Exploring and exploiting the aberrant DNA methylation profile of endocrine-resistant breast cancer

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KEYWORDS: biomarker ■ breast cancer ■ DNA methylation ■ endocrine resistance

The steroid hormone estrogen is critical for the development and maintenance of the female reproductive system and also has a fundamental role in breast cancer pathogenesis. In 70% of all breast cancer cases, activation of the estrogen receptor (ER) drives cancer cell proliferation and subsequent tumor development [1,2]. ER-positive breast cancer subtypes (luminal A and luminal B) are associated with better overall survival compared with ER-negative subtypes (basal, HER2$^+$ and claudin^{low}), which is in part due to the widespread use of adjuvant endocrine therapy [3]. Endocrine therapies that serve to inhibit ER signaling, such as the selective ER modulator tamoxifen, have been used effectively for almost 40 years and have been proven to reduce the risk of disease recurrence [2,4]. Nevertheless, 28% of luminal A and 43% of luminal B breast cancer patients will exhibit intrinsic or acquired drug resistance and develop distant metastases up to 15 years after initial diagnosis [4]. Metastases most commonly form in the bone, brain, lungs or liver, ruling out surgical intervention for most patients. Current second-line therapeutic strategies remain limited and responses are often short-lived. As such, the median duration of survival from time of relapse is 2.2 and 1.6 years for luminal A and luminal B breast cancer patients, respectively [5]. Endocrine-resistant disease makes up almost a quarter of all breast cancer cases and represents one of the most significant obstacles in breast cancer treatment. Therefore, there is an obvious and urgent need to improve both the way ER-positive breast cancer patients are stratified as responders to endocrine therapy and how endocrine-resistant disease is managed therapeutically. Ideally, this could be achieved with robust biomarkers predictive of treatment response that, in the case of metastatic disease, could be profiled using noninvasive assays of the blood in the absence of a tumor biopsy. Here, we consider how exploring the DNA methylation profile of endocrine-resistant cancer could potentially provide clinically relevant biomarkers to guide new approaches to detection and therapy, as well as advance our understanding of the mechanisms underlying this disease phenotype.

It is well established that profound alterations to the genome-wide DNA methylation landscape occur in early stages of cancer initiation, during cancer progression and also throughout the acquisition of drug resistance [6,7]. These alterations are characterized by global DNA hypomethylation, which promotes genome instability and oncogene activation, and local DNA hypermethylation, typically at CpG island promoters of tumor suppressor genes, which is associated with gene silencing. The detection of aberrant DNA methylation has emerged as the most promising and best developed class of epigenetic biomarkers for cancer detection [8]. Differentially methylated regions (DMRs) of DNA have been successfully characterized as biomarkers for early detection of disease, tumor classification and response to treatment in numerous cancer subtypes (as reviewed in [8,9]). The major advantage of DNA methylation biomarkers is that DNA is inherently stable and can be obtained from numerous sources including tissue, plasma, saliva and urine [9,10]. In patients with metastatic breast cancer, genomic DNA fragments, most likely derived from necrotic or apoptotic cancer cells that carry cancer-specific epigenetic alterations, can be detected and isolated from serum. The concentration of cell-free circulating DNA in plasma from healthy individuals ranges from 10–20 ng/ml, which can increase up to 1000 ng/ml in patients with metastatic disease [11]. Using cell-free circulating DNA, multiple regions of cancer-specific hypermethylated DNA can be readily amplified using

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bisulphite PCR-based technology for diagnostic purposes [12].

Some of the most promising DNA methylation biomarkers are those that are predictive of chemotherapeutic response [13]. In this instance, the methylation status of critical components of key regulatory signaling networks, such as the DNA repair pathway, can be assessed to predict the 'apoptotic threshold' of cancer cells. For example, hypermethylation of *MGMT* is associated with increased sensitivity to the alkylating agent temozolomide in glioblastoma patients [14]. In addition, hypermethylation of *WRN*, another DNA repair gene, is indicative of increased sensitivity to topoisomerase-I inhibitor, irinotecan, in primary colorectal cancer patients [15]. Similarly, hypermethylation of the DNA repair gene *BRCA1* is being assessed as a biomarker of sensitivity to PARP inhibition in breast and ovarian cancer [16].

> *"...the endocrine-resistant cell phenotype is underpinned by remodeling of the epigenome."*

In accordance with these studies, we recently reported that hypermethylation of the estrogen-regulated prosurvival factor, *BCL-2*, provides a potential biomarker of response to cytotoxic therapy in endocrine-resistant breast cancer [17]. While high levels of *BCL-2* are associated with resistance to chemotherapy in multiple cancer-subtypes, its expression in breast cancer is associated with favorable prognosis, ER positivity and low tumor grade. By contrast, low *BCL-2* expression is a recurring feature of endocrine-resistant disease [18]. Using multiple models of endocrine resistance, we determined that the diminished expression of *BCL-2* was associated with hypermethylation of the second exon, in a region that overlapped a CpG island and an ER-binding site. Diminished expression of this prosurvival factor conferred increased sensitivity to current and next-generation antimitotic chemotherapies, paclitaxel and PLK1 inhibitor, BI2536 [17]. Although hypermethylation of the *BCL-2* CpG island has not yet been properly assessed in serum from a large cohort of breast cancer patients, we propose that it could potentially provide a useful positive biomarker of response to cell cycle-specific chemotherapy in endocrine-resistant disease. Interestingly, the detection of *BCL-2* methylation has previously been reported to be a marker of early carcinogenesis in colon cancer tissue [19], in serum samples derived from pancreatic cancer patients [20] and in urinary samples from bladder cancer patients [21], although no association with adjuvant treatment response was assessed.

Significantly, hypermethylation of the *BCL-2* gene is not the only change in DNA methylation to have been reported in endocrine-resistant breast cancer. In fact, recent evidence would suggest that the endocrine-resistant cell phenotype is underpinned by remodeling of the epigenome [22,23]. We hypothesize that if a DNA-methylation signature specific to the endocrine-resistant breast cancer cell phenotype was defined, it could be profiled in patients as a predictive measure of response to endocrine therapy. This would have multiple clinical applications. First, patients that fail on endocrine therapy are often given an alternative endocrine therapy as second-line treatment. For example, if a patient acquired resistance to a selective ER modulator, they would then receive an aromatase inhibitor or selective ER downregulator, since the majority of patients retain expression of the ER [24]. If it were possible to demonstrate whether a cancer had an endocrine-sensitive or -resistant epigenetic signature, the success of such a therapeutic approach could be maximized. Even more importantly, interrogating the status of a panel of endocrine-resistant specific DMRs could prove particularly useful for identifying primary ER-positive breast cancer patients likely to exhibit intrinsic endocrine resistance, or those more likely to acquire resistance throughout treatment, for whom treatment regimens could be suitably modified.

> *"...there is considerable potential for DNA-methylation biomarkers to become the next generation of molecular diagnostics."*

Our studies, and others like them, indicate that the DMRs present in endocrine-resistant breast cancer cells occur within promoter or enhancer regions [17,22,25]. More specifically, we hypothesized that hypermethylation occurs precisely at the ER-binding sites of ER-regulated genes, as is the case for *BCL-2* [17]. It is entirely conceivable that inactivation of these ER-responsive elements on a global scale could facilitate endocrine resistance. Currently, it is not determined whether the DNA methylation profile of endocrine-resistant breast cancer is acquired over the time taken to develop endocrine resistance, or whether it represents the clonal expansion of a colony of cancer cells that feature a lower dependency on ER signaling for survival. One hypothesis is that sparse or 'seeding' methylation

at the DMRs of interest could reflect a propensity to gain extensive methylation that spreads as resistance develops. This in turn could reflect a patient's predisposition to endocrine resistance and, hence, quantification of DNA methylation at these regions could provide a 'resistance risk factor'. If this hypothesis were proven to be correct, methylation could be monitored in a neoadjuvant setting, permitting the identification of patients destined to fail on endocrine therapy. Although it has not been previously reported that seeding methylation can be used to predict treatment response, we have shown that this occurs in a model of early breast cancer [26]. Seeding methylation has been used to predict the early onset of colorectal cancer by monitoring the methylation of the *MLH1* gene and the development of *BRCA1*-like breast cancer using the methylation of *BRCA1* as a risk factor [27,28]. Critically, such low levels of methylation did not affect gene expression in these studies, highlighting a limitation of current molecular diagnostic tools that are based on gene-expression assays.

A major strength in the field of epigenomic research and specifically, epigenetic diagnostics, is the associated technology and resources currently available to researchers. DNA methylation profiling platforms, such as methylated DNA immunoprecipitation sequencing and Illumina's (CA, USA) Infinium® Human Methylation 450K array, have given researchers an efficient and reliable way to characterize and compare global DNA methylation profiles [29,30]. Results from experimental systems, such as endocrine-resistant cell lines, can be validated using genome-wide methylation data from large breast cancer patient cohorts, made publically available by international consortia, such as The Cancer Genome Atlas [31]. The methylation status of candidate biomarkers can then be interrogated at much higher resolution in DNA derived from a variety of different materials including tissue, blood and even formalin-fixed paraffin-embedded samples using a variety of techniques, including clonal-bisulphite sequencing and targeted-bisulphite sequencing or numerous PCR-based procedures, such as methyl-specific PCR, MethyLight, HeavyMethyl or sensitive melting analysis after real-time methyl-specific PCR (as reviewed in [9]). While the sensitivity of these techniques is a major advantage in terms of translation into a clinical application, various technical challenges have hindered the development of standardized diagnostic tools and, to date, very few DNA methylation markers have been implemented in the clinic. Despite this, with the discovery pipeline now in place, there is considerable potential for DNA-methylation biomarkers to become the next generation of molecular diagnostics.

The question remains whether DNA methylation biomarkers will be useful for predicting endocrine sensitivity in primary ER-positive breast cancer patients and chemosensitivity in patients with resistant disease. However, what is truly exciting and should not be overlooked is that, while these studies have significant potential for clinical translation, they will also provide an unprecedented insight into the molecular etiology of endocrine-resistant disease.

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