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 ERα 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 ERα mutation. To our knowledge, this is the first study to search for K303R ERα 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 oophorectomy 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. ERα 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 ERα 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 *ERα* 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 *ERα* mutation has been identified in more than a few invasive breast cancers (13-17).

The K303R *ERα* mutation has been found to confer resistance to tamoxifen treatment (11,18). Recently, this mutation was investigated in stably wild-type or K303R ERα-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 *ERα* 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 (Arimidex™; 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 ≥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 *ERα* exon 4 fragment (216 bp), including the site of the mutation. Oligonucleotide sequences were PCR-ERαF, 5'-GACCGAAGAGGAGGGAGAAT-3' and PCR-ERαR, 5'-GGAATAGAGTATCGGGGGCT-3'. PCR was carried out in a reaction volume of 25 μl, containing ~100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-Hcl (pH 8.3), 1.5 mM $MgCl_2$, 0.1 ng/μl bovine serum albumin (BSA), 200 μM dNTPs, 0.3 μM each primer and 0.1 U/μl *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 *ERα* 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 ERα 1 (described by Herynk *et al*) was too near to the site of the mutation for a clear sequencing. Thus, Reverse PCR ERα 2 was used (11). All DNAs were amplified by PCR and 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.

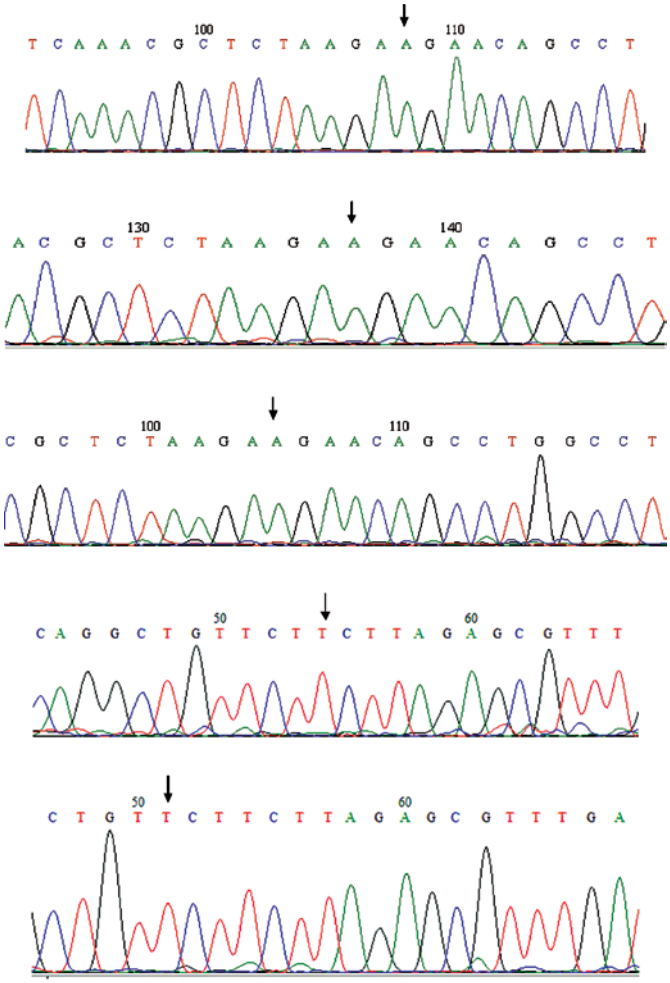

Figure 1. Examples of sequencing of ERα exon 4, as described in Materials and methods. The sequences were shown in forward and reverse orientation; the arrow indicates the wild-type 908 base, A in the forward sequence (TAAGA**A**GAAC) and T in the reverse sequence (GTTC**T**TCTTA).

Discussion

A somatic mutation at the nucleotide 908 of *ERα* (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 *ERα* 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 *ERα* 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		% Response	ERα status
			ER (%)	PgR (%)	ki67 (%)	Size (mm)	Histotype	Grading	ER (%)	PgR (%)	ki67 (%)	T	N	M	Positive no.	Total no.		
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 *ERα* 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 *ERα* 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 *ERα* 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 *ERα* 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 *ERα* mutation; no tumor in our cohort showed similar characteristics, as they were all lobular or ductal carcinomas. Moreover, K303R *ERα* 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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References

1. Chalbos D, Vignon F, Keydar I and Rochefort H: Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). J Clin Endocrinol Metab 55: 276-283, 1982.
2. Russo J and Russo IH: The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol 102: 89-96, 2006.
3. Beatson G: On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. Lancet 147: 101-107, 1986.
4. Smith IE and Dowsett M: Aromatase inhibitors in breast cancer. N Engl J Med 348: 2431-2442, 2003.
5. Osborne CK: Tamoxifen in the treatment of breast cancer. N Engl J Med 339: 1609-1618, 1998.
6. Allred DC, Mohsin SK and Fuqua SA: Histological and biological evolution of human premalignant breast disease. Endocr Relat Cancer 8: 47-61, 2001.
7. Elledge RM and Fuqua SAW: Estrogen and progesterone receptors. In: Diseases of the Breast. Harris JR, Lippman ME, Morrow M, *et al* (eds). Lippincott, Williams & Wilkins, Philadelphia, pp471-488, 2000.
8. Hopp TA, Weiss HL, Parra IS, Cui Y, Osborne CK and Fuqua SA: Low levels of estrogen receptor β protein predict resistance to tamoxifen therapy in breast cancer. Clin Cancer Res 10: 7490-7499, 2004.
9. Pike MC, Spicer DV, Dahmoush L and Press MF: Estrogens, progestins, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev 15: 17-35, 1993.
10. McGuire WL, Chamness GC and Fuqua SA: Estrogen receptor variants in clinical breast cancer. Mol Endocrinol 5: 1571-1577, 1991.
11. Herynk MH, Parra I, Cui Y, Beyer A, Wu MF, Hilsenbeck SG and Fuqua SA: Association between the estrogen receptor α A908G mutation and outcomes in invasive breast cancer. Clin Cancer Res 13: 3235-3243, 2007.
12. Fuqua SA, Wiltschke C, Zhang QX, Borg A, Castles CG, Friedrichs WE, Hopp T, Hilsenbeck S, Mohsin S, O'Connell P and Allred DC: A hypersensitive estrogen receptor-α mutation in premalignant breast lesions. Cancer Res 60: 4026-4029, 2000.
13. Tebbit CL, Bentley RC, Olson JA Jr and Marks JR: Estrogen receptor α (ESR1) mutant A908G is not a common feature in benign and malignant proliferations of the breast. Genes Chromosomes Cancer 40: 51-54, 2004.
14. Tokunaga E, Kimura Y and Maehara Y: No hypersensitive estrogen receptor-α mutation (K303R) in Japanese breast carcinomas. Breast Cancer Res Treat 84: 289-292, 2004.
15. Davies MP, O'Neill PA, Innes H and Sibson DR: Hypersensitive K303R oestrogen receptor-α variant not found in invasive carcinomas. Breast Cancer Res 7: R113-R118, 2005.
16. Zhang Z, Yamashita H, Toyama T, Omoto Y, Sugiura H, Hara Y, Haruki N, Kobayashi S and Iwase H: Estrogen receptor α mutation (A-to-G transition at nucleotide 908) is not found in different types of breast lesions from Japanese women. Breast Cancer 10: 70-73, 2003.
17. Conway K, Parrish E, Edmiston SN, Tolbert D, Tse CK, Geradts J, Livasy CA, Singh H, Newman B and Millikan RC: The estrogen receptor-α A908G (K303R) mutation occurs at a low frequency in invasive breast tumors: results from a population-based study. Breast Cancer Res 7: R871-R880, 2005.
18. Cui Y, Zhang M, Pestell R, Curran EM, Welshons WV and Fuqua SA: Phosphorylation of estrogen receptor α blocks its acetylation and regulates estrogen sensitivity. Cancer Res 64: 9199-9208, 2004.
19. Giordano C, Cui Y, Barone I, Ando S, Mancini MA, Berno V and Fuqua SA: Growth factor-induced resistance to tamoxifen is associated with a mutation of estrogen receptor alpha and its phosphorylation at serine 305. Breast Cancer Res Treat 119: 71-85, 2010.
20. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, van Glabbeke M, van Oosterom AT, Christian MC and Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92: 205-216, 2000.
21. Barone I, Cui Y, Herynk MH, Corona-Rodriguez A, Giordano C, Selever J, Beyer A, Andò S and Fuqua SA: Expression of the K303R estrogen receptor-α breast cancer mutation induces resistance to an aromatase inhibitor via addiction to the PI3K/Akt kinase pathway. Cancer Res 69: 4724-4732, 2009.
22. Conway K, Parrish E, Edmiston SN, Tolbert D and Tse CK: Risk factors for breast cancer characterized by the estrogen receptor alpha A908G (K303R) mutation. Breast Cancer Res 9: R36, 2007.
23. Li CI, Malone KE, Porter PL, Weiss NS and Tang MT: Reproductive and anthropometric factors in relation to the risk of lobular and ductal breast carcinoma among women 65-79 years of age. Int J Cancer 107: 647-651, 2003.