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Molecular analysis of hormone receptor positive (luminal) breast cancers – What have we learnt?

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ABSTRACT

Recently, whole-genome molecular profiling of cancers has revealed that breast cancer consists of a number of distinct diseases at the biological level, each of which will require independent research into the most suitable therapy for that patient. In particular, this has long confirmed the clinician's impression that the clinical behaviour of oestrogen receptor (ER)-positive breast cancer can be markedly heterogeneous despite similar levels of expression of the oestrogen receptor. At present, it seems that there are at least two distinct diseases of luminal origins. In the future, it is likely that we will be treating the luminal-A tumours, characterised by high expression of the ER and related genes, differently from the non-luminal-A tumours, which are characterised by low expression of the ER and related genes, high expression of proliferation genes and a poor clinical outcome. This article reviews the progress thus far in producing a framework for defining the ER-positive luminal subtypes and for our current understanding of the genetic aberrations that may be contributing to the poor prognosis of the non-luminal-A breast cancers.

1. Introduction

Clinicians have long been aware of the clinical and biological heterogeneity associated with oestrogen receptor (ER)-positive breast cancers. Until recently, the ER (referring to ERalpha) has been only molecular characteristic routinely measured in the evaluation of newly diagnosed breast cancers as it is known to be prognostic (albeit weak) but more importantly, predictive of benefit to anti-oestrogen therapy. However, not all ER-positive breast cancers respond to anti-oestrogen therapy. The expression of ER is known to vary amongst breast cancers according to age (increasing with older age) and with proliferation activity (inversely related) and is known to influence tamoxifen response.[1,2] However, the expression of the ER alone does not solely explain the considerable differences in clinical outcome for tumours that have apparently similar histopathological features. Recently, with the completion of the human genome project and the advent of high throughput microarray technology, whole-genome gene expression studies have proposed that the heterogeneity of clinical response can be correlated with different molecular 'portraits' of breast cancers.[3] In particular, the ER and HER2 status have been observed to predominate in defining distinct molecular subtypes of breast cancer with significantly different clinical outcomes. In this article, we review what we have learnt about heterogeneity of ER-positive breast cancers from these molecular studies and, how this information may help us to eventually individualise the therapy for our breast cancer patients with ER-positive tumours.

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2. Molecular classification of breast cancers revealed through microarray profiling and unsupervised cluster analysis

In a landmark study, on the basis of microarray data, Perou and colleagues[3,4] used an unsupervised or class discovery approach to determine if breast cancers could be divided into subgroups based on similar transcriptional profiles. These subgroups were then assessed as to whether they had different clinical outcomes as a result of their diverse biological make-up. The clustering algorithm produced three main groups of breast cancer: oestrogen receptor positive ('luminal-like'-associated with expression of the oestrogen receptor (ER) and ER-regulated genes), oestrogen receptor negative ('basal-like'-associated with overexpression of the basal cytokeratins) and HER2 overexpressing tumours. The luminal tumours had a significantly better clinical outcome than the basal-like and HER2 overexpressing group. Of particular interest, the luminal breast cancers could be divided further into 2 or 3 different subgroups (luminal-A, -B and -C). The luminal-A subgroup had the highest expression of ER and ER-regulated genes and a better clinical outcome compared with the luminal-B group, suggesting that the molecular 'portrait' of breast tumour at an early stage may give clinicians considerable insight into its prognosis and treatment response. These results were extremely provoking as they suggested that molecular profiling may help us elucidate the biology underlying the heterogeneity of clinical outcome in breast cancers, and may aid clinicians in eventually realising the highly desired aim of individualising therapy for their patients. Shortly after this study was published, and despite the potential instability of these class discovery, cluster-based approaches, similar subgroups were produced by independent investigators, supporting the concept that breast cancer consists of a number of biological distinct diseases at the molecular level.[5,6] Whilst this molecular classification is currently of limited clinical implications due to the technical difficulty in applying a microarray clustering algorithm to an individual sample, conceptually these early microarray studies were important as they proved for the first time that breast cancer is indeed a biologically heterogeneous disease and its behaviour could not be simply explained by variation in ER level.

3. Definition of luminal subtypes and elucidating the underlying biology behind prognosis in ER-positive breast cancer

Although the basal and the HER2 subtypes are easily identified in the clinical setting, a consistent definition of clinically relevant ER-positive subgroups through molecular profiling is lacking. A number of different gene signatures producing classifications in ER-positive breast cancer have been proposed (discussed below). These are yet to be reconciled, and a consistent definition of the luminal subgroups that can be routinely and easily clinically implemented is yet to be agreed upon. These numerous classifications are mainly the result of the large number of genes analysed relative to the number of samples available (i.e. tens of thousands of genes to maybe a couple of hundred samples) in microarray studies. In addition, it has become increasingly evident to the researchers in the field that the gene sets reported in the literature that provide good prediction of prognosis in breast cancer may not necessarily contain the genes that are important biologically for tumour progression and therefore cannot provide realistic therapeutic targets. A stable definition of clinically relevant luminal subtypes would significantly help clinical and laboratory researches in the area. It would lead to prospective stratification in clinical trials that evaluate endocrine therapy, chemotherapy and biological therapies and more specific research into the biological pathways involved in each subtype: determining if any of these could serve as therapeutic targets and the development of appropriate experimental models.

3.1. *Intrinsic gene set*

Based on their initial cluster analysis discussed above, Perou and colleagues have proposed an 'intrinsic gene set' and have developed a classifier for single sample predictions using the mean expression profile (i.e. the centroids) for each of the 5 subtypes defined initially.[3,5,7] The intrinsic gene set was defined as a set of genes which showed the most variation across tumour samples, but the least variance amongst tumour profiles from the same sample.[3] Independent validation of a new, refined intrinsic gene list in 311 breast cancer samples found that each subtype was associated with different relapse free and overall survival, with the basal, HER2 overexpressors and the luminal-B subgroups having the worse clinical outcome.[7] Of biological interest, the intrinsic gene list contains a significant proportion of genes involved in cellular proliferation and interferon-regulated genes. High expression of these gene groups was linked to the poor prognosis subtypes (basal, HER2 and luminal-B), suggesting that these groups of genes could play a significant role in the biology of these clinically aggressive subgroups.

3.2. *Genomic Health 21-gene recurrence score (RS)*

Genomic health, using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), has developed an algorithm based on the expression of 16 genes that can separate node negative, ER-positive breast cancers into 3 groups of low, intermediate and high risk of recurrence.[8] This assay was derived from analysing the results from three preliminary studies involving 447 samples from a heterogeneously treated population (chemotherapy and tamoxifen) and 250 candidate genes selected after a literature search of the most important microarray experiments relating to breast cancer prognosis. Of these, 21 genes were chosen, 5 of which were control genes. The predictor was subsequently validated prospectively on 675 archival patients who had received tamoxifen only in the NSABP B-14 trial. The RS accurately predicted patients at high versus low risk of recurrence on tamoxifen ($p < 0.00001$). The advantage of this test, compared with the intrinsic gene set classification, is that it can be easily performed on paraffin embedded tissue with a high degree of accuracy using a small amount of tissue. However, many argue that this genomic test does not encompass more than quantitative oestrogen, progesterone receptor status,

proliferation and HER2 measurements in an unnecessarily complex mathematical algorithm. The measurements of these variables are all already widely used and are freely available and, importantly, this test provides no new biological insights into prognosis in ER-positive breast cancer. Those patients classified as low risk seem to have high expression of the ER-related genes and low expression of the proliferation and HER2 gene groups. These characteristics seem to be common with the luminal-A subtype defined by the intrinsic gene set. The RS is currently commercially available (Oncotype Dx™; Genomic Health Inc., Redwood City, CA) and is the subject of a randomised clinical trial that evaluates its ability in ER-positive, HER2 negative, node negative breast cancer patients as a predictor of response to chemotherapy (TAILORx) being conducted by the United States (US) Intergroup.[9]

3.3. *Oestrogen-regulated genes*

Oh and colleagues have used oestrogen-induced genes identified from MCF-7 cells treated with 17β-estradiol to define two prognostic groups of patients within 400 ER-positive breast cancer samples.[10] These oestrogen-regulated genes were, as expected, found to be highly expressed in the luminal/ER-positive cluster produced by the intrinsic gene set. Two prognostic groups could be produced by this oestrogen gene set within the ER-positive breast cancer samples examined, with the good prognostic group noted to have high expression of FOXA1, progesterone receptor-related and ribosomal genes, and the poor prognostic group observed to have high expression of genes involved in proliferation, MAGE-A, interferon and apoptosis (FLIP, AVEN, *Survivin*) pathways. The subgroups produced were highly correlated to the luminal-A/B classification produced by the intrinsic gene set. The advantage of this study was that it produced biological hypotheses about potential mechanisms which could contribute to the luminal-B subgroup having a poorer outcome, apart from reduced expression of oestrogen-regulated genes. Investigation of dysfunctional apoptosis, proliferation and interferon pathways may therefore provide insight into the molecular interactions that are important in regulating this subgroup's tumour growth.

3.4. *Genomic grade-defined luminal subtypes*

Histologic grade has long been known to be a strong prognostic factor in breast cancer; however, difficulties in reproducing grading amongst pathologists and a large proportion of breast cancers being graded as 'intermediate' have limited its usefulness in the clinical setting. A grade gene expression signature (GGI) was therefore developed, with the aim that it could objectively and quantitatively determine the similarity of a tumour's profile to low (well differentiated and hence, good prognosis) or high (poorly differentiated and hence, poor prognosis) histologic grade.[11] Despite tracking a single biologic pathway – cellular proliferation – the GGI was able to divide over 600 ER-positive breast cancers into two prognostic groups. These groups were highly correlated to those produced by the intrinsic gene set and the 21-gene RS.[12]

The ability to define a poor prognostic group of patients with ER-positive breast cancer allowed us to further investigate the upstream oncogenic pathways that could be driving increased cellular proliferation. Gene set enrichment analysis (GSEA)[13,14] proposed a number of hypotheses that could be investigated in the laboratory setting. Of interest, we noted that a number of apoptosis-related, DNA damage response, ERBB2 and PI3K/AKT gene sets were significantly enriched in the luminal-B, high genomic grade subgroup. We have subsequently shown using MCF-7 cells that activating the ERBB2 pathway results in tamoxifen resistance, suggesting that HER2 pathway is important for oestrogen-independent growth even without HER2 overexpression.[15] We have also found that a gene set encapsulating PI3K/AKT signalling produced by PIK3CA mutations can define prognostic groups in ER-positive breast cancer and importantly, *within* the luminal-B subgroup, suggesting that this pathway may be important for tamoxifen response and oestrogen signalling.[16] Currently, there are a number of small molecular inhibitors of both the HER2/3 and PI3K/AKT pathways, and given that there are gene sets which may identify those ER-positive breast cancers which may benefit from targeting these activated pathways, these findings may have important implications for the selection of therapies for evaluation in ER-positive breast cancer clinical trials in the future.

3.5. *Two-gene ratio*

Ma and colleagues have proposed two-gene expression signature which can define 2 groups of patients with ER-positive breast cancer at risk for relapse after treatment with adjuvant tamoxifen. The signature was developed from the whole-genome gene expression profiles of 60 breast cancer samples that were treated with adjuvant tamoxifen monotherapy and were subsequently reduced to a two-gene expression ratio: homeobox 13 (HOXB13) over interleukin 17B receptor (IL17BR).[17] The expression ratio was initially validated on an independent set of 20 patients using PCR-based analysis on standard formalin-fixed paraffin embedded tissue (FFPET). The overall accuracy was 80% (95% confidence interval (CI): 56–94%). The breast cancers with low expression of HOXB13 or a low HOXB13:IL17RB ratio have a good prognosis compared with those with high expression. The performance of the two-gene ratio has been assessed on several larger series of patients, using both frozen and standard formalin-fixed paraffin embedded tissues. In general, it seems that the ratio can define two prognostic groups in node negative ER-positive breast cancer; however, the validation studies varied considerably in the technologies used (microarray expression values versus RT-PCR), different cut-points and patient cohorts of different stages of breast cancer (primary versus metastatic).[18–21] The two-gene ratio seems to perform less well in node-positive patients, for reasons that are unclear[22] and is correlated with other predictors of poor prognosis-HER2 amplification, S-phase fraction, histological grade, tumour size and a number of positive lymph nodes.[19,20]

These studies have led to *in vitro* experiments to determine the role of HOXB13 and IL17BR in ER-positive breast cancer. In HOXB13-infected MCF-10A cells, HOXB13 seems to have a role in stimulating cell invasion. Recently, it has been shown that both HOXB13 and IL17BR are oestrogen-regulated genes and are involved in oestrogen signalling; however, the expression

of these genes differs from the classic ER-regulated genes suggesting that these genes may be useful as a marker of dysfunctional ER signalling.[23]

4. Is there concordance amongst luminal group classifications?

The above discussion briefly highlights a number of gene sets available in the literature that claim to be able to produce prognostic groups in ER-positive breast cancer. The overlap in the genes contained in these lists is small; however, despite this, significant concordance in the risk groups produced by many of these signatures has been shown (the intrinsic gene set, the GGI, the 21-gene RS, oestrogen-regulated genes).[11,12,24] This observation provides reassurance that the actual genes contained in prognostic gene lists seem to be identifying a dominant, common biologic phenomenon – cellular proliferation rates. We have previously shown the importance of proliferation genes in predicting prognosis in ER-positive breast cancer and the high correlation of subgroups produced by the GGI with the 21-gene RS in classifying ER-positive breast cancers.[12] Overall, these observations suggest that these particular classifiers are consistent in identifying luminal-A (good prognosis with tamoxifen) and non-luminal-A (luminal-B and potentially other poor prognostic subtypes) tumours. However, in a study comparing the risk groups that produced 21-gene RS and the two-gene ratio in tamoxifen-treated patients, the concordance was poor,[25] supporting *in vitro* studies that the two-gene ratio identifies another biological mechanism apart from proliferation rates.[23] This suggests that combining an assessment of proliferation status and the two-gene ratio may improve risk stratification for women with ER-positive breast cancers and define a very poor prognostic group of ER-positive breast cancers.

From a biological viewpoint, it is clear that the luminal-B or poor prognostic ER-positive cancers are characterised by low expression of the ER and ER-regulated genes and high expression of proliferation genes. From the studies described above, it is seems that abnormal apoptosis function, interferon regulation, DNA damage response, ERBB2 and PI3K/AKT pathways may also contribute, and the extent of their involvement in the luminal-B or poor prognostic ER-positive breast cancers should be investigated further in the laboratory and clinical settings. Furthermore, the proliferation signature contains a mixture of genes involved in cell cycle, cell death, transcription, protein synthesis and ribosome biogenesis. Elucidating which of these functions is most critical for tumour growth will help in pinpointing an effective therapeutic strategy.

5. Current implications for therapy prescription

Tamoxifen has been the standard adjuvant endocrine treatment for many years for women with ER-positive breast cancer and has resulted in significant improvements in disease-free and overall survival.[2] However, despite this, recurrences still occur with many women developing resistance to tamoxifen. As a result, there has been considerable effort to improve outcomes for these women using newer endocrine agents, particularly the aromatase inhibitors, and different scheduling – in combination with tamoxifen, upfront aromatase inhibitors or prolonged treatment for greater than the standard 5 years (reviewed by [26]). In many parts of the world, aromatase inhibitors have now become a standard part of adjuvant therapy for post menopausal women with ER-positive breast cancer, though the question remains on the best way to prescribe endocrine therapy given the many options available, and which women would still do well from a period of tamoxifen treatment, given that it is still a highly cost-effective and efficacious treatment for many women.

Recent results, which examined the role of progesterone receptor (PR) expression and HER2 overexpression in predicting differential endocrine therapy benefit, have been somewhat disappointing in that neither factor seems to be predictive. In other words, both of these factors are mainly prognostic in this setting.[27–29] Consequently, most experts recommend that women with ER-positive tumours with poor prognostic features ((1) suggestive of the luminal-B subtype with absence of PR expression, high tumour grade (and/or high Ki67); (2) HER2 overexpression; (3) $\geqslant$4 lymph nodes positive and (4) lympho-vascular invasion) consider an aromatase inhibitor upfront instead of tamoxifen as the absolute benefits are likely to be greater. It has been noted that most of the sequential tamoxifen-aromatase inhibitor trials required the patients enrolled to be disease-free at the time of 'switching', implying that a degree of sensitivity to endocrine agents was required for this strategy to be of benefit (positive for expression of both ER and PR or suggestive of the luminal-A group).[27] Also, this implies that it may be this tumour phenotype that tends to late rather than to early recurrences.

At this stage, whilst molecular profiling of breast tumours has shown the existence of different prognostic tumour phenotypes within ER-positive breast cancer, determining if any of these gene signatures provide any insight into the benefit of endocrine agents rather than just prognosis has been more difficult. Many of the studies involved tumours that had been treated with tamoxifen; however, a poor prognosis may still imply inherent tumour aggressiveness rather than tamoxifen resistance.[8,10,12] Hence, it is unclear at present if the luminal-B subtype will benefit more from alternative endocrine agents apart from tamoxifen (i.e. aromatase inhibitors) or if chemotherapy and/or biologic agents are required to effectively counteract its intrinsically aggressive biology. Interestingly, studies evaluating the two-gene ratio's (HOXB13:IL17BR) performance have suggested that these two genes may be prognostic as well as having the potential to be a biomarker of responsiveness to tamoxifen.[20,21]

A number of gene sets have shown the potential to predict response to chemotherapy. In particular, the 21-gene RS, particularly the proliferation group of genes, has been reported to predict chemotherapy benefit in ER-positive, node negative patients,[30] implying that the GGI and oestrogen-regulated genes defined by Oh et al. are likely to have similar abilities. Of note, tumour grade has long been observed to predict high response to chemotherapy. These observations imply that the luminal-B group may do well if chemotherapy is prescribed. However, in a study examining the pathological complete response rates of breast cancer subtypes (as defined by the intrinsic gene set), the luminal/ER-positive group had a poor

response (7%) to neoadjuvant taxane and anthracycline-based therapy compared with the basal (45%) and HER2 (45%) subtypes, despite these groups having high expression of proliferation genes.[31] Obviously, further research in this area is urgently required.

6. Future directions

At present, clinicians can conclude that ER-positive breast cancer is indeed heterogeneous, and molecular profiling suggests that there are at least two distinct diseases of luminal origins. Thus, it is likely that in the future we will be treating the luminal-A and non-luminal-A subtypes quite differently from each other. The research performed thus far highlights that it is essential that future studies performed in ER-positive breast cancer be prospectively stratified for molecular subtypes as important therapy effects may be diluted or missed by analysing an unselected ER-positive population. It is possible that we have already missed a significant therapy as demonstrated by the negative results from studies evaluating trastuzumab in breast cancer populations not over-expressing HER2. Efforts to determine the best treatment options for the individual ER-positive subgroups will require considerable efforts to (1) agree on a framework for defining luminal subtypes and (2) agree on a reliable and reproducible pathological test that will be able to define the luminal subtypes, and include accurate quantification of the ER, grade and HER2 status. As such, it is paramount that we identify the important pathways that characterise the luminal subtypes as this would provide more useful information than simply using a gene set, even if it provides good classification and has undergone adequate independent validation. At present, it is unclear if these assays will require fresh frozen tissue or be converted to a small number of genes that can be assessed using RT-PCR from FFPET.

Neoadjuvant studies may be the most rapid and efficient way to advance clinical research in this area. At present, the lack of suitable archival tissue makes it difficult to evaluate the effect of any therapy on altering the natural history of the poor prognosis non-luminal-A subtypes compared with tamoxifen. Neoadjuvant studies are ideal as they can provide fresh frozen tissue that can be used to determine the molecular subtype of the breast cancer being evaluated, and the final surgical specimen can provide an assessment of the response of the breast cancer to a given therapy. The final pathological response of the breast cancer to therapy is an excellent surrogate for survival, i.e. women who achieve pathological complete response (pCR), defined as the absence of tumour in the breast or lymph nodes after treatment, have an excellent long-term clinical outcome compared with those who do not.[32] Clearly, further work also needs to be performed in the laboratory setting. Target discovery, developing and assessing small molecular inhibitors in suitable *in vitro* and *in vivo* models of the luminal-A and -B subtypes would be highly beneficial prior to validation and evaluation in early phase human clinical trials.

Formal, prospective clinical trials assessing therapies in relation to molecular subtypes in breast cancer are currently lacking. As mentioned previously, the 21-gene RS is presently the subject of a major international trial that assesses its ability to predict response to chemotherapy in ER-positive, node negative breast cancer. This trial was initiated after a study using retrospective archival tissue from a clinical trial which suggested that there was a linear relationship between the RS and the degree of benefit from chemotherapy.[30] In the TAILORx trial, patients who are designated an intermediate risk of relapse will be randomly assigned to chemotherapy than to hormonal therapy alone.[9] While the final analysis will not be available for many years, it is expected that its results will shed some light on the role of various functional groups of genes, in particular the proliferation genes, in predicting response to chemotherapy in ER-positive breast cancer patients.

In conclusion, much work still needs to be done to (1) define the luminal subtypes for clinical implementation and (2) discover and understand the molecular aberrations and growth networks involved in the individual subgroups. Molecular profiling has advanced considerably in the last decade and has changed our view of breast cancer from a single disease to a number of distinct entities that will require different therapies. Furthering our understanding of the biological basis of ER-positive subtypes using microarray data remains a major challenge for researchers in the field today. In addition, advancing clinical research in the area will require extensive collaboration between clinicians and researchers in the laboratory, and a commitment by pharmaceutical companies to test their pathway and small molecule inhibitors in selected populations with appropriate correlative translational studies.

Conflict of interest statement

None declared.

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