Genomic Changes in Normal Breast Tissue in Women at Normal Risk or at High Risk for Breast Cancer

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ABSTRACT: Sporadic breast cancer develops through the accumulation of molecular abnormalities in normal breast tissue, resulting from exposure to estrogens and other carcinogens beginning at adolescence and continuing throughout life. These molecular changes may take a variety of forms, including numerical and structural chromosomal abnormalities, epigenetic changes, and gene expression alterations. To characterize these abnormalities, a review of the literature has been conducted to define the molecular changes in each of the above major genomic categories in normal breast tissue considered to be either at normal risk or at high risk for sporadic breast cancer. This review indicates that normal risk breast tissues (such as reduction mammoplasty) contain evidence of early breast carcinogenesis including loss of heterozygosity, DNA methylation of tumor suppressor and other genes, and telomere shortening. In normal tissues at high risk for breast cancer (such as normal breast tissue adjacent to breast cancer or the contralateral breast), these changes persist, and are increased and accompanied by aneuploidy, increased genomic instability, a wide range of gene expression differences, development of large cancerized fields, and increased proliferation. These changes are consistent with early and long-standing exposure to carcinogens, especially estrogens. A model for the breast carcinogenic pathway in normal risk and high-risk breast tissues is proposed. These findings should clarify our understanding of breast carcinogenesis in normal breast tissue and promote development of improved methods for risk assessment and breast cancer prevention in women.

KEYWORDS: breast cancer, breast cancer risk, normal breast tissue, carcinogenic pathway, cancerized fields, preneoplastic breast tissue

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Introduction

Breast cancer develops from the progressive accumulation of mutations in "driver" and other genes that confer a proliferative advantage to the cells. The initiation of these changes and carcinogenesis necessarily begins in normal breast tissue, and the emerging consensus is that the most common subtypes of breast cancer, including luminal A/B and basal-like tumors, likely arise as a result of transformation of a luminal progenitor cell (LP) of origin.[1–3] Multiple factors have been implicated in the etiology of sporadic breast cancer,[4,5] and among the most prominent are estrogens. Estrogen and its metabolites are major carcinogens for breast cancer.[6–9] Estrogen is metabolized to catechol estrogens, quinones, superoxide, and hydroxyl radicals, and these metabolites have been associated with a range of DNA damage effects, including depurinating DNA adducts, DNA single- and double-strand breaks, point mutations and deletions, and epigenetic changes.[7,10,11] The presence of genomic abnormalities in critical growth-controlling genes may in turn lead to the initiation of carcinogenesis, and the presence of promotional agents allows clonal expansion of these cells into a larger cancerized field (Fig. 1). Within this expanding population of altered cells, new molecular alterations (genetic or epigenetic) might be acquired, promoting greater growth autonomy of the cells and resulting in the outgrowth of clonal populations from common founder cells or from within common founder populations.[12] Continued expansion of these clonal populations may eventually lead to cancerized fields that occupy large portions or even the entire breast.[13–15]

The normal breast tissue in women is exposed to estrogens over a considerable period of time, beginning at menarche and extending to menopause and beyond, and this is under physiological conditions (ie, without additional risk factors) through normal regulation of the menstrual cycle. This continuing exposure to estrogens, one would anticipate, may lead to the development and accumulation of genomic abnormalities in breast tissue which, while considered to be at normal risk (because of the absence of known risk factors), may still acquire increased susceptibility for progression to malignancy. The incidence of breast cancer begins to increase at 30–40 years of age.[16] Assuming a normal age of menarche (12 years), this is consistent with a considerable period of time for exposure to estrogens and other carcinogens. Importantly, it has been shown that 50%–70% of women who develop

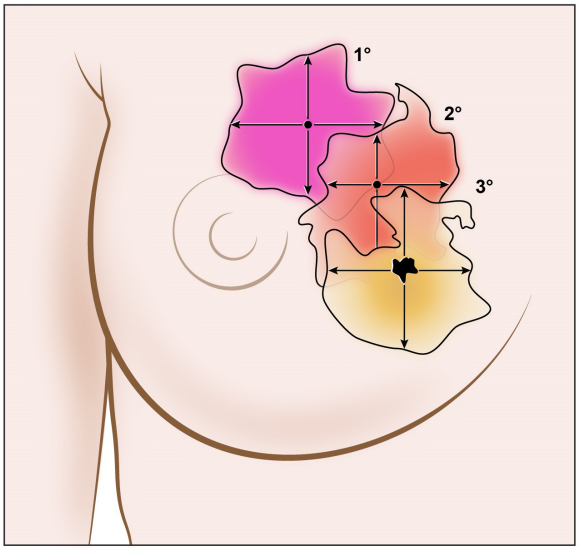

Figure 1. Development of cancerized fields within the breast. Acquisition of initial genomic changes results in clonal expansions of cells to form a field of altered cells with increased genomic instability (rose field, 1°). Acquisition of additional molecular changes in altered cells within this field leads to focal clonal expansion and the sequential development of additional cancerized fields (orange 2° and yellow 3°), with a continued increase in genomic instability and the ultimate transition to cancer in a tertiary field (3°). The presence of multiple cancerized fields with different patterns of genomic alterations may contribute to genomic heterogeneity within the breast. Clonal expansion is accompanied by displacement of surrounding normal breast tissue (often with irregular boundaries), which may contribute to varying frequencies of mutational changes with increasing distance from the center of a developing tumor. Figure 1 is after the concept of Rivenbark and Coleman.[12]

breast cancer have no identifiable risk factors,[17,18] supporting the proposal that many women considered to be at normal risk for breast cancer are in fact accumulating carcinogenic genomic changes in their breast tissue. Identification of the genomic changes in normal breast tissue and an understanding of their role in breast carcinogenesis may have important implications, including clarification of the early and even initiating changes in breast carcinogenesis, defining the breast carcinogenic pathway, development of molecular profiles for risk assessment (which would be of particular value in women at "normal" risk for breast cancer), and identification of new targets for the early and effective prevention of breast cancer.

The risk for breast cancer in women may be increased by many factors, including those that increase exposure to estrogens (early menarche, nulliparity, late first full-term pregnancy, oral contraceptive pills, late menopause, postmenopausal obesity),[19] the presence of histologic proliferative lesions (atypical hyperplasia, lobular carcinoma in situ), or those in which normal breast tissue is associated with an established breast cancer (such as contralateral breast tissue or normal breast tissue adjacent to a breast cancer). With progression to these higher risk states, one would anticipate an increase in genomic instability and more extensive genomic changes, qualitatively and/or quantitatively. The nature and relative sequence of development of these changes, however, is not well understood. Elegant in vitro studies of human mammary epithelial cells (HMECs; derived from reduction mammoplasty [RM])[20] have demonstrated that as cells progress from an early phase of genomic integrity, diploid karyotype, and an intact cell cycle checkpoint,[21–23] through a proliferative phase (post-stasis),[24,25] this is accompanied by one or more genetic/epigenetic abnormalities (such as *p16*^{INK4A} inactivation, *HOXA* inactivation, *cyclin D1* overexpression), telomere erosion and lack of cell cycle checkpoint control, and subsequently, the rapid development of gross chromosomal rearrangements (GCRs).[22,24,25] Ultimately, with activation of telomerase, cells may proceed to immortalization and malignancy.[26] These findings emphasize the diversity in genomic changes including numerical and structural chromosomal changes, epigenetic changes, and changes in the expression of a wide range of genes, which occur with progression through the carcinogenic pathway to a higher risk state.

The present review of the literature was conducted to better define the two major phases in the breast carcinogenic pathway: genomic changes in normal breast tissue in women at normal risk and those in women at high risk for sporadic breast cancer. All publications relevant to these two categories were reviewed and genomic changes in four major molecular categories were identified, numerical chromosomal changes, structural chromosomal changes, epigenetic changes, and changes in gene expression. The two risk categories (normal risk and high risk) are considered separately, Part I, describing genomic changes in normal breast tissue at normal risk, and Part II, describing genomic changes in normal breast tissue at high risk for breast cancer. The findings indicate rather a remarkable range of genomic abnormalities in normal risk breast tissue, including segmental chromosomal changes of loss of heterozygosity (LOH), DNA methylation of tumor suppressor and other genes, and telomere shortening, all of which are consistent with years of exposure to estrogens. High-risk normal breast tissue demonstrates persistence and progression of these changes with the addition of aneuploidy, increased genomic instability, and evidence of large cancerized fields in the breast. Together these findings indicate a dynamic and continuing pattern of genomic changes from normal risk to high risk for breast cancer in these normal breast tissues. A model of the carcinogenic pathway in normal risk breast tissue and in high-risk breast tissue, and their relationship to each other, is proposed. The relationship of genomic changes in each of these two risk categories to the in vitro HMEC life cycle is discussed. The implications of these findings for management of women at risk for breast cancer are considered.

Materials and Methods

Literature search and criteria for identification of tissue specimens. A literature search was conducted through PubMed and cross-references to identify all reports that

described molecular changes in normal breast tissue in women considered to be either at normal risk or at increased risk for sporadic breast cancer by standard risk assessment criteria, and without histologic evidence of breast pathology in the examined tissue. Breast tissues from women at normal risk were all procured from women without a personal history of breast cancer and included RM specimens, breast biopsies of benign or normal tissue, nipple aspirate fluid, or random periareolar fine-needle aspirate from women at low risk. Specimens analyzed could also include cases of benign breast disease, such as simple apocrine metaplasia, classified as College of American Pathologists' [CAP][27] category 1 (no increased risk for invasive breast cancer). Women without evidence of an increased risk for breast cancer may also be designated as being at low risk for breast cancer in this review.

Normal breast tissues considered to be at high risk for breast cancer were typically procured from (A) normal breast tissue adjacent to either a preneoplastic high-risk lesion (atypical hyperplasia) or a malignant lesion (in situ or invasive breast cancer); (B) normal breast tissue in the contralateral breast in women with an ipsilateral in situ or invasive breast cancer; (C) normal breast tissue in women known to develop a future breast cancer (eg, cases in case/control studies); (D) normal breast tissue in women considered to be at high risk as determined by standard risk estimates;[14,28–30] or (E) ductal lavage or fine-needle aspirate samples from women at high risk by any of the above criteria.[31,32] The focus of the present review is the molecular changes in normal breast tissue. The molecular changes in specific premalignant lesions at increased risk (florid hyperplasia without atypia, radial scar, complex fibroadenoma, atypical ductal, or atypical lobular hyperplasia) are not considered.

Abnormalities of DNA content and chromosome number. The DNA content or chromosome number (ploidy) has been studied in the literature by a variety of methods, including DNA index,[29] DNA content,[33] karyoptype,[34] or Fluorescence In Situ Hybridization (FISH).[15] FISH studies were conducted using pericentromeric-specific or gene-specific probes.[15,35]

Structural chromosomal abnormalities. Structural chromosomal studies of normal breast tissue were commonly conducted by karyotyping analysis, DNA allelotyping, or array comparative genomic hybridization (arrayCGH). Chromosomal loci selected for analysis in the literature were typically polymorphic microsatellite repeats known to have a high frequency of LOH in ductal carcinoma in situ (DCIS) or invasive breast cancer or relevance to breast tumorigenesis (LOH in early-stage carcinogenesis or at sites of identified or putative tumor suppressor genes [TSGs]).[36–40] Allelic imbalance (AI) suggestive of LOH was identified by allelotyping and defined as either an imbalance of allele intensities greater than 25%–50%,[38,41] a change in allele ratio of >50% relative to the normal control,[40] or the unequivocal loss of intensity of one allele seen at heterozygous loci.[37,38] Corresponding stromal tissue or lymphocytes served as a control.[40,42] Partial chromosome gains or losses were also studied by arrayCGH, utilizing either normal male metaphase spreads or microarray.[43,44] Telomere shortening, sequencing analysis of p53, DNA amplification, and immunohistochemistry were conducted as described in the references accompanying the respective data in Table 4. The normal risk tissues which were used as the material for the studies are indicated, with the accompanying references, in Table 3 (normal risk for breast cancer) and Table 7 (high risk for breast cancer).

Epigenetic abnormalities. DNA methylation was measured in normal and tumor tissues by methylation-specific PCR,[28,45,46] and the degree of methylation expressed as the percentage of samples demonstrating methylation of the respective gene.

Part I: Normal Breast Tissue at Normal Risk for Breast Cancer

Genomic changes.

Numerical chromosomal abnormalities in normal breast tissue at normal risk for breast cancer. The majority of series examining normal/benign breast tissues at normal risk, including RM, normal breast ductal epithelium, and benign nonproliferative breast lesions, did not find evidence of increased DNA content, aneuploidy, or other numerical chromosomal changes in these tissues (Table 1). There have been occasional series that demonstrated numerical chromosomal changes in chromosome 1,[47–49] or other chromosomes,[49] indicating that even some of the benign breast lesions already showed a tendency toward aneuploidization.[47,48] Clonal chromosomal changes have also been observed in the benign tumor fibroadenoma by karyotype,[50] but not by arrayCGH analysis.[44] The former include numerical chromosomal abnormalities in chromosome 22 (50%), X (40%), 14 (40%), 20 (40%), and 21 (30%),[50] as well as structural alterations in other chromosomes. These have been noted in a review by others.[51] Demographic information, however, was not provided for these lesions, and it is important to note that fibroadenomas with complex features, proliferative disease in the parenchyma adjacent to the fibroadenoma, or fibroadenomas in women with a family history of breast cancer may be associated with an increased risk of breast cancer of 3.1-fold or more.[52] Taken together, the preponderance of evidence suggests that aneuploidy, even involving single chromosomes, is an uncommon finding in normal/benign normal risk breast tissue from a variety of sources, with maintenance of a normal diploid chromosomal content.

Structural chromosomal abnormalities in normal breast tissue at normal risk for breast cancer. The predominant chromosomal structural abnormality that has been studied in normal risk breast tissue is AI/LOH/microsatellite instability (MSI; Table 2). These are important indicators of genomic instability and may reflect partial or complete loss of function of tumor suppressor and other genes.[4,53,54] Larson et al[37,41] studied normal-appearing breast ducts or terminal ductal lobular units (TDLUs) in RM specimens histologically at nine genomic

Table 1. Numerical chromosomal changes in normal breast tissue at normal risk and at high risk for breast cancer.

TISSUE SOURCE	ANALYSIS	NORMAL RISK*	HIGH RISK*	REFERENCES
Normal breast tissue	DNA content/index	Diploid	1.3%–4%	33, 48, 90
Normal breast tissue	DNA content/index	Diploid	25%	29
Normal breast tissue	Karyotype	Diploid		220
Normal breast tissue	Karyotype		18%	34
Normal breast tissue	Karyotype	Diploid	66%	136
Normal breast tissue	Karyotype	Diploid		221
Normal breast tissue	Karyotype		16.7%	137
Intraductal proliferation without atypia	DNA histograms	Diploid		105
Hyperplasia adjacent to cancer	FISH		3.8%–33.9%	47
ADH adjacent to cancer	FISH		4.9%–40.0% chromosome 1 copy number	47
Fibroadenoma	ArrayCGH	Diploid		44
Fibroadenoma	Karyotype	65.1% clonal chromosomal alterations		50
Chromosome 1				
Benign breast disease#	FISH	1.8%–6.7%		47, 48
Normal breast tissue, chromosome 1	FISH	0%		15
Ductal hyperplasia adjacent to cancer	FISH		55.6%	222
Breast cancer	FISH		100%	15
Adjacent to cancer	FISH		0.0%	222
Adjacent to cancer	FISH		0%	48
Adjacent to cancer, 2 cm	FISH		70.4%	15
Adjacent to cancer, 3 cm	FISH		56.3%	15
Adjacent to cancer, 4 cm	FISH		22.2%	15
Contralateral breast	FISH		90.0%	15
Chromosome 17				
Normal breast tissue, chromosome 17	FISH	0%		15
Ductal hyperplasia adjacent to cancer	FISH		0%	222
Breast cancer	FISH		92.9%	15
Adjacent to cancer	FISH		0.0%	222
Adjacent to cancer, 2 cm	FISH		78.6%	15
Adjacent to cancer, 3 cm	FISH		58.8%	15
Adjacent to cancer, 4 cm	FISH		33.3%	15
Adjacent to LCIS	FISH		27.0% loss	223
Contralateral breast	FISH		80.0%	15
Chromosomes 1, 8, 11, 17 low risk normal	FISH	0%		135, 224, 225
Chromosomes 1, 8, 11, 17 benign	FISH	11%		49
Chromosomes 1, 8, 11, 17 high risk hyperplasia	FISH		89%	135
Chromosomes 1, 8, 11, 17 high risk nonproliferative	FISH		69%	135
Chromosome 1, 16–18, X	FISH		Monosomy 29.5%, trisomy 2.3%	223
Chromosomes 7–12, 17, 18, X nonproliferative	FISH	40%		35

Notes: *Incidence of aneuploidy in respective series. #Benign breast disease = epitheliosis, sclerosis, adenosis.
Abbreviations: ADH, atypical ductal hyperplasia; LCIS, lobular carcinoma *in situ*.

Table 2. Structural chromosomal changes in normal breast tissue at normal risk for breast cancer.

LOCUS	GENE	TISSUE SOURCE	MOLECULAR ABNORMALITY	INCIDENCE IN NORMAL RISK BREAST TISSUE*	INCIDENCE IN HIGH RISK BREAST TISSUE*	REFERENCES
1q D1S549–213/1q41		Reduction mammoplasty	LOH/AI	11.0%	39%	41
1p32	*MYCL1*	Apocrine metaplasia	LOH/AI	7.1%		56
3p11–12		Reduction mammoplasty	Clonal deletion	18.0%		34
9p D9S1748, D9S171	*p16 CDK inhibitor*	Normal TDLU	LOH	7.4%		40
9p D9S1748, D9S171	*p16 CDK inhibitor*	Apocrine metaplasia	LOH	26.3%		40
11p15	*TH01*	Reduction mammoplasty	LOH/AI	13.0%	21.2%	37, 41
11p15, D11S4046	*TH01*	Normal TDLU	LOH	7.4%		40
11p15, D11S4046	*TH01*	Apocrine metaplasia	LOH	26.3%		40
11q13	*INT-2*	Apocrine metaplasia	LOH/AI	14.3%		56
11q22–24	*pYGM/ATM*	Reduction mammoplasty	LOH/AI	6.0%	6.0%	41
13q12–14	*BRCA2, RB1*	Normal TDLU	LOH	7.4%		40
13q12–14	*BRCA2, RB1*	Apocrine metaplasia	LOH	10.5%		40
13q12.3, D13S267		Apocrine metaplasia	LOH/AI	6.7%		56
16q22: D16S496, D16S512, D16S421		Normal TDLU	LOH	18.5%		40
16q22: D16S496, D16S512, D16S421		Apocrine metaplasia	LOH	42.1%		40
16q (D16S265, D16S402/16q23.3)	*H-cadherin*	Reduction mammoplasty	LOH/AI	11.0%	17.0%	41
16q24.1–24.3, D16S539		Apocrine metaplasia	LOH/AI	13.6%		56
17p: D17S1788, D17S1880	*TP53*	Normal TDLU	LOH	18.5%		40
17p: D17S1788, D17S1880	*TP53*	Apocrine metaplasia	LOH	15.8%		40
17p13.1	*TP53*	Apocrine metaplasia	LOH/AI	8.7%		56
17p13, D17S513		Apocrine metaplasia	LOH/AI	9.1%		56
17p15.1–15.2	*TP53*	Reduction mammoplasty	MSI/LOH AI	16.7%		37
17q11.2–12, D17S250		Apocrine metaplasia	LOH/AI	18.8%		56
17q23		Reduction mammoplasty	LOH/AI	6.0%	24.0%	41
Chromosomes 6, 9, 11, 13,1 4, 20, X		Fibroadenoma	Structural alterations	60.0%		50

Note: *Percentage incidence in samples of respective series.
Abbreviations: LOH, loss of heterozygosity; AI, allelic imbalance; MSI, microsatellite instability; TDLU, terminal ductal lobular unit.

loci: 1p (*MYCL1*), 1q, 2p, 7q, 11p (*TH01*), 17p (*TP53*), 17q, 18q, and *Xq* (androgen receptor [AR]). They identified genetically abnormal clones in 15.6% of samples, including sites of two TSGs, such as *TH01* and *TP53*. They estimated a somatic mutation rate of 1.2% in these tissues, which was greater than the expected baseline somatic mutation rate of <0.5%. Their data indicated the presence in these normal breast tissues of multiple, genetically distinct abnormal clones that could progress independently and simultaneously, providing a possible explanation for the genetic heterogeneity noted in many breast tumors.[37] They concluded that genetic abnormalities that may be critical to breast tumorigenesis start accumulating far before pathologic detection even in high-risk lesions. These findings were expanded in a second study in which AI was examined in a larger number of microdissected histologically normal TDLUs in RM specimens.[41] This study indicated that 5% of TDLUs, or 28% of cases of RM, demonstrated AI at loci on 1q (11%), 11p (13%), 11q (6%), 16q (11%), or 17q (6%). This suggested that the RM tissues with abnormalities in their normal epithelium constitute a subgroup of seemingly low-risk women who are actually at increased risk of developing breast cancer.[41] The pattern of MSI seen in normal-appearing tissues was similar to the type of instability reported in breast cancers, with a substantial proportion of changes seen in tri- and tetranucleotide repeat markers.[37] Confirmation of the presence of LOH and/or AI in multiple chromosomal loci in breast tissue at normal risk for breast cancer is provided by other studies.[34,38,40,55] Selim et al[56] also considered these abnormalities to be clonal, noted abnormalities on chromosomes 1p, 11q, 13q, 16q, 17p, and 17q, and found no evidence of complete loss or aneusomy of chromosomes 1 and 17, consistent with the overall observation (discussed above) of a lack of numerical chromosome change in normal risk breast tissues. Washington et al[40] identified LOH at 3p14, 9p (*p16* cdk inhibitor), 11p15 (*TH*, tyrosine hydroxylase), 13q12–14 (*BRCA2 and RB1*), 16q22, and 17p (*p53*), all of which are also abnormal in high risk tissues (see below, Table 7), suggesting a role for each of these abnormalities in breast tumorigenesis. Two studies did not find any evidence of LOH,[39,57] which may reflect the focal nature (such as may occur with a limited number or extent of cancerized fields), sampling variability, and/or heterogeneity in the incidence of these chromosomal abnormalities among normal risk tissues.

The presence of LOH from segmental deletions in normal breast tissues would also be consistent with estrogen carcinogenesis: estrogen metabolites may cause DNA double-strand breaks and chromosomal deletions,[10] and these in turn are a prominent factor in the development of LOH.[53,58] In this regard, it is noteworthy that the mean age of women providing specimens for these studies is consistent with considerable estrogen exposure. For example, the mean age of women providing RM specimens for the analysis by Larson et al[41] was 41 years (range 31–50 years), and the mean age of women providing benign tissue specimens for the study by Washington et al[40] was 53.7 years (range 35–74 years). Assuming a normal age of menarche (12 years), the breast tissue of these women would have been subjected to, on average, 30 years or more of estrogen exposure and the potential for DNA damage and double-strand breaks. Importantly, several of these series also included tissue from young women ≤20 years of age,[37,56,59,60] and thus, the age of onset of genomic abnormalities is unclear. Sequencing, arrayCGH, or other higher resolution analyses have not been reported for these tissues, and thus, the true extent of structural changes is not clear. However, karyotypic studies, which typically have a resolution of 10–100 Mb,[61] do not indicate any evidence of large-scale deletions or amplifications or GCRs in these normal risk tissues.

Telomere dysfunction in normal breast tissue at normal risk for breast cancer. Another important structural change present in normal risk breast tissues is the shortening of telomeres. Telomeres are nucleic acid–nucleoprotein complexes comprising T_2AG_3 nucleotide repeats protected by a nucleoprotein cap at the terminal ends of linear chromosomes.[62,63] Loss of the cap or telomere erosion results in chromosome instability, with telomeric fusions and breakage-fusion-bridge (BFB) cycles leading to GCRs through nonreciprocal translocations, causing chromosome arm gains and losses, further genomic instability, and ultimately, acquisition of the tumor phenotype.[62,64] Accordingly, evidence of significant telomere shortening is an important indicator of chromosome instability and potential carcinogenesis.[69] The earliest evidence of telomere shortening may be in a phenotypically defined cell population derived from RM specimens, which are enriched in LPs and characterized by unusually short telomeres.[65] In addition, 25% of these LPs were still capable of exhibiting robust clonogenic activity in vitro, which may be partially explained by the elevated telomerase activity that was also seen only in LPs.[65] This is particularly relevant to breast carcinogenesis, as it is currently thought that the most common tumor subtypes, including luminal A/B and basal-like tumors, likely arise as a result of transformation of a LP of origin.[1–3] The presence of telomere shortening in these cells may, therefore, allow it to play a role in the initiation and/or promotion of carcinogenesis.[64] At the same time, the conditions in normal breast tissue appear to be conducive to the development and promotion of telomere shortening. Cyclical proliferation due to estrogens during the menstrual cycle can cause moderate telomere shortening due to the end-replication problem.[66] Oxidative stress from estrogen metabolites may also cause telomere shortening,[67] and bypass of a telomere-sensitive checkpoint (perhaps involving *p16* inactivation [see below]) may allow further proliferation with additional telomere loss.[66] Meeker et al[68] presented important evidence of telomere shortening in TDLUs from normal risk breast tissue in their studies of RM tissues. They reported that 50% of normal risk breast epithelium contained focal areas of moderate telomere shortening, and this was restricted to luminal (secretory) cells of the TDLUs and was not seen in myoepithelial cells, normal large lactiferous ducts, or male breast

ducts. They postulated that such shortening may delineate a population of epithelial cells at risk for subsequent malignant transformation.[66,68] Others reported that the telomere DNA content (TC) of RM tissue was comparable to TC from peripheral blood lymphocytes (PBLs) of cancer patients; however, the cell type analyzed in RM was not specified.[60] It is noteworthy that in vitro studies of HMECs (developed from RM tissue) demonstrate that, as cells emerge from a period of initial stasis and proceed through a second proliferative period (corresponding to the clinical development of hyperplasia and atypical hyperplasia associated with increased risk),[70] this is accompanied by progressive telomere erosion at a rate of 30 bp/population doubling.[22] These HMECs exhibiting eroding telomeric sequences ultimately enter telomere-based crisis to generate the types of chromosomal abnormalities seen in the earliest lesions of breast cancer.[22] Together, these findings and those of Meeker et al[68] and Kannan et al[65] suggest that the telomere shortening observed in normal risk breast tissue, such as RM specimens, may be early changes of breast carcinogenesis.

Epigenetic changes in normal breast tissue at normal risk for breast cancer. DNA methylation of CpG islands in the promoter region of genes is an important means of silencing gene transcription of tumor suppressor and other genes. DNA gene promoter CpG hypermethylation, if present early in breast cells at normal risk for breast cancer, may facilitate initiation and promotion of breast cancer. Epigenetic changes are heritable, and thus will be incorporated into subsequent cancerized fields that develop in the breast, persisting throughout carcinogenesis with long-lasting effects on the breast. DNA methylation of tumor suppressor and other genes also affects multiple cellular processes and pathways, and all of the six novel capabilities a cell has to acquire to become a cancer cell are as follows: limitless replicative potential, self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, sustained angiogenesis, and tissue invasion and metastasis.[71,72] Alteration of any of these processes in normal breast epithelium through DNA methylation might therefore have the potential to promote clonal expansion, increase genomic instability, and expand cancerized fields in the breast. Multiple genes have been identified, which are methylated in normal breast tissue at normal risk (Table 3). It can be seen that several studies have identified methylation of multiple genes in individual normal risk tissues,[28,45] suggesting more widespread disruption of signaling pathways. Importantly, many of these genes are frequently hypermethylated in breast cancer, including *BRCA1, APC, p16^{INK4A}*, and *RARβ*,[73] and many of these genes affect the novel capabilities a cell has to have to become malignant, especially limitless replicative potential. The potential to promote proliferation through inactivation of these genes could thus have an important early effect on both clonal expansion and the development of autonomous growth and widespread cancerized fields in these normal breast cells. Among these genes, the TSG *p16^{INK4A}*, for example, is considered to play an important role in early breast carcinogenesis and has been shown to be hypermethylated in HMECs from RM specimens.[74] In HMECs in vitro, the progression during early carcinogenesis from a period of stasis and cell cycle arrest to a period of increased proliferation occurs with the concomitant spontaneous DNA methylation of *p16^{INK4A}*.[75] Cells containing hypermethylated *p16^{INK4A}* promoters have the ability to not only accumulate genomic instability but also induce critical oncogenic phenotypes such as angiogenesis and inhibition of apoptosis.[75] These cells are considered to represent a potent precursor population for oncogenic progression.[75] Loss of the *p16^{INK4A}* tumor suppressor has also been shown to stimulate upregulation of polycomb group of proteins, which in turn bind and transcriptionally silence specific loci involved in cell-fate processes (eg, self-renewal and terminal differentiation), ultimately leading to de novo DNA hypermethylation of multiple downstream genes.[76,77] This could be an important mechanism for further deregulation of tumor suppressor and other genes in these normal risk tissues. The importance of hypermethylation of these genes is also shown by their contribution to the risk for breast cancer. Lewis et al[28] found that promoter methylation of *APC* and *RASSF1A* occurred frequently in benign breast epithelium and was associated with epidemiologic markers of increased breast cancer risk. *APC* and *RASSF1A* were associated with calculated breast cancer risk, particularly in women with prior benign breast biopsies. In addition, hypermethylation of these genes reflects an important degree of chromatin instability that, when combined with chromosomal instability from LOH and small segmental deletions, may provide for a significant degree of overall genomic instability in these normal breast cells. In view of the multiple signaling pathways regulated by these TSGs, one would anticipate DNA methylation that has been observed in these normal cells to have a significant effect on early breast carcinogenesis.

Last, reactive oxygen species (ROS) are an important metabolite of the carcinogen estrogen and may contribute to hypermethylation. ROS-induced oxidative stress can contribute to gene silencing by mechanisms that involve aberrant hypermethylation of TSG promoter regions and thus lead toward progression to a malignant phenotype.[11] For example, studies have showed that when exposed to oxidative stress, TSGs *p15^{INK4B}* and *p16^{INK4A}* accrued aberrant methylation patterns, and ultimately, their expression was silenced.[11,78] Other TSGs, such as *CDKN2A, RB, VHL*, and *BRCA1*, have also been identified in cancer cells as being inactivated via oxidative-induced aberrant CpG island promoter methylation.[11] These findings further support the potentially important consequences of long-term exposure to estrogens in these women.

Gene expression abnormalities in normal breast tissue at normal risk for breast cancer. The expression pattern of genes in a wide range of categories, including cell cycle regulation, oncogenes, angiogenic and other growth factors, apoptotic/anti-apoptotic factors, invasion and metastasis, and cell signaling,

Table 3. DNA methylation of genes in normal breast tissue at normal risk, at high risk, and in breast cancer.

GENE	CELLULAR FUNCTION	HALLMARKS OF CANCER*	METHYLATION IN NORMAL RISK BREAST TISSUE#	METHYLATION IN HIGH RISK BREAST TISSUE#	METHYLATION IN BREAST CANCER#	REFERENCES
APC	Cell adhesion, signal transduction, stabilization of the cytoskeleton, regulation of cell cycle and apoptosis	Tissue invasion and metastasis	26%	33%	57%	28
			0.0%	11%	44%	226
				83.3%	83.3%	227
				0%	36%	228
				44.4%	52.5%	229
				0.0%	31.7%	230
			10%		28%	231
ARH1 CpG I				30%	47%	188
ARH1 CpG II				38%	36%	188
ARF	Cell cycle regulator			8.3%	9.1%	227
BRCA1	DNA repair, recombination, checkpoint control of the cell cycle and transcription	Limitless replicative potential		32%	39.5%	46
				7.4%	17.5%	229
				0.0%	28.6%	233
				5.0%	10.0%	230
				18.2%	29.0%	189
			14.1%	31.6%		59
			20%		17%	231
CALCA	Promotes angiogenesis			100.0%	93.9%	227
DAPK	Mediator of interferon-γ induced apoptosis	Evading apoptosis		75%	78.8%	227
				0.0%	7%	235
CDH1 E-cadherin	Epithelial cell-cell adhesion, suppresses invasion and metastasis	Tissue invasion and metastasis		60%	65.8%	46
				4%	4%	188
				18.5%	22.5%	229
			0.0%	100.0%	92.9%	236
			0.0%		52.0%	237
			0.0%		39%	231
			0.0%		45%–70%	238
CDH13 H-cadherin	Cell-cell adhesion	Tissue invasion and metastasis		3%	14%	188
				5.9%	33.0%	182
			17%	28%	36%	28
				5.0%	23.3%	230
				0.0%	22.2%	232
p16^{INK4A} (CDKN2A)	Cell cycle regulation, involved in senescence	Limitless replicative potential		60.0%	68.4%	180
				100%	48%	156
				2%	1%	188
				0.0%	17%	235
			47%			74
			20%		18%	231
				0% distant NABT	16%	227
				0%	1.7%	230
				33.7%		189
			32.1%	25.8%		59

Table 3. (*Continued*)

GENE	CELLULAR FUNCTION	HALLMARKS OF CANCER*	METHYLATION IN NORMAL RISK BREAST TISSUE#	METHYLATION IN HIGH RISK BREAST TISSUE#	METHYLATION IN BREAST CANCER#	REFERENCES
CDKN2B				28.3%	25.0%	230
CTNNB1				8.3%	16.7%	227
Cyclin D2	Cell cycle regulation	Limitless replicative potential	2%	0%	57%	28
			0%–7%		52%	45, 239
				11%	30%	240
			0%	0%	46%	241
				0%	50%	242
				83.3%	87.9%	227
				18.5%	30.0%	229
			0.0%		11%	231
				<1.0%	6.3%	243
ERα/ESR1	Regulation of cell proliferation	Self-sufficiency in growth signals		7%	5%	188
			5.1%	26.3%		59
			0.0%		52.0%	237
				8.3%	13.6%	227
				25.9%		189
			0.0%		25% (ER–)	245
			40%		46%	231
ERβ			0.0%	0.0%	70.3%	244
FHIT	Control proliferation and apoptosis	Evading apoptosis		25.0%	22.7%	227
				1.7%	6.7%	230
GSTP1	Carcinogen detoxification	Limitless replicative potential		52%	57.9%	46
				16.7%	50.0%	227
				3.3%	16.7%	230
			0.0%		13%	231
HIC1			30%		48%	231
HIN-1	Putative cytokine, inhibits cell growth	Insensitivity to anti-growth signals		5%	20.0%	188
			14%		57%	45
				91.7%	97%	227
				70.4%	75.0%	229
IGF2	Regulation of cell proliferation		50.0%	48.9%	35.5%	246
IGFBP7				4.3%	25.9%	232
Line-1				68%	60%	188
MGMT				8.3%	12.1%	227
MT1G				50.0%	51.5%	227
PDLIM4				23.9%	35.2%	232
RARβ M4				27.8%		189
RARβ2	Apoptosis, involved in senescence, inhibition of proliferation	Limitless replicative potential	9%	32%	43%	28
				92.9%-adjacent 0%-distant from tumor	92.9%	174
				12.5%	30.0%	247
				16%	34.2%	46

(*Continued*)

Table 3. (*Continued*)

GENE	CELLULAR FUNCTION	HALLMARKS OF CANCER*	METHYLATION IN NORMAL RISK BREAST TISSUE#	METHYLATION IN HIGH RISK BREAST TISSUE#	METHYLATION IN BREAST CANCER#	REFERENCES
				25%	53.0%	227
				0%	34%	242
				2%	7%	188
				25.9%	25.0%	229
				4.3%	24.1%	232
				6.8%	35.9%	243
			0.0%		41%	239
			0.0%			59
			0.0%		20%	231
				0.0%	38%	235
RASSF1A	Reduces colony formation, suppresses anchorage-independent growth, and inhibits tumor formation, apoptosis	Self-sufficiency in growth signals	37%	29%	59%	28
			7%		68%	45
				3%	19%	188
				100.0%	93.9%	227
				85.2%	82.5%	229
				23.9%	42.6%	232
				8.3%	43.3%	230
				11.2%	63.7%	243
				7.5%	62.0%	177
				0.0%	62.0%	235
RIL	Apoptosis, cell cycle regulation, inhibition growth and invasion			10%	27%	188
SCGB3				13.0%	38.9%	232
S100A2	Regulate cell cycle progression and differentiation			91.7%	90.9%	227
TIG1				91.7%	95.5%	227
TIMP3	Inhibits matrix metalloproteinases	Tissue invasion and metastasis		50.0%	68.2%	227
TMS1		Evading apoptosis		7%	32%	248
TWIST	Inhibits oncogene- and p53-dependent cell death	Evading apoptosis		0%	42%	242
			6%		67%	45
				11.1%	17.5%	229
14-3-3σ	Cell cycle regulation	Limitless replicative potential	0%	81.8%	96%	181
				75%	60%	188
				100%	100%	227
			0.0%		91.0%	249
hMLH1, NKD2, PGRB RIZ1				2%–4%	1%–4%	188

Notes: *Adapted from Widschwendter and Jones;[39] #incidence among samples in respective series.

has also been studied in normal breast tissue at normal risk for breast cancer. The inactivation of TSGs by either allelic deletion or DNA methylation has been reviewed above. The expression of genes (principally protein) studied in normal risk tissues and comparison with high-risk normal tissue and breast cancer are summarized in Table 4. The genes in these studies were generally selected in the respective publications because of their relevance to breast carcinogenesis and were studied individually or as part of a small panel of genes by immunohistochemistry rather than as part of microarrays. Some genes

Table 4. Gene expression changes normal breast tissues at normal risk, at high risk, and in breast cancer.

GENE	ANALYSIS	EXPRESSION IN NORMAL RISK BREAST TISSUE	EXPRESSION IN HIGH RISK BREAST TISSUE	EXPRESSION IN BREAST CANCER	REFERENCES
BCl-	IHC	100%	100%	76%	250
	IHC	7% premenopausal 40% postmenopausal	80%		251
Cyclin A	ISH	35.3%	77.8%	77.8%	201
Cyclin B1	IHC	2.0%	2.0%	10.0%	252
Cyclin D1	IHC	Not expressed	43% (ADH)	57%	89
	ISH	54.5%	16.7%	16.7%	201
		No expression		35% amplified	253
	IHC	11.7%	39.4% (ADH)	43.6%–48.3%	202
	IHC		25% weak positive	100.0%	179
	IHC	Weak, undetectable		>50%	254
		Weak staining	Occasional weak stain	42.9%	255
	IHC	Premenopausal <1% Postmenopausal <10%	<10%	82%	251
Cyclin D3	IHC		0.0%	90%	179
Cyclin E1	IHC	No expression	No expression	28%	256
	Western blot		Very Low levels of expression	88.9%	129
	mRNA		Increased in 11%, ER pos	Increased in 23%	206
Cyclin E2	mRNA		Increased in 17%, ER Pos	Increased in 38.0%	206
β-catenin	IHC		Strong expression	Reduced in 41.8%	257
Cathepsin-D	AU score	40.3	37.7	129.9	258
E-cadherin (CDH1)	IHC		Strong expression	66.7%	257
	FISH			69.2%–84.6% loss	259
CHFR	IHC		Strong expression	Negative 36%	260
COX-I	IHC		Weak/moderate	Weak/moderate	261
COX-2	IHC		Negligible expression	56%	261
COX-2			Increased		234
$p16^{INK4A}$	IHC	(See DNA methylation)	16% low/negative	100.0%	179
	IHC		Patchy/heterogeneous	50% loss of express	262
$p21^{waf1/cip1}$	IHC		10%	90%	179
			<1.0%	90.0%	207
	IHC		Negative	49%	203
$p27^{kip1}$	IHC	90.0%		22.7%	263
	IHC	Strong staining-85.2%		33.8%	264
	IHC		High levels expression	80%	265
	IHC		65%	92.5%	179
$p34^{cdc2}$	IHC		16.0%	85.0%	264
$P34^{cdc2}$	IHC	2.5%		22.9%	252
TP53	IHC	No expression		39%	33
	IHC	12.7%	20.2 %		101
	Mutation (exonic)	28.1%	31.7%		101
	IHC	6.6%	14.1%		83
	IHC	3.0%	29.0%		29
	IHC		Not expressed	19.0%	203

(*Continued*)

Table 4. (*Continued*)

GENE	ANALYSIS	EXPRESSION IN NORMAL RISK BREAST TISSUE	EXPRESSION IN HIGH RISK BREAST TISSUE	EXPRESSION IN BREAST CANCER	REFERENCES
	IHC	Not expressed			103
	IHC	No expression	No expression	24%	250
	IHC		No expression	25.0%	143
	IHC/mutation	No expression	No expression	22% immunopositive 100% mutation in immunopositive	266
	IHC		30% mild expression	100% immunopositive in p53 mutations	267
	IHC	25.0%			84
	Mutation	8.3%			84
	Mutation in immunopositive	59.2%			100
	Mutation in immunonegative	26.7%			100
	IHC	16.0%		30.0%	268
	IHC		27%		31
	IHC	0%	12.9%	50%	269
	IHC		9.7%		208
	IHC	28.6%	0.0%		86
RB	IHC	Not expressed	Not expressed (ADH)	29.0%	89
	IHC		75%	58%	270
EGF	IHC	Not expressed		30.3%	271
EGFR	IHC	3%	37%		29
	IHC	33.3%	33.3%	9.1% (overexpressed)	272
		1.0%	5%	7.0%–26.0%	191
	IHC	Negative—weak positive		Negative	273
Fas	IHC	90.9%	87.5%	56.9%	208
FasL	IHC	22.6%	41.6%	45.8%	208
BASIC-FGF	IHC	100%		93%–100%	274
Her-2/neu		Not expressed (c-erbB2)		23%–59%	90
	IHC	No overexpression	20.0%		29
	IHC	6.25%	40.0%		86
	IHC	13.9%	12.7%		83
	IHC	0.0%	0.0%	26.4%	208
	IHC	No expression	No expression	88%	88
	Amplified	4.5%	9.5%	18%	82
	IHC	0%		30% overexpressed	82
	IHC		0.0%	37.5%	143
	IHC	8.3%			84
	IHC		Not expressed	45%	195
	IHC	Negative		41%	87
IGF-1	IHC	59%		61%–72%	274
	IHC	0.0%		38%	87
IGF-II	IHC	83%		78%–89%	274

Table 4. (*Continued*)

GENE	ANALYSIS	EXPRESSION IN NORMAL RISK BREAST TISSUE	EXPRESSION IN HIGH RISK BREAST TISSUE	EXPRESSION IN BREAST CANCER	REFERENCES
IGF-1R	Radiolabeling	1.0%	50%	50.0%–67%	191
Ki67	IHC	<3%		6.1%–17.3%	113
	IHC	0.93%			86
	Radiolabeling	<2%			124
	IHC	3.1% premenopausal 0.33% postmenopausal			125
	IHC	1.1%–5.00%			86
	IHC		8.06%		86
	IHC		0.91%	12.76%	195
	IHC		43%	100%	179
MYC	FISH	No amplification	No amplification	13%–33%	196
	IHC		63.6%—weak	100.0%	195
	IHC		65% with grade III tumors	100%	179
	Multiplex PCR		No amplification	21%	275
	FISH	No amplification		91.7%	276
	IHC	Negative		57.4%	271
	FISH			46.2%–76.9% gain	259
Proliferation index	IHC	2.5% premenopausal 0.5% postmenopausal			251
	IHC	2.0%		8.5%	252
PDGFβ	IHC	74%		57%–69%	274
PD-ECGF	IHC	49%		75%–85%	274
VEGF	IHC	96%		100%	274
RARβ	mRNA expression		98.0%	51.6%	277
RAS-p21	IHC	Weak, heterogeneous	Weak, heterogeneous	Strong	198
	IHC	Low/mild	6.7%	91.9%	199
	IHC	Intense stain	Intense stain	Heterogeneous	200
	IHC	Not expressed		64.8%	271
	Mutation		None	8.3%	278
c-Src		Trace amounts		Increased 4–30 fold	279
	IHC		Negative	50.7%	197
	Tyrosine kinase activity for src	Low		Increased 70%	280
Survivin	IHC		No expression	70.7% expressed	281
	IHC	Not expressed		79.1%	282
	IHC		Focal, weak	60.0%	283
Telomerase activity		14% low	56%	94% strong	284
		17%		76%	285
		Not detected	50% expression	67% expression	152
Telomere shortening		Shortening in 25%	58.3%	70%–88.9%	68
TGFα	IHC	Negative		49.0%	87
	Staining score	Negative 0.6		2.35	286
TGFβ	IHC	Negative		38%	87

Abbreviations: IHC, immunohistochemistry; ISH, *in situ* hybridization.

(*p16*^{INK4A}, *RARβ*) that were described in DNA methylation studies are included. It can be seen that the expression of most of these genes, with the possible exception of *Her-2/neu* and *p53* (which are discussed below), do not appear to be significantly altered (either increased or decreased) in normal breast tissue at normal risk based on comparison with expression patterns in high-risk normal tissue or breast cancer. We do not have sequencing data for these genes, and thus, the presence of mechanisms of gene inactivation such as indels or point mutations, or other mechanisms of gene activation including amplification, chimeric genes, fusion genes, point mutations, or mechanisms of conversion of proto-oncogene to oncogenes such as retroviral transduction or proviral insertion,[79,80] cannot be excluded. Many of the mechanisms of gene activation also require GCRs such as translocations, chromosomal fusions, or episome formation. However, we do not see evidence of these changes in karyotypic studies, and based on a comparison to respective expression values for high-risk normal tissue and/or breast cancer, gene activation appears to be uncommon in these normal risk tissues. If this supposition is correct then, while loss of function of TSGs and other genes by LOH or DNA methylation is present early in breast carcinogenesis, gene activation and amplification and associated GCRs do not appear to occur early but rather are much later events. Whole genome microarray and sequencing studies represent an important need for future studies of normal risk tissues to further define the timing of these changes in breast carcinogenesis.

One gene that may be overexpressed in normal risk breast tissue is *Her-2/neu,* which is amplified or overexpressed in approximately 10%–34% of cases of breast cancer.[81] Stark et al[82] found *Her-2/neu* amplification (increased relative copy number) in 4.5% of nonproliferative benign tissues, indicating that *Her-2/neu* amplification may occur as a relatively early event in the development of breast cancer (Table 4). Further, they found that the estimated risk of developing breast cancer for women with *Her-2/neu* amplification in their benign tissues was more than twofold increased; however, none of the benign tissues were positive for *Her-2/neu* (c-erbB2) protein overexpression. Rohan et al[83] found that 13.9% of low-risk benign lesions showed overexpression of c-erbB2 protein; however, overexpression was not associated with an increased risk for breast cancer. In a study by Millikan et al,[84] low levels (1%–5%) of staining were identified in nonproliferative lesions, but there was no instance of associated amplification (overexpression of *HER-2/neu* has been noted in malignant tissues having diploid copies of the gene, indicating that mechanisms other than gene amplification can contribute to elevated expression of the c-erbB2 protein).[82] Pechoux et al[85] reported overexpression of c-erbB2 in 47.1% of benign lesions, and Wells et al[86] noted positive staining for c-erbB2 in 62.5% of cases of apocrine adenosis, 40% of which were classified as intermediate or strong; normal breast epithelium was negative for c-erbB2 staining. In contrast, five other studies examining normal/benign low-risk breast tissue did not find any evidence of increased expression of *Her-2/neu* in these tissues.[29,87–90] Together, these studies indicate that expression of *Her-2/neu* in normal risk tissues is heterogeneous, and when increased, it is generally at levels lower than that observed in breast cancer. Whether the same criteria used to designate overexpression in breast cancer are applicable for risk assessment of normal breast tissue needs to be determined; however, the study by Stark et al[82] described above suggests that in some cases expression of *Her-2/neu* may have utility for these purposes. Importantly, the biological significance (and not simply the prognostic or therapeutic significance) of increased *Her-2/neu* expression must also be considered: while increased levels of *Her-2/neu* in normal tissues may not have prognostic significance, if present in the context of a cancerized field, may nevertheless still be sufficient to enhance chromosomal instability and susceptibility to additional mutations, thereby promoting progression through the carcinogenic pathway.

The *p53* gene, located on 17q13.1, has multiple cellular roles and is one of the most commonly altered genes in breast cancer.[91] Expression of *p53* can be altered in breast cancer by either mutation (occurring, on average, in 24%–27% of breast cancers)[92] or allelic deletion (occurring in up to 64% of cases).[93–99] Mutant *p53* has a long half-life with higher steady-state levels than wild type and is more readily detected by immunostaining. Three studies have demonstrated p53 mutations in benign low-risk lesions (Table 4),[84,100,101] and in addition, Rohan et al[83] found that positive *p53* immunostaining in benign breast disease was associated with 2.55-fold (95% confidence interval [CI] = 1.01–6.40) increased risk of progression to breast cancer. These three studies also demonstrated that p53 mutations may be present and not be associated with immunostaining. There have been 10 studies examining *p53* expression by immunohistochemical (IHC) alone and all found *p53* staining to be low or negative;[29,33,83,86,89,102–106] however, the possibility that some of the subjects in these latter reports might have unrecognized *p53* mutations cannot be excluded. It is interesting that structural chromosome studies described above suggest a significant incidence of AI or deletion at or around the *p53* locus in normal risk tissues (Table 2). This raises the possibility that in some cases low/absent IHC staining may also represent loss of *p53* rather than simply rapid clearance of a wild-type protein. Allred and Hilsenbeck,[107] in an editorial, suggested that low levels of p53 protein may be a marker of cells exposed to a carcinogenic environment, leading to increasing genetic instability, tumor initiation, and disease progression. Last, it should be emphasized that *p53* regulates many cellular processes, including apoptosis, cell cycle arrest, survival, DNA repair, genomic stability, and senescence.[91] Any loss of *p53* function, even partial, in these normal breast cells, therefore, has the potential to cause widespread cellular disruption and promotion of carcinogenesis.

Estrogens and estrogen receptor in normal breast tissue at normal risk for breast cancer. Estrogens play a prominent role in breast carcinogenesis through both their genotoxic and their mitogenic effects. The genotoxic effects of estrogens in normal breast tissue include the potential for single-strand breaks and telomere shortening,[67] double-strand breaks and segmental deletions with LOH,[58] and induction of DNA methylation by ROS,[11] as well as the potential for DNA adducts, point mutations, and MSI.[10] These effects contribute to enhancement of genomic instability and alterations in growth-controlling genes. At the same time, estrogens may have prominent mitogenic actions on the cell that are mediated by the nuclear transcription factors, estrogen receptor alpha (ERα) or beta (ERβ). ERα and ERβ signal in opposite ways from an AP1 site when complexed with the natural hormone estradiol: with ERα, 17beta-estradiol activates transcription, whereas with ERβ, it inhibits transcription, has antiproliferative effects, and has been considered a potential TSG.[108,109] The expression of ERα in normal breast tissue at normal risk has consistently been found to be low, generally <30% of women in most series (Table 5; range, 0%–57.0%).[110–116] One study found the ER gene to be methylated in four cases of

Table 5. Estrogen receptor alpha/beta expression in breast tissue at normal risk, at high risk, and in breast cancer.

ESTROGEN RECEPTOR	NORMAL RISK BREAST TISSUE*	HIGH RISK BREAST TISSUE*	DCIS	BREAST CANCER	REFERENCES
ERα		52%		81%	287
ERα					112
Nulliparous	43%				
Multiparous	54%				
ERα	31%			61.4%	288
	4.0%		0.7% (high grade DCIS)		113
	6.8%–42%		87%–92%		114, 115
ERα					251
Premenopausal	6%	23%			
Postmenopausal	35%	45%			
ERα	17.3%			31.5%	121
	6%	5%	31%		116
	7%				289
	7.0%	3.0%			290
	0%	20.3%		61.5%	291
	0%	10.2%			13
		Minority of cells		76%	262
		18.7×10^9 copies mRNA		16.7×10^9 copies mRNA	292
	57.0%	84.0%			293
	Unmethylated				294
	8.3% methylated				227
	40% methylated				231
	Unmethylated			25% methylated (ER negative)	245
ERβ		100%		86%	262
	94.3%		82.5%	85.0%	295
	85%	88.0%	3.0%		113
		82%		69.5%	117
	93%	>91%		31%	251
	Unmethylated	Unmethylated			244
		50%		35%	287
		12.3×10^7 copies mRNA		53.9×10^7 copies mRNA	292

Note: *Expression by immunohistochemistry unless stated otherwise.

fibrocystic disease (40% of benign breast lesions);[231] however, other studies reported the incidence of methylation to be low (5%) or absent.[59,237,245] Together, this would suggest that the low expression is generally not the result of silencing of the gene by epigenetic mechanisms. The expression of ERβ, conversely, is high being generally >75%.[113,117,118] The actions of estradiol on these tissues may be ERα dependent or independent. ER-dependent actions stimulate cell proliferation and initiate mutations arising from replicative errors occurring during premitotic DNA. The promotional effects of estrogen then support the growth of cells harboring mutations. Over a period of time, sufficient numbers of mutations accumulate to induce neoplastic transformation.[119] There is supporting evidence that ERα in normal breast tissue is functional and mediates both proliferative and metabolic responses in normal breast epithelium. Shekhar et al,[120] using a MCF10AT breast epithelial xenograft model, examined the early effects of estradiol exposure on morphological progression of preneoplastic lesions and defined the step(s) in the morphological sequence at which estrogen may act. They found that estrogen exerted a growth-promoting effect on benign or premalignant ductal epithelium by enhancing (1) the frequency of lesion (hyperplastic, atypical, ductal carcinoma in situ, invasive carcinoma) formation, (2) the size of lesions, (3) the speed of transformation from normal/mild hyperplasia to those with atypia, and (4) the degree of dysplasia.[120] Other studies have shown that, while 96% of steroid receptor-positive breast epithelial cells synthesized both ERα and progesterone receptor,[121] ER-positive cells were less likely to be dividing than ER-negative cells in normal breast tissue.[115] Clarke et al[121] have shown that ER-positive cells do not in general proliferate in the normal human breast. They describe a model in which proliferation of ER-negative cells is controlled by paracrine factors released from ER-positive cells under the influence of estradiol.[121] This also raises the important question of whether breast epithelial cells, following acquisition of mutations in growth-promoting genes, may become more sensitive to the mitogenic effects of estrogens during early carcinogenesis. Overall, the proliferative nature of normal breast tissue at normal risk, as measured by proliferative, mitotic or labeling indices, and apoptotic indices, appears to be low/normal.[86,106,113,122–125]

Summary and Conclusions

The present review examined published studies of molecular abnormalities in normal breast tissue considered to be at normal risk for breast cancer. These studies indicate several prominent molecular changes in these normal breast tissues, including LOH or AI from small segmental deletions at loci of potential TSGs, DNA methylation of tumor suppressor and other genes, telomere shortening, and potentially, overexpression of Her-2/neu and p53 mutations. There are several reasons to suggest that these molecular abnormalities may represent the early changes of breast carcinogenesis as follows: (1) many of these changes occur in loci or in genes known to be important in breast carcinogenesis. (2) Multiple cellular processes are potentially altered by inactivation of these sites including five of the six components of Hanahan and Weinberg's capabilities necessary for a cell to become cancer.[72] The presence of these abnormalities also indicates an important degree of early chromosomal and chromatin instability. (3) These changes have been identified in multiple reports examining these normal breast tissues. (4) Many of these changes persist and are observed in normal breast tissue at high risk for breast cancer (see below, Part II). (5) Although studies of normal breast tissue in very young women or teenagers are limited, available studies of RM tissue from girls aged 16, 19, or 21 years do not suggest any genomic abnormalities,[20] and thus, the above molecular abnormalities are new and acquired. (6) The genomic abnormalities are consistent with known DNA damage effects of the carcinogen estrogen, and the types of changes (LOH, DNA methylation, telomere shortening) are important in carcinogenesis. (7) These abnormalities were observed in normal breast tissue from women in their 30s or 40s (such as in RM specimens), which would allow for considerable exposure to, and damage from, estrogens. (8) The detection of these changes requires the presence of a clonal population of cells.[36,42,60] This in turn would indicate the requirement for initiation and promotion in these cells, and the presence of alterations in at least some of the growth-promoting genes, an important feature of early carcinogenesis. Many of the authors of these studies have interpreted these findings to suggest that the molecular abnormalities identified in these normal breast tissues may represent early carcinogenesis or an increased risk for breast cancer.[28,41,68,74,82,83,107] The degree to which these changes are associated with an increased risk is unclear, but intuitively they would increase the genomic instability of these tissues and their susceptibility to acquiring additional mutations. Last, the presence of these abnormalities in women in their 30s and 40s also suggests that the initiation of these changes probably occurred at a much younger age, also consistent with early exposure to estrogens.

The presence of genomic abnormalities in these normal risk tissues may provide an important corollary to observations on the life cycle of HMECs in vitro (Fig. 2). It has been shown that HMECs in culture (developed from RM tissue of young women aged 16, 19, and 21 years) and placed under conditions of increased stress (serum free) enter a period of proliferation arrest (stasis), followed by emergence into a post-stasis period of increased proliferation, which culminates in a period of marked genomic changes and growth arrest (agonescence). Cells in stasis are characterized by a normal karyotype, arrest in G1, expression of $p16^{INK4A}$, intact cell cycle checkpoint, and genomic stability,[20,23,126,127] whereas cells in early post-stasis are characterized by normal karyotype, loss of expression of $p16^{INK4A}$, DNA methylation of multiple genes, lack of cell cycle checkpoint control, and lack of genomic integrity.[22,24,127,128] By comparison, normal breast tissue from women at normal risk (described in Part I

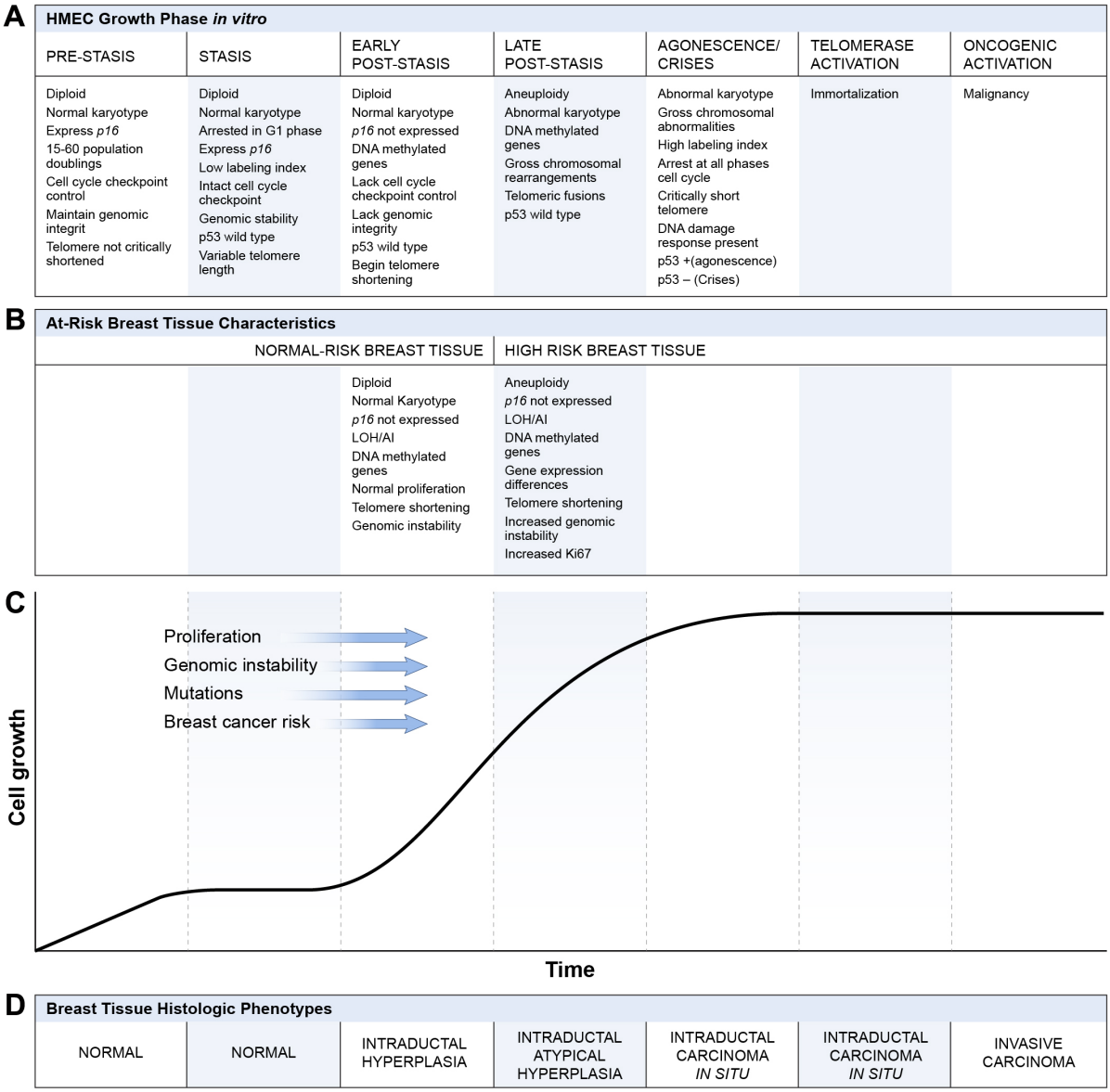

Figure 2. Characteristics of breast carcinogenesis in vitro and in at-risk breast tissues. (**A**) The molecular and cellular characteristics of disease-free HMECs observed in vitro are indicated for each life cycle growth phase.[20,22–24,126–128] (**B**) The genomic changes observed in normal breast tissue at normal risk and at high risk for breast cancer (as described in Part I and Part II, respectively, of this review) are indicated. The phases of the HMEC life cycle to which the genomic changes in normal risk and high-risk breast tissues correspond are shown. (**C**) The growth curve of HMEC in vitro is depicted (adapted from Tlsty et al,[75,127] Romanov et al,[22] and Novak et al).[24] Progression through the carcinogenic pathway is accompanied by increased proliferation, genomic instability, mutations, and breast cancer risk. (**D**) The histologic phenotypes which have been proposed to correspond to the HMEC phases are indicated.[75]

Abbreviations: LOH, loss of heterozygosity; AI, allelic imbalance; P16, *p16*^{INK4A}.

of this review) are characterized by normal karyotype, loss of expression of *p16*^{INK4A}, DNA methylation of multiple genes, loss of cell cycle genes, and loss of genomic integrity with LOH and telomere shortening, features which are comparable to those observed in early post-stasis in vitro. It was postulated that growth of HMEC in vitro in early post-stasis may mimic ductal hyperplasia;[70] ductal hyperplasia has in fact been identified in normal risk RM specimens.[130] Together, these findings suggest an important parallel between the genomic changes of normal breast tissue at normal risk for breast cancer, and the in vitro changes in HMECs during the early post-stasis period. If correct, this may have important implications for understanding and defining the molecular changes of early breast carcinogenesis.

A model describing the carcinogenic pathway in normal breast tissue at normal risk for breast cancer. A model is proposed describing the early genomic changes in the carcinogenic pathway of normal breast tissue at normal risk for breast cancer (Fig. 3). Exposure to estrogens, its metabolites, and other carcinogens elicit carcinogenic effects including

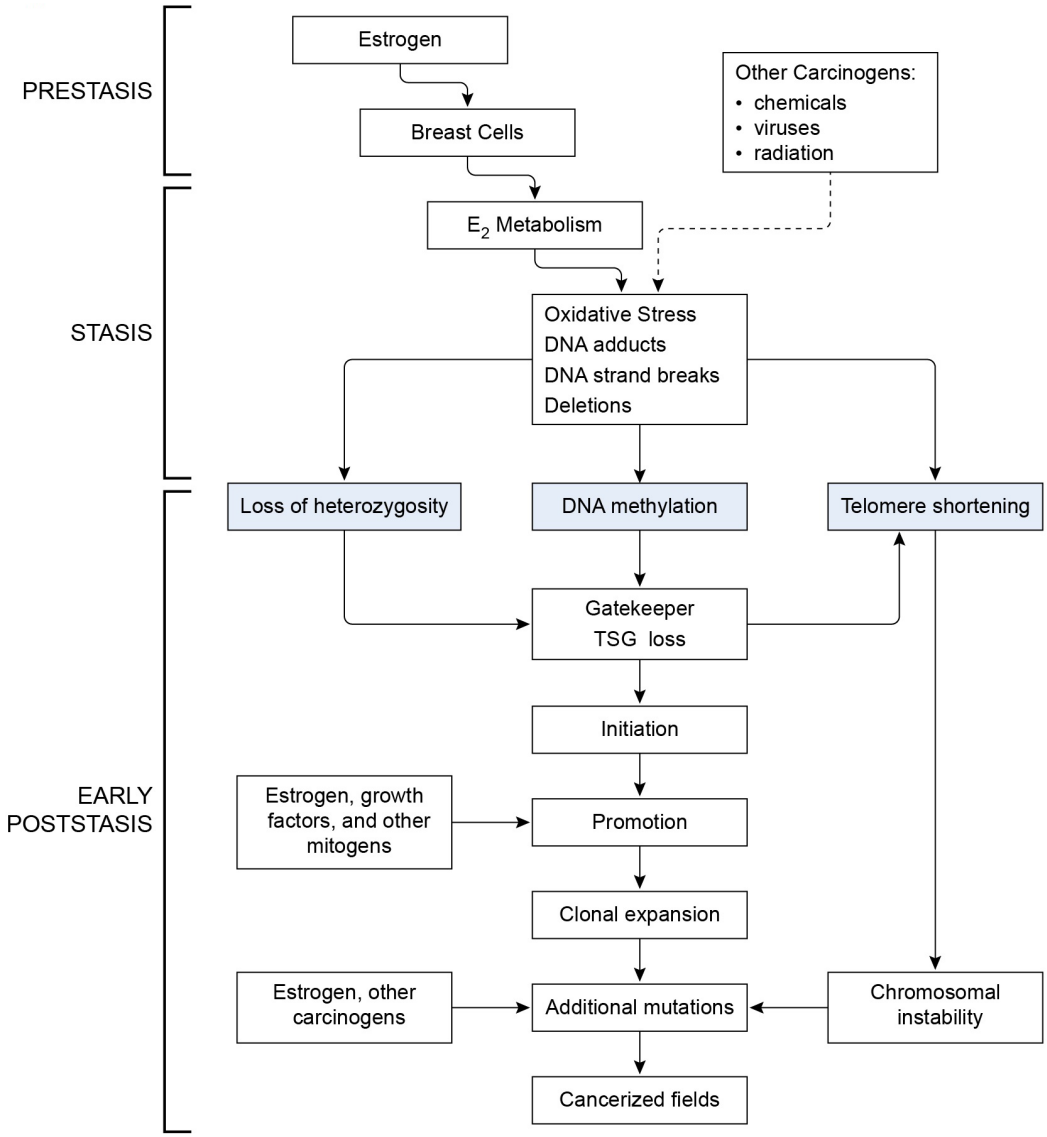

Figure 3. Model of the initiation and promotion of breast carcinogenesis in normal risk breast tissue. A proposed model for the early genomic changes in breast carcinogenesis, resulting from the carcinogenic effects of estrogens and other agents in normal breast tissue at normal risk for breast cancer. These effects include DNA methylation, chromosomal changes with LOH, and telomere shortening. Following initiation through abnormalities in growth-promoting genes, mitogenic stimulation may result in the promotion, clonal expansion, and the development of cancerized fields, with increased susceptibility to acquiring additional mutations. A correlation with phases of the HMEC life cycle in vitro is proposed.[24,75,127]
Abbreviation: TSG, tumor suppressor gene.

inactivation of gatekeeper TSGs by LOH and DNA methylation. These TSG losses represent an important loss of growth control, constituting the initiation of the carcinogenic process. Promotion of these conditions by estrogens and other mitogens encourages clonal expansion and development of a cancerized field, accompanied by increased genomic instability and increased susceptibility to the accumulation of additional mutations. Telomere shortening contributes to the genomic instability. These breast tissues remain histologically normal, with a normal karyotype and without gross chromosomal abnormalities. The relationship of this proposed pathway to phases of the HMEC life cycle is suggested (Fig. 2).

Part II: Normal Breast Tissue at High Risk for Breast Cancer

Introduction. The study of normal breast tissue at normal risk for breast cancer in Part I revealed the presence of genomic abnormalities associated with breast carcinogenesis including LOH, DNA methylation of TSGs, and telomere shortening. The presence of these abnormalities, with associated increased cell cycle progression, altered DNA repair, and other cellular alterations, would be expected to increase genomic instability and the susceptibility to acquiring additional abnormalities. In addition, exposure to estrogens through the normal menstrual cycle, and potentially other carcinogens, continues to inflict further damage on the cells. There are many factors

that have been identified, which increase the risk for breast cancer, including hormonal factors increasing estrogen exposure (early menarche, late menopause, late first full-term pregnancy, nulliparity, postmenopausal obesity),[19] family history, the presence of histologic abnormalities including atypical ductal hyperplasia or lobular carcinoma in situ, or the presence of breast cancer (Table 6). In the latter two situations, the presence of histologic abnormalities also indicates progression through the carcinogenic pathway, with the associated normal tissue (contralateral, or normal adjacent to the lesion) at increased risk for breast cancer. At the same time in each of the higher risk states, one would anticipate an increase, qualitatively and/or quantitatively, of genomic abnormalities and in genomic instability compared with lower risk tissues. Evidence of this instability is provided by the potential for rapid progression of the higher risk states to a state with still greater instability or even malignancy. For example, in HMECs, as the cells approach the second plateau (senescence), between 10 and 20 population doublings before the final passage of cells, there is the rapid accumulation of genomic abnormalities including translocations, deletions, other rearrangements, telomeric associations, polyploidy, and aneuploidy.[22] By analogy, in normal breast tissue adjacent to breast cancer, the recurrence rate for breast cancer (following excision in the absence of breast irradiation) is 35% at 12 years,[131] quite high with progression over a short period of time. Together, these observations indicate the importance of defining the genomic changes characteristic of high-risk tissue. The analysis of higher risk normal breast tissue will provide critical information about the sequence of events in breast carcinogenesis, promote identification of important intermediate targets for breast cancer prevention by identifying advanced but premalignant changes in breast carcinogenesis, facilitate development of a molecular signature for risk assessment, and facilitate selection of women who may be candidates for prevention therapy by virtue of the presence of targets or the presence of more advanced carcinogenic changes. Our ability to understand the breast carcinogenic pathway has been advanced considerably with recent studies that provided a detailed picture of genomic changes in breast cancer.[132] This will help in defining the importance and the timing of the appearance of earlier genomic abnormalities in normal breast tissue.

To provide a better understanding of molecular changes associated with increased risk for breast cancer, published reports of normal breast tissue at high risk for sporadic breast cancer were studied. The categories of tissues that are studied are described under the "Materials and Methods" section. The molecular categories selected for Part II were those studied in the previous section examining breast tissue at normal risk for breast cancer and included numerical chromosomal changes,

Table 6. Risk of high-risk breast tissues and histologic lesions for breast cancer.

TISSUE/HISTOLOGIC LESION	RISK/INCIDENCE	REFERENCES
Contralateral breast tissue	0.6% recurrence/year	296
	RR = 2–6 fold	297
	Incidence at 5 year = 4.1% RR = 1.4	298
Normal breast tissue adjacent to cancer: in-breast recurrence	1.4%–5.7%/year recurrence RR = 3.0*	299
	35% recurrence at 12 years	131
	25.1% locoregional recurrence at 10 years	300
High risk normal tissue cohort without epithelial atypia	4.0% risk of breast cancer at 3 years	31
High risk normal tissue cohort with epithelial atypia	15.0% risk at 3 years	
Atypical ductal hyperplasia	4.0 fold increased risk of breast cancer	305
	16.9%–18.0% risk at 20 years#	301, 302
	RR = 3.88–4.4	
	1 lesion 62.5% (Ipselateral)	303
	2 lesions 54.6% (Ipselateral)	
	≥3 lesions 59.1% (Ipselateral)	
	1 lesion –9.4%	302
	2 lesions 12.5%	
	3 lesions 25.1%	
Lobular carcinoma *in situ*/atypical lobular hyperplasia	7.1% at 10 years	304, 305
	RR = 4–5 fold	
Gail Index, high risk	1.67% risk of breast cancer at 5 years	

Notes: *Without vs. with whole breast irradiation. #Single focus of ADH.

structural chromosomal changes, epigenetic changes, gene expression changes, the estrogen receptor (ER), and estrogen responsiveness. This will provide an important opportunity to identify progression within these categories in the transition from normal risk to high risk, as well as to identify new and separate characteristics of the high-risk state. These categories are also thought to best provide a comprehensive picture of molecular abnormalities in normal breast tissue. It is important to note that characterization of high-risk normal tissue in this, Part II, often included a comparison to RM (normal risk) controls. It will be remembered that RM tissues were also characterized in studies of normal risk tissues in Part I, and together, this will provide further continuity in this overall review of the carcinogenic pathway.

A model for the development and progression of changes in the carcinogenic pathway of high-risk breast tissue is proposed, and the relationship of these changes to the progression of changes in vitro, which have been observed in the HMEC life cycle, is discussed.

Genomic changes.

Numerical chromosomal changes in normal breast tissue at high risk for breast cancer. Aneuploidy is an important indicator of genomic instability, with multiple consequences for cell proliferation, cell physiology, and tumorigenesis.[133,134] Examination of normal breast tissue at high risk for breast cancer revealed an increased incidence of aneuploidy with progression from normal risk to high-risk tissues (Table 1). Botti et al[15] examined the primary cancer and normal adjacent and contralateral breast tissue with centromeric-specific probes for aneusomy of chromosomes 1 and 17 and found the following: (1) almost all primary breast tumors were aneusomic for chromosomes 1 and 17. (2) The primary breast tumor and adjacent uninvolved parenchyma shared the same pattern of chromosomes 1 and 17 aneusomy in 66.7% of patients. (3) Chromosomes 1 and 17 aneusomy in contralateral benign breast samples from high-risk patients were not different from those in the primary breast tumor or adjacent tissue samples. The finding of identical chromosomal changes in the contralateral breast and adjacent normal tissue were both significantly higher than that observed in benign low-risk breast tissue, were consistent with a field effect, and indicated that the changes in the normal tissue adjacent to breast cancer do not simply represent a paracrine effect of the primary tumor. They concluded that chromosomes 1 and 17 aneusomy may represent an intermediate biomarker of breast tumorigenesis. Sneige et al[135] evaluated fine-needle aspirates of breast tissue from women at either low risk or at high risk for breast cancer and found chromosomal aberrations in 69% of high-risk nonproliferative cases. High-risk cases had significantly more monosomy of chromosomes 1, 11, and 17 and polysomy of chromosome 8 compared to low-risk cases, indicating that aberrations of chromosome number are common in normal tissue of high-risk women, irrespective of cytologic findings.[135] These are in agreement with other studies that observed aneuploidy in high-risk normal tissues by karyotypic analysis,[34,136,137] DNA index,[29] or FISH.[48,138] Aneuploidy in these tissues may be an important contributor to deregulation of the transcriptome, aneuploid-associated stresses, and production of a tumorigenic karyotype, all serving to promote continued progression in the carcinogenic pathway.[133,134,139]

The progression of normal breast tissue from a state of low risk to high risk for breast cancer is accompanied by several changes that may contribute to the development of aneuploidy. These tissues exhibit shortening of telomeres, and it has been shown that in primary epithelial cells, the chromosomes with short telomeres are more frequently involved in missegregation events than chromosomes of normal telomere length, with whole chromosome aneuploidy occurring through both nondisjunction and anaphase lagging of dicentric chromatids.[64] Aneuploidy may also be related to loss of critical TSGs. Abnormalities of *p16^{INK4A}* and *p53* may be altered in high-risk normal breast tissue, and abnormalities in these two genes, either alone or in combination, may promote telomere shortening (see Discussion below of Telomere dysfunction). Loss or mutational inactivation of p53 may lead to abnormal amplification of centrosomes, increasing the frequency of mitotic defects leading to unbalanced chromosome transmission to daughter cells.[140] In addition, as will be discussed below, the progression from low-risk to high-risk normal breast tissues is accompanied by widespread changes in gene expression. This could potentially include altered mitotic checkpoint, kinetochore, centrosome, or DNA repair genes, which could influence the development of aneuploidy.[141] Last, the presence of aneuploidy in high-risk normal breast tissue and its absence in low-risk tissues may also allow aneuploidy to potentially serve as a biomarker for high-risk breast tissue.

Structural chromosomal changes in high-risk normal breast tissue. Structural chromosome abnormalities have been identified in normal breast tissue at increased risk for breast cancer and are principally in the form of small segmental or microdeletions, which are frequently associated with AI or LOH.[42,142] Many of these changes are also concordant with changes in the associated breast cancer, indicating their importance in breast carcinogenesis. An important structural abnormality, telomere shortening, has also been identified in high-risk tissue and at an increased frequency compared with normal risk breast tissue. Together, these findings indicate a significant increase in chromosomal instability with progression to high-risk breast tissue. At the same time, GCRs, including large-scale deletions or gene amplification as well as telomeric fusions appear to be rare in the high-risk normal breast tissue.

AI and LOH or copy number gains or losses have been identified across a significant number of chromosomal loci and are among the most common structural chromosomal findings in normal high-risk breast tissue. Among the 16 series summarized in Table 7, the vast majority identified AI/LOH

Table 7. Structural chromosomal changes in normal breast tissue at high risk for breast cancer.

LOCUS	GENE(S)	TISSUES SOURCE	MOLECULAR ABNORMALITY	INCIDENCE IN HIGH RISK BREAST TISSUE*	INCIDENCE IN BREAST CANCER*	REFERENCES
1p31/1p36	*14-3-3σ*	Normal adjacent	LOH	18.0%	33.3%	142
1p36 (DIS2644)	*ATP13A2*	Contralateral breast	LOH	67% (CL breast)	68%	306
D1S2843		Contralateral breast	LOH	72%	93%	306
1p36.13 (D1S552)	*IFF02 LOH/MS markers*	Contralateral breast	LOH	60%	74%	306
1p36.32–34.1	*PLEKHG5* *TNFRSF9* *TNFRSF8* *TNFRSF1B* *NBL1* *FABP3*	Normal adjacent >2 cm	ArrayCGH	Copy number loss	Copy number loss	144
1q		Normal adjacent	LOH	22.2%	16.7%	42
		Normal adjacent	LOH	35.3%	64.7%	142
1q21.1–22	*LASS2, TNFAIP8L2, EFNA1*	Normal adjacent >2 cm	ArrayCGH	Copy number loss	Copy number loss	144
1q31, 1q32		Normal adjacent	LOH	56%	64.7%	142
1q42		Normal adjacent	deletion	14.3%	28.6%	137
2q11.1	*REV1L*	Normal adjacent	ArrayCGH-homozygous deletion	10.0% homozygous deletion 43.3% single copy deletion	10.0% homozygous deletion 43.3% single copy deletion	145
TPO (*2pter*)		Normal adjacent	LOH	16.5%	33.0%	39
3p14	*FHIT*	Apocrine metaplasia adjacent to cancer	LOH	28.6%	14.3%	40
3p11–26	*TRβ1 and RARβ*	Normal adjacent	LOH	27.1%	75%	175
3p22.1–21.1	*RBM5, TUSC2, TUSC4, CYB561D2*	Normal adjacent >2 cm	ArrayCGH	Copy number loss	Copy number loss	144
3p24 (3p22–25)		Normal adjacent	LOH	20.0%	33.3%	143
3p25.3	*CIDEC, VHL*	Normal adjacent >2 cm	ArrayCGH	Copy number loss	Loss	144
3q26.33–27.3		Normal adjacent >2 cm	ArrayCGH	Copy number loss	No change	144
3q29	*PAK2*	Normal adjacent >2 cm	ArrayCGH	Copy number loss	Loss	144
4p16.3		Normal adjacent >2 cm	ArrayCGH	Copy number loss	Copy number loss	144
7p12–15	*EGFR locus*	Normal adjacent	AI	30%	34%	190
8p22–p21.3	*RHOBTB2, PINX1, DLC1, TUSC3, LZTS1, PDGFRL*	Normal adjacent	AI	4.2%	37.5%	213
9p21	*$p16^{INK4A}$*	Apocrine metaplasia adjacent to cancer	LOH	14.3%	28.6%	40
9p21/D9S171		Normal adjacent	LOH	Present		307
9p22/D9S157		Normal adjacent	LOH	Present		307
9q33.3–34.3	*MAPKAP1, PTGES, TRAF2*	Normal adjacent >2 cm	ArrayCGH	Copy number loss	Copy number loss	144
10q21.3–22.2	*CCAR1, AMID, UNC5B*	Normal adjacent >2 cm	ArrayCGH	Copy number loss	Copy number loss	144

(*Continued*)

Table 7. (*Continued*)

LOCUS	GENE(S)	TISSUES SOURCE	MOLECULAR ABNORMALITY	INCIDENCE IN HIGH RISK BREAST TISSUE*	INCIDENCE IN BREAST CANCER*	REFERENCES
11p		Normal adjacent	LOH	11.1%	0.0%	42
11p15	TH01	Apocrine metaplasia adjacent to cancer	LOH	28.6%	28.6%	40
11p15.1	*XLKD1, MRVI1, TSG101, HTATIP2*	Normal adjacent	AI	66.7%	13.6%	213
11p15.5		Normal adjacent	LOH	3.3%	16.7%	143
11q12.2–13.1	*DDB1, BRMS1*	Normal adjacent	LOH	Copy number loss	Copy number loss	144
11q13.1		Normal adjacent	LOH	Copy number loss	Copy number loss	144
11q22 (D11S2000)	*GR1A4*	Contralateral breast	LOH	67%	80%	306
12q13.11–13.3	*DDIT3*	Normal adjacent	LOH	Copy number loss	Loss	144
12q24.23–24.31	*CDK2AP1*	Normal adjacent	LOH	Copy number loss	Copy number loss	144
13q (between BRAC2 and RB)		Normal adjacent	LOH	11.5%	53.9%	142
13q (D13S267)	*BRCA2*	Normal adjacent	LOH	20%	40%	57
13q12–14	*BRCA2, RB*	Normal adjacent	LOH	28.6%	71.4%	40
13q13		Normal adjacent	LOH	0%	33.3%	143
1611.2–q12.1	*DNAJA2*	Normal adjacent	CGH	Copy number loss	Copy number loss	144
16p13.3	*CIQTNF8, TNFRSF12A, Q8NFX8_human TRAP1*	Normal adjacent	LOH	Copy number loss	Copy number loss	144
16p13.3–11.2	*TNFRSF17, ERCC4, BFAR*	Normal adjacent	ArrayCGH	Copy number loss	Copy number loss	144
16q21–24.3	*TRADD, GAS8*	Normal adjacent	ArrayCGH	Copy number loss	Copy number loss	144
16q22	*CDH1*	Apocrine metaplasia adjacent to cancer	LOH	42.9%	57.1%	40
16q22–24	*CDH1*	Normal adjacent	LOH	16.7%	5.6%	42
16q23.3 (D16S402)	*CDH13*	Contralateral breast	LOH	63%	76%	306
16q23.3–q24.1 (D16S504)	*WW0X*	Contralateral breast	LOH	84%	92%	306
16q23.3–q24.1 (D16S518)	*WWOX*	Contralateral breast	LOH	71%	80%	306
16q23.1–24.2	*CDH13*	Normal adjacent	LOH	25%	100%	39
17p13 (D17S520)	*MYH13*	Contralateral breast	LOH	79%	82%	306
17p		Normal adjacent	LOH	13.9%	38.9%	142
17p13.1 (D17S786)	*PIK3R5*	Contralateral breast	LOH	82%	76%	306
17p13.1	*TP53*	Apocrine metaplasia adjacent to cancer	LOH	71.4%	71.4%	40
17p13.1		Normal adjacent	LOH	3.3%	53.3%	143
17p13.3–q25.3	*TUSC5, TP53, TNFRSF13B, TNFAIP1, TP53I13, BRCA1, RAD51C*	Normal adjacent	ArrayCGH	Copy number loss	Copy number loss	144
17q		Apocrine metaplasia adjacent to cancer	LOH	42.9%	42.9%	40
		Normal adjacent	LOH	10%	30%	57
		Normal adjacent	LOH	11.1%	0.0%	42

17q11.2–12	Normal adjacent	LOH	11.8%	58.8%	142
17q21	*BRCA1*	Normal adjacent	LOH	25%	50%	39
17q24/D17S785		Normal adjacent	LOH	40.0%	60%	39
19p13.3	*ZNF14*	Normal adjacent	ArrayCGH-homozygous deletion	6.7% normal adjacent 30% single copy deletion	6.7% normal adjacent 30% single copy deletion	145
19q13.3–q13.43	*VGLL3, GLTSCR1*	Normal adjacent	CGH	Copy number loss	Copy number loss	144
19q13.2	*NPAS1*	Normal adjacent	ArrayCGH-homozygous deletion	36.7% homozygous deletion 30% single copy deletion	36.7% homozygous deletion 30% single copy deletion	145
20p11.21–q11.23	*PDRG1, TP53INP2*	Normal adjacent	ArrayCGH	Copy number loss	Copy number loss	144
22q11.23–13.33	*FAM10A6, TNFRSF13C*	Normal adjacent	ArrayCGH	Copy number loss	Copy number loss	144
22q13.1	*APOBEC3B*	Normal adjacent	ArrayCGH	16.7% homozygous deletion	16.7% homozygous deletion	145
Sites of AI		Normal adjacent	AI	74% ≥2 sites of AI 57% ≥3 sites of AI	70% ≥2 sites of AI 40% ≥3 sites of AI	60
Clonal chromosomal abnormalities		Normal adjacent	Karyotype	18%		34

Note: *Percentage incidence in samples of respective series.
Abbreviations: AI, allelic imbalance; ArrayCGH, array comparative genomic hybridization; CL breast, contralateral breast.

in at least some cases. Many of these abnormalities are at loci for genes important in breast carcinogenesis. The incidence and frequency of AI/LOH in normal breast tissue also appears to increase with increasing risk of the tissue. This was demonstrated in studies by Larson et al[37,41,42] who examined AI/LOH in tissues at normal risk (RM), at high risk (normal tissue adjacent to atypical hyperplasia or adjacent to cancer), or in breast cancer. They observed that, as one progressed from low-risk RM to normal breast tissue at high risk, the percentage of all subjects with any abnormality increased, the mean percentage of abnormal alleles increased, and the mean percentage of abnormal ducts increased. These trends were more pronounced in women under the age of 50 years.[37] They concluded that the increased prevalence of AI in normal-appearing epithelium is associated with breast cancer and increased breast cancer risk and may reflect dysregulation, even in normal-appearing epithelium, of genomic processes contributing to cancer development.[41] In addition to increasing frequency in normal adjacent breast tissue (NABT), there is also a high degree of concordance for these changes between the normal adjacent tissue and the tumor,[40,57,60,142,143] emphasizing a role in breast carcinogenesis. Heaphy et al[60] observed that more than a third of unbalanced alleles in adjacent, histologically normal tissues were conserved in the matched tumors. In 8 of 30 cases in which LOH was detected in the adjacent morphologically normal TDLUs, Deng et al[143] found that the same allele was missing in the adjacent carcinoma in all eight cases. They concluded that the molecular heterogeneity that characterizes invasive breast cancers may occur at the earliest detectable stages of progression. They also confirmed that these findings did not represent contamination of the TDLUs with cancer cells. Forsti et al[142] observed a 33% concordance between tumor and normal tissue for LOH in chromosome 1, and 11% and 13% for chromosomes 13 and 17, respectively. They concluded that the LOHs seen in the morphologically normal tissue may reflect early, critical events required for the progression to invasive breast cancer. Further, these results demonstrated that tumor adjacent normal tissues already harbor genetic changes typical for tumors, and these alterations can reveal the earliest changes leading to tumorigenesis. Li et al[144] studied normal breast tissue >2 cm from adjacent carcinoma by arrayCGH (1 Mb resolution) and found multiple aberrant genomic regions, which were also shared with the primary carcinoma, including 1p36.32–34.1, 3p22.1–21.1, 9q33.3–34.3, 11q12.2–13.1, 16p13.3, 16q11.2–12.1, 16q21–24.3, 17p13.3–q25.3, 19p13.3–q13.43, and 22q11.23–13.33. Genes related to cell proliferation, DNA repair, cell cycle, and apoptosis regulation, such as *TP53* and the genes for cell division protein kinase 9, programmed cell death protein 5 (*TFAR19*), DNA-repair protein (*XRCC1*), and apoptosis regulator BAX, appeared to be involved.

The genomic chromosomal losses identified by microsatellite markers or arrayCGH in high-risk normal tissue appear to be primarily small segmental losses or microdeletions

(defined as deletions ≤5 Mb and containing <10–12 genes), occurring at a single or at most a few loci on a single chromosome arm.[41,42,142,145] Larson et al[42] found that LOH in normal-appearing high-risk ducts/TDLUs encompassed only single markers, ie, additional informative loci on the same chromosome arm showed no LOH. In contrast, LOH in cancers usually encompassed all informative markers on a chromosome arm. Deng et al[143] noted that the size of the LOH locus varied from one to three satellite markers. Komatsu et al,[145] using arrayCGH, observed homozygous deletions of *REV1L, ZNF14*, and *NPAS1* genes in normal mammary tissue obtained from patients who had homozygous deletions in cancer; these homozygous deletions were considered to be small-scale deletion polymorphisms. Studies using arrayCGH and metaphase spreads, however, did not detect any large-scale amplifications or deletions.[57]

Importantly, it can be seen in Table 7 that a wide range of chromosomal loci and genes may be altered by these segmental losses in high-risk normal breast tissues. Multiple TSGs may by involved—*14-3-3σ, FHIT, RARβ, p16^{INK4A}, TH01, BRCA1, BRCA2, p53, RB*, and *CDH1*. These in turn regulate important cellular processes, including cell cycle control (*14-3-3σ, FHIT, p16^{INK4A}, p53, RB*), apoptosis (*14-3-3σ, FHIT, RARβ, p16^{INK4A}*), DNA repair (*BRCA1, BRCA2, p53*), and cell–cell adhesion (*CDH1*). This emphasizes the heterogeneity and diversity of chromosomal effects in these normal tissues and that multiple genes may contribute to disruption of a given cellular process (cell cycle control, apoptosis, DNA repair) with potential for significantly enhancing genomic instability and progression of carcinogenesis.

Telomere dysfunction in high-risk normal breast tissue. Telomeres are the terminal ends of chromosomes, which play an important role in maintaining chromosome stability. Telomere loss can have significant consequences for the cell, including sister chromatid fusion and prolonged BFB cycles that result in DNA amplification and large terminal deletions, translocations from either nonreciprocal transfer or duplication of all or part of an arm of another chromosome,[146] or telomeric crises.[62,147] Telomere shortening can result from several causes, including spontaneous progressive shortening with each cell division (end-replication problem),[62] DNA single-strand breaks,[148] double-strand breaks, or loss of capping function.[146] Telomere shortening may be present throughout the breast carcinogenic pathway. It has been shown, for example, that shortened telomeres are present in LPs and mature luminal cells[65] and in normal risk RM tissue (see Part I above).[68] Examination of normal high-risk breast tissue has revealed both an increase in telomere shortening and an increase in telomerase expression compared with normal risk tissues, indicating progression of carcinogenesis and chromosomal instability in the transition from normal risk to high-risk tissues.[64,149] Heaphy et al[60] found shortened telomeres (to a level outside the range seen in >95% of all normal tissues) and unbalanced allelic loci present (a) in 50%–75% of tumor-associated histologically normal tissue specimens, (b) at sites at least 1 cm from the tumor margins, and (c) in a substantial fraction of the cells comprising the adjacent normal tissue. The extent of the altered telomeres decreased with increasing distance from the tumor, consistent with a cancerized field. Meeker et al[68] similarly noted that normal secretory cells in TDLU of NABT demonstrated moderate telomere shortening in 58% of cases, which was increased compared with a 25% incidence in normal noncancerous epithelium. Telomere length variation (TLV) in NABT has also been found to be a potential biomarker for local recurrence, with subjects having a small TLV showing a fivefold (95% CI = 5 1.2–22.2) higher local recurrence than those with a large TLV.[149] These studies provide strong evidence of increased and clinically significant telomeric shortening in high-risk normal breast tissues.

The enzyme telomerase stabilizes chromosomes by maintaining chromosome length, immortalizes mammalian cells, and is expressed in more than 90% of human tumors,[150] but is not expressed in normal risk tissues.[151] Three studies,[150,152,153] but not a fourth,[154] have found telomerase to be expressed in high-risk normal breast tissues. Expression was greatest in normal tissues within 1 cm of the cancer and diminished with greater distances.[150] The spatial relationship between human telomerase reverse transcriptase (*hTERT*) promoter activity and proximity to the tumor is identical to that reported previously for telomere length and AI in bulk breast tissues.[60,150] Trujillo et al[150] found that increased *hTERT* mRNA expression was associated with overexpression of many growth-promoting genes in cell cycle and mitosis, in agreement with others,[155] which would be expected to further exacerbate genomic instability and promote maintenance and immortalization of cells with telomeric-shortened chromosomes. Hines et al[153] observed that a subset of the epithelial cells within the normal tissue expressed higher cellular levels of *hTERT* mRNA and speculated that tumors of the breast arise from these *TERT* expressing epithelial cells.

Telomere dysfunction may be further enhanced by mutations in two TSGs, *p16^{INK4A}* and *p53*, which have been observed in high-risk normal tissue and may serve as predisposing mutations promoting telomere shortening and eventually aneuploidy. Loss of *p16^{INK4A}* is an early event in breast carcinogenesis and may occur by either deletion (LOH in 33% in normal breast tissue)[40] or DNA methylation.[156] Inactivation of *p16^{INK4A}* via hypermethylation, with continued replication, leads to further telomere erosion, resulting in loss of the capping function and initiation of the BFB cycle.[22,66,127,146] Disruption of telomere structure by erosion of telomeric DNA or loss of telomere-binding protein function also activates *p53* to initiate cellular senescence or apoptosis to suppress tumorigenesis.[157] Telomere damage can also elicit a G1/S arrest through the *RB*-regulator *p16^{INK4A}*, especially in cells lacking *p53* function.[157] Mutation of *p53 or RB* genes enables a precancerous cell to divide approximately 20–30 more times,

and if it is in the presence of telomerase reactivation, cellular immortalization may occur.[147] Telomere dysfunction can act synergistically with *p53* deficiency to initiate malignant transformation.[158] Importantly, *p16* and *p53* may act together to further enhance telomere dysfunction. Jacobs and de Lange,[159] for example, demonstrated that while *p53* deficiency alone only partially abrogated the telomere damage-induced cell cycle arrest, combined inhibition of *p16(INK4A)* and *p53* led to nearly complete bypass of telomere-directed senescence. They concluded that *p16(INK4A)* contributes to the p53-independent response to telomere damage. Inhibition of the *p16/Rb* and/or *p53/p21* pathways thus enables continuous cell division and critical telomere shortening, or telomere crisis.[160] Loss of $p16^{INK4A}$ and *p53* together in normal high-risk epithelium may thus be an important contributor to genomic instability and progression of carcinogenesis in these tissues.

Gene expression changes in high-risk normal breast tissue. Studies of gene expression provide further evidence of increased genomic abnormalities in normal high-risk breast tissue. Three types of studies have examined gene expression in these tissues: (a) gene expression profiling studies, (b) epigenetic inactivation of tumor suppressor and other genes, and (c) studies of oncogenes, cell cycle regulatory, and other genes. Each of these categories will be considered separately. Gene expression profiling studies identified genes differentially expressed either within high-risk tissue (such as cancer-adjacent normal) or between high-risk and normal risk tissues (eg, RM). Graham et al[161] directly compared normal adjacent tissues from ER-positive breast cancers and ER-negative breast cancers. A total of 198 genes were identified, which were differentially expressed between tissues of the two subtypes and which identified functional categories implicated in carcinogenesis, including cell adhesion, motility, transcription, cell cycle, immune response, and hormonal activity and regulation. The gene expression differences which they found in these normal high risk tissues also reflected the gene expression differences in the ER positive compared with the ER negative primary cancers. They concluded that genomic changes characteristic of specific breast cancer subtypes may be detectable in these normal tissues before histologic evidence of abnormalities, and further, that normal epithelium gene expression profiles could help define a breast cancer subtype-specific risk signature in normal tissue. Further support for differences according to subtype is found in the studies by Martini et al.[162] They identified genes differentially expressed between cancer and cancer-adjacent normal tissues for each of the four hormone receptor (HR) subtypes (ER+/Her2–, ER+/Her2+, ER–/Her2+, and ER–/Her2–). They found unique genes differentially expressed for each subtype, suggesting that gene regulation is dependent upon the *ER/HER-2* markers selected. Two additional studies provide further evidence of receptor subtype-specific expression patterns in the high-risk normal tissues. Tripathi et al[163] compared normal breast epithelium adjacent to ER-positive breast cancer with RM specimens and identified 105 genes differentially expressed, most commonly in immediate early genes, genes of MAPK signaling cascade, G-protein coupled and chemokine receptor activity, and transcription factors. A total of 32 genes (31%) were previously implicated in breast carcinogenesis, of which 25 exhibited reduced expression and 7 exhibited increased expression in the high-risk normal vs. RM tissues. These findings demonstrated that cancer-related pathways may be disturbed in normal breast epithelium and supported the high degree of genetic instability of these tissues. Radovich et al,[164] using RNAseq, provided a detailed analysis of the triple-negative breast cancer subtype in cancer-adjacent normal tissue. When they compared NABT to normal control (microdissected normal ductal epithelium), they found 933 genes differentially expressed (>2.0-fold, $P < 0.05$), with over 90% being upregulated in NABT, in contrast to the high degree of gene expression downregulated for luminal A tumors described in the study by Tripathi et al.[163] This is further evidence that the expression profile of the normal adjacent tissue relates to the subtype of the associated cancer and suggests that cancer subtype determination occurs early in the carcinogenic process. Importantly, these findings demonstrated that cancer-related pathways may be disturbed in normal high-risk breast epithelium, contributing expression abnormalities to the genetic instability of these tissues.

Recent studies have suggested that differentially expressed genes in high-risk tissues may also play a role in wound healing and inflammation. Troester et al[165] examined genes differentially expressed between cancer-adjacent normal tissue and RM and found the cancer-adjacent normal signature to be enriched for many genes previously shown to have an important role in wound healing and inflammation, including higher transcript levels of *F3* in cancer-adjacent normal samples, along with a number of other genes involved in extracellular matrix formation such as *ADAMTS4, OGN, PDGFRL, FBLN1, LUM,* and *EFEMP1*. They concluded that the prevalence of the wound response signature in histologically normal tissue adjacent to breast cancer suggests that microenvironment response is an important variable in breast cancer progression. Importantly, this "wound response" signature in cancer-adjacent normal tissue correlated with disease-free survival (DFS), providing important evidence that a molecular profile in normal breast tissue can have prognostic significance. Interestingly, Radovich et al[164] similarly found expression patterns of genes involved in edema and angiogenesis in cancer-adjacent tissue, in agreement with the findings by Troester et al[165] that adjacent normal tissue contains gene expression patterns indicative of wound healing. The etiology of the activation of a wound response signature is not known, although paracrine interactions among the diverse cell types that are present in the mammary gland during carcinogenesis might be considered.[165] Two distinct gene expression subgroups have been identified in the microenvironment of cancer-adjacent normal tissue as follows: an active subtype

defined by high expression of fibrosis and cellular movement genes and an inactive subgroup defined by high expression of claudins and other cellular adhesion and cell–cell contact genes.[166,167] These subtypes were found to influence the aggressiveness and outcome of ER-positive human breast cancers,[166] further supporting the prognostic significance of extratumoral gene expression signatures. Interestingly, the inactive signature was also associated with significantly higher percentage of mammographic density and dense area of the breast, important risk factors and indicating breast density reflects transcriptional changes in the normal breast tissue.[167] Together, these findings support an important role for the microenvironment, both through a potential influence of the initiation and development of breast cancer as well as paracrine stimulation within the microenvironment and by adjacent cancer cells on epithelium and stromal cells in cancer-adjacent normal tissue.

Last, Chen et al[168] examined breast cancer and adjacent normal tissue and identified a *malignancy-risk* signature in the normal tissue. Pathway analysis of the malignancy-risk gene set showed a remarkable overexpression of proliferative function genes, with the majority of the malignancy-risk genes classified to be primarily associated with DNA replication and mitosis. These findings also support the possibility of a malignancy-risk signature with predictive value in normal high-risk breast tissue.

MicroRNAs (miRNAs) are small noncoding RNA molecules that function to silence mRNA molecules. Multiple miRNAs have been identified in breast cancer and at levels either downregulated or upregulated from normal breast tissue and are considered to play an important role in breast cancer development.[169–171] For example, overexpression of miRNA-22 is associated with reduced ERα and increased[172] breast cancer cell growth, whereas downregulation may enhance proliferation through increased activity of c-Myc.[169] Multiple miRNAs have been identified in normal breast tissue adjacent to cancer,[170] but the changes in their expression and the influence of preneoplastic carcinogenesis are not known, representing important subjects for future studies.

Epigenetic inactivation of tumor suppressor and other genes. Epigenetic inactivation of genes through DNA methylation of promoter regions occurs frequently in high-risk normal breast tissue and is an important contributor to genomic instability in these tissues. The incidence of DNA methylation of tumor suppressor and other genes in normal risk, high-risk, and breast cancer tissues is summarized in Table 3. It can be seen that multiple genes are affected and that both the number of genes and the incidence of methylation is increased in high risk compared with normal risk tissues, indicating progression in the carcinogenic pathway. Genes inactivated in high-risk normal breast tissue by DNA promoter methylation affect a wide range of cellular processes, and multiple genes are present in the categories representing the acquired capabilities of cancer:[71] limitless replicative potential (*cyclin D2, RARβ, p16^{INK4A}, 14-3-3σ, GSTP1, BRCA1*), tissue invasion and metastasis (*APC, CDH1, CDH13*), self-sufficiency in growth signals (*RASSF1A*), insensitivity to antigrowth signals (*HIN-1*), and evading apoptosis (*DAPK*). Several of these genes (*RASSF1A, APC, p16^{INK4A}, CDH13, BRCA1, HIN-1*) were also found to be methylated in low-risk/benign tissues (see above, Part I), and thus, these epigenetic changes are not only early but also appear to represent persistence in the carcinogenic pathway. Interestingly, there appear to be few, if any, genes altered, which are involved in DNA mismatch repair (caretaker genes) in normal high-risk breast tissue. This is consistent with the findings from gene expression profiling (see above) and with the conclusion that DNA mismatch repair defects involving *hMLH1* and *hMSH2* underexpression are extremely rare events in sporadic and familial breast cancer.[173]

RARβ, located on chromosome 3p24, is a nuclear steroid receptor that induces growth inhibition with cell cycle arrest and stimulates apoptosis. Table 3 summarizes series examining methylation of *RARβ* in high-risk normal tissue, and it can be seen that there is a wide range in the incidence of methylation from the absence of methylation to >92.0% loss of *RARβ* transcripts.[174] Lewis et al[28] found *RARβ2* to be methylated in 32% of benign breast samples from cancer patients, but only 9% of similar samples from unaffected women. In a study by Widschwendter et al,[174] *RARβ* expression was absent in 93% of tumors and adjacent normal tissue, but present in normal tissue localized distant from the tumor. Sequencing analysis of DNA did not detect any mutation in the retinoic acid responsive element, consistent with an epigenetic modification. Importantly, *RARβ* inactivation may also result from LOH. Deng et al[143] reported LOH at the 3p24 locus in normal tissue adjacent to cancer, and Li et al,[175] in a further analysis, found that the region of LOH was at 3p24.3 corresponding to the gene *RARβ2*. In an elegant study, Yang et al[176] examined the *RARβ2*/3p24 locus for both LOH and methylation. They found that, in 11 breast cancer samples with LOH at this locus, 7 showed *RARβ2* expression, suggesting that *RARβ2* can be expressed in a monoallelic fashion. The other four cases with LOH, however, showed methylation with complete loss of *RARβ2* transcripts, indicating that biallelic inactivation of the *RARβ2* gene could result from either epigenetic inactivation of both parent alleles or epigenetic modification of one allele and deletion of the remaining allele. These studies, in agreement with Widschwendter et al,[174] suggest a crucial role for *RARβ* in the carcinogenesis of breast cancer. *RASSF1A* (Ras associated domain family protein), located within the minimal homozygous deletion region 3p21.3, was found to be methylated in 3.0%–29% of cases in normal breast adjacent to cancer[28,45,177] and was found to have a very high degree of inactivation (100%)[177] in grade I breast cancer, suggesting that methylation of *RASSF1A* may be an early event in breast cancer pathogenesis. The high degree of methylation in breast cancer also suggested that either both alleles were

methylated or one allele was methylated and the other one was lost. Dammann et al,[177] using a satellite marker 140 kb proximal to the *RASSF1* gene, found LOH in 16% of cases of breast cancer, and two of these cases were methylated. Others have found a 33% incidence of concurrent AI/LOH and DNA methylation in *RASSF1A* promoter, providing another example of potential biallelic inactivation of a TSG important in breast tumorigenesis.[178] The *APC* gene is methylated in 33% of cases of normal breast tissue with cancer.[28] The importance of methylation of *RARβ, RASSF1A*, and *APC* is underscored by the finding that promoter methylation of these three genes in benign breast epithelium was associated with epidemiological markers of increased breast cancer risk: *RARβ2* methylation was correlated with a personal history of breast cancer, whereas *APC* and *RASSF1A* methylation was associated with calculated breast cancer risk, particularly in women with prior benign breast biopsies.[28]

The TSG $p16^{INK4A}$ inhibits activity of the CDK4-cyclin D complex, a key regulator of progression through the G1 phase of the cell cycle. $p16^{INK4A}$ is frequently methylated in normal tissues adjacent to cancer with, in some cases, most of the ductal cells adjacent to all grades of tumors failing to show expression.[156,179,180] Di Vinci et al[180] found the gene predominantly methylated in normal tissue adjacent to cancer and raised the possibility that hypermethylation of *p16* promoter could be a common and early event in breast carcinogenesis that, in keeping with the multistep model of initiation and progression of breast cancer, necessitates additional epigenetic or genetic lesions to manifest the malignant phenotype. *14-3-3σ*, located at chromosome 1p36.11, is a member of a family of proteins capable of binding to a variety of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors. The *14-3-3σ* gene is induced by DNA damage and is required for a stable G2 cell cycle arrest in epithelial cells. *14-3-3σ* was found to be methylated in normal tissue adjacent to both invasive and in situ breast cancer,[181] and interestingly, LOH has also been observed in this region,[142] suggesting additional mechanisms for inactivation of this TSG. *H-cadherin,* a cell surface protein involved in cell–cell recognition is methylated in 6%–28% of normal breast tissue associated with cancer.[28,182] Interestingly, whereas the role of certain genes such as *APC, E-cadherin*, and *H-cadherin* that contribute to tissue invasion and metastasis is apparent in cancer, one might speculate that loss of cell–cell adhesion might also influence clonal expansion and development of the cancerized field.

Several of the tumor suppressor genes are known to interact with each other, and thus, loss of one TSG may alter other signaling pathways. For example, the *14-3-3σ* gene is directly regulated by *p53*.[183] *14-3-3σ,* which contributes to G2 arrest in epithelial cells, binds to *p53* and activates its sequence-specific transactivation, resulting in the blockade of cell cycle progression.[67,184] The $p16^{INK4A}$ gene encodes two proteins, one of which is the TSG *ARF*, which acts in the *p53* pathway to block degradation of *p53*.[185] *TP53* and *p16* act cooperatively to increase HMEC growth,[128] overcome telomere damage-induced cell cycle arrest,[159] and increase the proliferative activity of tumors.[186] Retinoic acid receptor may regulate expression of EGFR, Erk1/2 phosphorylation, c-Jun, and COX-2.[187] *APC,* through binding of *β-catenin*, regulates *myc* and *cyclin D1* expression, cell cycle entry, and progression.[28] In a study by Feng et al,[188] a positive correlation was found between breast cancer and normal tissue for methylation of four genes, *RASSF1A, RIL, HIN-1,* and *CDH13*. When these four genes were defined as two panels of two genes each, it was found the methylation of the *HIN-1/RASSFIA* panel strongly correlated with the expression of ERs, PRs, and HRs. Conversely, the methylation of the *RIL/CDH13* panel strongly correlated with negative ER, PR, and HR expression.[188] Finally, it has been observed that *INK4A/ARF* promoter hypermethylation in high-risk breast epithelium is associated with an increased frequency of *RARB, ESR1, and BRCA1* promoter hypermethylation ($P = 0.001$), suggesting that the combination of *INK4A/ARF* promoter hypermethylation and increased promoter hypermethylation of these three genes may set the stage for further tumor progression.[189]

Oncogenes and cell cycle regulatory genes. Several oncogenes or genes that are traditionally amplified in breast cancer have been studied for expression changes in high-risk normal breast tissue, including *EGFR, HER-2/neu, c-Myc, c-Src, Ras, and cyclin D1* (Table 4). Two members of the *Erb* family, *ErbB1 (EGFR)* and *ErbB2 (Her-2/neu)*, may be overexpressed in normal/high-risk tissues. Tidow et al[190] examined 163 primary breast cancer cases by microsatellite analysis and identified AI at the *EGFR* locus in 55 cases (33.7%). They confirmed that the AI represented an amplification, rather than a deletion of one allele with a quantitative 5′ nuclease assay. A total of 75% of the patients bearing AI of this locus in the tumor also showed AI at normal, nontumorous tissue, supporting the assumption that distinct amplification of intronic sequences of the *EGFR* gene, which enhance the basic transcriptional activity of the gene, represents one of the first steps in breast carcinogenesis. Pekonen et al[191] noted that the epidermal growth factor receptor *EGFR* protein expression was increased in normal adjacent tissue in 5% of cases and was equivalent to expression in the cancer,[191] and other studies reported the expression to be increased in 21% of nonproliferative high-risk cytology, but this did not correlate with either cancer development or time to cancer development.[31]

Normal breast tissue at high risk for breast cancer have been studied for expression of *Her-2/neu,* and as indicated in Table 4, these tissues generally do not express, or have low levels of expression of this oncogene. Among the latter, *Her-2/neu* protein overexpression was present in 12.7% of cells of benign breast disease in women who subsequently developed breast cancer,[83] and in 10% in nonproliferative aspirates from high-risk women,[29] but expression was not associated with an increased risk for progression to breast cancer.

Other studies found that both nuclear and cytoplasmic signals were often detected in morphologically normal-appearing epithelium adjacent to breast cancer[192] and that these tissues contained 1.87 ± 0.1 copies per cell, but this did not suggest amplification.[138] It is well known that activation of signaling cascades from the EGFR receptor family members involves receptor dimerization between members of the *Erb* family, such as between *ErbB1 (EGFR)* and *ErbB2 (Her-2/neu).*[193] Overexpression of one or both of these genes may therefore potentially further enhance receptor signaling and cellular proliferation by cooperativity with each other.[194] As noted in the discussion of low-risk/normal tissue, it is quite possible that increased expression of these oncogenes, even at lower levels, may have biological significance by enhancing cellular processes or proliferation without yet having prognostic significance. Among other oncogenes that have been studied, 65% of normal tissues adjacent to carcinoma expressed *c-Myc* in one study, with a detectable elevation of *c-Myc* in the normal tissues adjacent to grade 3 tumors,[179] while another[195] reported weak staining in normal ductal epithelium in 63.6% of cases, with the percentage of cells expressing *c-Myc* protein ranging from 2% to 8%. Corzo et al,[196] however, found no evidence of *c-Myc* amplification in these high-risk tissues. *Hras* and *c-Src* are not overexpressed in high-risk normal breast tissue.[197–200]

Another prominent category of genes whose expression may be increased in normal high-risk breast tissue are genes regulating cell cycle progression, including *cyclin A, cyclin D1*, *cyclin E1/2,* and $p34^{cdc2}$. Cyclin A activates *CDK2* kinases and promotes cell cycle G1/S and G2/M transitions. Expression of cyclin A in NABT was equivalent to that of the carcinoma in 77.8% of cases and significantly greater than that of normal risk tissue.[201] Cyclin D1 functions as a key regulator of progression through the G1 phase of the cell cycle.[202] Cyclin D expression was detected at levels significantly greater than normal breast epithelium in atypical ductal hyperplasia, suggesting that overexpression of cyclin D1 protein is important at the earliest stages of breast oncogenesis and continues to have a crucial role throughout oncogenesis.[202] $p34^{cdc2}$ is a cell cycle regulatory protein important in controlling G2/M transition. Kourea et al[203] found significant expression in normal breast tissue adjacent to cancer in 16.0% of cases, and this was associated with longer DFS ($P = 0.0030$) and overall survival ($P = 0.0046$) in univariate analysis, whereas in multivariant analysis, $p34^{cdc2}$ was the only independent predictor of DFS ($P = 0.001$). While expression of most of these genes has not been found to be associated with (or have not been studied for) an increased risk for breast cancer, increased expression may have biological or oncogenic significance for several reasons, including promotion of clonal expansion of altered cells, enhanced accumulation of genetic errors, reduced time for DNA repair, as proposed by Stopper et al,[204] or by forcing responsive cells through the cell cycle to promote an override of checkpoints operating under homeostatic control of the cell cycle, resulting in genomic instability. Cyclins E1 and E2 regulate transition from the G1 phase to the S phase[205] and may be modestly elevated (E1—11%, E2—17%) in normal tissue associated with ER-positive tumors.[206] The gene $p21^{waf1/cip1}$ inhibits cyclin-dependent kinases. Three studies have found low or negative expression of $p21^{waf1/cip1}$ in NABT, raising the possibility that this may promote proliferation in these tissues.[179,203,207] Other genes involved in cell cycle regulation, including *cyclin D3,* and the cyclin-dependent kinase inhibitor $p27^{kip1}$ were found to have normal expression in high-risk tissue (Table 4).

Last, but not least, the *p53* gene is critical in breast carcinogenesis, regulating multiple cellular processes and being one of the most commonly mutated genes in breast cancer (see above, Part I). Mild-to-moderate expression of *p53*, suggesting mutational abnormalities, has been observed in several reports of high-risk/normal tissue (range 0%–30.0%; Table 4). Rohan et al[101] studied *p53* by immunohistochemistry and sequencing in cases of benign breast tissue in women who subsequently developed breast cancer. They found that 43.9% of sequenced cases had *p53* nucleotide changes, and 20.2% had increased expression by IHC. Nonpolymorphic intronic changes were associated with a 2.8-fold increase in risk for breast cancer (odds ratio = 2.84; 95% CI = 1.09–7.41). They concluded that *p53* protein accumulation and nonpolymorphic intronic changes in *p53* are associated with increased risk of progression to breast cancer in women with benign breast disease. Mottolese et al[208] reported *p53* expression in 9.7% of peritumoral tissue, which suggested that the presence of *p53* nuclear accumulation in benign tissue of cancer-containing breasts could reflect genomic damage due to long-term carcinogenic exposure. *p53* expression has also been shown to be increased in cases with atypical hyperplasia and was significantly associated with subsequent cancer development/detection ($P = 0.0026$),[29] while others have proposed that *p53* gene alteration might be an early or even an initiating event in breast cancer carcinogenesis.[209] *P53* abnormalities, however, are not present in all high-risk normal tissues (Table 4), indicating the heterogeneity of these changes and consistent with the heterogeneity of *p53* mutational changes in breast cancer.[92] Importantly, it is worth reemphasizing that *p53* has many cellular functions[91] and that the increased *p53* expression noted in cancer-adjacent normal tissues in some studies, while it may be mild, may still have important biological consequences; in tissues already showing increased genomic instability from LOH, DNA methylation and gene expression changes, telomere shortening, and aneuploidy, any additional dysfunctional *p53* protein could have significant consequences for promotion through the carcinogenic pathway.[107]

Hormone receptor subtypes in high-risk normal breast tissue. The ER content of high-risk normal breast tissue appears to be comparable to that of normal risk breast tissue and is predominantly ER-negative (Table 5). While the presence of the ER has been demonstrated in high-risk normal breast tissue, there is also evidence that the incidence of ER in these tissues

is significantly less than that in breast cancer. The incidence in high-risk normal tissue is summarized in Table 5 and is, on average, 30.3% in these series, whereas the incidence in luminal A/B breast cancers ranges from 52.1% to 76.7%, depending on ethnicity.[210] The disparity in the incidence of ER positivity between cancer-adjacent normal tissue and breast cancer is intriguing and unexplained, but suggests that progression to malignancy may be accompanied by the presence of multiple factors that either select for transformation of ER-positive cells or enhance the presence and expression of the ER in cells. There is also good evidence that the ER in these normal cancer-adjacent tissues is functional: (A) treatment with antiestrogens reduces breast cancer recurrence in the contralateral breast in women with invasive carcinoma and DCIS[211] and reduces in-breast recurrence in patients with DCIS;[212] (B) the gene expression profile in normal adjacent tissue for ER+ tumors is comparable to that for the respective ER-positive cancer,[161] with the ER in breast cancer widely recognized as halving biological and prognostic significance; and (C) evidence was presented in Part I of this review to indicate that ER-positive normal tissues respond to estrogens with the secretion of growth factors, which then act in a paracrine manner to stimulate adjacent ER-negative cells. This responsiveness of ER-containing normal breast tissue could therefore be another important factor in promoting progression of these high-risk cells through the carcinogenic pathway.

The cancerized field in normal breast tissue. The concept of the cancerized field of the breast was discussed in the "Introduction" section of this review. It was noted that, with the accumulation of molecular abnormalities, clonal populations of altered breast cells develop and expand to form a cancerized field of altered cells (Fig. 1). The subsequent acquisition of molecular changes and clonal expansion allows secondary, tertiary, or more cancerized fields to develop, which occupy progressively greater portions of the breast, and with greater and greater genomic instability, which promotes progression and eventual malignant transformation. Because of varying expansion at the perimeter of the field, irregular contours and volumes of the fields emerge. In an effort to determine the extent and character of the cancerized field, studies examining breast tissue for the presence of molecular changes (including aneuploidy, LOH, AI, DNA methylation, gene expression, or telomere length) at different distances from the primary tumor have been performed. These studies indicate that the cancerized field can extend for up to 5 cm from the primary tumor,[15,42,57,166,178,213] but that in some cases, the frequency of the changes diminish,[15,215] or are absent[60,143,174] with greater distances from the tumor. The greater proximity of certain changes to the primary tumor may indicate a later acquisition in breast carcinogenesis and possibly greater involvement in the transformation process. At the same time, the presence of some changes at considerable distances from the primary tumor indicates the size of a cancerized field and the extent of breast tissue at risk for further abnormalities. It has been shown, for example, that telomere lengths in tumor or paired normal adjacent tissues at 1 cm are significantly shorter than in histologically normal tissues from paired tissues at 5 cm or RM.[60] The frequencies of AI in tumor and paired tissues at 1 cm were also higher than at 5 cm in that study. These findings are felt to be consistent with the interpretation that cells within 1 cm adjacent tissue have a telomere-dependent mechanism of generating genomic instability, an early event in breast tumorigenesis.[66,68,69] Others have found that the field of normal tissue proximal to the breast tumors contained a population of HMECs similar in *hTERT* expression levels and in gene expression to the HMECs within the tumor mass and that this population was significantly reduced in tissues more distal to the tumor.[150] These authors concluded that a significant number of cells within 1 cm of adjacent tissue may potentially possess limitless replicative capacity and a telomere-dependent mechanism of generating chromosomal rearrangements, overexpress genes involved in proliferation, and are exposed to a tumorigenic microenvironment.[150] The concept of field cancerization also extends to gene expression changes in the microenvironment. Roman-Perez et al[166] have identified a molecular signature defined by high expression of fibrosis and cellular movement genes, which was present in a peritumoral location but not a distant location, suggesting that distance to tumor may be an important source of intraindividual variation in expression of this phenotype. Together, the presence of cancerized fields in the breast provides a basis for the heterogeneity as well as the incidence of genomic changes within the breast. Genomic changes in the immediate vicinity of the tumor may play a particularly important role in, and even be influenced by, the transformation process.

Prognostic significance of chromosomal changes in high-risk normal breast tissue. The relative risk of certain high-risk tissues for breast cancer is summarized in Table 6. In addition, several studies have identified molecular changes such as AI/LOH and DNA hypermethylation in high-risk normal tissue, which may contribute to this risk. Important examples of this are the following. (A) The prevalence of AI in TDLU of high-risk tissue is increased and is associated with breast cancer and increased breast cancer risk.[41] (B) The presence and high incidence of 3pLOH in histologically normal TDLU adjacent to early stage breast cancer was significantly related to local tumor recurrence, leading to a 3.9- to 5.2-fold increase in the hazard ratio.[175] The time to recurrence was longer in such cases than in those without *3pLOHn,* suggesting de novo tumor development. (C) The presence of LOH across 20 highly polymorphic satellite markers relevant to breast cancer in breast epithelial cells obtained by fine-needle aspiration from 30 asymptomatic women was associated with a 22.9% mean lifetime risk for developing breast cancer, as calculated by the Gail model, compared with 16.7% for women with no LOH ($P = 0.05$).[14] (D) Promoter methylation of *RARβ*, *RASSF1A*, and *APC* in benign breast epithelium was associated with epidemiological markers of increased breast

cancer risk.[28] More specifically, *RARβ2* methylation was correlated with a personal history of breast cancer, whereas *APC* and *RASSF1A* were associated with calculated breast cancer risk, particularly in women with prior benign breast biopsies. Together these studies, in addition to providing important information about breast carcinogenesis in these tissues, support the proposal that molecular changes in normal high-risk breast tissue may be useful for risk assessment and prognosis of these tissues. These studies frequently examined a limited number of genes or loci, and it will, therefore, be of interest to examine these tissues with whole genome techniques, such as gene expression profiling or DNA methylation arrays, to further define this concept.

Summary and Conclusions

In Part I of this review, normal breast tissue at normal risk for breast cancer was examined and genomic changes were identified, which were consistent with early carcinogenesis, including LOH, DNA methylation of tumor suppressor and other genes including $p16^{INK4A}$, and telomere shortening. A clonally expanded population of cells was required for identification of these changes, suggesting both the occurrence of initiation and promotion of these cells and the early development of a cancerized field. In Part II of this review, normal breast tissue at high risk for breast cancer was examined. In these tissues, persistence and progression of LOH, DNA methylation, and telomere shortening, which was evident in the normal risk tissues, was observed, and in addition, the acquisition of aneuploidy, increased genomic instability, telomerase expression, widespread abnormalities in gene expression, and loss of cell cycle control were present. These findings are consistent with ongoing carcinogenesis from continuing exposure to estrogens and other carcinogens, and increased genomic instability promoting acquisition of additional mutations. In addition, many of these changes in the high-risk tissue were present over a wide cancerized field, in some cases extending 5 cm from the primary breast cancer, while also being concordant with changes in the breast cancer, and in some cases, such as aneuploidy, the frequency of aneuploidy was found to be equal in cancer-adjacent normal tissue and in tissue of the contralateral breast, suggesting that the molecular profiles are not tissue specific but may be representative of a wide range of high-risk tissues in general.

An important finding in this review is the absence of gross chromosomal abnormalities such as amplifications, large-scale deletions, telomeric associations, or gross rearrangements in the normal high-risk tissue. These have been shown to be important characteristics of breast cancer.[132] It has been noted that, in the end stages of the in vitro HMEC life cycle as cells approach senescence, gross chromosomal abnormalities accumulate rapidly.[22] By analogy to the HMEC life cycle, many earlier changes have been observed in high-risk normal breast tissues, which would appear to set the stage for the development of GCRs and appear to place these high-risk tissues potentially on the verge of senescence and malignant transformation: DNA double-strand breaks, an important component of GCR;[214,215] telomere shortening and telomere-driven instability, an important mechanism of GCR;[6] gross deregulation of the transcriptome from aneuploidy and evidenced by widespread differentially expressed genes on gene expression profiling;[134,164] loss of critical TSGs by DNA methylation and segmental deletions, and loss of cell cycle control; expression of telomerase with the potential for unlimited cellular proliferation; and altered DNA repair with promotion of error-prone nonhomologous end joining from loss of *BRCA1* and *BRCA2*.[216–218] The significant degree of genomic instability in high-risk tissues conferred by these changes would contribute to its risk for further progression and the potential for rapid development of malignancy such as may occur in cancer-adjacent breast tissue. By analogy, these changes might place these high-risk cells in the mid-to-late portion of the post-stasis period of the HMEC life cycle, as depicted in Figure 2.

The progression of breast cells through the carcinogenic pathway to malignancy is also accompanied by determination of particular receptor subtype characteristics of the tumor (luminal A/B, basal-like, claudin-low, and metaplastic). Several theories have been proposed for these developments, which have been reviewed by Skibinski and Kuperwasser.[1] The emerging consensus is that the most common subtypes, including luminal A/B and basal-like tumors, likely arise as a result of transformation of a LP of origin.[1] In contrast, rare metaplastic and claudin-low breast tumors may have a different origin, either from a unipotent myoepithelial stem cell or from a mammary stem cell or basal/myoepithelial cells. While basal and luminal A/B tumors can both arise in similar precursors, the nature of the oncogenic signal determines the eventual phenotype of the tumor, with the basal gene set consisting of *TP53, BRCA1, MAGI3-AKT, RB, YAP/TAZ,* and *SLUG* and the luminal gene set consisting of *PI3K, MAP3K1, GATA3, TBX3, FOXA1,* and *CDH1*.[1] Many other frequently mutated genes may act as determinants of tumor differentiation in addition to exerting oncogenic effects which, through both epigenetic and genetic influences, can serve as engines of tumor diversity.[1] An important question, especially for the characterization of normal at-risk tissue, is the timing of the development of tumor subtype. In the present review of cancer-adjacent high-risk normal tissue, it was found that the ER content was low (as well as being much lower than associated ER-positive cancers) and not significantly different from that of normal risk tissue. Examination of gene expression patterns of these high-risk normal tissues, however, revealed expression profiles that were similar to the expression profiles of the respective, associated, ER-positive, or ER-negative breast cancer.[161] This implies that genomic changes characteristic of specific breast cancer subtype may be detectable before histologic evidence of abnormality. Importantly, when genomic changes (expression

differences, structural, or epigenetic changes) for specific genes were examined, it was found that changes in some of the genes in the luminal gene set (including *GATA3*, *CDH1*, *FOXA1*[46,132,161,308]) were present in the normal tissue associated with adjacent ER-positive breast cancer, and expression differences in some of the genes in the basal gene set (including changes in or near *TP53* [Table 4], *BRCA1,*[39,144] *and RB*)[40] were present in normal tissue associated with adjacent ER-negative breast cancer. This would suggest that the emergence toward tissue-specific subtype may be occurring in high-risk normal breast tissue and that further or complete development of subtype occurs with transformation to malignancy. It will be important to examine normal high-risk breast tissue, especially cancer-adjacent, for mutational changes in each of the genes of the respective basal or luminal gene sets to further define the timing of this transition.

In conclusion, progression through the breast cancer carcinogenic pathway beginning with early changes in tissue at normal risk for breast cancer and proceeding through high-risk cancer-adjacent normal tissue is associated with structural and numerical chromosomal changes, extensive epigenetic DNA methylation changes, and multiple gene expression abnormalities. The presence of similar changes, including DNA methylation and LOH, in normal risk and high-risk breast tissues accompanied by additional changes in high-risk tissue, and concordance of these changes with those in cancer, suggests that the clonal evolution of these changes was consistent with the progressive development of cancerized fields. In view of the progressive nature of carcinogenesis over a range of tissue types, these findings also encourage the study of tissues from women at intermediate risk to further define the sequence of events and the relationship of these changes to risk for breast cancer.

A model describing the carcinogenic pathway in normal breast tissue at high risk for breast cancer. To clarify the relationship of genomic changes to each other in high-risk normal breast tissue, a model has been proposed summarizing the major molecular events in the carcinogenic pathway for normal breast tissue at increased risk for breast cancer. This model takes as its origin the presence of cancerized fields, which result from carcinogenic events in normal breast tissue at normal risk for breast cancer (Fig. 4), and represents continuing progression of many of the events in those tissues. Central to progression in the pathway is the continuing action of estrogens and other carcinogens, causing DNA single- or double-strand breaks, deletions, DNA methylation and point mutations, and DNA adducts. Each of the resulting abnormalities may contribute to genomic instability and facilitate acquisition of additional mutations. Importantly, while each abnormality (such as telomere shortening, LOH, DNA methylation) may play an important role in carcinogenesis, there is considerable interaction between many of these events and processes, and the development of malignancy should be viewed as the collective action of many or most of these abnormalities, which would also explain the wide range of genomic abnormalities and diversity present in a breast cancer. At the same time, one might also speculate that certain "critical" abnormalities such as *TP53* inactivation, *p16*^{INK4A} inactivation, telomerase activation, aneuploidy, or gene activation may play a larger role, and in their absence, development of breast cancer may be delayed or avoided. Identification of key intermediates or gene expression profiles should promote development of risk assessment signatures, identify new targets for development of prevention drugs, and aid in the selection of women for prevention therapy. All these are major goals in the management of women at risk for breast cancer.

Limitations to the Study, and Future Directions

The following are considered to be limitations to this review. (A) The studies conducted in normal risk and high-risk tissues frequently examined panels of genes or satellite markers, and (with the exception of gene expression profiling and RNAseq in high-risk normal tissues) whole genome approaches such as DNA methylation arrays, SNP arrays, or even DNA sequencing were not conducted. Thus, the true extent of genomic abnormalities in these tissues was most likely underestimated. (B) Detailed demographic data for the normal risk tissues such as RM was not available, and thus, further assessment of risk and correlation of early molecular findings with any risk factors, if present, could not be performed. (C) Inherent heterogeneity within and between tissue sources would provide for a range of molecular values for the different categories. The degree to which this heterogeneity might also reflect an influence of different risk factors is unclear.

These findings encourage a number of future studies. (A) Expanded molecular characterization of "normal risk" and "high-risk" normal breast tissue with whole genome studies, including DNA sequencing and DNA methylation, to more fully define the nature and extent of structural and epigenetic changes in these tissues. (B) Analysis of "normal risk" tissues, ideally in a prospective manner and supplemented with inclusion of demographic data for different risk factors, in subjects to allow accurate assessment of risk and facilitate correlation with genomic findings in early carcinogenesis. (C) Analysis of ductal epithelial cells from young women in their 20s with more limited exposure to estrogens to provide important information about early changes in breast carcinogenesis. A recently developed improved method of ductal sampling is well suited for collection and downstream analysis of cells from normal risk (and high-risk) women of all ages.[219] (D) Increased sampling and analysis of normal tissue adjacent to high-risk lesions, including atypical ductal and atypical lobular hyperplasia and LCIS, to provide a broad spectrum of genomic changes and to define the later phases of preneoplastic breast carcinogenesis. (E) Establish a tissue repository of normal breast tissue from women who are truly normal risk (Tyrer-Cuzick high-risk index of = 1.0)

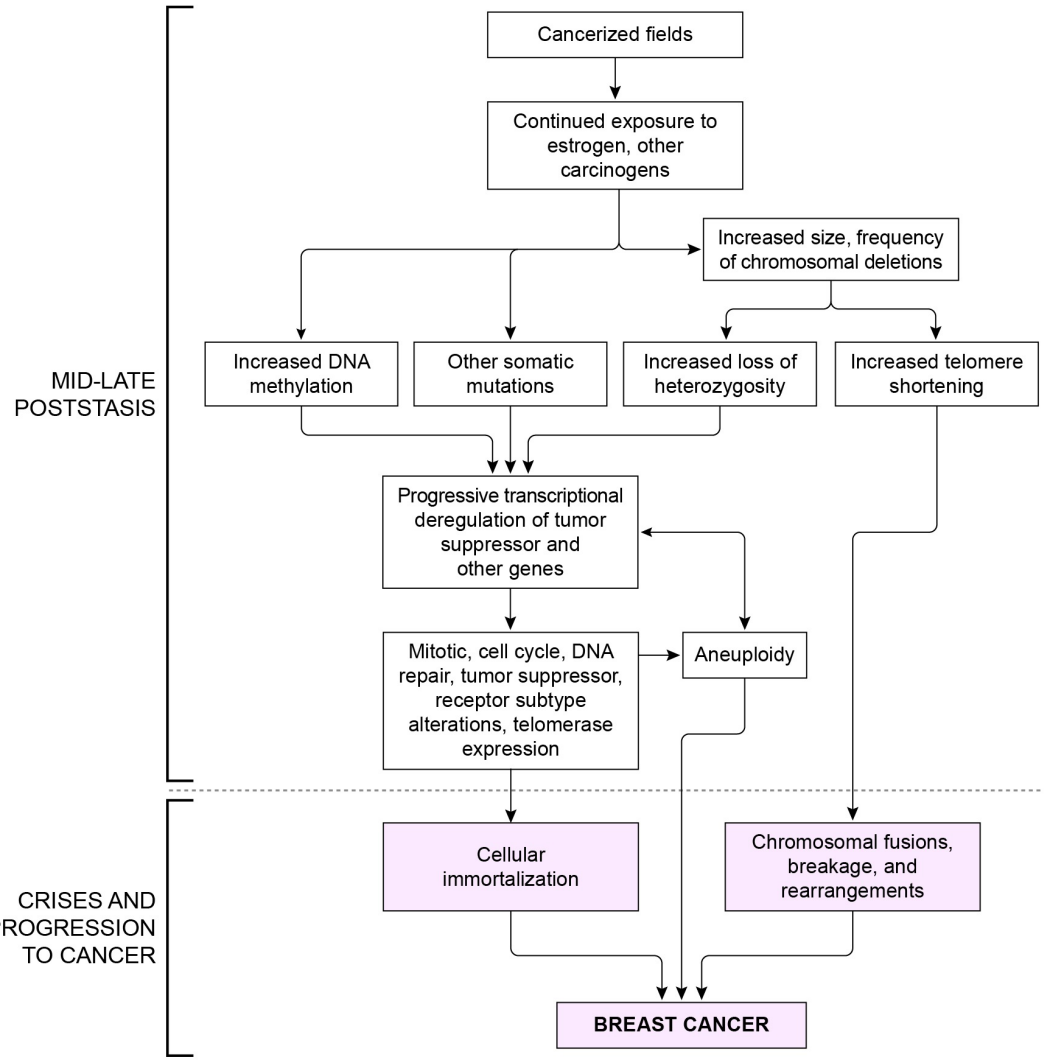

Figure 4. Model of the carcinogenic pathway for progression of genomic changes and development of breast cancer in normal breast tissue at high risk. Carcinogenesis was initiated with genomic changes and the development of cancerized fields in normal breast at normal risk for breast cancer (Fig. 3). Continuing exposure to carcinogens results in progressive accumulation of mutations including DNA methylation, point mutations and insertions/deletions (indels), segmental deletions with LOH, and telomere shortening. Genomic instability is further increased by the acquisition of aneuploidy, widespread transcriptional deregulation, altered DNA repair, and expression of telomerase. Continued progression may be associated with the accumulation of GCRs and under appropriate conditions,[21,26] cellular immortalization, malignant transformation, and breast cancer. The correlation of events with the post-stasis period of the HMEC in vitro life cycle is proposed.

to facilitate, by comparison, analysis of higher risk tissues. (F) Define the genomic differences in normal breast tissue according to ethnicity to clarify disparities in risk among different ethnic groups.

Abbreviations

ADH, atypical ductal hyperplasia; LPs, luminal progenitor cells; HMEC, human mammary epithelial cells; arrayCGH, array comparative genomic hybridization; LOH, loss of heterozygosity; AI, allelic imbalance; MSI, microsatellite instability; TDLU, terminal ductal lobular unit; TC, telomere DNA content; RM, reduction mammoplasty; ROS, reactive oxygen species; IHC, immunohistochemical; ER, estrogen receptor; NABT, normal adjacent breast tissue.

Author Contributions

Conceived and designed the experiments: DND. Analyzed the data: DND. Wrote the first draft of the manuscript: DND. Developed the structure and arguments for the paper: DND. Made critical revisions and approved final version: DND. Author reviewed and approved of the final manuscript.

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