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Commentary

## Premalignant Breast Neoplasia: A Paradigm of Interlesional and Intralesional Molecular Heterogeneity and Its Biological and Clinical Ramifications

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### Abstract

As is well established in invasive breast disease, it is becoming increasingly clear that molecular heterogeneity, both between and within lesions, is a prevalent, distinct phenotype of premalignant lesions of the breast. Key pathways of tumorigenesis modulate critical features of premalignant lesions such as proliferation, differentiation, stress response, and even the generation of diversity. Current studies show that evaluation of these lesions may provide clinically useful information on future tumor formation as well as biological insights into the origin and functional significance of this distinct phenotype. *Cancer Prev Res*; 3(5); 579–87. ©2010 AACR.

### Introduction

Premalignant lesions of the breast are becoming an increasing focus of clinical breast care and translational and basic science. Detection of these lesions has increased because of widespread screening programs and increasing numbers of biopsies. Although a premalignancy diagnosis is a risk factor for future development of invasive breast cancer, the majority of so-diagnosed individuals will remain free of invasive disease following excision of the lesion. Therefore, continued scientific insights into the origins and behavior of *in situ* carcinomas and atypical lesions are essential to achieving the goal of distinguishing the higher-risk minority of patients, who are destined to develop invasive carcinomas and thus need aggressive prevention and screening, from the lower-risk majority of patients, who could be directed to more limited interventions.

Particularly promising efforts toward realizing this goal of risk stratification are recent studies to elucidate the molecular heterogeneity of premalignant lesions such as ductal carcinoma *in situ* (DCIS). DCIS has long been known to be heterogeneous in its morphologic features, presentation, and clinical behavior. The emergence of genomic and gene expression profiling has estab-

lished that DCIS is heterogeneous at a level of molecular complexity that is comparable with or perhaps greater than that of invasive disease (1–10). Although certainly less studied, the heterogeneity observed in the microenvironment surrounding individual premalignant lesions may also be a critical determinant of their future behavior. Recent studies have begun to identify heterogeneity within the microenvironment surrounding invasive tumors that could be linked to outcome (11–14). The genesis of these stromal changes may precede the formation of invasive lesions and reflect causes or consequences of early tumor progression. This discussion will focus on the epithelial cells within a premalignant lesion and the biological and clinical ramifications of its intralesional and interlesional heterogeneity.

### Interlesional Heterogeneity of Premalignant Lesions

Comparative studies have shown that DCIS has a range of molecular subtypes similar to those identified through gene expression profiling of invasive ductal carcinomas (IDC; refs. 1–4, 7). Important molecular distinctions are observed in studies of DCIS versus IDC that may provide clues to the origins of molecular diversity in breast carcinogenesis. For example, Allred et al. initially observed that human epidermal growth factor receptor 2 (HER2) is overexpressed at a higher frequency in DCIS than in IDC (15). An increased prevalence of HER2 protein overexpression (and gene amplification) in DCIS versus IDC has been confirmed in a number of subsequent studies (1, 16–18). HER2 overexpression seems to be most significant in high-grade DCIS, in which its prevalence is nearly 75% (17). In contrast to this relatively higher prevalence of HER2 overexpression in DCIS, the prevalence of marker expression indicative of the basal-like subtype is relatively lower in DCIS compared with IDC (1, 3, 4, 17, 18).

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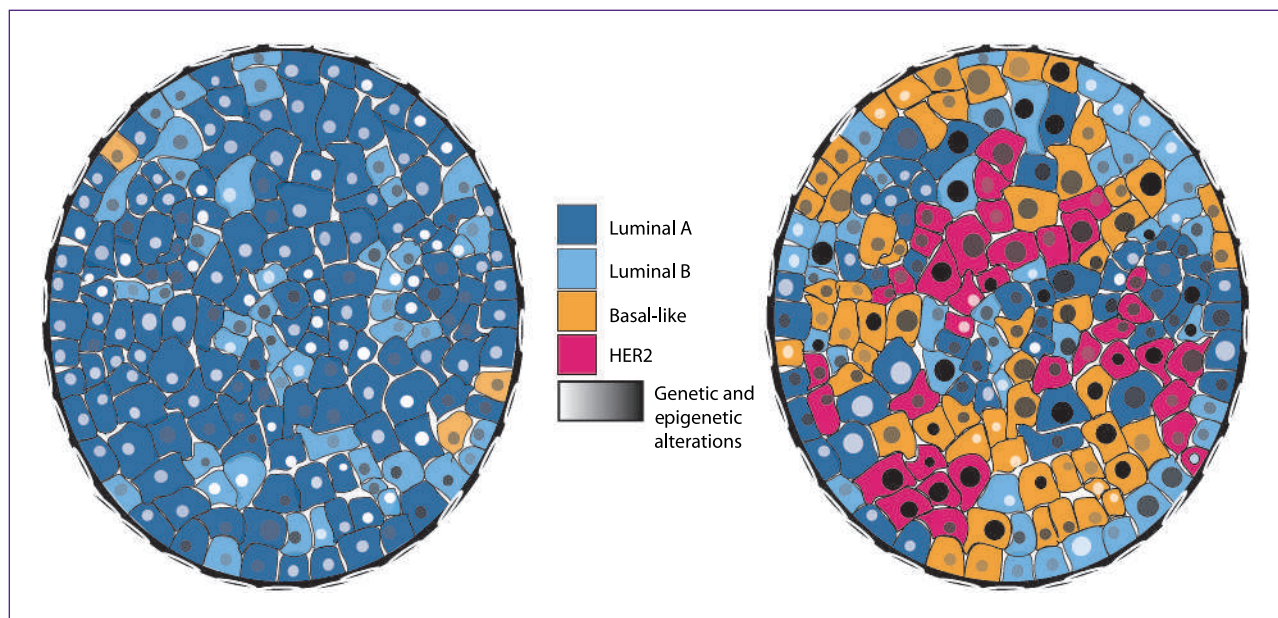
The apparent imbalance between DCIS and IDC in molecular subtype prevalences suggests that there is no simple linear relationship of progression from DCIS to invasive disease. These observations further suggest that different DCIS subtypes might be associated with different malignant potentials. For example, DCIS with basal marker expression may give rise to invasive carcinoma at a higher frequency than does DCIS exhibiting HER2 overexpression. This lack of a linear relationship might explain the relatively higher frequency of the basal-like subtype recently observed in studies of IDC versus DCIS (2). Alternatively, immunohistochemical detection of protein markers may not always reflect microarray gene expression profiles and therefore may not provide a faithful representation of subtype distribution (7, 19). Most important, immunohistochemistry and microarray studies to define molecular subtypes of DCIS (and even IDC) typically fail to address intralesional heterogeneity, or the possibility of more than one subtype within a single lesion. As discussed below, a more thorough analysis or identification of intralesional heterogeneity may provide new insights into the potential for progression to malignancy.

### Intralesional Heterogeneity of Premalignant Lesions

Examination of individual premalignant lesions illustrates a second level of premalignant heterogeneity, intralesional heterogeneity. The sectorized areas of a single lesion

can evidence characteristic staining for different subtypes of breast neoplasia. For example, a single lesion might have areas that express proteins associated with a luminal subtype distinct from other areas that express proteins associated with a basal-like subtype (Fig. 1). In addition to cellular manifestations of heterogeneity, intralesional heterogeneity also occurs in nuclear morphologic assessments of nuclear grade, which is an important factor in the clinical assessment of tumors. Components of nuclear morphology that underlie variations in nuclear grade are complex and extend beyond size (20, 21). Variations occur in nuclear shape, contour of nuclear membranes, and number and size of nucleoli. Chromatin can be dispersed or margined and could form coarse and variably asymmetrical aggregates of heterochromatin. To contend with intralesional heterogeneity at the morphologic level, histologic grading systems assign a score based on properties of selected cell subpopulations. For example, the nuclear grade of a breast IDC is clinically assessed based on nuclear size and shape in areas of highest grade representing as little as 10% of the cell population. Nuclear morphometry, a quantitative approach for assessing heterogeneity of size and other nuclear characteristics, has prognostic value in IDC and DCIS and correlates with nuclear grade (22, 23).

In an early study of intralesional heterogeneity at the molecular level, Ma et al. observed distinct patterns of gene expression within premalignant and malignant breast lesions that suggested intralesional heterogeneity of histologic grade (24); for example, most grade 2 lesions



**Fig. 1.** Theoretical spectrum of molecular heterogeneity in DCIS. The two ducts represent theoretical comparisons of DCIS between different individuals (i.e., interlesional heterogeneity) or between two regions within a patient's single DCIS lesion (i.e., intralesional heterogeneity). DCIS could range from homogeneity (left) to a surprising degree of heterogeneity (right) within a single lesion. The two ducts depict the ends of a continuum of heterogeneity in nuclear and cell size, presence and number of coexisting molecular subtypes, and genetic and epigenetic alterations.

exhibited a hybrid of grade 1 and grade 3 molecular signatures. More recently, Allred et al. elegantly addressed intralesional diversity at the molecular level in a large series of 120 cases of pure DCIS (25). Almost half of these cases showed variable regions of nuclear grade within an individual biopsy. Intralesional areas of variable histologic grade were microdissected to allow for thorough molecular characterization with a panel of immunohistochemical markers (hormone receptors, GATA3, HER2, p53, and basal cytokeratins) and with gene expression profiling in a subset of samples. A strikingly high proportion (nearly three-quarters) of the DCIS samples with intralesional heterogeneity in nuclear grade showed more than one molecular subtype, with nearly all possible combinations represented (e.g., basal-like and luminal-A subtype in one lesion).

The presence of molecularly and morphologically distinct subpopulations of cells within DCIS raises important questions about the origins of molecular variability and the pathways of breast cancer progression (26). Although DCIS is considered a nonobligate precursor to invasive breast cancer, mathematical models of progression suggest that DCIS and IDC may develop through separate but parallel pathways from a common progenitor (27). Different models that have accurately incorporated progression data collectively suggest multiple pathways of progression (28). Genome-wide profiling of invasive and premalignant lesions of the breast has revealed that the pathway of progression for phenotypically low nuclear grade lesions is molecularly distinct from the pathway for high nuclear grade lesions (10, 29). Low nuclear grade premalignant lesions of the breast include low-grade DCIS, atypical ductal hyperplasia, flat epithelial atypia, and some lobular neoplasias. These lesions all tend to share characteristic alterations such as loss of 16q and gains of chromosome 1q (reviewed in ref. 30). In contrast, high nuclear grade lesions are genetically more complex with varied chromosomal losses and gains. The coexistence of low-grade and high-grade cells in some DCIS lesions therefore suggests the presence of clonal heterogeneity. Mathematical models of progression have not yet considered the implications of intralesional heterogeneity because the primary data typically are derived from established histologic grading systems. Accurate models of progression may depend on consideration of the presence and evolution of clonal diversity. Given that clonal heterogeneity in premalignancy might be an independent risk factor for invasive tumor development (reviewed in ref. 31), future genomic characterization of areas of intralesional heterogeneity within DCIS might also be clinically significant.

### Deregulated *p16/pRb* Signaling: Phenotypic Consequences for DCIS

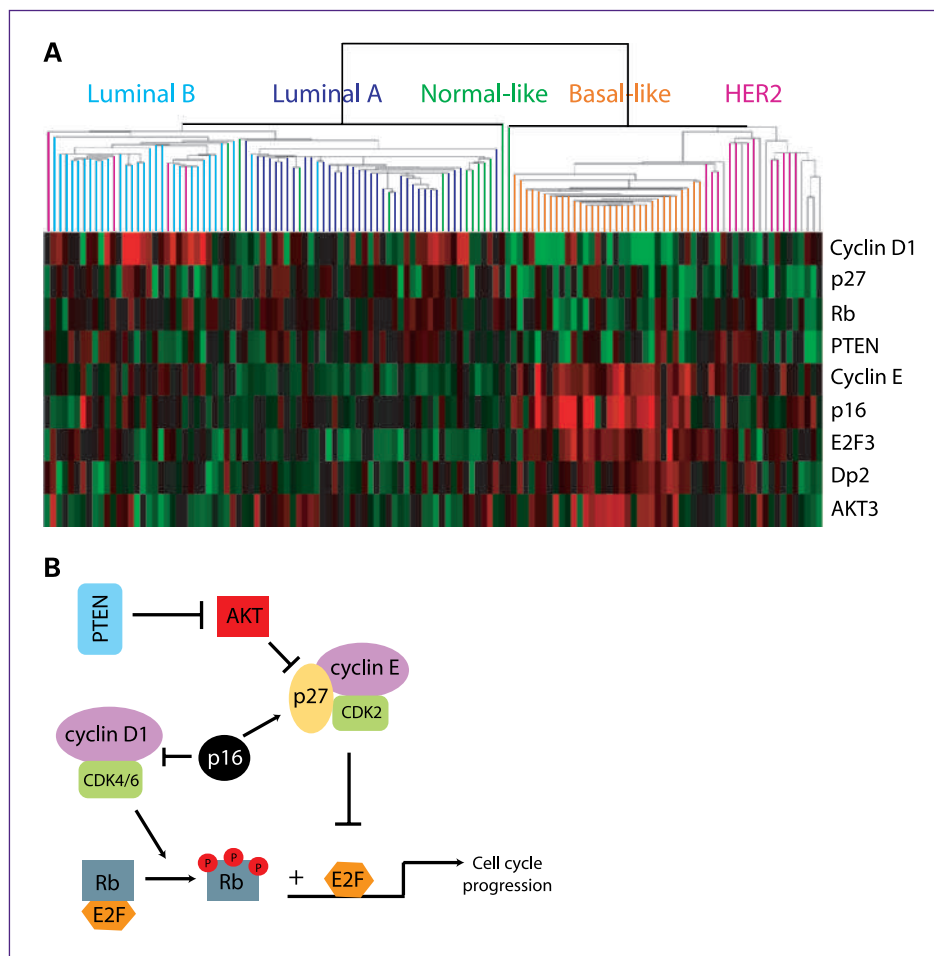
To understand the behavioral consequences of intralesional heterogeneity, it is useful to examine the molecular programs that dictate the different phenotypes of DCIS.

DCIS lesions are often classified as low, intermediate, or high grade. Several grading systems have been developed and are based on evaluating several phenotypes within the premalignant lesion. The mitotic rate is routinely evaluated in invasive carcinoma and has also been well studied in DCIS. The most commonly evaluated clinical phenotypic features of DCIS are (a) the degree of differentiation, (b) degree of nuclear atypia, and (c) extent of necrosis. The role of the *pRb* pathway is particularly interesting in the context of histologic grading because the *p16/pRb* signaling pathway controls a coordinated ensemble of phenotypes that influence grade (i.e., genes that regulate cell cycle progression, differentiation, chromatin remodeling, and cell death). Although not currently used for clinical assessment of DCIS, the status of stress responses in premalignant lesions may provide clinically useful information. These processes contribute to the clinical manifestations of carcinogenesis and may contribute molecular markers that have clinical utility.

The *pRb* pathway plays a key role in mitotic rate (proliferation), one of the critical phenotypes in determining histologic grade in invasive breast carcinomas. Repression of E2F-dependent transcriptional targets that control cell cycle arrest is assumed to be the major mechanism by which *pRb* exerts antitumorigenic effects. This focus on cell cycle effects and antiproliferative functions was substantiated by mutation studies showing that removal of *pRb* allowed accelerated entry into the G<sub>1</sub>-S phases of the cell cycle and that exogenously introduced unphosphorylated pRb caused cell cycle arrest (32–35). This alteration in cell cycle control would be expected to manifest itself via an elevated mitotic rate, such as a higher fraction of cells expressing Ki67, and consequently, via hyperplastic morphologic alterations.

Abrogation of cyclin-dependent kinase 4/6 (*CDK4/6*), cyclin D1, *p16*, and/or *pRb* seems to be required for malignancy, and tumor types are often distinguished by particular alterations in one member of the pathway. Although the abrogation of each member of this pathway imparts tumorigenic potential, emerging evidence in breast carcinogenesis suggests that different mechanisms of *p16/pRb* pathway inactivation might lead to diverse tumor subtypes (Fig. 2A). For example, cyclin D1 is overexpressed (primarily through gene amplification) predominantly within tumors of the luminal B subtype (2, 36–39), whereas basal-like tumors typically show overexpression of p16 and elevated levels of E2F target genes, accompanied by loss of pRb expression (2, 40). Although we do not know if deregulation of *pRb* signaling (Fig. 2B) has implications for the origins of the basal-like subtype, these data do support that deregulation of *pRb* is mechanistically linked to it.

Although undoubtedly important to neoplasia, the mitotic rate is not the only phenotype providing prognostic information that is influenced by abrogation of the *pRb* pathway. For example, it is becoming widely appreciated that the pRb protein integrates cell cycle arrest with cellular differentiation. Both *in vitro* and *in vivo* model systems have provided evidence that pRb plays a role in



**Fig. 2.** Distinct molecular subtypes of invasive breast tumors; p16/pRb signaling in breast neoplasia. A, hierarchical clustering (by gene expression) of 130 primary invasive breast tumors identifies distinct molecular subtypes, which are color-coded (top of diagram) as follows: light blue, luminal B; dark blue, luminal A; green, normal-like; orange, basal-like; pink, HER2-positive; gray, unclassified. Loss of functional pRb signaling defines the basal-like subtype of invasive tumors. Expression levels of *cyclin D1*, *p27*, *pRb*, phosphatase and tensin homologue (*PTEN*), cyclin E, *p16*, *E2F3*, *Dp2*, and *AKT3* are shown. The level of expression of each gene is relative to the median expression of this gene across all 130 samples. Adapted from Gauthier et al. (ref. 2; reprinted with permission from Elsevier). B, regulation of p16/pRb signaling is represented diagrammatically. pRb plays an integral role in cell cycle regulation and coordinates multiple signal transduction pathways that regulate proliferation. Deregulation of pRb could occur through multiple mechanisms including loss of heterozygosity, promoter hypermethylation, and gene mutation. Methods of inactivation of the p16/pRb signaling pathway include aberrant phosphorylation through amplification of cyclin E, loss of p27, activation of AKT signaling, and/or loss of PTEN through gene mutation or promoter hypermethylation.

determining cell fate (41–43). Work in a growing number of tissues has shown that functional expression of pRb transcriptionally modulates genes that are important in achieving complete cellular differentiation. These regulatory properties seem to be independent of pRb effects on cell cycle progression. For example, pRb mutants that uncouple cell cycle properties from differentiation effects have been identified (44).

In normal cells, the pRb protein is upregulated during differentiation and interacts with selected coregulators of transcription to activate or repress gene loci. Absence of pRb function allows morphologic differentiation but prevents full functional maturation of cells. In human breast epithelial cells, removal of pRb function allows cells to form characteristic acinar structures in three-dimensional

culture conditions, but the expression of lactoferrin and serum cytokeratin 19 (CK19), which are markers for luminal differentiation, are not expressed (45). Molecular studies have shown that the upregulation of pRb transcription in some tissue systems is regulated by p53 and by mechanical forces in the surrounding stroma. Manipulation of these conditions could induce lactoferrin production in human mammary epithelial cells (46). The consequences of these regulatory circuits in breast premalignancy are interesting. In tumors in which the pRb pathway has been abrogated by mutation of other members of the pathway, e.g., *CDK4* mutation, *p16* silencing or *cyclin D1* overexpression, inactivation of pRb interactions with E2F would still affect cell cycle arrest and allow an increased mitotic rate. However, tumors with these mutational alterations would

still express pRb protein, which could coregulate genes important in differentiation. In contrast, basal-like tumors, which are missing pRb function, not only experience a loss of cell cycle control but also fail to express markers of cellular differentiation.

Recent studies indicate that *pRb* may also play a pivotal role in the generation of nuclear atypia through the regulation of genetic and epigenetic alterations and changes in cellular polarity. Nuclei of an abnormal shape, size, and texture are a hallmark of malignancy. These nuclear properties are governed by the packaging of chromatin within the nucleus and the proper partitioning of chromosomal elements. Epigenetic systems that dictate chromatin structure are regulated by *pRb*, most often through the downstream regulation of transcriptional targets that activate histone methyltransferases, DNA methyltransferases, or acetylases. The repression of *p16* activity, accompanied by the inactivation of *pRb* as a transcriptional repressor, leads to the overexpression of chromatin-remodeling proteins such as EZH2 and SUZ12 (47, 48). In addition to executing the silencing of selected loci, overexpression of these proteins has been identified in approximately half of DCIS lesions and indicate a poor prognosis when detected in invasive breast cancers (49, 50). In addition to epigenetic controls on nuclear size and shape, genetic changes may also play an important role in generating nuclear atypia. The role of the *pRb* pathway in disrupting genomic integrity is discussed below. Last, coordination of centrosome duplication with DNA synthesis is choreographed by the *pRb* pathway. Abrogation of this pathway leads to the generation of aneuploidy and the loss of proper polarity (51).

Potential mechanisms of *pRb* pathway alterations, including deletion, mutation, and/or promoter hypermethylation, may have differential effects on the nucleus. Studies by our group and others show that *pRb* gene expression levels are lowest in basal-like tumors (2, 40, 52, 53). Tumors with loss of functional *pRb* signaling have a higher frequency of chromosome copy number alterations than do tumors with aberrations of other members of the *p16/pRb* pathway (54).

Finally, in the assessment of DCIS lesions, increased cell death is associated with a more aggressive behavior. The control of cell death pathways is complex, but studies have linked *pRb* alterations with the control of p53 function and the regulation of cell death (55). Therefore, alteration of the *pRb* pathway modulates multiple phenotypes, including proliferation indices, differentiation, nuclear atypia, and death indices that are important in malignancy. A careful analysis of the mechanisms of deregulating this pathway may provide useful clinical information.

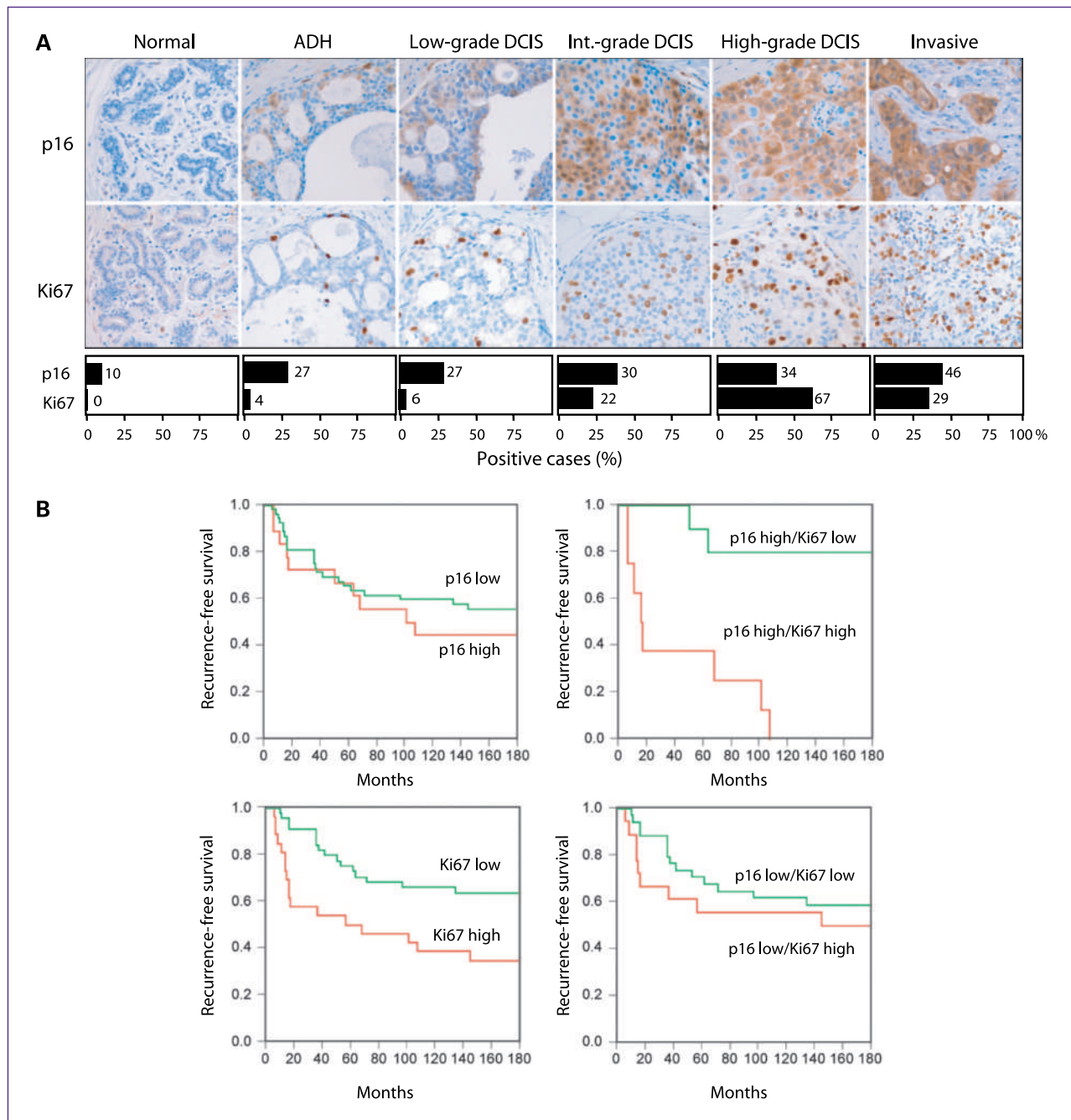
### Intralesional Heterogeneity of Premalignant Lesions: Clinical Consequences for DCIS

In addition to the typical phenotypes used to classify DCIS in the clinic, several molecular markers might prove useful in stratifying risk for future invasive tumor formation and may also relate to *pRb* biology. There is a notable

link between the *pRb* pathway and the activation of cellular senescence. Although well-studied *in vitro*, senescent cells are just beginning to be studied *in vivo*, in which the understanding of their presence and significance is rapidly emerging. Studies in human samples (2) and murine models of prostate carcinogenesis (56), melanocytic nevi (57–59), lung adenomas (60, 61), and lymphoma (62) show that overexpression of p16 protein and other markers of cellular senescence preferentially identify indolent premalignant and benign lesions. In studying *p16/pRb* signaling within premalignant lesions such as DCIS and atypical ductal hyperplasia, it is important to consider that p16 overexpression is conditional on the functional state of *pRb*. Unlike cells of most malignant tumors, cells within premalignant lesions may have intact *pRb* signaling. The p16 overexpression in premalignancy may be a physiologic response to stress activation leading to cell cycle arrest and a senescence program. Indications of an intact response to cellular stress in DCIS seems to be of clinical significance because overexpression of p16 in the absence of proliferation (i.e., activation of a senescence program) identifies women less likely to develop subsequent breast cancer (2). These observations support the hypothesis that cellular senescence is a barrier to tumorigenesis, which is maintained in some premalignant lesions.

In normal cells, activation of the *pRb* pathway in response to cellular aging or cellular stress such as oxidative, metabolic, hypoxic, and/or oncogenic stress drives cell cycle arrest and plays an important role in premature and replicative senescence-response pathways (63, 64). Through inhibition of CDK4/6, p16 blocks the phosphorylation of pRb and thus inhibits E2F transcription factors from binding to promoter sequences essential for cell cycle progression (65–69). Furthermore, the *pRb* pathway regulates the expression of stress proteins known to be important in conferring malignant phenotypes. Human mammary epithelial cells typically do not express the proinflammatory protein cyclooxygenase-2 (COX-2). However, mutations within the *pRb* pathway affect the expression of this protein in distinct ways. As recently reported, abrogation of the *pRb* pathway through *p16* repression enhanced the induction of COX-2 when cells were exposed to a variety of exogenous stresses (2). In contrast, cyclin D1 overexpression did not sensitize cells to the stress signals and did not induce COX-2. Of interest, removal of pRb function itself caused a constitutive overexpression of COX-2. It is intriguing to consider that subpopulations of normal human mammary epithelial cells *in vivo* and *in vitro* show *p16/pRb* inactivation through promoter hypermethylation, and consequently, COX-2 overexpression (70, 71). Because COX-2 enhances cell migration, angiogenesis, and proliferation and inhibits apoptosis and immune surveillance, it is clear that different mutational mechanisms for abrogating the *pRb* pathway have important phenotypic consequences for tumors.

Premalignant cells that have abrogated *pRb* signaling and bypass stress-induced senescence may also manifest



**Fig. 3.** p16 Overexpression coupled with proliferation increases the risk of subsequent tumor events among women with DCIS. A, immunohistochemistry staining for p16 and Ki67 in normal breast tissue ( $n = 40$ ), atypical ductal hyperplasia (ADH;  $n = 33$ ), low-, intermediate (Int)-, and high-grade DCIS ( $n = 142$ ), and IDC ( $n = 100$ ). Each bar graph represents the percentage of immunopositive cases. p16 staining was scored on a 0, 1, 2, 3 scale based on the extent of immunopositive cells (0, no staining; 1, <25%; 2, 25-75%; 3, >75%). Positive/high p16 staining is defined as a score of  $\geq 2$ . The Ki67 index was determined by manually counting a minimum of 1,000 nuclei within at least three 40 $\times$  fields. Positive/high Ki67 is defined as greater than 10% immunopositive cells. B, Kaplan-Meier plots of recurrence-free survival by p16 status, Ki67 status, and p16/Ki67 coexpression status among 70 patients diagnosed with and treated for pure DCIS. Adapted from Gauthier et al. (2); reprinted with permission from Elsevier.

overexpression of p16. In this setting, elevated levels of p16 protein in highly proliferative cells suggest that the growth-suppressive effects of p16 are short-circuited downstream of p16. Despite exhibiting hallmarks of

stress activation, such as overexpression of p16, p53, p21, and COX-2 and phosphorylation activation of stress-activated kinases such as p38, which are usually associated with the induction of a proliferative arrest,

cells with dysfunctional *pRb* undergo unobstructed proliferation (2, 55, 72). An abrogated response to stress signals in DCIS also seems to have clinical value because overexpression of p16 in the presence of ongoing proliferation identifies women more likely to develop subsequent breast cancer (Fig. 3).

In support of *pRb* biology linking multiple malignant phenotypes, tumors with the lowest expression of pRb exhibit illuminating characteristics. Increased immunodetection of p16 correlates with decreased pRb levels in breast carcinomas (2, 53, 73, 74). Tumors with p16 overexpression at the immunohistochemical level have been identified as poorly differentiated tumors through associations with high nuclear grade, high Ki67, increased p53, and low estrogen receptor/progesterone receptor expression (75–79). Of importance, these tumors also overexpress COX-2. Therefore, although the activation or inactivation of cellular stress and senescence pathways are not currently used clinically, this information may be helpful in the future.

### Deregulated *p16/pRb* Signaling: Clues to the Origins of Molecular Heterogeneity in Breast Tumors

The acquisition of genomic instability through the deregulation of *p16/pRb* signaling provides the potential for generating heterogeneity. Despite the well-established role of *pRb* dysfunction in deregulating cell cycle progression and desensitizing cells to damage-induced cell cycle arrest or apoptotic signals, only more recent studies have directly addressed the causal relationship between loss of *pRb* signaling and genomic instability. The loss of *pRb* deregulates the expression of genes involved in mitotic progression and chromosome segregation generating genetic diversity. Furthermore, the loss of functional *pRb* not only leads to inappropriate proliferation but also to whole-chromosome gains and losses (aneuploidy) due to aberrant expression of spindle checkpoint proteins such as mitotic arrest-deficient 2 (MAD2; refs. 80, 81). It is interesting to note that loss of *p16* expression also results in chromosome instability and aneuploidy but through a *pRb*-independent mechanism. Indeed, it was recently shown that loss of p16

expression in genomically stable, normal mammary epithelial cells results in the production of supernumerary centrosomes in a CDK2/p21-dependent fashion (51). These collective results suggest that the loss of *p16/pRb* signaling may cause chromosome and genomic instability early during tumorigenesis (i.e., prior to the acquisition of oncogenic events) and might contribute to the genesis of molecular heterogeneity in tumors.

However, differences in marker expression might represent the end result of increased mutations or, alternatively, may represent the emerging result of ecological interactions. Consistent with this speculation, our studies of DCIS (2) found that heterogeneity was the prevalent phenotype, with few lesions showing homogeneous expression of a given marker. In DCIS lesions expressing a senescence signature, differences in the extent of p16 expression might represent regional differences in cellular stress or different “snapshots in time” of the stress response rather than the consequence of mutation.

Last, concepts ordinarily used in evolutionary or ecological studies might provide insights into the functional significance of heterogeneity in premalignant lesions. The significance of intralesional heterogeneity may be analogous to that of the generation of diversity observed in ecological interactions developed by stressed populations. In these populations, exposure to stress activates evolutionary and ecological interactions that result in diversity which buffers against complete loss of viability under rapidly changing conditions (i.e., the insurance hypothesis; ref. 82). Conceptually speaking, the generation of heterogeneity therefore serves a function that might extend beyond direct induction of mutational changes, serving, for example, to preserve the survival of the population as a whole.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Cancer Prevention Research

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