Pathological definition of triple negative breast cancer

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Introduction

We are witnessing tremendous advances in the understanding of the biological and clinical implications of heterogeneity of breast carcinomas. Indeed, taking advantage of the many peculiar clinical, histopathological and biological features of the different tumour types, we have learnt how to evaluate more reliably the risk of disease progression, to better tailor systemic interventions and to target specific molecular pathways with new therapeutic agents. Together with the current strategies for early detection of breast carcinoma and the refinements of the local treatments by surgery and radiation therapy, the improved quality of systemic therapies have resulted in the reduction of the mortality rate for a disease whose prevalence is continuously increasing worldwide [1,2]. Basic, translational and clinical researchers have started coordinating their efforts to unveil new biological features of breast cancer that might become either targets for novel specific drugs or new predictive parameters to better tailor existing therapies. New clinical questions are being addressed, with the aim of improving the selection of the candidate patients to tailored interventions and eventually identifying those who will actually respond to these therapies. A new generation of randomised clinical trials for pre-defined subpopulations of breast cancer patients selected according to the biological characteristics of their tumours are being conducted and newly launched to prove the efficacy of tailored treatments. The design and the conduct of these new clinical trials require an unprecedented coordination of the activity of clinical investigators, pathologists and translational researchers worldwide.

The patients who are less likely to have benefited from the advances in the systemic treatments of breast carcinoma are those with the so-called triple negative (TN) tumours (i.e. immunohistochemicallly negative for oestrogen receptor (ER) and progesterone receptor (PgR) and lacking over-expression and amplification of the HER2 gene). They account for some 10–20% of all breast cancer patients, but they also account for the most lost lives due to breast cancer [3]. The increasing clinical interest for these tumours of the breast stems from their alleged poor prognosis despite high response rates to conventional chemotherapeutic regimens in the neoadjuvant setting [4], and from the lack of any targeted therapies. Indeed, patients with TN tumours are not offered either hormonal or anti-HER2 interventions, being candidates for non-tailored cytotoxic chemotherapy.

The lack of any positive immunophenotypical marker has long prevented these tumours from being investigated more thoroughly. Only the advent of more sophisticated assays, enabling the exploration of the whole universe of gene expression of the tumour cells, has shed some light into the black box of TN breast cancer [5]. The pioneering studies of gene expression profiling have documented that the majority of TN tumours belong to the molecular class of the basal-like breast cancers, although these two tumour types do not overlap completely. Far from being limited to a new taxonomic approach to breast cancer, the molecular investigations have provided new insights into the biological pathways driving the neoplastic transformation and tumour progression of TN carcinomas. Some positive markers for these tumours have eventually been identified (EFGR, c-Kit) which may represent possible targets for new therapeutic approaches.

Prototypical features of TN breast cancer (Table 1)

The definitional hallmark of TN breast cancer is their negative status for both ER and PgR and the lack of over-expression and amplification of the HER2 gene. Unfortunately, a consensus on what constitutes a negative hormone receptor status is still lacking. Indeed, both in daily practice and clinical studies, different thresholds for an ER and PgR negative tumour are used, including absence of any immunoreactivity, or <1%, <5%, <10% and <20% immunoreactive cells [4,6–9]. It is worth noting here that according

Table 1
Prototypical features of triple negative breast cancer

Morphological features	High histological grade Lack of tubule formation Prominent nuclear abnormalities High mitotic count Broad pushing borders Necrotic and fibrotic areas Prominent lymphocytic infiltrate
Biological features	Lack of ER and PgR immunoreactivity Negative HER2 status High Ki-67 labelling index p53 mutations Immunoreactivity for basal cytokeratins, vimentin, P-cadherin, EGFR, PDGFR, IGF-IR and c-kit
Molecular classification	Basal-like (most commonly)

to the Allred's combined score, tumours with even less than 1% neoplastic cells showing moderate or intense immunostaining qualify for a positive hormone receptor status. The lack of consensus in the assessment of hormone receptor status makes it very difficult to compare data stemming from different studies and inevitably it will also jeopardise the conclusions of the current clinical trials on TN breast cancer.

With the caveats related to the definition of the triple negative status, the prototypical features of TN tumours include both clinical and histopathological characteristics. Comparing TN with non triple negative breast cancers, several clinical differences are noticeable [6]. First of all, TN tumours reportedly affect younger (mean age 47–55 years) women, are more prevalent in the population of women carrying BRCA-1 mutations [10], their mean size at diagnosis is larger than for non TN tumours, and the prevalence of lymph node metastases is higher. Interestingly, for TN tumours there is no apparent correlation between tumour size and nodal status, and even small tumours with a triple negative phenotype have a higher rate of nodal positivity. TN tumours are more common in the African-American population, often manifest clinically as "interval" cancer between consecutive mammograms, and are more likely to be diagnosed by clinical examination, probably due to a more rapid growth rate.

The clinical course of the disease is particularly aggressive, with a tendency to early dissemination to distant organs (brain and lung) irrespective of the axillary lymph node status, and the time from the appearance of distant recurrence to death is much shorter in this type of malignancy than in other types of breast cancer. The appearance of the distant metastases is rarely preceded by a local recurrence, and if a local relapse occurs it is not predictive of systemic metastases. Moreover, the risk of recurrence is particularly high during the first 3 to 5 years after diagnosis, and it decreases thereafter. Overall survival is shorter than for other breast malignancies, and most deaths occur within the first 5 years after diagnosis.

Histologically, TN tumours are more commonly high-grade neoplasms, which lack tubule formation, have prominent nuclear abnormalities and high mitotic count. They have broad pushing tumour borders, and are often characterised by large areas of central or geographic necrosis and fibrosis, often associated with a ribbon-like tumour architecture, and show a prominent lymphocytic infiltrate. Less commonly, TN breast carcinomas show metaplastic changes, with squamous and spindled tumour cells, chondroid or osseous metaplasia, or features of carcinosarcoma [11]. Some of the above histopathological features are reminiscent of the typical characteristics of the medullary and medullary-like cancers, which indeed belong to the TN category of breast carcinomas.

Central necrosis and fibrosis have long been identified as a peculiar feature of some high-grade invasive ductal carcinomas with a poor prognosis and early appearance of pulmonary and cerebral metastases. More recently, the biological and clinical implications of the fibrotic foci in breast cancers have been extensively investigated [12]. These foci have been considered as surrogate markers of hypoxia and neoangiogenesis. Typically, they are encountered in the centre of the tumour and consist of radially expanding fibrous bands arranged in irregular or storiform patterns, with increased fibroblastic cellularity and/or collagenisation. Their size exceeds 1 mm, and they may occasionally contain a few tumour cells and areas of coagulative necrosis. The occurrence of a fibrotic focus is an independent prognostic parameter and a risk factor for nodal metastases. Conversely,

tumours with increased lymphocytic infiltrate are associated with a favourable prognosis. Patients with tumours showing no/minimal (less than 10 lymphocytes/high power field) lymphocytic infiltrate in combination with central fibrosis have the worst 5-year metastases-free survival (39% according to Kreike and colleagues [7]), as compared to patients with tumours showing moderate/extensive lymphocytic infiltrate in combination with central fibrosis (78%), or no/minimal lymphocytic infiltrate without central fibrosis (94%). All the patients whose tumours showed moderate/extensive lymphocytic infiltrate and lack central fibrosis in the above investigation remained free of metastases. A multivariable Cox proportional hazard analysis of 97 patients with TN tumours revealed that only lymphocytic infiltrate and central fibrosis are independent risk factors for metastases-free survival [7].

The immunohistochemical profile of TN breast cancer has been extensively investigated [3,6–8,11,13] and it is characterised by the variable expression of several markers, including cytokeratins 5,14 and 17 (at least one of these cytokeratins is expressed in the vast majority of TN tumours), vimentin (55%), P-cadherin (93%), EGFR (27–37%), PDGFR (31%), IGF-IR (36%), c-kit (11–38%), S-100 protein (22%), p63 (10%), smooth-muscle actin (8%) and by the nuclear accumulation of p53 (50–56%). Cyclin D1 is overexpressed in as many as 51% of the tumours, whereas down-regulation of p27 expression and loss of PTEN has been documented in 56% and 14% of the TN tumours, respectively [13]. The median Ki-67 labelling index is 35% (range 10–90%), and only 20% of the tumours have Ki-67 labelling indexes less than 20% [13].

The highly heterogeneous immunohistochemical profile of TN breast carcinomas suggests that at least some of these neoplasms exhibit markers of myoepithelial derivation or differentiation (e.g. S-100 protein, smooth muscle actin, p63) [14], and that they show a complex derangement of growth factor receptors with tyrosine kinase activity, and of the proteins regulating the cell cycle.

Also, the immunophenotypical characterisation of pure ductal carcinomas *in situ* (DCIS) of the breast has recently identified a variant of high-grade DCIS with the triple negative phenotype associated with expression of basal cytokeratins and of EGFR as a putative precursor lesion of TN invasive carcinomas *in situ*. Accordingly, it is postulated that TN breast cancers arise *de novo* from these precursor lesions, and are not the result of a de-differentiation process during the progression of pre-existing carcinomas.

TN breast carcinomas are heterogeneous

Though the vast majority of TN breast carcinomas are invasive duct carcinomas of no special type and share the above prototypical features, it should be emphasised that the triple negative phenotype is also a feature of some special types of breast cancer that have remarkably different morphological and clinical characteristics. These include the pleomorphic subtype of invasive lobular carcinomas, the myoepithelial carcinomas, the metaplastic carcinomas, the "oat cell" neuroendocrine carcinomas, the apocrine carcinomas, the medullary carcinomas, the secretory (juvenile) carcinomas and the adenoid-cystic carcinomas. It is important to identify these special types within the family of TN breast cancer, because some of these entities are associated with a better prognosis and do not benefit from aggressive chemotherapeutic regimens. This is particularly true for adenoid-cystic and medullary carcinomas, and for the low-grade metaplastic carcinomas (i.e. the low-grade adenosquamous carcinomas and the fibromatosis-like carcinomas). Apocrine carcinomas are almost invariably characterised by a triple negative phenotype, but their clinical outcome is more closely correlated with their histological grade and the disease stage.

The TN phenotype does not overlap with the "basal-like" expression profile

"Basal-like" breast carcinomas have been identified by the hierarchical clustering of the variations in the expression of 496 genes ("intrinsic" gene subset) [5]. This approach resulted in the identification of 4 to 6 molecular subtypes of breast cancer (2 or 3 luminal types, HER2 overexpressing, normal breast-like and basal-like). Basal-like tumours account for 14% to 26% of the investigated samples; they are characterised by the expression of basal markers, like cytokeratins 5 and 17, EGFR, KIT, laminin, collagen type XVII, calponin 1 and caveolin 2, whereas they do not express ER and HER2. Basal-like carcinomas show an aggressive clinical course and a poor outcome. Due to the clinical, biological and histological similarities with TN breast cancers, it has been tempting to consider the triple negative phenotype as the immunohistochemical surrogate for the molecularly-defined basal-like tumours [7]. This view, however, is oversimplistic [15], because only 71–91% of TN have a basal-like gene expression profile (according to the threshold used for a negative hormone receptor status), and only 77% of basal-like

carcinomas have a TN immunophenotype (they may express ER and/or HER2) [16]. In addition, almost 20% of non-TN tumours do have a basal-like gene expression profile.

To identify better immunohistochemical surrogates of basal-like breast cancers, several putative positive markers of basal-like tumours have been investigated, alone or in combination with the triple negative phenotype. The list of the most commonly proposed positive markers includes, among others, basal cytokeratins (cytokeratin 5,14,17), EGFR, c-Kit, P-cadherin, p63, vimentin and smooth muscle actin. None of these markers, however, alone or in combination, proved to be 100% specific and sensitive for the immunohistochemical identification of basal-like tumours. Indeed, only 60% of the molecularly defined basal-like carcinomas express CK 5 (and surprisingly they do not express CK14) and only 57 to 72% of them express EGFR.

Interestingly, however, the several attempts to correlate the intrinsic expression profile with the morphological and immunohistochemical features of breast cancer have provided new insights into the heterogeneity of these tumours. First, it has been documented that the molecular class of basal-like cancers also includes a spectrum of diseases, among which there are tumours with a better prognosis, such as medullary and adenoid-cystic carcinomas, the apocrine carcinomas and the metaplastic carcinomas [17]. Conversely, it has been shown that it is possible to identify the four major molecular classes (luminal A and B, HER2 enriched and basal-like) within several cohorts of tumours, immunohistochemically positive or negative for ER and HER2. These data emphasise that the evaluation of breast carcinomas with a combined approach, including morphology, immunohistochemical characterisation and molecular profiling, may well improve our capability of identifying tumour classes more homogeneous from a biological and clinical point of view.

A clinically meaningful approach to TN breast cancer

To retain the peculiar clinical implications of the triple negative phenotype in breast cancer, we should first identify those histotypes that – despite being triple negative – have a better prognosis. These include the low grade apocrine and metaplastic carcinomas, the medullary carcinomas and the adenoid cystic carcinomas. In addition to the immunohistochemical triple negative status, these tumours also share an intrinsic basal-like signature, and therefore they can only be identified by the histopathological assessment of their peculiar morphological features. This is but another example of the need for a truly combined and comprehensive approach to a clinically meaningful classification of breast carcinoma, as opposed to the perception that the new and more sophisticated assays should simply replace the established ones.

So as not to deny a patient the possible benefit of endocrine interventions, a consensus should therefore be reached about the definition of a hormone receptor negative status. In our opinion, only tumours lacking any immunoreactive cell qualify for a negative ER and PgR status. This definition could well improve intra- and interlaboratory reproducibility of the assessment, because it avoids any possible disagreement on the estimation of the percentage of immunostained cells, and it is independent of the actual scoring system, be it based upon the crude percentage of stained cells or on the combined evaluation of the percentage of stained cells and of the staining intensity.

A major drawback of the immunohistochemical assays for hormone receptor status, however, is the still unacceptable high rate of false-negative and false-positive results, which also precludes the correct identification of TN tumours. It has been repeatedly shown that the false-negative rate for ER and PgR may be as high as 20%, whereas the false-positive rate is 2–4% for ER but much higher (approximately 15%) for PgR. The higher rate of false-positive results with PgR assays is likely related to the use of the newly developed monoclonal antibodies raised in rabbits [18]. An inaccurate assessment of the hormone receptor status has dramatic implications for patients, who can be denied a potentially useful endocrine therapy in case of a false-negative result, or may be offered an ineffective and potentially harmful treatment because of a false-positive assay.

Also, the HER2 negative status should be carefully assessed so as not to miss candidate patients for anti-HER2 tailored therapies. Luckily, the definition of a HER2 negative status is not controversial, and it is based on the lack of over-expression of the protein (as evaluated by immunohistochemistry) and on the lack of gene amplification (as documented by *in situ* hybridisation techniques, with either fluorescent [FISH] or chromogenic [CISH or SISH] probes). Again, as for the evaluation of the hormone receptor status, the need for an accurate assessment of HER2 status cannot be over-emphasised. Unfortunately, despite the availability of standardised reagents and protocols for both immunohistochemical and *in situ* hybridisation assays, and the publication of several guidelines and

recommendations for optimal testing, the assessment of HER2 status is still biased by a high rate of false-positive and false-negative results [19].

All the pathologists involved in the care of breast cancer patients should become increasingly aware of the tremendous clinical implications of the assessment of hormone receptor and HER2 status of breast cancer. Too often the "expert" pathologists devote all their efforts to the noble task of reaching the correct histopathological diagnosis of breast cancer, and the more challenging it is then the more excited they are. When it comes to the assessment of the receptor status, they feel much less involved, and they happily leave this task to less experienced colleagues. Too often it seems intolerably tedious to critically evaluate the immunostaining results, to update the staining protocols, to check for the consistency of the results over time, or to supervise the internal and external quality control schemes. Too often there is not enough time to attend the multidisciplinary sessions, and the chance of discussing the clinical implications of the pathological report with the treating oncologists is therefore missed. Undoubtedly, given the pre-eminent role of a complete and accurate assessment of all the morphological and biological features of the tumours for an adequate treatment of the patients, the attitude of the pathologists towards the assessment of the biological features of breast cancer has to be changed.

Finally, it may be rewarding to search for prognostic and predictive markers that might be clinically useful for better evaluating the risk of recurrence of TN tumours and hopefully open up new therapeutic options [20]. Several investigations have dealt with the prognostic role of the expression of basal cytokeratins (particularly cytokeratin 5/6 and 14) with conflicting results. Indeed, some reports have shown an independent correlation of the expression of basal cytokeratins with a worse DFS and OS, either in node-negative or in node-positive disease, whereas other studies did not confirm such a correlation, or even reported a better outcome for patients with tumours expressing cytokeratin 14 [21]. Again, one of the problem issues with the immunohistochemical studies for the expression of basal cytokeratins is the lack of consensus about the definition of a positive tumour. Some authors have used a threshold of 10% immunoreactive cell to identify a positive tumour, whereas others have considered positive all the tumours showing immunoreactivity even in a single tumour cell.

The epidermal growth factor receptor (EGFR) is another potentially useful prognostic parameter in TN breast carcinoma. We have recently documented immunoreactivity for EGFR in 50% or more neoplastic cells in 36 (13%) of 284 consecutive patients with TN breast carcinomas. In multivariate analysis, these patients showed a significantly reduced DFS and OS (HR 2.39, 95% CI, 1.32–4.34, $P = 0.004$ for DFS; HR 2.34, 95% CI, 1.20–4.59, $P = 0.01$ for OS) at a median follow-up of 70 months (interquartile range 59–94 months), as compared with the patients whose tumours lacked or showed less extensive EGFR immunoreactivity [22]. In the same investigation, we did not find any correlation of the expression of basal cytokeratins (cytokeratin 5/6 and 14) with the survival. Further studies using database analyses or prospective trials are required to confirm the prognostic value of EGFR expression and to justify the exploitation of selective EGFR inhibitors in the adjuvant treatment of TN tumours. If confirmed, EGFR expression should be taken into account when designing a proper treatment choice for patients with these breast malignancies.

Conflict of interest statement

None declared.

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