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

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Abstract:

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 first line AI therapy using sequential ctDNA sampling in patients with advanced breast cancer.

Patients and Methods: 83 patients on 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 were tracked back through samples prior to progression to study the evolution of mutations on therapy. The frequency and impact of *KRAS* mutations were validated in an independent cohort of available baseline plasma samples in the SoFEA study, which enrolled patients with prior sensitivity to AI.

Results: Of the 39 patients who progressed on 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%CI 3.7-NA) prior to 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) of patients, although there was no impact of *KRAS* mutations on progression free or overall survival.

Conclusions: Cancers progressing on first line AI show high levels of genetic heterogeneity, with frequent sub-clonal mutations. Sub-clonal *KRAS* mutations are found at a high frequency, although the detection had no impact on progression on subsequent endocrine therapy in the SoFEA study. The genetic diversity of AI resistant cancers may limit subsequent targeted therapy approaches.

Key words:

Breast cancer, *ESR1*, *KRAS,* ctDNA

Key message:

Breast cancers progressing on first line aromatase inhibitors show high levels of genetic heterogeneity, with frequent sub-clonal mutations that may limit subsequent targeted therapy approaches.

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

We investigated the development and evolution of genetic resistance to first line AI therapy in a prospective study using sequential ctDNA sampling in patients with advanced breast cancer. Samples were analysed with *ESR1* multiplex droplet digital PCR (ddPCR) assays and by enhanced tagged-amplicon sequencing (eTAm-Seq, InVision™) to investigate for mutations that may contribute to AI resistance. Sub-clonal *KRAS* mutations were found relatively frequently in ctDNA of patients progressing on first line AI therapy, suggesting that *KRAS* mutations could be selected as a potential mechanism of resistance. We validated the frequency of *KRAS* mutations in baseline plasma of the SoFEA study, a large phase III trial of patients who had progressed on prior AI therapy.

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, REC 10/H0805/50) to collect plasma samples for ctDNA analysis every three months on therapy, and at disease progression. All plasma samples were analysed with *ESR1* multiplex ddPCR assays, and samples at disease progression were analysed by eTAm-Seq. Mutations identified by eTAm-Seq were tracked back through samples prior to 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 by the RMH 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 end of treatment in EDTA Blood Collection Tubes (BCT). Samples were processed within 2 hours of collection by centrifugation at 1600g for 20 minutes at room temperature. Plasma was separated from buffy coat and red blood cells, aliquoted and stored at -80^{o}C until DNA extraction.

In the SoFEA trial, baseline blood was collected in EDTA BCT and processed within 0 to 9 days of sample collection. Plasma was separated by centrifugation 1600g for 20 minutes. We have previously demonstrated that archival EDTA plasma samples can be used for ctDNA analysis with ddPCR (8).

DNA extraction

Following thawing, ctDNA was extracted from 2 or 4ml 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^{o}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.1138G>C*), L536R (*c.1607T>G*), Y537C (*c.1610A>G*), D538G (*c.1613A>G*). Multiplex 2 contained FAM-labelled probes for S463P (*c.1387T>C*), Y537N (*c.1609T>A*) and Y537S (*c.1610A>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 7 commonly occurring hotspot mutations on codons 12 and 13 of *KRAS*.

Samples were analyzed using DNA extracted from 1 ml plasma (1ml plasma equivalent). Multiplex reaction volumes were made up to 20µl with 10µl of Bio-Rad ddPCR Supermix for probes, 1µl of assay and 9µl 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 then 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 10minutes followed by 40 cycles of 94°C for 30 seconds then 55°C for 60 seconds, ramp rate 2.5°C/second, and final incubation 98°C for 10 minutes. The subsequent analysis was done on a Bio-Rad QX200 droplet reader, and analyzed using QuantaSoft software v1.7.4.0917 (Bio-Rad). A multiplex assay was called as mutation positive if there were at least 2 FAM-positive droplets. Samples were only called negative if there were at least 300 wild type alleles detected and <2 FAM-positive droplets. If this criterion was not met the sample was repeated or failed if there was insufficient material to repeat.

InVision™ / eTAm-Seq analysis

The InVision liquid biopsy platform combines efficient next-generation sequencing library preparation and statistical algorithms to identify and quantify low frequency tumor-derived single nucleotide variants (SNVs), insertions/deletion (Indels) and CNVs in cell-free DNA, based on methods previously described (10)(3). Next generation sequencing libraries were prepared using a two-step amplification process, with primers targeting 36 cancer-related genes (Supplementary figure 1) designed to hotspot and entire coding regions of interest. The panels (v1.4/v1.5) are optimised for amplification of highly fragmented DNA with amplicon sizes ranging 72bp –154bp.

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 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 <0.25 of driver mutation allele frequency identified in the analyzed samples.

Orthogonal validation of the 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 previously described (11).

Statistical analysis

All statistical analysis was performed with GraphPad Prism version 6.0, Stata or R. Lead time was calculated using the Turnbull estimator. For analyses of progression free survival (PFS), Kaplan-Meier curves were plotted and groups compared using the log-rank test.

Results

***ESR1* mutations are frequently subclonal and polyclonal at progression on AI**

83 patients with ER positive metastatic breast cancer on 1st line AI therapy were enrolled into a prospective study to collect plasma samples for ctDNA analysis every 3 months and 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 7 commonly occurring *ESR1* mutations to track these mutations in plasma until clinical progression. Of the 39 patients who progressed on first line AI, 56.4% (22/39) had *ESR1* mutations detectable at progression. In the patients with *ESR1* mutations detected, the mutations were polyclonal in 40.9% (9/22) of patients (Figure 2). In serial tracking prior to progression, *ESR1* mutations were detectable in plasma prior to progression in 86.4% (19/22) of patients, with *ESR1* mutations detectable a median of 6.7 months (95% CI 3.7-NA) prior to clinical progression (Figure 4). In patients who progressed on AI, all patients who had *ESR1* mutations

detected prior to progression also had *ESR1* mutations detected at progression, suggesting the 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. Consistent with other studies (5) (12) (13), *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 allele fraction (AF) of 0.002, whereas 9 *ESR1* mutations detected only by ddPCR occurred at AF's ranging from 0.0004 to 0.032. For 1 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 to concordant cases (median 14.3 vs 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) of the patients (Figure 2), found at aggregate relative allele frequency <0.25, with *ESR1* mutation diversity increasingly detectable at the point of progression compared to samples taken prior to 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. Deep sequencing or ddPCR of ctDNA obtained from plasma identified *RAS* mutations in 15.4% (6/39) of progressing patients (4 *KRAS* (2 of which were polyclonal), 1 monoclonal *HRAS* and 1 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 (14) (15). 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 prior to 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. 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.

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) of patients, with no *KRAS* mutations detected in controls (Supplementary table 2). 19.0% (8/42) 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 3).

We assessed the impact of *KRAS* mutations on progression free and overall survival (Figure 6). For patients with *KRAS* mutations the median PFS was 3.7 months (95% CI, 2.7, 11.5) and for patients with wild type *KRAS* a median PFS of 4.6 months (95% CI, 3.0, 6.5) (HR=1.04 95% CI (0.65, 1.67)

p=0.86). There was no significant difference in overall survival in those with and without *KRAS* mutations.

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 previ 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 prior to 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 prior to progression (9). This prior study reported a lower frequency of *ESR1* mutations at progression on an AI but only 4 *ESR1* mutations were analysed using 4ng 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 finding emphasises the importance of assessing clonal dominance of *ESR1* mutations in clinical development. Also, due to the sub-clonal nature of these mutations, the amount of plasma DNA analysed may have a major impact on frequencies of *ESR1* mutations identified.

In this study, we identify selection of *KRAS* activating mutations as 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) (12), and *KRAS* mutations are undetectable in the primary of two patients with selected *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 (16, 17), 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 of 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 that selected genetic mechanisms of resistance are frequent in 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.

Acknowledgments:

We thank the patients who participated in the plasmaDNA AI and SoFEA studies, along with the investigators, study nurses, and staff who supported the trials.

Funding:

This research was funded by Le Cure, Breast Cancer Now and Inivata. The SoFEA trial was funded by Cancer Research UK (grant C1491/A10962, reference numbers CRUKE/03/021 and CRUK/09/007). ICR-CTSU receives programme grant funding from Cancer Research UK (grant C1491/A15955). We acknowledge National Institute for Health Research funding to the Royal Marsden and Institute of Cancer Research Biomedical Research Centre.

Disclosures: KH, ME and NR and EG are employees, consultants or shareholders of Inivata Ltd., and/or inventors of patent applications related to sequencing strategies described in this manuscript. AR has an advisory role for Novartis, Roche and Genomic health.

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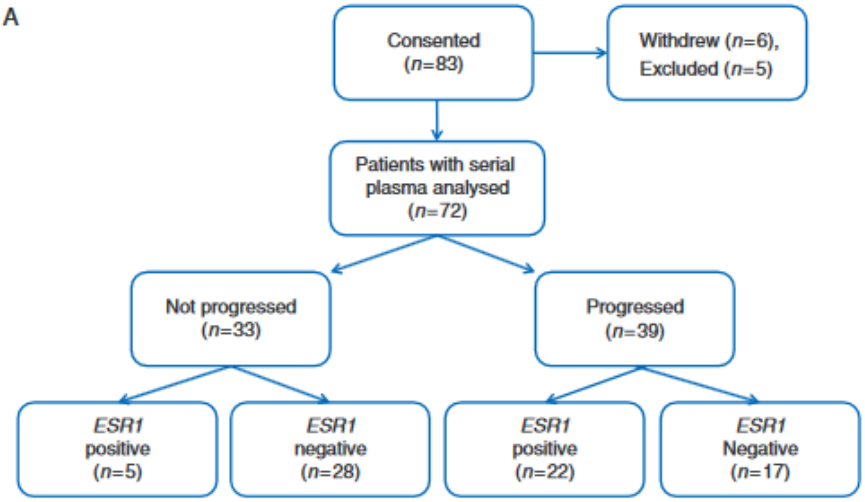
A



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.

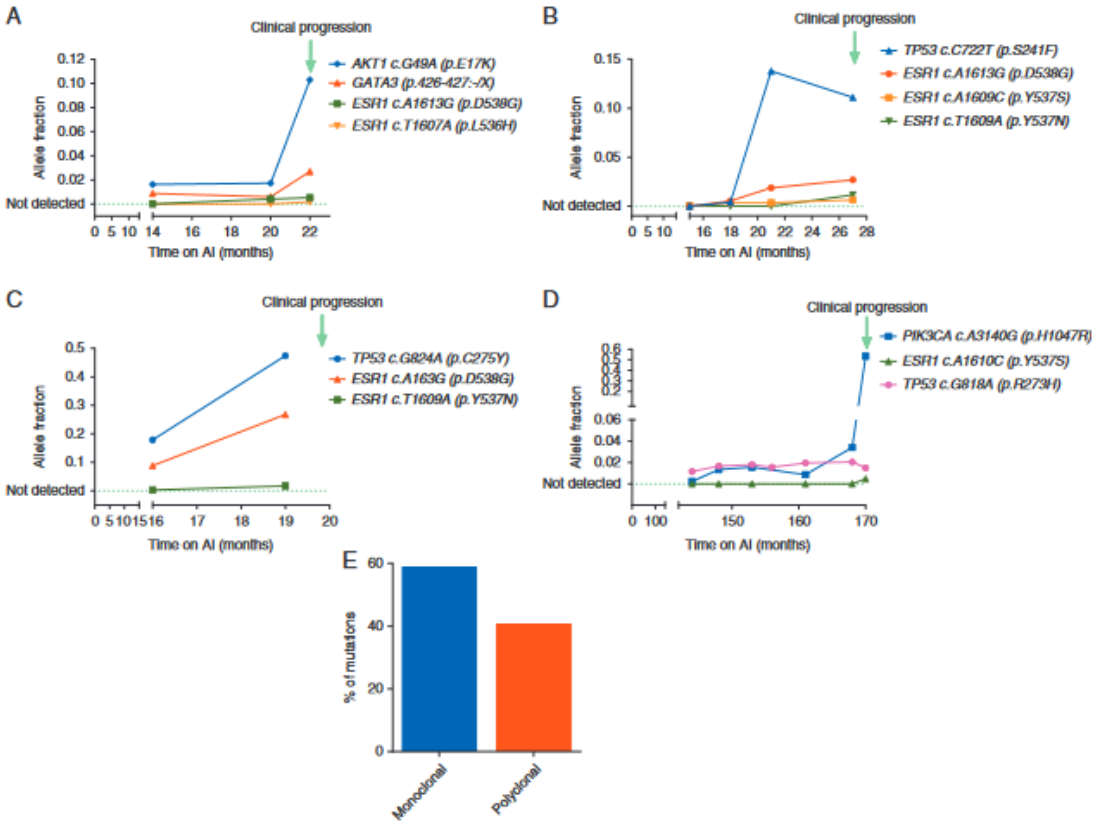


Figure 2. Evolution of *ESR1* mutations during aromatase inhibitor (AI) therapy. (A–D) Mutation tracking in ctDNA collected during first line AI therapy. Data from four patients with *ESR1* subclonal mutations detectable in ctDNA tracked until clinical progression. Allele fractions are shown as determined 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%).

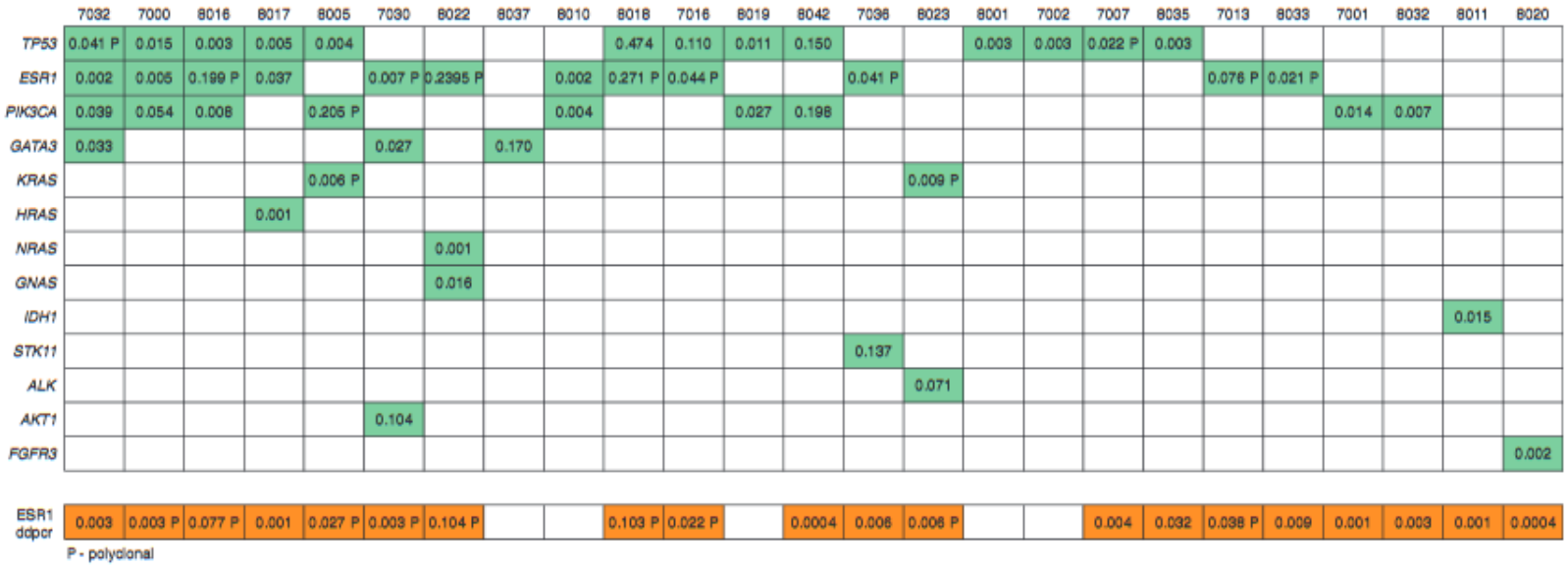


Figure 3. Error corrected ctDNA sequencing of plasma samples taken after progression on the first-line aromatase inhibitor (AI). Mutations identified in plasma DNA by eTAm-Seq error corrected sequencing, with *ESR1* mutation analysis by ddPCR. Discordant cases for *ESR1* between ddPCR and ctDNA sequencing 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.

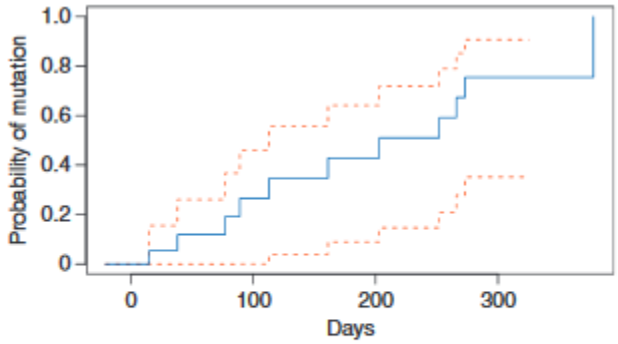


Figure 4. Lead time to development of *ESR1* mutations. Serial tracking before progression, *ESR1* mutations were detectable in plasma median 6.7 months [95% confidence interval (CI) 3.7–NA] before clinical progression.

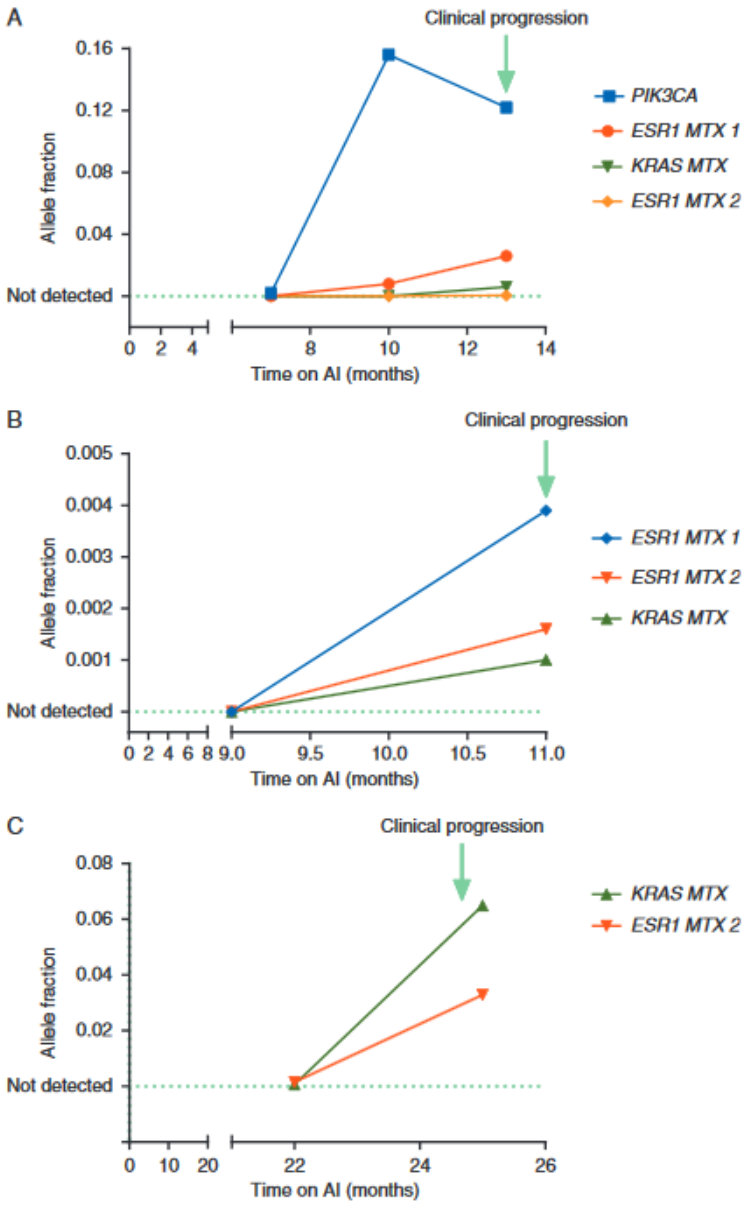


Figure 5. Evolution of *KRAS* mutations during the first-line aromatase inhibitor (AI) therapy. (A–C) Mutation tracking in ctDNA collected during first line AI therapy. Data from three patients with *KRAS* subclonal mutations detectable in ctDNA tracked until clinical progression. Allele fractions are 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.

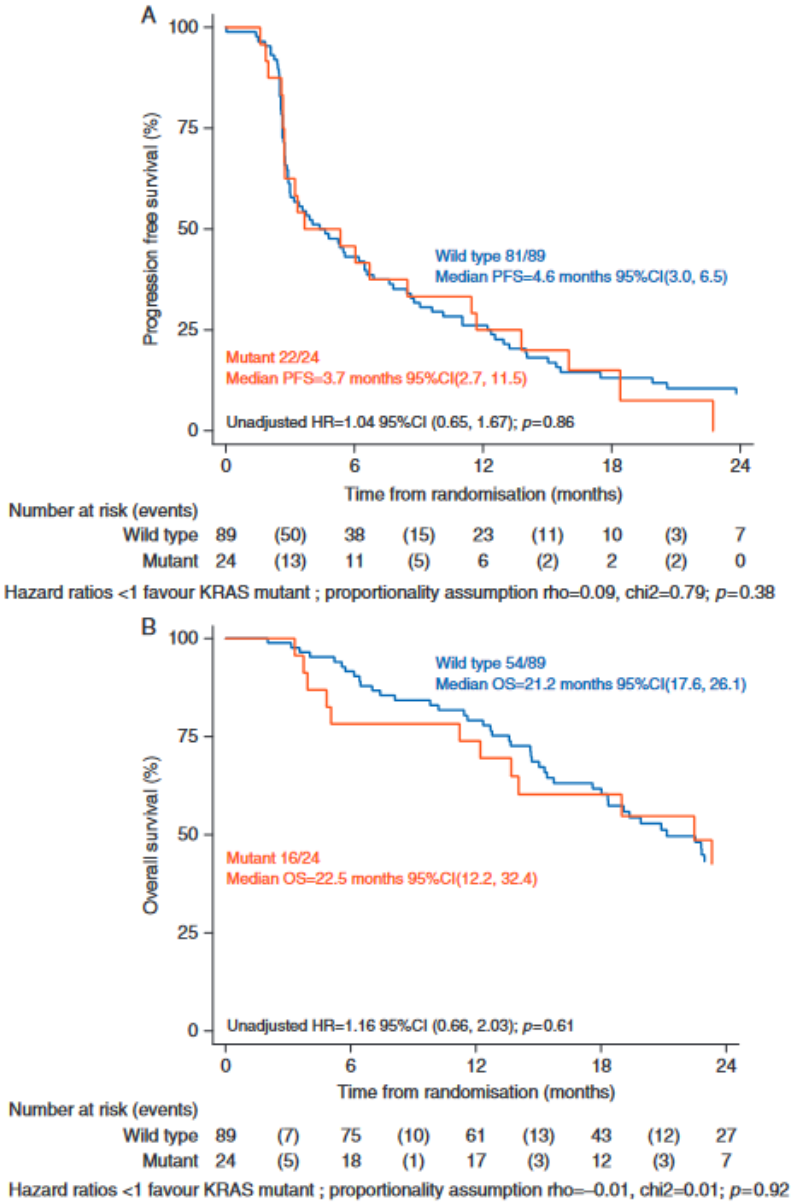


Figure 6. Independent validation of *KRAS* mutations in baseline plasma from the SoFEA study. (A) Progression-free survival (PFS) in SoFEA by *KRAS* mutation status. HR, hazard ratio. (B) Overall survival (OS) in SoFEA by *KRAS* mutation status.