

Absence of the K303R estrogen receptor α mutation in breast cancer patients exhibiting different responses to aromatase inhibitor anastrozole neoadjuvant treatment

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Abstract. Aromatase inhibitors, such as anastrozole, are established in the treatment of hormone-dependent breast cancer. However, approximately 20% of patients treated with anastrozole do not respond, and it remains impossible to accurately predict sensitivity. Thus, novel markers to predict response are required. The K303R estrogen receptor (ER) α mutation confers resistance to tamoxifen treatment. Moreover, K303R-expressing MCF-7 cells, transfected with an aromatase expression vector and stimulated with androstenedione (an aromatase substrate), were found to be resistant to the inhibitory effect of anastrozole. The aim of this study was to verify whether the presence of the K303R ER α mutation is associated with response to 3-month neoadjuvant treatment with anastrozole (Arimidex) in a cohort of post-menopausal breast cancer patients. Of 37 patients with ER⁺ tumors, 19 showed a clinical response to anastrozole and 18 were resistant. Biopsies were obtained from tumors responding to the therapy or from non-responding tumors. None carried the K303R ER α mutation. To our knowledge, this is the first study to search for K303R ER α mutations in tumors clinically responsive or resistant to an aromatase inhibitor. Lack of the mutation leads us to believe that this mutation has *in vivo* biological significance in only a subset of breast cancers.

Introduction

Estrogens are important regulators of cell growth and differentiation in a variety of tissues, including normal mammary epithelium (1). In addition, estrogens play an important role in breast cancer promotion and progression (2). This is based on observations that tumors regress in response to oophorec-

tomy or after hormone therapies, such as the anti-estrogen tamoxifen or aromatase inhibitors (AI) that block estrogen biosynthesis in post-menopausal women (3-5).

Estrogens affect cellular processes by binding to their cognate receptors, estrogen receptor (ER) α and β , which function as transcription factors mediating the mitogenic effects of estrogen. ER α expression in normal breast epithelium is generally low; however, significantly higher expression has been reported in pre-malignant lesions (6), with the majority of breast tumors expressing both receptors (7,8). Since prolonged endogenous estrogen exposure is a potential risk factor for invasive breast cancer (9), overexpression of ER α or the emergence of mutated receptors could be early events in tumor progression (10,11). Fuqua *et al* (12) identified an A to G somatic mutation at ER α nucleotide 908 (A908G) from several usual ductal hyperplasias (early pre-malignant lesions), resulting in a lysine to arginine transition at residue 303 (K303R). To our knowledge, no other ER α mutation has been identified in more than a few invasive breast cancers (13-17).

The K303R ER α mutation has been found to confer resistance to tamoxifen treatment (11,18). Recently, this mutation was investigated in stably wild-type or K303R ER α -overexpressing MCF-7 cells, transfected with an aromatase expression vector, stimulated with androstenedione (an aromatase substrate), with or without the AI anastrozole. Anastrozole treatment decreased androstenedione-stimulated growth of the wild-type cells, whereas K303R-expressing cells were resistant to the inhibitory effect of the drug (19). These findings suggest that, since K303R-mutant cells may escape from growth inhibition when treated with AIs, genetic assays for the mutation might offer a novel predictive marker for hormonal response (19).

The aim of this study was to verify whether the presence of the K303R ER α mutation is associated with response to 3-month neoadjuvant treatment with anastrozole (Arimidex) in a cohort of post-menopausal breast cancer patients.

Materials and methods

Patients. All tumor samples and clinical data were collected with approval of the Fondazione 'Salvatore Maugeri' Ethics

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Committee and with the informed consent of the patients. The 37 patients enrolled in this study between July 2004 to November 2007 were post-menopausal and had breast cancer stage T2 or T3, tumor size >2.5 cm, variable lymph node status and no distant metastasis. All of the tumors were HER2/neu⁻ and ER⁺/PgR⁺, apart from two that were ER⁺/PgR⁻ (Table I). The patients received neoadjuvant therapy with anastrozole (ArimidexTM; Astra Zeneca) 1 mg/day for 3 months. Clinical response was evaluated by serial tumor clinical examination and mammary ultrasound bidimensional measurements, performed by a single operator (L.R.) before, during and after treatment. Patients with a decrease in tumor volume $\geq 30\%$, according to RECIST criteria (20), were classified as responders.

Sequence analysis. Total DNA was extracted from 37 formalin-fixed, paraffin-embedded breast cancer biopsies using the High Pure PCR Template Preparation kit (Roche Molecular Biochemicals, Basel, Switzerland), according to the manufacturer's instructions.

SNaPshot primer extension sequencing was performed as described by Herynk *et al* (11).

For dye-labeled terminator sequencing, all DNA samples were amplified by PCR with primers for an ER α exon 4 fragment (216 bp), including the site of the mutation. Oligonucleotide sequences were PCR-ER α F, 5'-GACCGAAGAGGAGGGAGAAT-3' and PCR-ER α R, 5'-GGAATAGAGTATCGGGGGCT-3'. PCR was carried out in a reaction volume of 25 μ l, containing ~ 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-Hcl (pH 8.3), 1.5 mM MgCl₂, 0.1 ng/ μ l bovine serum albumin (BSA), 200 μ M dNTPs, 0.3 μ M each primer and 0.1 U/ μ l Taq polymerase. Amplification consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 45 sec at 58°C and 1 min at 72°C, with a final extension at 72°C for 7 min.

PCR products were purified by Wizard SV Gel and the PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced using the Big Dye Terminator V3.1 Cycle Sequencing kit and Prism Model 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Results

Using the response criteria as described in Materials and methods, 19 patients were classified as responders and 18 as non-responders (Table I). Only 1 patient (10050) showed disease progression during the treatment.

K303R mutation analysis. As Herynk *et al* argued that SNaPshot primer extension sequencing is more sensitive than dye-labeled terminator sequencing for the detection of the ER α K303R mutation (11), an identical approach on an initial group of 10 DNA samples was attempted. Unfortunately, interpretation of the SNaPshot primer extension sequencing results was very difficult, as a very high background was present (data not shown). We then decided to design a new forward primer for PCR amplification, as the Forward PCR ER α 1 (described by Herynk *et al*) was too near to the site of the mutation for a clear sequencing. Thus, Reverse PCR ER α 2 was used (11). All DNAs were amplified by PCR and

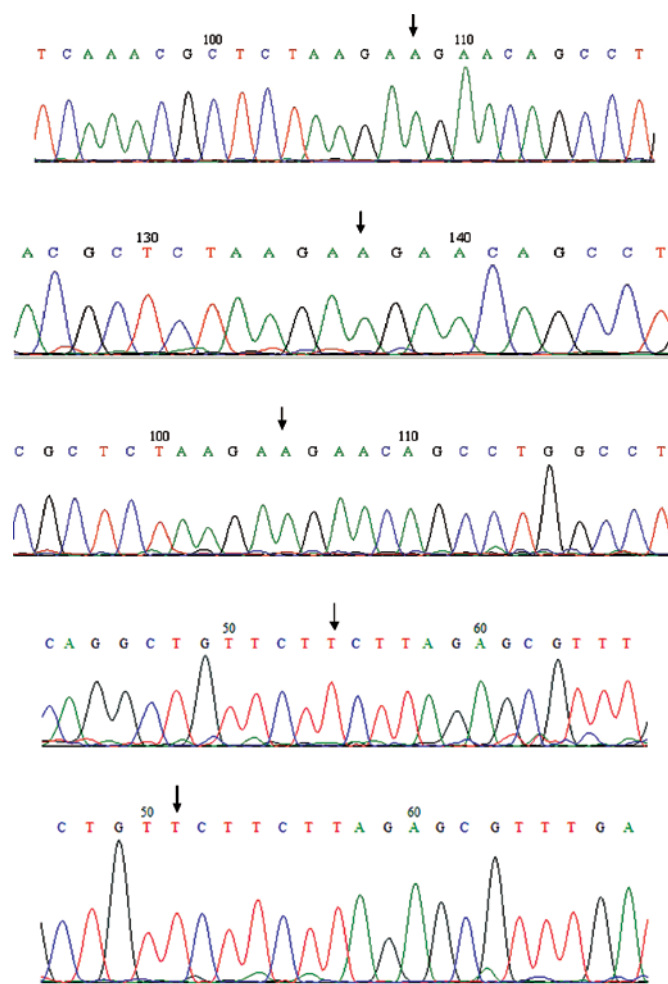


Figure 1. Examples of sequencing of ER α exon 4, as described in Materials and methods. The sequences were shown in forward and reverse orientation; the arrow indicates the wild-type 908 base, A in the forward sequence (TAAGAAGAAC) and T in the reverse sequence (GTTCTTCTTA).

sequenced as described in Materials and methods. Seven DNA samples showed no suitable PCR product or had no results in sequencing. Forward and reverse sequences were readable for 30 out of the 37 DNA samples, and all showed absence of the K303R mutation. An example of sequencing is shown in Fig. 1.

Discussion

A somatic mutation at the nucleotide 908 of ER α (A908G) has been identified in pre-malignant breast lesions and invasive breast cancers (11,12). This mutation, resulting in a lysine to arginine transition at residue 303 (K303R), confers hypersensitivity to estrogen and resistance to tamoxifen (11,18,21). Giordano *et al* hypothesized that the mutant K303R ER α provides a proliferative advantage to breast tissue through a continuous mitogenic stimulus, even during phases of low circulating hormone (as menopause) and demonstrated that its expression conferred resistance to the aromatase inhibitor anastrozole *in vitro* (19).

The present study is the first to investigate K303R ER α mutations in a cohort of post-menopausal breast cancer patients treated with anastrozole neoadjuvant therapy. The

Table I. Pathophysiological characteristics and percentage of response to therapy of the studied patients.

Patient	Age (years)	Initial size (mm)	Tru-Cut			Surgical sample				Stage			Lymph nodes		ERα status			
			ER (%)	PgR (%)	ki67 (%)	Size (mm)	Histotype	Grading	ER (%)	PgR (%)	ki67 (%)	T	N	M		Positive no.	Total no.	% Response
10050	80	35	60	80	8	37	IDC	2	80	40	5	2	1	0	1	13	-6	WT
10055	80	27	60	70	10	25	IDC	2	80	40	8	2	NV	0	NV	NV	7	WT
10041	76	36	70	50	5	33	IDC	2	70	50	5	2	1	0	3	13	8	WT
10038	77	30	80	70	20	26	ILC	3	70	30	15	1C	0	0	0	12	13	WT
10051	70	29	80	0	10	25	ILC	2	80	5	5	2	1	0	1	20	14	WT
10022	79	26	70	80	5	22	IDC	2	80	60	5	2	NV	0	NV	NV	15	WT
10027	78	45	80	30	40	38	IDC	3	80	30	40	2	0	0	0	6	16	WT
10054	52	37	80	80	8	30	ILC	2	60	40	40	1C	1	0	1	14	19	WT
10052	80	25	80	80	8	20	IDC	2	80	60	5	1C	1	0	1	7	20	NV
10024	75	31	80	80	5	24	IDC	2	80	60	5	1C	0	0	0	3	23	WT
10003	84	30	90	40	5	23	IDC	2	90	40	5	2	1	0	2	6	23	WT
10018	74	30	80	60	8	23	ILC	2	80	20	10	2	1	0	2	15	23	WT
10000	70	53	70	40	10	40	IDC	3	70	20	8	3	1	0	14	14	25	WT
10028	73	26	80	70	5	19	IDC	1	80	60	5	2	1	0	2	10	27	WT
10013	71	25	80	70	5	18	IDC	2	80	70	5	1C	0	0	0	6	28	WT
10029	61	25	80	70	5	18	IDC	2	80	70	8	1C	1	0	6	16	28	WT
10064	83	25	80	80	5	18	IDC	1	80	40	5	1C	NV	1	NV	NV	28	NV
10056	75	39	70	10	15	28	IDC	1	80	10	8	2	NV	0	0	22	28	WT
10004	82	25	80	80	5	17	ILC	2	80	80	5	2	0	0	0	4	32	NV
10040	72	28	80	80	5	19	ILC	2	80	70	5	1C	0	0	NV	NV	32	WT
10026	76	30	80	80	10	20	IDC	2	90	70	10	2	NV	0	NV	NV	33	WT
10035	73	26	70	70	5	17	IDC	2	80	60	5	1C	1	0	2	8	35	WT
10049	74	43	80	20	15	28	ILC	3	80	20	15	2	1	0	1	2	35	NV
10010	66	25	70	90	8	16	ILC	2	80	80	8	1C	0	0	0	8	36	WT
10001	79	30	70	10	5	19	ILC	2	80	10	5	2	0	0	0	4	37	WT
10017	73	26	90	90	40	16	IDC	2	90	80	10	1C	0	0	0	12	38	WT
10043	71	38	70	70	5	23	ILC	1	70	60	5	1C	1	0	1	13	39	WT
10007	75	35	80	80	5	21	ILC	2	80	60	5	2	NV	0	NV	NV	40	NV
10048	58	35	90	5	30	21	ILC	2	90	5	15	2	1	0	NV	NV	40	NV
10032	72	32	80	80	5	19	ILC	2	80	50	5	2	0	0	0	1	41	WT
10039	85	27	80	80	10	16	ILC	3	80	70	5	1C	NV	0	NV	NV	41	WT
10025	75	41	80	90	5	23	IDC	1	80	80	5	2	0	0	0	12	44	WT
10037	65	25	80	80	5	14	IDC	2	80	70	5	1C	0	0	0	6	44	WT
10047	76	28	80	40	10	15	ILC	2	80	80	5	1C	0	0	0	6	46	WT
9999	78	40	80	70	5	21	IDC	2	80	40	5	2	1	0	8	9	48	WT
10030	85	29	80	10	8	15	IDC	2	80	10	8	1C	1	0	7	16	48	WT
10061	79	27	90	90	10	13	ILC	2	90	80	10	1C	NV	0	NV	NV	52	NV

ILC, infiltrating lobular carcinoma; IDC, infiltrating ductal carcinoma; NV, not evaluable; WT, normal sequence. Responders are indicated in bold.

purpose of this study was to verify whether the K303R ER α mutation is associated with response to treatment. Notably, no DNA extracted from the post-treatment biopsies demonstrated the alteration, neither in tumors responding to therapy, nor in non-responding tumors.

The absence of the K303R ER α mutation in our tumor cohort could be attributed to the relatively limited number of samples. Nevertheless, this alteration was either not detected in invasive cancer in four other studies (13-16), or was found with a low frequency (37 of 653 breast tumors, 5.7%) (17). Herynk *et al* argued that dye-labeled terminator sequencing was not adequate for the detection of the A908G ER α mutation, and that the alteration was detected at a high frequency in invasive breast tumors using only primer extension sequencing (SNaPshot) (11). We attempted the same approach on an initial group of 10 DNA samples. Unfortunately, interpretation of the results was very difficult, as a very high background was present; therefore, dye-labeled terminator sequencing with a different forward primer was used. With this approach, very clean sequences and low background were obtained. Conway *et al* attributed inferior sensibility to automated sequencing due to suppression of peaks, particularly G following A (17). Thus, we used Big Dye terminators and sequenced in the two senses, where the mutated sequence transforms a T into a C. No mutation was able to be found.

An alternative explanation could be that the *in vivo* K303R ER α mutation has biological significance in only a subset of breast cancers, as hypothesized by Conway *et al* (17). In that study, mixed lobular/ductal tumors were more likely than ductal tumors to carry the K303R ER α mutation; no tumor in our cohort showed similar characteristics, as they were all lobular or ductal carcinomas. Moreover, K303R ER α mutation-positive breast cancer was significantly associated with longer duration and recent use of oral contraceptives (OCs) and OC use has been more strongly associated with the development of lobular and mixed lobular/ductal breast tumors (22,23). In our cohort, all of our patients were post-menopausal, and 31 out of the 37 (84%) were treated at an age older than 70 years; in Italy, the use of OCs was introduced in 1971, so it is likely that many of our patients never practiced their use.

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