Retention of the p53 Codon 72 Arginine Allele Is Associated with a Reduction of Disease-Free and Overall Survival in Arginine/Proline Heterozygous Breast Cancer Patients

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ABSTRACT

Purpose: The arginine to proline substitution at codon 72 represents a common aminoacidic polymorphism of the p53 protein. Recent data suggest that p53 codon 72 may modulate the response to cancer therapy. The aim of this study was to test the hypothesis that the p53 codon 72 genotype, evaluated in the tumor tissue and in the disease-free lymph node, is related to differences in disease-free and overall survival among breast cancer-affected patients.

Experimental Design: We assessed the p53 codon 72 genotype in DNA from disease-free lymph nodes and neoplastic tissues obtained from 67 women affected by breast cancer who underwent surgical resection at the Bologna Breast Cancer Surgical Unit from 1993 to 1995.

Results: We found that the retention of the p53 codon 72 arginine allele in the tumor tissue of proline/arginine heterozygous breast cancer patients is associated with statistically significant reduced disease-free and overall survivals.

Conclusions: Our findings suggest that the genotyping for p53 codon 72 locus in both the tumor tissue and in the lymph node of breast cancer patients could contribute to identify a subset of arginine/proline heterozygous patients who have a reduced survival that is associated with the specific retention of the arginine allele in the tumor tissue.

INTRODUCTION

The p53 gene is characterized by a common polymorphism because of an arginine to proline aminoacidic substitution at codon 72 (1). The two alleles are quite common in a variety of populations (2–4). Functional data suggest that the two variants are not biochemically equivalent (5), because they differ in the capability to bind the transcription machinery component TAF30, to physically interact with p73, to be targeted to the proteasome, and to modulate the susceptibility to apoptosis in a variety of experimental systems (1, 5–9).

Case-control studies have suggested that p53 codon 72 polymorphism modulates the susceptibility to various malignancies, including breast cancer, but contrasting data have been obtained thus far (3, 10–12).

Recent studies found that the p53 codon 72 arginine allele is preferentially retained in the neoplastic tissue of arginine/proline heterozygous patients affected by common carcinomas, such as vulval, esophageal, urinary tract, and lung cancer (13–17). Furthermore, in at least the case of head and neck cancer, the presence of an arginine-mutated allele was found to be related with a reduced sensitivity to cancer therapy (18).

On the basis of this literature the aim of this study was to test the hypothesis that p53 codon 72 genotype, evaluated both in the tumor tissue and in the disease-free lymph node, is related to disease-free survival and overall survival of breast cancer-affected patients.

MATERIALS AND METHODS

Patients. Sixty-seven primary invasive breast carcinomas collected from 1993 to 1995 at the Bologna Breast Cancer Surgical Unit were included in this study. Patient mean age was 60.3 ± 12.6 years (age range, 28–86 years). Seventeen patients underwent conservative breast surgery followed by radiation therapy, and 50 underwent radical mastectomy. Systemic chemotherapy (cisplatinum, methotrexate, and 5-fluourouracile) was given to 26 patients. Hormonotherapy (tamoxifen, 20 mg daily) was given to 35 patients. The median follow-up of the living patients was 109 months (range, 53–120 months).

Samples. The tumors were histologically classified and staged according to WHO criteria and Union Internationale Contre le Cancer Tumor-Node-Metastasis system, respectively. Histologically, 60 cases were ductal not otherwise specified carcinomas, 6 were classified as infiltrating lobular carcinomas, and 1 as mucoid carcinoma. Invasive ductal carcinoma and, when predominant, the ductal component of the mixed ductal/lobular type were histologically graded (G) following Elston and Ellis’s method.
The tumors were also typed by NG2 as follows: mild (NG1), moderate (NG2), and severe (NG3) nuclear atypia. Tumor size (pT) and the presence or absence of regional lymph node involvement (pN+ and pN0, respectively) was also recorded.

**Immunohistochemistry and Image Cytometry.** Serial sections from formalin-fixed, paraffin-embedded tissue blocks were collected on 3-aminopropyltriethoxy-silylane (Sigma Chemical Co., St. Louis, MO) coated slides, dried overnight at 37°C, and processed for immunohistochemistry according to a streptavidin-biotin-peroxidase preformed complex technique. The following MoAbs, all from BioGenex Laboratories (San Ramon, CA), were used: anti-ER clone 1D5, diluted 1:120; anti-PR clone 1A6, diluted 1:65; anti-Ki-67 protein clone Ki-67, diluted 1:100; and anti-ErbB-2 protein clone CB11, diluted 1:200. Microwave pretreatment was performed with citrate buffer solution (pH 6.0) using anti-ER, -Ki-67, and -PR MoAbs, or with EDTA buffer solution (pH 8.0) for anti-ErbB-2 MoAb (19).

Eight serial sections were obtained from each block of normal lymph nodes, histologically selected for DNA extraction. The first and last section (4-μm thick) were immunostained using an anti-wide spectrum cytokeratin MoAb, clone MNF116 (DakoCytomation, Glostrup, Denmark), diluted 1:160 with a Proteinase K treatment, to exclude micrometastasis.

Nuclear immunostaining of ER, PR, and Ki-67 was quantified by image cytometry with Cytometry software (C&V, Bologna, Italy) as detailed previously (19). A labeling index was obtained and expressed as the percentage of the labeled nuclear area of the total neoplastic nuclear area (%LIa), and was categorized using the following cutoff values: negative = ER and PR %LIa <10%, Ki-67 <20%Lia; and positive = ER and PR %LIa ≥10%, Ki-67 ≥20% Lla.

ErbB-2 overexpression was evaluated according to a semiquantitative method and categorized as follows: negative if ≤25% neoplastic cells were positive; and positive if >25% neoplastic cells were positive (20).

**DNA Extraction and Labeling.** Neoplastic DNA was extracted from 67 breast carcinoma tissue fragments. Part of each neoplastic tissue specimen was immediately frozen and stored at −80°C for subsequent DNA extraction using the Qiamp Tissue kit (Qiagen, GmbH, Hilden, Germany). Only samples showing a percentage of neoplastic cells >50% were used for DNA extraction, and both CGH and PCR analysis (21, 22). The percentage of neoplastic versus stromal and inflammatory cells was evaluated in the H&E-stained sections of each sample.

Normal DNA from each patient was obtained from noninfiltrated (cytokeratine negative) lymph nodes. Briefly, five to six sections, 10-μm thick, were cut from each sample. After deparaffinization by xylene and absolute ethanol washing, the sections were scraped by a sterile razor blade, put in tubes, and digested by Proteinase K (0.02 mg/ml) in 50–200 μl of DNA extraction buffer [Tris-EDTA (pH 8.0)] for 3 h at 55°C. The tubes were put in boiling water for 10 min to stop the reaction.

**CGH.** One μg of normal reference DNA and 1 μg of tumor DNA were labeled by nick translation. Normal DNA was labeled by Digoxigenin-11-dUTP (Roche, Basel, Switzerland) and tumor DNA by Biotin-16-dUTP (Roche), and analyzed as described previously (22).

In those cases in which CGH analysis was not available, the allele loss at p53 codon 72 locus was assessed by genotyping the p53 intron 1 VNTR, using primers p53VNTRFWS’-ACTCCACGCCCTGGGCCAAATAGAGCT-3’ and p53VNTRRW5’-ACA- AAACATCCCTACCAACAGC-3’ (10 pmol each) following a standard PCR protocol. The analysis allowed for the detection of six different alleles.3

**Genotyping.** p53 codon 72 alleles were amplified using the primer pair 5’-GCA GAGACC TGT GGG AAG CGA-3’ and 5’-ACC GTA GCT GCC CTG GTA GTT-3’. PCR cycling conditions were as follows: 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, for 31 cycles. The protocol was also carried out for 35 cycles by an independent operator to obtain a replicated assay for each sample. In no cases were discrepancies found between repeated samples. Fifteen μl of PCR products were digested with BsrUI restriction enzyme (New England Biolabs) as recommended by the supplier, and fragments were separated on 2% agarose gel. The *arginine* allele was identified by the presence of the restriction BsrUI enzyme recognition sites. In six samples, three for each genotype, p53 codon 72 genotype was confirmed by PCR amplification of p53 exon 4 (primers pairs: 5’-GCA AGAGACC TGT GGG AAG CGA-3’ and 5’-ACC GTAG CTGC CCGTG TGAGT-3’) followed by Automatic sequencing in a CEQ2000 Automatic Sequencer (Beckman).

**Statistical Analysis.** The relationship between a continuous and a categorical variable was tested using the unpaired *t* test. Fisher’s exact test was performed when differences in the distribution of categorical variables between groups were tested. DFS and OS were computed and compared using Kaplan-Meier and log-rank (Mantel-Cox) tests, respectively. Prognostic significance was evaluated using Cox proportional hazards model.

OS was calculated from the date of surgery to last contact if alive (censored) or death (event). DFS was calculated from the date of surgery to last contact if alive (censored) or to date of relapse or death, whichever come first (event). Patient death of other diseases was recorded, and patient follow-up was considered censored at this time. The analysis was performed in January 2003. The analysis was conducted using StatView 5.0 statistical software (SAS Institute Inc., Cary, NC).

**RESULTS**

p53 codon 72 genotype was assessed on DNA extracted from noninfiltrated (cytokeratine negative) lymph nodes and from the neoplastic tissue of 67 breast cancer patients. The analysis of the p53 codon 72 genotype on the DNA from the

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2 The abbreviations used are: NG, nuclear grading; MoAb, monoclonal antibody; ER, estrogen receptor; PR, progesterone receptor; %Lia, percentage labeling index area; CGH, comparative genomic hybridization; VNTR, variable number of tandem repeats; DFS, disease-free survival; OS, overall survival; AR, allelic retention.

3 M. Bonaţe, C. Barbi, F. Olivieri, E. Marzi, and C. Franceschi, unpublished observations.
lymph node tissue revealed 6 (8.9%) proline homozygotes (Pro/Pro), 29 (43.3%) arginine/proline heterozygotes (Arg/Pro), and 32 (47.8%) arginine homozygotes (Arg/Arg). In 9 of 29 Arg/Pro patients, only 1 allele was detected in the DNA from the neoplastic tissue: in 7 cases it was the arginine one, and in 2 cases it was the proline one. In these patients, the loss of heterozygosity at the p53 locus was confirmed by CGH or by p53 intron 1 VNTR genotyping (data not shown).

The clinical-pathological features, namely tumor size (pT), nodal involvement (pN), grading (G), NG, and biopathological parameters, namely ER, PR, ErbB-2, and Ki-67 expression were evaluated in the group of patients who retained the arginine allele in the tumor tissue (n = 7, named as ALp53Arg) and in those who showed no changes between the genotype in the lymph node and in the tumor tissue (n = 58, named as GLp53; Table 1). No significant differences in the distribution of clinical and biopathological parameters between ALp53Arg and GLp53 patients were found. Interestingly, all of the ALp53Arg tumors were found to be positive for ER staining even if this difference was not significant (P = 0.06; Table 1).

The survival data of ALp53Arg and GLp53 were analyzed according to Kaplan-Meier method. An extreme reduction in the DFS and OS was found in ALp53Arg patients compared with GLp53 patients (DFS = 14.3% versus 65.0% censored, log rank test; P = 0.003; Fig. 1A; OS = 28.6% versus 68.3% censored; P = 0.005; Fig. 1B).

The prognostic significance of the p53 arginine AR was then evaluated by Cox proportional hazards regression analysis, in which age, as well as clinical and bio-pathological parameters, were considered as covariates (Table 2). Simple regression analysis suggested that AR is a significant prognostic factors for both OS (P = 0.005) and DFS (P = 0.009), and multivariate regression analysis confirmed that AR is an independent prognostic factor for DFS (P = 0.039) and OS (P = 0.032). In addition, multivariate regression analysis showed that pN is the most significant prognostic factor (DFS, P = 0.005; OS, P = 0.007), whereas Ki-67 staining reaches a borderline significance (DFS, P = 0.058; OS, P = 0.057) in the patient series.

**DISCUSSION**

In this investigation we found that the retention of the arginine allele at p53 codon 72 locus in arginine/proline heterozygous breast cancer-affected women (ALp53Arg patients) is
associated with reduced DFS and OS. In this regard Cox regression analysis indicates that the retention of the *arginine* allele affects OS and DFS independently from other, more established prognostic factors, such as the presence of lymph node metastasis and Ki-67 staining. Interestingly we found also that all of the *Alp53* Arg patients are positive for ER expression, and that *Alp53* Arg allele retention (AR) on disease-free survival (DFS) and overall survival (OS) is significant (Table 2).

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<th>Variable</th>
<th>DFS $\chi^2$ value</th>
<th>DFS p value</th>
<th>OS $\chi^2$ value</th>
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Univariate analysis

Multivariate analysis

Table 2 Cox regression analysis for the prognostic significance of the arginine allele retention (AR) on disease-free survival (DFS) and overall survival (OS)

In conclusion, genotyping for *p53* codon 72 locus in both the tumor tissue and in the lymph node of breast cancer patients contributes to identify a subset of *arginine/proline* heterogeneous patients who, despite the fact that they seem to display the same clinical features as the rest of the patients at the time of the diagnosis, have a reduced survival, which is associated with the specific retention of the *arginine allele* in the tumor tissue.

REFERENCES


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