

## **Association between the Estrogen Receptor $\alpha$ A908G Mutation and Outcomes in Invasive Breast Cancer**

Matthew H. Herynk,<sup>1</sup> Irma Parra,<sup>1</sup> Yukun Cui,<sup>1,2</sup> Amanda Beyer,<sup>1</sup> Meng-Fen Wu,<sup>1</sup> Susan G. Hilsenbeck,<sup>1</sup> and Suzanne A.W. Fuqua<sup>1</sup>

**Abstract Purpose:** Estrogen receptor  $\alpha$  (ER $\alpha$ ) predicts the natural history of breast cancer without intervening therapy. Here, we have optimized the detection of a somatic mutation, an A908G transition of ER $\alpha$ , and examined its association with clinical and biological features of invasive breast cancer.

**Experimental Design:** We compared two methods of sequencing to detect the A908G ER $\alpha$  mutation. We then used primer extension sequencing with genomic DNA isolated from invasive breast tumors to determine whether the mutation was associated with clinical outcome in 267 axillary node – negative and axillary node – positive breast tumors. The presence of the mutation and clinical variables were analyzed for association with recurrence-free survival and overall survival by Cox proportional hazards regression models.

**Results:** We determined that dye-labeled terminator sequencing was not adequate for detection of the A908G ER $\alpha$  mutation. The mutation was detected at a high frequency (50%) in invasive breast tumors using primer extension sequencing, and was found to be associated with clinical measures of poor outcome, including larger tumor size and axillary lymph node positivity. Although the mutation was associated with recurrence-free survival in univariate analysis, it was not an independent predictor of outcomes in multivariate analysis.

**Conclusions:** Consistent with our previous finding of this somatic ER $\alpha$  mutation in breast ductal hyperplasias, we now present evidence that the A908G mutation is present in invasive breast tumors using an optimized sequencing method. We find that the mutation is significantly associated with aggressive biological tumor features, and with an unfavorable prognosis, but was not an independent prognostic marker in untreated patients.

Estrogens play a crucial role in regulating the growth and differentiation of normal breast epithelium and breast cancers, with many of these cancers dependent on these hormones for their growth. Estrogens affect cellular processes by binding to their cognate receptors, estrogen receptors (ER)  $\alpha$  and  $\beta$ , which function as transcription factors mediating the mitogenic effects of estrogen. ER $\alpha$  expression in normal breast epithelium is generally low; however, significantly higher expression has been reported in premalignant lesions (1), with the majority of breast tumors expressing both receptors (2, 3). Because prolonged endogenous estrogen exposure is a potential risk

factor for invasive breast cancer (4), we originally hypothesized that overexpression of ER $\alpha$  or the emergence of mutated receptors could be early events in tumor progression (5). Subsequently, using manual genomic Sanger dideoxysequence analysis, we identified an A to G somatic mutation at ER $\alpha$  nucleotide 908 (A908G) from several usual ductal hyperplasias, which are early premalignant lesions. This mutation results in a lysine to arginine transition at residue 303 (K303R ER $\alpha$ ; ref. 6). To date, no other ER $\alpha$  mutation has been identified in more than a few invasive breast cancers (for a review, see ref. 7).

Dye-labeled terminator genomic automated fluorescent sequencing has been used to screen for the A908G ER $\alpha$  mutation in human breast specimens from women in the United States and Japan, but the mutation was not detected using this methodology (8–10). In another recent study, the mutation was detected, but at a low frequency, using single strand conformation polymorphism analysis, confirming our preliminary data that it was indeed present in invasive tumors (11). In the current study, we first compared two genomic sequencing approaches [dye-labeled terminator automated fluorescent sequencing (12) and primer extension sequencing (13)], and then extended the study using primer extension sequencing.

Our first objective was to determine the optimum sequencing method for detection of the mutation, and then to determine its prognostic utility in untreated patients. Our overall purpose

**Authors' Affiliations:** <sup>1</sup>Breast Center, Baylor College of Medicine, Houston, Texas and <sup>2</sup>College of Life Science, Zhejiang University of Science and Technology, Hangzhou, China

Received 10/30/06; revised 3/8/07; accepted 3/12/07.

**Grant support:** National Cancer Institute R01 CA58183 and Department of Defense Fellowship DAMD17-02-1-0278 (Y. Cui) and DAMD17-03-1-0417 (M.H. Herynk).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Suzanne A.W. Fuqua, Breast Center, Baylor College of Medicine, One Baylor Plaza, 1220 Alkek, Houston, TX 77030. Phone: 713-798-1600; Fax: 713-798-1673; E-mail: sfuqua@bcm.edu.

© 2007 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-06-2608

was to determine whether the A908G mutation was associated with clinical outcome and natural history in a cohort of breast cancers without intervening adjuvant therapies. We present results showing that the presence of the mutation identifies a subgroup of women with a worse outcome.

### Materials and Methods

**Patient population and tumor specimens.** In this study, we used 267 invasive breast cancers obtained from women in the United States, and maintained in an archived tumor bank of the Breast Center at Baylor College of Medicine. The patients in this study were derived from a prospectively assembled tumor bank. Tumor samples were archived in the form of formalin-fixed, paraffin-embedded medium density tissue microarrays as described (14). All samples were originally stored as fresh frozen tissues, and were fixed and arrayed relatively recently (2001). At the time of DNA extraction, the microarrays were ~4 years old. Patients were diagnosed between 1973 and 1993 with primary breast cancer and were treated with mastectomy or lumpectomy plus axillary dissection, with or without postoperative radiation therapy; none of the women underwent adjuvant tamoxifen therapy. Tumor and clinical characteristics of the cohort are summarized in Table 1. The median follow-up time was 76 months, and the tumors have previously been described as part of our Program Project database (15). This study was approved by the Baylor College of Medicine Institutional Review Board according to NIH guidelines.

**Other biological factors.** Several biomarkers have been previously measured on the tumor samples used in this study. Total ER and progesterone receptor (PR) protein levels were measured by ligand-

binding assay, and PR-A and PR-B protein isoforms were measured by immunoblot analysis as described elsewhere (16). For the ligand-binding assay, tumors with an ER content of at least 3 fmol/mg protein, and with a PR content of at least 5 fmol/mg protein, were considered positive. ER $\alpha$  protein status was also determined by immunohistochemistry using the ER-6F11 antibody from Novocastra as described previously; Allred scores of 3 to 8 were considered positive for ER $\alpha$  expression (17). AIB1 levels were previously determined by immunoblot, with high levels associated with a better outcome in untreated patients (18). S-phase fraction was calculated by flow cytometry at the time of original tissue collection (19); cases were classified as low (<6% S phase), intermediate (6-10% S phase), or high (>10% S phase). Levels of HER2 were also determined by immunohistochemistry as previously described using a semiquantitative estimate of the proportion of positive staining on the entire slide with estimates ranging from 0 to 4, and higher values correlated with shorter disease-free survival (20).

**Tumor DNA isolation, PCR amplification, and sequencing.** Tumor DNA was isolated from a 2- $\mu$ m-thick, 0.6-mm core tissue microarray using Qiagen DNeasy Tissue kits according to the manufacturer. The primer sequences used for PCR amplification and sequencing are shown in Table 2. For PCR amplification of ER $\alpha$ , an initial amplification using primers ER $\alpha$  1 and 2 was done with a denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 60°C for 30 s, and primer extension at 72°C for 30 s. Upon completion of the cycling steps, a final extension at 72°C for 5 min was done before the reaction was stored at 4°C. To remove unincorporated PCR primers and deoxynucleotide triphosphates from the PCR amplification, the samples were treated by adding 2 units of exonuclease I (U.S. Biochemical), and 5 units of shrimp alkaline phosphatase (Roche Applied Science), for 1 h at 37°C and

**Table 1.** Clinical characteristics of breast cancer cases and by A908G ER $\alpha$  mutation status

	All (n = 267)	Mutant (n = 133; %)	WT (n = 134; %)	P*
Age (y)				
≤50	72	37.5	62.5	0.015
>50	195	54.4	45.6	
Tumor size (cm)				
0-2	89	36.0	64.0	0.002
>2-5	149	54.4	45.6	
>5	27	70.4	29.6	
Missing	2			
Nodes				
Node negative	161	34.8	65.2	<0.0001
Node positive				
1-3	60	70.0	30.0	
>3	46	76.1	23.9	
S phase				
Low (0% to <6%)	61	41.0	59.0	0.256
Intermediate (≥6% to ≤10%)	62	53.2	46.8	
High (>10%)	101	53.5	46.5	
Missing	43			
Ploidy				
Diploid	91	49.5	50.5	0.732
Aneuploid	143	51.7	48.3	
Missing	33			
ER (fmol/mg)				
Negative (<3)	51	49.0	51.0	0.900
Positive (≥3)	216	50.0	50.0	
PR (fmol/mg)				
Negative (<5)	108	50.0	50.0	0.791
Positive (≥5)	149	48.3	51.7	
Missing	10			
Median follow-up time (mo)	76	72	78	

\* $\chi^2$  test.

**Table 2.** Primer sequences for PCR amplification, dye-labeled terminator sequencing, and primer extension sequencing (*SNaPshot*)

Primer	Use	Sequence (5' to 3')
ER $\alpha$ 1	Forward PCR	ACATGAGAGCTGCCAACCTT
ER $\alpha$ 2	Reverse PCR	GGAATAGAGTATCGGGGGCT
ER $\alpha$ 3	Forward extension	TTCATGATCAAACGCTCTAAGA
ER $\alpha$ 4	Reverse extension	ACAAGGCCAGGCTGTTC

15 min at 80°C. These PCR products were used for both dye-labeled terminator and primer extension sequencing.

A negative control consisting of a PCR reaction without genomic DNA to ensure that no contaminating DNA was present, and a positive control of wild-type (WT) ER $\alpha$  genomic DNA from MCF-7 human breast cancer cells (previously determined to be WT sequence; ref. 6), were run in parallel with all tumor PCR reactions. Plasmids containing either WT or the mutant A908G ER $\alpha$  sequence (6) were used for PCR amplification in DNA mixing experiments to compare the dye-labeled terminator and primer extension sequencing methods.

Dye-labeled terminator automated fluorescent sequencing was done with an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (Perkin-Elmer/Applied Biosystems Division) according to manufacturer's recommendations using either the ER $\alpha$  1 primer (forward) or the ER $\alpha$  2 primer (reverse). Primer extension sequencing was done using the ABI PRISM *SNaPshot* sequencing method (Perkin-Elmer/Applied Biosystems Division), which involves the extension of a primer that ends one nucleotide 5' of the ER $\alpha$  908 nucleotide, using fluorescently labeled dideoxynucleotide triphosphates. The ER $\alpha$  3 (forward) and the ER $\alpha$  4 (reverse) primers were used for the extension *SNaPshot* reactions. All fluorescent sequencing products were analyzed on an ABI PRISM 310 Genetic Analyzer capillary sequencer (Perkin-Elmer/Applied Biosystems Division). Data were analyzed with the ABI Gene Scan software package. Manual verification of sequencing results was also done. All tumor DNAs were first sequenced using *SNaPshot* in the reverse direction. To confirm this result, another aliquot of DNA from mutation-positive tumors was then PCR amplified again and resequenced using *SNaPshot* in the forward direction. Only those tumor samples with the A908G mutation detected on both the reverse and forward strand with *SNaPshot* sequencing were considered mutation positive.

**Statistical methods.** Descriptive statistics were used to summarize tumor and clinical characteristics by mutation status. Differences between WT and A908G ER $\alpha$  mutation were compared using the  $\chi^2$  test. Associations between mutation status and biological factors, for which the sample size was smaller, were assessed using Fisher's exact test. Comparisons of ER positivity rates between immunohistochemistry and ligand-binding assay were made using the McNemar's test.

Recurrence-free survival (RFS) was calculated from the time of diagnosis to the date of the first proved recurrence, or censored at last follow-up or death not due to cancer. Overall survival (OS) was calculated from the time of diagnosis to death from any cause or censored at last follow-up. Follow-up was truncated at 120 months for purposes of plotting. Survival curves were estimated by the Kaplan-Meier method and compared using the log-rank test.

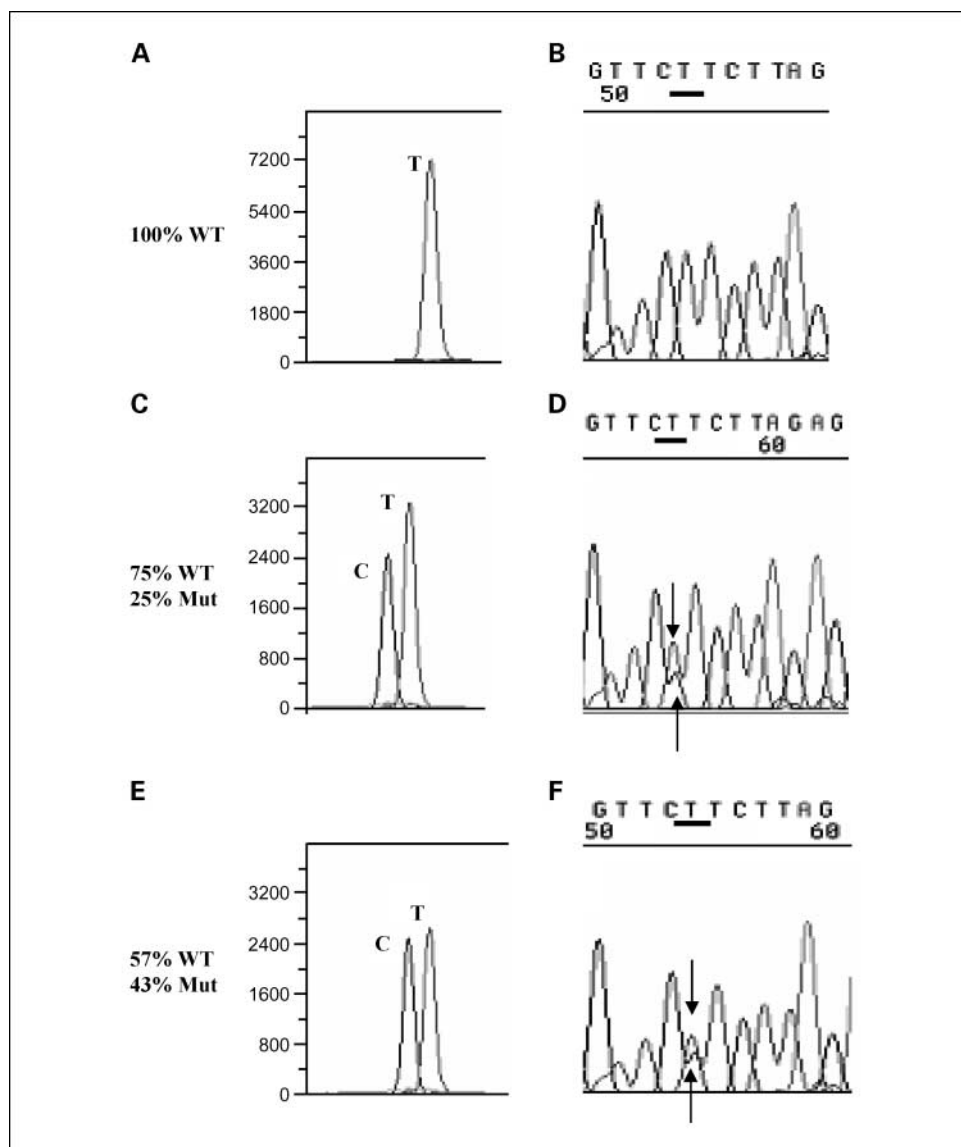
Cox proportional hazards regression was used to assess the associations between clinical characteristics and RFS or OS. Factors found to be significant as single exploratory variables were entered into a multivariate Cox model (21). Mutation status and clinical characteristics included in the model were categorized as indicated in Table 1. The assumption of proportional hazards was tested for each candidate exploratory variable by incorporating a time-dependent interaction into the Cox regression models. As has been reported previously by us (22) and others, ER was found to violate the assumption of proportional hazards for RFS. To correct for this in the context of the Cox model, we

therefore opted for a Cox regression model incorporating a time-dependent covariate for ER. As an alternative approach, we constructed accelerated failure time models with various distributions (Weibull, log normal, etc.) assumed for the failure time. All accelerated failure time models yielded essentially identical results to the Cox regression model (data not shown).

## Results

*SNaPshot* primer extension sequencing is sensitive for detection of the A908G ER $\alpha$  mutation. We compared two sequencing approaches, *SNaPshot* primer extension sequencing and dye-labeled terminator sequencing, using primers oriented in the reverse direction, because we had previously observed that reverse-strand DNA sequencing was most robust (data not shown). As a control for our ability to detect the mutation, we did mixing experiments of PCR-amplified DNA from either WT or mutant-containing plasmids (Fig. 1), simulating all the experimental conditions to be used for the clinical samples. In this experiment, we sequenced DNA series by varying the ratio of mutant DNA to WT DNA. As expected, 100% WT DNA showed a single *SNaPshot* T reverse sequence base peak (A), and the correct automated base call of T using dye-labeled terminator sequencing (B). With 25% or 43% mutant-containing DNAs, both WT and mutant bases were correctly genotyped using *SNaPshot* (C and E, respectively). However, in these 25% and 43% mutant-containing DNA mixes, dye-labeled terminator sequencing called the samples homozygous WT A (*top arrows*, D and F), although a mutant C peak was visible in the chromatograms (*bottom arrow*); the peak height of the WT T base was visibly reduced due to the presence of two bases at this position. As expected, the *SNaPshot* method was not a quantitative procedure, but rather a yes/no base call (C and E), compared with the automated fluorescent sequence algorithm which miscalled or missed the smaller mutant peak. When WT and mutant DNAs were equally present, dye-labeled terminator sequencing correctly called a mutant C base, but the WT T base was visible under the C peak (*arrow*, H). In all of the series, even with 76% mutant-containing DNA mixes (I), *SNaPshot* correctly identified both WT and mutant peaks. We conclude that the ER $\alpha$  A908G base peak heights were reduced in the dye-labeled terminator sequencing profiles. One complication with the dye-labeled terminator technology is that the pattern of termination can be nonuniform from differential incorporation of the dideoxyterminators, resulting in uneven peak heights (12, 23).

To further compare the two methods, genotyping data obtained from DNA of a representative invasive ductal tumor are shown in Fig. 2. We compared the two sequencing approaches using sequencing primers oriented in both the forward (A and C) and reverse directions (B and D) so as to sequence both DNA strands of the tumor. The tumor exhibited heterozygosity at ER $\alpha$  908 with a WT sequence (A nucleotide) and the mutated base (G nucleotide) using the *SNaPshot* sequencing method on the forward strand (A). The reverse strand *SNaPshot* sequencing data confirmed heterozygosity at the 908 ER $\alpha$  residue (B). Interpretation of the *SNaPshot* sequencing data for this tumor was straightforward because a positive/negative answer on heterozygosity was obtained using this method. The dye-labeled terminator sequencing of this heterozygote tumor revealed only the WT A nucleotide



**Fig. 1.** Experiment varying the amount of A908 WT and mutant A908G ER $\alpha$  plasmid DNAs, which were sequenced using *SNaPshot* (A, C, E, G, I, K) and dye-labeled terminator (B, D, F, H, J, L) with reverse sequencing primers. The TTCT (WT) and TTCC (Mut) base pair combination (*overlined*) and the WT or mutant C 908 reverse strand bases (*underlined*).

sequence on the forward strand (C, *arrow* and *underlined*), and a WT T nucleotide in the sequencing reaction of the reverse strand (D, *arrow* and *underlined*). We conclude that the ER $\alpha$  A908G mutation can occur in invasive breast tumors, and that the primer extension method is more sensitive for detection of the mutation at this location within ER $\alpha$ .

**The A908G ER $\alpha$  mutation and correlations with other clinical and biological variables.** We next did *SNaPshot* sequencing on primary breast tumors from a cohort of 267 untreated patients with known clinical outcomes with the goal of examining the role of the mutation in the natural history of these patients (Table 1). Most patients were >50 years of age and had tumors that were 2 to 5 cm in size with an intermediate to high S-phase fraction. Approximately 40% of the cases were node-positive at diagnosis, a frequency that is consistent with the Surveillance Epidemiology and End Results population-based database.<sup>3</sup>

Most of the tumors were also aneuploid, and ~81% of tumors expressed ER and 58% expressed PR (as determined by ligand-binding assays) at the time of diagnosis.

Tumor DNAs were first sequenced in the reverse direction using *SNaPshot*, and then all of the mutation-positive cases were confirmed using sequencing of the forward DNA strand. Only tumors with the A908G mutation detected on both strands were considered positive. The mutation was detected in 133 of 267 (49.8%) of the breast tumor samples; the distribution of clinical variables of tumors containing either the WT sequence and the A908G mutation are also shown in Table 1. When we first reported the mutation in premalignant breast lesions, we hypothesized that the mutation might confer a selective advantage in postmenopausal women due to its ability to respond with increased proliferation to the low levels of hormone present in postmenopausal women (6). In this cohort of patients with invasive lesions, we found the mutation to be significantly more frequent in women >50 years of age (54.4% versus 37.5%,  $P = 0.015$ ). This result is consistent with our hypothesis that the mutation may play a

<sup>3</sup> <http://www.seer.cancer.gov/seerstat>

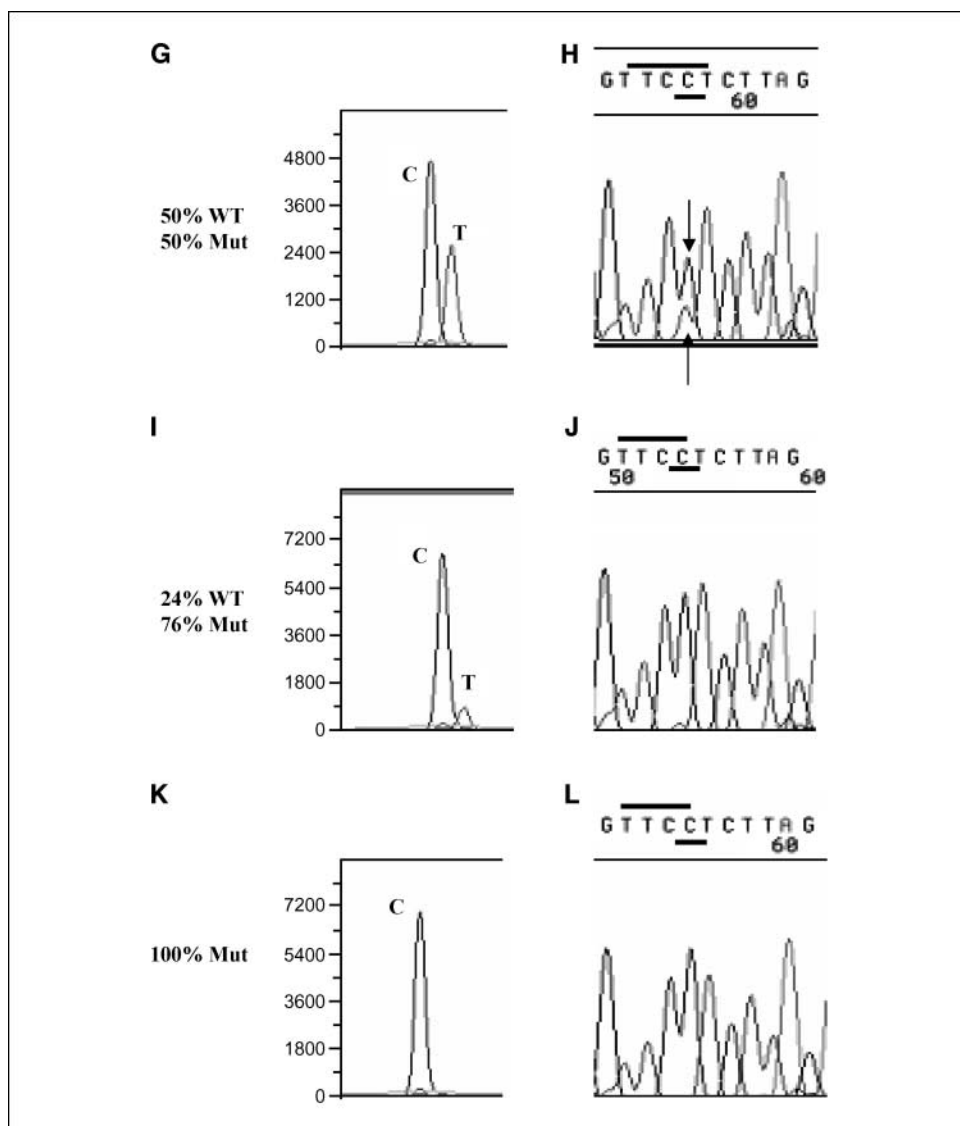


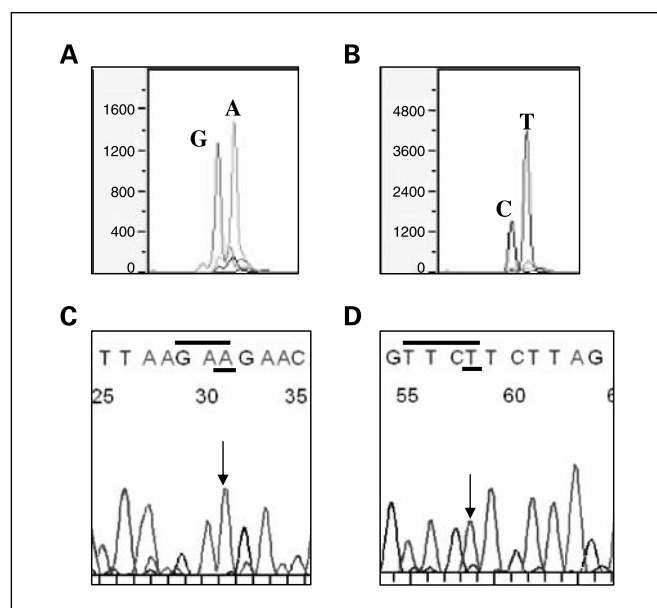
Fig. 1 Continued.

role in postmenopausal women, but might be of less importance in the biology of tumors from premenopausal women.

Similarly, we predicted that if the A908G ER $\alpha$  mutation indeed conferred a selective advantage, it might be present in tumors from patients with worse outcomes (24, 25). In a small pilot study that we previously did using manual Sanger dideoxysequencing of 50 invasive tumors, we found that the mutation was more frequent in lymph node-positive than node-negative tumors (26). The higher frequency of the mutation in node-positive tumors in the present study (70% and 76.1% versus 34.8% in node-negative,  $P < 0.0001$ ) is consistent with our earlier results. Furthermore, in the current study, we found that the presence of the mutation was significantly associated with larger tumor size ( $P = 0.002$ ; Table 1). Both axillary lymph node positivity and larger tumor size are established clinical variables associated with a poorer outcome (27). The mutation was not associated with S-phase fraction, ploidy, or with the levels of ER or PR as measured by ligand-binding assay. Thus, those tumors that contained the

mutation were associated with worse clinical characteristics compared with tumors with only WT ER $\alpha$ .

The majority of cases were clinically ER positive by ligand-binding assay for both the mutant and WT (Table 1). Immunohistochemistry data was also available on a subset of the tumors (Table 3). As classified by immunohistochemistry, fewer patients in the mutant-positive group were classified as being ER $\alpha$  positive (56.4% versus 73.8%, respectively; Table 3). When comparing immunohistochemistry to ligand-binding assay, ER positivity rates were similar by both methods in WT tumors ( $P = 0.77$ , McNemar's test), whereas the rate of ligand-binding assay ER positivity was higher than immunohistochemistry detected ER positivity in mutant tumors ( $P < 0.01$ , McNemar's test). These results suggest several possibilities. Perhaps the immunohistochemistry method is not as sensitive for detection of the mutant protein, or alternatively the localization of the mutant protein may be different from WT (because only nuclear protein is classified as ER-positive using immunohistochemistry, whereas total ER protein is measured in ligand-binding assays). We and others have shown that the



**Fig. 2.** *SNaPshot* sequencing of a representative invasive breast tumor in the forward direction (A) and reverse direction (B). The same tumor DNA sequenced using dye-labeled terminator in the forward direction (C) and the reverse direction (D). The WT nucleotide at residue 908 in the forward direction is A, and the reverse direction it is T. The mutant residues is a G (forward direction) or a C (reverse direction). Arrow, 908 base call using the automated fluorescent technique.

mutation resides adjacent to a protein kinase A and p21-activated kinase 1 phosphorylation site at ER $\alpha$  S305 (24, 28) and that phosphorylation of the receptor may affect its localization in the cell (29). Regardless of the reason for this discordance in ER $\alpha$  status, however, the results suggest that the mutation can occur in ER-negative cases. It is known that ER $\alpha$  can be lost during tumor progression; ~18% of recurrences present as ER negative when the primary lesion was ER positive (30). Therefore, the mutation may play a role early during tumor progression in some tumors, whereas loss of ER $\alpha$  expression is a later event as has been seen in HER2 and BRCA-1 model systems (31, 32).

The mutation was not correlated with ER $\beta$  levels, the PR-A isoform, or the proliferation marker Ki67 (Table 3). However, there was a weak but nonsignificant correlation between the mutation and the absence of the PR-B isoform ( $P = 0.057$ ). This result is interesting in that we have previously reported that low PR-B levels were associated with a poorer outcome in tamoxifen-treated breast cancer patients (16). The presence of the mutation was also more frequent in HER2-positive tumors, but this association did not quite reach statistical significance ( $P = 0.055$ ). The significance of the potential associations with PR-B and HER2 are currently under study.

**A908G ER $\alpha$  mutation status and prognosis.** To examine the relationship between the mutation and prognosis, or natural progression of the disease, we included in our study only those patients who did not receive adjuvant therapy. The mutation was significantly associated with shorter time to recurrence (Fig. 3A, log-rank test,  $P = 0.002$ ); 10-year RFS was 46% [95% confidence interval (95% CI), 36-56%] for patients with the A908G mutation in their tumors compared with a RFS of 70% (95% CI, 60-78%) for WT. Age and PR status by ligand-binding assay were not prognostic in this group of patients (data not shown). However, several of the other

clinical variables investigated in this study had statistically significant associations with a worse RFS, including tumor size ( $P < 0.0001$ ), lymph nodes ( $P < 0.0001$ ), S-phase fraction ( $P = 0.029$ ), ploidy ( $P = 0.024$ ), and ER status ( $P = 0.014$ ). ER was found to violate the assumption of proportional hazards, and to adjust for this we also added a time-dependent term. When the A908G mutation status and these other variables were included in a Cox multivariate analysis, only tumor size, lymph nodes, and ER status were significantly associated with RFS and remained in the model (Table 4). The model revealed that the mutation was not associated with poor RFS after adjusting for tumor size, lymph nodes, ER status, and a time-dependent covariate of ER status (Table 4;  $P = 0.526$ ). In this final model, positive nodes  $>3$  (hazard ratio, 3.28; 95% CI, 1.97-5.44), tumor size  $>5$  cm (hazard ratio, 2.65; 95% CI, 1.40-5.03), and ER negative (hazard ratio, 4.37; 95% CI, 2.16-8.85) were significantly associated with RFS. These results are consistent with previous observations that axillary lymph node status is a powerful prognostic factor in breast cancer (27).

Differences in OS between the ER $\alpha$  mutant and WT tumor groups (Fig. 3B;  $P = 0.098$ ) did not achieve significance, although disease-specific survival was significant when patients were censored at death from other causes or last follow-up ( $P = 0.0028$ , data not shown). This is because early deaths tend to be disease related, whereas later deaths often are not. Tumor size ( $P < 0.0001$ ), lymph nodes ( $P < 0.0001$ ), and S-phase fraction ( $P = 0.003$ ) were significantly associated with OS in the univariate Cox regression analyses. Thus, the A908G ER $\alpha$  mutation was not an independent prognostic factor for RFS or OS, most probably due to its clear association with tumor size and positive nodal status.

## Discussion

This is the first study to evaluate the clinical utility of the A908G ER $\alpha$  mutation in human breast cancer. Our data help to reconcile our earlier findings in hyperplasias (6), and these current results demonstrating the mutation in invasive breast cancers also help to reconcile results reported by others who used dye-labeled terminator sequencing methods for detection of the A908G ER $\alpha$  mutation (8–10). The mutation was clearly visualized in our current study using *SNaPshot* sequencing. It has been shown that there are reproducible peak height errors using the dye-labeled terminator sequencing method (33, 34), and that specific 3- or 4-bp combinations can affect base pair heights of the 3' base. For instance, the three-base combination GAA results in a small peak height for the 3' base (A; refs. 33, 35), and this three-base combination is the same sequence in the ER $\alpha$  908 WT forward direction (*sequence overlined* in Fig. 2C). Similarly, sequence-dependent incorporation may be complicating the discrimination of the mutant C base on the reverse strand as well. The sequence TTCC is problematic in that the 3' C peak can be smaller using dye terminator chemistry (34), and this is the same sequence preceding the mutation in the ER $\alpha$  reverse strand (*sequence overlined* in Fig. 2D). Conway et al. (36) have also reported the problem of using the dye-labeled terminator sequencing and restriction digestion methods for detection of this mutation. Thus, the identification of ER $\alpha$  908 heterozygote individuals may be difficult because of uneven peak heights, especially in

heterogeneous tumor samples where contaminating normal cells could further dilute the mutant signal as was reported by one group of investigators (9). Therefore, the dye-labeled terminator sequencing method may not be the most suitable method for discriminating the ER $\alpha$  A908G mutation in mixed tissue types.

We have previously reported that the A908G ER $\alpha$  mutation was present in ~30% of typical breast hyperplasias, a type of early but nonobligate premalignant breast lesion (6). Our demonstration herein that the mutation is present at a high frequency (~50%) in invasive breast cancers is suggestive that the mutation might play a role in cancer progression. Of course, a direct test of this hypothesis will require large epidemiologic studies of patients with premalignant lesions and long-term follow-up. It is known that at diagnosis, ~40% of patients with breast cancer will have histologic evidence of axillary lymph node involvement and that lymph node involvement is highly correlated with patient prognosis. Our data showing a statistically significant increase in the A908G ER $\alpha$  mutation in node-positive cancers provides supportive evidence that the mutation is correlated with poor prognosis, and in our univariate analyses, the mutation was significantly correlated with a worse outcome. The mutation may be a biomarker of increased risk in some tumors for tumor progression and metastatic dissemination, especially in postmenopausal women where cells that express the mutation might be at a growth advantage during the natural course of the disease. Tumor size and nodal status are independent prognostic factors, with nodal status being our most reliable prognostic factor (27). The mutation did not remain a significant independent variable in

our multivariate models, which weakens the effect of mutation as a single breast cancer prognostic biomarker. Clearly, the mutation does not independently predict RFS or OS in untreated patients. The failure of the mutation status to remain an independent prognostic variable in our multivariate models might reflect its strong relationship to nodal status and tumor size. Although Conway et al. (36) did not have follow-up available on their patients, they did find a significant correlation between the A908G ER $\alpha$  mutation and higher-grade breast tumors, suggestive that the mutation is associated with a more aggressive tumor type. The occurrence of the mutation in ER $\alpha$ -positive premalignant hyperplastic lesions (6), but also in more advanced lesions that may be ER negative, is reminiscent of reports from HER2 and BRCA1 mutant transgenic mouse models, where hormones can influence early tumorigenesis, whereas ER loss is a frequent event in the progression of these tumors (31, 32).

Another obvious difference between our data and some other reports is the racial background of the population studied; the A908G ER $\alpha$  mutation was not detected in Japanese breast cancers using genomic sequencing or RFLP analysis (9, 10). This disparity could also be related to the lower incidence of premalignant ductal hyperplasia and tumor ER $\alpha$  positivity in Japanese women (37), or other ethnic and hormonal differences in etiology between the two countries (38, 39). This interesting possibility certainly warrants further study.

It has been reported that the A908G ER $\alpha$  mutation can be detected using single strand conformation polymorphism followed by either manual radioactive sequence confirmation of tumors with abnormal band patterns or *SNaPShot*

**Table 3.** Correlation of the A908G ER $\alpha$  mutation with other biological variables

	All (n)	Mutant (%)	WT (%)	P*
ER $\alpha$ <sup>†</sup>				
Negative (0,2)	53	73.6	26.4	0.032
Positive (3-8)	133	56.4	43.6	
ER- $\beta$ <sup>‡</sup>				
Negative (<3)	44	77.3	22.7	1.000
Positive ( $\geq$ 3)	38	78.9	21.1	
PR-A <sup>‡</sup>				
Negative (0-<1)	47	83.0	17.0	0.282
Positive ( $\geq$ 1)	35	71.4	28.6	
PR-B <sup>‡</sup>				
Negative (0-<1)	49	85.7	14.3	0.057
Positive ( $\geq$ 1)	33	66.7	33.3	
HER2 <sup>§</sup>				
Negative (<2)	83	68.7	31.3	0.055
Positive ( $\geq$ 2)	21	90.5	9.5	
AIB1 <sup>  </sup>				
High (>1.61)	24	79.2	20.8	1.000
Low ( $\leq$ 1.61)	58	77.6	22.4	
Ki67 <sup>¶</sup>				
Negative ( $\leq$ 5%)	21	47.6	52.4	1.000
Positive (>5%)	40	50.0	50.0	

NOTE: A number of biomarkers have been previously studied on many of the cases included in this study; not all cases were assessed for all markers.

\*Fisher's exact test.

<sup>†</sup>ER $\alpha$  was previously determined by immunohistochemistry and contains both node-negative and node-positive tumors.

<sup>‡</sup>ER $\beta$  and PR-A and PR-B isoforms were previously determined by immunoblot analysis and contain only node-positive tumors.

<sup>§</sup>HER2 was previously determined by immunohistochemistry and contains only node-positive tumors.

<sup>||</sup>AIB1 was previously determined by immunoblot analysis and contains only node-positive tumors.

<sup>¶</sup>Ki67 was previously determined by immunohistochemistry and contains only node-negative tumors.

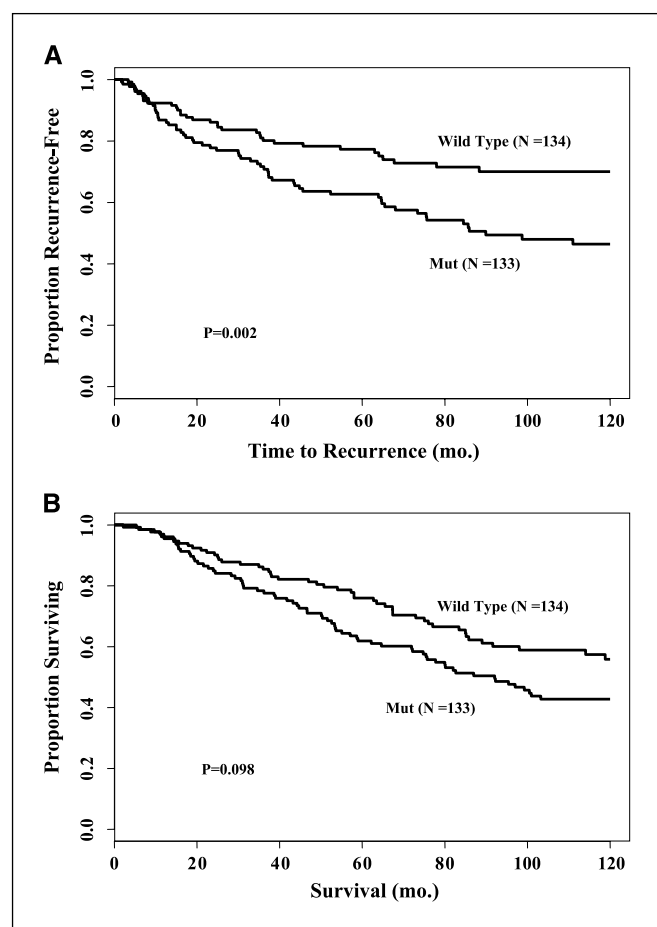
sequencing, but at a much lower frequency (5.7%; ref. 36). These authors also reported that the A908G ER $\alpha$  mutation may be more frequent in mixed lobular/ductal breast tumors, and they confirmed our earlier finding that the mutation represents a somatic change in the breast. We had insufficient lobular cancers in this data set to examine for histologic correlations with the A908G ER $\alpha$  mutation. We have similarly used single strand conformation polymorphism and a number of other screening techniques, such as oligonucleotide array hybridization and mismatch cleavage, to detect the A908G ER $\alpha$  mutation in clinical breast samples (data not shown). However, we did not find that these alternative genotyping methods (40–42) were preferable to *SNaPshot* because of the laborious optimization required and decreased mutation detection sensitivity with some of these, such as single strand conformation polymorphism (43). The true population-based estimate of the frequency of the A908G will await further validation studies using optimized sequencing methods.

There are few examples of genes involved in breast cancer that are mutated and confer a gain-of-function phenotype (hypersensitivity and enhanced substrate for phosphorylation), as we have shown for this mutation (6, 24). The high frequency of the A to G transition in ER $\alpha$  is intriguing. Because the majority of spontaneous mutations are single base pair changes

**Table 4.** Cox regression model of A908G ER $\alpha$  mutation with time-dependent covariate for ER on RFS

Variable	Hazard ratio (95% CI)	P
Mutation status		0.5258
WT	1.00	
Mutant	1.14 (0.76-1.71)	
Tumor size (cm)		0.0119
0-2	1.00	
>2-5	1.42 (0.92-2.18)	
>5	2.65 (1.40-5.03)	
Node no.		<0.0001
Node negative	1.00	
Node positive (1-3)	1.31 (0.81-2.11)	
Node positive (>3)	3.28 (1.97-5.44)	
ER		<0.0001
Positive ( $\geq 3$ )	1.00	
Negative (<3)	4.37 (2.16-8.85)	
Time-dependent ER*		0.0058
Positive ( $\geq 3$ )	1.00	
Negative (<3)	0.97 (0.94-0.99)	

\*Decreasing detrimental effect of ER negativity over time (months).



**Fig. 3.** Univariate survival analyses according to mutation status. Kaplan-Meier survival curves for proportion recurrence-free (A) and proportion surviving (B) for patients stratified by WT or mutant sequence in ER $\alpha$ . Number of events in each group and P values.

(44), it will be important to determine whether the A908G ER $\alpha$  transition is at a spontaneous mutation “hotspot,” or alternatively is driven by exogenous carcinogen exposures or endogenous DNA damage processes.

It is known that ER $\alpha$  is posttranslationally modified by protein acetylation and phosphorylation by a number of secondary messenger signaling cascades. We and others have shown that the K303R ER $\alpha$  mutation resides adjacent to a protein kinase A and p21-activated kinase 1 phosphorylation site at ER $\alpha$  S305 (24, 28), and it has been shown that S305 phosphorylation promotes ligand hypersensitivity and ligand-independent activity of ER $\alpha$ , as well as up-regulation of the cell cycle regulatory protein cyclin D1 (45). The K303R ER $\alpha$  mutation exhibits enhanced substrate efficiency for protein kinase A signaling to and phosphorylation of the ER $\alpha$  S305 site. Further evidence of the importance of the ER $\alpha$  S305 site comes from Michalides et al. (46), who have shown that protein kinase A signaling to this site confers resistance to the antiestrogen tamoxifen. We do not yet know the role of the A908G ER $\alpha$  mutation as a predictive marker in breast cancer because this study focused on a prognostic evaluation of the mutation in untreated patients, where we did not find it to be an independent factor predicting RFS or OS. It remains to be determined whether the mutation is a predictive marker in clinical trials using either tamoxifen or aromatase inhibitors. Hopefully, with a resolution of the technical differences between detection methods, and the use of sensitive and optimized methods for A908G ER $\alpha$  mutation detection as described here, larger studies to determine the potential clinical relevance of this mutation in different races, histologic types, and treated tumor populations are justified.

**Acknowledgments**

We thank Robin Brown for excellent administrative assistance and Dr. Gary Chamness for editorial assistance.

## References

1. Allred DC, Mohsin SK, Fuqua SA. Histological and biological evolution of human premalignant breast disease. *Endocr Relat Cancer* 2001;8:47–61.
2. Elledge RM, Fuqua SAW. Estrogen and progesterone receptors. In: Harris JR, Lippman ME, Morrow M, et al. editors. *Diseases of the breast*. Philadelphia: Lippincott, Williams & Wilkins; 2000. p. 471–88.
3. Hopp TA, Weiss HL, Parra IS, et al. Low levels of estrogen receptor  $\beta$  protein predict resistance to tamoxifen therapy in breast cancer. *Clin Cancer Res* 2004;10:7490–9.
4. Pike MC, Spicer DV, Dahmouch L, et al. Estrogens, progestins, normal breast cell proliferation, and breast cancer risk. *Epidemiol Rev* 1993;15:17–35.
5. McGuire WL, Chamness GC, Fuqua SAW. Estrogen receptor variants in clinical breast cancer. *Mol Endocrinol* 1991;5:1571–7.
6. Fuqua SAW, Wiltshcke C, Zhang QX, et al. A hypersensitive estrogen receptor—a mutation in premalignant breast lesions. *Cancer Res* 2000;60:4026–9.
7. Herynk MH, Fuqua SA. Estrogen receptor mutations in human disease. *Endocr Rev* 2004;25:869–98.
8. Tebbit CL, Bentley RC, Olson JA, Jr., et al. Estrogen receptor  $\alpha$  (ESR1) mutant A908G is not a common feature in benign and malignant proliferations of the breast. *Genes Chromosomes Cancer* 2004;40:51–4.
9. Tokunaga E, Kimura Y, Maehara Y. No hypersensitive estrogen receptor- $\alpha$  mutation (K303R) in Japanese breast carcinomas. *Breast Cancer Res Treat* 2004;84:289–92.
10. Zhang Z, Yamashita H, Toyama T, et al. Estrogen receptor  $\alpha$  mutation (a-to-g transition at nucleotide 908) is not found in different types of breast lesions from Japanese women. *Breast Cancer* 2003;10:70–3.
11. Fuqua SAW, Cui Y, Mohsin SK, et al. The estrogen receptor  $\alpha$  A908G mutation is present in invasive breast cancer. *Proc Am Assoc Cancer Res* 2005;87.
12. Rosenblum BB, Lee LG, Spurgeon SL, et al. New dye-labeled terminators for improved DNA sequencing patterns. *Nucleic Acids Res* 1997;25:4500–4.
13. Sokolov BP. Primer extension technique for the detection of single nucleotide in genomic DNA. *Nucleic Acids Res* 1990;18:3671.
14. Martin MD, Hilsenbeck SG, Mohsin SK, et al. Breast tumors that overexpress nuclear metastasis-associated 1 (MTA1) protein have high recurrence risks but enhanced responses to systemic therapies. *Breast Cancer Res Treat* 2006;95:7–12.
15. Bardou VJ, Arpino G, Elledge RM, et al. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *J Clin Oncol* 2003;21:1973–9.
16. Hopp TA, Weiss HL, Hilsenbeck SG, et al. Breast cancer patients with progesterone receptor PR-A-rich tumors have poorer disease-free survival rates. *Clin Cancer Res* 2004;10:2751–60.
17. Harvey JM. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* 1999;17:1474–81.
18. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2003;95:353–61.
19. Brown R, Allred D, Clark G, et al. Prognostic value of Ki-67 compared to S-phase fraction in axillary node-negative breast cancer. *Clin Cancer Res* 1996;2:585–592.
20. Molina R, Ciocca DR, Tandon AK, et al. Expression of HER-2/neu oncoprotein in human breast cancer: a comparison of immunohistochemical and Western blot techniques. *Anticancer Res* 1992;12:1965–71.
21. Collett D. *Modeling Survival Data in Medical Research*. New York: Chapman & Hall; 1994.
22. Hilsenbeck SG, Ravdin PM, de Moor CA, et al. Time-dependence of hazard ratios for prognostic factors in primary breast cancer. *Breast Cancer Res Treat* 1998;52:227–37.
23. Kronic MN. Heterozygote determination using automated DNA sequencing technology. In: Taylor GR, editor. *Laboratory methods for the detection of mutations and polymorphisms in DNA*. New York: CRC Press; 1997. p. 175–89.
24. Cui Y, Zhang M, Pestell R, et al. Phosphorylation of estrogen receptor  $\alpha$  blocks its acetylation and regulates estrogen sensitivity. *Cancer Res* 2004;64:9199–208.
25. Fuqua SAW. The role of estrogen receptors in breast cancer metastasis. *J Mammary Gland Biol Neoplasia* 2002;6:407–17.
26. Fuqua SAW, Hopp T, Van M, et al. An estrogen receptor  $\alpha$  mutation that predicts metastatic breast cancer clinical behavior. 9th SPORE investigators' workshop 2001;174.
27. Carter C, Allen C, Henson D. Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. *Cancer* 1989;63:181.
28. Wang RA, Mazumdar A, Vadlamudi RK, et al. P21-activated kinase-1 phosphorylates and transactivates estrogen receptor- $\alpha$  and promotes hyperplasia in mammary epithelium. *EMBO J* 2002;21:5437–47.
29. Lee H, Bai W. Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. *Mol Cell Biol* 2002;22:5835–45.
30. Hull DF, Clark GM, Osborne CK, et al. Multiple estrogen receptor assays in human breast cancer. *Cancer Res* 1983;43:413–6.
31. Bershtein LM, Alimova IN, Tsyrlina EV, et al. Mammary tumors in HER-2/NEU mice are characterized by low content of estrogen receptors- $\alpha$  and absence of progesterone receptors. *Bull Exp Biol Med* 2003;135:580–1.
32. Katiyar P, Ma Y, Fan S, et al. Regulation of progesterone receptor signaling by BRCA1 in mammary cancer. *Nucl Recept Signal* 2006;4:e006.
33. Parker LT, Zakeri H, Deng Q, et al. AmpliTaq DNA polymerase, FS dye-terminator sequencing: analysis of peak height patterns. *Biotechniques* 1996;21:694–9.
34. Parker LT, Deng Q, Zakeri H, et al. Peak height variations in automated sequencing of PCR products using Taq dye-terminator chemistry. *Biotechniques* 1995;19:116–21.
35. Lee LG, Connell CR, Woo SL, et al. DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. *Nucleic Acids Res* 1992;20:2471–83.
36. Conway K, Parrish E, Edmiston SN, et al. The estrogen receptor- $\alpha$  A908G (K303R) mutation occurs at a low frequency in invasive breast tumors: results from a population-based study. *Breast Cancer Res* 2005;7:R871–80.
37. Stemmermann GN. The pathology of breast cancer in Japanese women compared to other ethnic groups: a review. *Breast Cancer Res Treat* 1991;18 Suppl 1: S67–72.
38. Deapen D, Liu L, Perkins C, et al. Rapidly rising breast cancer incidence rates among Asian-American women. *Int J Cancer* 2002;99:747–50.
39. Maskarinec G. Breast cancer—interaction between ethnicity and environment. *In Vivo* 2000;14:115–23.
40. Elledge RM, Fuqua SAW, Clark GM, et al. Prognostic significance of p53 gene alterations in node-negative breast cancer. *Breast Cancer Res Treat* 1993;26:225–35.
41. Moul JW, Theune SM, Chang EH. Detection of RAS mutations in archival testicular germ cell tumors by polymerase chain reaction and oligonucleotide hybridization. *Genes Chromosomes Cancer* 1992;5:109–18.
42. Cotton RG, Rodrigues NR, Campbell RD. Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. *Proc Natl Acad Sci U S A* 1988;85:4397–401.
43. Tolbert DM, Noffsinger AE, Miller MA, et al. p53 immunoreactivity and single-strand conformational polymorphism analysis often fail to predict p53 mutational status. *Mod Pathol* 1999;12:54–60.
44. Loeb LA. A mutator phenotype in cancer. *Cancer Res* 2001;61:3230–9.
45. Balasenthil S, Barnes CJ, Rayala SK, et al. Estrogen receptor activation at serine 305 is sufficient to upregulate cyclin D1 in breast cancer cells. *FEBS Lett* 2004;567:243–7.
46. Michalides R, Griekspoor A, Balkenende A, et al. Tamoxifen resistance by a conformational arrest of the estrogen receptor  $\alpha$  after PKA activation in breast cancer. *Cancer Cell* 2004;5:597–605.