Constitutional Epimutations: From Rare Events Toward Major Cancer Risk Factors?

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ABSTRACT

Constitutional epimutations are epigenetic aberrations that arise in normal cells prenatally. Two major forms exist: secondary constitutional epimutations (SCEs), associated with *cis*-acting genetic aberrations, and primary constitutional epimutations (PCEs), for which no associated genetic aberrations were identified. Some SCEs have been associated with risk of cancer (*MLH1* and *MSH2* with colon or endometrial cancers, *BRCA1* with familial breast and ovarian cancers), although such epimutations are rare, with a total of <100 cases reported. This contrasts recent findings for PCE, where low-level mosaic *BRCA1* epimutations are recorded in 5%-10% of healthy females across all age groups, including newborns. *BRCA1* PCEs predict an elevated risk of high-grade serous ovarian cancer and triple-negative breast cancer (TNBC) and are estimated to account for about 20% of all TNBCs. A similarly high population frequency is observed for mosaic constitutional epimutations in *MGMT*, occurring as PCE or SCE, but not in *MLH1*. Contrasting *BRCA1* and *MLH1*, a potential association with cancer risk for *MGMT* epimutations is yet unclear. In this review, we provide a summary of findings linking constitutional epimutations to cancer risk with emphasis on PCE. We also highlight challenges in detection of PCE exemplified by low-level mosaic epimutations in *BRCA1* and indicate the need for further studies, hypothesizing that improved knowledge about PCE may add significantly to our understanding of cancer risk, carcinogenesis, and potentially development of other diseases as well.

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INTRODUCTION

The incidence of most cancer forms is increasing worldwide.[1,2] Although lifestyle factors including occupational exposure to toxic agents, dietary habits, alcohol, and other factors have all been associated with cancer risk,[2] the exact roles of individual factors as well as the mechanism by which they execute their effects remain incompletely understood. Some toxic agents (such as cigarette smoking and ultraviolet light exposure) are associated with distinct genomic signatures in certain cancer forms such as lung cancer and melanomas[3]; however, for most cancer forms, defined mutational signatures related to exogenous agents have not been identified. Although cancer risk increases with aging, carcinogenesis evolves over time, and indirect evidence, including immigration studies from different countries revealing a strong impact on cancer risk in second-generation immigrants,[4-7] points toward early-life, potentially prenatal events to be of importance.[8-11]

Cancers are predominantly characterized by their genomic aberrations, but over the past decades, there has also been an increasing focus on epigenetic alterations in cancer biology.[12-15] Although epimutations are frequently observed in cancers, an unresolved question has been to what extent early epimutations in healthy tissue may be cancer risk factors. Elucidating such a role for epimutations may have significant implications to our understanding of the underlying biology of carcinogenesis.

EPIMUTATIONS

An extensive description of the mechanisms governing epigenetic regulation is not the subject of this review, and the readers are referred to other contemporary sources.[16,17] As gene promoter hypermethylation and histone modulation hindering transcription often appear in concert,[18] and since promoter hypermethylation is the epigenetic mechanism, most extensively characterized in human cancers, here, we will use the term epimutation synonymous to aberrant promoter hypermethylation.

CONSTITUTIONAL EPIMUTATIONS

Constitutional epimutations refer to normal tissue epimutations occurring prenatally, generally affecting all three germline layers.[19,20] In brief, these epimutations can be classified into two major groups: primary constitutional epimutations (PCEs), not associated with any genetic aberrations, and secondary constitutional epimutations

(SCEs), associated with a genetic aberration, often located within the promoter or its vicinity, in *cis*.[21-23] Genetic variants that lead to secondary epimutations include single-nucleotide variants and larger structural variants, such as deletions, insertions, and duplications.[23-29] SCEs in *MLH1* and *MHS2* have been identified in families diagnosed with Lynch syndrome, and SCEs in *BRCA1* in a few families with a high incidence of breast/ovarian cancer.[20,25,30] Although SCEs may be found with a high variant epiallele frequency (VEF) in normal tissue (approaching 0.5) reflecting soma-wide hemiallelic methylation, both SCE and PCE have shown mosaicism with variable VEF ranging from <1% to <50% for particular genes, for example, *MLH1*. Therefore, VEF cannot be taken as an indicator of the underlying mechanism.

Normal tissue epimutations likely representing PCE have been detected in promoter regions of many genes.[31-33] However, aside from a small number of patients with documented *MLH1* PCE, including those exhibiting low-VEF epimutation patterns,[34-38] *BRCA1* is the only gene in which PCEs have been confirmed to be associated with an elevated risk of cancer.[39] Notably, these *BRCA1* PCEs all present in a low-level mosaic pattern (Fig 1) with a VEF often below 1%.[39] It should be emphasized that the incidence of low-level mosaic constitutional epimutations remains unknown for most tumor suppressor genes, underlining the need for further studies. The key differences between PCEs and SCEs identified so far are summarized in Table 1.

Constitutional epimutations in the O-6-methylguanine-DNA methyltransferase (*MGMT*) constitute an intermediate case, revealing characteristics of both PCEs and SCEs. Here, low-level mosaic epimutations have been detected with a strong propensity for (but not exclusively located to) the rs16906252 T-variant allele. This raises the question of whether epimutations affecting other genes may also have an allelic skewness with propensity for a particular variant allele.

CONSTITUTIONAL *MLH1* EPIMUTATIONS

Constitutional epimutations affecting *MLH1* have been related to colorectal cancer risk but also to a few cases of endometrial cancers. Although both cases of PCEs and SCEs have been reported, in some individuals, it is unclear whether a genomic aberration exist; thus, *MLH1* PCEs and SCEs will be discussed together.

In an initial report by Gazzoli et al,[40] WBC DNA methylation of the *MLH1* promoter was demonstrated in a Lynch syndrome family member diagnosed with a microsatellite instability (MSI)–positive colon cancer presenting loss of MLH1 protein staining by immunohistochemistry (IHC). Subsequently, *MLH1* methylation has been detected in WBCs of a subset of patients with incident MSI-positive colorectal cancers, in the absence of family history, with VEFs in the range of 20%-50% as well as low-level mosaic epimutations.[35,41-44] Interestingly, Sloane et al[38] reported a young male diagnosed with colorectal cancer harboring constitutional epimutations in about 50% of his alleles, while his mother revealed mosaic *MLH1* methylation in <5% of the alleles.

In 2011, Hitchins et al[24] identified a haplotype harboring tandem nucleotide substitutions, where a c.-27C>A variant was the likely cause of *MLH1* methylation and cancer diagnosis in a family with Lynch syndrome. Subsequently, this variant has been detected in several independent families of European ancestry, with a haplotype indicating a common ancestor,[45] and is now subject to panel screening. In addition, cases revealing *MLH1* epimutations in concert with other large rearrangements of the *MLH1* gene have been reported[23,28,29,46] as well as individuals harboring high-VEF *MLH1* normal tissue epimutations without any associated genetic factor and negative for any familial cancer history.[43,47] Thus, the quantitative contribution of genetic aberrations to *MLH1* constitutional epimutations remains open.

Although up to 15% of all colon cancers are defined as MSI+, and the majority of the MSI+ colon cancers carry methylation of the *MLH1* promoter,[48,49] constitutional epimutations only account for a minor fraction: to this end, <100 individuals with constitutional *MLH1* methylation and concurrent colorectal or endometrial cancer have been reported in the literature.[24,27-29,34,35,41,42,44,47,50-56] However, many patients with constitutional *MLH1* methylation are diagnosed at younger age and the possibility of constitutional epimutations should be considered among young patients diagnosed with a MSI+, *MLH1*-hypermethylated colon cancer.[35]

Regarding endometrial cancer, *MLH1* epimutations are found in the tumor tissue of up to 30% of cases,[57,58] but their constitutional origin has been confirmed in a handful of cases only,[34,44,51] with a preponderance for young age.

Taken together, among colorectal and endometrial cancers harboring tumor *MLH1* epimutations, the epimutation has been proven constitutional in a minor fraction of cases.[34,42,44] Notably, <1% of newborns reveal *MLH1* epimutations in their umbilical cord blood (Nikolaienko et al, unpublished data).

CONSTITUTIONAL *MSH2* EPIMUTATIONS

Constitutional methylation of *MSH2* was first described by Chan et al.[59] In a subsequent study,[30] the same family was further characterized together with an additional set of nine Dutch and Chinese families. Patients in these families all revealed loss of MSH2 staining by IHC and hypermethylation of the *MSH2* promoter within the colorectal cancers, as well as methylation of the *MSH2* promoter across various normal tissues, although to a variable extent. Importantly, all patients carried a deletion in a gene upstream of *MSH2*, *EPCAM*, causing *MSH2* promoter methylation and reduced *MSH2*

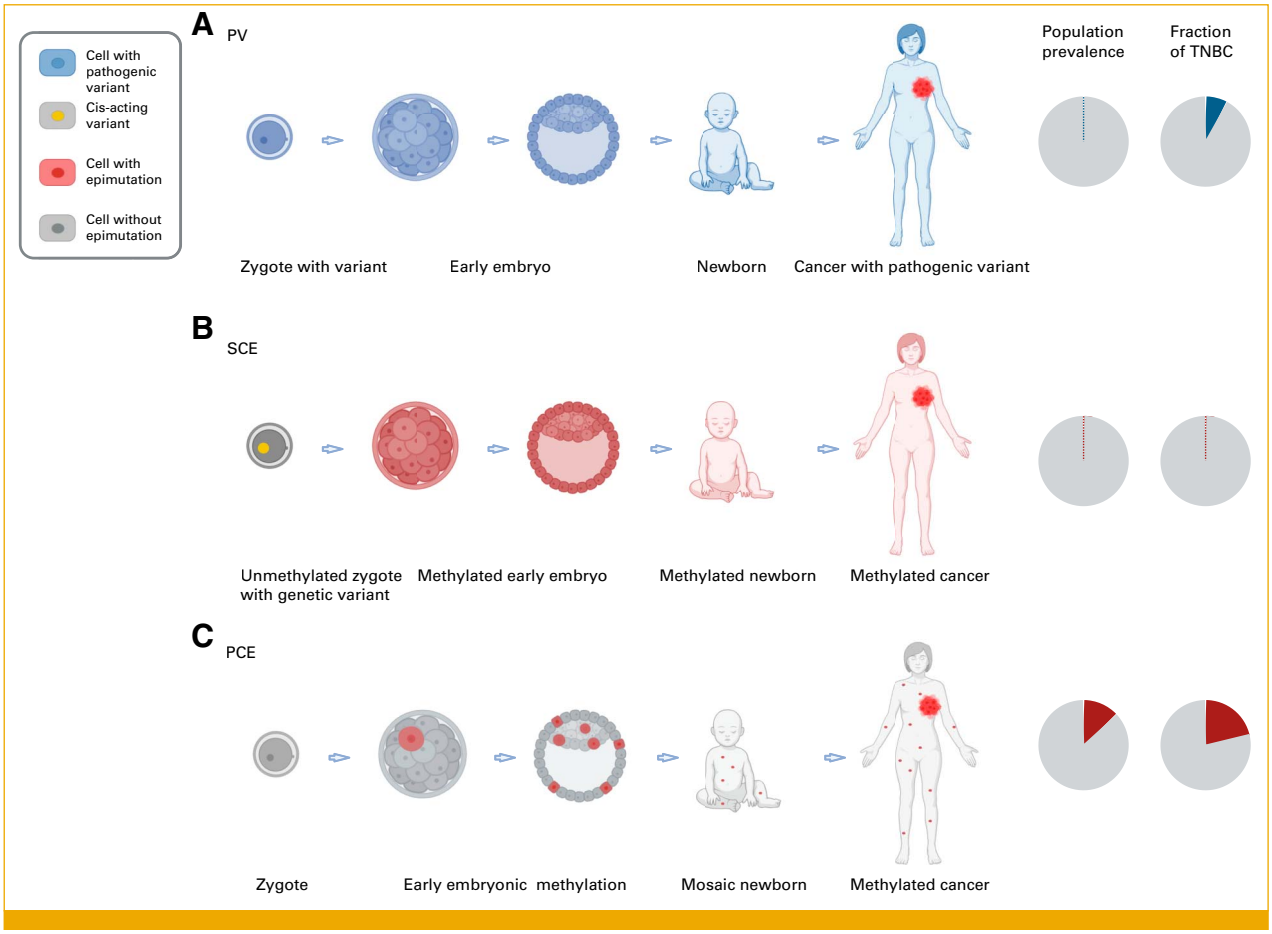

FIG 1. Contribution of (A) germline genetic pathogenic variants (blue), (B) SCEs (red), and (C) PCEs (red) to cancer risk. Contrasting genetic variants and certain SCE, both with a VAF or VEF of 0.5, PCE in *BRCA1*, the most frequent PCE characterized so far, exhibit a low-level mosaic pattern. Furthermore, *BRCA1* PCEs are associated with a lower cancer risk per individual compared with germline mutations and SCE but have a much higher population prevalence and contribute to a higher fraction of cancers. PCEs, primary constitutional epimutations; PV, pathogenic germline variant; SCEs, secondary constitutional epimutations; TNBC, triple-negative breast cancer; VAF or VEF, variant (epi)allele frequency.

transcription in the colon mucosa and subsequent colorectal cancer cells. Further studies have recorded *EPCAM* deletions with different breakpoints to be the underlying cause in about 10% of *MSH2*-deficient colon cancers. These present a variant of the Lynch syndrome with a lifetime risk of colon cancers mirroring the risk associated with pathogenic germline variants in *MLH1* or *MHS2*, albeit with a lower, though significant, risk of endometrial cancers.[60,61]

TABLE 1. Main Differences Between PCEs and SCEs

Characteristic	PCE	SCE
Underlying genetic aberration	No	Yes
Mendelian inheritance	No	Yes
Incidence	High (*BRCA1*), low (*MLH1*)	Low (*BRCA1*, *MLH1*, *MSH2*)
VEF	Low (*BRCA1*), low/high (*MLH1*)	High
Cancer risk (HR)	Moderate	High

NOTE. *MGMT* represents an in-between case, with a high incidence for both PCE and SCE.[151] Also, in WBC, the VEF is low among individuals homozygous for the reference rs16906252 allele or carrying the rs16906252 T variant allele.
Abbreviations: HR, hazard ratio; PCEs, primary constitutional epimutations; SCEs, secondary constitutional epimutations; VEF, variant epiallele frequency.

CONSTITUTIONAL *MGMT* EPIMUTATIONS

MGMT is a tumor suppressor downregulated by promoter methylation in various types of cancers.[62-70] Although germline pathogenic variants (PVs) in *MGMT* have not been detected so far, the T-allele of the SNP rs16906252, located in the first exon of *MGMT*, has been associated with elevated promoter methylation across a panel of solid malignancies.[64,66,67,71,72]

The potential role of *MGMT* constitutional epimutations for cancer risk is unclear. Mirroring findings for *MLH1* (see above), Shen et al[65] detected *MGMT* methylation not only in cancer tissue, but also in normal colon mucosa located 10 cm from the tumor borders. More recently, mosaic *MGMT* methylation associated with the rs16906252 T-allele has also been detected in WBCs of adults as well as newborns.[73,74] In a large study of germline genotypes (WBCs) including a validation cohort, Kuroiwa-Trzmielina et al[69] found the rs16906252 T-allele to be associated with an odds ratio (OR) of 3-4 for developing *MGMT* promoter–methylated colorectal cancer but also a significantly *reduced* risk of developing colorectal cancers without *MGMT* methylation. However, while the authors confirmed a significant association between rs16906252 T-allele and *MGMT* epimutations in normal and cancer tissues, the association of constitutional *MGMT* epimutation status in WBC and cancer risk was not assessed. See recent paper by Nikolaienko et al.[151]

CONSTITUTIONAL *BRCA1* EPIMUTATIONS

The percentage of breast cancers classified as triple-negative breast cancer (TNBC) varies between 10% and 20% among ethnic groups.[75] Sixty percent to 80% of TNBCs reveal the basal-like gene expression signature.[76-78] In addition, mutational signatures reflecting homologous recombination DNA repair deficiency, strongly associated with impaired *BRCA1* function,[79] are reported in between 60% and 80% of all TNBCs.[80-82] However, only between 8% and 40% of all TNBCs (number pending on ethnic group) carry a *BRCA1* PV as an underlying cause of their disease.[81,83,84] Although studies have identified germline PVs in several other genes involved in homologous recombination repair, such as *BRCA2*, *PALB2*, *BRIP1*, *RAD51C*, and *RAD51D*, such mutations,[85] similar to somatic mutations in *BRCA1*, are rare.[81,86] The fact that TNBCs have been meticulously characterized by whole-genome sequencing leaves the likelihood of identifying new unknown genetic aberrations low, indicating that there must be other underlying causes of a large fraction of cases.

Contrasting *BRCA2* tumor epimutations that are rare events,[19] recent studies have shown 25%-30% of primary TNBCs to harbor *BRCA1* epimutations in the cancer tissue.[87-89] In addition, *BRCA1* hypermethylation was frequently observed among the so-called estrogen-receptor (ER)-low tumors,[88] tumors revealing ER immunostaining between 1% and 10% and shown to have gene expression signatures mirroring TNBCs.[90] By contrast, *BRCA1* epimutations were found to be rare among luminal and human epidermal growth factor receptor 2-overexpressing breast cancers.[91] Primary TNBCs harboring *BRCA1* epimutations have been shown to present gene expression and mutational signatures mirroring those in TNBCs from patients harboring *BRCA1* germline or somatic PVs.[80,81,86,87] Furthermore, conflicting evidence has linked *BRCA1* epimutations to response to PARP inhibitors and platinum-based therapies in primary breast and ovarian cancers,[77,92-95] consistent with epimutations causing *BRCA1* deficiency.[96,97]

In high-grade serous ovarian cancer (HGSOC), germline *BRCA1* and *BRCA2* mutations, respectively, are detected in 8%-15% and 4%-8% only,[98-101] despite approximately 50% revealing homologous recombination repair defects.[102] Among patients not harboring *BRCA1/2* PVs, 9%-20% have been reported to harbor *BRCA1* promoter methylation in the tumor tissue,[103-106] contrasting a low incidence in low-grade tumors.[107]

Secondary constitutional *BRCA1* epimutations have been identified but in a few families only.[25] As for primary epimutations, the presence of mosaic *BRCA1* epimutations in WBCs from patients with breast cancer was first reported in 2008,[108] but this and subsequent studies on patients with breast and ovarian cancers[108-114] enrolled a limited number of participants, preventing risk calculations. In 2018, analyzing a large cohort of patients with ovarian cancer and controls with subsequent validation cohorts,[115] we found low-level mosaic *BRCA1* epimutations to be associated with an OR of 2.2-2.9 for HGSOC, but no increased risk for other types of ovarian cancer. This study, however, like all previous studies, was conducted on WBC samples drawn after diagnosis. Thus, a potential influence of the disease on the *BRCA1* methylation in blood could not be excluded.

In a subsequent population-based nested case-control study in the Women's Health Initiative (WHI), we found WBC *BRCA1* mosaic epimutations in healthy women to be associated with an elevated hazard ratio (HR) for both incident HGSOC (HR, 1.93) and TNBC (HR, 2.35).[39] Similar HRs were found in subgroup analysis of patients from whom WBC samples were collected >5 years before their diagnosis. The findings represent proof-of-concept for *BRCA1* PCE being a cancer risk factor.

The exact mechanism behind these epimutations has not been identified. Although a potential trans-acting genetic aberration may not be excluded, the finding that methylation was independent of *BRCA1* promoter genotype[39,115] as defined by the rs799905 SNP status (Fig 2) together with allelic concordance of methylation between WBC and tumor tissue and a lack of transgenerational association[88] argues against a *cis*- or *trans*-acting factor.

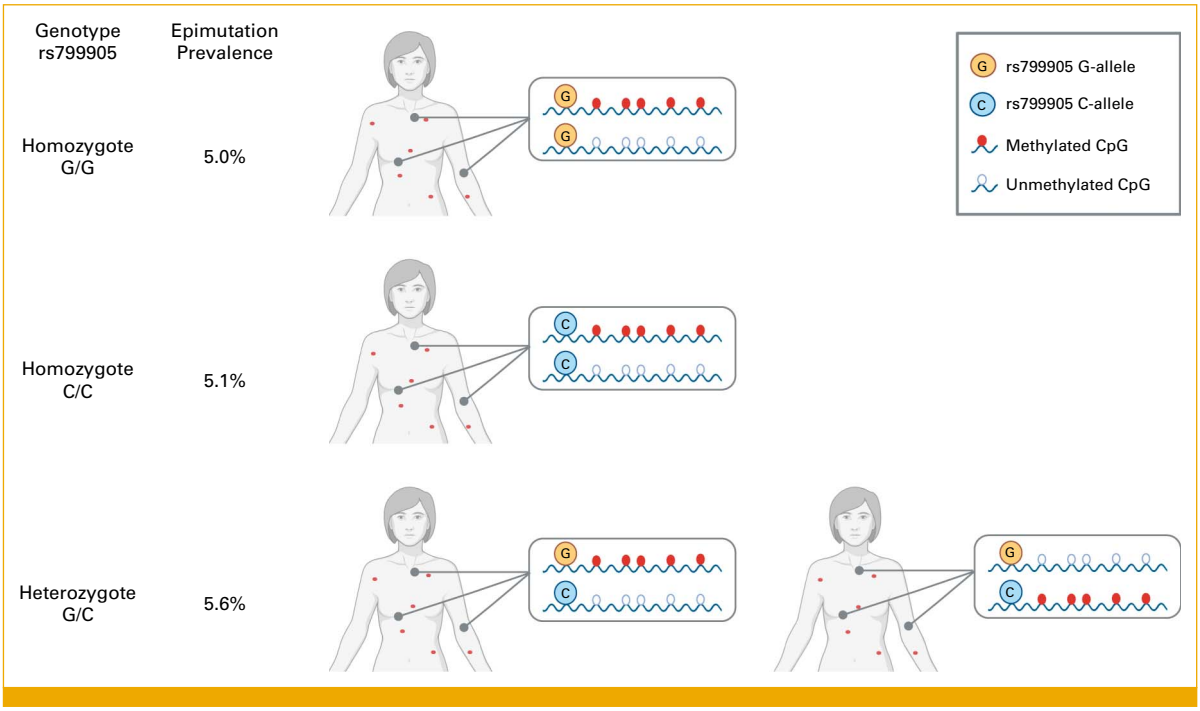

FIG 2. Allele specificity of *BRCA1* epimutations. Interindividually, *BRCA1* epimutations are equally distributed between individuals with different rs799905 genotypes. Intraindividually, in rs799905 heterozygotes, the epimutations are on the same allele in different samples/tissues.

Although low-level mosaic constitutional epimutations are detected also in WBCs in individuals harboring germline *BRCA1* PVs,[39,115] *BRCA1* mutations and epimutations seem to be mutually exclusive in breast cancer tissue from such individuals,[88,89] indicating mutations and epimutations to be independent risk factors.

The somewhat lower HR for HGSOC in the WHI study[39] compared with our previous results[115] raises the question of whether the HRs in the WHI study may represent underestimates. With a median age of 62 years at enrollment for the participants in the WHI study,[39] it is likely that a number of TNBCs and HGSOCs may have been diagnosed before study inclusion. This aligns with the findings by Prajzendanc et al,[116] who reported a HR of 4.7 between hospital-based TNBC patients and healthy controls and a recent study by us,[88] suggesting that about 20% of all TNBCs and ER low-expression tumors may arise from cells harboring constitutional *BRCA1* hypermethylation.

FURTHER CHARACTERISTICS OF PCE

Contrasting SCEs that seem to be a side effect of genetic variants, our understanding of the etiology and dynamics of PCE as well as knowledge about genes affected remains limited. Although PCEs in genes such as *BRCA1*[88] and *MGMT* (Nikolaienko et al, unpublished data) are detected in umbilical cord blood, we do not know whether they may be associated with endogenous or exogenous factors during pregnancy. Furthermore, we do not know whether such epimutations also may arise and/or be eradicated later in life. We detected *BRCA1* WBC epimutations among 8% and 9% of young Norwegian females (age 25-35 years) and newborns, respectively. For reasons unexplained, the incidence in adult and newborn males were about half the incidence in females. No concordance between newborn and parental epimutation status was recorded, excluding Mendelian inheritance of *BRCA1* PCE.[88]

Although conflicting data indicate an age-related drop in epimutation frequency in females,[39,115] more data, preferably including longitudinal samples from normal tissues other than blood, may be needed. If such a drop is confirmed, it may be due to actual demethylation of a promoter, or related to clonal shifts where *BRCA1*-unmethylated clones displace methylated ones, supported by the fact that X-chromosome inactivation skewness and clonal hematopoiesis are known to increase with aging.[117,118] A selective loss of WBC epimutations may have significant implications to our interpretation of tumor methylation data. Apart from TNBCs harboring constitutional epimutations, we found about 10% of TNBCs to harbor tumor but not WBC *BRCA1* epimutations.[88] In case *BRCA1* epimutations may be selectively lost in WBC, even more TNBCs than currently estimated may arise from *BRCA1*-epimutated breast cells (Fig 3).

As noted above, WBC constitutional *MLH1* epimutations are detected in <1% of adults and newborns. Although several

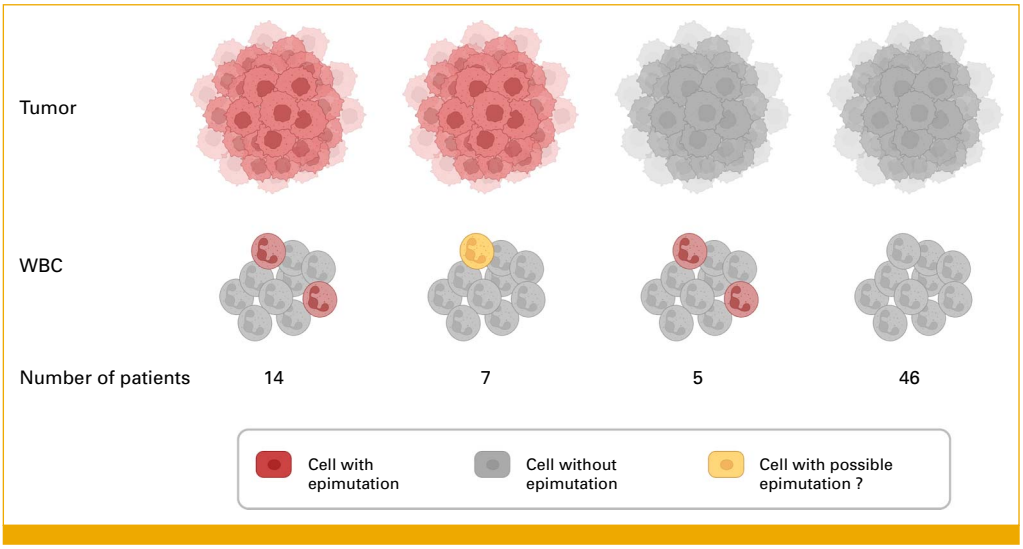

FIG 3. *BRCA1* epimutations (red) in tumors and matched WBC. Data from samples of n = 72 patients with TNBC or ER-low breast cancers (ER <10%) reported in the study by Nikolaienko et al.[88] Tumor biology in the n = 7 *BRCA1*-epimutated tumors from individuals without WBC *BRCA1* epimutations was not different from the n = 14 *BRCA1*-epimutated tumors in individuals *with* concordant WBC epimutations. With the low-VEF mosaic *BRCA1* epimutations approaching detection limit in many patients, it remains a possibility that some (or all) of the n = 7 patients may actually harbor WBC *BRCA1* epimutations, but with a VEF below current detection limits (yellow), meaning that these tumors may have arisen from constitutionally *BRCA1*-epimutated cells as well. ER, estrogen receptor; TNBC, triple-negative breast cancer; VEF, variant epiallele frequency; XXX, triple X-chromosome.

studies have shown similar intraindividual *MLH1* epimutation status in WBCs and other normal tissue types,[50-52] low-level *MLH1* epimutations have also been detected in some patients harboring MSI+ colon cancers without concordant WBC epimutations.[119] This might be due to differences in clonal expansion of epimutation-carrying cells in different tissues. On the contrary, the possibility exists that some of these cases may reflect epimutations occurring later (after the split of germ layers), resulting in tissue-specific epimutations.

METHODOLOGICAL CHALLENGES IN DETECTING LOW-LEVEL MOSAIC PCE

The observed incidence of low-level mosaic PCE depends on the sensitivity of the assay and the VEF cutoff for sample positivity applied. Using a highly sensitive NGS-based assay,[120] we detected WBC *BRCA1* epimutations with a VEF as low as 0.1% in 5.5% of noncancer US women (WHI; median age, 63 years; range, 50-79 years).[39] By contrast, applying commonly used methylation arrays such as the Illumina 450K or 850K (EPIC), most of these low-level *BRCA1* epimutations would remain undetectable.[120]

A second issue is potential disease-associated alterations in WBC fractions. Thus, studies applying genome-wide methylation analyses have detected differences in WBC DNA methylation related to incident cancers, likely because of changes in WBC fractions.[121-128] Although no variation in *BRCA1* promoter methylation related to WBC subfractions was recorded neither in newborns nor adults,[115,125,126] a similar validation is warranted for all PCE-affected genes.

EVALUATING PCE AND SCE AS CANCER RISK FACTORS

The differences in molecular characteristics between low-level mosaic PCEs and SCEs make it necessary to apply different study designs when evaluating their potential associations with cancer risk. Considering SCEs, their association with cancer risk is detected by studies confirming (1) an association between a genetic variant and the epimutations, and (2) family segregation between the variant/epimutation and cancer. This has been exemplified both for cancers of the colon[24,30] and the breast/ovary.[25] By contrast, mosaic low-VEF PCEs such as those recorded in the *BRCA1* gene are associated with a moderately increased cancer risk (HR of 2-5; see below). The fact that PCEs occur independently of genetic variants means that such epimutations are unlikely to cause familial aggregation of cancer. Thus, an elevated cancer risk associated with primary epimutations must be assessed in population-based studies (Fig 4) like nested case-control studies.[39] Also, to confirm a PCE to be a cancer risk factor, a number of additional characteristics need to be confirmed, as outlined in Figure 5.

Proof of concept for a PCE to be a cancer risk factor warrants at least one study confirming incident cancer risk related to epimutations in normal tissue DNA sampled from patients

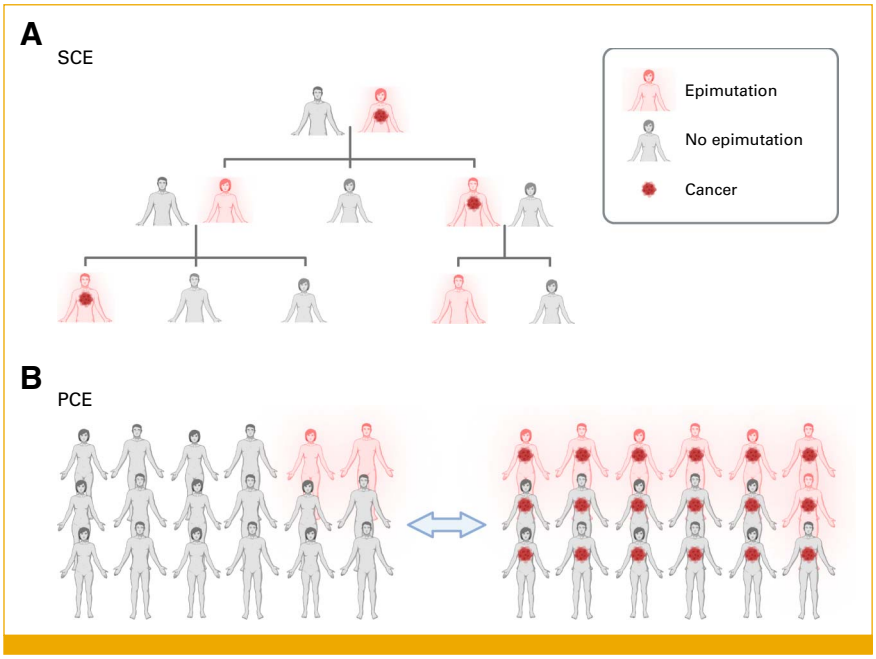

FIG 4. Different models to explore potential contribution of SCE and PCE to cancer risk. (A) SCEs, similar to germline pathogenic variants, are likely detected by their familial segregation with cancer. (B) PCE, not inherited in a Mendelian pattern and associated with a modestly elevated risk, must be characterized population-wide, for example, in nested case-control studies. Importantly, to confirm a PCE to be a cancer risk factor, there should be at least one study confirming an association with subsequent incident cancers in individuals not diagnosed with malignant disease at the time of sampling. PCE, primary constitutional epimutation; SCE, secondary constitutional epimutation.

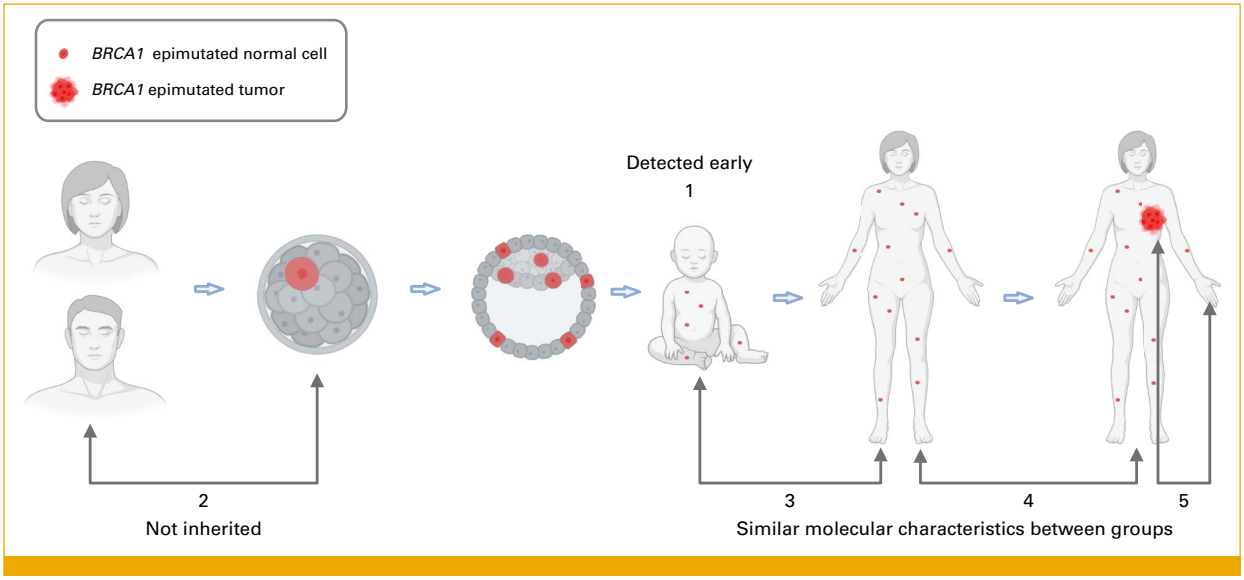

FIG 5. Strategies for defining a constitutional epimutation as a PCE. We believe the following characteristics (apart from lack of association to a genetic aberration and association to cancer risk) must be fulfilled: (1) detection of epimutation in newborns, (2) lack of transgenerational association, indicating lack of Mendelian heritage, (3) qualitatively similar epimutation pattern in newborns and adults, (4) qualitatively similar epimutation pattern in normal tissue from healthy controls, individuals subsequently diagnosed with incident cancer, and patients with cancer, and (5) allelic concordance of epimutations between cancer and normal tissue from the same patient. PCE, primary constitutional epimutation.

before a diagnosis of incident cancer to eliminate potential secondary disease-related effects. We believe, however, that a positive finding indirectly validates use of normal tissue samples collected after diagnosis for further epimutation analysis of the same gene, provided that a similar methylation pattern and incidence is recorded in patients diagnosed with their cancers as recorded in noncancer individuals developing incident cancers. Collection of WBCs from patients after diagnosis may be useful when studying paired tumor and WBC samples from the same patients.[88] Although the risk of potential tumor DNA contamination, either from cell-free DNA or circulating tumor cells, in patients with an established cancer should be recognized, the risk is low taking into account the fraction of circulating tumor cells among all blood cells, which is estimated to be <1 in a million.[129-131]

As for epimutations with a high VEF approaching 0.5 in normal tissue, a pathogenic role in tumor tissue can be inferred from loss of heterozygosity (LOH) or other marks of inactivation of the unmethylated allele. In case a tumor arises from an epimutated cell in a low-level mosaic individual, one would expect to see a clonal expansion into a tumor revealing a high VEF of the same allele as affected in the normal tissue, in addition to LOH.[88]

Finally, the fact that a tumor appears in an individual carrying a genetic PV or epimutation does not prove a causal relationship. Among *BRCA1/2* germline PV carriers, gene signatures associated with a homologous DNA repair defect are limited to tumors for which an elevated risk has been confirmed with respect to the exact tumor form.[132] The fact that genetic inactivation of some genes (eg, DNA repair genes) leaves discoverable marks such as mutational signatures revealing functional inactivation of these genes proves a mechanistic explanation of carcinogenesis. Thus, epimutations in such genes should be expected to cause the same tumor characteristics as the pathogenic genetic variants, which has been shown for MSI+ in respect to *MLH1* and *MHS2* epimutations in colon and endometrial cancers,[35,57,58,60] and *BRCA1* epimutations in the breast.[87]

AIMS FOR FUTURE STUDIES

The findings that PCEs and SCEs in *MLH1* and *BRCA1* and SCEs in *MSH2* are associated with increased risk of cancer confirm constitutional epimutations to be an underlying cause of cancer. Yet, a number of important questions remain to be addressed:

First, *how* do constitutional epimutations arise? As for SCEs, clearly these are secondary to genomic aberrations. As for PCEs, like those in *BRCA1*, Mendelian inheritance has been excluded.[88] Although PCEs may have arisen randomly, some small studies have indicated family clustering.[128,133] It should be recalled that environmental agents are known to influence DNA methylation prenatally as well as during lifetime[128,134-137]; thus, further studies are warranted to assess a potential role of exogenous as well as endogenous factors.

Second, *when* do PCEs arise? Mosaic mutations with an early embryonic origin have been detected in multiple genes related to different disease conditions including cancer.[138-145] The fact that *BRCA1* primary epimutations seem to affect tissues derived from all germ layers[88,115] is consistent with an early origin, probably occurring during the first 2 weeks after gestation,[146] a time period involving several methylation/demethylation waves.[147]

Third, why are *some genes*, such as *BRCA1*, subject to PCE, while WBC epimutations in other genes, such as *BRCA2*, seem absent?

Fourth, what is the reason for the *gender difference* in PCE frequency for *BRCA1*? And does this relate to PCEs in general?

Fifth, do *germ layer–specific or*, *even*, *tissue-specific PCEs* exist? Although some data from studies on *MLH1* PCEs support this hypothesis, more research into this issue is warranted.

Sixth, how may knowledge on constitutional epimutations influence our *models of genetic influence on phenotype*? A key tool exploring overall genetic contribution to cancer risk, or any other phenotype, is comparing concordance in monozygotic versus dizygotic twin pairs.[148] But the timing of epimutations relative to monozygotic twin split will affect epimutation concordance in monozygotic twins.[146] Therefore, in case epimutations arise before splitting, the current models for genetic contribution to any phenotype, on the basis of twin studies, may be overestimates and should be revised on the basis of epigenetic knowledge.

Seventh, what may be the impact on *prevention*? The currently estimated HRs for TNBC and HGSOC may provide basis for future stratification in personalized screening programs. In a short-term perspective, relevant impact may be achieved for women diagnosed with spontaneous breast cancer, as they are known to have an increased risk for subsequent cancer of the ovary and second breast cancer.[149,150] On the basis of the HR for TNBC and HGSOC related to *BRCA1* constitutional methylation,[39] women diagnosed with TNBC and harboring constitutional *BRCA1* epimutations may be considered at increased risk of a secondary tumor and assessed for special surveillance.

Eighth, are epimutations in other tumor suppressor genes risk factors for *other cancers*? A rational approach would be to start by assessing genes that are epimutated in a certain fraction of a specific tumor type and reveal mosaic normal tissue epimutations at a population frequency enabling testing of the hypothesis.

Ninth, are constitutional epimutations affecting the risk of *diseases other than cancer*?

In conclusion, the finding of *BRCA1* PCEs in 5%-10% of healthy females and their association with TNBC and HGSOC have shown that constitutional epimutations may be a common risk factor for some cancer forms. Further research is warranted for exploring the mechanisms behind PCEs and the potential role of PCEs affecting other genes as risk factors for other cancer forms. Further characterization of PCEs may have significant implications to our understanding of carcinogens and cancer risk with implications to prevention as well as screening.

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