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WAF1/Cip1 Gene Polymorphism and Expression in Carcinomas of the Breast, Ovary, and Endometrium

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The p53 gene is altered in approximately 50% of human cancers and is considered to be important in the pathogenesis of these malignancies. The p53 protein product regulates the transition from G1 to S phase of the cell cycle and entry to the DNA damage repair pathway. As alterations in this pathway appear to be important in a variety of human cancers, downstream effector proteins of p53 are potential sites for somatic alterations. WAF1/Cip1, also known as WAF1, Cip1, sdi1, or CAP20, codes for a 21-kd protein ($p21^{WAF1/Cip1}$), which was recently described as a universal inhibitor of cyclins and is thus critical in cell cycle control. Mutations in WAF1/Cip1 are potentially important in human malignancies because they could affect the control of the cell cycle. To understand whether mutations of WAF1/Cip1 occur in cancer, we screened 53 cases of invasive breast carcinoma, 35 cases of ductal carcinoma in situ (DCIS), 53 ovarian carcinomas, and 47 endometrial carcinomas in the second exon of WAF1/Cip1 (90% of the open reading frame). $p21^{WAF1/Cip1}$ expression was characterized with immunohistochemistry. Cells from the blood of 21 normal individuals were also characterized using single-strand conformational polymorphism analysis, DNA sequencing and restriction analysis. Single-strand conformational polymorphism analysis demonstrated an altered mobility pattern for exon 2 in 12 invasive breast cancers (22.6%), 5 DCIS of the breast (14%), 8 invasive ovarian carcinomas (15%), and 9 endometrial carcinomas (19%). In total, 209 samples were screened, and 38 cases (18.2%) had an altered codon 31. Each case with altered single-strand conformational polymorphism, analyzed by DNA sequencing and/or restriction analysis, showed the same alteration of codon 31, a C to A transversion encoding a change in amino acid sequence from serine to arginine (31Ser → 31Arg). DNA from the blood of 21 normal individuals showed the same alteration in WAF1/Cip1 in 4 cases (19%). Furthermore, paired normal tissue was available for 3 of 20 breast carcinomas with the Ser31Arg transversion. Normal DNA from all 3 cases showed the same 31Arg alteration as found in the tumor tissue. These results indicate that codon 31 is a polymorphic site and that the serine to arginine shift is a polymorphism. $p21^{WAF1/Cip1}$ expression, identified by immunohistochemistry, was found to vary in a pattern that depended both on the tissue type and on the presence or absence of the codon 31 polymorphism. Using pair-wise comparisons in breast DCIS, we found higher protein expression in tumor nuclei as compared with benign stromal cell nuclei (P = 0.002) or normal ductal epithelium (P = 0.005).

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Invasive breast cancer specimens showed a trend in p21$^{WAF1/Cip1}$ immunostaining similar to DCIS but did not reach statistical significance (P = 0.12). However, when cases with extensive desmoplastic reaction were excluded, a statistically significant association (P = 0.019) similar to that in DCIS was noted. In contrast to the breast tumors, ovarian carcinomas exhibited significantly greater p21$^{WAF1/Cip1}$ expression in the benign stromal (fibroblast) nuclei surrounding the tumor than in the carcinoma cell nuclei (P = 0.016). Endometrial carcinoma revealed no difference in staining when comparing benign tissue with carcinoma (P = 0.99); however, unlike breast and ovarian carcinomas in which there was no correlation between p21$^{WAF1/Cip1}$ expression and the presence or absence of the alteration at the 31st codon, endometrial carcinomas showed an increased percentage of immunopositive nuclei associated (P = 0.056) with 31Arg. These results demonstrate tissue-specific expression patterns of WAF1/Cip1 in different tumors which appears to be characteristic of the tumor type. (Am J Pathol 1997, 150:167–175)

The *p53* tumor suppressor gene product is a transcription factor that is expressed after DNA damage and induces transcription of downstream genes to cause either G1 arrest or apoptosis. The genes involved in the DNA damage response pathway are not fully understood; however, one of these genes, referred to as *WAF1/Cip1*, has been isolated and characterized.[1–5] *p53* directly induces p21$^{WAF1/Cip1}$ expression through a *p53* binding element 2.4 kb upstream of the *WAF1/Cip1* open reading frame. Expression of p21$^{WAF1/Cip1}$ and subsequent binding of a cyclin-dependent kinase (CDK), a cyclin, and proliferating cell nuclear antigen (PCNA) forms a quaternary complex, which is able to either activate or inhibit the CDK activity.[6] A single p21$^{WAF1/Cip1}$ molecule stimulates phosphorylation of factors facilitating the transition between G1 and S phase. At a greater concentration, p21$^{WAF1/Cip1}$ appears to inhibit the kinase reaction of the quaternary complex and halt the cell cycle. *p53*-independent p21$^{WAF1/Cip1}$ induction has also been described.[7] Inactivation of p21$^{WAF1/Cip1}$ through alteration could result in unchecked growth and destabilize the normal phenotype. This possibility was investigated in a series of human cancers and selected normal tissues.

Materials and Methods

Tissues

The use of human tissue in this study was reviewed and approved by the University of Southern California Institutional Research Committee. Frozen tissue from eighty-eight cases of breast cancer from the University of Southern California Breast Tumor Bank were used in this study. Samples were stored at −80°C in a Revco freezer. Fifty-three cases were classified as strictly invasive disease, with invasive ductal carcinomas making up 92% and invasive lobular carcinomas accounting for 8% of the total. Thirteen cases had evidence of both invasive and *in situ* carcinoma and twenty-two cases had solely ductal carcinoma *in situ* (DCIS). Among cases with DCIS components, 57% were classified as comedocarcinoma, 19% papillary DCIS, 11% cribriform DCIS, 8% DCIS not otherwise specified, and 5% solid carcinoma *in situ*. Frozen tissue sections stained with hematoxylin and eosin (H&E) were used to assess the histological composition of the specimens. Normal tissue was also obtained from paraffin-embedded tissue blocks of our institutional archives in two of these cases and from frozen tissue in one case.

Frozen tissue from forty-seven cases of endometrial cancer, including endometrioid carcinomas (80%), malignant Müllerian mixed tumors (14%), clear-cell carcinomas (3%), and serous papillary tumors (3%) were used in this study. Fifty-three epithelial ovarian carcinomas were also characterized for *WAF1/Cip1*. The carcinomas included papillary serous carcinomas (57%), endometrioid carcinomas (15%), poorly differentiated adenocarcinomas (14%), clear-cell carcinomas (6%), mucinous carcinomas (4%), and mixed carcinomas (4%). Thirty milliliters of blood from a random sample of twenty-one normal women (twelve cases) and normal men (nine cases) were analyzed.

DNA Isolation and PCR (Polymerase Chain Reaction)

In the cases of breast DCIS, a dissecting microscope was used to separate different tissue components from 10 μm thick, frozen, tissue sections that were then fixed in 95% ethanol and stained with ethyl green. The different components, invasive cancer, DCIS, and benign tissues were separately subjected to DNA extraction. Tissue was digested in a buffer of 10 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K for 24 hours at 50°C. A mixture of

phenol/chloroform/isoamyl alcohol was used to extract the DNA, which was then precipitated with ethanol. DNA was extracted from paraffin-embedded tissue sections (20 μmol/L) in two cases of breast DCIS, which were then deparaffinized with xylene, rehydrated through graded alcohols, and digested with a proteinase K solution (100 mmol/L Tris, 4 mmol/L EDTA, 0.5 mg/ml proteinase K for 24 hours at 58°C).

The 450-bp second exon of *WAF1/Cip1* was selected for analysis because it codes for 90% of the protein product, including highly conserved sequences such as the putative zinc finger motif and part of the nuclear localization signal. The following oligonucleotide primers were used: 5′-GGCGCCATCTCAGAACCGGC-3′ and 5′-TGTCATGCTGGTCTGCCGCC-3′.

Single-Strand Conformational Polymorphism (SSCP)

Alterations in the second exon of *WAF1/Cip1* were screened by SSCP technique for altered mobility. SSCP was performed as previously described,[8] with certain modifications.[9] To increase the autoradiographic signal of SSCP, two ^{35}S-labeled nucleotides, dATP and dCTP (Amersham, Arlington Heights, IL) were incorporated with PCR. Separation of the PCR products was performed with mutation detection enhancement gels (AT Biochem, Malvern, PA) according to the manufacturer's specifications. These gels are reported to offer improved separation of different conformations by SSCP.

DNA Sequencing

DNA sequencing was performed using the Circum-Vent thermal cycle sequencing kit (New England Biolabs, Beverly, MA), according to the manufacturer's instructions. All cases with altered mobility by SSCP were reamplified from the original genomic DNA and sequenced. The sequences were confirmed by a second round of PCR amplification and DNA sequencing to rule out the possibility of PCR-generated artifacts.

Restriction Analysis

Restriction analysis was also performed to confirm the sequence alterations. Two restriction enzymes, *Blp*I and *Bsm*BI (New England Biolabs), distinguish the normal sequence from an altered sequence at codon 31. *Blp*I recognizes the normal 5′-GCTNAGC-3′ site and *Bsm*BI recognizes the sequence 5′-GCAGAGN-3′. Samples (approximately 200 ng) of PCR-amplified DNA was digested separately with each enzyme.

Immunohistochemistry

The peroxidase anti-peroxidase technique[10] was used to identify the p21$^{WAF1/Cip1}$ protein product in tissue sections. Frozen sections, 4 μm thick, were cut and fixed in picric acid/paraformaldehyde for 15 minutes. Initially, a mouse monoclonal antibody (EA10; Oncogene Science, Uniondale, NY) and a rabbit polyclonal antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, CA.) were used for immunostaining of the *WAF1/Cip1* protein. Preliminary results of C-19 immunostaining showed background cytoplasmic, membrane, and extracellular stromal staining. Thereafter, only EA10 was used for immunostaining of all of the carcinomas in this report. One hundred nuclei were counted in each sample in random high-power fields. Nuclear immunostaining was considered positive. The positively immunostained percentage was derived from the number of nuclei containing immunoreaction product divided by the total number of nuclei, both stained and unstained. The primary antibody (EA10) was diluted to 5 μg/ml in 10% normal rabbit serum. The secondary antibody was diluted to 20 μg/ml in 10% normal rabbit serum. All cases were evaluated for the presence or absence of p21$^{WAF1/Cip1}$ immunostaining.

Statistical Analyses

Fisher's exact test was used to compare the differences in p21$^{WAF1/Cip1}$ staining in cases with and without the codon 31 alteration. Paired *t*-test was used to compare the difference in percent of p21$^{WAF1/Cip1}$ stained cells between tumor and benign populations. All *P* values reported are two sided.

Results

An alteration in codon 31 of *WAF1/Cip1*, consisting of a C→A transversion that codes for a 31Ser→31Arg shift, was observed in approximately 18% of all samples analyzed by SSCP and DNA sequencing or restriction endonuclease digestion. Seventeen of the eighty-eight (19%) breast carcinomas had altered mobility by SSCP with a similar pattern of migration in all cases (Figure 1). Twelve of fifty-three invasive breast cancers (22.6%) and five of thirty-five DCIS

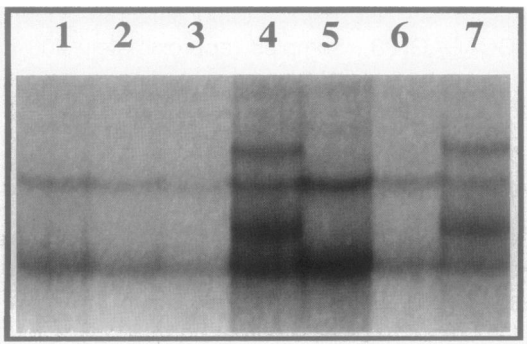

Figure 1. *SSCP analysis of exon 2 from* WAF1/Cip1 *in endometrial carcinomas.* **Lanes 1** *to* **3, 5,** *and* **6,** *typical two-band conformation, representing the two complementary strands of DNA;* **lanes 4** *and* **7,** *typical pattern observed with four DNA bands, two migrating the same as normal DNA and two migrating more slowly.*

cases (14%) were identified as altered by SSCP. Eleven of the twelve invasive breast cancers were ductal carcinomas and one was a lobular carcinoma. Among the DCIS cases, at least one case of each histological type showed altered SSCP mobility. Similarly, eight of fifty-three cases (15%) of ovarian cancer and nine of forty-seven cases (19%) of endometrial carcinomas had altered WAF1/Cip1 by SSCP (Figure 1). All cases, characterized by sequence and/or restriction analysis, were heterozygous with the exception of two cases of invasive breast cancer that were homozygous for the alteration (Figure 2). Five cases of breast cancer, not altered by SSCP, were confirmed as having a normal DNA sequence. There was no association between endometrial carcinoma or ovarian carcinoma histological type and the *WAF1/Cip1* alteration.

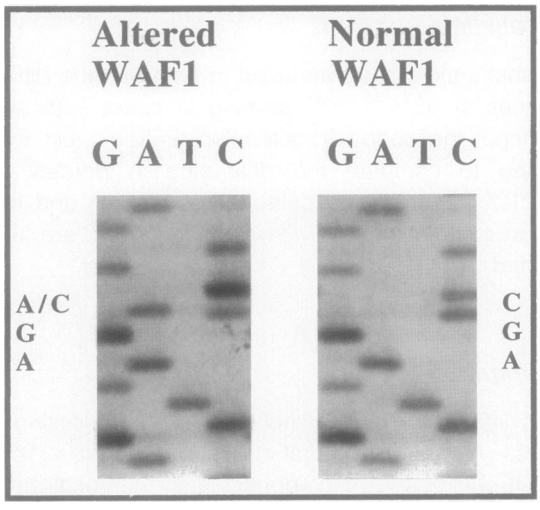

Figure 2. *DNA sequence analysis of breast carcinomas* in situ. *The normal* WAF1 *gene contains an AGC codon 31, whereas the altered* WAF1 *gene contains an AGA codon 31. The presence of both A and C nucleotides at the third position of codon 31 in the altered* WAF1 *is consistent with either contamination by normal stromal components or heterozygosity at this site. As stromal cells were not present within the duct lumen and DCIS cells were microdissected from the duct lumen, the case shows heterozygosity at this site.*

The consistent nature of the alteration, observed at only a single codon, was suggestive of a polymorphism. To confirm this possibility, normal tissue of three breast cancers with altered *WAF1/Cip1* were sequenced and the 31Ser→31Arg transversion was observed in all three samples. In addition, SSCP and restriction analysis was performed on the DNA from peripheral blood cells of normal individuals. Four of twenty-one samples (19%) had an altered codon 31 in the *WAF1/Cip1* gene.

Expression of $p21^{WAF1/Cip1}$ was characterized in these tissues using immunohistochemistry. One hundred nuclei were counted in tumor cells and benign stromal elements in invasive breast cancer, ovarian, and endometrial carcinomas. In breast DCIS, one hundred nuclei from the DCIS component, from normal ductal epithelium, and from benign stroma were scored. $p21^{WAF1/Cip1}$ immunostaining was identified exclusively in the nucleus of both tumor cells and normal cells. Comparisons were made between the percentages of immunostained nuclei in carcinomatous and benign cells in a pair-wise fashion for each case.

A substantial proportion of breast, ovarian, and endometrial cancer specimens showed immunostaining for $p21^{WAF1/Cip1}$ in either the tumor cell nuclei, benign stromal cell nuclei, or both. Among the cases of DCIS, $p21^{WAF1/Cip1}$ was expressed in at least some nuclei in 33 and 35% of samples with normal ducts and benign stromal nuclei, respectively, whereas $p21^{WAF1/Cip1}$ immunostaining was noted in at least some carcinomatous nuclei in 56% of DCIS samples. Among the invasive breast cancer samples, $p21^{WAF1/Cip1}$ expression was observed in at least some stromal cell nuclei in 45% of cases, whereas $p21^{WAF1/Cip1}$ expression was observed in at least some tumor cell nuclei in 83% of invasive breast cancer cases.

In both breast carcinoma *in situ* and invasive breast cancer, the overall percentage of nuclei immunostained was also higher in carcinoma nuclei than the percentage of immunostained benign nuclei. When the percentages of cells immunostained were averaged, there was a difference in staining between tumor and benign cells. On average, in DCIS cases, 9% of carcinomatous nuclei were immunostained for $p21^{WAF1/Cip1}$, whereas only 3% of normal ductal nuclei were immunostained and 4% of benign stromal nuclei were immunostained for $p21^{WAF1/Cip1}$. Samples of invasive breast cancer showed a similar trend. Tumor cells had, on average, 10% of nuclei immunostained whereas the surround-

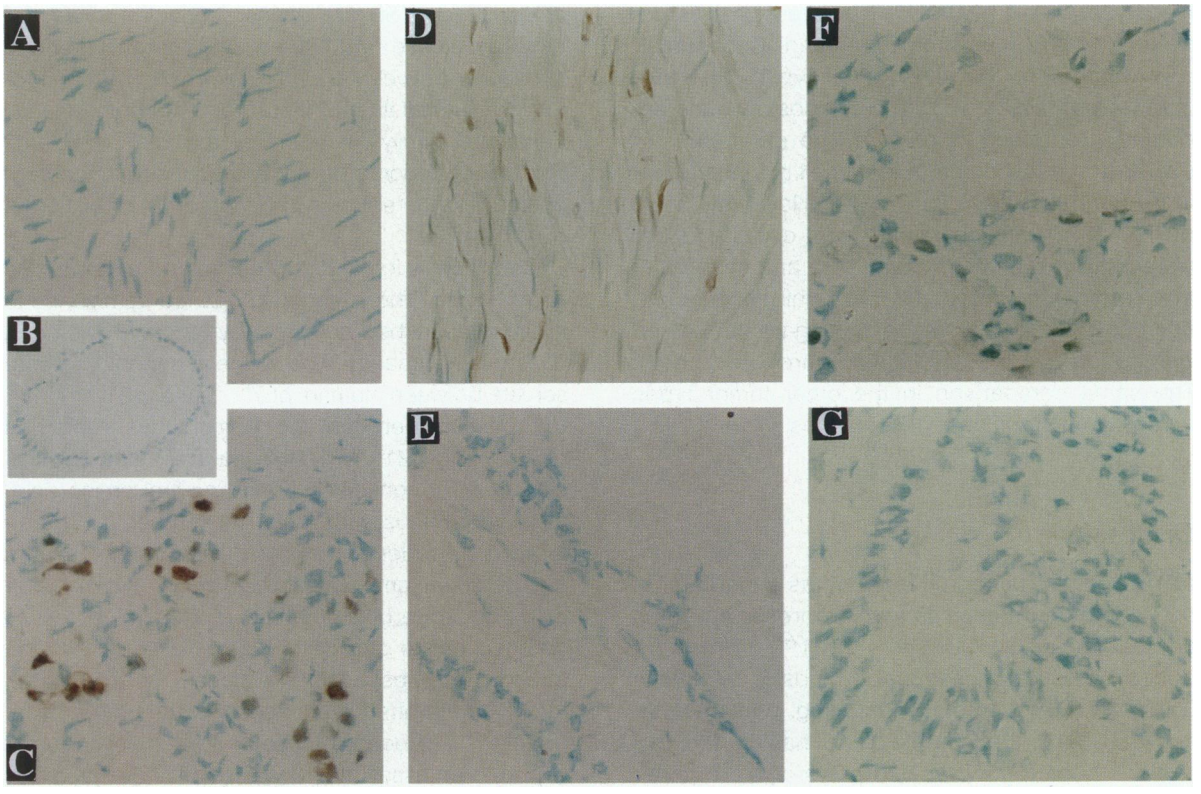

Figure 3. *p21$^{WAF1/Cip1}$ immunostaining in breast DCIS, invasive ovarian carcinoma, and endometrial carcinoma. Frozen tissue sections were immunostained by the peroxidase anti-peroxidase technique with an anti-*p21$^{WAF1/Cip1}$ *antibody, Ab1.* **A** *to* **C**: *Comparison of* p21$^{WAF1/Cip1}$ *immunostaining of benign stroma* (**A**), *benign ductal epithelium* (**B**), *and intraductal breast carcinoma cells* (**C**) *from a case of DCIS with increased immunostaining in the nuclei of tumor cells relative to the nuclei of benign epithelial and stromal cells.* p21$^{WAF1/Cip1}$*-containing nuclei are identified by brown diaminobenzidine immunoprecipitate. Other nuclei are counterstained green by ethyl green histological stain. Magnification, ×1400* (**A** *and* **C**) *and ×460* (**B**). **D** *and* **E**: *Comparison of benign stromal cells* (**D**) *containing* p21$^{WAF1/Cip1}$*-immunopositive nuclei and carcinoma cells* (**E**) *without* p21$^{WAF1/Cip1}$ *immunostaining from an ovarian carcinoma. Tissue counterstained with ethyl green; magnification, ×1400.* **F** *and* **G**: p21$^{WAF1/Cip1}$ *immunostaining of an endometrial carcinoma with codon 31Arg alteration of* WAF1 *showing immunopositive nuclei* (**F**) *compared with a low level of* p21$^{WAF1/Cip1}$ *immunostaining in an endometrial carcinoma with codon 31Ser* (**G**). *Magnification, ×1400.*

ing benign tissue had a lower average staining of 5%. Reactive desmoplasia, identified as broad sheets of dense connective tissue with fibroblasts, was observed in 13 invasive breast carcinomas and contained a high percentage of p21$^{WAF1/Cip1}$-stained nuclei. When cases exhibiting extensive reactive desmoplasia were excluded, tumor cells still had, on average, 10% of nuclei positive for p21$^{WAF1/Cip1}$, whereas the benign stromal elements expressed p21$^{WAF1/Cip1}$ in only 2% of cells. Using pair-wise comparisons, a statistically significant difference was observed between p21$^{WAF1/Cip1}$ immunostaining in DCIS nuclei and benign stromal ($P = 0.002$) or epithelial cell ($P = 0.005$) nuclei (Figure 3, A, B, and C). The findings for invasive breast carcinoma showed the same trend but did not achieve statistical significance ($P = 0.12$). When the cases with marked reactive desmoplasia were excluded from statistical analysis, a significant difference in immunostaining was noted between invasive carcinoma and stromal elements ($P = 0.019$), similar to the difference noted in breast DCIS. Extensive reactive fibrosis was found only in invasive breast cancers and not in breast DCIS.

In contrast to the breast cancers, the pattern of staining in ovarian cancer cases showed higher p21$^{WAF1/Cip1}$ immunostaining in the benign tissue as compared with invasive tumor tissue; 80% of the ovarian cancer cases showed p21$^{WAF1/Cip1}$ immunostaining in either the carcinoma cell nuclei, the benign stromal cell nuclei, or both. There was a higher degree of p21$^{WAF1/Cip1}$ expression in benign stromal nuclei compared with tumor nuclei. On average, 13% of benign stromal cells expressed p21$^{WAF1/Cip1}$ whereas only 7% of tumor nuclei immunostained positively for p21$^{WAF1/Cip1}$. A pair-wise comparison of tumor/benign differences demonstrated a higher percentage of benign nuclei than carcinomatous nuclei exhibiting p21$^{WAF1/Cip1}$ immunostaining ($P = 0.016$; Figure 3, D and E). Similar to the pattern of immunostaining in the cases of invasive breast carcinomas with markedly reactive stromal fibrosis, a

higher degree of $p21^{WAF1/Cip1}$ expression was observed in the fibrous stroma surrounding the tumor.

In contrast to the other tumor types, pair-wise comparison of $p21^{WAF1/Cip1}$ immunostaining in endometrial carcinomas revealed no differences in expression between carcinoma cell nuclei and benign stromal nuclei ($P = 0.99$). However, when $p21^{WAF1/Cip1}$ immunostaining was compared between cases with the codon 31Arg and cases with the more common codon 31Ser, a higher percentage of $p21^{WAF1/Cip1}$ immunostaining was observed with codon 31Arg ($P = 0.056$; Figure 3, G and H). The same comparison in the other tumor types revealed no significant associations.

Discussion

$p21^{WAF1/Cip1}$ has been described as a mediator of *p53*-dependent tumor growth suppression and as an inhibitor of G1 CDKs.[11–13] $p21^{WAF1/Cip1}$ is induced by *p53* expression and binds to the cyclin E-CDK2-PCNA complex, inhibiting phosphorylation of Rb by the complex and halting the cell cycle.[14,15] $p21^{WAF1/Cip1}$ also plays a role in other cyclin-CDK-PCNA complexes.[16] Independent of *p53*, $p21^{WAF1/Cip1}$ expression is observed in cells undergoing terminal differentiation in embryonic mice.[17] In adult mouse tissues with or without functional *p53*, $p21^{WAF1/Cip1}$ is expressed in the brain and fully differentiated columnar epithelium of the gut as well as the lungs, heart, and skeletal muscle.[17] Thus, $p21^{WAF1/Cip1}$ appears to function as an inducible growth inhibitor in embryonic differentiation as well as in the adult, and the role of *p53* appears to be important only in the adult at the G1 checkpoint.

Recent work has shown that the $p21^{WAF1/Cip1}$ protein functions both as a positive and negative regulator of the CDKs through its binding in the quaternary complex.[6] A single $p21^{WAF1/Cip1}$ molecule activates the complex, whereas multiple copies of $p21^{WAF1/Cip1}$ inhibit the function of the complex. Zhang[6] speculates that the association of $p21^{WAF1/Cip1}$ with active kinase implies that there is another function for $p21^{WAF1/Cip1}$ in the cell cycle as a CDK assembly factor.

The second exon of *WAF1/Cip1* contains 90% of the open reading frame. It is 75% identical and 79% similar at the amino acid level between mouse and human. This exon contains a putative zinc finger motif between amino acids 13 and 41 as well as a potential nuclear localization signal between amino acids 140 and 163.[3] The sequences between amino acids 13 and 56 are almost perfectly conserved between mouse and human, and there is a strong homology between $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ protein[18,19] as well as $p57^{Kip2}$ protein,[20,21] both recently described family members of the CDK inhibitors. This conservation of the amino acid sequence suggests that this region is important to the function of $p21^{WAF1/Cip1}$.

Codon 31 is in the highly conserved putative zinc finger region. The amino acid change from serine to arginine described here and by others[22,23] is not a conservative change. The serine at codon 31 is conserved between human $p57^{Kip2}$ and $p21^{WAF1/Cip1}$. Comparison between the homologous region in $p27^{Kip1}$ and $p21^{WAF1/Cip1}$ reveal that, although this codon is not conserved, it is a neutral amino acid, threonine, that is substituted for the serine. The alteration at codon 31 exchanges a serine, an uncharged polar amino acid with a single hydroxyl side chain, for an arginine, which is a basic, positively charged amino acid with a seven-membered side chain. These observations suggest that this change may create a phenotypic variant of the $p21^{WAF1/Cip1}$ protein.[24–26] Recently Chedid et al[23] used a tumor suppression assay that revealed no functional difference between the different proteins to show that the codon 31 alteration is a polymorphism. However, their assay used mouse mammary tumor virus promoter-driven constructs to produce the two proteins. This assay would not reproduce *in vivo* protein levels as neither the cells nor the experimental conditions can be considered equivalent to the normal cellular environment.[27]

The observed pattern of $p21^{WAF1/Cip1}$ expression in this study was different in each tumor type. In the two cohorts of breast cancer tissue, there was a similar pattern of $p21^{WAF1/Cip1}$ staining. Both *in situ* and invasive breast carcinoma cells showed an increase in $p21^{WAF1/Cip1}$ staining in pair-wise comparisons of tumor and normal cell immunostaining (Figure 4). Invasive breast carcinomas showed a trend ($P = 0.12$) toward a higher percentage of $p21^{WAF1/Cip1}$ staining in the tumor cells as compared with the normal tissue. Exclusion of cases with reactive desmoplasia revealed a statistically significant increase in staining in the invasive carcinoma as compared with benign stroma ($P = 0.019$). Similarly, breast DCIS showed a statistically significant association between high staining in tumor nuclei and lower expression in normal ductal epithelium ($P = 0.005$) and benign stromal nuclei ($P = 0.002$). The pair-wise comparisons were determined by subtracting the percentage of positive nuclei in benign tissue from the

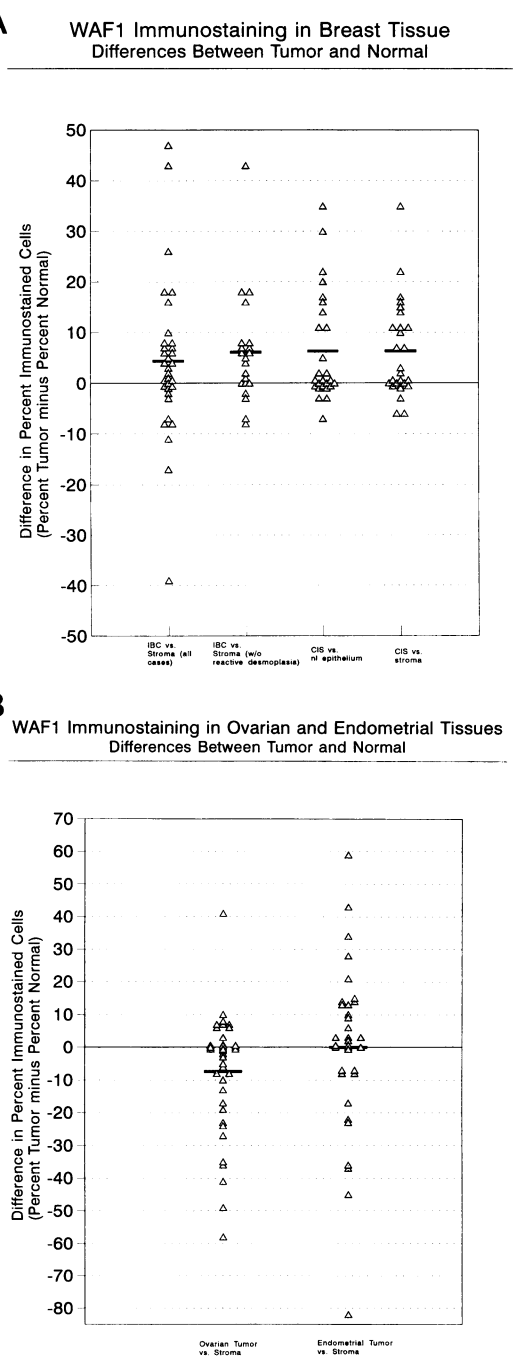

Figure 4. *Pair-wise differences in* $p21^{WAF1/Cip1}$*-immunostained cells of carcinoma nuclei and of benign nuclei on a case-by-case basis. Each data point is the value of the percentage of* $p21^{WAF1/Cip1}$ *immunostaining in benign nuclei subtracted from the percentage of nuclei immunostained in the carcinoma nuclei. The bold line indicates the mean value of data points for each tumor type.* **A:** *Comparison of the values generated in two subgroups of invasive breast cancer cases (IBC) and two subgroups of breast carcinoma* in situ *(CIS). The invasive carcinoma groups show differences between invasive carcinoma cells and benign stroma for either all cases or cases with limited reactive desmoplasia. The breast carcinoma* in situ *differences are for either benign ductal epithelium or for benign stromal cells and DCIS immunostaining percentages.* **B:** *Differences between percentage of* $p21^{WAF1/Cip1}$*-containing nuclei observed in benign stromal cells and carcinomas for both ovarian and endometrial tumors.*

percentage of immunopositive nuclei in tumor cells (Figure 4). Both invasive breast carcinoma and breast DCIS had mean values of the differences that were above zero, indicating greater staining in the tumor nuclei. Ovarian tissue exhibited the opposite result. The mean of the pair-wise differences was less than zero, indicating higher $p21^{WAF1/Cip1}$ immunostaining in the stromal elements. The mean of the pairwise differences in endometrial tissues was −0.05; thus, there was essentially no difference in staining between the endometrial carcinoma and stroma. Interestingly, we noted an increasing percentage of nuclei expressing $p21^{WAF1/Cip1}$ in breast cancer overall. Whereas 33% of samples had $p21^{WAF1/Cip1}$ expression in benign nuclei, 56% of samples had immunostained nuclei in DCIS and 83% of samples showed evidence of $p21^{WAF1/Cip1}$ expression in the nuclei in invasive disease. Conversely, a pair-wise comparison of $p21^{WAF1/Cip1}$ staining in ovarian carcinomas showed the opposite association (P = 0.016) with a higher percentage of benign cell nuclei stained compared with carcinoma cells. We found no significant differences overall between staining in either tumor or normal tissue nuclei in endometrial carcinoma. However, a pair-wise comparison of immunostaining in the presence of codon 31Arg with immunostaining in the presence of codon 31Ser demonstrated increased immunostaining associated with codon 31Arg (P = 0.056). No differences were observed between immunostaining in the presence of 31Arg and immunostaining in the presence of 31Ser in breast and ovarian cancers.

P53 expression was evaluated by immunohistochemistry and *p53* mutations were evaluated by SSCP and DNA sequencing in our laboratory for the endometrial carcinoma cases (B. Saffari, D. Hong, T. Varkey, L. A. Jones, A. El-Naggar, and M. F. Press, unpublished), ovarian carcinoma cases (W.-H. Wen, A. Reles, L. A. Jones, A. El-Naggar, J. Felix, J. Halley, L. Bernstein, I. Runnebaum, and M. F. Press, unpublished), and breast carcinoma *in situ* cases (J. Lukas, N. Niu, and M. F. Press, unpublished). The Mantel-Haenszel test was used to evaluate potential associations between the presence of *p53* mutation and *WAF1/Cip1* alteration, stratifying for the type of tumor. The scatterplots and *F*-test associated with the partial correlation coefficient were used to evaluate the relationship between P53 and $p21^{WAF1/Cip1}$ immunohistochemical results. There was no association between *WAF1/Cip1* alteration and *p53* mutation, nor was there any statistically significant association between $p21^{WAF1/Cip1}$ expression and P53

expression or mutations in breast, ovarian, or endometrial carcinomas. However, an inverse trend was noted in immunostaining in both endometrium and in breast DCIS. In these tissues, scatterplots suggested that high $p21^{WAF1/Cip1}$ immunostaining corresponds with low P53 staining. High P53 immunostaining similarly tended to correlate with low $p21^{WAF1/Cip1}$ staining, but these results were not significant. Thus, in these carcinomas, $p21^{WAF1/Cip1}$ expression did not appear to be solely controlled by p53 expression.

Expression of $p21^{WAF1/Cip1}$ in fibrous stroma of tumors is not expected as part of a DNA-damage repair pathway as the DNA content of the connective tissue is expected to be normal. Inflammatory fibrosis, frequently noted in invasive breast carcinomas, is thought to be in great part caused by release of transforming growth factor (TGF)-β. TGF-β is a multifunctional polypeptide that has an array of cellular effects. It is reported to inhibit growth of normal breast epithelium *in vivo*, but it stimulates fibroblast chemotaxis and fibrogenesis. The effects of TGF-β on cells are variable. TGF-β was very recently found to up-regulate $p21^{WAF1/Cip1}$ expression through a TGF-β response element near the transcription initiation site in *WAF1/Cip1*. This site is physically and functionally separate from the *p53* consensus sequence.[28] It is possible that TGF-β expression mediates the ingrowth of fibroblasts with concurrent up-regulation of $p21^{WAF1/Cip1}$ in the stroma in ovarian carcinomas and in invasive breast cancers.

In conclusion, *WAF1/Cip1* showed a nonconservative serine-to-arginine substitution at codon 31 that was identified in approximately 18% of individuals and is interpreted as a polymorphism. $p21^{WAF1/Cip1}$ expression was variable in different cancers both with regard to expression in carcinoma cells and with regard to expression in benign cells in the tumor. The differences between $p21^{WAF1/Cip1}$ expression in tumor cells and benign stromal cells also varied in a fashion that appeared to be characteristic of particular tumor types.

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