**Molecular characterisation of ER+ breast cancer dormancy and acquired resistance using a clinical model: potential involvement of epigenetic regulation**

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Target journals

Clinical Cancer Research

Cancer Research

Abbreviating as:

LT long-term

ET early-on

Helpful or confusing?

**Abstract**

Understanding more about the biology of dormant tumours can help to minimise and treat cancer recurrence. Preclinical experimental studies …

**Keywords**: breast cancer, dormancy, endocrine treatment, letrozole, sequential samples, resistance, microarray, proteomics

Acquired=secondary resistance

**Introduction** Dormancy degil resistance odakli.dormant ornekler karsilastirma icin kullanildi diye anlatcaz. How it is done in the past? Bunu hasta orneklerine sinirla. How people determined resistants. Highlight no one has done before…and by saying This is the first study showing …. We validated the findings using independent series of cohorts. Independent cohort of matched samples of primary breast tumours and distant metastasis (liver and bone marrow) from the same patients that endocrine therapy failed.

Endocrine therapy, mainly tamoxifen has clearly improved outcomes for estrogen receptor alpha positive (ER+) breast cancer patients. Although five years of adjuvant tamoxifen produces a 26% proportional reduction in mortality {EBCTCG, 1998 #39}, many recur later and develop advanced ER+ breast cancer, an heterogeneity disease with limited therapy options remaining as an incurable disease {Hart, 2015 #309}. While the annual risk of mortality reduces for ER-negative breast cancer after five years following initial diagnosis and treatment, the annual rate remains constant for patients with ER+ disease {Demicheli, 2010 #59}. However, the cumulative incidence of recurrence and death continues at a steady rate. This is due to dormant cells remain in the body …

Recurrences still develop in 40-50% of ER+ patients, often many years or even decades following surgery and an apparently successful endocrine therapy.

Dormant cancer cells are thought to persist either by completely withdrawing from the cell cycle, or by continuing to proliferate at a slow rate that is counterbalanced by cell death {Uhr, 2011 #238}. Mention cellular mechanisms.. {Sosa, 2014 #243}{Dittmer, 2017 #299}. Dormancy is one of main mechanisms underlying resistance to therapy.

Resistance to endocrine therapy can occur at disease inception (*de novo* resistance) but a larger number of patients acquire resistance during treatment (acquired or secondary resistance) {Selli, 2016 #272}. Several mechanisms of endocrine resistance have been described previously {Clarke, 2015 #170} {Ma, 2015 #187}. Majority of these findings based on preclinical data obtained from cell lines and animal models. Recently, profiling of clinical samples to measure the effect of treatment or to predict response to treatment … Experimental design issues such as the lack of paired samples for comparison may overshadow the validation of findings {Sims, 2008 #5}.

For example, in a study, samples from patients who failure therapy and require salvage surgery were compared with pre-treatment samples of a separate group of disease-free patients to identify candidate genes for tamoxifen failure {Vendrell, 2008 #301}.

We aimed to identify treatment-induced changes in dormant and resistant tumours to characterise ER+ breast cancer dormancy and acquired resistance using extended-neoadjuvant endocrine treatment as a novel clinical model. This is the first study to demonstrate the extended (>4 months) letrozole-induced molecular changes using multiple sequential samples from a unique cohort of patients.

**Methods**

*Patients and samples*

Clinical characteristics of patients are given in **Table 1**. Patient and sample sizes in each study cohort with inclusion and exclusion criteria are given in **Supp. Fig.1**.

Samples were from breast cancer patients treated with neoadjuvant letrozole (Femara, 2.5 mg; Novartis Pharma AG, Basel, Switzerland). The study was approved by the local regional ethics committee (07/S1103/26, August 2007) and all patients gave informed consent. Sequential samples were taken with a 14-gauge needle before and after letrozole treatment and at the time of surgery. Fresh samples were snap-frozen in liquid nitrogen. Each tumour was confirmed to contain 50% cellularity and at least 60% tumour tissue histopathologically (H&E sections). Following pulverisation of tissue with membrane disruptor (Micro-Dismembrator U, Braun Biotech), phase separation was performed by guanidinium thiocyanate-phenol-chloroform extraction (Qiazol Lysis Reagent, Qiagen).

*Gene expression profiling*

RNA was extracted from the aqueous phase by column-based purification (miRNeasy mini kit, Qiagen). Then, RNA was labelled and hybridized to chips (HumanHT-12 v4 Illumina BeadChip) according to the manufacturer’s protocol (NuGEN) for amplified samples as previously described {Arthur, 2014 #11}{Turnbull, 2012 #51}. Raw data was detection filtered, log2 transformed, and quantile normalized using lumi package {Du, 2008 #307}.

*Proteomics analysis*

Protein was extracted from the organic phase using… (protocol).

*Data analysis*

Raw gene expression data is available from the National Centre for Biotechnology Information Gene Expression Omnibus (accession no GSE…..). Validation dataset GSE numbers Cross-platform correction was performed using Combat (Jonhson 2007, ref 26 in JCO paper). MDS and hierarchical clustering analysis was performed using ….ref. (R/Bioconductor).

Paired Rank Product analysis was performed using Multi Experiment Viewer (MeV, V4.9.0).

Proteomics data is available …

**Results**

**Long-term endocrine therapy as a model to investigate dormancy and resistance**

Sequential clinical samples from the same patient with no surgery and extended letrozole treatment were used to model clinical ER+ breast cancer dormancy and acquired resistance (**Fig. 1A**). A total of 62 patients were classified in two groups based on dynamic change in tumour size by USS and in proliferation (**Fig. 1B**). Patients with >40% initial decrease in tumour size by 4 months of treatment and with no progression by the latest biopsy were classified as “dormant”, if otherwise they were classified as “acquired resistant”. For patients latest USS measurement were taken more than a month before surgery (latest sampling), changes in three proliferation markers were used to assist classification decision. For this, dynamic changes in the widely used markers of proliferation Ki-67 (MKI67), PCNA and MCM2 levels were used (**Fig. 1B**) {Jurikova, 2016 #305}. MCM2 has recently suggested as an alternative to Ki-67 for measuring clinical and treatment outcome in breast cancer {Yousef, 2017 #304}. Classifications based on USS and mean change in proliferation markers were concordant for the majority of the patients with a few exceptions where USS classification has been used for the final decision (**Fig. 1B**). Dormant and resistant samples distributed uniformly independent of time. Duration of treatment at each time point was not significantly different between dormant and resistant samples. For long-term timepoint, mean and range values for dormant and resistant patients were 186 (121-884) days and 226 (121-1366) days, respectively (**Fig. 1C**). There were no significant differences in clinico-pathological features of patients from different response classes (**Table 1**).

Intrinsic molecular subtypes (pam50, genefu, R) were found to change under endocrine treatment (**Fig. 1D**). At diagnosis, all tumours were LumA or LumB except one resistant tumour being classified as Her2 enriched. Molecular subtypes was ordered based on their prognostic significance, from good to poor diagnosis: Normal-like> LumA > LumB > Her2/Basal {Weigelt, 2010 #306}. After long-term treatment, a total of 5 out of 20 (25%) tumours switched to a worse prognosis subtype whereas change in dormant patients was always towards a better prognosis subtype (**Fig. 1D**).

Kaplan-Meier analysis demonstrated significantly lower disease free survival for resistant patients compared to dormant (log rank, P=0.026, **Fig. 1E**). The time between removal of primary tumour and either event time or latest follow-up time was used for disease-free survival analysis. Recurrence rates for dormant and resistant patients were 21% (9/42) and 45% (9/20), respectively. Resistant patient suffered earlier recurrence compared to dormant patients (P=0.05, range=26-947 vs 136-2042 days, **Fig. 1E**). In addition, 2-year recurrence-free survival was significantly different between groups (log rank, P=0.021, **Fig. 1E**).

**Distinct transcriptomic changes under long-term letrozole treatment**

We performed unsupervised analysis to see whether samples will be separated based on their response class and/or treatment duration. Most variant 500 (mv500) genes were used for unsupervised clustering of gene expression. Hierarchical dendrograms in **Fig. 2A** show that samples cluster into two main subclasses forming mixed clusters, resistant tumours being spread among dormant patients. However, long-term treatment samples clustered together and the separation improved with the use of only long-term treated samples for most variant feature selection and clustering where two clusters with different percentage of resistant among dormant samples (52% vs 21%) were determined (**Fig. 2A)** suggesting that long-term treatment induces expressional changes.

In order to determine whether samples varied over time under treatment, we utilised multidimensional scaling (MDS) analysis with mv500 features. MDS plot showed a significant (P<0.001) shift from left to right after long-term treatment. This trend was significant (P<0.001) for dormant but not for resistant samples (**Fig. 2B**) showing that dormant and resistant patients respond to treatment differently.

Also, interpatient (between patients) and intra-patient (comparison of samples from the same patient) Pearson correlation coefficients were calculated to determine variability. Interpatient correlation coefficients were similar at each time point and was not different between response classes (not shown). Long-term treated samples were significantly (P=0.01, Wilcoxon) less correlated with pre-treatment samples (median=0.89, range=0.74-0.95) compared to correlation between early-on and pre-treatment samples (median=0.91, range=0.84-0.95). The decrease in correlation over time was significant (P….) for dormant but not for resistant patients (**Fig. 2C**).

As unsupervised approach showed that treatment does induce changes with more consistent changes in dormant patients, we further investigated what changes over time using differential expression analysis.

**Changes in genes/pathways under long-term letrozole treatment**

To determine the differences in gene expression apparent after long-term treatment compared to diagnosis (pre-), pairwise Rank Product analysis (FDR <0.01) was used.

In dormant patients, a total of 2319 genes significantly differentially expressed (1063 down- and 1256 up-regulated) in long-term treated dormant samples compared to pre-treatment. These down and up genes were significantly enriched (ReactomePA, P <0.01) for a total of 62 and 26 pathways, respectively (Table). Briefly mention about pathways…

In resistant patients, a small number of genes (238 genes) were differentially expressed (653 down and 175 up-regulated) between long-term treated and pre-treatment samples. Down- and up genes were significantly enriched (ReactomePA, P <0.05) for 2 and 9 pathways (Table). Briefly mention about pathways…

We then investigated whether these changes occurs early-on treatment or specific to long-term treatment. In dormant patients, significant changes started early-on treatment but more dramatic after long-term (**Fig. 3A**). For resistant patients, down-regulation was significant at early-on timepoint whereas up-regulation was significant only after long-term which suggest that these lately up-genes may mediate acquired resistance (**Fig. 3B**).

We further determined whether differentially expressed genes identified in each class were shared between dormant and resistant patients. Down- and up-regulated genes identified in resistant tumours were significantly changed (P <0.01) in dormant patients (**Fig 3C**). Up genes identified in dormant patients were significantly upregulated in resistant patients. rebel NDs ===bunlar belki farkli bir alt gruptur

These suggest that genes and pathways identified using within class comparison may be common features of long-term treatment rather than being specific to dormancy/resistance. This led us to perform comparative analysis (dormant vs acquired resistant) which can provide better characterisation and class separation.

EMT Estimate Imsig

**Comparative analysis of dormant and resistant patients identifies robust classifiers (if possible) potential role of epigenetic regulation in acquired resistance**

Fig4 must mention about genes and don’t give heatmaps (Andy)

Connectivity Map (cMap) analysis

A negative score means that the signature can be reversed by the drugs.

An HDAC inhibitor Trichostatin A had the second lowest score (Fig 4..). Letrozole had a positive score of 0.893 further confirming the reliably of the hypothetical scores calculated bu cMap.

S100P methylation blood-tissue correlation

**Also mention about comparative proteomics**

**Which pathways changes globally?**

We further implemented a wider approach and explored a total of 1943 different Reactome database pathways in the dataset. A total of 105 pathways were significantly (FDR <0.01) differentially expressed between dormant and resistant tumours after long-term treatment. Among those were cell cycle, cellular senescence, DNA repair and replication, acetylation and deacetylation of histones, mitochondrial translation, Rho GTPases signalling, and TP53 activity related pathways.

A total of … pathways were initially at early-on treatment significantly downregulated whereas upregulated after LT treatment suggesting possible acquired escape mechanisms.

**Can any currently available gene signatures predict response class in the dataset?**

BCI

Oncotype

Others

**Discussion**

Here we demonstrated the results of the biggest clinical dataset studied so far to investigate ER+ breast cancer dormancy and resistance.

Unsupervised analysis did not provide a clear separation suggesting that variability between dormant and resistant tumours was subtle and determined by other factors than the most variant features. However, MDS plots and Pearson correlations, showed that dormant tumours significantly change under long-term treatment whereas acquired resistant patients are heterogeneous in their response.

# LT is making difference compared to early-on timepoints.

#But some changes are apparent at early-on and can be used to predict later-on changes. Dormancy can be predicted within first 4 months. Andy said don’t say prediction.

Genetic alterations observed in long-term treated resistant tumours are independent from resistance behaviour rather than being descriptive as they were significantly apparent in all dormant samples. Similarly, transcriptomics changes identified in long-term treated dormant tumours were shared by some but not all resistant tumours. This might be a consequence of resistance heterogeneity where some tumours share similar changes with dormant tumours while having their individual way of escape from treatment-induced dormancy. Re-growing tumours with opposite profile of dormants …..

Change in ECM ==meaning==is it tumour ECm or stromal ECM? How to differentiate?

Decent separation when you look at timepoint 4 (or these pathways genes) ….

They appeared to seem different

Resistance heterogeneity

tumour heterogeneity….

Molecular mechanisms that mediate epigenetic regulation include DNA methylation and chromatin modifications.

Epigenetic alterations are recognized to occur in various developmental disorders and cancer,

**Conclusion**

**Acknowledgements**

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**References**

**Fig. 1.** **Long-term endocrine therapy as a clinical model to investigate ER+ breast cancer dormancy and acquired resistance. A)** Extended (>4 months) letrozole treatment was utilised as a clinical model of breast cancer dormancy and resistance. Sequential clinical samples from the same patient with no surgery and extended treatment were used to model clinical breast cancer dormancy and resistance. Pre-treatment (<0 days), early-on treatment (0-120 days) and long-term (extended: >120 days) neoadjuvant letrozole treatment. **B)** Dynamic change in tumour size by USS and mean expression of proliferation markers MKI67, PCNA and MCM2 were used to classify patients into two categories, dormant (blue) and resistant (red). Overall comparisons of classifications per patient based on USS and mean change in proliferation markers with final classification are shown. **C)** The duration of letrozole treatment (days, log2 transformed) for samples, each bar represents a sample. **D)** Intrinsic subtype classification by PAM50 at each timepoint. **E)** Kaplan-Meier plots showing overall survival and 2-year recurrence-free survival probability in dormant vs resistant patients (log-rank test). Density plot shows the distribution of time to recurrence.

**Fig. 2.** **Distinct transcriptomic changes under long-term letrozole treatment**. **A)** Unsupervised hierarchical clustering with most variant 500 features. **B**) Multidimensional scaling (MDS) plot using most variant 500 genes across all timepoints. Each dot corresponds to a sample and sizes represents the duration of treatment. **C)** Intra-patient (comparison of samples from the same patient) correlations are shown. Dormant (blue), resistant (red), pre (<0 days, pre-treatment), early (0-120 days-on treatment) and long (long-term treatment, >120 days), \*\*\*P<0.001.

**Fig. 3. Enrichment for cell cycle, senescence, epigenetic regulation and ECM-associated pathways.** Differentially expressed genes between pre-treatment and long-term treated sample of dormant (**A**) and resistant (**B**) patients were determined.Heat-maps showing change in down-and up-regulated genes’ expression in dormant (**A**) and resistant (**B**) samples. Each column represent a sample and each row a gene. Colours are log2 mean-centred values with red indicating high values and blue indicating low expression. Bar plots on top of heat-maps represent the time on treatment (log2, days) for each sample. Graphs on the left show dynamic changes in mean expression of down- and up-regulated genes. **C)** Dynamic change in mean expression of differentially expressed genes across patient groups. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05.

**Fig.4. Comparative analysis of dormant and resistant tumours. A)**

Top 5 drugs with the lowest scores in connectivity map analysis with differentially expressed genes.





