

## A Mitochondrial Target Sequence Polymorphism in Manganese Superoxide Dismutase Predicts Inferior Survival in Breast Cancer Patients Treated with Cyclophosphamide

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**Abstract Purpose:** Manganese superoxide dismutase protects against oxidative damage and modulates the efficacy of chemotherapeutic drugs. A functional single-nucleotide polymorphism in codon 16 of *SOD2* (rs4880), which encodes manganese superoxide dismutase, results in a substitution of valine by alanine (Val16Ala). We hypothesized that this single-nucleotide polymorphism affects breast cancer survival of patients receiving chemotherapy.

**Experimental Design:** Two patient populations from the United States ( $n = 248$ ) and Norway ( $n = 340$ ) were genotyped for Val16Ala. Kaplan-Meier survival and Cox proportional hazards regression analyses were used to examine the relationship between Val16Ala and disease-specific survival.

**Results:** Val16Ala was significantly associated with breast cancer outcome in both patient populations. Carriers of the Ala allele had inferior survival rates in the multivariate analysis [hazard ratio (HR), 2.44 and 95% confidence interval (95% CI), 1.11-5.37 in U.S. cohort; HR, 1.91 and 95% CI, 1.06-3.45 in Norway cohort for Ala/Ala versus Val/Val]. In an analysis of the combined cohorts, this association was significant for patients receiving adjuvant therapy (HR, 2.47; 95% CI, 1.46-4.19), but not for patients without it (HR, 1.47; 95% CI, 0.57-3.74). After further stratification by type of chemotherapy, the effect of the Ala allele was mostly restricted to cyclophosphamide-containing chemotherapy regimens (HR, 22.0; 95% CI, 5.22-92.9; Ala/Ala versus Val/Val).

**Conclusion:** The Val16Ala polymorphism affects survival of patients receiving cyclophosphamide-containing chemotherapy. The findings provide the first evidence pointing toward a mechanism for cyclophosphamide resistance in breast cancer patients.

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Excessive reactive oxygen species (ROS) have been shown to promote tumor development (1-4). Persistent high production of ROS in cancer cells leads to oxidative stress, which increases mutation rates, accelerates tumor progression, and activates cancer-related signal transduction pathways. Subsequent adaptation of these cells to oxidative stress is thought to cause resistance to chemotherapy and radiotherapy (5).

Manganese superoxide dismutase (MnSOD) is a key mitochondrial antioxidant enzyme that protects against ROS and lipid peroxidation. Its main function is the conversion of endogenously produced superoxide into hydrogen peroxide (6). MnSOD also protects against oxidative damage by exogenous factors, such as radiation, and can modify the effects of chemotherapeutics (7, 8). In cell culture and animal models, increased MnSOD activity protects against the toxicity of doxorubicin (9, 10), which is a widely used anticancer drug for treatment of patients with breast cancer and other malignancies.

MnSOD is encoded by the *SOD2* gene. A mitochondrial target sequence polymorphism in *SOD2* (11) results in a substitution of valine (Val) by alanine (Ala). The substitution disrupts the mitochondrial target sequence secondary structure and affects mitochondrial targeting, leading to retention of the Val variant in the mitochondrial membrane whereas the Ala

## Translational Relevance

This study examined the association between a single-nucleotide polymorphism (Val16Ala) in the *SOD2* gene, which encodes the manganese superoxide dismutase, and breast cancer survival in two patient populations from the United States and Norway. The authors found that Val16Ala was significantly associated with breast cancer outcome in both populations. In particular, carriers of the Ala/Ala genotype had inferior survival rates when treated with cyclophosphamide-based therapies. The finding provides the first evidence pointing toward a mechanism for cyclophosphamide resistance in breast cancer patients and may have important clinical implications because 20% to 25% of the general population in the United States and Europe are carrying this genotype. Although preliminary, these data suggest that patients with the Ala/Ala genotype should be considered for alternative treatment.

variant localizes to the mitochondrial matrix (12, 13). This also affects cellular MnSOD activity levels. Cells expressing the Ala variant have been found to have a 30% to 40% higher enzymatic activity than cells expressing the Val variant (13). However, it remains to be shown whether Val16Ala has a dominant effect on MnSOD activity *in vivo* (14). Although results from multiple studies indicate that the Ala variant may confer an increased risk of breast cancer in interaction with the environment (15–20), results from two studies evaluating Val16Ala as a prognostic factor are at variance (21, 22).

Any influence of Val16Ala on breast cancer outcome may result from a therapy effect. Val16Ala may solely predict sensitivity to therapy, which is in contrast to a classic prognostic factor (23). To address this issue, we investigated the effect of Val16Ala in cohorts of breast cancer patients receiving either no chemotherapy or different chemotherapy regimens given as adjuvant or neoadjuvant chemotherapies. With this approach, we found Val16Ala to be predictive of a long-term outcome among patients receiving chemotherapy, but not among untreated individuals. Importantly, the effect of Val16Ala on outcome was largely restricted to patients treated with cyclophosphamide-containing regimens. Our findings provide the first data pointing to a mechanism of resistance to cyclophosphamide in breast cancer patients.

## Materials and Methods

### Study population

This study contained two major patient cohorts. Institutional review board approval was obtained at the participating institutions in the United States and Norway.

**U.S. cohort.** Incident breast cancer cases ( $n = 248$ ) were recruited between February 1993 and August 2003 in the greater Baltimore area, as described previously (24, 25). All patients were identified through surgery lists and enrolled into the study before surgery. None of them participated in a clinical trial. Information to determine the estrogen receptor (ER) status, disease stage, treatment, and survival was obtained from medical records and pathology reports, Social Security Death Index, and National Death Index. Disease staging was done according to the tumor-node-metastasis system of American Joint Committee on

Cancer/Union Internationale Contre le Cancer. Survival was determined for the period from the date of hospital admission to the date of the last completed search for death entries in the Social Security Death Index (February 3, 2006). Median and mean follow-up times for overall survival in this cohort were 58 and 65 mo, respectively.

**Norway cohort.** Norwegian breast cancer patients ( $n = 340$ ) were participants of four previously published studies. Ninety-one patients were recruited at the Haukeland University Hospital between 1991 and 1997 as part of a trial evaluating the effect of adjuvant doxorubicin on locally advanced breast cancer (26). Thirty-six patients were recruited at the same hospital between 1993 and 2001 as part of a trial to evaluate the response to adjuvant 5-fluorouracil (5-FU) and mitomycin in locally advanced breast cancer (27). One hundred and nine patients participated in a larger study of 920 breast cancer cases recruited at several Norwegian hospitals between 1995 and 1998 as part of a trial evaluating the presence of tumor cells in the bone marrow of patients with primarily stage I and stage II breast cancers (28). One hundred and four patients were recruited into a larger study of 212 breast cancer cases at the Ullevål University Hospital between 1987 and 1994 as part of an investigation evaluating the relationship between an abnormal tumor p53 status and tumor p21 protein expression in breast cancer patients (29).

### Genotyping

For the U.S. cohort, Val16Ala (rs4880) was genotyped at National Cancer Institute Genotyping Core Facility, using the Taqman assay conditions described in the SNP500 cancer database (30). We genotyped genomic DNA from fresh-frozen breast tissue (170 non-tumor tissues and 28 tumors) and buffy coat ( $n = 50$ ). The genotype assay contained negative and positive controls, with 10% blinded duplicates. We successfully genotyped 98% of the cases ( $n = 244$ ) and had 100% concordance among blinded duplicates. The Norway cohort was genotyped using the 7900HTFast Real-Time PCR System (Applied Biosystems) at Norwegian Radium Hospital using the standard assay conditions for Applied Biosystems assay ID: C\_8709053\_10. We genotyped genomic DNA from 56 fresh-frozen breast tumor tissue and 284 buffy coat samples. We successfully genotyped 97% of the cases ( $n = 329$ ) and had 100% concordance among blinded duplicates.

### MnSOD activity assay

Human lymphoblast cells were obtained from Coriell and maintained in suspension with RPMI 1640 with 2 mmol/L L-glutamine and 15% fetal bovine serum. Five of the cell lines were homozygous for the Val allele, and the other five were homozygous for Ala allele. The MnSOD activity of these cells was measured as described (31). This assay is based on the reduction of nitroblue tetrazolium to blue formazan by the superoxide radical. In the assay, MnSOD activity is discerned from CuZnSOD activity by the addition of 5 mmol/L sodium cyanide.

### p53 mutational analysis

*TP53* mutations were identified in the U.S. and Norway cohorts, as previously described (24, 26, 27).

### Statistical analysis

Intercooled Stata 9.0 (Stata Corp) statistical software was used for data analysis. All statistical tests were two-sided, and an association was considered statistically significant with a  $P$  value of  $<0.05$ .  $\chi^2$  and Student's  $t$  tests were done to assess differences in the frequency of characteristics between patient subgroups. We did survival analyses to determine breast cancer-specific survival and censored all causes of death that were not related to breast cancer in our analyses. The Kaplan-Meier method and the log-rank test were used for univariate survival analysis. The Cox proportional hazards regression was used for multivariate survival analysis to calculate adjusted hazard ratios (HR) for breast cancer-specific survival. A statistical test for interaction was done in Stata to determine if the effect of Val16Ala on breast cancer

survival is modified by other factors. We tested for interactions in the Cox models containing main effects and an interaction term, assuming a dominant effect of the Ala allele (Ala/Ala and Ala/Val versus Val/Val).

## Results

**Study population characteristics and genotyping.** We studied the relationship between the Val16Ala polymorphism and disease outcome in 248 U.S. and 340 Norwegian breast cancer patients. Four U.S. and 11 Norway patients were excluded because of missing genotype information. Patient characteristics are described in Table 1. The genotype frequency distribution for Val16Ala showed a statistically insignificant variation between U.S. and Norway patients, and the survival rates were similar between the two cohorts. Significant differences existed for tumor size, node involvement, tumor grade, and tumor ER and p53 mutation status. The differences in ER status and tumor grade between U.S. and Norway patients were explained by race/ethnic differences in these cohorts. After adjusting for race/ethnicity, no significant association between cohort and either tumor grade [odds ratio, 1.03; 95% confidence interval (95% CI), 0.71-1.47] or ER status (odds ratio, 1.09; 95% CI, 0.68-1.75) remained. Fifty-seven percent of women from the U.S. cohort were African-Americans. To further examine possible differences by race/ethnicity, we

compared the distribution of the clinical characteristics among Norwegians, European-Americans, and African-Americans (Supplementary Table S1). The Val16Ala genotype distribution in the U.S. patient population did not significantly differ between African-Americans and European-Americans ( $\chi^2$ ;  $P = 0.588$ ). African-American patients were significantly more likely to have ER-negative and high-grade tumors than patients of European descent. Other differences between the Norway and the U.S. cohort were not explained by race/ethnic differences within the U.S. cohort, e.g., tumor size, node involvement, and p53 mutation status, and similar numbers of the Norwegian, European-American, and African-American patients received adjuvant chemotherapy. We also examined the effect of race/ethnicity on survival and compared survival of African-American patients with those of European descent (Supplementary Table S2). Consistent with previous studies (32–34), African-American patients tended to have poorer 5-year and 10-year survival rates than patients of European descent, although the differences were not statistically significant at the  $P$  value of  $<0.05$  level.

**Val16Ala polymorphism and breast cancer-specific survival.** The Val16Ala polymorphism was significantly associated with breast cancer-specific survival in both cohorts and in the combined analysis (Fig. 1). Univariate and multivariate Cox regression survival analysis (Table 2) indicated that the Ala

**Table 1.** Patient characteristics

	U.S. cohort (n = 244)	Norway cohort (n = 329)	P*
<i>Val16Ala</i> genotype			
Val/Val	76 (31%)	75 (23%)	0.060
Val/Ala	121 (50%)	174 (53%)	
Ala/Ala	47 (19%)	80 (24%)	
Tumor parameters †			
Race			
African descent	139 (57%)	—	<0.001
European descent	105 (43%)	329 (100%)	
Age at diagnosis (mean ± SD)	55.0 ± 13.9	62.5 ± 13.8	<0.001
Survival			
Alive	161 (66%)	223 (68%)	0.651
Deceased	83 (34%)	106 (32%)	
Tumor size			
T1	53 (27%)	90 (28%)	<0.001
T2	93 (47%)	95 (30%)	
T3	41 (21%)	78 (24%)	
T4	10 (5%)	56 (18%)	
Node involvement			
No	141 (62%)	136 (45%)	<0.001
Yes	85 (38%)	167 (55%)	
Grade			
1	33 (16%)	41 (13%)	<0.001
2	74 (35%)	177 (56%)	
3	104 (49%)	100 (31%)	
Estrogen receptor status			
Negative	100 (41%)	96 (31%)	0.011
Positive	143 (59%)	216 (69%)	
p53 mutation status			
Negative	198 (81%)	226 (70%)	0.002
Positive	46 (19%)	98 (30%)	
Adjuvant chemotherapy			
No	97 (43%)	98 (27%)	<0.001
Yes	130 (57%)	239 (73%)	

\* $\chi^2$  test and Student's t test (age at diagnosis) were used to compare differences in patient characteristics.

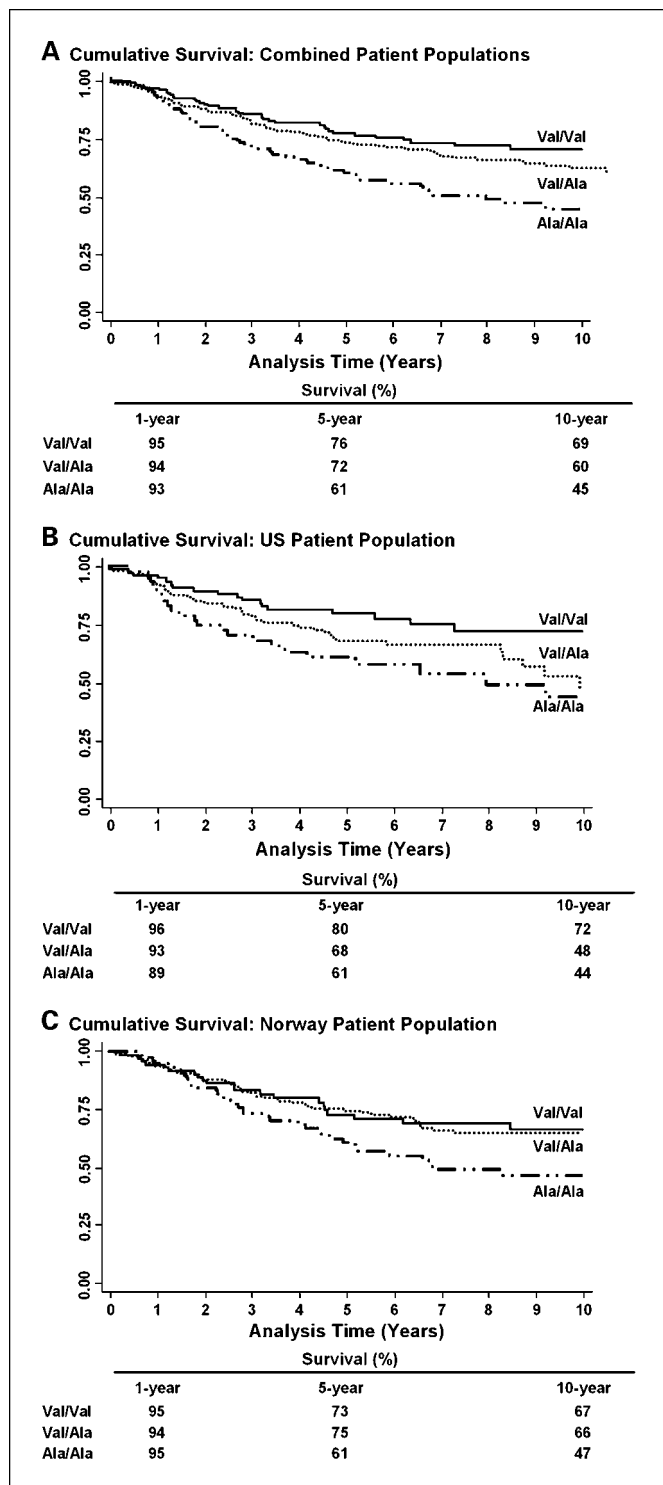
† Cases with missing information are not included.

allele is a predictor of poor outcome. The estimated HRs for the Ala/Ala genotype, when compared with the Val/Val genotype, were remarkably similar between the U.S. and Norwegian cohorts. We also modeled the survival analysis to compare

dominant (Val/Val versus Val/Ala and Ala/Ala), recessive (Val/Val and Val/Ala versus Ala/Ala), and additive effects of Val16Ala. Although the Ala/Ala genotype was associated with decreased survival of patients regardless of which model was used, an additive effect by the Ala allele seemed to be the best model for the data.

**Tumor ER and p53 status influences the association between Val16Ala and breast cancer-specific survival.** Because MnSOD function can be modulated by estrogen and p53 (35, 36), we assessed their effect on the relationship between the Val16Ala polymorphism and breast cancer survival (Table 3). This analysis was done on the combined data set due to sample size constraints. In the univariate and multivariate analyses, the Ala/Ala genotype was significantly associated with poor survival in ER-positive patients when compared with the Val/Val genotype. Similar but statistically insignificant trends existed for ER-negative patients. Stratification by tumor p53 mutational status showed that the effect of the Val16Ala polymorphism on breast cancer survival is strongest among patients without p53 mutations. Because the prevalence of p53 mutations was significantly lower in ER-positive than ER-negative tumors ( $P < 0.001$ ;  $\chi^2$  test), we conducted further analyses and observed that the increased risk of poor survival in ER-positive patients was only significant when tumors were negative for p53 mutation (HR, 3.90; 95% CI, 1.79-8.51; Ala/Ala versus Val/Val;  $n = 119$ ). Patients positive for ER and p53 mutations did not experience an increased risk of poor survival (HR, 0.94; 95% CI, 0.28-3.13;  $n = 25$ ). These findings suggest that Val16Ala could be functionally most important among those patients with ER-positive and p53 wild-type tumors. Because hormone replacement therapy may affect the relationship between Val16Ala and survival in ER-positive patients, we further stratified ER-positive patients into those who had received tamoxifen and those who did not. All ER-positive Norwegian patients and about half of U.S. patients received tamoxifen (Supplementary Table S3). Because of the small number of ER-positive patients who did not receive hormone replacement therapy ( $n = 64$ ), any finding has to be treated with caution, but it appeared from our analysis that Val16Ala may specifically influence survival in ER-positive patients receiving hormone replacement therapy (Supplementary Table S3).

**Val16Ala predicts survival in patients receiving chemotherapy.** To determine whether the effect of Val16Ala on survival is dependent on the type of chemotherapy, we combined the cohorts and stratified breast cancer patients by treatment status and did univariate and multivariate survival analyses within the strata (Table 4). Val16Ala was only significantly associated with disease outcome among patients receiving chemotherapy. In a subanalysis of these patients, we examined the effect of Val16Ala on patient survival, depending on the type of chemotherapy received: doxorubicin-based, 5-FU-based, or cyclophosphamide-based chemotherapy. In all therapy groups, the Ala/Ala genotype significantly increased the risk of poor outcome. Additional modeling of the survival analysis, e.g., using dominant, additive, or recessive models, pointed to a recessive effect of the genotype in patients with doxorubicin-based chemotherapy and an additive effect in those receiving 5-FU-based or cyclophosphamide-based therapy. However, the effect of the genotype was greatest among patients receiving cyclophosphamide-based therapies.



**Fig. 1.** Association between Val16Ala polymorphism and breast cancer survival. Kaplan-Meier survival curves for 10-y survival. *A*, combined analysis of the U.S. and Norwegian cohorts ( $n = 573$ ). Log-rank test,  $P = 0.003$ . *B*, U.S. cohort ( $n = 244$ ). Log-rank test,  $P = 0.038$ . *C*, Norwegian cohort ( $n = 329$ ). Log-rank test:  $P = 0.039$ .

**Table 2.** Association of Val16Ala with 10-y breast cancer survival

	Univariate		Multivariate*	
	HR (95% CI)	P	HR (95% CI)	P
Combined analysis (n = 573)			(n = 465)	
Val/Val	1 (reference)		1 (reference)	
Val/Ala	1.27 (0.87-1.84)	0.215	1.26 (0.83-1.91)	0.274
Ala/Ala	1.96 (1.30-2.94)	0.001	2.19 (1.39-3.34)	0.001
Val/Ala and Ala/Ala	1.47 (1.03-2.09)	0.034	1.52 (1.03-2.25)	0.033
	<i>P</i> <sub>trend</sub>	0.001		
Val/Val and Val/Ala	1 (reference)		1 (reference)	
Ala/Ala	1.67 (1.22-2.27)	0.001	1.91 (1.36-2.67)	<0.001
U.S. cohort (n = 244)			(n = 183)	
Val/Val	1 (reference)		1 (reference)	
Val/Ala	1.62 (0.93-2.81)	0.088	1.58 (0.84-2.96)	0.155
Ala/Ala	2.24 (1.20-4.18)	0.011	2.44 (1.11-5.37)	0.027
Val/Ala and Ala/Ala	1.78 (1.05-3.00)	0.031	1.75 (0.96-3.18)	0.067
	<i>P</i> <sub>trend</sub>	0.011		
Val/Val and Val/Ala	1 (reference)		1 (reference)	
Ala/Ala	1.63 (1.00-2.66)	0.050	1.71 (0.96-3.06)	0.066
Norway cohort (n = 329)			(n = 282)	
Val/Val	1 (reference)		1 (reference)	
Val/Ala	1.04 (0.62-1.73)	0.880	0.88 (0.49-1.56)	0.662
Ala/Ala	1.73 (1.01-2.97)	0.047	1.91 (1.06-3.45)	0.031
Val/Ala and Ala/Ala	1.24 (0.77-2.01)	0.369	1.22 (0.73-2.05)	0.448
	<i>P</i> <sub>trend</sub>	0.033		
Val/Val and Val/Ala	1 (reference)		1 (reference)	
Ala/Ala	1.68 (1.12-2.52)	0.012	1.98 (1.30-3.01)	0.002

\*Cox proportional hazards regression with adjustments for age at diagnosis, cohort, race, tumor size, nodal involvement, tumor grade, estrogen receptor status, and p53 mutation.

Because many patients had received combination therapies, an additional analysis was done to examine the possibility of confounding effects by multiple chemotherapeutics on the relationship between Val16Ala and breast cancer survival (Table 5). For that, four patient groups were identified: (a) doxorubicin monotherapy, (b) cyclophosphamide/doxorubicin or cyclophosphamide/doxorubicin/5-FU combinations, (c) mitomycin/5-FU, or (d) combination of cyclophosphamide/methotrexate/5-FU. The combinations of cyclophosphamide and doxorubicin (b) and of cyclophosphamide, doxorubicin, and 5-FU (c) are also commonly called AC and FAC regimens, respectively, in clinical practice. Multivariate survival analyses revealed that patients receiving doxorubicin monotherapy were at a significantly increased risk of poor survival in the recessive model. Addition of cyclophosphamide to doxorubicin containing chemotherapy regimens (AC and FAC) resulted in a significantly increased risk of poor survival if patients were carriers of the Ala/Ala genotype (unadjusted HR, 6.33; 95% CI, 1.58-25.7; adjusted HR, 139.5; 95% CI, 2.77-7017; Ala/Ala versus Val/Val). In patients who received mitomycin/5-FU chemotherapy, there was no significant association with survival in any model. Patients receiving cyclophosphamide/methotrexate/5-FU had an increased risk of poor survival if they were carriers of the Ala/Ala genotype, similar to AC/FAC patients (unadjusted HR, 6.02; 95% CI, 1.35-26.8; adjusted HR, 24.27; 95% CI, 2.84-207; Ala/Ala versus Val/Val). Together, these results suggest that Val16Ala affects most significantly the disease outcome of patients who received a cyclophosphamide-based regimen.

**Interaction analysis.** We applied a test for interaction and examined interactions between Val16Ala and known risk factors in

breast cancer survival. We did not find an interaction with age at diagnosis, race/ethnicity, tumor size, node involvement, tumor grade, p53 mutation status, or tumor ER and progesterone receptor (PR) status. However, we observed a significant interaction between this genotype and cyclophosphamide-based therapies (AC, FAC, and cyclophosphamide/methotrexate/5-FU) on breast cancer survival ( $P_{\text{interaction}} = 0.023$ ), assuming a dominant effect of the Ala allele (Ala/Ala and Ala/Val versus Val/Val). This observation is consistent with our previous data that the relationship between Val16Ala and breast outcome is due to a specific interaction between this genotype and cyclophosphamide-based therapy.

**MnSOD activity is higher in lymphoblastoid cell lines with the Ala/Ala genotype.** We compared MnSOD activity between five lymphoblastoid cell lines carrying the Val/Val genotype and five lymphoblastoid cell lines carrying the Ala/Ala genotype (Supplementary Fig. S1). We observed an approximate increase of 50% in MnSOD activity in cells with the Ala/Ala genotype (mean  $\pm$  SD,  $34.8 \pm 17.7$  relative units) when compared with the Val/Val genotype ( $23.3 \pm 8.1$ ).

## Discussion

In our study of 248 U.S. and 340 Norwegian women with incident breast cancer, a polymorphism in the SOD2 gene, Val16Ala, was associated with breast cancer survival. This association was restricted to patients receiving chemotherapy and was most significant for those patients who received a cyclophosphamide-containing therapy. Our findings suggest that Val16Ala influences the effect of chemotherapy in breast cancer and modifies disease-specific survival.



Although several studies have reported that Val16Ala is associated with the risk to develop breast and prostate cancer (15–20, 37–40), only few studies examined the effect of this polymorphism on breast cancer outcome. One study found an association between the Val allele and an increased risk of disease progression and breast cancer-specific death among 95 patients with metastatic breast cancer who received high-dose chemotherapy and autologous stem cell support (22). A second study, conducted in 279 breast cancer patients undergoing either adjuvant chemotherapy, radiation, or both, did not find a significant association between Val16Ala and outcome (21). In contrast, we observed a significant association of the Ala/Ala genotype with decreased breast cancer survival in two independent patient populations. Perhaps, differences in therapy and patient population may account for the opposite finding between the first study and ours. However, many patients in the second study were treated with cyclophosphamide, and there is no obvious difference in the patient populations other than that the Arkansas patients were recruited through a tumor registry, which could explain the difference in the findings between the second study and ours. Another possible explanation for the conflicting results may relate to the tumor redox status. It is possible that this status changes with disease progression, which may affect intrinsic

ROS production and the relative effect of the Val16Ala genotype on therapy outcome and overall survival. Thus, future studies in larger data sets are required to further examine the relationships between Val16Ala, patient survival, and response to therapy in breast cancer.

We tested the hypothesis that Val16Ala would affect breast cancer survival when ROS levels are increased, which is seen in patients receiving chemotherapy. Our cell culture studies with human lymphoblastoid cells showed that the Ala allele encodes a higher MnSOD activity, which is consistent with previous studies on the effect of this genotype on MnSOD expression and activity using overexpression of a transgene (12, 13). Alkylating agents, such as cyclophosphamide, and anthracyclines, such as doxorubicin, are examples of chemotherapeutic drugs that generate ROS in cancer cells during treatment (41). Cyclophosphamide generates abundant ROS when metabolically activated in cells, inducing oxidative stress and lipid peroxidation (42–44). The mechanism by which cyclophosphamide increases ROS is not fully understood, and it may involve glutathione depletion and activation of signaling pathways, such as p66Shc. Consistent with cyclophosphamide-induced ROS generation, we detected a significant interaction between Val16Ala and cyclophosphamide in breast cancer survival and found that the Ala/Ala genotype predicts a

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**Table 3.** Effect of ER and p53 mutation on the association of Val16Ala with 10-y breast cancer survival

	Univariate		Multivariate*	
	HR (95% CI)	P	HR (95% CI)	P
ER negative (n = 196)			(n = 166)	
Val/Val	1 (reference)		1 (reference)	
Val/Ala	0.96 (0.54-1.74)	0.907	1.11 (0.56-2.19)	0.760
Ala/Ala	1.76 (0.93-3.32)	0.080	1.64 (0.83-3.26)	0.157
Val/Ala and Ala/Ala	1.17 (0.68-2.04)	0.563	1.24 (0.67-2.29)	0.487
	<i>P</i> <sub>trend</sub>	0.060		
Val/Val and Val/Ala	1 (reference)		1 (reference)	
Ala/Ala	1.84 (1.13-2.87)	0.014	1.76 (1.03-2.99)	0.035
ER positive (n = 359)			(n = 299)	
Val/Val	1 (reference)		1 (reference)	
Val/Ala	1.54 (0.93-2.552)	0.095	1.45 (0.83-2.54)	0.192
Ala/Ala	2.14 (1.24-3.70)	0.007	2.54 (1.35-4.78)	0.004
Val/Ala and Ala/Ala	1.73 (1.07-2.79)	0.024	1.76 (1.04-2.99)	0.035
	<i>P</i> <sub>trend</sub>	0.005		
Val/Val and Val/Ala	1 (reference)		1 (reference)	
Ala/Ala	1.60 (1.07-2.42)	0.024	2.06 (1.32-3.21)	0.001
P53 mutation negative (n = 424)			(n = 340)	
Val/Val	1 (reference)		1 (reference)	
Val/Ala	1.38 (0.87-2.20)	0.171	1.64 (0.98-2.73)	0.060
Ala/Ala	1.98 (1.20-3.26)	0.007	2.58 (1.49-4.45)	0.001
Val/Ala and Ala/Ala	1.58 (1.02-2.44)	0.039	1.90 (1.18-3.53)	0.008
	<i>P</i> <sub>trend</sub>	0.007		
Val/Val and Val/Ala	1 (reference)		1 (reference)	
Ala/Ala	1.60 (1.09-2.35)	0.017	1.89 (1.25-2.85)	0.002
P53 mutation positive (n = 144)			(n = 125)	
Val/Val	1 (reference)		1 (reference)	
Val/Ala	0.82 (0.42-1.58)	0.547	0.76 (0.36-1.56)	0.458
Ala/Ala	1.97 (0.95-4.08)	0.069	1.38 (0.58-3.26)	0.468
Val/Ala and Ala/Ala	1.03 (0.55-1.93)	0.915	0.89 (0.45-1.76)	0.734
	<i>P</i> <sub>trend</sub>	0.038		
Val/Val and Val/Ala	1 (reference)		1 (reference)	
Ala/Ala	2.40 (1.40-4.12)	0.001	1.81 (0.99-3.33)	0.054

\*Cox proportional hazards regression with adjustments for age at diagnosis, study site, race, tumor size, tumor grade, nodal involvement, estrogen receptor status, and p53 mutation.

**Table 4.** Modification of the association of Val16Ala with 10-y breast cancer survival by receipt of chemotherapy

	Univariate			Multivariate*		
	HR (95% CI)	P	n	HR (95% CI)	P	n
No chemotherapy	(n = 221)			(n = 161)		
Val/Val	1 (reference)		58	1 (reference)		42
Val/Ala	0.96 (0.49-1.87)	0.898	111	1.28 (0.56-2.93)	0.563	81
Ala/Ala	1.31 (0.62-2.75)	0.480	52	1.47 (0.57-3.74)	0.424	38
Val/Ala and Ala/Ala	1.06 (0.57-1.99)	0.846	163	1.24 (0.58-2.64)	0.569	119
	<i>P</i> <sub>trend</sub>	0.488				
Val/Val and Val/Ala	1 (reference)		169	1 (reference)		123
Ala/Ala	1.33 (0.73-2.43)	0.345	52	1.60 (0.81-3.16)	0.175	38
Chemotherapy	(n = 322)			(n = 293)		
Val/Val	1 (reference)		85	1 (reference)		77
Val/Ala	1.42 (0.90-2.25)	0.130	166	1.24 (0.76-2.02)	0.385	153
Ala/Ala	2.41 (1.41-3.94)	<0.001	71	2.47 (1.46-4.19)	0.001	63
Val/Ala and Ala/Ala	1.68 (1.09-2.58)	0.018	137	1.58 (1.01-2.49)	0.047	216
	<i>P</i> <sub>trend</sub>	0.001				
Val/Val and Val/Ala	1 (reference)		251	1 (reference)		230
Ala/Ala	1.87 (1.29-2.71)	0.001	71	1.95 (1.32-2.90)	0.001	63
Doxorubicin-containing therapy	(n = 160)			(n = 149)		
Val/Val	1 (reference)		44	1 (reference)		41
Val/Ala	1.12 (0.60-2.10)	0.709	84	0.90 (0.47-1.75)	0.775	79
Ala/Ala	2.76 (1.41-5.40)	0.003	32	2.90 (1.33-6.32)	0.007	29
Val/Ala and Ala/Ala	1.50 (0.84-2.67)	0.169	116	1.21 (0.67-2.22)	0.523	108
	<i>P</i> <sub>trend</sub>	0.003				
Val/Val and Val/Ala	1 (reference)		128	1 (reference)		120
Ala/Ala	2.57 (1.53-4.35)	<0.001	32	2.64 (1.48-4.69)	0.001	29
5-FU – containing therapy	(n = 121)			(n = 112)		
Val/Val	1 (reference)		25	1 (reference)		24
Val/Ala	2.95 (1.03-8.50)	0.045	64	2.87 (0.94-8.74)	0.064	59
Ala/Ala	4.63 (1.56-13.7)	0.006	32	5.99 (1.71-21.0)	0.005	29
Val/Ala and Ala/Ala	3.49 (1.25-9.74)	0.017	96	4.12 (1.40-12.1)	0.010	88
	<i>P</i> <sub>trend</sub>	0.004				
Val/Val and Val/Ala	1 (reference)		89	1 (reference)		83
Ala/Ala	1.90 (1.05-3.42)	0.033	32	2.07 (1.10-3.88)	0.023	29
Cyclophosphamide-containing therapy	(n = 142)			(n = 123)		
Val/Val	1 (reference)		35	1 (reference)		31
Val/Ala	2.72 (1.03-7.14)	0.042	74	1.92 (0.69-5.29)	0.208	64
Ala/Ala	5.82 (2.17-15.51)	<0.001	33	22.0 (5.22-92.9)	<0.001	28
Val/Ala and Ala/Ala	3.56 (1.41-8.98)	0.007	107	3.22 (1.24-8.38)	0.016	92
	<i>P</i> <sub>trend</sub>	<0.001				
Val/Val and Val/Ala	1 (reference)		109	1 (reference)		95
Ala/Ala	2.86 (1.62-5.06)	<0.001	33	4.60 (2.29-9.21)	<0.001	28

NOTE: Analysis included 543 patients. Information on receipt of chemotherapy was not available for 12 Norwegian patients and 18 U.S. patients.  
\*Cox proportional hazards regression with adjustments for age at diagnosis, study site, race, tumor size, tumor grade, nodal involvement, p53 mutation, and estrogen receptor status.

poor outcome among patients treated with this drug. Carriers of this genotype represent 20% to 25% of the patient population, which would be candidates for therapies other than cyclophosphamide. Mechanistically, the Ala allele may increase resistance to chemotherapeutic drugs, because it increases MnSOD activity and detoxification of drugs, whose anticancer activity involves generation of ROS. Previous *in vitro* studies have indicated that high expression of MnSOD increases resistance to doxorubicin (9, 10) and 5-FU (45–47), suggesting that increased MnSOD activity may have similar effects. Because our data indicate that Val16Ala has its strongest outcome effect with cyclophosphamide therapy, future studies that examine the relationship between MnSOD and cyclophosphamide cytotoxicity in human cancer cells are needed.

We observed that Val16Ala could be functionally most significant among patients with ER-positive and p53 wild-type

tumors. MnSOD is induced by p53 in response to stress (36) and can suppress radiation-induced neoplastic transformation and induction of hypoxia-inducible factor-1 $\alpha$  and vascular endothelial growth factor (7, 48, 49). From these data, we hypothesize that Val16Ala may become functional in the p53-induced stress response when breast tumors are treated with chemotherapeutic drugs, e.g., cyclophosphamide and doxorubicin, with a subsequent induction of p53 and MnSOD. This pathway is consistent with previous research observing resistance to anthracycline therapy among breast cancer patients with a mutant p53 tumor status (50). Our analyses did not find that race/ethnicity is a confounder of the relationship between Val16Ala and survival. However, because of the higher frequency of ER-negative tumors among African-American breast cancer patients, Val16Ala may affect African-American women differently than it is affecting women of European ancestry.

**Table 5.** Subanalysis of the association of Val16Ala with 10-y breast cancer survival by specific chemotherapy regimen

Therapy*	Univariate			Multivariate †		
	HR (95% CI)	P	n	HR (95% CI)	P	n
Doxorubicin monotherapy (n = 86)						
Val/Val	1 (reference)		22	1 (reference)		22
Val/Ala	0.65 (0.30-1.45)	0.300	43	0.70 (0.31-1.61)	0.402	43
Ala/Ala	1.73 (0.77-3.87)	0.180	21	2.03 (0.84-4.87)	0.115	21
Val/Ala and Ala/Ala	0.95 (0.47-1.92)	0.893	64	1.05 (0.50-2.20)	0.892	64
	<i>P</i> <sub>trend</sub>	0.189				
Val/Val and Val/Ala	1 (reference)		65	1 (reference)		65
Ala/Ala	2.18 (1.12-4.22)	0.020	21	2.35 (1.18-4.66)	0.015	21
Cyclophosphamide/doxorubicin or cyclophosphamide/doxorubicin/5-FU (n = 63)						
Val/Val	1 (reference)		21	1 (reference)		18
Val/Ala	2.61 (0.71-9.51)	0.145	32	2.49 (0.39-15.8)	0.330	28
Ala/Ala	6.33 (1.58-25.7)	0.010	10	139.5 (2.77-7,017)	0.013	8
Val/Ala and Ala/Ala	3.37 (0.98-11.59)	0.054	42	2.60 (0.54-12.5)	0.233	36
	<i>P</i> <sub>trend</sub>	0.005				
Val/Val and Val/Ala	1 (reference)		53	1 (reference)		46
Ala/Ala	3.51 (1.32-9.33)	0.012	10	16.1 (3.22-80.3)	0.001	8
Mitomycin/5-FU (n = 35)						
Val/Val	1 (reference)		8	1 (reference)		8
Val/Ala	2.44 (0.52-11.4)	0.256	19	2.80 (0.54-14.4)	0.218	19
Ala/Ala	1.62 (0.27-9.75)	0.598	8	1.91 (0.26-13.9)	0.521	8
Val/Ala and Ala/Ala	2.20 (0.49-9.85)	0.305	27	2.20 (0.47-10.3)	0.316	27
	<i>P</i> <sub>trend</sub>	0.797				
Val/Val and Val/Ala	1 (reference)		27	1 (reference)		27
Ala/Ala	0.79 (0.22-2.85)	0.721	8	0.57 (0.14-2.26)	0.428	8
Cyclophosphamide/methotrexate/5-FU (n = 77)						
Val/Val	1 (reference)		14	1 (reference)		14
Val/Ala	3.05 (0.69-13.4)	0.141	41	2.15 (0.45-10.3)	0.338	36
Ala/Ala	6.02 (1.35-26.8)	0.018	22	24.27 (2.84-207)	0.004	19
Val/Ala and Ala/Ala	3.99 (0.95-16.8)	0.059	63	4.61 (1.06-20.1)	0.042	55
	<i>P</i> <sub>trend</sub>	0.004				
Val/Val and Val/Ala	1 (reference)		55	1 (reference)		50
Ala/Ala	2.53 (1.21-5.26)	0.013	22	4.17 (1.77-9.81)	0.001	19

\*Eleven patients who received doxorubicin and three patients who received cyclophosphamide as part of other therapy regimens than described were not included in the analyses.

†Cox proportional hazards regression with adjustments for age at diagnosis, study site, race, tumor size, tumor grade, nodal involvement, p53 mutation, and estrogen receptor status.

Our study has strengths and limitations. We conducted the analysis in two independent populations, limiting the likelihood of a false-positive discovery. We were also able to assess the implication of the tumor p53 mutational status on the association between Val16Ala and breast cancer survival in both patient populations. However, the existing sample size did not allow a more in-depth examination of the effect of Val16Ala using stratification of subsets, e.g., analysis of interactions between Val16Ala and specific chemotherapeutics after stratification of patients by ER and p53 status. We also pooled the cohorts to increase power when we did subgroup analyses, e.g., to examine whether the effect of Val16Ala on survival is dependent on the type of chemotherapy. Even in this combined analysis, some subgroups consisted of small patient numbers, leading to wide confidence intervals and perhaps unstable risk estimates. Furthermore, information on the therapy of patients was obtained from medical records in the U.S. cohort and from some of the Norway patients. This therapy information and the therapy regimens across both study populations were grouped by type of combination therapy, but we are aware that there is substantial heterogeneity

among the patients by choice of combination (e.g., combination of hormone replacement therapy and chemotherapy), dosage, and treatment duration within each subgroup.

In conclusion, we found that a functional polymorphism in the SOD2 gene affects the survival of breast cancer patients. Carriers of the common Ala/Ala genotype were at a significantly increased risk of poor survival when treated with specific chemotherapeutic drugs, especially cyclophosphamide. Our findings suggest that Val16Ala modifies the response of patients to chemotherapy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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