Epigenetics of Circulating Tumor Cells in Breast Cancer

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Abstract

Liquid biopsy based on the analysis of circulating tumor cells (CTCs) has emerged as an important field of research. Molecular characterization of CTCs can provide insights into cancer biology and biomarkers for the clinic, representing a non-invasive powerful tool for monitoring breast cancer metastasis and predict the therapeutic response. Epigenetic mechanisms play a key role in the control of gene expression and their alteration contributes to cancer development and progression. These epigenetic modifications in CTCs have been described mainly related to modifications of the DNA methylation pattern and changes in the expression profile of noncoding RNAs. Here we summarize the recent findings on the epigenetic characterization of CTCs in breast cancer and their clinical value as tumor biomarkers, and discuss challenges and opportunities in this field.

Keywords

Epigenetics · DNA methylation · Noncoding RNAs · Circulating tumor cells · CTC · Liquid biopsy · Breast cancer

8.1 Introduction

Breast cancer is the most common tumor diagnosed in women, with 2.1 million newly diagnosed cases in 2018, and it is the main cause of cancer death in females worldwide [1]. Although

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deaths related to this type of tumor have decreased in last years, in part due to the early diagnosis, unfortunately some patients present distant metastasis at the time of diagnosis reducing the possibilities of effective therapy [2]. Breast cancer is considered a multifactorial disease where there is an association with several factors including environmental, hormonal, genetic and epigenetic, diet and lifestyle [3, 4]. According to the gene expression profile, it can be classified into different subtypes and it has been described as a complex and heterogeneous disease with distinct clinical behavior and histopathological features [5, 6].

Although there are some circulating biomarkers (e.g. CA15.3 or BR27.29) to evaluate breast cancer, due to their low sensitivity [7] it is necessary to find new non-invasive biomarkers and mechanisms for the evaluation and characterization of breast cancer. In this sense, in recent years liquid biopsy has emerged as a very important non-invasive tool useful for the clinic and the characterization of tumors [8]. Liquid biopsy refers to the analysis of circulating material in biological fluids that comes from tumors. This methodology incorporates great advantages to the clinical practice, since it allows with high sensitivity and specificity a non-invasive detection of the tumors, the monitoring of therapy response, quantification of minimal residual disease and evaluation of the development of resistances to therapy [9, 10]. Among the tumor material that can reach bloodstream containing tumor-derived information we can find circulating tumor cells (CTCs), circulating DNA (ctDNA), circulating noncoding RNAs (ncRNAs) and microvesicles like exosomes [11–14].

In recent years CTCs have emerged as an important field of cancer research with great implications in cancer progression and metastasis of different tumors, including breast cancer [15, 16]. CTCs are rare cells shed from a primary tumor or metastatic site that circulate through blood to establish in a new tissue to form a metastatic lesion. These cells have variable morphology depending on the cancer type and stage and in blood appear in frequency of 1 or less CTC per 10^6–10^7 leukocytes depending on the disease stage and aggressiveness of the tumor [17, 18]. In addition, CTCs can appear in circulation as single cells or clusters of cells (CTC-clusters), which are associated with higher metastatic potential [19]. Nowadays there are different systems to isolate CTCs mainly based on (i) EpCAM based enrichment, (ii) leukocyte depletion and (iii) size-based enrichment [20–22]. Once isolated, CTCs can be enumerated or characterized at molecular level to provide insights into cancer biology and biomarkers for the clinic [15, 23]. One of the molecular mechanisms that can be disrupted in CTCs is the epigenetic machinery, such as DNA methylation and ncRNAs [24, 25]. Epigenetic mechanisms regulate gene expression in different types of cells and conditions [26], showing in cancer an aberrant epigenetic pattern associated with cancer progression and metastasis [27, 28].

In the field of breast cancer, CTCs have shown a key role to evaluate the disease. Thus, the enumeration of CTCs by the CellSearch® system was approved by FDA as a prognostic biomarker for metastatic breast patients [11]. Beyond abundance of cells, different molecular alterations have been evaluated in CTCs as potential biomarkers in breast cancer. These studies have been mainly focused in non-epigenetic molecular mechanisms, however, recent studies have also evaluated the potential of epigenetic marks in CTCs of breast cancer patients [29, 30], showing to be a hallmark of CTCs. Therefore, in this review we provide an overview of the epigenetic mechanisms in CTCs of breast cancer, mainly DNA methylation and ncRNAs, and their implication in tumor progression and metastasis, as well as their value as clinical biomarkers.

8.2 The Epigenetic Machinery: DNA Methylation and Noncoding RNAs

The term epigenetics was first proposed by Waddington et al. in 1942 [31]. Epigenetics refers to hereditary changes in the activity and expression of genes that occur without altering the DNA sequence [32, 33]. This mechanism plays an important role in regulating the gene expression of many biological processes [26]. Epigenetic mechanisms show several levels of regulation (Fig. 8.1): DNA methylation, histone modifications, posi-

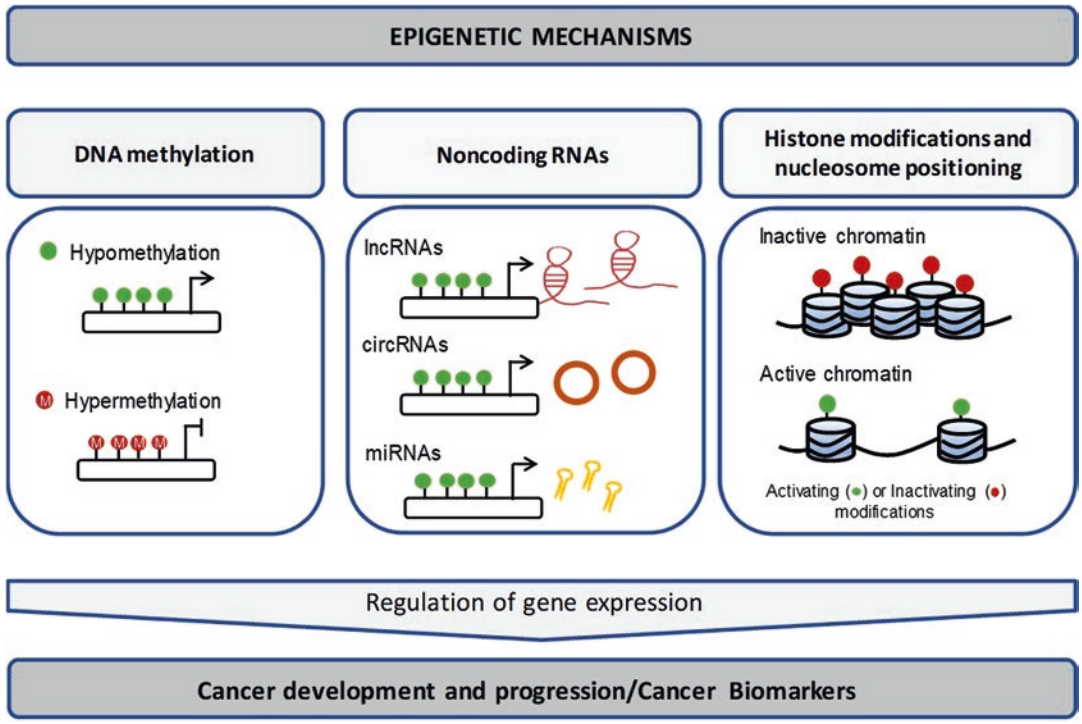

Fig. 8.1 Schematic representation of the epigenetic machinery. Epigenetic mechanisms play a key role in the regulation of gene expression of both coding and noncoding genes. In cancer these epigenetic modifications can be deregulated inducing development and progression of tumors. These epigenetic players can be used as cancer biomarkers for breast cancer and other types of tumors

tioning of the nucleosome and non-coding RNAs (ncRNAs) [34]. In particular, DNA methylation and ncRNAs are two of the most widely studied epigenetic players with important implications in cancer development and progression [9].

8.2.1 DNA Methylation

The best-known epigenetic mechanism is DNA methylation, which is a covalent modification of the DNA resulting from the addition of a methyl group (CH_3) to the 5′ carbon of cytosines in cytosine-phosphate-guanine (CpG) dinucleotides leading to 5-methylcytosine (5mC) [35]. This process is enzymatically regulated by DNA methyltransferase (DNMT) enzymes (DNMT1, DNMT3A and DNMT3B) that catalyze the transference of methyl groups from the S-adenosil-L-metionine (SAM) to the cytosines. The establishment of the DNA methylation profile needs a *de novo* methylation process that is controlled by the enzymes DNMT3A and 3B. On the other hand, the enzyme DNMT1 is responsible for maintaining the methylation patterns during cell division [36, 37]. DNA methylation generally occurs in certain areas of the genome, such as gene promoters, that present a high concentration of CpG dinucleotides defined as CpG islands. However, DNA methylation also occurs in other different genomic regions to maintain the conformation and integrity of the chromosomes, as well as to avoid the potential damage of the mobile genetic elements [38].

DNA methylation mechanism plays an important role in regulating gene expression, which can undergo alterations inducing the development of several diseases, such as cancer [28]. Thus, there are certain regions of the DNA that can gain methylation (hypermethylation) whereas other sequences can loss this methylation mark (hypomethylation) [35]. In cancer, hypermethylation of

promoters in CpG islands is usually linked to the silencing of both coding and noncoding genes [39, 40]. However, genome-wide hypomethylation has been associated with the expression of proto-oncogenes, genomic instability and malignant transformation of tumors [41, 42]. In breast cancer there are some studies that have shown the promoter hypermethylation of certain tumor suppressor genes. Some of these epigenetically regulated genes are Ras-associated domain family member 1A (*RASSF1A*), cyclin D2 (*CCND2*), glutathione S-transferase P1 (*GSTP1*), hypermethylated in cancer 1 (*HIC1*), retinoic acid receptor beta (*RARβ*), and death-associated protein kinase (*DAPK*) [43–48]. For example, the methylation of *RASSF1A* has been associated to the progression of breast cancer and metastasis development [49]. On the other hand, the methylation of GSTP1 has shown to be related with differential response to chemotherapy and the survival of the patients with breast cancer [50].

It is also important to note that DNA methylation is a reversible epigenetic mechanism that can be reversed in human cells by ten-eleven translocations (TET) enzymes. TET enzymes play central roles in regulating gene expression catalyzing the conversion of 5mC to 5-hydroxy-methylcytosine (5hmC) in several tissues [51]. The function of these enzymes can be altered in cancer leading to an imbalance in genomic 5mC/5hmC levels that is associated with oncogenic transformation, including in breast cancer [52]. Importantly, there are also epigenetic-based drugs (epidrugs) that are able to reverse the methylation status of genes inducing hypomethylation [53]. One example is the group of DNA methyltransferase inhibitors (DNMTi), such as the nucleoside analogues 5-azacytidine (5-AZA-CR) and decitabine (5-AZA-CdR), which were the first FDA-approved epidrugs for the treatment of patients with myelodysplastic syndromes and certain leukemias [54].

8.2.2 Noncoding RNAs

In addition to DNA methylation, noncoding RNAs also play an important role in the control of gene expression [55, 56]. It has been postulated that almost 98% of the transcriptome correspond with noncoding transcripts [57]. These noncoding RNAs (ncRNAs) are mainly classified according to their length using 200 nucleotides (nt) as a cutoff. Thus, we can find small ncRNAs (sncRNAs) with less than 200 nt, including microRNAs (miRNAs), small interfering RNA (siRNA) and piwi-interacting RNA (piRNA). And there also long ncRNAs (lncRNAs) with more than 200 nt, including long intergenic ncRNAs (lincRNAs), long intronic ncRNA (intronic lncRNAs) and circular RNAs (circRNA) [58–60].

Among the sncRNAs, microRNAs (miRNA) are the most widely studied. miRNAs (18-25 nt) are single-stranded molecules that bind to specific regions of target messenger RNA (mRNA) and mediate posttranscriptional gene silencing by blocking transcription or degrading mRNA [61]. Through these mechanisms, a single miRNA can regulate the expression of hundreds of genes regulating important features for cancer tumorigenesis [62]. Therefore, microRNAs in cancer can show tumor suppressor ("suppressor-miRs") or oncogenic ("onco-miRs") properties, where onco-miRs are usually over-expressed whereas suppressor-miRs are downregulated [63, 64]. In addition, miRNA signatures have shown to be specifically associated with different types of cancers leading to define the molecular characteristics of tumors [65].

The number of ncRNAs identified in recent years is increasing rapidly. In particular, it has been recently described that lncRNAs constitute the vast majority of the non-coding transcriptome [66]. Although lncRNAs lack the potential to encode proteins, they may exhibit some mRNA-like properties, such as multiexonic gene structures, polyadenylation, presence of 5´ cap and transcription by RNA polymerase II [67, 68]. LncRNAs have important functions controlling gene expression and are associated with a great variety of regulatory functions, such as splicing control and transcriptional regulation [69, 70]. Although most of the lncRNAs have not yet been studied in detail, some of these molecules have been characterized in cancer, showing that they can act as oncogenes (e.g. HOTAIR and

MALAT1) [56, 71] or as tumor suppressor genes (e.g. TP53TG1, LED, LINC-PINT) [40, 70, 72].

Both microRNAs and lncRNAs can be deregulated in breast cancer. In 2005 Iorio et al. identified for the first time the disruption of microRNAs associated to breast cancer. In this work they identified the expression of several microRNAs (e.g. *miR-125b, miR145, miR-21*, and *miR-155*), associated with relevant characteristics of breast cancer including estrogen and progesterone receptor expression, stage of the disease, invasion or proliferation [73]. Since this study several microRNAs have been identified in relation to different breast cancer subtypes [74], as well as the regulation of stemness [75]. Similarly, some lncRNAs have shown aberrant expression associated to breast cancer tumorigenesis. For example, the oncogenic lncRNA *HOTAIR* is highly expressed in breast tumors promoting cancer metastasis [56], invasion [76] and cell proliferation [77]. Some other lncRNAs have shown tumor suppressor functions in breast cancer, such as *GAS5*, which is downregulated in breast tumors inducing proliferation due to the inhibition of apoptosis [78].

8.3 Methods for the Detection of Epigenetic Mechanisms in CTCs

There are a variety of techniques that can be used to detect epigenetic mechanisms either at genome-wide scale or in a specific locus [79–82]. DNA methylation can be analyzed using different approaches based on methods that use bisulfite conversion, restriction enzymes, specific antibodies or nanopore-based single DNA sequencing [83, 84]. Combined with these approaches DNA methylation can be assessed for genome-wide screening with NGS or microarrays systems [40, 85, 86], or for locus-specific assays using different technologies including pyrosequencing, methylation-sensitive high resolution melting (MS-HRM), MethyLight assay, quantitative methylation-specific PCR (qMSP), methylation-specific PCR (MSP) or MethylBEAMing, among others [39, 87–91]. On the other hand, the expression of ncRNAs can be detected at transcriptomic level with NGS (RNA-seq) and microarrays or by means of the analysis of specific transcripts with quantitative methods such as qRT-PCR [40, 92–94]. Due to the differences between methodologies, it is important to consider their advantages and limitations for the selection of the appropriate option [95].

Some of these well-known technologies have already been used in CTCs (Table 8.1) for locus-specific DNA methylation analysis such as MSP, qMSP, HRM and pyrosequencing [24, 96, 97]. However, other new methodologies to analyze DNA methylation in CTCs are emerging. This is the case of the development of a single-cell protocol based on agarose embedded bisulfite treatment (scAEBS) that allows the analysis of DNA methylation of multiple loci using multiplex PCR (multiplexed-scAEBS) [98]. This method is an adaptation of the agarose embedded bisulfite treatment (AEBS) protocol previously described [99] and it is based on bisulfite conversion single-cell methylation analysis. Importantly, the multiplexed-scAEBS allows the detection of allele-specific methylation in different genes of single CTCs [98].

In addition to specific locus, DNA methylation of CTCs can be analyzed at genome-wide level both with microarrays systems and NGS. In this sense, DNA methylation microarrays were used for the analysis of invasive CTCs (iCTCs) after the isolation of these cells with the Vitatex cell-adhesion matrix (CAM) platform [100]. In addition, NGS after bisulfite conversion of DNA has recently shown to be useful for CTC analysis, allowing the detection of multiple CpGs differentially methylated between single CTCs and CTC-clusters [101].

Regarding the analysis of ncRNAs, mainly miRNAs have been analyzed in CTCs. Some of the studies have detected individual transcripts or a panel of specific transcripts using qRT-PCR after the isolation of CTCs with CellSearch® system or immunomagnetic beads [25, 102]. Interestingly, qRT-PCR can also be used after the extraction of miRNAs from CTCs using a Flinders Technology Associates (FTA) Elute Card [103], which is a cellulose paper able to

Table 8.1 Methods more frequently used for detecting epigenetic mechanisms in CTCs

Epigenetic mechanism	Method	Approach	References
DNA methylation	MSP	Target specific	[24, 108]
	qMSP	Target specific	[96]
	HRM	Target specific	[97]
	Pyrosequencing	Target specific	[97]
	Multiplexed-scAEBs	Multiple targets	[24]
	Methylation arrays	Genome-wide	[100]
	NGS	Genome-wide	[101]
miRNAs	qRT-PCR	Target specific	[25, 102]
	ISH-LNA	Target specific	[105, 106]

MSP Methylation-specific PCR, *qMSP* Quantitative methylation-specific PCR, *HRM* High resolution melting, *scAEBS* single-cell agarose-embedded bisulfite sequencing, *NGS* Next-generation sequencing, *qRT-PCR* Quantitative reverse transcription PCR, *ISH-LNA* in situ hybridization combined with LNA probes, *LNA* Locked-nucleic-acid

immobilize cells for the extraction of nucleic acids [104]. Due to its high sensitivity this technique could be useful for the detection of miRNAs in a low number of CTCs [103]. However, other studies have focused on the analysis of miRNAs in CTCs using in situ hybridization (ISH) methodologies. Thus, Ortega et al. developed the first protocol to detect miRNAs in CTCs using ISH (MishCTC) [105]. This method combines the ISH with the immunomagnetic selection of cytokeratins, immunocytochemistry and locked-nucleic-acid (LNA) probes to detect miRNAs expression in CTCs. Other group was also able to adapt an in situ hybridization (ISH) protocol using LNA probes in combination with the CellSearch® CTC detection system, which allows the detection of miRNA expression in individual CTCs [106]. One of the advantages of these methods is the use of LNA probes, which increases the efficiency of hybridization improving the ability to detect miRNA expression [107].

8.4 Deregulation of Epigenetic Mechanisms in CTCs of Breast Cancer

Several studies have shown (Table 8.2) that tumor suppressor genes can be epigenetically disrupted in CTCs of breast cancer patients [15, 30], suggesting that epigenetics is a hallmark of CTCs. This epigenetic alterations in CTCs have been mainly described related to modifications of the DNA methylation pattern of genes [24, 96, 109] and changes in the expression profile of non-coding RNAs, especially microRNAs [25, 106] (Fig. 8.2). DNA methylation and ncRNA expression may provide insights into the molecular mechanisms of metastasis and epithelial-mesenchymal transition (EMT), with important therapeutic implications [110, 111]. This is a very promising field with many classes of epigenetic modifications little or nothing explored in CTCs that could significantly contribute to decipher the mechanisms underlying cancer progression and metastasis [101].

8.4.1 DNA Methylation in CTCs

Chimonidou et al. provided for the first time that tumor suppressor and metastasis suppressor genes can be methylated in CTCs [24], opening new avenues in the field for the study of DNA methylation in CTCs of cancer patients. After isolating CTCs from peripheral blood of metastatic breast cancer patients using an EpCAM immunomagnetical based assay, this group analyzed the promoter methylation status of a panel of three tumor suppressors by methylation-specific PCR (MSP). One of the genes analyzed was cystatin E/M (*CST6*), which has been described as a tumor suppressor gene in breast cancer [112] inhibiting proliferation, migration and invasion related to breast cancer bone metastasis [113]. The other genes studied were, SRY-box containing gene 17 (*SOX17*) and breast cancer metastasis suppressor gene 1 (*BRMS1*),

Table 8.2 Epigenetic alterations and biomarkers in CTCs of breast cancer

Gene	CTC approach	Epigenetic approach	Epigenetic alteration and relevance	References
CST6 *BRMS1* *SOX17*	EpCAM immunomagnetical based assay	MSP	CpG methylation. Association with disease stage	[24]
BRMS1	Peripheral bood cytospins	MSP	CpG methylation. Prognostic biomarker	[108]
CST6 *ITIH5* *RASSF1*	Size-based microfilter	Pyrosequencing	CpG methylation. Prognostic biomarker	[150]
ESR1	EpCAM+ CTCs and CellSearch®	qMSP	CpG methylation. Predictive biomarker of therapy response	[23]
miR-200c/141 *miR-200b/a/429* *CDH1*	CellSearch® and FACS sorting	Multiplexed-scAEBS	CpG methylation. Epigenetic regulation of EMT-associated genes	[98]
Binding sites for: *OCT4* *NANOG* *SOX2* *SIN3A*	Microfluidic-based method	NGS	CpG methylation. Different methylation in single CTCs and CTC-clusters. Potential therapeutic target	[101]
Panel of miRNAs	CellSearch®	qRT-PCR	Overexpression. Potential as epigenetic biomarkers	[25]
miR-21 *miR-146a* *Mir-200c* *miR-210*	EpCAM immunomagnetical based assay	qRT-PCR	Overexpression. Potential as epigenetic biomarkers	[102]
miR-10b	CellSearch®	ISH-LNA	Overexpression. Potential as epigenetic biomarkers	[106]

MSP Methylation-specific PCR, *qMSP* Quantitative methylation-specific PCR, *scAEBS* single-cell agarose-embedded bisulfite sequencing, *NGS* Next-generation sequencing, *qRT-PCR* Quantitative reverse transcription PCR, *ISH-LNA* in situ hybridization combined with LNA probes, *LNA* Locked-nucleic-acid, *LNA* Locked-nucleic-acid, *EpCAM* Epithelial cell adhesion molecule

with important tumor suppressor functions in breast cancer through the regulation of Wnt/beta-catenin signaling pathway [114] and chromatin remodeling [115, 116], respectively. Importantly, the methylation analysis of these three tumor suppressor genes revealed that CST6, SOX17 and BRMS1 were hypermethylated in CTCs of breast cancer patients [24], which was later confirmed in another work of the same group [117]. In addition, the methylation status of these genes also showed differences between individual patients, indicating that CTCs are characterized by the presence of a heterogeneous methylation pattern [24].

DNA methylation regulates the expression of genes in normal and tumor cells of different types of tumors [42, 118]. However, at this time this issue is not well characterized in CTCs and there are few studies that have evaluated this association. In breast cancer one work revealed some correlation between the methylation of BMRS1 promoter analyzed by MSP and the protein expression levels [108]. In other type of tumor other study showed a high correlation between the loss of methylation in c-Met promoter and gene expression in a CTC cell line [97].

The study of single cells provides the opportunity to analyze the complexity and heterogeneity of cells [109]. In this sense, a recent work was able to analyze the promoter methylation status of three EMT-associated genes (miR-200c/141, miR-200b/a/429 and CDH1) in individual CTCs of breast cancer patients [98]. Using multiplexed-scAEBS they analyzed the methylation status of 159 single CTCs from 11 patients with metastatic breast cancer, evidencing a heterogeneous level

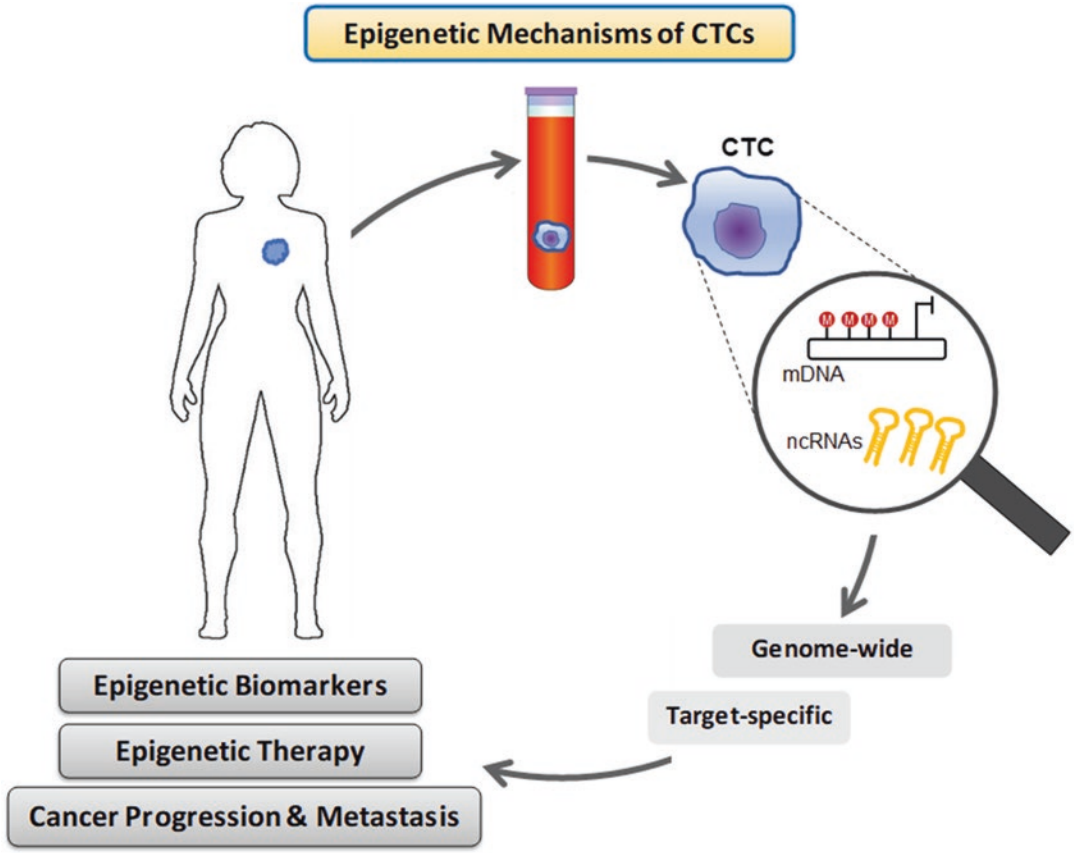

Fig. 8.2 Epigenetic mechanisms in CTCs of breast cancer patients. The CTCs of breast cancer patients undergo alterations of the epigenetic mechanisms, such as DNA methylation and ncRNA expression. These type of epigenetic players can be characterized in CTCs using epigenomic approaches (genome-wide) or target-specific assays. The identification of aberrant epigenetic profiles can provide insights into cancer biology and render tumor biomarkers and epigenetic therapeutic targets with an important clinical value for breast cancer patients. *mDNA* methylated DNA, *ncRNAs* noncoding RNAs

of methylation in CTCs, which is in line with previous studies [24].

In different types of cancers, including breast cancer, CTCs can be present in bloodstream as single cells or aggregates of CTCs (CTC-clusters) [19, 119, 120]. In a very recent study the DNA methylation profile of single CTCs and CTC-clusters captured by a microfluidic-based method from breast cancer patients and mouse models was evaluated following a genome-wide DNA methylation approach [101]. The analysis in patient derived-CTCs by NGS revealed a different DNA methylation profile between clusters and single cells, representing a potential therapeutic target. Although the global methylation pattern was similar, they found specific differentially methylated regions in CTC-clusters, showing a hypomethylation pattern in DNA binding sites for transcription factors related to stemness and proliferation (OCT4, NANOG, SOX2, and SIN3A). Importantly, in vitro CTC-cluster dissociation into single cells with the individual treatment of CTC cluster-dissociating compounds (ouabain and digitoxin) induced the DNA methylation reprograming resulting on the hypermethylation of binding sites for OCT4, SOX2, NANOG, and SIN3A, which correlated with a decreased expression of their target genes and metastasis burden. These results also suggested that DNA methylation remodeling was

due to the failure in cell-cell junctions after the treatment with CTC cluster-dissociating compounds [19, 121]. Altogether these results linked the epigenetic regulation of CTC-clusters with and increased accessibility for transcription factors relevant for stemness and promoting metastasis, opening a new scenario to reduce cancer metastasis.

8.4.2 Non-coding RNAs in CTCs

In breast cancer, CTCs have shown to have alterations in the microRNA expression profile. In this sense, Sieuwerts et al. analyzed the profile expression of microRNAs by qRT-PCR in CTCs isolated with the CellSearch® system from metastatic breast cancer patients collected before starting first-line systemic therapy in comparison with healthy blood donors [25]. With this approach they identified the overexpression of 10 miRNAs in CTCs, highlighting the relevance of microRNAs molecular characterization. This study was performed in a bulk of CTCs, however, the detection of microRNAs in individual CTCs is also possible. For this purpose Gasch et al. adapted an in situ hybridization (ISH) protocol using LNA probes combined with the CellSearch® CTC detection system [106]. With this methodology they were able to analyze the expression of miR-10b in individual CTCs isolated from the blood of metastatic breast cancer patients and other types of tumors. They demonstrated for the first time a heterogeneous expression of microRNAs in CTCs isolated from the same patient. Importantly, the analysis of miR-10b+ CTCs could be important for breast cancer patients due to miR-10b has shown association with the development of metastasis [122].

MicroRNAs are key regulators of gene expression involved in cancer metastasis by means of different mechanisms [123]. In addition to mir-10b, other microRNAs related to metastasis have been shown to be altered in CTCs of breast cancer patients. This is the case for miR-21, miR-146a, miR-200c, and miR-210 whose expression in CTCs of breast cancer patients is deregulated controlling important functions of the multistep metastatic process related to migration and invasion. In a recent study the expression of these miRNAs was analyzed using qRT-PCR in CTCs isolated from 55 metastatic breast cancer patients by anti-EpCAM-coated immunomagnetic beads [102]. Interestingly, all miRNAs showed significantly overexpression in CTCs of metastatic breast cancer patients compared to healthy controls, which offers the possibility of better understanding the biology of CTCs.

8.5 Connection Between Epigenetic Alterations of CTCs and Circulating Nucleic Acids

The molecular profile of CTCs and circulating DNA can both present alterations related to tumor disease [102, 108, 124]. In breast cancer several studies have shown that there is an association between the molecular pattern of CTCs and circulating DNA or ncRNAs. For example, mutations in circulating DNA are able to reflect the heterogeneity observed in single CTCs, providing a reflection of the molecular profile observed in CTCs [125]. In this sense, breast cancer patients have shown concordance and complementary information between molecular alterations of CTCs and circulating nucleic acids [126], suggesting that CTCs could contribute to the release of epigenetic and other molecular alterations to bloodstream of cancer patients [96, 127, 128].

The methylation status of particular genes in CTCs has shown correlation with the methylation level of the same genes in circulating DNA and tumor tissue. In breast cancer this connection has been confirmed analyzing the methylation status of the gene *SOX17*, which was highly methylated in primary tumors, and in matched CTCs and circulating DNA [96]. In particular, this study showed significant correlation between *SOX17* methylation in circulating DNA and CTCs in patients with operable breast cancer after surgical removal of the primary tumor. Other study evaluated the gene *BRMS1*, which is a candidate metastasis-suppressing gene with an important

function in promoting migration and invasion [129]. The methylation analysis of *BRMS1* promoter revealed that this gene is hypermethylated in primary tumors of early stage patients and in their corresponding CTC samples, however not in non-tumoral breast tissues [108]. In addition, the methylation status of the genes *APC* and *GSTP1* in circulating DNA correlated with the presence of CTC in the blood of breast cancer patients. Importantly, both methylated DNA and CTC showed association with a more aggressive tumor biology and advanced disease [130]. In line with this, the methylation of other genes in circulating DNA, including *RASSF1A* and *ESR1*, was associated with the detection of CTCs in circulation of breast cancer patients [127].

Similar to DNA methylation, there is a connection between the profile of circulating ncRNAs and CTCs. In this sense, the overexpression of metastasis-related miRNAs, such as *miR-21*, in CTCs of breast cancer patients was associated with the upregulation of these miRNAs in the corresponding plasma [102]. In other work, Madhavan et al. evidenced for the first time that circulating miRNAs can predict the CTC status of patients with metastatic breast cancer. They identified a panel of circulating miRNAs able to differentiate between metastatic breast cancer patients with presence or absence of CTC in blood, showing potential to evaluate the progression-free and overall survival of metastatic breast cancer patients [131].

8.6 Epigenetic Biomarkers in CTCs

Epigenetic mechanisms can be measured in body fluids and are useful as tumor biomarkers in clinical practice mainly to assess the risk of cancer development, detect the presence of a type or subtype of tumor (diagnosis biomarker), evaluate the risk of relapse or disease progression (prognostic biomarker), predict the response to certain therapies (predictive biomarkers) and follow the response to the treatment (monitoring biomarker) [132, 133]. This type of epigenetic biomarkers has an important role for the implementation of a more personalized medicine and precision oncology in different types of tumors, including breast cancer [84, 134, 135] (Fig. 8.2).

Epigenetic biomarkers have relevant characteristics to be useful as tumor biomarkers for the clinic due to their reliability, sensitivity, stability, frequency and noninvasive accessibility in biological fluids [132, 136]. Until now several epigenetic biomarker candidates have been proposed in breast cancer. For example, some genes (*BRCA1* and *RAD51C*) have been described in association with risk assessment and early-onset sporadic disease [137]. In addition, epigenetic biomarkers have also shown to be useful in breast cancer for detection (e.g. *APC*, *RASSF1A*, *DAPK1, miR-21/miR-155/miR-365, HOTAIR*) [138–140], prognosis (e.g. CpG island methylator phenotype, *RASSF1A, miR-21, MALAT1*) [141–144] and for evaluating therapy response (e.g. *BRCA1*, *FERD3L* and *TRIP10* signature, *miR-21*, *miR-125b, HOTAIR*) [145–149].

Epigenetic biomarkers in liquid biopsy are especially important for clinical purposes in cancer in part due to the possibility of analyzing noninvasive samples. Until now most of the epigenetic studies in liquid biopsy have focused in circulating nucleic acids. However, the clinical significance of CTCs has also been studied, suggesting that they are surrogate biomarkers of tumor prognosis and may serve to evaluate the response to chemotherapy [29, 125]. In breast cancer patients the hypermethylation of some genes in CTCs has revealed potential as biomarkers (Table 8.2). This is the case of *CST6*, *SOX17* and *BRMS1* whose methylation status has shown a positive association with the stage of the disease [24]. Importantly, the methylation levels of *BRMS1* promoter in CTCs was also able to provide prognostic information for disease free survival in early breast cancer [108]. In particular, the hypermethylation of *BRMS1* was associated with a lower disease-free survival and worse prognosis, showing a significantly association with a higher incidence of relapses. Similarly, other group identified the methylation status of several genes in CTCs associated with poor progression-free survival (PFS)

in metastatic breast cancer patients [150]. In this work patients with hypermethylation in CTCs of the genes *CST6*, *ITIH5*, or *RASSF1* showed poor PFS compared to those ones with unmethylated CTCs, which could be useful to identify patients at high risk for disease progression. DNA methylation marks have also showed connection with the therapy response in breast cancer [147]. Thus, the hypermethylation of the gene *ESR1* in CTCs was associated with the lack of response to everolimus/exemestane therapy in patients with ER+/HER2- advanced breast cancer [23]. This result evidence the great potential of epigenetic marks of CTCs to evaluate therapy response in cancer. Although there are currently few studies evaluating the potential of ncRNAs in CTCs as biomarkers for breast cancer, the deregulation of microRNA expression observed in CTCs (Table 8.2) also suggests great potential as epigenetic biomarkers of the disease [25, 106].

8.7 Conclusions and Future Perspectives

The field of circulating tumor cells has emerged in recent years as an important topic in cancer research, with great implications in cancer progression and metastasis of breast cancer and other tumors [11, 101, 151]. The molecular characterization of CTCs can be useful to provide insights into cancer biology and to identify tumor biomarkers for the clinic. Epigenetic mechanisms, such as DNA methylation and ncRNAs, have shown to play an important role in metastasis and have also an important clinical value as biomarkers for the detection, prognosis and the evaluation of therapy response [9]. In addition, epigenetic mechanisms have the potential to be reversed representing interesting targets for cancer therapy [53, 111].

There are several methods that can be used to detect epigenetic mechanisms, however only a few of them have been used for the epigenetic characterization of CTCs in breast cancer. Some of these approaches are useful for detecting DNA methylation and miRNA expression in CTCs, based on locus-specific assays or genome-wide analyses. Thus, in the field of breast cancer, DNA methylation and miRNAs have shown to be deregulated in association with cancer progression and metastasis. Interestingly, there is an association between epigenetic alterations of CTCs and the corresponding epigenetic profile detected in bloodstream. This connection suggests that CTCs could contribute to the release of tumoral material with epigenetic alterations to the bloodstream of breast cancer patients [96]. This kind of approach represents an important non-invasive tool for the management and therapy of the breast cancer patients. Although there are relevant advances in the field, studies to evaluate the clinical potential of epigenetic biomarkers in the CTC of patients with breast cancer are still lacking.

The epigenetic characterization of CTCs has been mainly focused in the molecular study of DNA methylation and miRNAs. This type of mechanisms has shown great relevance in breast cancer but there are also other epigenetic players that could bring some light on this tumor, including 5hmC, other types of ncRNAs (e.g. lncRNAs and circular RNAs) and epitranscriptomic modifications (e.g. N6-methyladenosine) [152]. CTCs are rare cells in circulation, therefore the development and improvement of single-cell methods and high sensitive technologies is of great importance to address in depth the complexity of epigenetics in CTCs of breast cancer patients. However, despite the number of existing challenges, the research field on epigenetics of CTCs opens a new scenario to elucidate the mechanisms of metastasis and personalize the management of breast cancer patients.

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