

# Centrosome Abnormalities and Chromosome Instability Occur Together in Pre-invasive Carcinomas<sup>1</sup>

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## ABSTRACT

Centrosomes play critical roles in processes that ensure proper segregation of chromosomes and maintain the genetic stability of human cells. They contribute to mitotic spindle organization and regulate aspects of cytokinesis and cell cycle progression. We and others have shown that centrosomes are abnormal in most aggressive carcinomas. Moreover, centrosome defects have been implicated in chromosome instability and loss of cell cycle control in invasive carcinoma. Others have suggested that centrosome defects only occur late in tumorigenesis and may not contribute to early steps of tumor development. To address this issue, we examined pre-invasive human carcinoma *in situ* lesions for centrosome defects and chromosome instability. We found that a significant fraction of precursor lesions to some of the most common human cancers had centrosome defects, including *in situ* carcinomas of the uterine cervix, prostate, and female breast. Moreover, centrosome defects occurred together with mitotic spindle defects, chromosome instability, and high cytologic grade. Because most pre-invasive lesions are not uniformly mutant for p53, the development of centrosome defects does not appear to require abrogation of p53 function. Our findings demonstrate that centrosome defects occur concurrently with chromosome instability and cytologic changes in the earliest identifiable step in human cancer. Our results suggest that centrosome defects may contribute to the earliest stages of cancer development through the generation of chromosome instability. This, together with ongoing structural changes in chromosomes, could accelerate accumulation of alleles carrying pro-oncogenic mutations and loss of alleles containing wild-type tumor suppressor genes and thus accelerate the genomic changes characteristic of carcinoma, the most prevalent human cancer.

## INTRODUCTION

CIN<sup>3</sup> is the most common form of genetic instability in human cancer and thought to be caused by continuous chromosome missegregation during mitosis (1, 2). Together with structural chromosome changes caused by chromosome breakage and misrepair, CIN is thought to be important to promote the Darwinian genomic evolution characteristic of cancer, whereby proto-oncogene mutations accumulate, and normal alleles of mutated tumor suppressor genes are lost (2–4). In fact, loss of heterozygosity in cancer primarily affects whole chromosomes or large chromosomal domains, suggesting that it results from gains or losses of entire normal or rearranged chromosomes (5). CIN is thought to facilitate the inexorable evolution of cancers toward cellular states that support tumor cell growth, dissemination, and resistance to therapy (1–3, 6, 7). A common element in the chain

of events associated with loss of fidelity in chromosome segregation is centrosome dysfunction (for review, see Refs. 7–12).

Centrosomes are the primary microtubule-organizing centers in animal cells. They contribute to the organization of microtubule spindles in mitosis and appear to control progression through cytokinesis and entry into S phase (9, 13–15). Our laboratory and another first detected centrosome defects in aggressive carcinomas of multiple origins (2, 16). Several subsequent studies confirmed these observations and extended them to other tumor types and animal models (17–22). The discovery of centrosome defects in essentially all carcinomas sparked interest in this organelle as a global contributor to the development and progression of tumors that exhibit genetic instability (2, 8–11, 23). The established role of centrosomes in organizing mitotic spindles suggested a model in which tumor cells with multiple centrosomes organize multipolar spindles that missegregate chromosomes and contribute to genetic instability. This phenomenon could occur in diploid cells or cells that failed previously in cell division to create polyploid cells with supernumerary centrosomes (24). Despite the occurrence of centrosome defects in most common human cancers and their known role in the assembly of mitotic spindles and chromosome segregation, a role for centrosomes in the earliest steps of human tumor development has not been well established.

Recent results from our laboratory have shown that centrosome defects and genetic instability occur in some low-grade prostate tumors, suggesting that they are present before development of aggressive tumors (21). Moreover, overexpression of some centrosome-associated proteins, including pericentrin, TACC, polo, and aurora (21, 24–28), induces tumor-like features. Centrosome defects have also been observed during the early stages of tumor development in a rat mammary carcinogenesis model (29), suggesting that centrosome defects may also occur in pre-invasive human tumors. A recent study of invasive human carcinoma showed that centrosome abnormalities occurred in some pre-invasive breast lesions (20). The authors analyzed seven cases of breast tissue and reported on one parameter of centrosome defect (size) but did not examine the relationship between centrosome defects and CIN in the pre-invasive lesions. It is important to perform a comprehensive analysis of centrosome defects in pre-invasive lesions for several reasons. A comparative analysis of pre-invasive lesions from tissues with different propensities to develop aggressive cancers may provide important information about the role of centrosomes in the development and progression of cancer. This type of analysis could also identify centrosome defects as a universal diagnostic indicator of most, if not all, carcinomas. The presence of centrosome defects in pre-invasive lesions may also provide a prognostic marker for tumor development, especially in prostate cancer, where the relationship of pre-invasive lesions to aggressive cancer is unclear.

In this study, we analyzed 116 pre-invasive lesions from three different human tissues (breast, cervix, and prostate). We show that centrosome defects occur in all tissue tissues and that they cosegregate with other tumor-like features associated with centrosome dysfunction, including spindle abnormalities, cytologic changes, and CIN (2, 21).

Received 8/15/02; accepted 1/17/03.

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<sup>1</sup> Supported by Grants PC000018, GM51994, and CA82834 from the Department of Defense (G. A. P., S. J. D.), NIH, and the National Cancer Institute (S. J. D.), respectively.

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<sup>3</sup> The abbreviations used are: CIN, chromosomal instability; CIC, carcinoma *in situ* of the uterine cervix; DCIS, ductal carcinoma of the female breast; PIN, prostate intraepithelial neoplasia.

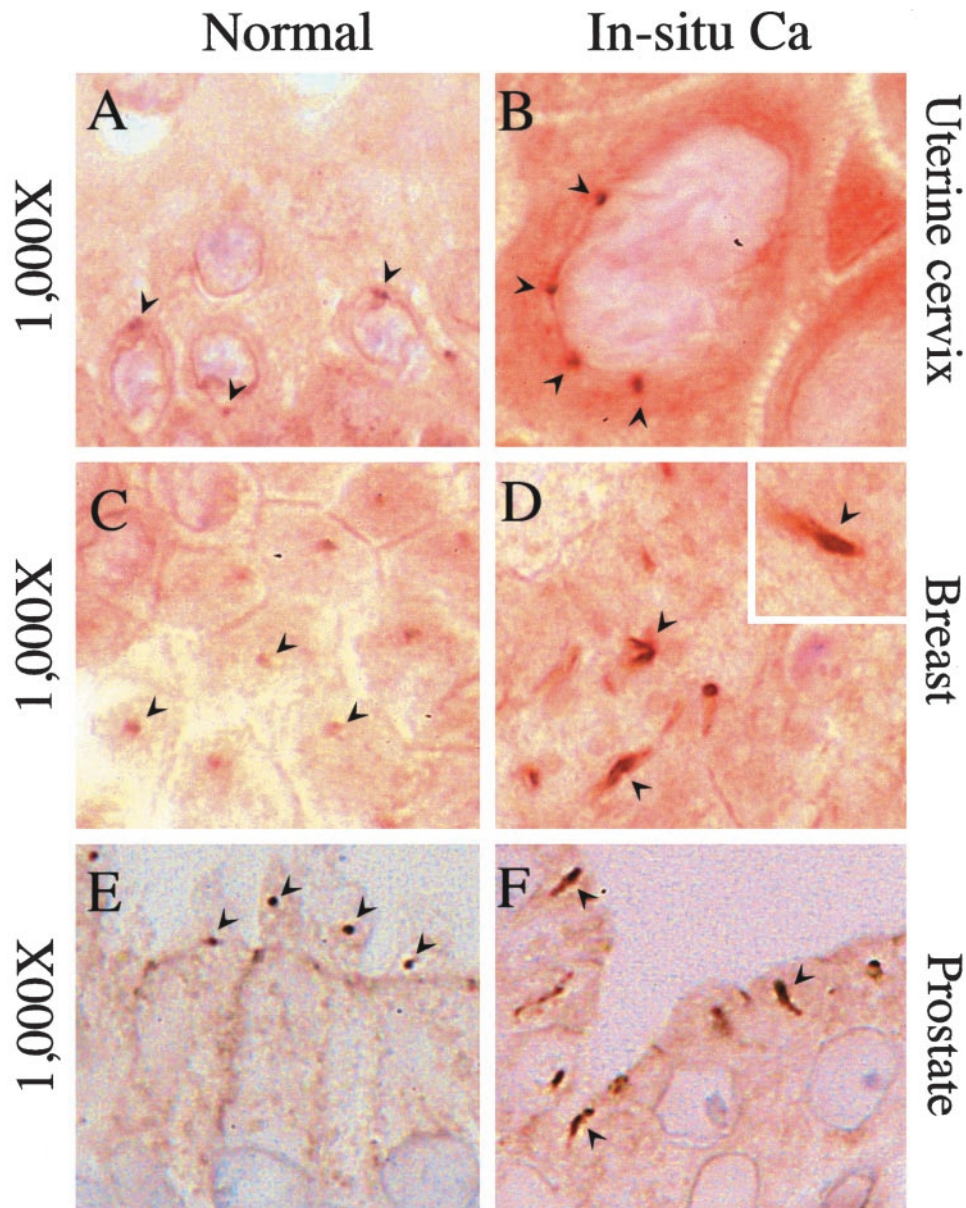


Fig. 1. Centrosome defects occur in carcinoma *in situ*. Photomicrographs of normal epithelium (A, C, and E) and adjacent *in situ* carcinoma (B, D, and F) immunostained with antibodies to pericentrin to visualize centrosomes. Magnifications are all the same ( $\times 1000$ ). In normal epithelia, centrosomes are round and uniform in size (arrowheads, A, C, and E), whereas in carcinoma *in situ*, they are larger (arrowheads in B, D, and F), multiple (B), or structurally abnormal (arrowheads in D and F). Nuclei are stained light blue with hematoxylin. Inset in D shows higher magnification of an elongated centrosome. Note that the nucleus and cell in B are considerably larger than those in adjacent normal epithelial cells (A).

## MATERIALS AND METHODS

**Immunohistochemical Staining and Analysis.** Formalin-fixed, paraffin-embedded tissue from carcinoma *in situ* of the uterine cervix, female breast, and male prostate was selected from the files of the Pathology Department at UMass Memorial Health Care. Samples were immunostained with pericentrin antibodies as described (2, 21, 27). Standard histopathologic criteria were applied to newly prepared H&E-stained sections to confirm the presence of carcinoma *in situ* in the specimen (30). Centrosomes were considered abnormal if they had a diameter greater than twice the diameter of centrosomes present in normal epithelium within the same section, if the ratio of their greatest and smallest diameter exceeded 2 or if there were more than two centrosomes in  $>5\%$  of the cells examined (21).  $\gamma$ -tubulin was chosen to stain mitotic spindles in archival formalin-fixed, paraffin-embedded tissues because it decorates spindle poles, whereas a large fraction of  $\alpha$  and  $\beta$  tubulins is cytoplasmic and obscures the spindle microtubule signal.<sup>4</sup> Multipolar mitoses, an obvious consequence of supernumerary centrosomes, are common in carcinoma cell lines with abnormal centrosomes, as we *et al.* (2, 22, 31, 32) have shown previously.

**CIN Analysis.** Tissue sections parallel to those used for pericentrin immunohistochemistry were used to stain centromeres of chromosome 1 and 8 (2). Briefly, after deparaffinization, sections were codenatured with biotinylated centromeric probes specific for chromosomes 1 or 8 and hybridized overnight at  $37^{\circ}\text{C}$  in a Hybrite oven (Vysis, Chicago, IL) in the hybridization buffer recommended by the probe manufacturer. After appropriate stringency washes, sections were placed on the automatic immunostainer, and an avidin-biotin complex method/3,3'-diaminobenzidine protocol similar to the one used above for immunohistochemistry was used to reveal the hybridized probe. Nuclei were lightly counterstained with hematoxylin. For quantitative analysis, the number of hybridization signals in 100–200 nuclei from *in situ* carcinoma and morphologically normal adjacent epithelium was recorded (2). Using these probes, it has been shown that normal diploid tissue has 10–15% cells with more than three signals per nucleus (2, 33). In tissue sections, some nuclei are truncated, leading to artificially increased numbers of diploid cells with apparently less than two signals per nuclei. For this reason, we primarily computed signal gains (greater than two) and not apparent losses. We also separately analyzed cells with only one copy of a given chromosome. Because of limitations imposed by truncation artifacts, we did not attempt to obtain an absolute measure of chromosome instability in sections, as it can be done

<sup>4</sup> G. A. Pihan, unpublished observations.

on cell lines (2, 6). Rather, we defined tumors with likely aneuploidy/CIN as those in which the fraction of nuclei with more than two signals exceeded 20% (33) and used this measurement as an index of chromosome instability/aneuploidy. Cells with only one chromosome were recorded and discussed separately. CIN and centrosome defects were also recorded in cell lines derived from normal prostate and *in situ* carcinoma of the prostate (34).

## RESULTS

**Centrosome Defects Are Present in Pre-invasive Cancerous Lesions.** Using antibodies to the centrosome protein pericentrin (35), we examined centrosomes in carcinoma *in situ* of the uterine cervix (CIC), female breast (DCIS), and prostate (PIN) as described (2, 21). Several distinct centrosome abnormalities were detected in these lesions, including supernumerary centrosomes (Fig. 1B, arrowheads), abnormally shaped centrosomes, such as elongated or corkscrew forms (Fig. 1, D and F), and centrosomes of larger diameter than those in normal epithelium within the same tissue section (Fig. 1, B and D). Thirty to 72% of all precancerous lesions had abnormal centrosomes (Fig. 2, A–C), whereas such abnormalities were rarely, if ever, detected in nontumor cells (Fig. 2, A–C). Centrosome defects were more prevalent in DCIS and CIC than in PIN lesions. This difference was consistent with differences in histological, cytological, and genetic features of these lesions, *e.g.*, DCIS and CIC show a high degree of nuclear atypia, cytologic disarray, loss of cell polarity, and genetic instability. In fact, on cytologic features alone, they are often indistinguishable from invasive breast and cervical cancers (36, 37). In contrast, PIN lesions show remarkable preservation of cell polarity and glandular architecture and can only be distinguished from normal glands by subtle changes in nuclear and nucleolar features. Similar levels of centrosome defects in pre-invasive lesions were identified using antibodies to  $\gamma$ -tubulin, another core protein of the centrosome (data not shown).

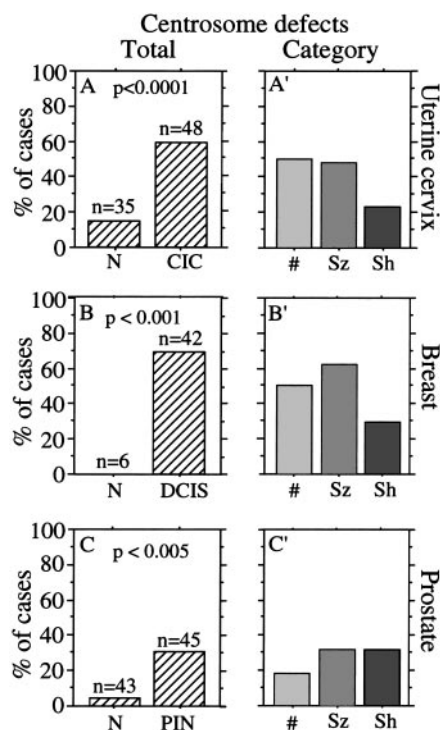


Fig. 2. Centrosome defects are prevalent in carcinoma *in situ*. Centrosome defects are present in 62, 75, and 28% of CIC (A), DCIS (B), and PIN (C) lesions, respectively (N, normal epithelia). First column (A–C), cumulative defects; second column (A'–C'), breakdown of centrosome defects by category (#, number; Sz, size; Sh, shape).

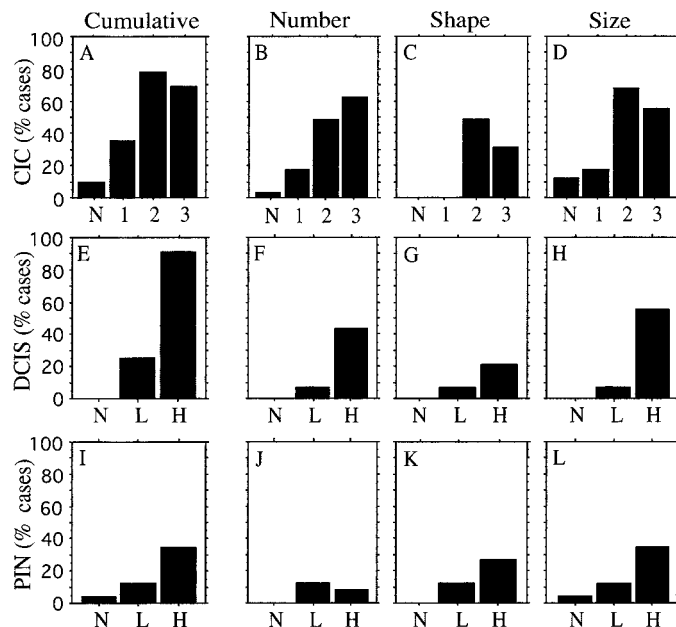
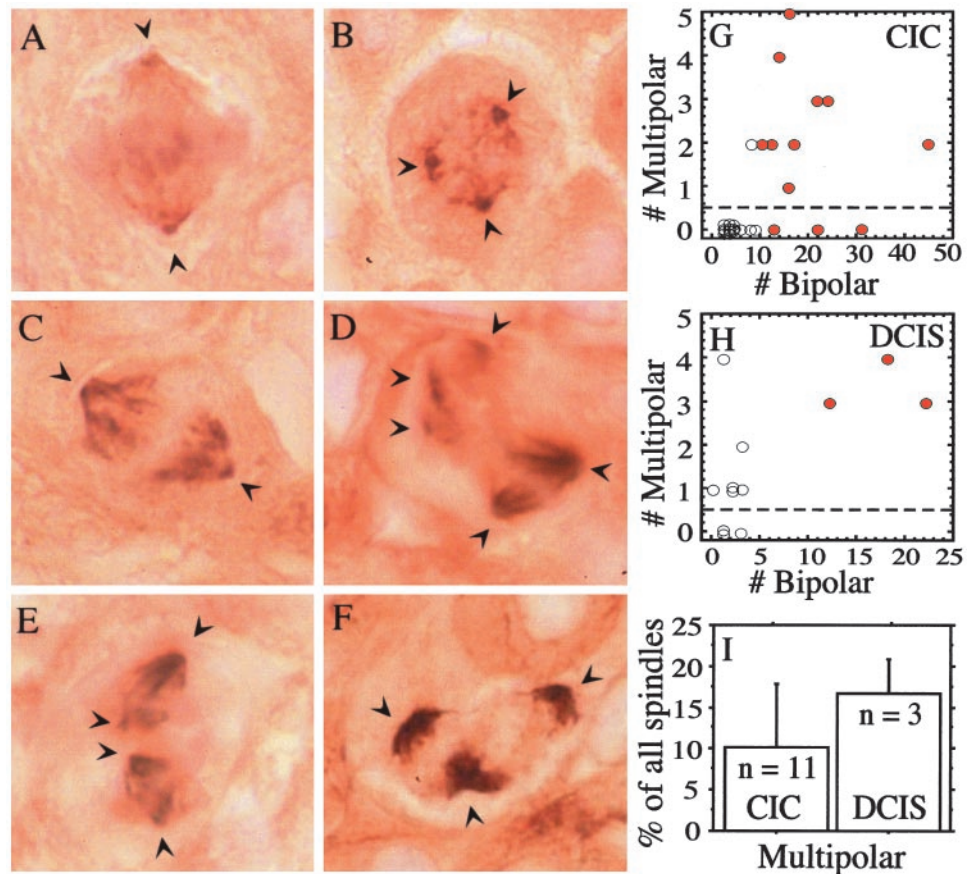


Fig. 3. The incidence of centrosome defects increases with increasing histological grade. The cumulative incidence of centrosome defects in each pre-invasive lesion (left column) includes grades 1–3 for CIC (A, I–3) and low (L) and high (H) grades for DCIS (E) and PIN (I). N, normal epithelium. Each subcategory of centrosome defects increases with grade, including increased centrosome number (B, F, and J), shape abnormalities (C, G, and K), and size (D, H, and L).

**The Incidence of Centrosome Defects Increases with Higher Histological Grade of *in situ* Carcinomas.** *In situ* carcinomas of different histological/cytologic grade differ in their associated risk of progression to invasive carcinoma (36, 38, 39). We observed a higher incidence of centrosome defects in the higher grades of all three precancerous lesions (Fig. 3). The increase in centrosome abnormalities was greater in the lesions associated with a higher propensity to evolve into invasive carcinoma and is consistent with a model where centrosomes contribute to the cytologic and genetic changes that occur during progression of precancerous lesions.

**Mitotic Spindle Abnormalities Are Frequent in Carcinoma *in Situ*.** One expected consequence of supernumerary centrosomes in mitotic cells was the development of multipolar mitotic spindles (2, 27). To identify abnormal spindles, we stained sections with  $\gamma$ -tubulin, which provided the best marker for spindle poles in our immunohistochemical procedure (Fig. 4; see “Materials and Methods”). The total number of mitotic figures was generally low. The percentage of samples that contained spindles was 74 (29 of 39, CIC), 35 (12 of 34, DCIS), and 0% (0 of 42, PIN). The low incidence of spindles in PIN lesions is likely the result of delayed fixation of these tissues and the relatively slow growth of prostate tumor cells compared with the other *in situ* lesions. Of the tumors with spindles, 75% (9 of 12) of DCIS and 34% (10 of 29) of CIC had at least one abnormal spindle (Fig. 4, H and G). Defective spindles included multipolar spindles (three or more poles; Fig. 4, B, D, and F), multiple bipolar spindles in single cells (Fig. 4E), and asymmetric bipolar and multipolar spindles (Fig. 4, D and F). In lesions with  $\geq 10$  spindles, a number chosen to avoid the inherent bias introduced in data by low spindle counts, the average number of multipolar spindles was 10–16% (Fig. 4J). Monopolar spindles were also detected, but they could not be authenticated because of the compounding effect of truncation artifacts induced by tissue sectioning. Mitotic figures were infrequently observed in normal epithelium adjacent to *in situ* lesions, a likely consequence of the low mitotic rate of these tissues. When present, these spindles were normal (symmetric, bipolar,  $n = 4$ , data not shown). The

Fig. 4. Mitotic spindle defects are common in CIC and DCIS. Examples of bipolar mitotic spindles immunostained with  $\gamma$ -tubulin in CIC and DCIS (A and C, respectively). Examples of multipolar spindles (B, C, D, F, and DCIS) and multiple spindles (E and DCIS). Quantitative analysis of the number of bipolar (X axis) and multipolar (Y axis) spindles in each CIC (G) and DCIS lesion (H). Each circle represents a single lesion. Filled circles represent lesions with  $\geq 10$  mitoses and were included in the estimation of the extent of mitotic spindle defects in CIC and DCIS. On average, 10 and 17% of the spindles, in CIC and DCIS lesions with  $>10$  immunostained spindles (red circles in G and H), are abnormal.



absence of spindle abnormalities in normal tissues was consistent with our previous results in nontumor tissues (5, 20) and was confirmed in another epithelial tissue with a high proliferative rate. In samples of celiac sprue, a form of intestinal malabsorption in which the intestinal epithelium has increased mitotic activity caused by increased rates of mucosal regeneration, we never observed abnormal mitoses ( $n = 45$ ). Taken together, these data indicated that spindle defects were specific for *in situ* lesions.

**Centrosome Defects Correlate with CIN in Precancerous Lesions.** Both chromosome instability (2, 6, 16) and centrosome defects are common features of epithelial cancers (11, 16, 21, 31). We have demonstrated previously a correlation between the extent of centrosome defects and CIN in invasive prostate cancer (2, 21). To determine whether a correlation exists between centrosome defects and CIN in carcinoma *in situ*, we examined consecutive serial tissue sections for these anomalies (2, 21). Although CIN was observed in many *in situ* lesions, it was never seen in normal epithelium in the same tissue section (Fig. 5, A, C, and E). Moreover, in all three *in situ* carcinomas, there was a statistically significant nonrandom association (Fisher's exact test,  $P < 0.005$ ) between centrosome defects and CIN (Fig. 5, G-I). In fact, most lesions with centrosome defects showed CIN (63–71%; Fig. 5). Conversely, the fraction of cases that lacked centrosome defects lacked CIN (81–95%; Fig. 5). The correlation between centrosome defects and CIN was significant despite the vastly different degrees of centrosome defects between DCIS, CIC, and PIN (Fig. 2). Interestingly, there were more lesions that had centrosome defects and lacked CIN (~30%) than lesions with CIN that lacked centrosome defects (~10–20%), consistent with a model where centrosome defects precede CIN in the progression of the tumor-like phenotype in precancerous lesions (2, 9). Comparison of the fraction of cells with one chromosome signal (hypoploids) across

all three precancers showed that carcinoma *in situ* invariably contained fewer cells with one chromosome signal than control tissues (DCIS,  $28.9 \pm 12$  versus control,  $38.1 \pm 10$ ,  $P = 0.023$ ; CIC,  $31.2 \pm 12$  versus control  $41.3 \pm 9$ ,  $P = 0.0023$ ; and PIN,  $24.6 \pm 13$  versus control  $31.2 \pm 10$ ,  $P = 0.03$ ). In conclusion, *in situ* lesions have a lower frequency of single chromosome copy number and a higher frequency of multiple chromosome copy number, suggesting that cells in these early lesions are mostly polyploid and almost never hypoploid.

## DISCUSSION

Our results demonstrate that centrosome defects are present in a significant fraction of *in situ* carcinomas of the breast, cervix, and prostate. These results extend our previous observations that centrosome defects are present in low-grade tumors and increase in more aggressive carcinomas (21). They also expand on other studies showing centrosome defects in a limited number of *in situ* lesions from human breast and rat tissues (20, 29). Because p53 mutations are not universal in these pre-invasive lesions (see below), we conclude that abrogation of p53 function is not a prerequisite for the development of centrosome defects early in tumor development. These observations are consistent with a role for centrosome defects in the establishment of carcinoma and perhaps the progression of early lesions to more aggressive cancers.

Centrosome defects occur frequently in advanced forms of some of the most common human cancers and may contribute to genetic instability by impairing the fidelity of chromosome segregation during mitosis (1, 3, 8, 9, 11, 16). It is currently held that carcinoma *in situ* is the immediate precursor of invasive epithelial cancers and that it shares some but not all genotypic and phenotypic characteristics of

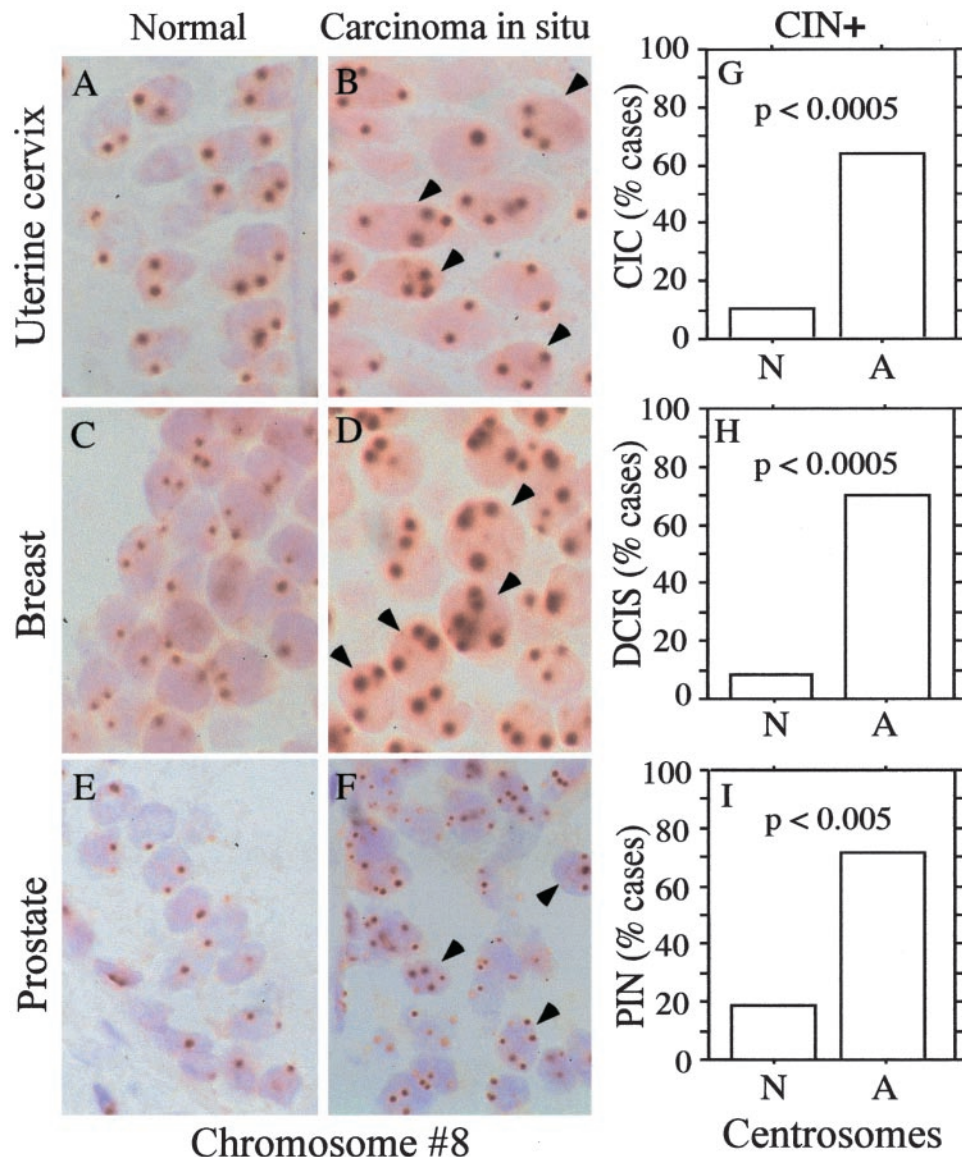


Fig. 5. Centrosome abnormalities correlate with chromosome instability in carcinoma *in situ*. Examples of *in situ* hybridization reactions performed on samples of CIC (B), DCIS (D), and PIN (F) are shown. Many cells have more than two signals for chromosome #8 (arrowheads in B, D, and F) and thus exhibit chromosome instability (CIN+). Cells in adjacent normal epithelium (A, C, and E) rarely have more than two signals. Quantitative analysis of CIN+ in CIC (G), DCIS (H), and PIN (I) lesions with normal centrosomes (N) or abnormal centrosomes (A). CIN is present in most lesions with abnormal centrosomes and a small fraction of lesions lacking centrosome abnormalities.

invasive cancer (38, 40, 41). Our results show that centrosome defects are present at the earliest morphologically recognizable stages of tumor development in some of the most common human cancers. They provide a mechanistic explanation for the commonly observed CIN and aneuploidy observed in most lesions found in human carcinoma *in situ* and experimental models of carcinogenesis (33, 42–45). These data are consistent with a role for centrosome defects in the generation of genetic instability during the early stages of the tumorigenic process.

Our study also demonstrates that centrosome defects correlate with the histological/cytologic grade of the *in situ* lesion and thus support a role for the centrosome in the induction of the morphological phenotype characteristic of carcinoma *in situ*. Centrosomes have been shown to play a role in cell polarity (46), shape (47, 48), and motility (47), all of which are perturbed in all *in situ* cancers examined in this study. Moreover, the presence of mitotic spindle defects in many CIC and DCIS lesions and the cosegregation of centrosome abnormalities with CIN strongly suggest that centrosome defects have a functional impact in *in situ* carcinoma.

Our results are consistent with a role for centrosome defects in the development of aggressive tumors, rather than those that remain

benign. This idea is supported by the high prevalence of centrosome abnormalities in lesions with a high rate of progression to high-grade cancer (DCIS and CIC) and the low prevalence of centrosome defects in PIN lesions, the majority of which progresses to low-grade invasive cancers. Because DCIS and CIC are usually indistinguishable cytologically from aggressive cancers (36, 49), it is believed that they give rise to these aggressive cancers. In contrast, cancers of the prostate are usually low grade (50), consistent with the low-grade appearance of most PIN lesions. These results support our centrosome-mediated model of tumor genesis (2), where centrosome defects induce dramatic and persistent changes in chromosome number (CIN), thereby shuffling the genome and allowing selection of the most aggressive phenotypes, such as those seen in invasive cancers.

The presence of centrosome abnormalities at the earliest stages of disease may also have the potential to predict evolution of *in situ* lesions into high-grade invasive cancers. This is of particular interest for the management of prostate cancer, because the majority of these tumors are biologically low grade but with time may progress to aggressive form. Currently, these cancers are often treated by prostatectomy, because there is no effective prognostic indicator of aggressive disease. If centrosome abnormalities can predict development to

high-grade cancer, they would provide a sorely needed surrogate marker for aggressive disease. We are currently testing if centrosome defects correlate with aggressive prostate cancer by examining PIN lesions from patients that subsequently progress to invasive cancer. On the basis of our previous work showing that the incidence of centrosome defects are higher in more aggressive tumors (21), we are hopeful that their incidence will also be higher in precancerous lesions that subsequently progress to aggressive tumors.

An interesting observation made in this study was the presence of low, yet measurable levels of centrosome defects in morphologically normal epithelium adjacent to CIC lesions (Fig. 2A). We speculate that this may be attributable to the presence of human papilloma virus infection. It is well established that papilloma virus is the cause of nearly all carcinomas of the cervix and present in all precursor lesions (51). Moreover, it has been demonstrated recently that papilloma virus can rapidly induce centrosome abnormalities in squamous epithelial cells *in vitro* (52).

Our observations also suggest a mechanism for centrosome-mediated generation of genetic instability in carcinoma *in situ*. The excellent correlation between centrosome defects, aberrant spindles, and CIN indicates that abnormal centrosomes contribute to spindle disorganization, chromosome missegregation, and genetic instability in these lesions. These data also suggest that supernumerary centrosomes lead to multipolar spindles and do not merely coalesce to form bipolar spindles as it has been suggested from work in cell lines (8, 53).

Although our study answers the important question of whether centrosome defects occur in pre-invasive cancers, it also leaves a number of interesting issues unanswered. One of the most important issues is whether centrosome defects are a cause or consequence of the *in situ* carcinoma phenotype. This is an issue of overriding importance in that the identification of the mechanism by which centrosome abnormalities arise may lead to both predictive testing and cancer-specific therapeutic interventions. There are many ways in which centrosome defects can arise. These include changes in proteins involved in cell cycle control, centrosome structure or function, and DNA repair, *e.g.*, mutation or elimination of p53 (54–56) or p53 downstream effectors/regulators, such as Mdm2 (57), p21<sup>Waf/Cip1</sup> (54, 57, 58), and GADD45 (44, 59), induces centrosome abnormalities. Abrogation of postmitotic p53-dependent checkpoints may be critical in allowing tetraploid cells with supernumerary centrosomes to continue to cycle (60–64). Similarly, alteration in the levels of centrosome-associated proteins, such as pericentrin (21, 27),  $\gamma$ -tubulin (65), aurora (24, 25, 28), polo (66), TACC (67), and RanBP (68), leads to abnormal centrosomes. Moreover, mutation or functional abrogation of proteins involved in DNA repair, such as Xrcc3 (69), Xrcc2 (69), BRCA1 (70, 71), BRCA2 (70, 72, 73), Mre11 (74), DNA polymerase  $\beta$  (75), or genome damage-signaling proteins such as ATR (76), can also lead to centrosome abnormalities. Lastly, centrosome abnormalities can arise by mutation of the *adenomatous polyposis coli* gene whose product interacts with microtubules (77), by cytokinesis failure (24), and by ectopic assembly of centrosome components into acentriolar microtubule-organizing centers (9, 21, 27).

We do not know which of these mechanisms is responsible for inducing centrosome defects in carcinoma *in situ* or if another as yet unidentified mechanism/pathway is involved. We believe it is unlikely that p53 mutations can account for our findings: (a) p53 mutations are not common in DCIS (78) and PIN (79); (b) centrosome defects in carcinoma *in situ* are not only numerical but also structural; (c) human somatic cells rendered p53<sup>-/-</sup> by targeted homologous recombination do not develop CIN or centrosome abnormalities unless challenged (80); (d) overexpression of endogenous p53, which correlates highly with mutated p53, occurred in <20% of CIC and PIN lesions (data not shown); and (e) supernumerary centrosomes in p53<sup>-/-</sup> or

p53 mutant cells (54, 55) may be secondary to the combined effects of cytokinesis failure and abrogation of postmitotic checkpoints, thus allowing polyploid cells to reenter the cell cycle and undergo mitosis with supernumerary centrosomes (24). Under these conditions, cells with supernumerary centrosomes have the potential to perpetuate chromosome instability by missegregating chromosomes through multipolar mitoses.

In conclusion, we have shown that centrosome defects are present in a significant percentage of pre-invasive carcinomas and that they occur together with mitotic spindle defects and chromosome instability. We propose that centrosomes may contribute directly to chromosome missegregation and genetic instability and, through this process, accelerate the accumulation of genes with oncogenic mutations and loss of genes encoding tumor suppressors, as characteristically observed in human carcinoma.

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