

## Clinical Investigation

# Common Variants of GSTP1, GSTA1, and TGF $\beta$ 1 are Associated with the Risk of Radiation-Induced Fibrosis in Breast Cancer Patients

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## Summary

The genetic determinants that underlie normal tissue radiosensitivity are poorly understood. This retrospective study evaluated the association between the risk of subcutaneous fibrosis in breast cancer patients receiving radiation and eight polymorphic variants in six candidate genes related to DNA repair mechanisms, oxidative stress response and fibroblast proliferation and differentiation. Results suggest that functional polymorphisms in GSTP1, GSTA1 and TGF $\beta$ 1 may contribute to the occurrence of skin fibrosis.

**Purpose:** To provide new insights into the genetic basis of normal tissue radiosensitivity, we evaluated the association between eight polymorphic variants located in six genes related to DNA repair mechanisms, oxidative stress, and fibroblast proliferation (XRCC1 Arg399Gln, XRCC1 Arg194Trp, TP53 Arg72Pro, GSTP1 Ile105Val, GSTA1 C-69T, eNOS G894T, TGF $\beta$ 1 C-509T, and TGF $\beta$ 1 T869C) and the risk of subcutaneous fibrosis in a retrospective series of patients who received radiotherapy after breast-conserving surgery.

**Methods and Materials:** Subcutaneous fibrosis was scored according to the Late Effects of Normal Tissue—Subjective Objective Management Analytical scale in 257 breast cancer patients who underwent surgery plus adjuvant radiotherapy. Genotyping was conducted by polymerase chain reaction—restriction fragment length polymorphism analysis on genomic DNA extracted from peripheral blood. The association between genetic variants and the risk of moderate to severe fibrosis was evaluated by binary logistic regression analysis.

**Results:** Two hundred thirty-seven patients were available for the analysis. Among them, 41 patients (17.3%) developed moderate to severe fibrosis (Grade 2–3), and 196 (82.7%) patients displayed no or minimal fibrotic reactions (Grade 0–1). After adjustment of confounding factors, GSTP1 Ile105Val (odds ratio [OR] 2.756; 95% CI, 1.188–6.393;  $p = 0.018$ ), GSTA1 C-69T (OR 3.223; 95% CI, 1.176–8.826;  $p = 0.022$ ), and TGF $\beta$ 1 T869C (OR 0.295; 95% CI, 0.090–0.964;  $p = 0.043$ ) polymorphisms were found to be significantly associated with the risk of Grade 2–3 radiation-induced fibrosis. In the combined analysis, carriers of three risk genotypes were found to be at higher odds for the development of Grade 2–3 fibrosis than were patients with two risk genotypes (OR 4.415; 95% CI, 1.553–12.551,  $p = 0.005$ ) or with no or one risk genotype (OR 8.563; 95% CI, 2.671–27.447;  $p = 0.0003$ ).

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**Conclusions:** These results suggest that functional variations in genes involved in oxidative stress response and fibroblast proliferation may modulate the development of radiation-induced fibrosis in breast cancer patients. The results of the combined analysis support the notion that approaches based on the combination of different genetic markers have the potential to predict normal tissue responses. © 2011 Elsevier Inc.

**Keywords:** Single-nucleotide polymorphisms, Radiosensitivity, Subcutaneous fibrosis, Radiotherapy, Breast cancer

## Introduction

Radiotherapy is commonly applied after breast-conserving surgery to reduce the risk of locoregional recurrence of breast cancer and has been accepted as a well-established standard of care (1). However, patients vary considerably in their normal tissue responses to radiotherapy even after similar treatments (2). As a consequence, standard radiation doses may induce different levels of early and late side effects. Although acute effects occur during or shortly after treatment completion and are usually reversible, late radiation toxicities can be permanent and occur typically 6 months to several years after radiotherapy through a variety of clinical manifestations, including subcutaneous fibrosis, atrophy, and vascular damage (3). Interindividual variability in normal tissue radiosensitivity is a multifactorial trait, which depends on treatment parameters including total radiation dose and schedule (4, 5); on clinical factors such as age, acute skin reaction, and lifestyle factors (6–10); and on a genetic component (11). Early indications of an inherited basis for clinical radiosensitivity came from the observation that patients harboring defects in genes such as ATM (ataxia telangiectasia), the FANCD1 gene family (Fanconi's anemia), NBN (Nijmegen breakage syndrome), and BLM (Bloom's syndrome) cannot tolerate standard radiotherapy (12). Predisposition to adverse responses to radiotherapy in such disorders is dominated by a strong effect of a few high-penetrance mutations in these susceptibility genes. However, these mutations are rare and can explain only a minority of radiosensitive patients. Therefore, in the current view, normal tissue radiosensitivity is regarded as a complex polygenic trait that results from the combined effects of multiple common single-nucleotide polymorphisms (SNPs) with modest functional effects (low-penetrance variants) (13). The role of candidate polymorphic genes involved in the base-excision repair mechanisms (XRCC1 and TP53), oxidative stress response (GSTP1, GSTA1, and eNOS), and fibroblast proliferation and differentiation (TGF $\beta$ 1) has been investigated by several studies; however, the results obtained are often difficult to compare directly because the studies are rather methodologically heterogeneous in terms of patient selection, cancer site, treatment characteristics, and normal tissue endpoints (14).

The aim of this retrospective study was to investigate the association between common gene variants and the risk of subcutaneous fibrosis in breast cancer patients who received radiotherapy after breast-conserving surgery. More specifically, we evaluated the association between eight polymorphic variants located in six genes (XRCC1, eNOS, GSTP1, GSTA1, TGF $\beta$ 1, TP53) and the risk of moderate to severe subcutaneous fibrosis in 237 breast cancer patients. The selected candidate genes represent diverse pathways in radiation response and have been previously described as potentially associated with late normal tissue radiosensitivity (13, 15–20).

## Methods and Materials

### Study subject and data collection

Two hundred fifty-seven patients affected by histologically confirmed breast cancer who underwent conservative surgery plus adjuvant radiotherapy between 1989 and 2009 participated in this study. The surgery was quadrantectomy in 96% of patients and lumpectomy in 4%. The 257 patients represented a subset of patients treated at our center and, to avoid any operator bias, were selected sequentially during regular follow-up from October 2009 to October 2010 at the Department of Radiotherapy of the University Hospital "Maggiore della Carità" in Novara, Italy. The radiotherapy technique consisted of two opposed tangential wedged fields without intensity modulation, followed by a boost on tumor bed. Radiation was planned on computed tomography slices in all cases: from 1989 to 1999 using three to four slices and afterward using contiguous slices 5 to 10 millimeters thick. All patients were given whole breast radiotherapy with conventional fractionation (50–50.4 Gy; 1.8–2 Gy/fraction), with photons (6–18 MV) or <sup>60</sup>Co  $\gamma$ -rays (1.25 MV). Treatment was followed by electron (9–10.5 MeV) in 235 of 257 cases (91.4%) or orthovoltage (200 kV x-rays) boost (9–16 Gy; 1.5–3 Gy/fraction) in 22 of 257 cases (8.6%) according to tumor characteristics. All treatments were equivalent in terms of biologic effective dose. Physical examinations for detection of late normal tissue toxicities (telangiectasia and subcutaneous fibrosis) were given and scored according to the Late Effects of Normal Tissue—Subjective Objective Management Analytical (LENT-SOMA) scale (21, 22). Grade 2 fibrosis of the breast was defined as definite increased density and firmness and Grade 3 as very marked density, retraction, and fixation. Considering that fibrosis could be induced by either surgery or radiation, the patients were scored Grade 2–3 when the alterations were detectable not only in the surgical site but also in the adjacent tissue included in the treated volume. Inasmuch as the objective of the present study was to identify predictors of subcutaneous fibrosis, 20 patients who experienced skin telangiectasia (LENT-SOMA Grade  $\geq$ 2) but not fibrosis (LENT-SOMA Grade <2) were excluded from the study to avoid a confounding effect of telangiectasia. Among the remaining 237 patients, 125 (52.7%) did not develop fibrosis (Grade 0), 71 patients (30.0%) developed Grade 1 fibrosis, 36 patients (15.2%) Grade 2, and 5 patients (2.1%) Grade 3 fibrosis. For group comparison, 41 patients with moderate to severe fibrosis (Grade 2–3) were referred to as the radiosensitive group and compared with 196 patients with no or minimal fibrotic reactions (Grade 0–1, control group). This study was approved by the local ethics committee of the University Hospital "Maggiore della Carità" of Novara, Italy, and met the requirements of the Declaration of Helsinki. Informed consent was obtained from all patients before their participation in the study.

## Candidate genes and polymorphisms

The selected polymorphisms are located in genes related to DNA repair mechanisms (XRCC1 and TP53), oxidative stress response (GSTP1, GSTA1, and eNOS), and fibroblast proliferation and differentiation (TGFβ1). The SNPs selected and their National Center for Biotechnology Information (NCBI) dbSNP ID (rs) are the following: XRCC1 Arg399Gln (rs25487), XRCC1 Arg194Trp (rs1799782), eNOS G894T (rs1799983), GSTP1 Ile105Val (rs1695), GSTA1 C-69T (rs3957356), TGFβ1 C-509T (rs1800469), TGFβ1 T869C (rs1982073), and TP53 Arg72Pro (rs1042522).

## DNA genotyping

Genomic DNA was extracted from peripheral blood by use of the QiaAmp DNA Mini Kit (Qiagen Valencia, CA). Polymerase chain reactions (PCRs), conducted in a total volume of 30 μL containing 100 ng of genomic DNA, were performed using 0.4 μM of each couple of primers (Table 1). After 35 cycles of PCR amplification (denaturation at 94°C for 30 seconds, annealing at 55°C [XRCC1 Arg399Gln] or 56°C [GSTA1 C-69T] or 61°C [eNOS G894T; TGFB1 C-509T; GSTP1 Ile105Val], or 62°C [TGFβ1 T869C; XRCC1 Arg194Trp; TP53 Arg72Pro] for 30 seconds, extension at 72°C for 30 seconds), amplification products underwent electrophoresis in 2% agarose gel and visualized after staining with ethidium bromide. The PCR products (10 μL: GSTP1 Ile105Val, TP53 Arg72Pro, eNOS G894T, XRCC1 Arg399Gln, XRCC1 Arg194Trp, GSTA1 C-69T; 5 μL: TGFβ1 C-509T and TGFβ1 T869C) harboring the SNPs were digested overnight in a total volume of 20 μL by 2 to 10 U of restriction enzymes (New England Biolabs, Milano, Italy) at recommended temperatures. The digested products underwent electrophoresis in 2.5% (eNOS G894T, XRCC1 Arg399Gln, XRCC1 Arg194Trp, and GSTA1 C-69T) or 4% agarose gel (GSTP1 Ile105Val, TP53 Arg72Pro, TGFB1 C-509T, and TGFβ1 T869C) and visualized after staining with ethidium bromide. Primer sequences, restriction enzymes, and expected fragment sizes are shown in Table 1. All PCR reactions were set up in a dedicated PCR area with dedicated PCR pipettes and reagents. For quality control purposes, each PCR and restriction enzyme

digestion included both negative and positive controls. For validation, about 10% of the samples were regenotyped. The results were reproducible, with no discrepancies in genotyping.

## Statistical methods

Each polymorphism was tested for deviation from the Hardy-Weinberg equilibrium by use of Pearson's chi-square test as implemented in the Finetti's program (<http://ihg.gsf.de/cgi-bin/hw/hw1.pl>). The univariate association between categorical variables and radiation status of breast cancer patients (radiosensitive patients, Grade 2–3 fibrosis; control group, Grade 0–1 fibrosis) was evaluated by use of Pearson's chi-square test. In case of 2 × 2 contingency tables, Fisher's exact test was used when an expected cell value was less than 5. The Mann-Whitney *U* test (body mass index [BMI], breast diameter represented by the distance between the incidence points of the tangential beams axes, and follow-up time) or *t* test (age) was used for continuous variables. Binary logistic regression analysis was then performed to evaluate the association between genetic variants and the risk of radiation-induced subcutaneous fibrosis. For all the selected polymorphisms a dominant genetic model was considered (e.g., TGFβ1 –509 TT vs. –509 TC+CC). Potential covariates to be included in the adjusted model were age, BMI, and breast diameter that could be indirectly correlated with dose inhomogeneities and consequently with higher skin doses, follow-up, acute toxicity, diabetes, hypertension, history of vasculopathy, smoking status, alcohol consumption, adjuvant treatment, dose/fraction, radiation quality, and boost therapy type. A stepwise backward elimination technique was used to develop a final model and to control for potential confounders. The final model used was that giving approximately the same odds ratio estimates as in a fully adjusted model with the largest gain in precision and included adjustment for age, adjuvant treatment, follow-up, BMI, breast diameter, history of vasculopathy, smoking status, dose per fraction, radiation quality, and boost method. The McFadden's Rho<sup>2</sup> value for each logistic regression model was used to assess the goodness of fit. The odds ratios (OR) and 95% confidence intervals were calculated. All clinical and genotype data were managed with the statistical software package SYSTAT for

**Table 1** Primers and restriction enzymes used for genotyping

Polymorphism	Primer sequence (5'–3')	Product (bp)	Restriction enzyme	Allele phenotypes (bp)
XRCC1Arg399Gln	F: TTGTGCTTCTCTGTGTCCA R: TCCTCCAGCCTTTTCTGATA	615	MspI	Arg: 374, 271 Gln: 615
XRCC1 Arg194Trp	F: CCGTGTGAAGGAGGAGGATGA R: CCTCCAGACCTCTCAACCCTC	275	MspI	Arg: 213, 42, 20 Trp: 233, 42
eNOS G894T	F: CATGAGGCTCAGCCCCAGAAC R: AGTCAATCCCTTTGGTGCTCAC	206	MboI	G: 206 T: 119, 87
GSTP1 Ile105Val	F: ACCCCAGGGCTCTATGGGAA R: TGAGGGCACAAGAAGCCCCT	176	BsmAI	Ile: 176 Val: 91, 85
GSTA1 C-69T	F: CCCTACATGGTATAGGTGAAAT R: GTGCTAAGGACACATATTAGCA	821	HinfI	C: 525, 254, 42 T: 525,197, 57, 42
TGFβ1 C-509T	F: CAGTAAATGTATGGGGTCGCAG R: GGTGTCAGTGGGAGGAGGG	153	Bsu36I	C: 117, 36 T: 153
TGFβ1 T869C	F:TTCCCTCGAGGCCCTCCTA R: GCCGCAGCTTGGACAGGATC	294	MspAII	T: 161, 67, 40, 26 C: 149, 67, 40, 26, 12
TP53 Arg72Pro	F: TTGCCGTCCCAAAGCAATGGATGA R: TCTGGGAAGGGACAGAAGATGAC	199	BstUI	Arg: 113,86 Pro: 199

Windows (version 12; Systat Software Inc., Chicago, IL). Inasmuch as this was a confirmatory study and Bonferroni's correction does not apply in such cases, a nominal level of significance was considered at  $p < 0.05$ . To evaluate the Type II error, we used the basic standard applet for statistical power provided at [www.dssresearch.com/toolkit/spcalc/power\\_p2.asp](http://www.dssresearch.com/toolkit/spcalc/power_p2.asp). Given the sample size of the control group ( $n = 196$ ) and the radiosensitive

patient group ( $n = 41$ ), and assuming a relative risk of 1.3-fold increase in normal tissue complications in patients with the high-risk genotype, power calculations were comprised between 87% for GSTA1 C-69T polymorphism (frequency of -69T carriers in control patients of approximately 70%,  $\alpha$  level of 5%) and 12% for the XRCC1 Arg194Trp polymorphism (frequency of 194Trp carriers in control patients of approximately 12%).

**Table 2** Clinical and demographic characteristics in the whole set of breast cancer patients ( $n = 237$ ) and after stratification according to the radiosensitive status (LENT-SOMA Grade 2–3 fibrosis in 41 patients and Grade 0–1 fibrosis in 196)

Clinical variables	Total: $n = 237$ (%)	Grade 0–1: $n$ (%)	Grade 2–3: $n$ (%)	$p$ value*
Age (y): median (range)	60 (35–85)			
Age (y): mean (SD)		60.2 (9.5)	63.9 (10.4)	0.040
BMI, median (range)	24.2 (18–51.4)			
BMI, mean (SD)		24.6 (3.9)	26.1 (3.8)	0.010
Breast diameter (cm): $n = 236$				
Median (range)	12 (4.9–25)			
Mean (SD)		12.0 (2.6)	12.94 (2.3)	0.011
Follow-up (mo): median (range)	63 (9–222)			
Follow-up, mean (SD)		70.2 (42.5)	72.9 (39.6)	0.413
Acute toxicity (RTOG Grade >1), $n = 236$				
No	161 (68.2)	132 (67.7)	29 (70.7)	0.704
Yes	75 (31.8)	63 (32.3)	12 (29.3)	
Diabetes				
No	222 (93.7)	186 (94.9)	36 (87.8)	0.090
Yes	15 (6.3)	10 (5.1)	5 (12.2)	
Hypertension				
No	176 (74.3)	146 (74.5)	30 (73.2)	0.861
Yes	61 (25.7)	50 (25.5)	11 (26.8)	
History of vasculopathy				
No	218 (92.0)	179 (91.3)	39 (95.1)	0.541
Yes	19 (8.0)	17 (8.7)	2 (4.9)	
Smoking status				
Never	200 (84.4)	162 (82.7)	38 (92.7)	0.108
Current or former	37 (15.6)	34 (17.3)	3 (7.3)	
Alcohol (wine at meals)				
No	229 (96.6)	189 (96.4)	40 (97.6)	1.000
Yes	8 (3.4)	7 (3.6)	1 (2.4)	
Adjuvant treatment, $n = 227$				
None	24 (10.6)	21 (11.2)	3 (7.5)	0.915
Chemotherapy (C)	51 (22.5)	42 (22.5)	9 (22.5)	
Hormone therapy (H)	107 (45.8)	87 (46.5)	20 (50.0)	
C + H	45 (19.8)	37 (19.8)	8 (20)	
Dose/fraction, Gy				
2	229 (96.6)	189 (96.4)	40 (97.6)	1.000
1.8	8 (3.4)	7 (3.6)	1 (2.4)	
Radiation quality				
x-rays	218 (92.0)	179 (91.3)	39 (95.1)	0.541
$\gamma$ -rays	19 (8.0)	17 (8.7)	2 (4.9)	
Boost therapy type				
Electrons	202 (85.2)	168 (85.7)	34 (82.9)	0.886
Photons (x-rays)	14 (5.9)	11 (5.6)	3 (7.3)	
No boost	21 (8.9)	17 (8.7)	4 (9.8)	
Boost dose fractionation (Gy)				
3	59 (24.9)	50 (25.5)	9 (21.9)	0.883
1.5–2	157 (66.2)	129 (65.8)	28 (68.3)	
No boost	21 (8.9)	17 (8.7)	4 (9.8)	

Abbreviations: BMI = body mass index; SD = standard deviation; RTOG = Radiation Therapy Oncology Group.

\* Pearson's chi-square test or Fisher's exact test for categoric variables. Mann-Whitney  $U$  test (body mass index, breast diameter, and follow-up time) or  $t$  test (age) for continuous variables.

## Results

Two hundred thirty-seven patients were available for this study. After treatment with radiotherapy, 41 (17.3%) patients had moderate to severe fibrosis (Grade 2–3) and 196 (82.7%) patients had no or minimal fibrotic reactions (Grade 0–1). The demographic and clinical data of the whole set of breast cancer patients and after stratification according to their radiosensitive status are shown in Table 2. Univariate analysis showed that patients who experienced moderate to severe skin fibrosis (Grade 2–3) differed from control patients (Grade 0–1) for older age (63.9 vs. 60.2 years,  $p = 0.04$ ), higher BMI (26.1 vs. 24.6,  $p = 0.010$ ) and higher breast diameter (12.94 cm vs. 12.0 cm,  $p = 0.011$ ). No other clinical factor was found to be significantly different between the two groups of patients in the univariate analysis. The distribution of genotypes for each polymorphism in the whole set of patients is summarized in Table 3. Distributions of genotype frequencies for all the polymorphisms analyzed were in Hardy-Weinberg equilibrium ( $p > 0.05$ ). Complete datasets were available for 227 patients (186 patients with Grade 0–1 fibrosis and 40 patients with Grade 2–3 fibrosos). After adjustment for confounding factors (age, BMI, breast diameter, adjuvant treatment, follow-up, history of vasculopathy, smoking status, dose per fraction, radiation quality in terms of x-rays or  $\gamma$ -rays, and boost method), logistic regression analysis revealed that GSTP1 Ile105Val, GSTA1 C-69T, and TGF $\beta$ 1 T869C polymorphisms were significantly associated with the risk of radiation-induced fibrosis in breast cancer patients (Table 4). More specifically, GSTP1 105 val allele carriers (OR 2.756; 95% CI, 1.188–6.393,  $p = 0.018$ ) and GSTA1 –69T allele carriers (OR 3.223; 95% CI, 1.176–8.826,  $p = 0.022$ ) were at higher risk to develop skin fibrosis, whereas TGF $\beta$ 1 869C carriers were found to be at lower risk (OR 0.295; 95% CI, 0.090–0.964,  $p = 0.043$ ). None of the other investigated polymorphisms was found to be significantly associated with fibrosis risk.

To evaluate the combined effect of GSTP1 Ile105Val, GSTA1 C-69T, and TGF $\beta$ 1 T869C polymorphisms on fibrosis risk, the number of risk genotypes (GSTP1 105 Ile/Val or Val/Val, GSTA1 –69CT or –69TT, and TGF $\beta$ 1 869TT) for each patient was considered. Grade 2–3 fibrosis was developed by 7.1% (1 of 14) of patients not carrying a risk genotype, by 12.0% of patients (10 of 83) carrying one risk genotype, by 17.5% of patients (20 of 114) carrying two risk genotypes, and by 38.5% of patients (10 of 26) carrying 3 risk genotypes (Fig. 1). Logistic regression analysis adjusted for confounding factors (age, BMI, breast diameter, follow-up, smoking status, history of vasculopathy, adjuvant treatment, dose per fraction, radiation quality, and boost method) revealed that carriers of the risk genotypes were at higher odds for the development of Grade 2–3 fibrosis than were patients with two risk genotypes (OR 4.415; 95% CI, 1.553–12.551;  $p = 0.005$ ) or patients with no or one risk genotype (OR 8.563; 95% CI, 2.671–27.447;  $p = 0.0003$ ).

## Discussion

In the present study, we analyzed selected gene polymorphisms in a cohort of breast cancer survivors representing a subset population included sequentially and without any predefined selection criteria to avoid any operator bias, during regular follow-up, with the aim of investigating the association between common gene variants and the risk of subcutaneous fibrosis. A limited number of

**Table 3** Distribution of polymorphic variants in the study population

Polymorphism	Patients, <i>n</i> (%)	<i>p</i> value*
XRCC1 Arg399Gln		0.603
Arg/Arg	95 (39.9)	
Arg/Gln	113 (47.5)	
Gln/Gln	29 (12.2)	
XRCC1 Arg 194Trp		0.898
Arg/Arg	209 (87.8)	
Arg/Trp	27 (11.3)	
Trp/Trp	1 (0.4)	
ENOS G894T		0.452
GG	97 (40.8)	
GT	105 (44.1)	
TT	35 (14.7)	
GSTP1 Ile105Val		0.214
Ile/Ile	108 (45.4)	
Ile/Val	110 (46.2)	
Val/Val	19 (8.0)	
GSTA1 C-69T		0.580
CC	67 (28.2)	
CT	114 (47.9)	
TT	56 (23.5)	
TGF $\beta$ 1 C-509T		0.495
CC	103 (43.3)	
CT	110 (46.2)	
TT	24 (10.1)	
TGF $\beta$ 1 T869C		0.724
TT	90 (37.8)	
TC	110 (46.2)	
CC	37 (15.5)	
TP53 Arg72Pro		0.394
Arg/Arg	139 (58.4)	
Arg/Pro	88 (37.0)	
Pro/Pro	10 (4.2)	

\* Test for departure from Hardy-Weinberg equilibrium.

studies have been already published about the possible correlation of gene polymorphisms and onset of fibrosis after radiation for breast cancer, with equivocal results (16, 18–20, 23, 24).

We analyzed the association of polymorphic genes related to DNA repair mechanisms (XRCC1 and TP53) and oxidative stress response (GSTP1, GSTA1, and eNOS) with the risk of radiation-induced fibrosis in breast cancer patients who received radiotherapy after breast-conserving surgery. In addition, two polymorphic variants of TGF $\beta$ 1 were also evaluated because this pleiotropic cytokine is involved in wound healing, inflammation, angiogenesis, and fibrosis (25), and elevated TGF $\beta$ 1 levels in pretreatment plasma are correlated with an increased risk for the development of postradiotherapy fibrosis in breast cancer patients (26). In the logistic regression analysis, GSTP1 Ile105Val, GSTA1 C-69T, and TGF $\beta$ 1 C869T polymorphisms emerged as significant factors for moderate to severe subcutaneous fibrosis in breast cancer patients. In particular, GSTP1 105Val and GSTA1 –69T carriers were found to be at higher risk for subcutaneous fibrosis, whereas TGF $\beta$ 1 869C carriers were found to be at lower risk. Our results are in line with previous reports showing an association of GSTP1 105Val allele with an increased risk of pleural thickening (17) or an increased risk of acute skin side effects after radiation therapy for breast cancer (27).

**Table 4** Association between single-nucleotide polymorphisms and radiation-induced skin fibrosis in breast cancer patients

Polymorphism	Grade 0–1 <i>n</i> (%)	Grade 2–3 <i>n</i> (%)	Odds ratio*	95% confidence interval	<i>p</i> value
XRCC1 Arg399Gln					
Arg/Arg	77 (39.3)	18 (43.9)	1 (Ref)		
Arg/Gln + Gln/Gln	119 (60.7)	23 (56.1)	0.843	0.375–1.895	0.679
XRCC1 Arg194Trp					
Arg/Arg	173 (88.3)	36 (87.8)	1 (Ref)		
Arg/Trp + Trp/Trp	23 (11.7)	5 (12.2)	1.279	0.377–4.338	0.692
GSTP1 ILE105Val					
AA	95 (48.5)	13 (31.7)	1 (Ref)		
AG + GG	101 (51.5)	28 (68.3)	2.756	1.188–6.393	0.018
GSTA1 C-69T					
CC	59 (30.1)	8 (19.5)	1 (Ref)		
CT + TT	137 (69.9)	33 (80.5)	3.223	1.176–8.826	0.022
ENOS G894T					
GG	76 (38.8)	21 (51.2)	1 (Ref)		
GT + TT	120 (61.2)	20 (48.8)	0.478	0.215–1.065	0.071
TGFβ1 C-509T					
CC	85 (43.4)	18 (43.9)	1 (Ref)		
CT + TT	111 (56.6)	23 (56.1)	1.841	0.561–6.036	0.313
TGFβ1 T869C					
TT	71 (36.2)	19 (46.3)	1 (Ref)		
TC + CC	125 (63.8)	22 (53.7)	0.295	0.090–0.964	0.043
TP53 Arg72Pro					
Arg/Arg	110 (56.1)	29 (70.7)	1 (Ref)		
Arg/Pro + Pro/Pro	86 (43.9)	12 (29.3)	0.654	0.271–1.573	0.343

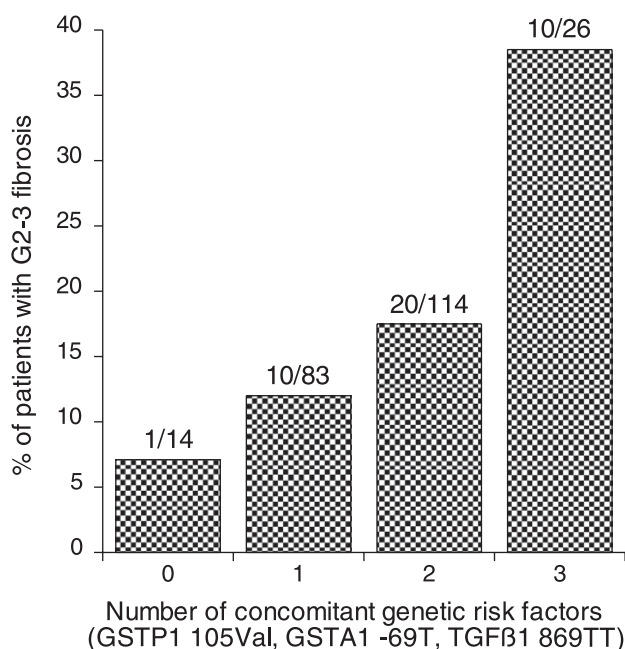
\* Odds ratio adjusted for age, body mass index, breast diameter, follow-up, adjuvant treatment, history of vasculopathy, smoking status, dose per fraction, radiation quality, and boost method.

Glutathione-S-transferases (GSTs), GSTP1 and GSTA1, are multifunctional enzymes involved in the protection of cellular components against anticancer drugs and in the detoxification of reactive oxygen species (ROS) generated by radiation-induced

oxidative (28). The GSTP1 isoleucine (Ile) to valine (Val) substitution at codon 105 and the C to T substitution at –69 position in the promoter region of the human GSTA1 have both been associated with a substantial reduction of enzyme activity (29–31). Thus, our data support the hypothesis that functional polymorphisms leading to lower detoxification activity of GSTP1 and GSTA1 may contribute to the predisposition of breast cancer patients to radiation-induced fibrosis.

On the other hand, TGFβ1 is a multifunctional growth factor with many different effects on cell proliferation, tissue differentiation, inflammation, and fibrosis (32, 33). Contrasting results have been reported on the impact of TGFβ1 C-509T and C869T polymorphisms on normal tissue radiosensitivity, and either an increased risk or a lack of association has been reported for both variants (14, 23). Our results are in line with two recent studies showing an association of the Pro allele in TGFβ1 codon 10 (869C) with a lower grade of radiation fibrosis in nasopharyngeal cancer patients (34) and a reduced risk of radiation pneumonitis in patients with non-small-cell lung cancer (35).

Inasmuch as interindividual variability in normal tissue radiosensitivity is a polygenic trait, which is probably influenced by several polymorphic variants, we also evaluated the combined effect of GSTP1 Ile105Val, GSTA1 C-69T, and TGFβ1 C869T polymorphisms on the risk of developing radiation-induced fibrosis. The concomitant presence of TGFβ1 869TT genotype, GSTP1 105Val, and GSTA1 -69T alleles emerged as a better predictor of radiosensitive status of patients than did SNPs. It is noteworthy that the association of the three combined polymorphisms remained significant after Bonferroni's correction



**Fig. 1.** Occurrence of moderate to severe fibrosis (Grade 2–3) in breast cancer patients according to the number of concomitant genetic risk factors (GSTP1 105Val and GSTA1 -69T alleles, TGFβ1 869TT genotype). See text for statistical analysis.

(threshold of significance required for Bonferroni correction for eight polymorphisms analyzed,  $p = 0.0063$ ).

Previous studies have reported a significant association between endothelial variant nitric oxide synthase eNOS G894T (18) and TP53 Arg72Pro polymorphism (19) and the risk of developing radiation-induced telangiectasia in breast cancer patients; however, we are unaware of any currently available data on the impact of these two polymorphisms on the risk of the development of fibrosis after radiotherapy. In the present study, a trend was observed for the G894T variant of the (eNOS) toward a lower risk for the development of radiation-induced fibrosis (OR 0.478; 95% CI, 0.215–1.065;  $p = 0.071$ ). This finding supports the possibility that a lower ability to generate ROS, as expected in eNOS 894T carriers (36), could result in a lower risk of late radiation reactions of normal tissues. However, larger prospective studies are needed for a conclusive evidence of association between eNOS G894T polymorphism and radiation-induced fibrosis in breast cancer patients. Furthermore, we failed to confirm a previously reported association of XRCC1 Arg399Gln polymorphism with the risk of radiation-induced subcutaneous fibrosis in breast cancer patients (13); however, this association was also not confirmed in a larger subsequent study (37).

## Limitations of the study

Our findings should be interpreted in the light of the following limitations. Given that normal tissue reactions such as skin fibrosis and telangiectasia develop gradually after a latency period, it is possible that not all patients have expressed their final level of radiotoxicity. However, follow-up of this cohort will continue, so reanalyses of late normal tissue complications at 5 years will be possible for all the patients. Second, the present study was underpowered to detect SNPs with a modest impact on fibrosis risk. Therefore, larger studies with sufficient statistical power are required for conclusive evidence of lack of association. Finally, further investigations based on a larger number of candidate polymorphic genes are strongly warranted for more comprehensive identification of a clinically useful SNP profile that will identify patients at greater risk for the development of radiation injury.

## Conclusion

In summary, in the present study we found a significant association between GSTP1 Ile105val, GSTA1 C-69T, and TGF $\beta$ 1 T869C genes and the risk for the development of radiation-induced fibrosis in breast cancer patients. In addition, the concomitant presence of GSTP1 105 val, GSTA1 -69T, and TGF $\beta$ 1 869TT emerged as a better predictor of the radiosensitive status of patients than did SNPs. These results support the notion that normal tissue radiosensitivity is a polygenic trait dependent on the combined effect of several polymorphic genes and suggest that approaches based on multiple genetic markers could have the potential to predict normal tissues responses after radiotherapy.

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