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Association between breast cancer subtypes and response to neoadjuvant anastrozole

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ABSTRACT

Considerable heterogeneity exists amongst oestrogen receptor positive (ER+ve) breast cancer in both its molecular profile and response to therapy. Attempts to better define variation amongst breast tumours have led to the definition of four main "intrinsic" subtypes of breast cancer with two of these classes, Luminal A and B, composed almost entirely of ER+ve cancers. In this study we set out to investigate the significance of intrinsic subtypes within a group of ER+ve breast cancers treated with neoadjuvant anastrozole. RNA from tumour biopsies taken from 104 postmenopausal women before and after 2 weeks treatment with anastrozole was analyzed on Illumina 48K microarrays. Gene-expression based subtypes and risk of relapse (ROR) scores for tumours pre- and post-treatment were determined using the PAM50 method. Amongst pre-treatment samples, all intrinsic subtypes were found to be present, although luminal groups were represented most highly. Luminal A and B tumours obtained similar benefit from treatment, as measured by the proportional fall in the proliferation marker Ki67 upon treatment (mean suppression = 75.5% vs 75.7%). Tumours classified as basal and Her2-like showed poor reductions in Ki67 upon treatment. Residual Ki67 staining after two weeks remained higher in the Luminal B group. ROR score was significantly associated with anti-proliferative response to AI and with clinical response. These results suggest that in the short-term, Luminal A and B tumours may gain similar benefit from an AI but that the higher residual Ki67 level seen in Luminal B is indicative of poorer long term outcome.

1. Introduction

Tumours expressing oestrogen receptor α (ERα) make up almost 80% of breast cancers diagnosed in the developed world. While the major molecular features of breast cancer segregate differentially between ER+ve and ER−ve disease [1,2], considerable heterogeneity also exists within the ER+ve group. Gene expression studies have led to tumours which express ERα being termed luminal type [1,3] with this group associated with response to antioestrogen therapy and improved survival.

Attempts to better define the variation within the luminal group has led to the description of Luminal A, B and for a short time Luminal C tumours [4], which are all typically ER+ve. Patients with Luminal B cancers show significantly poorer outcomes when compared to those with Luminal A [3,4]. Luminal tumours are characterised by expression of ER, PR, and genes associated with ER such as *GATA3*, *FOXA1*, and *XBP1*, as well as expression of luminal cytokeratins 8 and 18 [1,5,6]. At the gene expression level, the distinction between Luminal A and B is largely based on the degree of expression of these genes. Luminal A tumours typically have high expression of ER and ER-regulated genes and low expression of proliferation-associated genes such as Ki67 [1,5,6].

Large randomized trials have established the safety and efficacy of aromatase inhibitors (AIs) in patients with hormone receptor-positive breast cancer, and AIs are widely used in clinical practice [7]. Historical data suggests that quantitative expression of the oestrogen receptor influences the benefit gained from tamoxifen [8–10]. Differences in relative benefit for patients with increased expression of ER have not been seen in trials comparing aromatase inhibitors with tamoxifen [11,12]. In a short-tem presurgical study, higher ER expression has been shown to associate with improved Ki67 response to aromatase inhibitor treatment [13].

In this study we set out to investigate the significance of intrinsic subtypes within a group of ER+ve breast cancers treated with neoadjuvant anastrozole. We aimed to determine (1) the abundance of each subtype within an entirely ER+ve group; (2) the effect

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Table 1
Frequencies of subtypes amongst pre-treatment, two weeks and 16 weeks-post anastrozole.

	Pre-treatment		2 weeks post-anastrozole treatment		16 weeks post-anastrozole treatment	
	Number	%	Number	%	Number	%
LumA	37	36	38	45	7	26
LumB	20	19	4	5	3	11
Basal	5	5	2	2	1	4
Her2	12	12	11	13	3	11
Normal	30	29	29	35	13	48
	104		84		27	

of treatment on each subtype and; (3) the response of each subtype to treatment.

2. Experimental

2.1. Patient samples

Core-cut tumour biopsies (14-gauge) were obtained from 112 postmenopausal women with stages I–IIIB ER+ early breast cancer before and after two-weeks' anastrozole treatment in a neoadjuvant trial [14]. Tissue was stored in RNAlater at −20 °C. Two 4 μm sections from the core were stained with hematoxylin and eosin to confirm the presence of cancerous tissue and the histopathology. Total RNA was extracted using RNeasy (Qiagen, Sussex). RNA quality was checked using an Agilent Bioanalyser (Santa Clara, CA, USA): samples with RNA integrity values of less than 5 were excluded from further analysis. Immunohistochemical measurements of ER, Ki67 and PgR and objective tumour response for these patients were obtained from previously published data [14].

2.2. Gene expression analysis and data pre-processing

RNA amplification, labelling and hybridization on HumanWG-6 v2 Expression BeadChips were performed according to the manufacturer's instructions (http://www.illumina.com) at a single Illumina BeadStation facility. Tumour RNA of sufficient quality and quantity was available to generate expression data from 104 pre-treatment biopsies, 84 two-week biopsies and 27 16-week biopsies (Supplementary Fig. 1). Data was extracted using BeadStudio software and normalized with variance-stabilizing transformation (VST) and Robust Spline Normalization method (RSN) in the Lumi package [15]. Probes that were not detected in any samples (detection p value >1%) were discarded from further analysis.

2.3. Data analysis

Intrinsic subtypes were assigned using a 50-gene subtype predictor [16] using gene expression data from Illumina arrays derived as described above. We assigned a subtype to each tumour specimen tested by calculating the distances to each of the subtype centroids with the Spearman rank correlation test [16]. Data from the exclusively ER+ve tumour group in the current study were normalized prior to subtyping analysis with data from a further 64 tumours, including 23 ER−ve cancers, analyzed concurrently on Illumina arrays. Risk of relapse (ROR) scores were calculated using: ROR = 0.05·basal + 0.12·HER2 − 0.34·LumA + 0.23·LumB as described previously [16].

Statistical analyses were performed in SPSS for Windows (SPSS Inc., Chicago, IL) and Graphpad Prism (Graphpad Software Inc., La Jolla, CA). Mean suppression of Ki67 was calculated using: mean suppression = 100 − [geometric mean(post treatment Ki67/pre-treatment Ki67·100)].

Table 2
Changes in molecular subtype upon treatment with aromatase inhibitor.

	Number	Percentage
Luminal A		
Unchanged	20	63
→ Her2	1	3
→ Normal-like	11	34
Luminal B		
Unchanged	3	18
→ LumA	9	53
→ Her2	2	12
→ Normal-like	3	18
Basal-like		
→ Her2	1	50
→ Normal-like	1	50
Her2		
Unchanged	7	78
→ Luminal B	1	11
→ Normal-like	1	11
Normal-like		
Unchanged	12	60
→ LumA	7	35
→ Basal-like	1	5

3. Results

3.1. Frequency of molecular subtypes

To determine the relative proportions of each molecular subtype, gene expression data from tumour biopsies taken prior to treatment with anastrozole, two weeks after treatment and 16 weeks after treatment was used to obtain classifications for the tumours at each timepoint. Of the 104 ER-positive tumours analyzed, at baseline 55% were classified as Luminal A or B, 12% were Her2-enriched, 5% were basal and 29% were assessed as normal-like (Table 1). All the tumours classified as basal-like were confirmed as ER+ve by central analysis and 5 of the 9 Her2-like tumours were Her2 positive by immunohistochemistry. After two weeks of treatment with anastrozole, the apparent proportion of Luminal B tumours fell approximately 4-fold with only 5% of tumours classified as this type. After 16 weeks of treatment, only 27 tumours were able to be assessed. Despite this, a marked increase in the frequency of normal-like tumours was observed, while the proportion of Her2-classified tumours remained constant over 16 weeks of treatment.

3.2. Changes in assigned molecular subtype upon treatment

After two weeks of treatment with aromatase inhibitor, the assigned subtype of 53% of tumours remained unchanged (Table 2). However, the proportion of tumours that changed varied substantially according to the pre-treatment assignment of a sample. Sixty three percent of Luminal A tumours were still classified as Luminal A-type after treatment, whereas only 20% of Luminal B tumours remained unchanged after aromatase inhibitor treatment. Most Luminal B tumours changed to Luminal A (53%) with a smaller pro-

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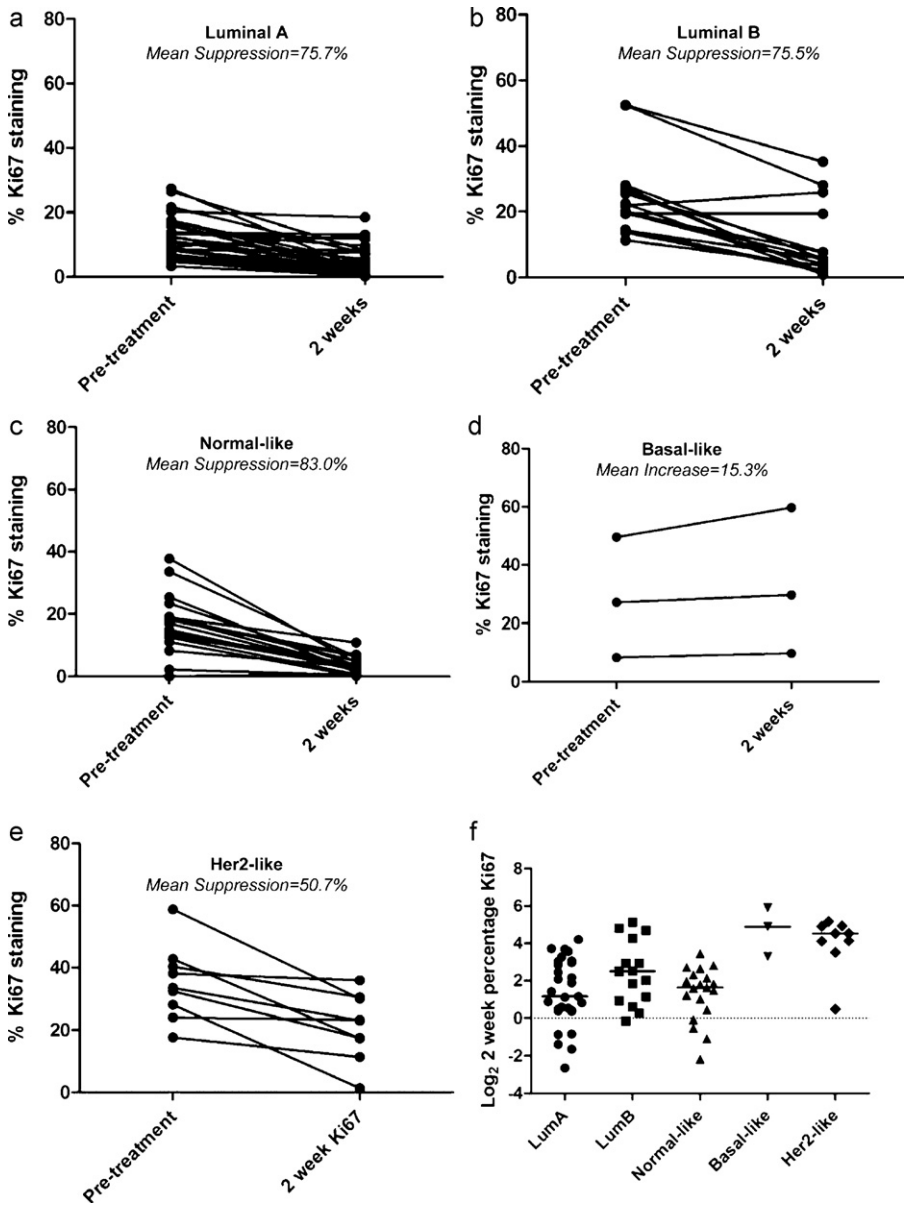

Fig. 1. Ki67 staining of tumours based upon pre-treatment subtype. Pre-treatment and two week staining in (a) Luminal A, (b) Luminal B, (c) normal-like, (d) basal-like and, (e) Her2-like tumours. (f) Ki67 staining in all tumours after two weeks of aromatase inhibitor. The median two week Ki67 staining of each subgroup is shown as a line.

portion changing to normal-like (18%). Of the Luminal A tumours that did change subtype, almost all changed to normal-like (11/12).

3.3. Changes in Ki67 and molecular subtype

The effect of 2 weeks of endocrine treatment on tumour cell Ki67 expression has previously been shown to be indicative of the effect of the treatment on long-term patient outcome [17,18]. To determine whether anti-proliferative response to aromatase inhibitor treatment is influenced by pre-treatment molecular subtype, changes in Ki67 upon treatment were examined in the five molecular subtypes (Fig. 1a–e). Proportional Ki67 suppression in Luminal A tumours was not significantly different to that observed in Luminal B tumours, despite Luminal B tumours initially having higher levels of Ki67 staining (geometric mean Ki67 suppression = 75.5% vs 75.5%; geometric mean pre-treatment Ki67 = 11% vs 22%). Normal-like tumours showed the greatest proportional reduction in Ki67 (83.0% suppression), while tumours classified as basal-like (15.3% increase) and Her2-like (50.7% suppression) generally showed poorer responses. Two week Ki67 also showed substantial variation between subgroups (Fig. 1f). Her2-like tumours showed significantly higher Ki67 at two weeks when compared with Luminal A, Luminal B and normal-like. Median two week Ki67 in Luminal B was higher than in Luminal A (5.6 vs 2.2) although this difference was not statistically significant ($p = 0.11$).

3.4. Risk of recurrence score analysis

The risk of relapse (ROR) score was developed to provide an assessment of prognosis by incorporating the gene expression-based "intrinsic" subtypes into a continuous score [16]. Pre-treatment ROR score was significantly correlated with two week change in Ki67 after two weeks of aromatase inhibition ($Rs = 0.35$, $p = 0.0019$) (Fig. 2).

To determine the relationship between ROR score and clinical response to aromatase inhibitor treatment, objective tumour response rates for these patients were obtained from previously published data [14]. The proportion of patients showing a com-

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Table 3
Relationship between risk score and clinical response. (A) Association between pre-treatment risk score and clinical response. (B) Association between 2 week risk score and clinical response.

Pre-treatment risk group	Complete or partial response	Total	Complete or partial response (%)	*p*-Value
A				
High risk	6	18	33	
Medium risk	21	43	49	0.04
Low risk	28	42	67	
	55	103		

2 week risk group	Complete or partial response	Total	Complete or partial response (%)	*p*-Value
B				
High risk	0	3	0	
Medium risk	9	24	38	0.03
Low risk	34	56	61	
	43	83		

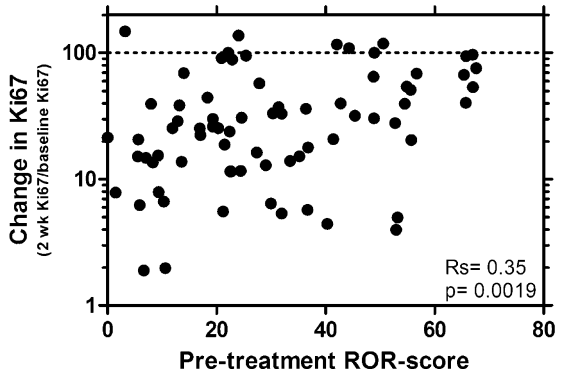

Fig. 2. Relationship between risk score and % change in Ki67 upon treatment with aromatase inhibitor.

plete or partial response by International Union Against Cancer (UICC)/WHO assessment criteria in each category of risk score (low, medium or high) was calculated. Using baseline classifications, the proportion of patients recorded as having a complete or partial response fell as risk score increased (Table 3A). Of the 18 patients with high pre-treatment ROR scores, only six experienced an objective clinical response whereas 28 out of 42 low-risk patients responded. Using risk groupings assessed from post-treatment samples, the trend towards improved response in patients with lower ROR scores strengthened. Of the three patients with high risk scores, none responded while 38% and 61% of the medium and low risk groups were observed to have an objective clinical response ($p = 0.03$).

4. Discussion

Over the last decade, the molecular taxonomy of breast cancer has been defined by the observation of intrinsic molecular subtypes with prognostic significance in multiple breast cancer cohorts [3,16,19]. However, the relationship between subtype and response to aromatase inhibitor treatment has not previously been characterised.

In this study we show that, contrary to our expectation, the anti-proliferative response to neoadjuvant aromatase inhibitor treatment is similar in Luminal A tumours to that in Luminal B tumours. However, ROR score, as a continuous measure of risk of recurrence, was significantly associated with anti-proliferative response to AI. ROR score was also associated with clinical response with the strongest association observed using two-week ROR score.

As a group, our solely ER+ve untreated samples had similar proportions of the five main subtypes of breast cancer to other ER+ve sets examined previously [16] (Table 1). The identification of basal-like tumours in an ER+ve set and Her2-like tumours without detectable Her2 overexpression suggests that tumours may display the transcriptional profiles associated with these states without alterations in the normally causative biomarker. It is notable that anti-proliferative response in these tumours was generally consistent with their molecular subtype rather than their immunohistochemical marker status. For example, all tumours identified as basal-like had poor responses while tumours identified as transcriptionally Her2-like had similarly poor outcomes, regardless of whether they had identified *ERBB2* amplification (Fig. 1e).

Although the assigned classification of a tumour upon aromatase inhibitor treatment is not directly comparable to a baseline intrinsic subtype, the frequency with which the various subtypes change gives an indication of the effect of AI on the transcriptome. The majority of Luminal A tumours remain as Luminal A with most of the remaining one third switching to normal-like or unclassifiable (Table 2). This may be a reflection of the changing cellular composition of the core biopsy or the quiescence of treated tumours. Conversely, the majority of Luminal B tumours change classification with most converting to Luminal A, thus reflecting the fall in proliferation induced by oestrogen deprivation. A smaller proportion changed to Her2-like, normal-like or remains unchanged. Of the other subtypes, Her2-like remain the least affected by treatment with over three quarters of these tumours retaining their pre-treatment classification. This is consistent with previous reports implicating Her2 in endocrine insensitivity [20].

Similar trends were observed in the antiproliferative response to AI therapy as measured by Ki67 staining. In general, Luminal A and B tumours showed similar proportional reductions in Ki67 while basal-like and Her2-like showed poorer response. The absence of a difference between Luminal A and B was unexpected given the higher expression of ER-related genes by Luminal A tumours that would be expected to mediate a greater response to oestrogen deprivation. Our interpretation of this is that in the short-term Luminal A and B tumours may gain similar benefit from an AI but that the higher residual (2 week) Ki67 level seen in Luminal B is indicative of poorer long term outcome [18].

ROR score, which is based upon intrinsic profiles, showed greater association with antiproliferative response (Fig. 2). A significant association was also seen with clinical response which was strengthened when two week ROR score was used. This finding is consistent with the observation that two week Ki67 is a better predictor of recurrence-free survival than pre-treatment Ki67, suggesting that on-treatment measurements may capture the combined effect of treatment upon the inherent prognosis of the tumour [18].

Overall, our analysis suggests that while conventional gene-expression based intrinsic subtyping provides a useful illustration of the variation in response to aromatase inhibitors exhibited by

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different types of tumours, its ability to predict which tumours will have better response to aromatase inhibitors is limited. Instead, the continuous ROR score appears to be better associated with both change in Ki67 and clinical response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2011.02.025.

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