Waf-1 (*p21*) and *p53* Polymorphisms in Breast Cancer

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Abstract

**p53 is a transcription factor for Waf-1/p21, a cyclin-
dependent kinase inhibitor. Certain polymorphic variants
of *Waf-1* and *p53* have been evaluated for their
association with cancer risk. Previous studies indicated
that certain *p53* polymorphisms confer an increased risk
of breast cancer [odds ratios (ORs) and 95% confidence
intervals (CIs) = 2.9, 1.4–6.3 Carcinogenesis (Lond.), *17*:
1313, 1996; 2.5, 1.3–4.8 Cancer Epidemiol. Biomark.
Prev., *6*: 105, 1997; and 1.5, 1.1–2.0, Anticancer Res., *18*:
2095, 1998). The primary objectives of this study were to
test the hypotheses that the serine variant (codon 31
polymorphism) of *Waf-1* is also involved in this process
and that there is an interaction between *Waf-1* and *p53*
polymorphisms. To do this, *Waf-1* and *p53* genotypes
were determined for women enrolled in a breast cancer
case-control study (Caucasians, African-Americans and
Latinas; 487 *Waf-1* and 504 *p53* genotypes were
obtained). Multivariate logistic regression was used to
evaluate possible associations between *Waf-1* and *p53*
polymorphisms, race, and menopause. The primary aim
was to determine whether an interaction between *Waf-1*
and *p53^{1–2-1}* existed. Whereas multivariate analysis
suggested associations between breast cancer and
inheritance of *Waf-1^{ser31}* in African-Americans (OR, 2.32;
95% CI = 0.66–5.60; n = 37 cases and 65 controls) and
Latinas (OR, 2.22; 95% CI = 0.71–6.89; n = 30 cases
and 75 controls), and inheritance of *p53^{1–2-1}* in
Caucasians (OR, 3.15; 95% CI = 1.14–8.89; n = 93
cases and 187 controls), we did not see an interaction
between *Waf-1^{ser31}* and *p53^{1–2-1}*. Consistent with the
finding that *p53^{1–2-1}* is a risk factor for Caucasian women
was the observation of a strong interaction between race
and p53 (P < 0.01).**

Introduction

Cell cycle control is critical for normal growth and differenti-
ation, and its disruption can lead to tumor growth and progres-
sion. Cell cycling is regulated by cdks,[2] catalytic partners of the
cyclins. A variety of cyclin-cdk complexes is formed during
distinct phases of the cell cycle and is necessary for progres-
sion. Waf-1 (p21), is a nonspecific cdk inhibitor (1). Cell cycle
arrest at the G_1-S phase restriction point is mediated through
up-regulation of *Waf-1* by *p53*, and the associated inhibition of
G_1 cyclins-cdk2 complexes. *p53* is a tumor suppressor gene that
maintains homeostasis through *Waf-1*-mediated induction of G_1
arrest or *Bax*-mediated apoptosis (2). In the presence of DNA
damage after exposure to a carcinogen, up-regulation of *p53*
and subsequently *Waf-1* could delay progression past the G_1
restriction point. Mutations in either *p53* or *Waf-1* may lead to
loss of this homeostatic control during human carcinogenesis.
The fact that 50% of all human cancers contain *p53* mutations
highlights its vital role in cell cycle regulation (3).

Polymorphisms in these cell cycle regulation genes have
been reported, and their frequencies are dependent on race
(4–6). At least four polymorphisms have been described for
Waf-1 (4, 5). A nucleotide substitution polymorphism (C/A
transversion) in the third base of codon 31 of *Waf-1* results in
a serine/arginine amino acid substitution. This polymorphism
has been implicated in breast cancer, cervical adenocarcinoma,
and endometrial cancer (7–9). At least 14 polymorphisms have
been confirmed for human *p53*. Five are in exons (codons 21,
36, 47, 72, and 213), and 9 are in introns (intron numbers 1–3,
6, 7, and 9; Ref. 6). There are >120 previous studies that have
sought an association between *p53* polymorphisms and cancer,
and though no clear consensus has been reached, 3 studies
suggest that a haplotype of 3 of these polymorphisms represent
a breast cancer risk factor (6, 10–13).

This study represents a first step to evaluate polymor-
phisms present in a series of genes that constitute a biological
pathway. In this case the pathway is that of cell cycle control
that is closely associated with cellular homeostasis that works
to prevent tissue overgrowth and tumorigenesis. This is analo-
gous to genes that constitute metabolic pathways where inher-
itance of a defective member results in an inborn error in
metabolism leading to common elements of pathology, *e.g.,*
glycogen storage diseases (14).

It is possible that different allelic variants of more than one
gene are more or less effective suppressors of the $G_1 \rightarrow S$
progression. The objective of this study was to test the hypoth-
esis that inheritance of minor allelic variants of either *Waf-1*
or *p53* is associated with increased susceptibility for breast
cancer either independently or together. Earlier studies indi-
cated *p53^{1–2-1}* is a risk factor for breast cancer in Caucasian
women (11, 12). This study is in continuation of the previous
study providing expansion of the *p53* database; it additionally
examines the *Waf-1* polymorphism and seeks a potential inter-
action between *p53* and *Waf-1*.

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[2] The abbreviations used are: cdk, cyclin-dependent kinase; OR, odds ratio; CI,
confidence interval; PCR, polymerase chain reaction; RFLP, restriction fragment
length polymorphism.

Table 1   Association of *Waf-1* polymorphism with breast cancer

Population (*n*)	Genotype			F(S)[b]	HW[c]
	R/R[a]	R/S	S/S		
Caucasian					
Controls (187)	155 (83)[d]	27 (14)	5 (3)	0.10	0.01
Cases (93)	82 (88)	7 (8)	4 (4)	0.08	2×10^{-6}
	$\chi^{2e} = 3.16, P = 0.21$				
African-American					
Controls (65)	36 (55)	23 (35)	6 (9)	0.27	0.42
Cases (37)	13 (35)	20 (54)	4 (11)	0.38	0.36
	$\chi^2 = 4.02, P = 0.13$				
Latinas					
Controls (75)	46 (61)	22 (29)	7 (9)	0.24	0.09
Cases (30)	17 (57)	9 (30)	4 (13)	0.28	0.15
	$\chi^2 = 0.41, P = 0.82$				

[a] R/R = homozygous arginine variant, R/S = heterozygote, S/S = homozygous serine variant.
[b] F(S) = frequency of serine variant.
[c] HW = Hardy-Weinberg equilibrium, *P*.
[d] Numbers in parentheses = percent.
[e] χ^2 difference, df = 2. Comparing control groups (χ^2 difference). Caucasian *vs.* African-American: $\chi^2 = 20.23, P = 0.001$, df = 2. Caucasian *vs.* Latinas $\chi^2 = 14.78$, $P = 0.001$, df = 2. Latinas *vs.* African American: $\chi^2 = 0.61, P = 0.74$, df = 2.

Materials and Methods

Human Samples. A detailed description of the population has been reported previously (11, 15). Breast cancer cases and controls were enrolled from patients presenting at the Mount Sinai Medical Center, New York, NY, between September 1994 and February 1996. Of 1690 eligible women identified, 1101 (65%) agreed to participate; participation rates were comparable regardless of race or diagnosis (15). From these, 175 incident breast cancer cases were matched on age and race with 175 women who had no diagnosis of breast disease and 181 women with a diagnosis of benign breast disease without atypia (women who had a diagnosis of benign breast disease with atypia were excluded). There were 303 Caucasians (102 cases and 201 controls), 117 African-Americans (41 cases and 76 controls), and 111 Latinas (32 cases and 79 controls). Informed consent was administered according to the Institutional Review Board Guidelines, and additional approval was obtained from the National Institute for Occupational Safety and Health, Human Studies Review Board. Ethnicity was self-described, and menopausal status was defined previously. Blood samples (30 ml) were obtained at the time of interview and were used as the source of DNA for genetic analyses.

Determination of *Waf-1* and *p53* Genotypes. The *Waf-1* codon 31 polymorphism was determined by PCR-RFLP according to a method published previously (5). A 100-bp genomic amplicon was generated with *Waf-1*-specific primers (forward 5′ AGA ACC CAT GCG GCA GCA AGG 3′, reverse 5′ TGG ATG CAG CCC GCC ATT AGC 3′; 100 pmol; Life Technologies, Inc., Rockville, MD), in a reaction mixture (50 μl) containing Amplitaq Gold (1 unit; Perkin-Elmer, Foster City, CA), deoxynucleotide triphosphates (100 μM; Promega, Madison, WI), and Tris-HCl/KCl/MgCl$_2$ (100/500/2.5 mM, respectively; Perkin-Elmer). Thermal cycling (35 rounds of denaturation, annealing, and extension) proceeded at 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min with a final extension of 5 min. The PCR products were digested with *Blp*I for 1 h at 37°C according to the manufacturer's instructions, and fragments were separated on a Nusieve agarose gel (4%). DNA fragments were stained with ethidium bromide (0.5 μg/ml) and analyzed using an Eagle Eye II image system (Stratagene, La Jolla, CA). Haplotypes for three *p53* polymorphisms were determined according to a PCR-RFLP method published previously (11).

Statistical Analysis. Exact methods and the χ^2 test were used to compare the gene frequencies of *p53* and *Waf-1* in the control populations of three racial groups, African-American, Latina, and Caucasian. The χ^2 test was also used to determine whether the genotype frequencies reported in our population conformed to Hardy-Weinberg population laws. SAS statistical software was used to conduct all of the statistical analyses (16).

Within racial groups, exact methods and χ^2 tests were used to compare *Waf-1* and *p53* allelic frequencies in breast cancer cases and controls. For the *Waf-1* minor variant, statistical comparisons were made initially by collapsing the heterozygous minor variants into one group (presence of at least one high risk allele) *versus* the presence of the major variant *Waf-1* A1 as the referent group. ORs and 95% CIs were also calculated comparing the homozygous only minor variant (presence of two *Waf-1* A2 alleles) to the presence of only one minor variant and the major variant (presence of only one *Waf-1* A2 or *Waf-1* A1). Similar analysis was conducted for the *p53*[1–2–1] allele.

Logistic regression was used to evaluate the significance of associations between breast cancer and the potential risk factors *Waf-1*, *p53*, race, and menopause. Each main effect term was first tested individually using the likelihood ratio test followed by two-way interactions for each pair of significant main effects. Resulting ORs were then calculated using the multivariable model with all of the significant main effects and interactions.

Results

The frequency of the minor (serine) allele of *Waf-1* in Caucasians was found to be 0.1, consistent with published reports (5, 17), but the allelic frequencies of *Waf-1* by race were found to vary (Table 1). The minor allele frequencies for African-Americans and Latinas were 0.27 and 0.24, respectively. These are significantly different when compared with Caucasians ($\chi^2 = 20.23, P = 0.001$ and $\chi^2 = 14.78, P = 0.001$, respectively). It should be noted that an excess of serine homozygotes was found in both Caucasian controls and in Caucasian cases ($P = 0.01$ and 2×10^{-6}; Table 1, *HW column*).

Table 2   Regression analysis for *Waf-1—p53* interaction for risk of breast cancer

Results of logistic regression model to evaluate potential associations between *Waf-1* and *p53* polymorphisms, race, menopause, and breast cancer risk. The likelihood ratio test was used to test each main effect term individually. Two-interactions for each pair of significant main effects were tested. All significant main effects and interactions were considered in a multivariable model to generate the ORs.

Population (*n*)	Inherited putative "at risk" alleles			
	None	*Waf-1*	*p53*	*Waf-1* + *p53*
Caucasians (280)	1.52 (0.48–3.90)	1.10 (0.35–3.50)	3.15 (1.14–8.89)	2.30 (0.30–17.63)
African-Americans (102)	1.0	2.32 (0.66–5.60)	1.29 (0.54–3.10)	2.99 (0.88–10.3)
Latinas (105)	1.86 (0.63–5.47)	2.22 (0.71–6.89)	0.52 (0.12–2.16)	0.62 (0.12–3.29)

Haplotypes, comprising three biallelic polymorphisms of *p53* were reported previously for 365 women enrolled in this study (11). We expanded this database and added results for an additional 139 women to reevaluate the association between *p53*$^{1-2-1}$ and breast cancer and to test the hypothesis of a gene-gene interaction between the minor *Waf-1* variant and polymorphisms in its transcription factor *p53*. Haplotypes were designated according to the original nomenclature (10). Thus, *p53* haplotypes were generated for 504 of the 531 women included in the age- and race-matched case-control study. When an association between the *p53*$^{1-2-1}$ implicated previously and breast cancer was reexamined in the expanded database no change in overall conclusions was observed. Inheritance of *p53*$^{1-2-1}$ conveyed a breast cancer risk of almost 2 (OR, 1.96; 95% CI = 1.14–3.40).

Logistic regression was then used to examine the question of interactions and significance associations between the potential breast cancer risk factors *Waf-1*, *p53*, race, and menopause. Interestingly, the results indicated that, without simultaneously considering other variables, *Waf-1*, *p53*, race, and menopause status were not significantly associated with risk of breast cancer. The two-way interactions between *p53* and race was statistically significant ($P < 0.01$), whereas that between *Waf-1* and race was not ($P = 0.15$). *p53* was strongly associated with increased odds of breast cancer among Caucasians (OR, 3.15; 95% CI = 1.14–8.89). No association was found between breast cancer and inheritance of *p53*$^{1-2-1}$ in either African-Americans (OR, 1.29; 95% CI = 0.54–3.10) or Latinas (OR, 0.52; 95% CI = 0.12–2.16). *Waf-1* was found to be potentially associated with breast cancer in African-Americans (OR, 2.32; 95% CI = 0.66–5.60) and Latinas (OR, 2.22; 95% CI = 0.71–6.89). Interestingly, *Waf-1* was not associated with breast cancer risk in Caucasians (OR, 1.10; 95% CI = 0.35–3.50). The two-way interaction between *Waf-1* and *p53* showed almost no association with disease, either before or after adjusting for race ($P > 0.9$ and $P = 0.15$, respectively). ORs for different combinations of race, *p53,* and *Waf-1* are given in Table 2. We recognize that a relatively small number of study subjects used to approach this type of analysis could have contributed to the failure to find an association.

Discussion

Waf-1 plays a direct role in mediating *p53*-induced G_1 arrest and *p53* is its transcription factor. Whereas *p53* is mutated in 20–40% of human breast cancers, *Waf-1* is mutated in relatively few (3, 7, 18). Moreover, relative phenotypic expression of *Waf-1* and *p53* appears to be important in breast cancer where tumors expressing both genes were less aggressive than those expressing only one (19). No formal case-control study has yet investigated a potential role for the *Waf-1* codon 31 polymorphism in human breast carcinogenesis, although it has

been studied in oral, esophageal, lung, ovarian, endometrial, and prostate cancers (4, 8, 20, 21).

This study tested the new hypothesis that the minor codon 31 *Waf-1* variant (serine, F = 0.10 Caucasians, 0.27 Latinas, and 0.34 African-Americans) is involved in human breast carcinogenesis. We have also attempted to investigate a potential gene-gene interaction between polymorphisms in the cdk inhibitor *Waf-1* and its transcription factor *p53*. To do this, *Waf-1* and the *p53* genotypes were determined for breast cancer cases (160 and 165, respectively) and controls (327 and 339, respectively) among three ethnic groups (Caucasians, African-Americans, and Latinas).

Earlier studies concluded that the *p53*$^{1-2-1}$ haplotype represents a breast cancer risk factor (10–13). This association was particularly strong for post-menopausal Caucasian women (OR, 2.5; 95% CI = 1.3–4.8; *n* = 365; Ref. 11). In addition, the haplotype frequencies were found to vary among different racial groups. This study has been extended here by increasing the database from 365 to 504, and the data remain consistent with the original findings.

When the age- and race-matched breast cancer case-control population was considered as a whole, *Waf-1* was not found to be associated with breast cancer. Interestingly, however, when each racial group was considered separately, the odds of breast cancer for the inheritance of the minor, codon 31, *Waf-1* allele (serine) was increased in both Latinas and African-Americans but not Caucasians. Although differences seen for *Waf-1* were not significant and are likely attributable to small numbers, when the study group is broken down by race, they are provocative.

For *p53*, the data are consistent with the original findings and those from independent studies (10, 11, 13). Inheritance of the *p53*$^{1-2-1}$ appears to be a breast cancer risk factor for Caucasian women and stronger in postmenopausal breast cancer (OR, 2.67; 95% CI = 1.35–5.28) than premenopausal breast cancer (OR, 1.09; 95% CI = 0.42–2.84). However, these data must be treated with caution, because the sample size is small and the CI for postmenopausal women are inclusive of that for premenopausal women.

Logistic regression was additionally used to seek interactions between the *p53* and *Waf-1* polymorphisms. No gene-gene interaction was found. Taking the implication of the *p53*$^{1-2-1}$ haplotype in Caucasian breast cancer and the trend toward inheritance of the *Waf-1*^{ser31} in Latina and African-American breast cancers together, these data still suggest that this point in the pathway to the G_1-restriction point is critical in breast cancer irrespective of race.

Furthermore, significant variation in *Waf-1* allele frequency between racial groups was observed, where the minor allele frequencies for Caucasians, African-Americans, and Latinas were 0.10, 0.27, and 0.24, respectively. This finding is

consistent with literature reports for the frequency of this *Waf-1* polymorphism (5, 17). Thus, any attempt to base power calculations on the data presented in this report needs to consider each racial group separately.

A previous study has linked the *Waf-1*, codon 31 polymorphism with phenotypic expression of the gene (7). In that study, frozen tissues were obtained from a Caucasian tumor bank, and the minor allele was associated with increased expression in endometrial cancer but not breast or ovarian cancer. However, the *Waf-1* genotypic distribution was similar to that observed here (7).

Studies of some other cancers have noted associations between the *Waf-1*, codon 31 polymorphism and cancer risk. These include prostate and cervical adenocarcinomas, cancers of the head and neck, and some lung cancers (5, 8, 22, 23). Although, *Waf-1* expression may be linked with prognosis in breast cancer and possibly progression in endometrial cancer, the codon 31 polymorphism has not been associated previously with risk of breast or ovarian cancers (7, 8, 19, 22, 23).

For *p53*, there are eight studies of the codon 72 polymorphism in breast cancer; however, none of them make strong claims of any association (6, 10–13). The overall data suggest that allelic variation at codon 72 of *p53*, as such, is not associated with breast cancer risk. In 1986 a group of genetic researchers in Sweden extended this approach beyond allelic classification by a single biallelic polymorphism. They were able to classify alleles by a haplotype consisting of three biallelic polymorphisms. This system of classification allows for eight possible independent alleles. Whereas it can be argued that such a strategy expands the candidate gene base leading to reduced power, it can be countered that increased specificity improves the ability to detect a detrimental allele when the overall lifetime risk associated with a relatively common variant is low. There are four studies that have used this approach, and consistent with the data presented here they suggest that the $p53^{1-2-1}$ allele is associated with increased breast cancer risk in Caucasian women (11–13, 24).

The results reported here are of interest for two reasons. Firstly, our expectation that both polymorphisms (*Waf-1* and *p53*) are intimately related to cancer risk through G_1-restriction leading to an additive or multiplicative effect was not supported. These results clearly demonstrate that the biological pathway is much more complex than we may have thought previously. Secondly, this interaction is made even more complex by the differences we see across racial groups. For this reason, the possibility that these risk factors operate through a common pathway, supported by the data, cannot be precluded. Additional studies with larger, multiracial populations will be necessary to elucidate the underlying mechanism that might tie these *Waf-1* and *p53* alleles to breast cancer risk.

Acknowledgments

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Correction

Polymorphisms in Breast Cancer

In the article on polymorphisms in breast cancer in the January 2002 issue of _Cancer Epidemiology, Biomarkers & Prevention_ (1), the authors mistakenly, but consistently, referred to the serine allele (S) of _Waf-1_ as the minor allele when in fact the minor allele is arginine (R) and the major allele is serine. This error appears in the Abstract (line 11), Introduction (last paragraph), Results (lines 1 and 8), and Discussion (paragraph 4, line 6). In addition, the headings to Table 1 are similarly incorrectly labeled. Because the authors stated hypotheses and analyzed data based on the ''minor'' allele frequencies, this error does not affect the Conclusions.

Reference

1. Keshava C, Frye BL, Wolff MS, et al. _Waf-1_ (_p21_) and _p53_ polymorphisms in breast cancer. Cancer Epidemiol Biomarkers Prev 2002;1:127–30.

BLOOD CANCER DISCOVERY

Waf-1 (*p21*) and *p53* Polymorphisms in Breast Cancer

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