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ORIGINAL ARTICLE

Tracking evolution of aromatase inhibitor resistance with circulating tumour DNA analysis in metastatic breast cancer

C. Fribbens[1,2], I. Garcia Murillas[1], M. Beaney[1], S. Hrebien[1], B. O'Leary[1], L. Kilburn[3], K. Howarth[4], M. Epstein[4], E. Green[4], N. Rosenfeld[4,5,6], A. Ring[2], S. Johnston[2] & N. Turner[1,2*]

[1]Breast Cancer Now Research Centre, Institute of Cancer Research, London; [2]Breast Unit, Royal Marsden Hospital, London; [3]Institute of Cancer Research Clinical Trials & Statistics Unit (ICR-CTSU), London; [4]Inivata Ltd., The Portway, Granta Park, Great Abington; [5]Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge; [6]Cancer Research UK Major Centre, Robinson Way, Cambridge, UK

Correspondence to: Prof. Nicholas C. Turner, The Royal Marsden and Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK. Tel: +020 7352 8133; E-mail: nicholas.turner@icr.ac.uk

Background: Selection of resistance mutations may play a major role in the development of endocrine resistance. *ESR1* mutations are rare in primary breast cancer but have high prevalence in patients treated with aromatase inhibitors (AI) for advanced breast cancer. We investigated the evolution of genetic resistance to the first-line AI therapy using sequential ctDNA sampling in patients with advanced breast cancer.

Patients and methods: Eighty-three patients on the first-line AI therapy for metastatic breast cancer were enrolled in a prospective study. Plasma samples were collected every 3 months to disease progression and ctDNA analysed by digital droplet PCR and enhanced tagged-amplicon sequencing (eTAm-Seq). Mutations identified in progression samples by sequencing were tracked back through samples before progression to study the evolution of mutations on therapy. The frequency of novel mutations was validated in an independent cohort of available baseline plasma samples in the Study of Faslodex versus Exemestane with or without Arimidex (SoFEA) trial, which enrolled patients with prior sensitivity to AI.

Results: Of the 39 patients who progressed on the first-line AI, 56.4% (22/39) had *ESR1* mutations detectable at progression, which were polyclonal in 40.9% (9/22) patients. In serial tracking, *ESR1* mutations were detectable median 6.7 months (95% confidence interval 3.7–NA) before clinical progression. Utilising eTAm-Seq ctDNA sequencing of progression plasma, *ESR1* mutations were demonstrated to be sub-clonal in 72.2% (13/18) patients. Mutations in *RAS* genes were identified in 15.4% (6/39) of progressing patients (4 *KRAS*, 1 *HRAS*, 1 *NRAS*). In SoFEA, *KRAS* mutations were detected in 21.2% (24/113) patients although there was no evidence that *KRAS* mutation status was prognostic for progression free or overall survival.

Conclusions: Cancers progressing on the first-line AI show high levels of genetic heterogeneity, with frequent sub-clonal mutations. Sub-clonal *KRAS* mutations are found at high frequency. The genetic diversity of AI resistant cancers may limit subsequent targeted therapy approaches.

Key words: breast cancer, *ESR1*, *KRAS*, ctDNA

Introduction

Selection of resistance mutations may play a major role in the development of resistance to therapy. Many examples are described, such as *KRAS* mutations emerging in colorectal cancer treated with anti-epidermal growth factor receptor (EGFR) therapy [1, 2] and the development of *EGFR* T790M mutations in patients with non-small-cell lung cancer treated with EGFR inhibitors [3, 4]. In breast cancer, *ESR1* mutations are rare in primary disease [5] but have a high prevalence in patients treated with aromatase inhibitor (AI) therapy in the advanced setting. *ESR1* mutations mainly occur within the ligand binding domain and result in ligand-independent activation of the estrogen receptor (ER) [6]. They are an acquired mechanism of resistance and mutations in ctDNA predict resistance to AI [7, 8]. In a retrospective study [9], circulating *ESR1* mutations were found in

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30.6% of patients at progression on an AI and were detectable in 75% of those patients before progression.

We investigated the development and evolution of genetic resistance to the first-line AI therapy in a prospective study using sequential ctDNA sampling in patients with advanced breast cancer. We find that as well as frequent acquisition of *ESR1* mutations, sub-clonal *KRAS* mutations were found relatively frequently in ctDNA of patients progressing on the first-line AI therapy, suggesting that *KRAS* mutations could be selected as a potential mechanism of resistance.

Materials and methods

Study design

Eighty-three patients on first-line AI therapy for metastatic breast cancer were enrolled in the prospective plasmaDNA AI study (CCR3297, London-Bromley Research Ethics Committee, REC 10/H0805/50) to collect plasma samples for ctDNA analysis every 3 months on therapy and at disease progression. The objective of the study was to determine the median lead-time between first detection of *ESR1* mutation in the plasma and clinical disease progression. All plasma samples were analysed with *ESR1* multiplex ddPCR assays. Samples at disease progression were analysed by eTAm-Seq, to investigate the genetics of breast cancer progressing on the first-line AI. Mutations identified by eTAm-Seq were tracked back through samples before disease progression, to study the evolution of mutations on therapy. Written informed consent was obtained from all patients. ER, progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) were assessed in a single laboratory at the Royal Marsden Histopathology department (or reviewed when reported from a referring hospital) using standard criteria.

Plasma collection and processing

In the plasmaDNA AI study, plasma samples were collected every 3 months and at the end of treatment in EDTA Blood Collection Tubes. Samples were processed within 2 h of collection by centrifugation at 1600 g for 20 minutes at room temperature. Plasma was separated from buffy coat and red blood cells, aliquoted and stored at -80 °C until DNA extraction.

In the SoFEA trial, baseline blood was collected in EDTA Blood Collection Tubes and processed within 0–9 days of sample collection. Plasma was separated by centrifugation 1600*g* for 20 minutes. SoFEA samples were analysed previously, demonstrating that archival EDTA plasma samples can be used for ctDNA analysis with ddPCR [8].

DNA extraction

Following thawing, ctDNA was extracted from 2 or 4 ml of plasma using the MagMax Cell-Free DNA Isolation kit (Thermo A29319) on a Kingfisher Flex Purification System (Thermo) according to manufacturer instructions. The DNA was quantified and stored at -20 °C until analysis.

Droplet digital PCR

DNA concentration was estimated in each sample as previously described [7].

For *ESR1* mutation analysis, we used two commercially available ddPCR multiplexes from Bio-Rad, multiplex 1 (dHsaMDXE91450042) and multiplex 2 (dHsaMDXE65719815). Multiplex 1 contained FAM-labelled probes for E380Q (*c.1138 G > C*), L536R (*c.1607 T > G*), Y537C (*c.1610 A > G*), D538G (*c.1613 A > G*). Multiplex 2 contained FAM-labelled probes for S463P (*c.1387 T > C*), Y537N (*c.1609 T > A*) and Y537S (*c.1610 A > C*).

For *KRAS* mutation analysis, we used a commercially available ddPCR multiplex from Bio-Rad (Cat Number 1863506). The multiplex assay contains FAM-labelled probes to seven commonly occurring hotspot mutations on codons 12 and 13 of *KRAS*.

Samples were analyzed using DNA extracted from 1 ml plasma. Reaction volumes were made up to 20 with 10 µl of Bio-Rad ddPCR Supermix for probes, 1 µl of assay and 9 µl nuclease-free water, then partitioned to a mean of 15 000 droplets using a ddPCR Auto Droplet Generator (Bio-Rad). For *ESR1* mutation analysis, the following conditions were used: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 52 °C for 60 seconds, ramp rate 2.5 °C/second and final incubation 98 °C for 10 minutes. For *KRAS* mutation analysis, the following conditions were used: 95 °C for 10 minutes followed by 40 cycles of 94 °C for 30 seconds, 55 °C for 60 seconds, ramp rate 2.5 °C/second and final incubation 98 °C for 10 minutes. Subsequent analysis was done on a Bio-Rad QX200 droplet reader and analysed using QuantaSoft software v1.7.4.0917 (Bio-Rad). A multiplex assay was called mutation positive if there were at least two FAM-positive droplets. Samples were called negative if there were at least 300 wild-type alleles detected and <2 FAM-positive droplets. If this criterion was not met, it was repeated or failed if there was insufficient material to repeat.

InVision^{TM}/eTAm-seq analysis

The InVision liquid biopsy platform combines efficient next-generation sequencing (NGS) library preparation and statistical algorithms to identify and quantify low frequency tumour-derived single nucleotide variants (SNVs), insertion/deletions (Indels) and copy number variations in cell-free DNA, based on methods described previously [3, 10]. NGS libraries were prepared using a two-step amplification process, with primers targeting 36 cancer-related genes designed to hotspot and entire coding regions of interest (supplementary Figure S1, available at *Annals of Oncology online*). The panels (v1.4/v1.5) are optimised for amplification of highly fragmented DNA with amplicon sizes ranging 72–154 bp. Pooled libraries were quantified using Kapa Library Quantification Kit, and 1.8pM libraries analysed on an Illumina NextSeq 500 (300 cycle PE). Sequencing files were analysed using the Inivata Somatic Mutation Analysis (ISoMA) analytical pipeline (V1.15-1.17), and sequencing reads were clipped, merged and aligned. Coding and splice-site mutations in SNVs and Indels were annotated using Variant Effect Predictor (VEP) using the canonical transcript for each gene. Sub-clonal mutations were defined as mutations with an aggregate allele frequency (AF) <0.25 of driver mutation AF identified in the analyzed samples.

Orthogonal validation of detected mutations by eTAm-Seq was performed using ddPCR as described above. *KRAS*, *PIK3CA* and *TP53* mutations were validated using either commercially available assays or in-house designed assays as described previously [11].

Statistical analysis

For each of the markers tested, the proportion of patients with the mutation were reported with 95% confidence interval (CI). The study was powered assuming a 15% incidence of activating mutation of *ESR1*, with a minimum of 50 patients to calculate the actual proportion that were *ESR1* mutation positive with 95% CI of ±9.9%. Validation of mutations found in the plasmaDNA AI prospective study, if recurrent mutations were identified at sufficiently high prevalence, was planned in the SoFEA phase III trial that has been described previously [12]. Briefly, the SoFEA study was a multicentre, randomized phase III trial in postmenopausal women with advanced, hormone receptor positive breast cancer who had progressed on a non-steroidal AI. All patients had demonstrated prior sensitivity to AIs, and were randomized to fulvestrant plus anastrozole, fulvestrant plus placebo, or exemestane.

The SoFEA validation analysis would provide a level of precision for mutation incidence around a 95% CI of ±5.5%, based on a proportion of 10%, or ±7.5% based on a proportion of 20% ($n = 117$ patients baseline plasma with sufficient quantity to analyse). Progression-free survival

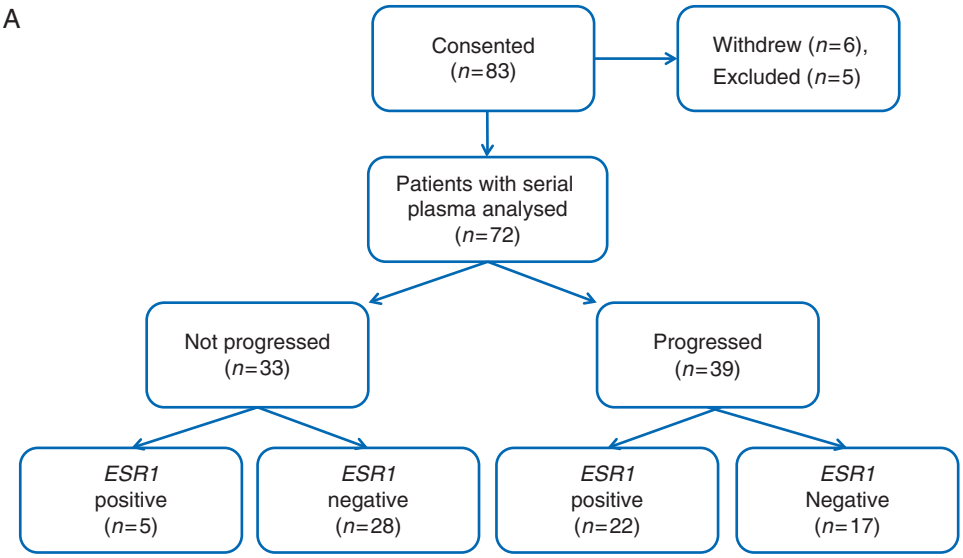
(PFS) and overall survival (OS) were defined in the main SoFEA trial [12]. To determine whether *KRAS* mutations were prognostic for PFS and OS, Cox proportional hazards models were used. The proportional hazards assumption made by using the Cox model was tested using Schoenfeld's residuals and was found to hold. Kaplan–Meier curves were plotted (mutant versus wild-type), median PFS time and unadjusted HR with 95% CI reported and groups compared using the log-rank test. All statistical analyses were performed with GraphPad Prism (version 6.0), Stata (version 13.1) or R. Lead-time was calculated using the Turnbull estimator.

Results

ESR1 mutations are frequently subclonal and polyclonal at progression on AI

Eighty-three patients with ER-positive metastatic breast cancer on first-line AI therapy were enrolled into a prospective study to collect plasma samples for ctDNA analysis every 3 months and

A



B

	n	%		n	%
n	72		Prior adjuvant endocrine therapy		
Median age	68 (37-94)		Tamoxifen only	40	55.6
Pathology			AI only	7	9.7
IDC	49	68.0	AI and tamoxifen	11	15.3
ILC	16	22.2	none	14	47.2
Mixed IDC/LIC	3	4.2			
NA/other	4	5.6	Prior chemotherapy		
Grade			Neo/adjuvant	29	40.3
1	7	9.7	Metastatic +/– adj	6	8.3
2	50	69.4	None	37	51.4
3	8	11.1			
NA	7	2.6	Metastatic sites		
Hormone receptor status			Bone	58	80.6
ER positive/PR positive	39	54.2	Visceral	41	56.9
ER positive/PR negative	10	13.9			
ER positive/PR NK	23	31.9			

Figure 1. PlasmaDNA AI study of sequential plasma DNA sampling during first line aromatase inhibitor therapy for advanced breast cancer. (A) Consort diagram of plasma samples analysed for *ESR1* mutations on the plasmaDNA AI study. (B) Baseline characteristics of patients in the plasmaDNA AI study.

at disease progression (Figure 1A). The clinical and pathological characteristics of the study cohort are described in Figure 1B.

We initially studied the evolution of *ESR1* mutations on AI therapy, using ultra-high sensitivity multiplex ddPCR assays for seven commonly occurring *ESR1* mutations to track these mutations in plasma until clinical progression. Of the 39 patients who progressed on the first-line AI, 56.4% (22/39, 95% CI, 39.6% to 72.2%) had *ESR1* mutations detectable at progression. In patients with *ESR1* mutations detected, the mutations were polyclonal in 40.9% (9/22, 95% CI, 20.7% to 63.7%) of patients (Figure 2). In serial tracking before progression, *ESR1* mutations were detectable in plasma before progression in 86.4% (19/22, 95% CI, 65.1% to 97.1%) patients, with *ESR1* mutations detectable a median of 6.7 months (95% CI 3.7–NA) before clinical progression (Figure 4). In patients who progressed on AI, all patients who had *ESR1* mutations detected before progression also had *ESR1* mutations detected at progression, suggesting early detection of *ESR1* mutations robustly predicted the presence of the mutation at progression. *ESR1* mutations were detectable in 15.2% (5/33) patients who had not yet clinically progressed (Figure 1B).

AI resistant breast cancers are genomically diverse

We investigated the genetics of breast cancers progressing on first-line AI, with eTAm-Seq deep sequencing of ctDNA from progression plasma samples (Figure 3). Consistent with other studies [5, 13, 14], *TP53* [36.1% (13/36)], *ESR1* [33.3% (12/36)] and *PIK3CA* [25.0% (9/36)] mutations were the most frequent mutations detected. *ESR1* mutations were identified in more samples by ddPCR than by eTAm-Seq. Of the 10 discordant cases, one *ESR1* mutation detected by eTAm-Seq but not ddPCR occurred at an AF of 0.002, whereas nine *ESR1* mutations detected only by ddPCR occurred at AF's ranging from 0.0004 to 0.032. For one case, there was weak evidence of an *ESR1* mutation but this was below the eTAm-Seq calling threshold. These cases had lower mutant copies per ml in ddPCR compared with concordant cases (median 14.3 versus 51.5, respectively, $P = 0.048$ Mann–Whitney U test), suggesting that ddPCR was detecting low levels of *ESR1* mutation in ctDNA. In patients with additional driver mutations detected in ctDNA, *ESR1* mutations were sub-clonal in 72.2% (13/18, 95% CI, 46.5% to 90.3%) of patients, found at aggregate relative AF <0.25, with *ESR1* mutation diversity increasingly detectable at the point of progression

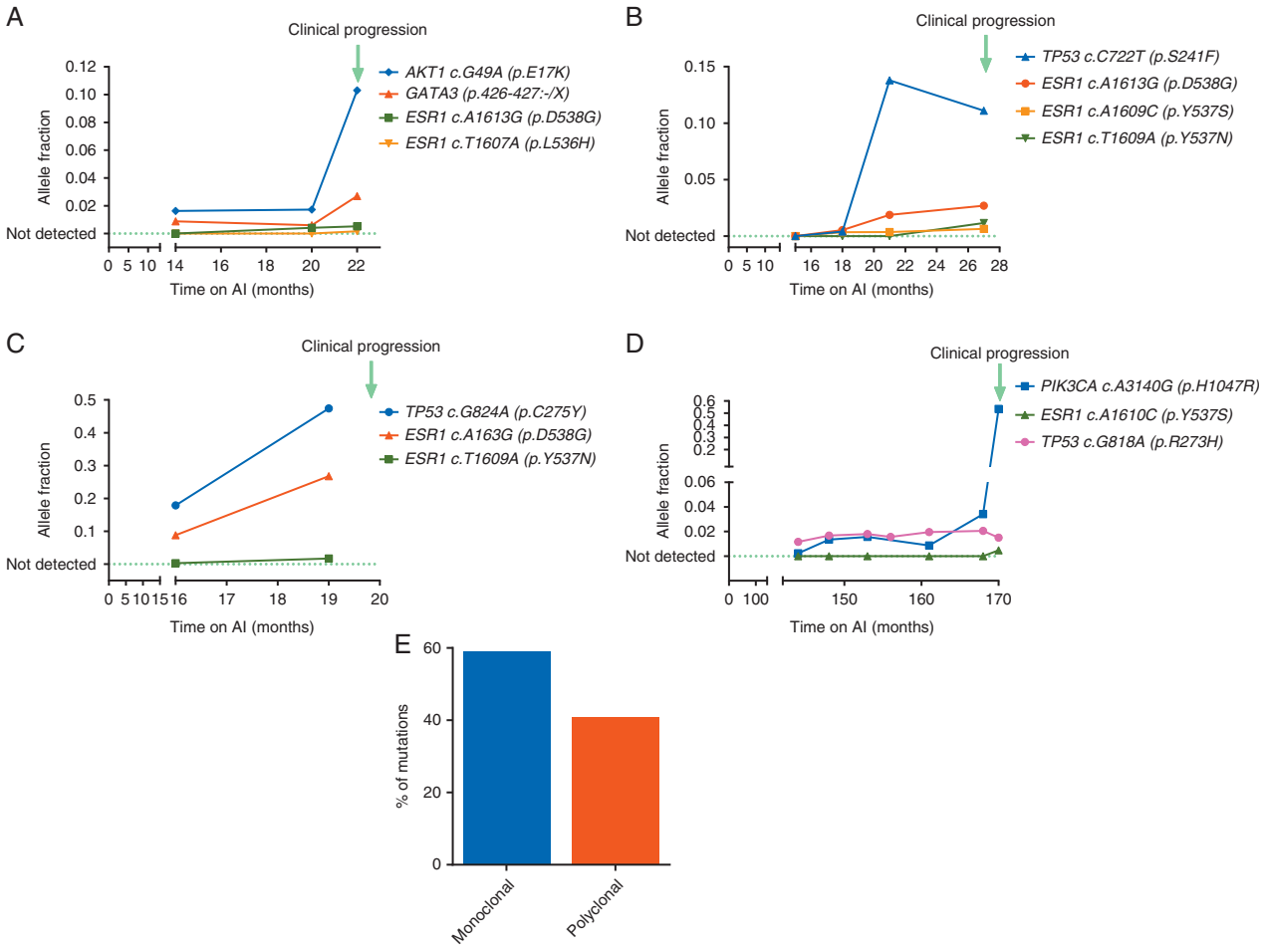


Figure 2. Evolution of *ESR1* mutations during aromatase inhibitor (AI) therapy. (A–D) Mutation tracking in ctDNA collected during first line AI therapy. Data from four patients with *ESR1* subclonal mutations detectable in ctDNA tracked until clinical progression. Allele fractions are shown as determined by sequencing. TP53 mutation in grey likely to have arisen from Clonal Haematopoesis of Indeterminate Potential (CHIP). (E) Percentage of cases with monoclonal (59.1%) or polyclonal *ESR1* mutations (40.9%).

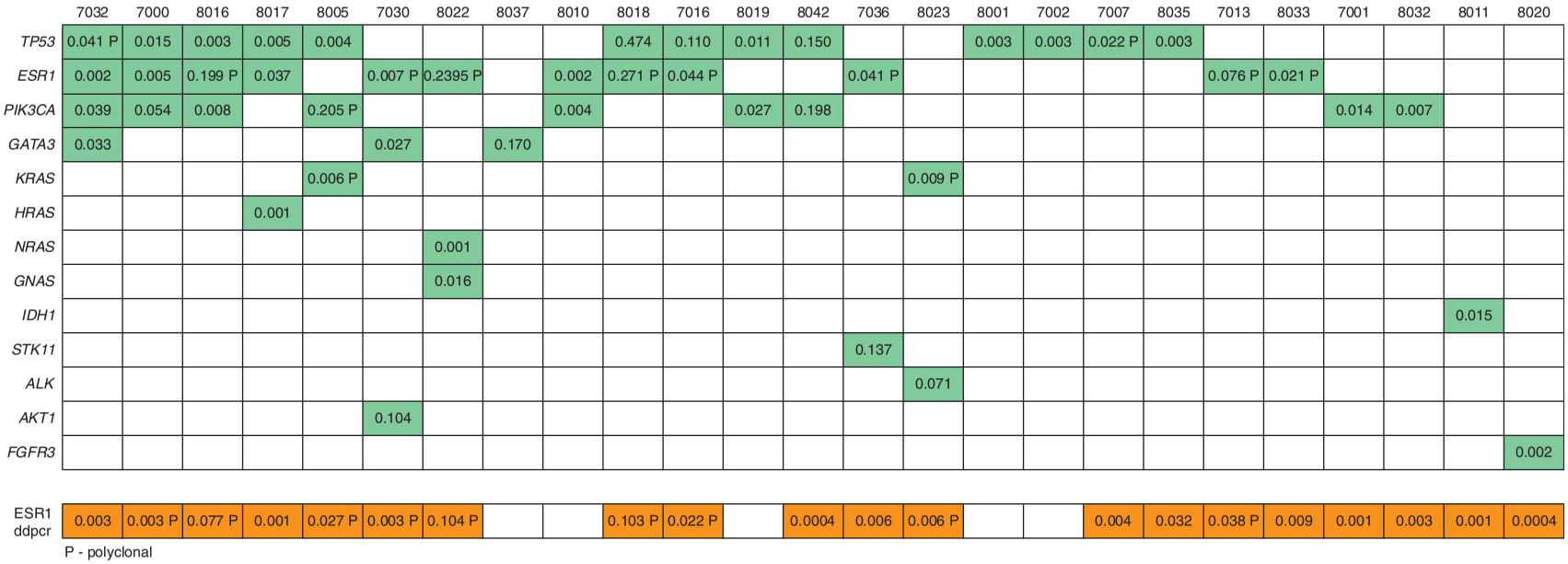


Figure 3. Error corrected ctDNA sequencing of plasma samples taken after progression on the first-line aromatase inhibitor (AI). Mutations identified in plasma DNA by eTAm-Seq error corrected sequencing, with *ESR1* mutation analysis by ddPCR. Discordant cases for *ESR1* between ddPCR and ctDNA sequencing had lower mutant copies per ml in ddPCR compared with concordant cases (median 14.3 versus 51.5, respectively, $P = 0.048$ Mann–Whitney *U* test) and likely represent very low levels of mutant copies and random sampling. 8037 also had *FGFR1* and *ERBB2* amplification identified. Of 36 progression plasma samples sequenced, 25 with mutations are displayed, 11 plasma samples with no mutations detected are not displayed. Numbers in boxes represent allele fraction for indicated gene. Where there are multiple mutations detected in the same gene, indicating polyclonality (P), aggregate allele fractions are given.

doi:10.1093/annonc/mdx483 | 149

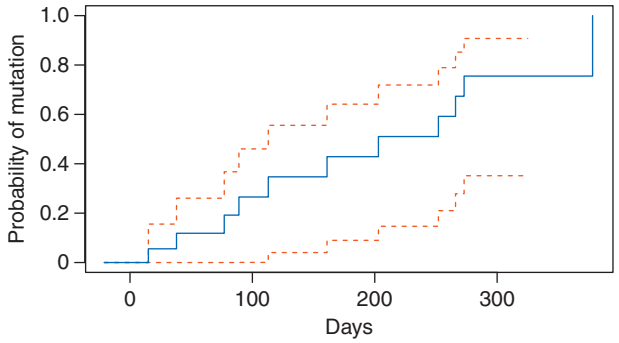


Figure 4. Lead time to development of *ESR1* mutations. Serial tracking before progression, *ESR1* mutations were detectable in plasma median 6.7 months [95% confidence interval (CI) 3.7–NA] before clinical progression.

compared with samples taken before progression (Figure 2). In patients with polyclonal mutations, individual mutations were observed to be on different DNA strands in eTAm-Seq, further supporting the sub-clonality of the observed *ESR1* mutations.

Deep ctDNA sequencing of progression samples identified previously unrecognized genetic diversity. Polyclonal *KRAS* mutations were identified in two patients, 8005 (p.G12V, p.G12S) and 8023 (p.G12V, p.G12C, p.G12R), a monoclonal *HRAS* mutation (p.G12V) in one patient and a monoclonal *NRAS* mutation (p.G12D) in another one. An activating p.R248C *FGFR3* mutation was identified in a further patient ctDNA sample. Sequencing or ddPCR of ctDNA obtained from plasma identified *RAS* mutations in 15.4% (6/39, 95% CI, 5.9% to 30.5%) of patients [four *KRAS* (two of which were polyclonal], one monoclonal *HRAS* and one monoclonal *NRAS*) (Figure 5). In patients where an additional driver mutation was detected in ctDNA, all identified *RAS* mutations were sub-clonal. In two patients with *KRAS* mutations detected at progression, primary tumour was available, with the *KRAS* mutation being undetectable in both patients.

Clonal Haematopoesis of Indeterminate Potential (CHIP) is an age-related clonal expansion that is detectable in a high proportion of ageing people [15, 16]. Mutations arising from CHIP may be detected in ctDNA analysis and present a potential confounder to discovery of resistance mutations in ctDNA. Although *KRAS* mutations are not a classic CHIP mutation, they are reported at low level. To ascertain whether detected *KRAS* mutations were arising from ctDNA or CHIP, we tracked *KRAS* mutations back through serial samples before progression (Figure 5). *KRAS* mutations arose in line with driver and *ESR1* mutations at disease progression, demonstrating that the *KRAS* mutations were detected in ctDNA. In contrast, a *TP53* mutation detectable at progression was shown to arise from CHIP with high-likelihood, as the AF of the mutation stayed constant through serial tracking, whilst mutations arising from ctDNA rose to the point of progression (Figure 2).

Identified *RAS* mutations are selected on AI therapy

To validate our novel discovery of *KRAS* mutations in AI resistant cancer, and to assess clinical significance of *KRAS* mutations in patients who progressed on endocrine therapy, we analysed baseline plasma samples from the phase III SoFEA study by ddPCR. We retrospectively analysed *KRAS* mutational status on 117 available baseline plasma samples of the 723 patients enrolled on the study. We investigated the association of *KRAS* mutations detected in ctDNA and clinical outcome. These samples had previously been analysed for *ESR1* mutation status [8]. *KRAS* mutational status was successfully interrogated in 96.6% (113/117) of available plasma samples, with *KRAS* mutations detected in 21.2% (24/113, 95% CI, 14.1% to 29.9%) of patients, with no *KRAS* mutations detected in controls (supplementary Table S2, available at *Annals of Oncology* online). 19.0% (8/42, 95% CI, 8.6% to 34.1%) of *ESR1* mutant cancers also had *KRAS* mutations. *KRAS* mutations were detected at low levels in the majority of patients. There were no significant differences in baseline characteristics between patients with and without *KRAS* mutations (supplementary Table S3, available at *Annals of Oncology* online).

There was no evidence that *KRAS* mutation status was prognostic for PFS or OS in patients who had progressed on an AI. Median PFS was 3.7 months (95% CI, 2.7–11.5) for patients with *KRAS* mutations and 4.6 months (95% CI, 3.0–6.5) for patients with wild-type *KRAS* (unadjusted hazard ratio = 1.04, 95% CI, 0.65–1.67; $P = 0.86$). Median OS was 22.5 months (95% CI, 12.2–32.4) for patients with *KRAS* mutations and 21.2 months (95% CI, 17.6–26.1) for patients with wild-type *KRAS* (unadjusted hazard ratio = 1.16, 95% CI, 0.66–2.03; $P = 0.61$) (Figure 6).

Discussion

In the prospective plasmaDNA AI study, we demonstrate that ER-positive advanced breast cancer progressing on AI shows substantial genetic diversity, with a high rate of *ESR1* mutations and a previously un-described high rate of mutations in *KRAS* and a classical activating mutation in *FGFR3*. Many selected mutations are demonstrated to be sub-clonal, although our findings identify a potential major role for selected *KRAS* mutations in resistance to AI therapy in the treatment of advanced breast cancer.

In this cohort of patients progressing on first line AI, *ESR1* mutations are found at high prevalence in plasma, detectable in over half of patients. Resistance to therapy can be anticipated with a long lead-time over clinical progression, with *ESR1* mutations detectable before progression in 86.4% of patients. These results are consistent with a prior retrospective study that reported *ESR1* mutations were detectable in 75% of patients before progression [9]. This prior study reported a lower frequency of *ESR1* mutations at progression on an AI but only four *ESR1* mutations were analysed using 4 ng preamplified DNA which likely explains the higher frequency reported here. The incidence of *ESR1* mutations we report is in line with the rate we previously reported in the SoFEA study, with *ESR1* mutations detected in 39.1% of baseline samples [8].

We show that many *ESR1* mutations detected in plasma are likely sub-clonal in the cancer, with the aggregate allele fraction of *ESR1* mutations frequently substantially lower than that of other identified driver mutations. This suggests that in an individual patient, *ESR1* mutations may not be the sole driver of resistance in the cancer. Multiple drugs that degrade the mutant ER are in early clinical, and pre-clinical development, and this

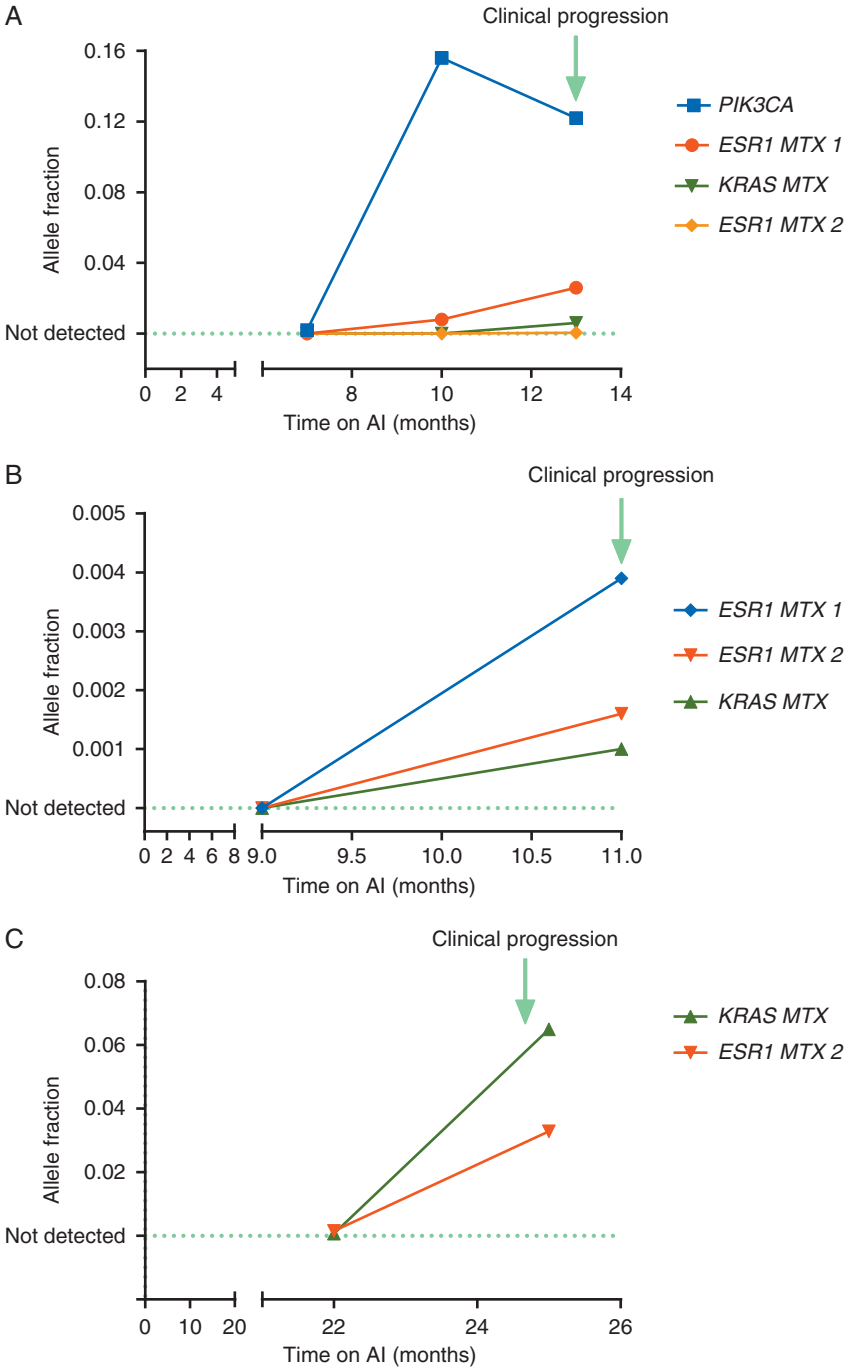


Figure 5. Evolution of *KRAS* mutations during the first-line aromatase inhibitor (AI) therapy. (A–C) Mutation tracking in ctDNA collected during first line AI therapy. Data from three patients with *KRAS* subclonal mutations detectable in ctDNA tracked until clinical progression. Allele fractions are shown as determined by ddPCR. Patient B had an *ALK* mutation detected on sequencing at progression with an allele fraction of 0.07. In two patients with *KRAS* mutations detected at progression primary tumour was available, with the *KRAS* mutation being undetectable in both patients.

finding emphasises the importance of assessing clonal dominance of *ESR1* mutations in clinical development. Also, due to the subclonal nature of these mutations, the amount of plasma DNA analysed may have a major impact on frequencies of *ESR1* mutations identified.

KRAS mutations are identified frequently in colorectal cancers with resistance to EGFR directed therapy [1, 2], but previously have not been identified as a major mechanism of resistance in ER positive breast cancer. We show that selection of *KRAS* activating mutations is a potential novel mechanism of resistance to AI, with a substantial prevalence of 21.2% (24/113) in the SoFEA validation series. *KRAS* mutations are identified in approximately 2% of primary ER-positive breast cancer [5, 13], and *KRAS* mutations are undetectable in the primary of two patients with selected

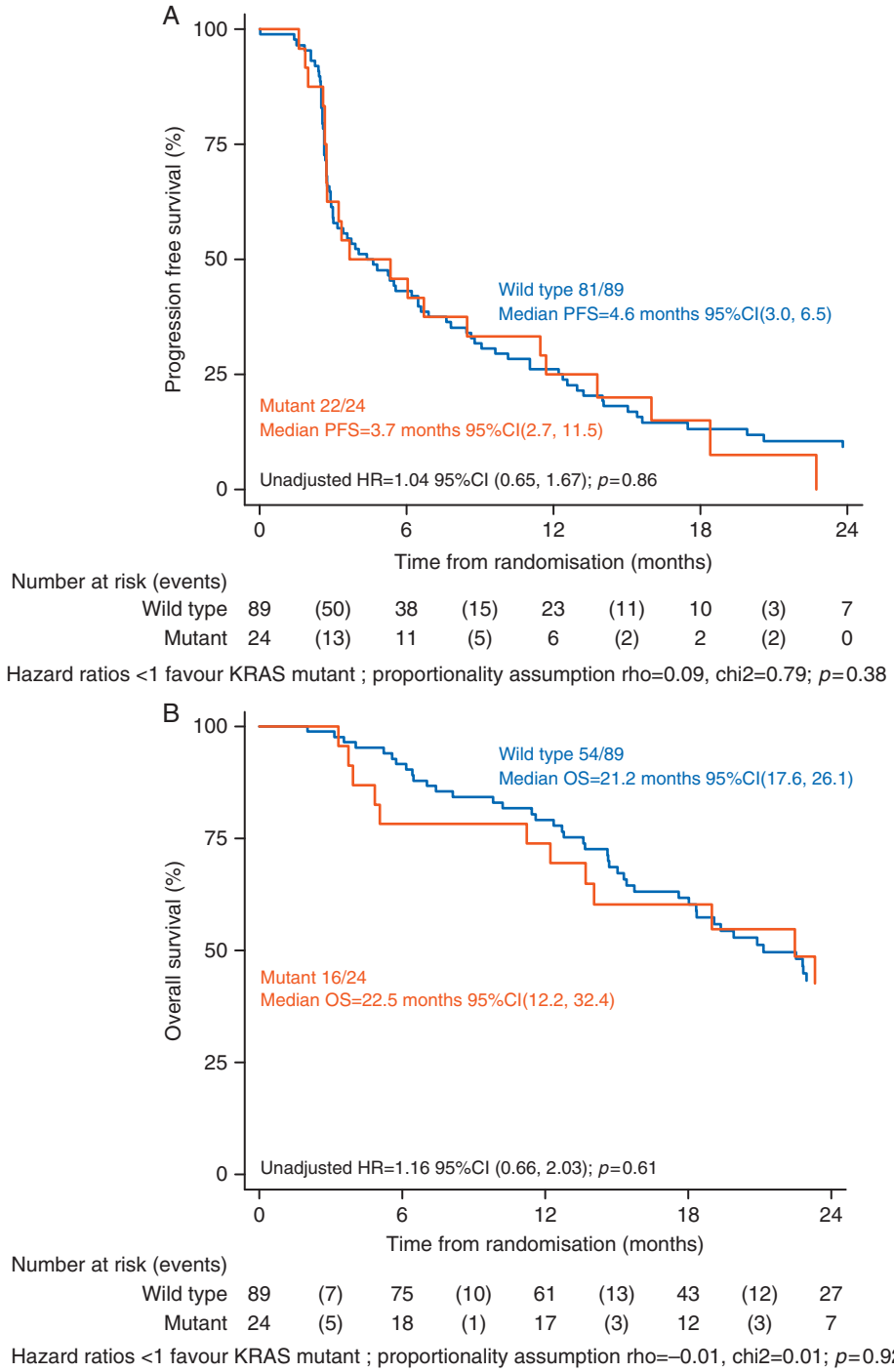


Figure 6. Independent validation of *KRAS* mutations in baseline plasma from the SoFEA study. (A) Progression-free survival (PFS) in SoFEA by *KRAS* mutation status. HR, hazard ratio. (B) Overall survival (OS) in SoFEA by *KRAS* mutation status.

KRAS mutations, suggesting selection by therapy. The *KRAS* mutations identified are frequently sub-clonal, possibly due to geographic development of *KRAS* mutations in individual metastases. Multiple prior studies have linked activation of MAP kinase pathway signaling to resistance to endocrine therapy [17, 18], suggesting the *KRAS* mutations may drive resistance to endocrine therapy in individual sub-clones. In SoFEA the presence of a *KRAS* mutation detected in ctDNA had no impact on PFS or OS, although this analysis used a relatively small number of samples and would need confirmation in a larger set. This finding suggests the importance of determining whether sub-clonal *KRAS* mutations continue to expand through subsequent therapy, or whether the mutations become undetectable once endocrine therapy is ceased, which will be an important area of future research.

This study has limitations. Some patients joined the study mid-AI therapy and had *ESR1* mutations detected at the first sample. Although this was taken into account when calculating

lead-time to progression, this adds imprecision to the median estimate of lead-time. There were a relatively small number of progression samples in the plasma AI study and it was not possible to perform sequencing on all progression samples due to amounts of DNA available. Although the ctDNA sequencing strategy we employ substantially expanded our ability to investigate the genetics of AI resistant cancer, leading to the discovery of *KRAS* mutations, the panel covered a limited number of genes. There may be other relevant selected mutations present at progression on AI that were not detected in this panel. Most of the *KRAS* mutations detected were present at low levels, and although some mutations were present at relatively high level it was not possible due to limited number of high level mutations to assess whether there is a different impact on outcome for those with high levels of *KRAS* mutation.

Our study demonstrates the potential of ctDNA analysis in describing the genetics of drug resistant disease, and the power of combining prospectively collected sample sets with high depth discovery ctDNA sequencing. Selected genetic mechanisms of resistance are frequent in resistance to first line AI therapy; *ESR1* mutations are found at high prevalence in this setting, along with high frequency sub-clonal *KRAS* mutations. AI resistant cancers are genetically heterogeneous and may consist of several clones that may limit the effectiveness of subsequent targeted therapies that target only one of the clones.

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Disclosure

KH, ME, NR and EG are employees, consultants or shareholders of Inivata Ltd. and/or inventors of patent applications related to sequencing strategies described in this article. AR has an advisory role for Novartis, Roche and Genomic health. SJ has research funding from Pfizer and an advisory role for Novartis, AstraZeneca and Genentech/Roche. NT has research funding from Roche, Pfizer and AstraZeneca and has an advisory role for Roche, Pfizer, Novartis and Astrazeneca. All remaining authors have declared no conflicts of interest.

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