Invasive Breast Cancer: Recognition of Molecular Subtypes

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Summary

Molecular profiling has fundamentally changed our understanding of breast cancer in the last 10 years, by creating a new taxonomy of breast cancers based on the expression patterns of so-called 'intrinsic genes'. Hierarchical clustering analyses performed on microarray-based gene expression profiles of breast cancers defined distinct breast cancer subgroups (luminal type A/B, HER2-enriched type, basal-like type). Since the initial landmark study by Perou et al., the concept of intrinsic breast cancer subtypes has been corroborated and expanded by several independent research groups. Further studies revealed individual properties of the intrinsic subgroups regarding the clinical course and the responsiveness to chemotherapy. The new gene expression profile-based taxonomy of breast cancer has been enthusiastically embraced by the scientific community and hailed as a major breakthrough on the way to individually tailored therapies. However, validation of the gene signatures in prospective studies is necessary before accepting these new technologies in daily clinical practice. In this review, the current data regarding the intrinsic subtypes and the associated clinical implications as well as the methodology of molecular profiling and possible use of immunohistochemistry in identifying intrinsic subtypes are discussed.

Schlüsselwörter

Mammakarzinom, invasives · Molekulare Subtypen · Luminaler Typ · Basalzell-Typ · HER2

Zusammenfassung

In den letzten 10 Jahren hat das Verfahren der molekularen Charakterisierung durch die Schaffung einer neuen, auf der Expression von sogenannten «intrinsischen Genen» basierenden Taxonomie unser Verständnis von invasiven Mammakarzinomen grundlegend verändert. Durch hierarchische Cluster-Analysen an mittels Microarrays bestimmten Genexpressionsprofilen konnten distinkte Subgruppen des Mammakarzinoms definiert werden (luminaler Typ A/B, HER2-angereicherter Typ, Basalzell-Typ). Seit der bahnbrechenden Arbeit durch Perou et al. wurde das Konzept der intrinsischen Subtypen des Mammakarzinoms von mehreren Arbeitsgruppen bestätigt und erweitert. Weitere Studien zeigten wesentliche Unterschiede zwischen den intrinsischen Subgruppen bezüglich des klinischen Verlaufs und des Ansprechens auf Chemotherapie. Die neue, auf Genexpression basierende Taxonomie der invasiven Mammakarzinome wurde von der Wissenschaftsgemeinde begeistert angenommen und als Durchbruch auf dem Weg zur individualisierten Therapie gefeiert. Dennoch ist eine Validierung der auf Gensignaturen basierenden Untersuchungen in prospektiven Untersuchungen erforderlich, bevor diese Technologien im klinischen Alltag eingesetzt werden können. Diese Übersichtsarbeit befasst sich mit den intrinsischen Subtypen des invasiven Mammakarzinoms, den hiermit assoziierten klinischen Implikationen wie auch mit der Methodik der molekularen Charakterisierung und der Frage, ob die intrinsischen Subtypen auch mittels Immunhistochemie identifiziert werden können.

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Introduction

Invasive breast carcinoma is the most common cancer of women and has been categorized by histomorphological criteria into invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), and other less common subtypes [1]. In most institutes, a 3-tiered grading scheme regarding growth pattern, nuclear grade and proliferative activity is used to further subdivide these subtypes [2]. Additionally, immunohistochemical analysis of estrogen receptor (ER), progesterone receptor (PR) and HER2 expression is carried out to determine if patients qualify for adjuvant therapy.

In recent years, DNA microarrays have been used to classify invasive breast carcinomas independently of histomorphological and immunohistochemical criteria. DNA microarrays represent a technology in which RNA is extracted from a tumor sample, transcribed into cDNA or cRNA and hybridized to microarray spots coated with DNA/RNA sequences representing distinct genes. With the aid of fluorescence labeling, the gene expression levels are determined in relation to a normal reference. With the advent of the DNA microarray technique, the determination of the expression levels of thousands of genes in one single tumor specimen has become possible [3].

Discovery of Intrinsic Subtypes in Breast Cancer: The Seminal Work

In their seminal work, Perou et al. [4] employed DNA microarrays for the investigation of the molecular profile of a collective of 65 surgical breast tumor specimens from 42 individual patients. A central concept of this study was that tumors could be characterized by the up- or down-regulation of special sets of genes, so-called 'intrinsic genes'. Intrinsic genes were defined as genes with significantly greater variation in expression between different tumors than between paired samples from the same tumor. On the basis of this algorithm, 496 gene probes representing an 'intrinsic gene list' were selected from an early microarray encompassing 8102 genes.

Numerous clusters representing genes associated with different breast cell types/functions were discovered. With the aid of hierarchical clustering analyses, 4 distinct molecular subgroups of invasive breast cancer were described: ER+/luminal-like, basal-like, HER2 enriched and normal breast-like. By defining these so-called 'intrinsic' subtypes, the groundwork for a new molecular taxonomy was laid [4]. Of note, it is highly probable that the normal breast-like subtype is an artifact caused by sampling errors. This point will be discussed further in the section 'Critique of Methodology'. Another important piece of information derived from the experiment by Perou et al. is that the group of ER-negative breast cancers is made up of at least two biologically distinct subtypes of tumors (basal-like, HER2 enriched), which are now understood as distinct diseases with different treatment options.

Molecular Subtypes, Mammary Development and Breast Cancer Evolution

Perou et al. [4] suggested that the intrinsic subtypes might reflect the different molecular features of mammary epithelial biology. This hypothesis was taken further by Prat and Perou [5] who suggested that the majority of invasive breast cancers could be assigned to different developmental points of the mammary epithelium. However, detailed analyses of gene mutation profiles amongst intrinsic subtypes revealed distinct gene mutation patterns for the different intrinsic subtypes, with a high index of p53 mutations in basal-like carcinomas [6–8]. These findings suggest that in breast cancer the molecular subtype of carcinoma might either be determined by the cell of origin or different genomic alterations, or possibly a combination of both.

Luminal Intrinsic Subtype

The defining molecular feature of luminal-type breast cancer is the expression of the ER [4]. Luminal-type breast cancer carries the best prognosis of all intrinsic subtypes [9] and responds to endocrine therapy [3]. Interestingly, invasive lobular breast cancer (ILC) overwhelmingly is classified as luminal type [10, 11]. Generally, ILC and low-grade IDC are subsumed as low-grade breast neoplasia family [12]. Molecular profiling seems to corroborate this concept. While basal-like and HER2-enriched breast cancers often respond well to neoadjuvant chemotherapy, the luminal subtypes show only limited chemosensitivity [13, 14]. Sorlie et al. [9] were the first to suggest a subdivision of luminal-type carcinoma into at least 2 distinct subgroups with characteristic molecular profiles and different prognoses, namely luminal type A breast cancer (fig. 1) and luminal type B cancer (fig. 2). The major difference between luminal A and luminal B breast cancers is the degree of proliferation and the HER2 expression signature, which is more pronounced in luminal B-type breast cancers [15, 16]. The distinction between luminal A and B carcinomas is of high clinical interest as luminal A-type breast carcinomas seem to be at a lower risk for relapse [13, 17] and luminal B-type breast carcinoma generally carries a worse prognosis but responds only slightly better to chemotherapy than luminal A-type carcinoma [18]. However, proliferation in ER+ carcinomas is a continuum, which makes a separation of luminal subgroups on the basis of proliferation – be it on an immunohistochemical level or on a molecular level – arbitrary and defining a cut-off difficult [13, 19, 20].

Basal-Like Intrinsic Subtype

The basal-like subtype of breast cancer (fig. 3) is characterized by the expression of cytokeratins, which are typically expressed in the basal cells of normal mammary gland epithelium, and by a very high expression of the proliferation cluster. It makes up approximately 15% of invasive breast cancers [21–23]. There is a striking prevalence of this mole-

Fig. 1. Example of luminal A-type carcinoma: Well differentiated IDC (grade 1) with nuclear ER and PR expression in over 90% of tumor cells, HER2/neu negativity and a proliferative index of < 14% of tumor cells.

Fig. 2. Example of luminal B-type carcinoma: Moderately differentiated IDC (grade 2) with nuclear ER and PR expression in over 90% of tumor cells, HER2/neu expression and a proliferative index of > 14% of tumor cells.

cular subtype in premenopausal African-American women [22], which may partially explain the poor outcomes characteristic of this patient group. Interestingly, BRCA1 mutation-associated breast carcinomas as a group strongly resemble sporadic basal-like tumors and might be regarded as a special subgroup in this intrinsic subtype [4, 24, 25]. It has been shown that, among the intrinsic subtypes as determined by molecular profiling, the basal-like subtype and the HER2-enriched subtype treated with trastuzumab showed the highest response rates to neoadjuvant chemotherapy [13, 14].

HER2-Enriched Intrinsic Subtype

The HER2-enriched subtype of breast cancer (fig. 4) is defined by overexpression of the *HER2/neu* gene and a lack of expression of genes characteristic of the luminal subtypes. Up

Fig. 3. Example of a basal-type carcinoma: Poorly differentiated invasive carcinoma with negativity for ER and PR and negativity for HER2/neu and expression of CK5 and EGFR.

to 25% of breast cancers are HER2 positive as defined by immunohistochemistry and fluorescence in situ hybridization/chromogenic in situ hybridization (FISH/CISH) [30]. It is intriguing that not all breast cancers defined as HER2 positive by immunohistochemistry are classified as HER2 enriched by molecular profiling. At the same time, not all intrinsic HER2 enriched tumors are diagnosed as HER2 amplified by immunohistochemistry or in situ hybridization [13, 31, 32]. Studies are needed to ascertain whether the molecular subtype HER2 enriched may profit from trastuzumab therapy in cases when HER2 amplification cannot be proven with conventional methods. Clinical experience demonstrates clearly that the collective of HER2-positive patients as defined by immunohistochemistry is a heterogeneous group, in particular regarding the response to HER2 blockers. It is well known that a significant number of patients with HER2-positive breast cancers possess a primary trastuzumab resistance or develop a secondary resistance to trastuzumab within 1 year [33]. Furthermore, it has been suggested that some patients with HER2-positive breast cancer might in fact even suffer a prognostic disadvantage from chemotherapy and trastuzumab therapy [34]. As of now, it is unclear whether the molecular subtyping with definition of the HER2-enriched intrinsic subtype performs better than immunohistochemistry or FISH/CISH in the clinical context. Extensive studies will be needed to reliably identify the patients who will profit from a HER2-targeting therapy, and hopes are high that molecular profiling will provide the solution of this therapeutical riddle.

Potential New Intrinsic Subtypes: Claudin-Low Subtype and Apocrine Subtype

Current data indicate that additional molecular subtypes will need to be incorporated into the molecular profiling system in order to render the biological picture more complete.

Fig. 4. Example of HER2/neu-enriched carcinoma: Poorly differentiated invasive micropapillary carcinoma with ER and PR negativity, strong membranous expression of HER2/neu in > 30% of tumor cells and a proliferative index of > 14% of tumor cells.

Currently, the molecular subtype ‘claudin-low’ is moving into the center of the molecular researchers’ attention. Neve et al. [26] reported a specific subtype in breast cancer cell lines that showed stem cell properties and was named ‘basal B’ subtype. The claudin-low subtype is characterized by a low expression of genes involved in tight junctions and cell-cell adhesion [27, 28]. Tumors of the claudin-low subtype resemble the basal-like subtype insofar as they show negativity for ER, PR and HER2. However, claudin-low tumors lack classical basal-like markers like cytokeratins 5/6 (CK5/6) and epidermal growth factor receptor (EGFR). According to Prat et al. [28], the claudin-low subtype represents 12–14% of the triple-negative carcinomas. As of now, data are lacking regarding the clinical implications of this new subtype. It is furthermore unclear whether the claudin-low subtype should be regarded as a distinct molecular and clinical subtype or rather as a component of the basal-like intrinsic subtype.

Another distinct molecular subgroup of breast tumors is characterized by ER negativity and androgen receptor (AR) positivity as well as by an apocrine morphology. The authors contend that tumors of the apocrine subtype may make up a substantial part of ER– tumors outside the basal subtype [29].

Can Intrinsic Molecular Subtypes Be Reliably Identified by Immunohistochemistry or Even Histomorphology?

Molecular profiling of cancer represents a time-consuming and expensive method and requires unfixed tumor tissue snap-frozen under standardized conditions immediately after surgery. Therefore, a question of great clinical relevance is

whether the intrinsic subtypes can be reliably identified by histomorphology alone or through a standard immunohistochemistry panel.

Histomorphological criteria can provide useful clues to the diagnosis of the different molecular subtypes, e.g. luminal tumors usually show a good or moderate differentiation with tubule formation whereas basal-like carcinomas reveal poor differentiation with central necrosis and prominent lymphoid infiltrates. However, the discriminative power of histomorphology is very limited regarding intrinsic subtypes.

Regarding immunohistochemistry, different study groups have tried to emulate molecular subtyping using a standard immunohistochemistry surrogate panel encompassing ER, PR, HER2/neu and Ki67. Generally, the luminal subtype can be identified by immunohistochemical expression of the ER or the PR. As described above, luminal B carcinomas are characterized by additional HER2 expression and/or higher proliferation. Cheang et al. [15] have shown that the differentiation between luminal A and B tumors with a sensitivity of 77% and a specificity of 78% is possible using a Ki67 cut-off of 14%. Recent studies have demonstrated that the combination of ER, HER2 and PR negativity and EGFR and CK5/6 positivity accurately identifies basal-like tumors from gene microarray data with 100% specificity and 76% sensitivity [23]. However, it has been shown that 9% of immunohistochemically triple-negative tumors were assigned to the HER2-enriched subtype on a molecular level, whereas 5% and 6% showed a molecular pattern corresponding to luminal A and B, respectively [28]. This heterogeneity at the molecular level of tumors with a similar immunohistochemical profile was corroborated by other studies, which showed that virtually all intrinsic subtypes are represented in the different clinico-pathological breast carcinoma categories [13, 35]. This data suggests that the intrinsic subtypes are closely, but not fully and accurately represented by the standard immunohistochemical markers used in clinical practice today.

Does Genetic Profiling Provide Additional Prognostic and Predictive Information beyond Standard Clinico-Pathological Parameters?

The vast individualized data set generated by the molecular profiling of breast carcinoma has very soon led to the question whether the knowledge of the molecular profile of individual breast carcinomas also conveys significant prognostic and predictive information.

The advent of gene profiling has given a significant boost to the efforts of pinpointing those patients with ER+ breast carcinoma who will – with high probability – profit from adjuvant chemotherapy. At the same time, it is of major medical and financial interest to identify patients with a low risk of recurrence who could be spared the toxicity of chemotherapy. To this end, several working groups have devised molecular

signature-based tests to better judge the risk of recurrence of ER-positive, lymph node-negative breast carcinomas. Amongst these tests, the most well known are the 70-gene good versus poor outcome model ('Mammaprint'; [36]), the 'wound response model' [37, 38], the recurrence score model ('OncotypeDX'; [39]), the intrinsic subtype model [4, 9, 24, 40] and the two gene ratio model [41]. Skepticism was induced by the fact that, although these tests were designed to be applied to the same clinical problem, only a small gene overlap exists between these tests [42]. However, when tested against the same data set, all tests but one (the two gene ratio model) showed similar prognostic values [17].

It has been argued that only high-throughput technologies are able to fully capture the biological diversity of breast carcinoma. In this context it needs to be mentioned that the proliferation (signature) is the mainstay in all classifiers to divide ER+, lymph node-negative tumors into subgroups with different prognosis [43]. In particular, OncotypeDX relies heavily on proliferation-related genes [20]. This raises the question whether immunohistochemical or even histomorphological parameters might not be equally efficient as surrogates for prognosis and prediction instead of costly molecular assays. However, until today, results of prospective randomized studies addressing the question whether better prognostic or predictive information can be achieved using molecular profiling in comparison to the classical clinico-pathological data sets are lacking. Ongoing prospective studies like the Mindact study will hopefully clarify to which extent molecular profiling provides additional prognostic and predictive information.

Molecular Profiling – a Critique of Methodology

Molecular profiling has been enthusiastically received as an exciting new technology by the scientific community. However, there were also some calls for caution which criticized the over-enthusiastic and uncritical embracing of a new method still lacking rigorous validation [44, 45]. First of all, the new molecular taxonomy was based on findings derived from different microarray platforms. To address the question of reproducibility of microarray technology, the US Food and Drug Administration (FDA) instigated the Microarray Quality Control (MAQC) project. The data collected in this extensive study showed that microarray measurements are highly reproducible within and across different microarray platforms. As a consequence, the FDA judged the microarray technology sufficiently reliable for clinical and regulatory purposes [46]. However, it needs to be pointed out that different profiling gene sets cannot be transferred from one microarray platform to another without extensive modification. As a consequence, the results of different microarray platforms cannot be directly compared. Another point of critique concerns the samples used for microarray technologies. As the tissue samples are not microdissected before analysis, the gene profiles

represent not only the tumor cells but also the peritumoral reaction and the host tissue. It seems probable that the subtype of normal-like breast carcinoma is an artifact of gene expression profiling, caused by the analysis of samples with a high content of normal breast epithelial cells and stromal cells [13, 32]. Furthermore, in the case of microinvasive carcinoma with extensive DCIS, there is no safe way of guaranteeing that the invasive carcinoma and not the in situ component are analyzed.

Beyond the level of methodology, the rather small sample sizes upon which the groundbreaking works of Perou and Sorlie were based were regarded with some uneasiness. Indeed, in his seminal work describing 'molecular portraits of human breast tumors', Perou analyzed a set of 65 surgical specimens derived from 42 different individuals [4]. In the following 2 publications by the Stanford group that further outlined the concept of molecular profiling of breast carcinoma, Sorlie et al. based their analyses on 78 and 115 breast carcinomas, respectively [9, 24]. Considering that in these studies a whole new molecular profile-based taxonomy of breast carcinomas was developed, a greater sample size would have validated the new concept with greater statistical power.

As molecular profiling is often hailed as a largely unbiased analysis tool that allows scientists to avoid the subjectivity of immunohistochemistry and histopathological grading, it is often forgotten that the hierarchical clustering method, far from being a completely automated analysis, is a method that requires the input of a human observer for the final interpretation of the data. In a test encompassing the 5 major intrinsic gene lists, Mackay et al. [47] demonstrated that none of the classification systems produced almost perfect interobserver agreement. The best interobserver agreement was documented for basal-type and HER2-enriched breast carcinomas, whereas poor interobserver agreement was found for luminal-type breast carcinomas.

It is also important to bear in mind that the analyzing tool of hierarchical clustering can only be applied retrospectively, to adequately sized collectives. The classical microarray-based hierarchical clustering method is therefore not suitable for assigning intrinsic subtypes to particular samples as the dendrogram changes with each additionally included sample. To circumvent this particular problem, single sample predictors (SSPs) have been devised [13, 24, 40]. SSPs are based on the median expression patterns of the different intrinsic subtypes (i.e. centroids). The SSP allows for any given sample to be assigned to the intrinsic subtype most similar in its molecular profile. However, the reliability of SSPs has been discussed controversially. While some groups have reported reliable and reproducible intrinsic subgroup assignments through SSPs [40], others have questioned the validity of this method and claimed that only basal-like breast carcinomas were safely identified by this method [32]. Further studies are needed to ascertain whether SSPs represent a valid method for molecular subtyping.

It must be noted that, while the technique of RNA-based molecular subtyping allows fascinating insights into the molecular properties of breast cancer, it is not (yet) a very good tool for assessing individual samples.

Despite the scientific and medial hype, we are therefore not yet at a point where this new technology has a relevant impact on day-to-day clinical practice.

Conclusions

The criticism inherent in the stringent validation of molecular profiling should not be misinterpreted as a rejection of an exciting new technology. Gene expression profiling carries an enormous potential and has taken us some way towards individualized therapy. The massive potential of this powerful high-throughput technology which allows investigators to better capture the molecular diversity of cancer cannot be overestimated, especially as the high-throughput technologies are continually being improved and expanded. It is to be expected that newer methods analyzing additional epigenetic factors such as non-coding RNAs and alternative splicing will complement and enrich molecular profiling in the future. High-throughput technologies have opened the way to the acquisition of unprecedented amounts of data regarding individual tumors. As our technical prowess in molecular profiling in-

creases, so will our understanding of breast cancer biology. However, the uncritical acceptance of new technologies may generate massive amounts of insufficiently validated data. In order to stay on top of this data flood, it is absolutely necessary to rigorously enforce standardization and validation of the new technologies. Furthermore, molecular profiling should be regarded as a component of the whole clinico-pathological picture and not be aggressively marketed as a superior substitute to the diagnostic, prognostic and predictive methods currently in use. Rather than furthering the cause of molecular profiling, the hybrid of calling pathology unsophisticated and subjective [48], together with the rather spectacular claim that microarrays will be the key to curing all human disease until 2050 [49], serve as a provocation not only to surgical pathologists. Retaliation has occurred in the form of growing criticism, cumulating in one expert in biomarkers claiming that it is statistically and mathematically possible to prove that any given gene affects survival 'even if it does not' [44]. In the interest of science and patient care alike it is time to take the competitive edge out of the discussion regarding molecular profiling.

Disclosure Statement

The authors declare that there is no conflict of interest.

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