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Pathological work up of the primary tumor: Getting the proper information out of it

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SUMMARY

The primary tumour of patients with early breast cancer is the main source of information to assess the risk of disease recurrence and to inform the choice of the most appropriate systemic treatment. Accordingly, it is the main responsibility of the pathologists to ensure the patients and treating physicians that all the relevant information is derived from the primary tumour with the highest accuracy and reproducibility. The morphological changes of the tumour cells reflect the aggregate effects of changes occurring in hundreds of genes and may be a very faithful mirror of the biological and clinical behaviour of breast cancer. According to the 2009 St. Gallen Consensus, the systemic therapy of early breast cancer is mainly informed by the expression of hormone receptors and by the HER2 status, and the assessment of Ki67 has been included among the useful parameters to inform the choice of adding chemotherapy to endocrine therapies for patients with ER-positive and HER2-negative disease. A comprehensive approach that includes the accurate evaluation of the morphological features of the tumour, with special reference to the histological type and grade, and the assessment of the main prognostic and predictive parameters (ER, PgR, HER2 and Ki67) should offer to the patients and the treating physicians a robust background upon which the final therapeutic decisions can be safely taken.

Introduction

Until novel and more sophisticated approaches, like for example serum proteomics or gene expression profiling of circulating tumour cells, will not be readily available, the primary tumour of patients with early breast cancer will remain the main source of information to assess the risk of disease recurrence and to inform the choice of the most appropriate systemic treatment. Accordingly, it is the main responsibility of the pathologists to ensure the patients and treating physicians that all the relevant information is derived from the primary tumour with the highest accuracy and reproducibility. Compliance with guidelines and recommendations issued by regulatory agencies and scientific bodies, as well as implementation and continuous participation in internal and external quality assurance programmes may assist the pathologists in coping with this unprecedented and very demanding task.

The following paragraphs are not intended to summarise the extensive body of literature on the pathological evaluation of breast cancer or to replace the recommendations and guidelines currently available. Instead, they are an opportunity to re-emphasise some of the aspects that may have been overlooked or not specifically addressed in the existing textbooks and articles. Some of the comments may be viewed as the personal opinion of the author, based on his experience with the pathology of breast cancer in the context of a truly multidisciplinary approach to the patients. Hopefully, the reader will find some useful hints to incorporate in the daily practice.

Handling and sampling of the surgical specimens

All the surgical specimens from mastectomy or breast-conserving surgery must be handled to ensure the best possible preservation of all the morphological and biological characteristics of the tumour cells. Inappropriate fixation (either caused by delay in placing the specimens into the fixative, or by use of insufficient volume of fixative relative to the size of the specimen) may cause extensive morphological artefacts, loss of tissue antigenicity and almost complete degradation of nucleic acids (especially mRNA). The tissue damage cannot be fixed by any means, and these specimens will not be suitable for a reliable assessment of prognostic or predictive parameters.

There are not stringent rules for an adequate sampling of primary breast cancer for histopathological examination. Whenever it is feasible, it is recommended[1] to sample the entire lesion (identified either by gross examination or by imaging), especially in case of specimens consisting predominantly of ductal carcinomas *in situ* not to miss any (micro)invasive component. In case of multiple lesions, all should be adequately sampled. If the entire lesion cannot be sampled, then I would recommend to take a minimum of 3 blocks for a 2-cm tumour, and an additional block for each

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additional cm of size. Academic centres and centres involved in basic and clinical research may well want to take extra-samples (so called "research blocks") from the primary tumour (and the surrounding nonneoplastic tissue) to be fixed and embedded in paraffin or snap frozen for banking purposes.

As anticipated, a proper fixation of the tissue samples is the essential prerequisite for an accurate assessment of all the morphological and biological features of the tumour. Despite its potential hazards for the exposed personnel, 10% neutral buffered formalin remains the fixative of choice. It should be freshly prepared and used in sufficient amount (at least 10 times the volume of the sample to be fixed) to ensure an effective fixation. Fixation time for surgical specimens should be 6 to 48 hours at room temperature. Shorter fixation times may lead to an alcoholic post-fixation of the tissue during the dehydration steps for paraffin embedding, and eventually affect the results of immunohistochemical reactions (especially for HER2). Longer fixation times may cause more extensive masking of tissue antigens, which will require the treatments for antigen retrieval in immunohistochemical assays be modified accordingly.

Formalin fixation will ensure an optimal preservation of the morphological details and the best results of immunohistochemical and *in situ* hybridisation assays for prognostic and predictive markers. Furthermore, nucleic acids may be extracted from formalin-fixed and paraffin-embedded tissues for additional testing (e.g., mutational analysis, reverse-transcription PCR, etc). Alternative fixatives should only be used after an extensive internal validation has assessed their effects on the preservation of tissue antigenicity and integrity of nucleic acids as compared to formalin fixation. Also, it should be kept in mind that all the commercially-available kits for immunohistochemistry and in situ hybridisation assays have been optimised (and eventually approved by the regulatory agencies) for use on formalin-fixed tissue samples. When using alternative fixatives, the results of these assays must be internally validated.

Assessing the histopathological features of breast cancer

It may appear outdated to spend time at the microscope in scrutinising the traditional morphological features of breast cancer in an age of more sophisticated approaches to the classification of the disease and to the assessment of prognostic and predictive variables. However, the morphological changes of the tumour cells reflect the aggregate effects of changes occurring in hundreds of genes, and may be a very faithful mirror of the biological and clinical behaviour of breast cancer. Overlooking the morphological features will prohibit to draw a truly comprehensive prognostic and predictive profile of the tumour, even when the results of more modern and fashionable assays are available. There are instances where the morphological identification of a special tumour type *per se* provides the whole set of information relative to the expected outcome and responsiveness to the therapy of the disease, without any need for performing additional investigations. Several tumour types (e.g., tubular or cribriform carcinomas) pursue an indolent clinical course and are invariably highly endocrine responsive.[2] If the immunohistochemical assays for oestrogen receptor (ER) turn out to be negative, or there is an apparent overexpression or amplification of the HER2 gene, or there is a high proliferative fraction, or the molecular classification is not luminal A, or the recurrence score is not low, then the morphological features are not consistent with the results of these assays, and either the histopathological diagnosis is wrong, or the results of the assays are false, and need to be double-checked.

Even the advent of the molecular classification of breast cancer did not diminished the role of the histopathological typing of the disease. As an example, it is now clear that the molecular class of "basal-like" carcinomas is heterogeneous and actually includes several tumour types, with very different prognostic features.[3] Indeed, adenoid-cystic carcinomas, low-grade apocrine and low-grade metaplastic carcinomas share the same molecular profile of "basal-like" carcinomas but are associated with a more favourable outcome.

Besides the identification of special tumour types, according to the WHO classification,[2] the histopathological scrutiny of breast cancer should always include the accurate assessment of the size and grade of the invasive component, the extent of any in situ component, the occurrence of peritumoral vascular invasion,[4] the margin status and the number of involved regional lymph nodes. All these parameters have prognostic implications and may be useful to inform the choice of the local and systemic treatments.

Grading of breast cancer, according to the extent of tubule formation, nuclear atypia and mitotic count, remains a powerful prognostic parameter. In a very recent article focusing on risk assessment by the OncotypeDX assay, it has been reported that only 1 of 36 low-grade tumours actually had an high recurrence score, and none of 7 high-grade tumours had a low recurrence score.[5] A major criticism to the histopathological grading system is that the intermediate grade (grade 2) includes a mixed population of tumours that are molecularly identifiable as either low- or high-grade.[6] This has been constructed, together with the alleged lack of reproducibility in grading among pathologists, to distrust the prognostic value of the traditional grading system. We may well agree that a three-tiers grading system has a number of disadvantages and does not reflect the actual molecular features of breast cancer. However, while waiting for the development and the clinical validation of a simplified grading system with only low- and high-grade tumours, we should be as accurate as possible at least in identifying the tumours of low grade and those of high grade using the current criteria,[7] because this information will be taken into account in the selection of a tailored treatment.

Defining the biological features of breast cancer

According to the 2009 St. Gallen Consensus,[8] the systemic therapy of early breast cancer is mainly informed by the expression of hormone receptors and the HER2 status. Furthermore, for patients with ER-positive and HER2-negative disease the option of adding chemotherapy to endocrine therapy is evaluated according to tumour size, grade, and proliferative fraction (most commonly assessed by immunohistochemal staining of the Ki67 antigen), occurrence of peritumoral vascular invasion, and nodal status.

It is therefore of primary importance for the systemic treatment of patients with early breast cancer to ensure the most accurate assessment of all these parameters in a reproducible and timely manner. In designing the systemic treatments, the treating physicians almost invariably have to completely rely on the data of the final pathological report, without any chance of verifying their accuracy. It is only when the final report includes inconsistent results that the physicians may be alerted to require a confirmatory check of the alleged features. This would be the case, for example, of an invasive lobular carcinoma, classic type, reported as ER-negative, or of a tubular carcinoma reported HER2-positive. More subtle inconsistencies, requiring re-testing, would be a tumour with a low proliferative fraction being reported HER2-positive, or an high-grade invasive duct carcinoma with the triple-negative phenotype (ER, progesterone receptor and HER2-negative) reported to have a low proliferative fraction.

One of the possible reasons why intrinsically inconsistent reports are still issued is that in some centres, especially where a multidisciplinary approach to the treatment of breast cancer patients is lacking, the assessment of the biological features of the tumours is performed by inexperienced pathologists, or even by

technical staff, without any final supervision by pathologists able to double check the consistency among the morphological features and all the biological data. It is advisable that, whenever feasible, the final pathological report includes both the morphological and the biological features of the tumour, and that the pathologist signing out the report takes the full responsibility of ascertaining the consistency of all the data.

The first important step for an optimal testing of the biological variables of the tumours is the choice of the block to be submitted for the assays. This must be representative of the invasive component of the tumour (except for cases showing *in situ* carcinoma only), taken at the periphery of the lesion, and always including a portion of nonneoplastic breast parenchyma (normally available when dealing with primary tumours). Whenever possible, the tissue that has been previously frozen for intra-operative diagnosis and then fixed and embedded should not be chosen for the biological assays. In case of bilateral breast cancer, samples from both tumours should be examined, because bilateral cancers often show a discordant phenotype; for multifocal or multicentric disease the decision whether to submit for the biological characterisation samples from one or more neoplastic foci is less straightforward. Ideally, all the different foci should be evaluated, but in the majority of the cases they all will show the same phenotype, raising the question whether this policy is truly cost-effective.[9] A reasonable compromise would be to assess first whether the different tumour foci show the same morphological features (i.e. tumour type and grade) or they are different. In the former case, it may be acceptable to test the biological variables in only one nodule, whereas in the latter it is mandatory to test all the foci that are morphologically different. An additional recommendation is to keep always in mind that the best scenario for the patient is to be candidate to a targeted therapy, be it an endocrine treatment or an anti-HER2 therapy. Therefore, if the testing of the first nodule results in a triple-negative phenotype, it is recommended to test additional blocks from the other nodules not to miss any ER-positive or HER2-positive component of the tumour, and not to deny the patients the option of a targeted intervention.

Assessing oestrogen and progesterone receptor status

The panellists of the 2009 St. Gallen Consensus took the seminal decision of defining ER-positive and progesterone receptor (PgR)-positive the tumours showing 1% or more immunoreactive cells.[8] This definition has been subsequently endorsed by the expert panel issuing the ASCO/CAP guideline recommendations for immunohistochemical testing of ER and PgR in breast cancer.[10] In case of ER or PgR-positive tumours, the actual percentage of neoplastic cells showing definite nuclear immunoreactivity must be reported, because the higher the number of positive cells the larger is the expected benefit of endocrine therapies. In addition to the actual percentage of the positive cells, it is recommended to report on the average intensity of the staining, whereas the use of a combined scoring system (like the H score or the Allred score) is considered optional.

The ASCO/CAP guideline recommendations cover not only the technical aspects of the pre-analytical and analytical steps of the immunohistochemical testing for ER and PgR, but also issues related to the interpretation, scoring and reporting of the results.[10] Compliance with these recommendations might well be instrumental in improving the accuracy and reproducibility of ER and PgR testing worldwide. Until now, the error rate in ER and PgR testing is unacceptably high, with as many as 20% false-negative results for ER.[11] This implies that almost one fifth of the patients who could benefit from an endocrine treatment are actually denied such therapy because of a false-negative assessment of ER status.

One of the most useful recommendations to avoid false-negative results in ER testing is to evaluate systematically the immunoreactivity of the nonneoplastic breast tissue surrounding the tumour to assess the sensitivity and the specificity of the staining, before looking at the tumour itself. The normal ducts invariably show an heterogeneous pattern of staining of the luminal cells, with a mixture of negative cells and a variable number of cells exhibiting very weak, moderate and intense immunoreactivity. If the assay only detects a few cells of the normal ducts with a homogeneous staining pattern, then the risk of a false-negative assessment of the ER status of the tumour is very high, because the assay is not sensitive enough to highlight cells with a weak to moderate immunoreactivity. The normal breast tissue also represents a useful built-in negative control of the staining, allowing to assess the specificity of the immunoreactions, because the myoepithelial cells and the stromal cells must invariably show a negative result for both ER and PgR.[10]

While ER-positive tumours may be negative for PgR, the reversed phenotype (ER-negative and PgR-positive) is very rarely – if ever – true. Almost all the cases with such an aberrant phenotype are due to a false-negative assay for ER or a false-positive assay for PgR, and the pathologists should be alerted to repeat the test on the same or a different block before rendering this most unusual report.

Testing HER2 status

Guidelines and recommendations describing how to optimally perform the immunohistochemical and in situ hybridisation (ISH) assays for assessing HER2 status and evaluate and score the results have been issued and recently updated.[12,13] These assays have been clinically validated in several studies demonstrating the high predictive value of a HER2 positive status for the efficacy of HER2-targeted treatments.

However, accuracy and inter-laboratory reproducibility of the assessment of HER2 status continue to be a concern worldwide, with a very high rate of inter-laboratory discordance both with immunohistochemical (with as many as 15% to 20% false-positive results) and ISH assays.[14–17] To minimize the risk of false-positive assessments it is important to verify that the nonneoplastic breast parenchyma remains unstained (or very weakly stained) with immunohistochemistry, and exhibits the normal number of copies of the HER2 gene when assayed by ISH techniques.

According to the regulatory agencies worldwide and the trastuzumab package insert, only patients whose tumors overexpress HER2 in more than 10% invasive tumor cells, or show HER2 gene amplification (4 or more copies of the gene/cell, or a ratio ≥2 between the gene copy number and the chromosome 17 centromeres) are candidate to trastuzumab treatment. The recommendations issued by ASCO/CAP[13] to raise the threshold for a positive immunohistochemical assay to >30% overexpressing tumour cells and for a positive ISH assay to a ratio of ≥2.2 or a gene copy number of >6/cell were not intended (and actually were not entitled) to replace the original thresholds, but especially to increase the concordance rate between the 2 assays.

It is worth re-emphasizing, however, that while immunohistochemical results are evaluated and scored at the individual cell level (by assessing the actual percentage of positive cells), ISH is a population-based assay, averaging the number of copies of the gene among all the neoplastic cells of one or more microscopic fields. The different criteria for scoring immunohistochemical and ISH results would not be an issue at all if the entire neoplastic cell population of every breast cancer were homogeneously HER2-positive or negative. There is now overwhelming evidence, however, that breast cancers often show heterogeneity in both HER2 overexpression and amplification, with only a (minor) fraction of invasive tumour cells being HER2 positive. That intratumoral

heterogeneity is not such an uncommon event as previously suggested and cannot be neglected any longer it is also witnessed by the recent guidelines issued by the College of American Pathologists[18] endorsing the definition of "genetic heterogeneity" to identify tumours with only 5% to 50% of the neoplastic cells showing amplification of the HER2 gene.

A feasible approach to tackle tumour heterogeneity without incurring in discordant results of immunohistochemical and ISH assays that would eventually imply a different treatment of the patients would be to use the same scoring system, based on the actual percentage of positive cells, for both assays. Accordingly, a tumour would be considered HER2-positive if more than 10% of the neoplastic cells are overexpressing the protein (3+ by immunohistochemistry) and/or carrying gene amplification.

The adoption of the HER2:chromosome 17 ratio to assess gene amplification was originally intended to avoid misclassification of polysomic tumours as amplified. In the last few years, however, different studies using alternative approaches to assess chromosome 17 status have consistently shown that true polysomy of chromosome 17 is an exceedingly rare (if at all possible) event in breast cancer. Almost all the cases with an increased number of chromosome 17 signals in ISH assays actually carry gain or amplification of the centromere region of chromosome 17, and not true polysomy.[19–21] If polysomy of chromosome 17 is such a rare event in breast cancer, all tumours showing an apparent polysomy at dual-color ISH assays should be classified according to the mean number of HER2 copies/cell as amplified (>6 gene copies/cell) or not amplified (<4 gene copies/cell).[22] Cases with 4 to 6 HER2 copies/cell and 3 or more CEP17 signals/cell would represent an equivocal category, and the prescription of HER2-targeted therapy would be eventually informed by the results of IHC assays.

Assessing the tumour proliferative fraction by Ki67 immunostaining

Tumour proliferation is one of the most important prognostic parameters in breast cancer, as it has also been documented by the predominant role of markers related to cell proliferation in the multigene prognostic signatures. In the clinical practice, the evaluation of the tumour proliferative fraction is most commonly performed by the immunohistochemical staining of the Ki67 antigen. This antigen is a nuclear protein with 2 isoforms (345 & 395 kDa) named after its immunoreactivity for the Ki-67 monoclonal antibody, originally raised in Kiel (Germany) against a nuclear antigen from a Hodgkin's lymphoma-derived cell line.[23] The gene size is of approximately 30,000 base pairs, with 15 exons and 14 introns on chromosome 10. The protein is expressed in all proliferating cells during late G1,S,G2 and M phases of the cell cycle, peaking in the G2-M, and it shows a rapid decline after mitosis. During interphase it is only detected within the nucleus; during mitosis most of the protein is relocated to the surface of chromosomes. Whereas the original Ki67 monoclonal antibody was not suitable for immunostaining formalin-fixed and paraffin-embedded tissue section, the commonly used MIB-1 monoclonal antibody[24] has been raised against a recombinant fragment of the protein, and it reacts with a formalin-resistant epitope.

The use of Ki67 immunolabeling as a prognostic and predictive markers has been extensively investigated in both the neo-adjuvant and adjuvant settings.[25,26] The panellists of the 2009 St. Gallen Consensus have included the assessment of Ki67 among the useful parameters to inform the choice of adding chemotherapy to endocrine therapies for patients with ER-positive and HER2-negative disease.[8] However, mainly due to the lack of standardisation in the performance of the assay and in the interpretation and scoring of the results, the measurement of Ki67 has not been considered a useful prognostic marker in the updated recommendations for the use of tumour markers in breast cancers issued by ASCO in 2007.[27]

Certainly to unveil the actual value of Ki67 as a prognostic and predictive marker in breast cancer we have to improve standardisation and reproducibility of its assessment. An *ad hoc* committee (the International Ki67 in Breast Cancer Working Group) has convened in London in March 2010 to share expertise and reach a consensus on the main technical and interpretative aspects of Ki67 immunolabelling, and eventually to issue recommendations for an optimal testing.

What can be anticipated is that there is a growing consensus among the scientists and treating physicians that the assessment of Ki67 may be an important addition to the available prognostic and predictive markers in breast cancer. In most of the published studies Ki67 labelling index has been evaluated by reporting the percentage of immunostained cells among 500–2,000 invasive neoplastic cells at the periphery of the tumour. It remains to be addressed whether immunoreactive cells in the hot spots should be included into the count or not, and whether Ki67 labelling index should be used as a continuous variable or be dichotomized according to the median value in a given population of patients.

Epilogue

The pathological work up of primary breast cancer continues to play an essential role in assessing the risk of tumour recurrence and in informing the local and systemic treatments for the patients. A comprehensive approach that includes the accurate evaluation of the morphological features of the tumour, with special reference to the histological type and grade, and the assessment of the main prognostic and predictive parameters (ER, PgR, HER2 and Ki67) should offer to the patients and the treating physicians a robust background upon which the final therapeutic decisions can be safely taken. The robustness of this background, however, depends on the expertise and knowledge of the pathologists, and on the accuracy and reproducibility of the assays for the assessment of the relevant markers. It is unacceptable that in many instances these prerequisites are still defective, with the neat result that many patients are denied an efficacious treatment.

The importance of the involvement of the pathologists in the multidisciplinary teams for the treatment of breast cancer patients, and in the design and conduct of the clinical trials cannot be overemphasised. They should also become more and more conscious of their role in the setting of translational research. It would be impossible to ascertain the actual value of the novel multigene prognostic or predictive signatures if these are not compared with the most accurate assessment of established parameters. Furthermore, because it seems unlikely that multigene signatures will ever be offered to all patients with breast cancer, it may be anticipated that the pathologists will have to cope with the new task of identifying those patients for whom the use of molecular assays would be a cost-effective option. Certainly, the pathologists will continue to have a tremendous role to play in fostering advances in the treatment of breast cancer patients. Please, let them know.

Conflict of interest statement

G. Viale: Consultancy: Roche, GSK; Travel expenses: Novartis.

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