Hormone Receptors and HER2 Expression in Primary Breast Carcinoma and Corresponding Lymph Node Metastasis: Do we Need Both?

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Abstract. *Background: Scattered studies report on controversial results concerning evaluation of primary breast tumors and their matched lymph node metastases. Aim. To investigate the molecular profile of primary breast tumors and corresponding lymph node metastases (LNM) based on estrogen receptor (ER), progesterone receptor (PR) and human epiderma growth factor receptor-2 (HER2 protein). Materials and Methods: Sixty-six primary tumors and corresponding axillary lymph node metastases were evaluated by immunohistochemistry for ER, PR and HER2 protein. According to these markers, cases were stratified as Luminal A, B, HER2 subtypes and triple-negative. Results. Thirteen out of 66 cases (19.7%) exhibited different tumor cell phenotypes in nodal metastases compared to primary breast tumors. All cases with hybrid phenotype had metastases with a pure HER2 phenotype. The most frequent switching was observed from luminal A to luminal B phenotype. Conclusion: The high rate of discrepancy between primary tumor and nodal metastasis phenotype imposes the need for a comparative assessment of both primary tumor and nodal metastasis before any therapeutic decision, in order to avoid recurrence and to improve patient prognosis and overall survival.*

Despite intensive research in the field and huge efforts to elucidate the heterogeneous nature of breast cancer, most cases not detected by breast screening are commonly assumed to have a poor prognosis (1). Breast cancer represents the most common cancer in women and the second leading cause of death among women. Several factors are believed to be involved in the aggressiveness and metastatic potential of breast cancer (2-4) but few of them are certified as true factors influencing these events (5-8). Morphologically similar breast carcinomas can display divergent clinical outcomes and responses to therapy. A major step in the development of specific therapy was the introduction of hormone therapy that significantly improved the natural evolution of breast cancer. Unfortunately, long-term follow-up studies have shown that hormone therapy did not greatly change overall survival (9, 10).

A new hope was born with the introduction of humanized monoclonal antibodies such as trastuzumab. Trastuzumab seems to be effective only in cases that strongly express HER2 at the membrane level; on the other hand, resistance to therapy has already been reported. Taken together, detection of ER, PR and HER2 shows a strong prognostic and therapeutic impact. These three markers are assessed virtually in all laboratories that process mammary biopsies. Hence the concept of triple-negative tumors was born and, a few years later, the molecular classification of breast cancer emerged.

Molecular classification of breast cancer represents an important step towards management of breast neoplasia because of better patient's stratification into six molecular sub-groups which have been shown to have different prognoses and therapeutic responses. This classification had a rapid impact on therapeutic protocols and seems to be a pathway towards personalized therapy.

Although two differential therapeutic schemes are now widely accepted based on this modern assessment of breast carcinoma, the prognosis and survival of patients with breast cancer has not significantly improved. Estrogens

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have long received most attention because of the success of selective ER modulators, such as tamoxifen, and aromatase inhibitors in breast cancer treatment (11). PR antagonists were dismissed because of severe side-effects (12), but awareness is now increasing that progesterone is an important hormone in breast cancer (13). It was shown that the androgen receptor (AR) also has an important role in the pathogenesis and outcome of breast cancer, but its significance in different subtypes of breast cancer is still under investigation (14). Furthermore, preliminary data suggest that the AR may play a role in breast cancer pathogenesis and may serve as a therapeutic target in certain more difficult-to-treat breast cancer subtypes, such as triple-negative breast cancer (15). For the HER2-positive molecular subtype, treatment is continuously refined to improve its efficiency and also to reduce side-effects. Early results from a phase III investigation (EMILIA) for the current use of trastuzumab emtansine demonstrated response rates of 25-35% in patients with breast cancer who had previously received trastuzumab (16). Previously described targeted-therapies are applied based on the molecular evaluation of the primary breast tumors but not on the assessment of the molecular profile of lymph node metastases (LNMs). Recent data suggested that the molecular subtypes of primary breast tumors exert an impact on nodal status rates and the local relapse rates are influenced by the molecular subtypes according to the nodal status (17). Synchronous evaluation of primary breast tumors and their corresponding LNMs is not a routine procedure in the clinical setting at present. Scattered studies involving a small number of patients reported controversial results regarding the evaluation of primary breast tumors and their matched LNMs (18, 19). All these articles limited their results to the evaluation of discordance between estrogen, progesterone, HER2/neu and Ki67 expression in primary breast tumors and their corresponding nodal metastases but did not provide any information about the mismatch of the molecular profile between primary tumors and their corresponding LNMs except for evidence of a high instability of tumor cells throughout tumor progression in breast cancer (20).

Currently, detection of ER, PR and HER2 is performed on tissue from the primary tumor and it is assumed that the lymph node or distant metastases have the same molecular profile.

Based on the previous limited findings, we speculated that some of the therapeutic failures are the result of metastatic tumor cells with a possible different phenotype insensitive to targeted therapy. Thus, we proposed to investigate the molecular profile of primary breast tumors and their corresponding nodal metastases with emphasis on the similarities and differences between primary and metastatic tumors based on ER, PR and HER2.

Materials and Methods

Patients and specimen processing. In the present retrospective study, 66 patients were included, ranging between 38 and 77 years old, diagnosed with breast cancer and axillary LNMs.

Primary tumors and corresponding lymph nodes detected with metastases were removed by open surgery, fixed in 10% buffered formalin for 48 h and paraffin embedded. Serial 5-μm sections were cut from each paraffin block, including both primary tumor and its corresponding LNM. Histopathological diagnosis was assessed by three experienced pathologists using conventional classification of breast cancer, and cases suitable for immunohistochemistry were carefully selected.

Immunohistochemistry. Additional slides were immunohistochemically-assessed for ER (mouse monoclonal antibody, clone 1D5, ready to use; DakoCytomation, Carpinteria, CA, USA), PR (mouse monoclonal antibody, clone Pgr636, ready to use; DakoCytomation), followed by the use of BOND Polymer Refine Detection Kit (Novocastra, Newcastle, UK) and for HER2/neu protein by using HercepTest PharmDx Kit (DakoCytomation). All immunohistochemical workflow was performed in a fully-automated manner by using a BOND Autostainer System. For a better characterization of the molecular profile of primary breast tumors, we additionally performed analysis of cytokeratin 5 (mouse monoclonal, dilution 1:100; Novocastra) and epidermal growth factor receptor EGFR (EGFR PharmDx; DakoCytomation).

Microscopic interpretation and data analysis. We evaluated ER and PR as brown positive signals restricted to the nucleus of tumor cells from primary breast tumors and LNMs. Ten microscopic fields (×40) of each immunostained section with the greatest number of positive cells were chosen. Positive nuclear signals were counted using a semi-quantitative method performed by the Lucia G software, previously described by Suciu *et al*. for Ki-67 assessment (21). We followed the guidelines of ER and PR assessment by Allred Score (22) combining the percentage of positive cells with intensity of nuclear staining but we modified the percentage of positive cells considering at least 10% of tumor cells as being positive relevant for diagnosis. We scored cases with fewer than 10% positive tumor cells as 0, 10 to 30% positive cells as +1, between 30 to 60% positive cells as +2, and cases with 60 to 100% positive cells as +3. For HER2/neu protein, status was assessed according to American Society of Clinical Oncology recommendations (23) as follows: 0 if no staining observed or weak, barely perceptible membranous staining up to 10% of cells; +1 in the case of weak membranous staining of >10%; +2 in cases of incomplete, weak/moderate circumferential membranous staining >10% of tumor cells or complete circumferential intense staining <10% of cells; +3 in the case of intense, circumferential staining of >10% of tumor cells. Cases scored as +2 and +3 were considered positive.

All microscopic procedures were performed by using Nikon Eclipse E600 Microscope equipped with a Canon Camera used for image capture.

An MS Access 2003 database was used to store and group the data. Data obtained from microscopic assessment of the slides were statistically assessed by applying SPSS software, version 17 (SPSS Inc. Chicago, IL, USA) We considered a *p*-value of less then 0.05 as significant.

Ethical issues. Patients were informed about the use of their tissue specimens for a research purpose and informed consent was obtained for each case. The Ethics Committee of our university evaluated and approved the present study (no. 9/672/22.01.2013)

Results

By conventional histopathological assessment, 53 (80.3%) out of 66 cases were classified as ductal invasive carcinomas, nine as lobular invasive carcinomas, three as medullary-type carcinoma and one as apocrine carcinoma. One of them was graded as G1, 24 out of 66 as G2, and 41 cases as G3.

The molecular profile of primary breast tumors was established by immunohistochemical surrogate markers for ER, PR, HER2/neu protein as main markers for molecular classification combined with cytokeratin 5 and EGFR as additional markers. Based on the molecular surrogate markers evaluation, 42 out of 66 (63.63%) primary breast tumor cases were luminal-A subtype, 10 (15.15%) were luminal type B, 5 cases (7.57%) had a hybrid phenotype luminal A/HER2 and another two cases had a hybrid phenotype luminal B/HER2. Six cases (9.09%) had a pure HER2 phenotype and one case (1.51%) had a basal-like phenotype.

In the case of primary tumors, we found a prevalence of ER+ cases (84.09%) to PR+ (76.14%). In LNMs, the distribution was ER+ in 67.05%, and PR+ in 60.23%.

Following the complete molecular assessment of the primary tumor, we compared the molecular subtype of the primary tumor to those of LNM. We found that the mean values of ER+ and PR+ cells were much higher in the primary tumor (ER+: 75.64%; PR+: 68.12%) than in LNM (ER+: 63.58% and PR+: 53.18%).

Ductal invasive carcinoma type was significantly correlated with luminal-A molecular subtype (p=0.0036) in primary tumors. No correlation was observed between tumor grade and molecular subtypes of breast cancer.

A significant correlation was found between the expression of ER, PR and HER2 protein in primary tumors and corresponding LNM (p<0.0001).

Assessment of hormone receptors in both primary tumors and LNMs revealed that the most common profile was luminal A (63.63% in primary tumor *versus* 59.09% in LNMs). The luminal B subtype was found in 15.15% in primary tumors compared with 19.69% in LNMs, respectively.

A slightly increased incidence of basal-like subtype was determined in LNMs compared to primary tumors and, a higher incidence of HER2 subtype in LNMs was found (7.57% in primary tumors compared with 10.6% for LNMs).

Thirteen out of 66 cases (19.7%) had different tumor cell phenotype in nodal metastasis compared to primary breast tumor. All cases with hybrid phenotype luminal A/HER2 or luminal B/HER2 had metastatic tumor cells with a pure HER2 phenotype (Figure 1). The most frequent switching was observed between luminal A phenotype (found in primary tumors) to a luminal B phenotype in tumor cells from the matched LNM.

Particular attention has been paid to medullary-type carcinoma. Even though there were only 3 cases, they had heterogeneous molecular phenotypes. One case of medullary carcinoma had luminal-B phenotype, the second had a hybrid luminal B/HER2 phenotypes and the third was classified as basal-like carcinoma. Interestingly, all had a different phenotype in their matched nodal metastases: hybrid luminal B/HER2 switched to pure HER2 phenotype, luminal B to basal-like, and basal-like to an unclassified phenotype.

Several factors influenced the results of the present study. Primary tumors showed a high heterogeneity of ER/PR and HER2/neu protein between areas of the same section. Thus, we considered all areas with the highest density of positive signals for assessed markers as relevant for our study, but a problem could arise for the negative areas of the primary tumors. For the LNM, metastatic areas were sometimes smaller but, despite this, we considered any positive area for ER, PR or HER2/neu protein relevant for interpretation.

Discussion

Breast cancer is the most frequent type of cancer among women and has unfavorable prognosis. It accounts for 23% of all cancer cases and 14% of cancer deaths (24). Despite several advances in genetic and histopathology of breast cancer, its incidence remains very high, accounting for 30-40% of all female cancers (25). Many factors are believed to contribute to this high rate of breast cancer, including lifestyle, environmental, genetic, and biological factors (26).

A new concept launched several years ago by Perou *et al*. (27), and recently revised (28), aimed to classify this disease into several molecular subtypes each with its own molecular signature, growth features and different invasive potential, in the belief that this new classification could improve therapy response, prognosis and overall survival. Despite these efforts, unfortunately, development of resistance to therapy is still frequent and often leads to cancer recurrence, sometimes with worse prognosis than the primary tumor (29, 30).

A common feature of malignant breast cancer, independent of their histopathology or molecular profile, is their ability to invade nearby tissues and spread through the lymphatic pathway. Nowadays, pathologists describe in detail the histopathology and molecular profile of the primary tumor but lack molecular assessement of LNM tumor cells. Few articles reporting controversial data about molecular profile of primary tumors and their matched LNM are available at this moment.

In 2003, Weigelt *et al*., using gene expression profiling, reported that human primary breast tumors are strikingly similar to matched distant metastases from lung, ovary, skin,

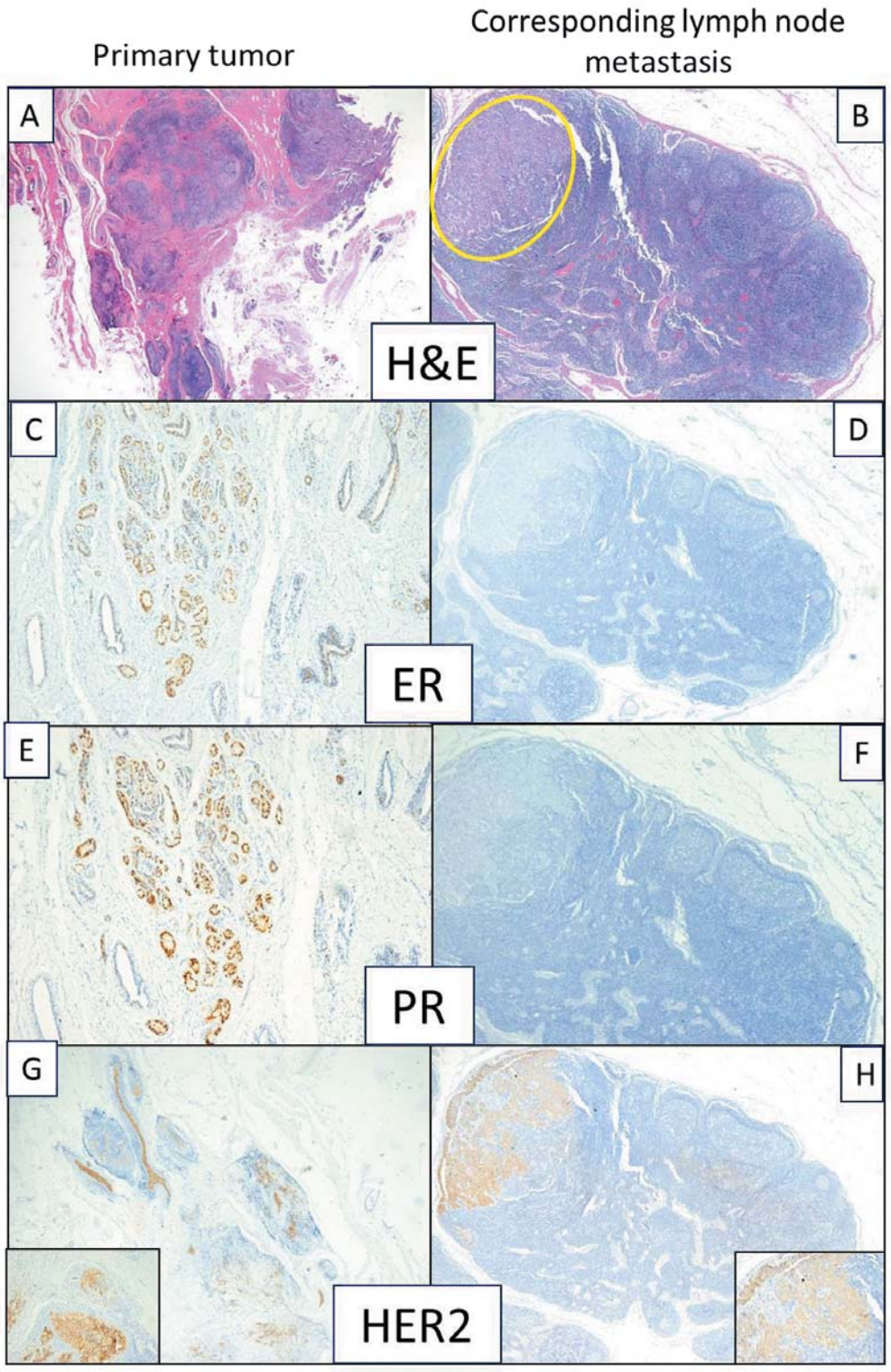

Figure 1. *Comparative view of the molecular profile from primary tumor (A, left column) and correspondent lymph node metastasis (B, right column) assessed by using surrogate immunohistochemical markers for ER, PR and HER2. Note the different expression of ER and PR, both positive in the primary tumor (C, E) and negative in the lymph node metastasis (D, F). HER2 protein was positive in both the primary tumor and its corresponding metastasis in a lymph node. Molecular profile based on ER, PR and HER2 assessment demonstrated the switch of the molecular profile from mixed luminal A/HER2 to a pure HER2 phenotype (D, F, H) (×10, inset ×20).*

lymph node of the same patient (31). They considered that the metastatic ability of breast cancer is an inherent feature and is not based on clonal selection.

In contrast, Montel *et al*. demonstrated several differences in the expression signatures of tumors derived from cloned weakly/non-metastatic human cell lines and from their isogenic metastatic counterparts from the same patient (32, 33). As the metastatic ability of the cell population increased, the receptor profiles changed. Almost 20% of our cases followed this rule and exhibited different hormone receptor prevalence between primary *versus* metastatic tumors, especially for ER, which seems to be the most unstable parameter during the metastatic process. This evidence supports the most frequent switching found in our study from the luminal A subtype in the primary tumor to the luminal B subtype in the matched LNM. It seems that primary cancer, containing many types of tumor cells, which can differ according to their expression profiles and their metastatic potential. Our observations concerning primary tumor heterogeneity of ER, PR and HER2 expression sustain the previous findings and suggest several tumor cell types with different metastatic potential. These demonstrate that the malignant phenotype and its molecular signature are not pre-determined and static, but continue to evolve in a tumor throughout its life history (34).

The present article reported an increased incidence of basal-like phenotype and HER2 phenotype in LNMs compared with primary tumors by switching of other phenotypes from primary tumors to basal-like or HER2 phenotype. Basal-like phenotype is known to have the most aggressive behavior and drug resistance. All hybrid luminal A or B/HER2 primary tumors converted to a pure HER2 phenotype in LNMs from our study. These findings suggest a more aggressive phenotype of nodal metastases and support therapeutic failure or drug resistance developed by patients who received therapy based exclusively on the primary tumor molecular phenotype assessment. Our study reported for the first time the instability of hybrid molecular forms of breast cancer and their matched nodal metastases. But we consider that special attention should be attributed to medullary-type breast carcinoma which exhibited the highest heterogeneity of both primary status and nodal metastases. Medullary-type carcinomas tend to have an unstable phenotype during their progression from primary tumor to nodal metastasis by acquisition of a more aggressive phenotype.

A therapeutic significant discordance in HER2 status was demonstrated by Santinelli *et al*. (35) between primary carcinoma and synchronous lymph node metastases (6.7%), local recurrence (13.3%) and metachronous distant metastases (28.6%). Similar results were found by Niikura *et al*. (36) who found that out of 40 HER2-positive cases (not treated), four metastases converted to an HER2-negative status. All cases with pure HER2 phenotype from our study retained their phenotype in the LNMs, but the incidence of HER2 type in LNMs increased based on the conversion of another phenotype to a metastatic HER2 phenotype.

Instability and heterogeneity of tumor cells from nodal metastases could be explained, in part, by the different microenvironment found in lymph nodes compared to the primary tumor site. The microenvironment of the tissue in which tumor cells have seeded is quite different from that where they originate and possibly creates a pressure on these cells, requiring them to develop adaptive responses which allow them to grow as a tumor in lymph nodes. Recently, the role of extracellular matrix components in breast cancer progression and metastasis was demonstrated (37).

Approximately 20% of our cases switched to a different phenotype from the primary tumor to their matched nodal metastasis. We consider that this high rate of discrepancy between the phenotype of the primary tumor and LNM imposes the need for a comparative assessment of both the primary tumor and LNM before any therapeutic decision. Based on this extensive and more complete breast tumor evaluation, future therapeutic strategies should be developed to target both primary and metastatic phenotypes. This would be the first step to avoid recurrence and to improve patient prognosis and overall survival.

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