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Molecular Aspects of Early Stages of Breast Cancer Progression

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Abstract It is clear that breast cancer progression is associated with inactivation of a number of different recessive oncogenes. The most widely evaluated tumor suppressor gene, p53, is mutated in approximately 30–50% of sporadic breast cancers. Mutations usually occur early in malignant progression. Loss of heterozygosity (LOH) studies have identified numerous chromosomal regions where other recessive oncogenes relevant to breast cancer may be located.

Each LOH is seen in a varying proportion of breast cancers and may appear either early or late in progression. High-grade ductal carcinoma *in situ* (DCIS) and invasive carcinoma have similar genetic lesions, showing that aberrations can occur before invasive disease. Direct evidence that the same aberrations can be acquired later in progression comes from a study of multiple metastases from the same patient; other studies found that primary invasive cancers are characterized by marked intratumor heterogeneity for each lesion examined.

The model we propose to account for these results hypothesizes that multiple genetic lesions can accomplish each phenotype required for malignancy (*i.e.*, dysregulated proliferation, invasion, angiogenesis, *etc.*) and that, for a given tumor, at least one aberrant gene for each phenotypic change is stochastically selected. Biological heterogeneity of breast cancer results from the stochastic acquisition of various genetic aberrations. We further propose that the lymphocytic reaction in high-grade DCIS may select for aggressive tumor subpopulations capable of escaping immune surveillance. Another aspect of tumor heterogeneity may be the multiple mechanisms employed by various tumors to escape immune surveillance.

Key words: Immune surveillance, loss of heterozygosity, oncogene, p53, tumor suppressor gene

There is much evidence suggesting that breast cancer is many different diseases. Supporting this hypothesis are the observations that molecular lesions causing aberrations of gene expression for oncogenes, tumor suppressor genes, growth factors, proteases, angiogenesis factors, and some stromal components have all been seen in a varying, and sometimes small, proportion of breast cancers.

Malignant progression in breast cancer is poorly understood. It is generally agreed that ductal carcinoma *in situ* (DCIS) usually becomes invasive with time; however, it is not certain that all breast cancers proceed through a clearly delineated *in situ* phase [1–5]. Furthermore, it is not clear which, if any, noninvasive lesions other

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than DCIS found in breast tissue are precursors of either invasive or *in situ* carcinoma.

Breast cancers are also unusual in that tumors of similar stage and histology can have very different clinical outcomes. At one extreme are the patients who succumb to metastatic disease within a year of diagnosis. At the other extreme, patients with cancers that appear similar may have 20 years of disease-free survival. It is likely that this biological heterogeneity among breast cancers at least partially results from the combination of genetic lesions present in a given tumor.

Figure 1 schematically illustrates a model of breast cancer progression which accounts for this extreme heterogeneity [6]. It is assumed that each phenotype required for malignant growth (*i.e.*, dysregulated proliferation, degradation of basement membrane, invasion, angiogenesis, *etc.*) can be accomplished by more than one genetic lesion, with some of the lesions causing a more aggressive phenotype than others. Thus, a cell progressing to malignancy stochastically acquires at least one aberrant gene for each phenotype required for malignancy. If a breast cancer cell is incapable of completing any crucial step, it will not successfully metastasize. For example, mammary cells capable of extensive and dysregulated cell proliferation still need other changes to become malignant. Likewise, a cell that has gained the capacity for degrading basement membrane will not be detected as cancer if it cannot continue proliferating.

The biological heterogeneity of breast cancers may arise from the many possible molecular changes accomplishing a given step with variable efficiency. Biological heterogeneity may also result from various possible orders of acquiring the steps necessary for malignant growth. Even after the invasive cancer phenotype has been acquired, the process may continue with additional lesions resulting in increasingly aggressive behavior.

This stochastic model of breast cancer views progression from a different perspective than the classic initiation and promotion paradigm used to explain chemical carcinogenesis. In classic carcinogenesis models, progression is a *separate* phenomenon which presumably occurs after promotion is completed. In contrast, the stochastic model for breast cancer postulates that promotion and progression are the same phenomenon and represent a continuum from normal to increasingly aggressive metastatic disease.

	DYSREGULATED PROLIFERATION	INVASION OF BASEMENT MEMBRANE	ANGIOGENESIS	ESCAPE FROM IMMUNE SURVEILLANCE
Increasing Aggressiveness				
↓	D-1*	I-1	A-1	E-1
↓	D-2	I-2	A-2	E-2
↓	D-3	I-3	A-3	E-3
↓	D-4	I-4	A-4	E-4

Fig. 1. Phenotypic Changes Characteristic of Malignancy. * Each number represents a gene(s) whose aberrant expression result in acquisition of the indicated malignant phenotype. A cell progressing to malignancy stochastically acquires these lesions; invasive carcinoma occurs when the tumor has acquired at least one aberration in each phenotypic change required for malignancy. Depending on which gene becomes abnormal, the resulting tumor can be more or less successful in accomplishing its indicated phenotype. Invasive carcinomas continue to acquire additional lesions resulting in increasingly aggressive behavior.

The stochastic model does not address whether initiation is also a part of the continuum or a discrete phase.

Fundamental to both the stochastic and classic carcinogenesis hypotheses is the assumption that cells populating a tumor at any given time are survivors of continuing selection pressure [7–9]. Most commonly, it is thought that one cell within a given tumor acquires an additional molecular lesion which provides it with a selective advantage. In the primary tumor, molecular aberrations with selective advantage would typically confer capability for either increased proliferation or decreased apoptosis. In contrast, molecular aberrations associated with invasion and/or metastases rather than tumor growth would cause a different type of selective pressure. These molecular aberrations would be seen in more cells comprising a metastasis compared to its primary lesion.

A third mechanism for inducing selective pressure could involve immune surveillance. Tumor cells whose molecular aberrations are recognized as abnormal will be rejected by the immune system. Acquisition of additional abnormalities that permit a tumor cell to escape this surveillance will enhance its ability to survive and subsequently predominate. In a manner similar to that proposed for other aspects of the malignant phenotype, we hypothesize that there may be multiple pathways for escaping immune surveillance. This hypothesis is indicated in Figure 1 as a separate phenotype termed "escape of immune surveillance."

Within this complex and heterogeneous biological framework, many studies have attempted to characterize the molecular lesions associated with breast cancer progression. Results from these studies provide the rationale for proposing the stochastic model. Evidence includes the generalizations that (1) each lesion can be seen in a proportion of breast cancers and differs in frequency of occurrence; (2) many lesions can be acquired early or late in progression; and (3) a particular phenotype (*i.e.*, rapid proliferation at the primary site) correlates with various molecular lesions. Examples of results leading to these generalizations come from research in various laboratories as well as our own studies on loss of heterozygosity (LOH), amplifications at the *bcl* and *erb*B-2 oncogene loci, and p53 mutations in breast cancer.

LOH IN BREAST CANCER

Loss of restriction fragment polymorphism is one of the most frequent genetic aberrations reported in breast cancers [for reviews see 10–12]. Classically, it has been measured by comparing tumor DNA with normal DNA from the same patient which has been cleaved with specific restriction endonucleases and run on Southern blots. In cases where the normal DNA is polymorphic, diminution of one of the polymorphic bands is called LOH. Usually the loss of one allele is incomplete because of contaminating normal cells in the tumor tissue. LOH is commonly interpreted to be the result of either a physical loss of one allele or a recombinational event which results in two copies of one allele. The length of the homozygous region varies among individual tumors, but is usually a substantial portion of the chromosome arm in question. Presumably, the remaining allele harbors an inactivated gene (by mutation, small deletion, hypermethylation, *etc.*) within the region which is deleted in common among all affected tumors.

LOH has been described in a varying proportion of breast cancers at chromosomal regions 1p, 1q, 3p, 6q, 7p, 11p, 13q, 16q, 17p, 17q, 18p, 18q, and 22q [13–37]. The incidence of loss varies for the different markers, with the more frequently lost regions being 3p, 6q, 7p, 16q, and 17p (40–60%) and the less frequently lost regions being 1p, 1q, 11p, 13q 18p, 18q, and 22q (15–20%). The baseline level of LOH for any randomly selected probe is approximately 5% [35].

LOH has not been evaluated systematically in carcinoma *in situ* because these lesions are usually only available as formalin-fixed, paraffin-embedded materials. This fixation results in DNA too small for Southern blot LOH studies. Recently developed PCR techniques permit evaluation of LOH in paraffin-embedded material [38]. Using PCR-based technologies, we have characterized DCIS cases for LOH at chromosomal loci 3p, 7q, 16q, and 17p. In preliminary studies, we found that a proportion of DCIS cases have LOH at each region tested, suggesting that these losses can occur early in malignant progression.

It is unclear whether a particular LOH could also be acquired late in malignant progression. We directly tested this question by characterizing three separate malignant effusions from the same patient acquired over a six month period [39,40].

All three samples had similar karyotypic markers, indicating that they were derived from the same tumor. However, only the last effusion had an LOH at 11p, suggesting that this gene aberration had been acquired subsequent to frank metastasis and might be conferring increasingly aggressive behavior to already metastatic cells.

We further determined that a particular LOH could be acquired late in progression by comparing the completeness of loss at more than one locus in DNA preparations from a number of tumors [38]. If a DNA sample with LOH at a given locus showed no residual staining of the lost allele, then we concluded that the tumor tissue had little if any normal cell contamination. Using the same DNA sample, we sometimes found LOH at another chromosome locus where there was some residual staining of the lost allele. Since we had already concluded that contaminating normal cells could not account for this residual density, we suggested that only some of the tumor cells had acquired the second loss. At each region examined, a proportion of tumors acquired a given LOH later than a different LOH. Hence, we proposed that LOHs are acquired stochastically, with one sometimes being acquired earlier in progression [35].

Tumor proliferative fraction has been evaluated in a number of different ways, including S-phase fraction measured cytometrically [41], assays of cell cycle parameters such as Ki-67 [42], and incorporation of DNA precursors such as radioisotopically labeled thymidine or bromodeoxyuridine (BrdU) [43,44]. The tumors analyzed for LOH in our studies were evaluated for proliferative fraction by incorporation of BrdU. In most cases, the BrdU was administered *in vivo* 30 minutes prior to surgery. Hence, very accurate measurements enabled us to evaluate the relationship between LOH at various chromosomal sites and tumor proliferative fraction. We found that rapid proliferation correlated with LOH at both 17p13.3 and 3p24–26 [45]. Surprisingly, the cases with LOH at 17p and high proliferative fraction were not more likely to have a LOH at 3p, suggesting the hypothesis that at least two different molecular lesions could be responsible rapid proliferation. These results are preliminary; clearly, additional studies are warranted to verify this observation. Furthermore, a statistical correlation does not prove a causal relationship; nevertheless, such associations can provide the basis for hypothesis building.

p53 MUTATIONS IN BREAST CANCER PROGRESSION

The p53 Gene

A number of recent articles have reviewed the rapidly expanding literature on the involvement of the p53 gene in human cancers, and breast cancer specifically [46–49]. p53 was discovered as a normal cellular protein bound to the viral transforming oncogene, large T-antigen [50,51]. Mutant p53 transforms cells like a dominant oncogene [53,53]; however, absence of p53 is also associated with transformation. The explanation for this paradox is that mutant p53 is a dominant suppressor gene which inactivates wild-type p53 by binding in a tetrameric configuration. The amino terminus of the p53 molecule contains a transcription-activating sequence [54,55] while the carboxy terminus contains a cluster of nuclear localization signals [56,57]. There are 11 exons in p53 with 5 domains (in exons 2, 5, 7, 8) which are highly conserved, indicating regions of important function in the molecule. Most of the p53 mutations in breast cancers are missence mutations distributed throughout the highly conserved regions of the molecule [48].

The p53 gene product is thought to be involved in a number of different cellular functions including cell cycle regulation [58–62], transcriptional regulation [54,55,63–66], differentiation [67–69], and apoptosis [70]. Most recently, in fibroblast model systems, wild-type p53 has been found to suppress entry into the S-phase of the cell cycle in cells with DNA amplifications associated with generation of drug resistance [71,72]. Thus, it has been suggested that wild-type p53 is a "watchdog" type of molecule which inhibits replication of cells with DNA damage (*i.e.*, potential malignantly transformed cells). In these studies, gene amplification only occurred when both p53 alleles were either mutated or lost. Whether a similar mechanism also holds for breast cancers remains to be established, particularly since breast cancers do not show a strong correlation between p53 mutations and LOH of the other p53 allele [25,45].

Wild-type p53 gene codes for a nuclear protein characterized by a short intracellular half-life which is increased by mutation [46]. Mutant p53 is known to bind a number of different molecules, including heat-shock protein 70 or the retinoblastoma gene product which apparently

stabilize the molecule, thus accounting for the protein's increased stability.

The increased protein stability of mutant p53 forms the basis of immunohistochemical assays for its detection [reviewed in 47,73]. In normal cells, the concentration of wild-type p53 is so low that it is undetectable by immunostaining. Increased molecular stability of the mutant protein enables it to be visualized under identical staining conditions [74]. All studies agree that there is heterogeneity in the percent positive cells within a given tumor, with some tumors containing only a few scattered immunopositive cells.

Among various studies, the percentage of breast cancers with nuclear immunopositivity varies from approximately 25–50% [47,75–79]. Incidences of 15–35% were reported by analyzing breast cancers for p53 mutations in the conserved regions of the gene [76,80–86]. Differing sensitivity of immunoassays may account for this variability. In most cases, when nuclear immunopositivity has been detected, mutation of the p53 gene has been confirmed [76,79]. However, immunopositivity may only detect a portion of p53 mutations. Obviously, it cannot detect nonsense mutations which prevent synthesis of any p53 protein. Depending on the immunoassay, it may not detect all of the missense mutations either.

In a number of reports, p53 mutations are associated with tumors that are negative for estrogen receptors (ERs), have a high histologic grade, and overexpress epidermal growth factor receptor (EGFR) [47,76,78,85,86–90]. In some reports, p53 immunopositivity also correlated with high S-phase fraction [87]. The biological significance of these associations is unclear. Since p53 is known to be a nuclear binding protein, perhaps it is involved in regulation of ER transcription. Association with high histologic grade and EGFR, and possibly high S-phase fraction, may be a consequence of negative ER. In fact, when ER status was accounted for, p53 immunopositivity no longer correlated with high S-phase [86]. The net result of high EGFR, ER negativity, and inactivation of p53 may be synergistic dysregulation of various growth-stimulatory pathways leading to rapid proliferation and/or other manifestations of aggressive behavior by the tumor. Unlike other tumors, breast cancer showed no correlation between LOH at the p53 locus and p53 mutations [90]. Thus, only one inactivated allele is sufficient to cause breast cancer. At least one other report [84] did find a correlation between mutation and LOH, although the number of cases was small. We recently found that there was a strong association between p53 mutations and LOH at chromosomes 3p and 7q [45].

Immunopositivity for p53 has also been detected in 13–25% of *in situ* carcinomas, suggesting that it can be acquired early in malignant progression [76,79]. As in invasive breast cancers, p53 immunopositivity strongly correlated with the presence of mutations, although additional mutations were detected in immunonegative tumors [45]. Immunopositivity was seen predominantly in the comedo type of *in situ* lesion, which is thought to be the most aggressive form of *in situ* breast cancer [76].

To determine whether p53 mutations can also be acquired later in progression, the presence of p53 immunopositivity was compared in primary breast cancers and lymph node metastases, subsequent recurrences or distant metastases from the same patient. In all cases, primary breast cancers and concomitant lymph nodes [77], or subsequent local recurrences, were identical [unpublished observation]. We have found a few cases where distant metastases were positive when the primary was negative, suggesting that p53 mutations can occasionally occur after invasive disease, perhaps conferring increasingly aggressive behavior to already existing breast cancers.

REACTIVE LYMPHOCYTES IN DCIS

We were led to our hypothesis that a mechanism for inducing selective pressure involves immune surveillance by reviewing both the literature and histologic sections of DCIS. In examining DCIS, we were struck by the nonuniform distribution of the lymphocytic host response among the different terminal ductal lobular units (TDLU). There was marked intralesion heterogeneity in distribution of these lymphocytes; one TDLU would be completely surrounded with lymphocytes while a second one in the same field had none. By carefully scoring the type of lesion and the percent of ductules with lymphocytic host response, we found that there was a strong correlation between TDLU that elicited a lymphocytic response and high-grade, comedo subtype DCIS.

Many of the lymphocytes surrounding the TDLU are T-helper cells, immunopositive for CD4. Additional studies will be needed to determine whether these T-helper cells are biologically functional and capable of rejecting the cells they surround within the TDLU. If so, we suggest that, in the process of eliminating those aberrant TDLU, the tumor inappropriately selects for cells which gain the ability to escape immune surveillance.

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