. We observed a coordinated change of some genes from this set in *BRCA1*-mutated samples. Following genes were downregulated: *PCYOX1, TMEM106B, ATP2B1, TBL1X, PTP4A2* and, less significant, also *NCOA3*. Among gene sets changed in 3 of 4 tests used, we found one set directly related to BRCA1 in breast cancer (BRCA1 reporter gene set from (6)). Other gene sets were related to induction of gene expression by TNFA or IFNA. In addition, two chromosome locations showed coordinated gene expression change: 16q24, with up-regulation of at least 25 genes, among them *LOC348180, SPG7, FANCA, C16orf7, GALNS, SLC7A5, FBXO31* in *BRCA1*-mutated samples; and 5q13, with down-regulation of at least 17 genes like *LOC653080, MARVELD2, SERF1ACOL4A3BP, TNPO1* and – of great interest – *PIK3R1*.

4.4. *BRCA1* expression signature in the context of differences between basal and luminal subtype of breast cancer

Molecular subtype (mainly basal-like and luminal-like) of breast cancer is also among the features that strongly influence its gene expression profile. Thus, we used microarray data to identify main molecular subtypes in our group of breast tumors, by clustering samples with subtype-related gene set specified by Sorlie *et al.* (7). It appeared that both subtypes are disproportionately distributed between *BRCA1*-mutated and non-mutated cancers (Table 1). Thus, we used two-way ANOVA to analyze *BRCA1* mutation effect in the context of molecular subtype. Both basal-like and luminal-like subtypes had distinctly different gene expression profile (5705 probesets with changed expression), while no transcripts showed changed expression between tumors with and without *BRCA1* mutation at FDR less than 20% and even at FDR less than 10%. When less stringent criteria were applied (non-corrected p-value less than 0.001), we obtained 1222 and only 37 probesets, respectively. The attempts to analyze signaling pathways and functional gene clusters differentially expressed between BRCA1 mutated and non-mutated basal tumors gave no significant results. Thus, it seems that BRCA1-mutated tumors probably do not differ significantly from other basal breast cancers, or the difference is so subtle that much larger numbers of samples are required to achieve informative results.

4.5. Q-PCR verifies whether changes in gene expression are related to the *BRCA1* mutation status or to other factors

We have chosen three genes for Q-PCR validation: *HIP2*, *TOB1* and *FANCA*. All three genes appeared on the list of 423 genes used in class prediction procedure in our dataset. *HIP2* gene occurred also among 37 genes obtained in the analysis of *BRCA1* mutation status in the context of the molecular subtype. *TOB1* gene was one of the only two genes which appeared both in the analyses done on our dataset, and on the dataset from (5).

In the validation step we first used only samples from ER-negative tumors to avoid potential bias related to the differences in gene expression between ER-positive and ER-negative tumors (Table 5). We found out that both *TOB1* and *HIP2* were differently expressed in *BRCA1*-mutated tumors compared to other tumors (p=0.04 in both analyses), confirming the results obtained by microarray study. However, when ER-positive samples were also considered, *TOB1* showed strong difference between ER-negative and ER-positive tumors (p=0.001), which resulted in high overall significance (p=0.004) between *BRCA1*-mutated and remaining tumors (non-mutated, both ER-positive and ER-negative). On the contrary, *HIP2* showed no differences between ER-positive and ER-negative samples and the overall difference between *BRCA1*-mutated and remaining tumors was also non-significant in this comparison (p=0.07).

Third gene, *FANCA,* was significantly changed in the context of *BRCA1* mutation status in the microarray data analysis corrected for the influence of ER status. It also appeared in the analyses of molecular pathways done on ER-negative samples (section 4.4.). Q-PCR analysis confirmed that *FANCA* expression does not depend on estrogen receptor status, however the difference between hereditary and sporadic cancers was also non-significant (expression changes were non-significant in all analyses).

4.6. The impact of *BRCA1* inactivation (mutation or methylation) on gene expression profile in breast cancer

In the next step of our analysis we checked by MS-PCR for *BRCA1* promoter methylation. In six breast cancer samples that were negative for *BRCA* mutations, we found *BRCA1* promoter methylation. This were: one sample of BRCAx cancer, two FCA samples and 3 samples of sporadic cancer. All tumors with *BRCA1* methylation were estrogen-negative and showed basal-like molecular profile (Table 1). There were no significant differences in gene expression pattern between samples with *BRCA1* mutation and samples with *BRCA1* promoter methylation (43 genes at non-corrected univariate p-value less than 0.001; insignificant in the global test: p=0.24). We thus assumed no biological difference between samples with the *BRCA1* gene truncated by either of two molecular events.

Next, we analyzed *BRCA1* inactivation in the context of ER-status of the tumor by two-way ANOVA. In this analysis *BRCA1* status was a stronger factor determining gene expression pattern than ER status: for *BRCA1* inactivation 259 probesets passed the criteria of FDR less than 10% (supplementary Table 4), while only 101 showed such differences in the context of ER. When non-corrected p-values were applied, these numbers were 260 and 185 probesets, respectively (p-value less than 0.001).

When *BRCA1* inactivation was analyzed in the context of molecular subtype, 2441 genes passed the FDR threshold of 20% for molecular cancer subtype while none of them reached this limit for *BRCA1* inactivation. With non-corrected p-value less than 0.001, 526 genes were found for the subtype, while 57 genes were significantly associated with BRCA1 inactivation (supplementary Table 6).

4.7. Unsupervised analysis confirms that main sources of molecular variability are ER status and molecular subtype

Principal Component Analysis is an unsupervised algorithm that produces graphical representation of samples in which the distance between the samples reflects differences in gene expression profile. Figure 1 shows that according to the first two principal components the tumor samples were split into two clusters. To investigate the nature of these two clusters we analyzed several features defined within the studied group of tumors. It appeared that all BRCA1 mutated tumors as well as *BRCA1* methylated samples, located within that cluster which was generally higher in first component and lower in second component (Figure 1A). All 5 medullary carcinomas were also located in this cluster (Figure 1D.). On the contrary, most BRCAx samples and the only BRCA2-mutated sample located

Table 4. Selected genes used in class prediction procedure

Gene symbol	Probe set	Gene description	Rank	Parametric p-value	*BRCA1*-mutated tumors[1]	Non-mutated tumors[2]	Ratio[3]
TOB1	**202704_at**	**transducer of ERBB2, 1**	**7**	**0,0003488**	**1583,9**	**4277,1**	**0,37**
DAPK1	203139_at	death-associated protein kinase 1	9	0,0003939	419	148,1	2,829
SMAD1	227798_at	SMAD, mothers against DPP homolog 1 (Drosophila)	18	0,0005539	740,1	1541	0,48
RASEF	1553986_at	RAS and EF-hand domain containing	22	0,0006355	19,9	158	0,126
EML1	204797_s_at	echinoderm microtubule associated protein like 1	36	0,0008215	21	60,2	0,349
GSTP1	200824_at	glutathione S-transferase pi	42	0,0010103	2294,4	847,6	2,707
MTA3	223311_s_at	metastasis associated 1 family, member 3	46	0,0010825	246,5	477,2	0,517
FLRT3	222853_at	fibronectin leucine rich transmembrane protein 3	53	0,0011724	3,8	25	0,152
RHOB	212099_at	ras homolog gene family, member B	57	0,0012658	1558,3	5337,6	0,292
RAB15	59697_at	RAB15, member RAS onocogene family	60	0,0013082	23,6	59,4	0,397
DNAJC1	242216_at	DnaJ (Hsp40) homolog, subfamily C, member 1	65	0,0013974	16,1	51,3	0,314
PIK3R3	202743_at	phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	69	0,0015051	1270,9	3043,1	0,418
HIST3H2A	221582_at	histone 3, H2a	73	0,0015822	1181	396,8	2,976
IL10RB	227125_at	Interleukin 10 receptor, beta	74	0,001599	367,4	177,4	2,071
S100A11	208540_x_at	S100 calcium binding protein A11 (calgizzarin)	88	0,001887	8431,2	6111,5	1,38
SEMA3C	203788_s_at	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	94	0,0020362	8,1	16,7	0,485
CASP9	203984_s_at	caspase 9, apoptosis-related cysteine peptidase	96	0,002097	163,9	245,3	0,668
LOC643998	227663_at	Similar to cadherin 12, type 2 preproprotein	99	0,0021606	140,5	575,2	0,244
PDPK1	224986_s_at	3-phosphoinositide dependent protein kinase-1	109	0,0022685	389,7	658,9	0,591
COL4A3BP	223465_at	collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	114	0,002372	41,3	92	0,449
RAPGEF2	238176_at	Rap guanine nucleotide exchange factor (GEF) 2	115	0,0023811	71,6	210,3	0,34
ARHGEF1	203055_s_at	Rho guanine nucleotide exchange factor (GEF) 1	116	0,0024038	48,7	33,7	1,445
IL20RA	219115_s_at	interleukin 20 receptor, alpha	120	0,0024981	5,8	21	0,276
AMACR	209426_s_at	alpha-methylacyl-CoA racemase	133	0,0027655	21,9	54,7	0,4
GRLF1	229394_s_at	glucocorticoid receptor DNA binding factor 1	137	0,002844	1078,5	2014,7	0,535
H2AFV	212205_at	H2A histone family, member V	140	0,0029347	1101,5	1710,8	0,644
MTA1	211783_s_at	metastasis associated 1	142	0,0029635	818,7	1368,4	0,598
SEPHS2	200961_at	selenophosphate synthetase 2	146	0,0030547	1454	2507,6	0,58
CA12	203963_at	carbonic anhydrase XII	148	0,0031106	49,3	562	0,088
RASGEF1A	242917_at	RasGEF domain family, member 1A	152	0,0032499	7,6	5,8	1,31
RAB15	221810_at	RAB15, member RAS onocogene family	159	0,0034652	29,4	77,5	0,379
RAB33B	221014_s_at	RAB33B, member RAS oncogene family	160	0,0034734	150,2	264,6	0,568
MARK3	202569_s_at	MAP/microtubule affinity-regulating kinase 3	161	0,0034856	338,9	507,7	0,668
MAP1S	218522_s_at	microtubule-associated protein 1S	164	0,0035946	312,9	196,4	1,593
TNFRSF19L	227060_at	tumor necrosis factor receptor superfamily, member 19-like	169	0,0036711	64,3	35,4	1,816
DIDO1	227335_at	death inducer-obliterator 1	182	0,0038991	199,4	387,2	0,515
TPD52L1	210372_s_at	tumor protein D52-like 1	195	0,0041787	44,8	163,5	0,274
RASSF4	221578_at	Ras association (RalGDS/AF-6) domain family 4	202	0,0043726	7,6	4,8	1,583
ALCAM	201951_at	activated leukocyte cell adhesion molecule	215	0,0045987	143,2	508,7	0,282
HIP2	**202347_s_at**	**huntingtin interacting protein 2**	**221**	**0,004798**	**498,5**	**844,9**	**0,59**
USP33	214843_s_at	ubiquitin specific peptidase 33	238	0,0051181	224,2	357,9	0,626
REEP6	226597_at	receptor accessory protein 6	259	0,0057829	3,7	27,2	0,136
BAG5	202984_s_at	BCL2-associated athanogene 5	288	0,0063224	34,9	103,6	0,337
STAT3	208992_s_at	signal transducer and activator of transcription 3 (acute-phase response factor)	291	0,0063687	1258,1	606,8	2,073
RASSF4	226436_at	Ras association (RalGDS/AF-6) domain family 4	294	0,0064893	1444,1	505,9	2,855
PAPPA	224940_s_at	pregnancy-associated plasma protein A, pappalysin 1	300	0,0066921	37,7	10	3,77
GATA3	209602_s_at	GATA binding protein 3	311	0,0070165	98,1	750,3	0,131
MAP4K3	218311_at	mitogen-activated protein kinase kinase kinase kinase 3	314	0,0071634	328,4	598,7	0,549
AKT1	207163_s_at	v-akt murine thymoma viral oncogene homolog 1	317	0,0073108	454,3	820,3	0,554
MRAS	206538_at	muscle RAS oncogene homolog	326	0,0075287	51,5	25,2	2,044
FANCA	**236976_at**	**Fanconi anemia, complementation group A**	**330**	**0,0075988**	**93,5**	**31,4**	**2,978**
LTB	207339_s_at	lymphotoxin beta (TNF superfamily, member 3)	334	0,0076744	193	55,1	3,503
MKNK2	218205_s_at	MAP kinase interacting serine/threonine kinase 2	335	0,0076756	1974	3836,5	0,515
SPOCK2	202524_s_at	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	342	0,0077511	146,3	52,7	2,776
IKBKB	209341_s_at	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	348	0,0078492	368,4	836,8	0,44
ICAM1	202637_s_at	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	354	0,0080214	716,1	232,7	3,077
PSMB9	204279_at	proteasome (prosome, macropain) subunit, beta type, 9	359	0,0081462	1940,7	737,3	2,632
MKI67	212020_s_at	antigen identified by monoclonal antibody Ki-67	368	0,0085212	146,1	47,2	3,095
USP53	231817_at	ubiquitin specific peptidase 53	372	0,0086144	97,9	252,8	0,387
STAT1	200887_s_at	signal transducer and activator of transcription 1, 91kDa	380	0,0087024	5674,1	3224,6	1,76
GPR176	227846_at	G protein-coupled receptor 176	389	0,0089517	268,5	110,5	2,43
ERBB2IP	217941_s_at	erbb2 interacting protein	391	0,0089844	1153,5	1731,7	0,666
SELENBP1	214433_s_at	selenium binding protein 1	410	0,0095837	136,6	566,8	0,241

Full list contains 423 genes (see Supplementary Table 8). Only characterized genes with potential or proven association with cancer are shown. [1]Geometrical mean of signal intensities observed in tumor samples from woman with *BRCA1* mutation; [2]Geometrical mean of signal intensities observed in samples from woman without *BRCA1* mutation; [3]Ratio of geometrical means of signals: "*BRCA1*-mutated" to "non-mutated".

Table 5. Results of real-time RT-PCR validation

Gene	*BRCA1*-mutated, ER-negative: n=10 median (IQR)	non-mutated, ER-negative: n=12 median (IQR)	non-mutated, ER-positive: n=5 median (IQR)	p-value ER-negative only: *BRCA1*-mutated *vs.* non-mutated	p-value all tumors: *BRCA1*-mutated *vs.* non-mutated	p-value all tumors: ER-positive *vs.* ER-negative
TOB1	1.01 (0.71)	1.65 (0.92)	2.61 (3.12)	0.04	0.004	0.001
HIP2	0.30 (0.19)	0.47 (0.27)	0.41 (0.44)	0.04	0.07	n.s.
FANCA	0.69 (2.24)	4.49 (5.51)	0.38 (2.32)	n.s.	n.s.	n.s.

Data are presented as median values, with interquartile range (IQR) given in parentheses. Kolmogorov-Smirnov non-parametric test with exact two-sided p-values was used to estimate significance of the differences; n.s. – non significant

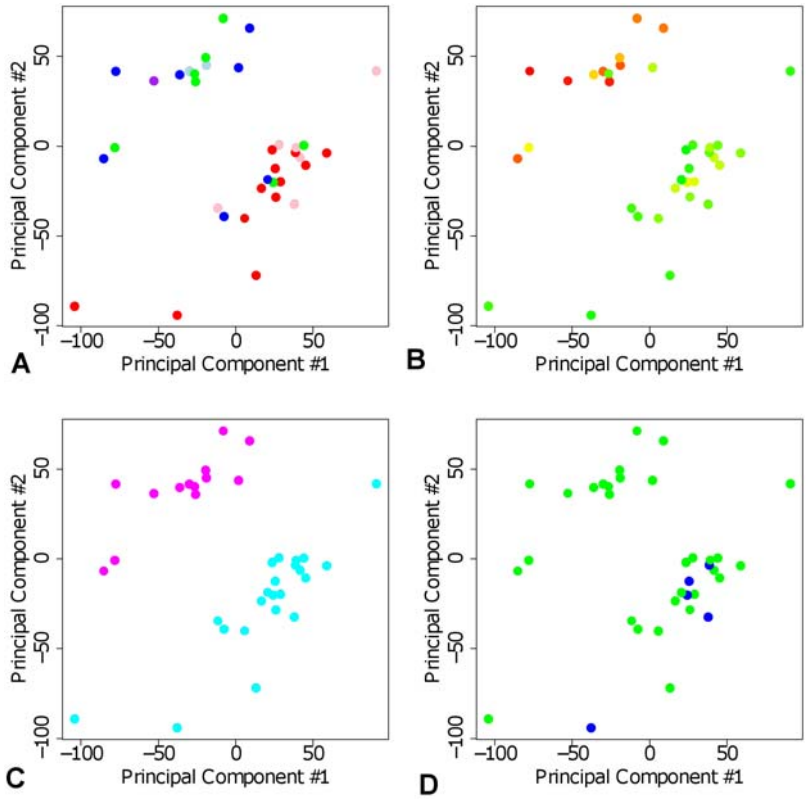

Figure 1. Principal Component Analysis reveals that major sources of variability in gene expression are related to the molecular subtype of tumor and estrogen receptor activity. Thirty five breast cancer samples were analyzed. First two principal components are shown. The distance between the samples reflects differences in gene expression profile. A. Tumor samples are colored according to hereditary *versus* sporadic status and BRCA genes truncation: BRCA1-mutated tumors – red, BRCA1-methylated tumors – pink, BRCA2-mutated – purple, BRCAx – blue, FCA – light blue, sporadic breast cancer – green. B. Samples are colored according to the estrogen receptor expression level, as measured by microarrays: red – highest expression, green – lowest expression. C. Samples are colored according to the molecular subtype: cyan – basal-like molecular subtype magenta – luminal-like molecular subtype (as defined by Sorlie *et al.* (7)). D. Samples are colored according to the histopatological type: medullary breast ca. – blue, ductal breast ca. – green

within the other cluster (Figure 1A.). When the samples were color-coded according to ER expression level as measured by microarrays, we observed that the clusters clearly differed in this aspect: the first cluster contained samples with low or negative ER expression, while the second consisted mostly of samples with high or medium expression level (Figure 1B.) When we marked the samples according to their molecular subtype (Figure 1C), it appeared that the basal *versus* luminal difference was the major factor that determined the distribution of the cancer samples into the two clusters. Thus, PCA confirmed that major sources of gene expression variability in breast cancer are ER expression level and molecular subtype, while tumors with BRCA1 gene inactivated by mutation or promoter methylation build the subgroup among other ER-negative, basal-like cancers.

5. DISCUSSION

The analysis presented by Hedenfalk *et al.* (5) is a landmark microarray study concerning hereditary cancer. These authors showed that hereditary breast tumors with proven mutation of *BRCA1* gene differ significantly in terms of general gene expression from samples with

BRCA2 mutation. The authors also pointed out that tumors from both *BRCA1* or *BRCA2* mutation-carriers differ from sporadic breast cancers. This problem was not directly addressed in any further microarray studies.

Thus, we aimed to verify the hypothesis of Hedenfalk and coauthors. However, in the Polish population *BRCA2*-induced breast cancer cases are very rare (e.g. sequencing of 100 DNA samples from patients with strong familial history of breast/ovarian cancer performed at the M. Sklodowska-Curie Memorial Cancer Center in Gliwice revealed 29 mutations in *BRCA1* gene and only 3 mutations in *BRCA2* gene, see also (17)). For this reason, we decided to ask, whether sporadic tumors differ from *BRCA1*-induced hereditary breast cancer in their gene expression profile.

In our study, the difference between *BRCA1*-mutated and sporadic tumors appeared to be of borderline significance: one could not exclude that the list of 41 probesets, selected in this comparison, was obtained by chance (p=0.065 in the global test). When we applied the same method of data analysis to the data from (5), we obtained an even shorter list of genes (11 clones) and its significance was also below the limit (p=0.065 in the global test). The same class prediction algorithm was used to classify *BRCA1*-mutated and sporadic tumors in both datasets. Classification accuracy was similarly relatively poor for our dataset and that from (5): 69% and 64%, respectively. Moreover, only two genes were common for the two classifiers: *TOB1* and *ALCAM*. Thus it may be concluded that the difference in gene expression profile of *BRCA1*-mutated and sporadic breast cancer is very discrete.

We were curious how it happened that in the previous study (5) the authors observed such a distinct difference in gene expression pattern between *BRCA1*-mutated, *BRCA2*-mutated and sporadic tumors. We assumed that other sources of variability and sample stratification might have significant impact on their results. One should take into account that *BRCA1* mutation-evoked breast cancer is typically ER-negative, while over a half of the population of sporadic breast cancer is ER-positive. Numerous microarray studies have shown ER status of breast cancer to be a very strong source of variability in gene expression pattern (18-20). In the meantime it also became obvious that ER-status and molecular subtype (as initially described by Sorlie *et al.* (7)) are tightly connected: lack of estrogen receptor expression is one of the hallmarks of basal-like tumors. In our study all tumors with *BRCA1* mutation were ER-negative. Among the remaining samples there were 6 ER-positive tumors (17% of all samples); however, we excluded them from most comparisons or corrected the analyses for ER status. On the contrary, in the study of Hedenfalk *et al.*, 45% of samples were ER-positive (5). In addition there were distinct differences in the frequency of ER-positive tumors between *BRCA1*-mutated group (none of 7 samples), sporadic group (4 of 7 samples) and *BRCA2*-mutated group (6 of 8 samples) in their study. We conclude that a weak concordance between our and their results comes from the fact that the pivotal influence of ER status have been regarded in the first study while not in the second.

The results of Q-PCR validation further supported our general conclusion that the differences in gene expression profile between *BRCA1*-mutation linked and sporadic breast cancers are of rather small scale and are strongly influenced by other sources of variability. The two genes, *TOB1* and *HIP2,* showed slightly different expression in *BRCA1*-mutated tumors in comparison to non-mutated ones (p=0.04) when we analyzed only the samples from ER-negative tumors. However, *TOB1* showed strong difference between ER-negative and ER-positive tumors (p=0.001). If ER-positive samples were also included in the analysis, the apparent difference between *BRCA1*-mutated and other tumors seemed to be an order of magnitude higher than previously (p=0.004). This phenomenon illustrates our opinion that majority of difference in gene expression profile usually ascribed to the presence of BRCA1 mutation may be a derivate of the difference in ER status and/or basal versus luminal difference. Second gene, *HIP2* showed no differences between ER-positive and ER-negative samples. Thus, when analyzing its expression in a mixed population of tumors, the difference between BRCA1-mutated and non-mutated samples was of the same significance like in the analysis done on ER-negative group (p=0,04). In conclusion, *HIP2* appears to be truly related to the *BRCA1* mutation status but difference in its expression level is weak.

Despite its probably higher dependence on the ER status than on *BRCA1* mutation status, *TOB1* gene may be of special interest. It was selected in the study of Hedenfalk and confirmed in our reanalysis of their dataset as well as in the analysis of our own dataset. TOB1 is an antiproliferative protein that probably acts *via* transcriptional repression of several signaling pathways and by controlling post-transcriptional stability of target mRNAs (21-23). Following an *in vitro* studies, TOB1 was proposed as a novel radio-sensitizer, suitable for breast cancer therapy (24). Altogether, these facts suggest that TOB1 may be considered as a potential marker and/or therapeutic target in a selected cases of breast cancers.

Interestingly, our BRCA1-related signature contained also several genes from the gene set described by Kang *et al.*, in the gastric cancer cell line resistant to DNA-damaging agent, doxorubicin (15). However, overexpression of these genes not obviously must correlate with doxorubicin resistance in BRCA1 mutated breast tumors, as they have impaired DNA repair and generally should be more sensitive to DNA-damaging chemotherapy. Thus, it may be proposed that this gene set may confer not only chemoresistance but some more general properties of cancer cells, although it requires confirmation in further studies.

It should be underlined that the results of our molecular analysis support the opinion of distinguished pathologists who have already suggested close relationship between *BRCA1*-mutated and basal breast cancers (24, 25). In fact, pathological and immunohistochemical characteristics of both types of breast cancer are nearly identical: both are described as predominantly grade 3 ductal or medullary carcinoma, ER-negative, PR-negative,

almost without HER2neu amplification, frequently characterized by p53 mutation and p53 positive IHC staining, lymphocyte infiltrate, comedo-like necrosis, pushing margins and high mitotic indices. Similar are also prognostic uncertainties in patients with *BRCA1*-linked or basal-like breast cancer, each group being of not uniformly poor prognosis (26, 4). At the molecular level, the kinship between BRCA1-mutated and ER-negative basal-like breast tumors is reflected by a nearly common gene expression pattern. Already van't Veer *et al.* noted that *BRCA1*-mutated breast cancer samples, included in their patient outcome analysis, all fell into the cluster of ER-negative tumors. When Sorlie *et al.* (7) reanalyzed the dataset of van't Veer according to the molecular subtype, they noted that *BRCA1*-mutated cancers clustered together with basal-like tumors. Similar results were probably achieved by Desper *et al.* (27) who analyzed the data from (5) using phylogenetic classification tree. They observed that the *BRCA1*-mutated tumors all clustered together in one sub-tree, while *BRCA2*-mutated tumors, in another sub-tree. These clusters were located far from each other, on the distal parts of the tree, while sporadic tumors laid between them, mostly as leaves off main branch. With our present knowledge we may suppose that sporadic tumors of ER-negative and/or basal-like phenotype were located closer to the *BRCA1*-mutated cluster while ER positive and/or luminal-like sporadic tumors were closer to the *BRCA2*-mutated cluster. However, this cannot be confirmed as Desper *et al.* used sample descriptions that differ from the original.

To explain why BRCA1-mutated breast tumors have molecular profile nearly identical as other basal-like tumors one can assume that in the latter the BRCA1 pathway is truncated as well. This may be caused either by mutations or epigenetic inactivation of several crucial genes. Indeed, it appeared that 6 of non-mutated basal-like tumors had BRCA1 promoter methylation. Further studies are needed to identify potential other molecular events affecting BRCA1 pathway in basal-like breast cancer. However, even if we assume that all basal-like tumors have somehow truncated BRCA1 pathway, we must take into account that these tumors are not absolutely homogenous. This conclusion emerged from the analysis of essential biological traits that could be affected in BRCA1-mutated versus other breast tumors. This approach revealed differentially expressed several pathways and gene sets known previously to be connected with the presence of BRCA1 mutation. This were immune response-specific genes and the genes engaged in the pathway "BRCA1-dependent Ub-ligase activity" or belonging to the BRCA1 reporter gene set specified by (6). Thus, it is possible that different events leading to BRCA1 pathway truncation are expressed as slightly different phenotypes of ER-negative and/or basal-like breast cancer.

In conclusion, it may be suggested that the majority of *BRCA1*-linked breast cancers have a molecular profile of basal-like cancer. As a practical consequence, clinical observations and therapeutic recommendations that are true for basal-like breast cancer may also apply to the *BRCA1*-mutated tumors. Further studies are needed to unravel whether and which of BRCA1 partners could be affected in non-mutated basal-like breast cancer, how such putative distinct molecular events account for the heterogeneity of basal-like tumors and why tumors with truncated *BRCA1* pathway develop mostly as basal-like subtype.

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