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DETECTABLE IN-BLOOD BRCA1 METHYLATION AS A BIOMARKER OF BREAST CANCER PREDISPOSITION

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ABSTRACT

Germline BRCA1 mutations are a well-established risk factor for the development of breast cancer. Nevertheless, many patients who present with a clinical phenotype typical of BRCA1-associated tumors do not carry pathogenic BRCA1 mutations. Current risk models are inadequate, highlighting the need for new biomarkers. In this context, blood-based epigenetic markers such as DNA methylation are being explored. Many studies have examined BRCA1 promoter methylation in blood DNA as a BC risk marker. Retrospective analyses report that BRCA1 methylation in blood correlates with higher risk in triple-negative tumors. However, findings remain inconsistent due to numerous technical issues, including methodological variability, assay limitations, and differences in targeted CpG sites. This review highlights the risk of developing breast cancer in women with a methylated BRCA1 promoter in peripheral blood-derived DNA, as well as the potential drawbacks and challenges in this area.

Methodology: Relevant studies were identified through a targeted search of the PubMed database using keywords such as "BRCA1," "methylation," "breast cancer," and "blood DNA." Inclusion criteria comprised studies evaluating BRCA1 promoter methylation in blood-derived DNA in relation to breast cancer risk. Studies analyzing BRCA1 promoter methylation exclusively in tumor tissue or other non-blood specimens were excluded.

KEYWORDS

BRCA1, Methylation, Epigenetics, Breast Cancer

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1. Introduction

Breast cancer (BC) continues to be the most common neoplasm in females, with an estimated 2.3 million new cases each year (Sung et al., 2021).Recent reports suggest that there is a rising incidence of BC, especially in young women who do not carry typical pathogenic mutations associated with BC and do not have family history of the disease that would qualify them for additional screening measures (Ahmad, 2019).

The introduction of germline genetic testing of mutations in intermediate and highly penetrant BC susceptibility genes has identified the genetic causes of 5-10 % of total BC cases leaving pathology of vast majority of cancers unknown. Moreover, with the number of the whole genome sequences increasing exponentially across different populations it is unlikely that new germline pathogenic mutations will be discovered.

This also indicates that other unidentified mechanisms apart from gene mutations underly many both familial and sporadic BCs. (Claus et al., 1996). Current BC risk scores are based on age, family history, reproductive factors (e.g., early menarche, late menopause, late age at first pregnancy), estrogen (endogenous and exogenous), and lifestyle (such as excessive alcohol consumption and too much dietary fat intake). However, the accuracy of these scores remains insufficient for assessing an individual's cancer risk. Identification of novel risk factors of breast cancer is necessary.

A significant volume of research shows that disease-related DNA methylation changes can be used as biomarkers at all stages of clinical disease management (Roy & Tiirikainen, 2020). The application of the methylation biomarkers in detection, personalization of treatment and monitoring of the disease requires the detection of these biomarkers in pathologically changed tissue, thereby restricting their widespread utility. However, detection of the methylation changes that increase cancer susceptibility can be successfully performed in surrogate tissues such as blood (Wong et al., 2020).

The first reports of links of in blood BRCA1 promoter gene methylation were reported in 2008 when Snell et al. have demonstrated the presence of the methylated BRCA1 promoter in patients with familial breast cancer.

In the following years, numerous other studies have reported the detection of in blood methylated BRCA1 promoter gene in BC patients. However, only a small fraction of these were able to find associations with higher BC risk, precluding its potential use as predictor of breast cancer early detection. Finally in 2022, Lønning et al. provided breakthrough results in the field of interests and showed that prospectively detection of in blood methylation of BRCA1 appears to predispose to triple-negative breast cancer (TNBC) (Lønning et al., 2022a).

Although the initial indication of associations between BRCA1 methylation detectable in blood was reported 15 years ago, it is only recently that substantial research evidence was generated to support assessment of epigenetic changes at tumor suppressor genes in healthy tissues as mechanism of cancer predisposition.

This review aims to summarize the findings of studies that assess methylation levels within the BRCA1 promoter region in peripheral blood-derived DNA from breast cancer patients. Additionally, we want to address the question of why convincing evidence for testing methylation of tumor suppressor genes in healthy tissues has only recently become available. This paper will be divided into two sections: the first section will comprehensively summarize the results, and the second section will be dedicated to identifying gaps in our understanding of this topic

2. Summary of the results

We have identified a total number of 23 studies investigating in blood DNA methylation of BRCA1 promoter region in breast cancer patients. Fourteen of those studies have described associations of the risk with the cancer predisposition, with 6 showing clear association. These studies also identified correlations with specific types of breast tumors that accompany BRCA1 pathogenic variants carriers.

Promoter DNA methylation detectable in peripheral blood was associated with a 3.5-fold (95% CI: 1.4–10.5, $p = 0.004$) increased risk of having early onset breast cancer (Wong et al., 2011a). The study has provided evidence for an associations of BRCA1 methylation with BRCA1-like tumors traits. From an initial group of 255 BRCA1 mutation negative early-onset BC patients, the authors created three groups based on having high (≥5), intermediate (4), or low (≤3) amounts of nine BRCA1 mutation-associated morphological features. The prevalence of detectable BRCA1 promoter methylation in peripheral blood DNA was 31% for the high group, 10% for the intermediate group, and 5% for the low group ($p=0.000002$). Methylation levels in the low group were comparable to unaffected controls.

Similar results, with significantly higher both tumor and blood DNA methylation for more than or equal to five BRCA1-like features reported another study (Daniels et al., 2016).

Interestingly, one study conducted on a group of 942 affected women have identified a strong link between methylation status and TNBC cases (adjusted OR 4.70; 95% CI: 3.13–7.07; $p < 0.001$) but did not find such an association in unselected BC cases (OR 1.44; 95% CI: 0.93–2.22; $p = 0.10$) (Glodzik et al., 2020)

It is noteworthy that all the mentioned studies were performed tissue obtained after the patients received their diagnosis and the lack of prospective studies was limiting.

Nonetheless, in 2022 year, Lonning et al provided conceptual breakthrough results strengthening associations with BRCA1-like tumors (Lønning et al., 2022b). In their nested case-control study, authors prospectively analyzed BRCA1 promoter methylation of white blood cells (WBC) DNA in a group of 637 women with TNBC, 511 with high grade serous ovarian cancer (HGSOC) and matched cancer-free controls (1841 for TNBC cases and 2982 for HGSOC, respectively). Median interval from blood withdraw to diagnosis was 9 years for TNBC and 10 years for HGSOC, respectively. The presence of BRCA1 methylation in WBCs was associated with higher risk of TNBC (HR, 2.35; 95% CI, 1.70-3.23; $P < .001$) and HGSOC (HR, 1.93; 95% CI, 1.36-2.73; $P < .001$). The HR was 1.83 (95% CI, 0.92-3.64; $P = .08$) for TNBC diagnosed 5 years before or less and 2.52 (95% CI, 1.75-3.63; $P < .001$) for those who received a diagnosis more than 5 years after blood sampling.

Given the trend of increased methylation frequency towards BRCA1-like tumors such as TNBC, it seems that in-blood BRCA1 promoter methylation should be most prevalent in women diagnosed at an earlier, pre-menopausal age (<50 years).

Our analysis of BC patient ages and methylation in the BRCA1 promoter did not identify a trend towards early onset of BC. Most studies reported no significant association between age at diagnosis and BRCA1

promoter methylation [PMID: 20882403, PMID: 29480000, PMID: 18642075, PMID: 32719340, PMID: 30634417].

Moreover, a considerable number of studies were unable to establish substantial associations between in-blood BRCA1 methylation and the risk of developing breast cancer. Notably, those studies that did not identify such associations generally did not categorize their cohorts based on characteristics typical of BRCA1 mutation carriers, aligning with the previously discussed connections to BRCA1-like tumors.

For example, Cho et ail. in their study on a group of 1021 in situ and invasive BC showed that methylation differences between sporadic breast cancer patients and controls were not statistically significant (Cho et al., 2015). Similar conclusions were reached by Bosviel et al., whose study conducted on 902 BC cases also failed to find evidence that supports the potential use of BRCA1 methylation in WBC DNA as a biomarker of increased risk of BC (Bosviel et al., 2012). These conflicting results were reported in another studies (Kontorovich et al., 2009), (Chen et al., 2006), (Cho et al., 2010).

3. Investigation of the underlying reasons of discrepancies between studies

Despite persistent efforts, there continues to be a lack of concordance about associations of in-blood BRCA1 promoter methylation. An increasing number of studies confirm the links with BRCA1-like tumors, however, there is still no clear evidence of associations with increased risk of BC development in general population. Numerous factors contribute to the existing inconsistencies in the field, with technical aspects of experimental procedures playing a significant role.

It is noteworthy that among the 23 studies analyzed a total number of 10 different technologies were used.

Each of these methods has its own set of characteristics, making the comparison of results obtained across diverse technologies inherently challenging. For example, MSP which is in principle, a simple, sensitive and cost-effective method has significant susceptibility to produce false-positive results (Lan et al., 2014), (Rand et al., 2002). Due to this, caution is warranted, and the results should preferably be validated with another technique. Moreover, MSP amplifies only fully methylated alleles, which limits the detection of heterogeneous methylation (Herman et al., 1996). In turn, pyrosequencing has a great advantage as it allows for quantification within each individual CpG and detection of heterogenous methylation (Colella et al., 2003). However, because of the complicated process of primer design, it is often problematic to analyze the exact regions of interest which may make it impossible to exactly replicate the findings.

Another drawback of pyrosequencing is the problematic dichotomization of the results. As there is no standardized cut-off threshold for classifying samples as methylated or unmethylated, authors on their own establish criteria for defining methylation status. This problem is not unique for pyrosequencing, it is also the case with other quantitative methods, such as Methylight. This is well-illustrated by Wong et al. who demonstrate that the frequency of methylation in women varied according to the cut-off point selected; proportions were 1.8% for threshold ≥1% of methylated regions, 3.5% for ≥ 0.5% and 12.4% for ≥0.1%, respectively (Wong et al., 2011b). When using cutoffs of ≥1% or ≥0.5%, the study revealed no associations between BRCA1 promoter methylation and BC risk. However, when authors altered the threshold and classified women with ≥0.1% methylation as methylated, they identified significant associations, including between hypermethylation of BRCA1 (OR: 1.31; 95% CI: 0.98-1.75) and an increased risk of BC.

Interestingly, Hansmann et al. proposed a practical threshold for the identification of constitutive BRCA1 epimutations (Hansmann et al., 2012). Authors conducted single molecule analysis of the BRCA1 promoter on 13 affected patients 10 with promoter methylation values ≥6% and three with values <6%, and as well on 10 healthy female controls with a methylation level <6%. Nine of the 10 patients with ≥6% BRCA1 promoter methylation had methylated alleles, whereas the 3 patients with <6% methylation and the 10 controls had only unmethylated alleles with single CpG methylation errors. Overall, 65 of 577 analyzed BRCA1 alleles were classified as epimutations in patients with ≥6% promoter methylation while none were found in patients with <6% promoter methylation or in healthy controls. In other words, epimutations were significantly more frequent in patients with ≥6% promoter methylation than in the two other groups ($\chi 2$ test; $P < 0.0000001$).

Another important consideration is the selection of the regions of the gene that were analyzed.

Methylation occurs at many CpG sites throughout DNA and it seems that many of them are not functionally equivalent, even within a single promoter region (Malley et al., 2011a), (van Vlodrop et al., 2011a). Studies report that selection of different, even closely located CpGs for analysis can lead to contradicting information between studies on the associations between DNA hypermethylation, gene expression, and clinical parameters (van Vlodrop et al., 2011b), (Jain et al., 2012), (Malley et al., 2011b).

Vos et al. investigated methylation levels in breast carcinomas tissues of 72 BRCA1/2 mutation carriers and 80 patients with sporadic BC (Vos et al., 2017). Authors examined three different CpGs within the BRCA1 promoter region and correlated each of them with the subtype of cancer and clinicopathological features. The methylation percentages and their distribution varied considerably between the three BRCA1 methylation probes. While two out of the three CpGs showed significantly higher methylation frequency in BRCA1/2 mutated cancers compared to sporadic ones, the third CpG showed more frequent methylation in sporadic cancers. Moreover, methylation within each CpG differed in terms of PR-receptor status, with more frequently detected methylation in only two of three analyzed CpGs in PR-negative tumors.

In turn, Pang et al. in their study carried out on 102 affected women noted differences between hereditary and sporadic BC at specific CpG sites (Pang et al., 2012). The authors investigated methylation levels of 30 independent CpGs within the region of BRCA1 promoter. Some of the investigated CpGs showed significantly higher DNA methylation levels in hereditary BC cases, while other showed opposite results with higher methylation levels in sporadic BC cases. Based on these findings, authors concluded that comparing two types of BC using two different CpG subgroups can yield different results.

There is significant discrepancy in the sites analyzed by individual studies in the field of interest to our group. Although many regions overlap between studies, there is a substantial difference that can directly affect the obtained results. It is also worth mentioning that in many current studies it was unclear or difficult to identify the exact CpGs analyzed by the authors.

Additionally, epigenome-wide studies have reported variation in methylation patterns between populations, including Caucasians, non-Caucasians (Blacks), Hispanics, Arabs, and numerous populations of the African continent (Pepin et al., 2021), (Song et al., 2021),. It is quite likely that the occurrence of BRCA1 promoter methylation varies across European countries as well, as is the case for BRCA1 mutations (Sekine et al., 2021). This fact underlines the necessity to conduct further studies across the under-represented races and illustrates importance of multi-center studies

Variations observed between studies may also be influenced by genetic variations, which can exert an effect on DNA methylation levels in specific genomic regions.

Evidence on inherited nature of methylation changes in BC patients provided Evans et al. study. (Evans et al., 2018). In their investigation, the authors analyzed lymphocyte-derived DNA of 49 unrelated individuals from families affected by breast and ovarian cancer. These individuals had no germline BRCA1/2 pathogenic genetic variants, however, they were showing high probability of harboring them (Manchester score > 34). Following identification hypermethylation in two of them (averages 43% and 41% methylation levels), analysis for epigenetic marks was performed on their families and eleven out of 15 family members demonstrated similar levels of methylation. Subsequent DNA sequencing upstream of the BRCA1 translation start site identified a heterozygous variant c.−107A>T in individuals affected by BRCA1 promoter hypermethylation in both families. This variant occurred in all tested individuals with the hypermethylated BRCA1 in both families and was absent in individuals lacking the hypermethylated allele. No members of the other 47 families carried this variant.

4. Conclusions

In recent years, there has been an increase in incidence of breast cancer, especially among young women. Current guidelines that determine screening frequency and preventive mastectomies are mainly based on genetic alterations, such as BRCA1 mutations. For those women who do not carry those pathogenic alterations, but are at increased risk of breast cancer, mainly extensive history of breast and ovarian cancer in first- and second-degree family members, the number of molecular biomarkers is limited. It seems that in this particular subpopulation, testing of in blood BRCA1 methylation might be appropriate. Moreover, epigenetic testing could allow for more precise identification of young patients (<50 years old) without clear risk factors, but who could develop triple-negative breast cancer in the future.

Recent studies suggest that BRCA1 methylation is associated with BRCA1-like cancers, with similar distinct histological features as in BRCA1 mutation carriers. Additionally, multiple other marks characteristic of this breast cancer subtype were detected, both in the blood and tumor-derived DNA, which underlines a possibility of an underlying predisposition and tumorigenic pathway (Scott et al., 2018). In a recent breakthrough study, BRCA1 methylation is present more than 5 years before the diagnosis of cancer, suggesting that it occurs before, and not as a result of cancer (Lønning et al., 2022c). This provides evidence to support the clinical application of BRCA1 methylation screening as it is detectable early enough to warrant altered screening protocols for its carriers.

However, several issues need to be addressed. Firstly, studies need to be replicated on a larger scale, ideally in prospective studies with diverse populations of patients. As for the detection of BRCA1 methylation itself, more data is required which regions within the promoter confer increased risk of breast cancer in order, along with the methylation threshold, above which there is significantly increased risk of cancer. Finally, there is necessity to identify new genetic variants that may be directly related to DNA methylation alterations.

It is likely that in the future risk stratification scores might be combined with methylation testing of high and intermediate penetrance breast cancer susceptibility genes, but presently more data is needed.

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