Abstract. Although breast cancer is a heterogeneous disease that is challenging to characterize and treat, the recent explosion of genetic and epigenetic research may help improve these endeavors. In the present review, we use genetic diversity to characterize and classify different types of breast cancer. We also discuss genetic and epigenetic changes that are involved in the development of different breast cancer types and examine how these changes affect prognosis. It appears that while a combination of mutations and copy number changes determine the type of breast cancer, epigenetic alterations may be the primary initiators of cancer development. Understanding these critical biomarkers and molecular changes will advance our ability to effectively treat breast cancer. Next, we examine potential drug therapies directed at epigenetic changes, as such epigenetic drug treatments may prove useful for treating patient-specific tumors, breast cancer progenitor cells, and drug-resistant cells. Lastly, we discuss on mechanisms of carcinogenesis, including a novel hypothesis outlining the role of epigenetics in the development of cancer progenitor cells and metastasis. Overall, breast cancer subtypes may have a similar epigenetic signal that promotes cancer development, and treatment may be most effective if both epigenetic and genetic differences are targeted.

One in eight American women will be diagnosed with breast cancer in her lifetime, making it the most common cancer in women and the second most common cause of cancer mortality among females. One of the challenges of treating breast cancer is the heterogeneity between various tumors (1). Therefore, understanding the critical biomarkers - their role in carcinogenesis and drug resistance as well as their use for diagnosis and treatment - is crucial to breast cancer treatment.

Breast cancer can be classified in multiple ways. Traditionally, classification was histological, separating breast cancers into ductal, lobular, nipple, or not otherwise specified (NOS). However, molecular classification using immunohistochemistry to reflect the hormone-responsiveness of the tumors and other cell markers has become increasingly useful for dictating treatment and prognosis. The molecular subtypes of breast cancer, which are based on the presence or absence of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor-2 (HER2), include: luminal A (ER+ and/or PR+; HER2–), luminal B (ER+ and/or PR+; HER2+), basa-like (ER–, PR–, and HER2–), and HER2-enriched (ER–, PR–, and HER2+) (2, 3).

It is important to note that not all tumors in a specific histological subtype belong in the same molecular classification. Additionally, the hormone responsiveness does not determine the molecular subtype. For example, not all tumors with HER2+ receptors are of the HER2-enriched mRNA subtype, and not all tumors in the HER2-enriched mRNA subtype are clinically HER2 receptor-positive (4).

In addition to the molecular subtypes discussed above, there has been a recent explosion of genetic data that redefines our thinking over the different types of breast cancers, both in terms of classification and treatment. A variety of genetic and epigenetic changes has been implicated in the development of breast cancer. In the present review, we discuss somatic gene mutations, copy number aberrations, exon sequencing changes, alterations in miRNA and protein expression levels, and changes in methylation...

Somatic Mutations

A recent study provides extensive evidence on how somatic mutations are involved in breast cancer. Stephens et al. examined the genomes of 100 tumors for somatic copy number changes and mutation in the coding exons of protein coding genes. They identified 7,421 somatic point-mutations across 21,416 protein-coding genes. The number of somatic mutations varied markedly between individual tumors (5) Many of the well-established cancer genes implicated in breast cancer development, including BRCA1, RB1, TP53, PTEN, AKT1, CDH1, GATA3, and PIK3CA, were found to have somatic mutations. These genes are involved in apoptosis, cell-cycle regulation, and transcription regulation. Other groups of genes responsible for signal transduction including APC, ARID1A, ARID2, ASXL1, BAP1, KRAS, MAP2K4, MLL2, MLL3, NF1, SETD2, SF3B1, SMAD4, and STK11, were also found to contain somatic mutations. Additionally, a new group of genes not previous implicated in breast cancer, were found mutated, including ARID1B, CASP8, MAP3K13, MAP2K13, NCO1, SMARC1, and CDKN1B (5). Among the tumors examined, there were mutations in at least 40 cancer genes with 73 different combinations of mutated cancer genes. This highlights the overwhelming genetic diversity of breast cancer tumors and helps us appreciate the complexity of classifying tumors based on genetic subtype.

Copy Number Aberrations

In another example using somatic mutations to classify different types of breast cancer, Curtis et al. examined the copy number aberrations (CNAs) in approximately 1,000 human breast tumors with long-term clinical follow-up (6). The study was based on the assumption that most heritable gene expression traits are governed by a combination of cis (proximal) loci, defined as those within a 3-megabase (Mb) window surrounding the gene of interest, and trans (distal) loci, defined as those outside of that window (7). They found deletions in the PPP2R2A, MTAP, and MAP2K4 genes and somatic CNAs in approximately 40% of genes, which could be a result of either cis- or trans-acting CNAs. The subtypes devoid of the CNAs had a better prognosis than those with multiple CNAs (6).

Somatic Mutations, Copy Number Aberrations, and Protein Expression

Additional research has revealed both the commonalities and differences of genes and mutation types implicated in various histological subtypes of breast cancer. The Cancer Genome Atlas Network analyzed primary breast cancers by genomic DNA copy number, DNA methylation, exon sequencing, mRNA arrays, microRNA sequencing, and reverse-phase protein arrays, and found that somatic mutations of three genes (TP53, PIK3CA, and GATA3) show a greater than 10% incidence across all breast cancers (4). In addition to identifying nearly all genes previously implicated in breast cancer (PIK3CA, PTEN, AKT1, TP53, GATA3, CDH1, RB1,
containing number changes, including focal amplification of regions mutations. They also found that different levels of copy basal-type had predominately non-sense and frameshift A and B subtypes had mostly missense mutations, while the significantly-mutated genes were identified, including TBX3, RUNX1, CBF2, AFF2, PIK3R1, PTPN22, PTPRD, NF1, SF3B1, and CCND3. They found that the different intrinsic mRNA subtypes (luminal A, luminal B, basal-like, and HER2-enriched) differ not only by mutation frequency but also by mutation type. For example, the TP53 mutations in the luminal A and B subtypes had mostly missense mutations, while the basal-type had predominately non-sense and frameshift mutations. They also found that different levels of copy number changes, including focal amplification of regions containing PIK3CA, EGFR, FOXA1 and HER2 genes, as well as focal deletions of regions containing MLL2, PTEN, RB1, and MAP2K4 genes, correlated with different mRNA subtypes. Additionally, quantified expression of cancer-related proteins was performed, and protein expression subtypes were highly concordant with mRNA subtypes. For instance, one protein sub-group with high protein expression of ER, PR, AR, BCL2, GATA3, and INPP4B contained predominantly luminal-A subtype cancers, while a second more heterogeneous protein subgroup consisted of both luminal A and luminal B cancers (4). This study provides evidence that the four different mRNA subtypes have distinct genetic characteristics, but are not clearly defined by one type of genetic change or mutation.

Epigenetic Changes

Epigenetic changes are also critical for the development and progression of many types of cancer, including breast cancer. Recently, there have been many studies focusing on defining these changes, and this has led to significant progress towards understanding how various epigenetic alterations, such as histone modifications, DNA methylation, and miRNA expression, ultimately affect gene expression.

For example, The Cancer Genome Atlas Network studied miRNA expression levels by identifying the top 3,662 variably expressed genes and seven different miRNA subtypes in 525 tumors and 22 tumor-adjacent normal tissues (4). These miRNA subtypes are correlated with the primary mRNA subtypes that are based on the clinical status of ER, PR, and HER2. Of note, miRNA groups 4 and 5 showed high overlapping with the basal-like mRNA subtype and contained many TP53 mutations. The remaining miRNA groups (1-3, 6, 7) contained a mixture of luminal A, luminal B, and HER2-enriched subtypes, and so these miRNA groups had little correlation with the PAM50-defined subtypes. With the exception of TP53, which showed a strong positive correlation, and PIK3CA and GATA3, which showed negative associations with groups 4 and 5, respectively, there was little correlation with mutation status and miRNA subtype.

In addition, other comprehensive studies have identified specific miRNAs with aberrant expression in breast cancer, confirming a difference in miRNA expression patterns between healthy breast tissue and breast cancer cells. Specifically, mir-12b, mir-145, mir-21, and mir-155, were all significantly down-regulated across multiple breast cancer types (8). More recent research has focused on the dynamics of miRNA expression throughout the progression of healthy tissue to invasive carcinoma. For example, Volinia et al. identified 9 miRNAs with significantly different expression in ductal carcinoma in situ (DCIS) as compared to invasive breast carcinoma. Three of these miRNAs (let-7d, mir-210, mir-221) were down-regulated in the transition from normal tissue to DCIS and subsequently up-regulated in the transition from DCIS to invasive carcinoma (9). Elucidating the targets of these miRNAs informs on their role in carcinogenesis. For instance, mir-210 appears to target BRCA1 and E-cadherin, among many other proteins.
In addition to the role of miRNA in breast cancer, other epigenetic changes, such as methylation, have been implicated in the pathogenesis of breast cancer. The Cancer Genome Atlas Network identified five distinct DNA methylation groups (group 1-5) based on methylation arrays of 802 breast tumors (4). Group 3 showed a hypermethylation phenotype and was significantly enriched in the luminal-B mRNA subtype, and group 5 showed the lowest levels of DNA methylation, which overlapped with the basal-like mRNA subtypes.

Furthermore, studies have demonstrated how methylation changes correlate with the formation of breast cancer and can be used to differentiate normal and benign breast cells from cancerous cells. A study by Elsheikh et al., which characterized 880 human breast carcinomas, suggested that changes in histone acetylation and methylation patterns might represent an early sign of breast cancer (10). Moderate-to-low levels of lysine acetylation and lysine and arginine methylation were linked to carcinoma subtypes with low prognostic levels, including basal carcinomas (triple-negative) and HER2-positive subtypes (10). In another study, Ordway and colleagues used the cytosine methylation-dependent restriction enzyme McrBC coupled with array hybridization to analyze methylation in 9 invasive ductal carcinomas and their adjacent normal tissues. They identified 220 differentially-methylated loci and analyzed 16 genes that were able to differentiate breast tumors from normal and benign tissues. One of these genes included GHSR, which is a member of the G-protein-coupled receptor family, and methylation of GHSR was able to differentiate invasive ductal carcinoma from normal and benign breast tissue with high sensitivity and specificity (11).

There is also evidence that the degree of methylation change is indicative of more aggressive metastatic breast cancer cells, compared to breast cancer cells that are less metastatic. Rodenhiser et al. compared the methylation status of upstream promoter region associated with genes from multiple pathways in a highly metastatic breast cancer cell line, MDA-MB-468LN, as compared to that in a less metastatic cell line, MDA-MB-468GFB (12). They observed hyper-methylation and hypo-methylation of genes that could be involved in carcinogenesis as well as in metastasis. Interestingly, the methylation status of 20-30% of genes differed between the two cell lines. The SWI/SNF gene, which is involved in chromatin remodeling, was shown to have altered levels of methylation, and these alterations could be involved in TGF-β pathway silencing and overexpression of MYC, which would implicate it as a gene involved in carcinogenesis and metastasis. Hypomethylation of ZEB2 and SNAIL2 indicate their overexpression, which in turn, can repress E-cadherin (CDH1), a gene that is involved in endothelial-mesenchymal transformation. Some of the integrin signaling pathway genes were hyper-methylated and some were hypo-methylated (12). This is not an unusual finding given that some of the integrin receptors, like E-cadherin, are involved in cell-cell attachment, which needs to be silenced via hypermethylation during metastasis, whereas other integrins, which are involved in cell motility, need to be up-regulated via hypomethylation. These results show the complexity behind metastasis and suggest that a distinct pattern of genes are silenced or re-expressed in more metastatic forms of breast cancer.

Epigenetic Drug Treatment

With the expanding field of knowledge on epigenetic changes involved in breast cancer development, there has been increasing focus on treating breast cancer cells with epigenetic drugs, such as AZA and/or HDAC inhibitors (HDACi), and subsequently examining the methylation status of known genes of interest (13).

De-methylating agents have been studied in order to assess their potential efficacy in the treatment of breast cancer. Radpour et al. showed that differential expression of oncogenes and tumor suppressor genes occurred after treatment of breast cancer cells with the de-methylating agent AZA (14). These results could have positive or negative implications in the treatment of breast cancer. Usually oncogenes are hypo-methylated and highly overexpressed in cancer cells, while tumor suppressor genes are hyper-methylated and aberrantly silenced. Ideally, the demethylating agent would preferentially affect the hypermethylated tumor suppressor genes, leading to decreased methylation and increased expression of these genes, without increasing the expression of oncogenes. However, there is a risk that these de-methylating agents will also demethylate the oncogenes and lead to increased expression. Radpour et al. confirmed that the de-methylating agent caused increased expression in both tumor suppressor genes and oncogenes. Consequently, it appears that demethylating agents alone will not be sufficient to destroy tumor cells. However, a combination of de-methylating agents along with HDACi, which are known to inhibit the cell cycle and induce apoptosis, and other cytotoxic agents, which can nullify the effect of activated oncogenes, may prove more effective.

Recent results from several laboratories showed that the combination of either AZA or HDACi with other cytotoxic drugs produces synergistic growth inhibition in breast cancer cells, both in cell cultures and in mouse models. For instance, Mataga et al. have shown that a combination therapy of HDACi with the protease calpain inhibitor, calpeptin, resulted in synergistic growth inhibition, cell-cycle arrest, and induction of apoptosis and cell death in breast and ovarian cancer cells (15). They also examined changes in methylation levels and expression of the maternally
imprinted pro-apoptotic gene, \textit{ARHI}, which is silenced by methylation in 30\% of breast and ovarian cancer cells (16). The paternal expression of \textit{ARHI} is down-regulated via CpG island DNA methylation silencing in the upstream region of the promoter, causing loss of heterozygosity. Mataga et al. have shown that using a combination therapy of HDACi and calpeptin, results in de-methylation and re-expression of \textit{ARHI}. Drug treatments that re-express silenced genes may do so by de-methylating-specific CpG islands that regulate expression. Targeting the de-methylation of aberrantly-methylated tumor suppressor genes, which leads to gene re-expression and allows for cell-cycle inhibition and apoptosis, could be an important key in the development of epigenetic cancer treatment.

Clinical trials have also supported the use of an epigenetic combination therapy. Yang \textit{et al.} demonstrated that HDACi in combination with DNMT inhibitors have shown superior ER re-expression in breast cancer cell lines compared to HDACi-alone (17). In a phase I clinical trial of phenylbutyrate in combination with the DNMT inhibitor Aza in myelodysplasia, the treatment response showed significant reversals of aberrantly-methylated genes (18). An ongoing phase II trial is currently testing the HDACi entinostat (also known as SNDX-275 and MS-275) in combination with Aza in patients with triple-negative metastatic breast cancer (19). In addition to epigenetic therapies, re-expression of the ER receptor by de-methylating agents could be exploited by using ER antagonists as one of the components of the combination therapy.

Studies have also indicated that the use of epigenetic drugs with other cytotoxic drugs may be effective. A previous study demonstrated that epigenetic drug treatment that includes DNMT inhibitors and HDACi with other known cytotoxic drugs like doxorubicin and cyclophosphamide are well-tolerated by patients and cause re-activation of genes involved in cell proliferation, cell differentiation, and apoptosis, invasion, metastasis, and immune-recognition of tumor cells (20). For example, cell-cycle inhibitors and pro-apoptotic genes \textit{p53} and \textit{p21} were up-regulated, and 18 members of the oxidative phosphorylation pathway, interferon regulatory factors, \textit{NM23}, and negative regulators of \textit{Wnt1} signaling pathway were up-regulated. Interestingly, a member of the ABC transporter family, \textit{ABCB5}, which is implicated in multi-drug resistance and expressed in tumor stem cells, was down-regulated. This study suggests that epigenetic drug treatment may sensitize drug-resistant breast cancer cells and breast cancer stem cells to other cytotoxic drugs. This idea is further supported by a new study performed by Johannessen \textit{et al.}, which showed that melanoma cells develop cAMP-mediated resistance when treated with \textit{RAF, MEK, ERK} or combined \textit{RAF-MEK} inhibitors, but combined treatment with the \textit{MAPK}-pathway and HDACi suppressed cAMP-mediated resistance (21).

\textbf{Carcinogenesis Model}

In the model proposed by Hanahan and Weinberg, six acquired capabilities – the hallmarks of cancer – were outlined as integral components in the multi-step development of cancer (22). They implied that as normal cells progressively evolve to a neoplastic state, they acquire a succession of these capabilities. Vogelstein \textit{et al.} have also proposed that step-wise mutations in genes, such as \textit{APC} and \textit{RAS}, are involved in the progression of colon cancer (23).

While the information regarding the genetic characteristics of different tumors gives us insight into possible mechanisms behind the development of carcinogenesis, it is almost impossible to fully-characterize which particular gene combination will occur in a certain patient. When all different germline mutations, somatic mutations, copy number aberrations, and methylation changes present in each individual tumor are considered, it becomes extremely difficult to distinctly define groups of cancer sub-type based on genetic characteristics. Moreover, any combination of these changes can be the underlying cause of breast cancer development. Therefore, in order to identify therapeutic targets for tumors with underlying genetic changes, first the cancer cell-specific mutations present within each patient need to be accurately predicted. This makes finding a single effective therapeutic genetic treatment tailored to an individual patient incredibly complex.

Sarkar \textit{et al.} suggest that a combination of all these genetic changes (germline and somatic), which are differentially present in the various cancer subtypes, are responsible for making a cell vulnerable to epigenetic alterations, which can initiate breast cancer progenitor cell development (23, 24). This initiating signal can originate from the breast-stromal interaction or from alterations in DNA methylation or histone modifications. Evidence from a study on chondroblastoma and giant-cell tumor of the bone has shown that genetic mutations can alter epigenetic signaling, which suggests that epigenetic changes may also be involved in the origin of bone cancer progenitor cells (26). Many cases of cancer relapse occur because traditional chemotherapy does not kill the cancer progenitor cells, and this leaves a window of opportunity for the development of further carcinogenesis. For lung cancer and different types of leukemia, pre-treatment with epigenetic drugs appears to reduce the rate of cancer relapse, which indicates that these drugs are capable of killing cancer progenitor cells. This supports the hypothesis that epigenetic changes act as the initiating signal in the formation of cancer progenitor cells and could provide us with a common pathway in the development of breast cancer progression across all subtypes (Figure 1).
Further Studies

The Roadmap Epigenomics Project, an initiative led by several NIH centers, aims to determine the methylation pattern of genes that are involved in carcinogenesis and metastasis. This research could be instrumental in determining specific changes in methylation of late-stage cancer cells compared to normal cells. However, it is important to consider methylation levels of genes at the early stages of cancer development as an important step in metastasis development. While these early changes may be instrumental in the formation of cancer progenitor cells with neoplastic potential, they may not be observed during the later stages of cancer progression, and therefore could be overlooked. We suggest that it is essential to gather information regarding the early epigenetic changes occurring in progenitor cells, because these could contribute to a common pathway in the initiation of carcinogenesis. In addition, we propose that differential activation and inactivation of specific genes controlled by epigenetic changes occur during the development of metastasis and that these lead to further rapid growth and spread of cancer (25). For example, it is known that TGF-β is necessary for epithelial-mesenchymal transition, and it is expressed during the early stages of metastasis. However, in stage-IV ovarian cancer cells, it was observed that TGF-β was silenced by methylation. Because TGF-β is also known for growth inhibition, it is natural that when the differentiation process is finished and the metastatic cells need rapid growth and invasion, TGF-β must be down-regulated. In this manner, TGF-β is required during the initial stages of metastasis to promote differentiation, but must be down-regulated during later stages of metastasis to decrease growth inhibition. The dual role of TGF-β is evidence that different methylation levels as metastasis progresses provide important information on targeting. All these findings point towards our hypothesis that predisposed breast cells of any histological subtype need a specific signal, likely to be epigenetic, to trigger the formation of breast cancer progenitor cell formation. In this case, the genetic diversity and subtypes described above may have a common pathway that leads to cancer development. If this is true, then we can target this common pathway in addition to the subtype-specific genetic changes in the treatment of breast cancer.

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References