Characterization of Molecular Subtypes of Paget Disease of the Breast Using Immunohistochemistry and In Situ Hybridization

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- **Context.**—Paget disease of the breast, in most cases, represents intraepidermal spread of ductal carcinoma in situ. Molecular subtypes of invasive carcinoma of the breast have prognostic and therapeutic significance and show characteristic distribution. Little is known about the distribution of molecular subtypes in Paget disease of the breast.

- **Objectives.**—To examine the distribution of molecular subtypes in Paget disease of the breast and to compare them to concurrent invasive carcinoma of the breast, if present.

- **Design.**—We examined 48 cases of Paget disease of the breast with immunohistochemistry and antibodies against estrogen and progesterone receptors, human epidermal growth factor receptor 2 (HER2), and Ki-67, as well as HER2 chromogenic in situ hybridization, to classify the cases into molecular subtypes. Then, we compared the results to the molecular subtypes of associated invasive carcinoma of the breast, if present.

Molecular subtypes (luminal A and B, human epidermal growth factor receptor 2 [HER2]-enriched, and basal-type) of invasive breast carcinoma have been defined using RNA microarray technology and demonstrate prognostic and predictive significance. Additionally, different risk factors predispose patients to develop different molecular subtypes of breast tumors. Interestingly, those molecular subtypes have also been demonstrated in the breast cancer precursor lesion ductal carcinoma in situ (DCIS). Importantly, the molecular subtypes can be reliably determined with immunohistochemical stains. Most cases of mammary Paget disease (MPD) are thought to represent an intraepidermal spread of neoplastic cells from an underlying DCIS. Frequently, MPD overexpresses HER2. However, little is known about the frequency of the different molecular subtypes found in MPD. In this study, we defined the different molecular subtypes in MPD, determined their relative distribution, and performed a comparison with concomitant, invasive carcinoma of the breast (ICB), if present.

- **Results.**—The HER2 subtype was the most common found in Paget disease of the breast, followed by the luminal B subtype and 2 cases of the triple-negative subtype. The associated invasive carcinoma cases were most often of the luminal B subtype, followed by the HER2 subtype and the triple-negative subtype. The molecular subtype of Paget disease and invasive carcinoma was congruent in most of the cases.

**Conclusions.**—Molecular subtypes of invasive carcinoma of the breast can already be detected in Paget disease. The distribution of molecular subtypes of Paget disease and of Paget disease–associated invasive carcinoma differs from invasive carcinoma without associated Paget disease, with the HER2 subtype overrepresented in Paget disease and associated invasive carcinoma and the luminal and triple-negative subtypes underrepresented.

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**MATERIALS AND METHODS**

With a computer-assisted search, cases of MPD diagnosed between 1996 and 2008 at the Institute of Pathology (University Hospital Erlangen, Erlangen, Germany) and at OptiPath (Joint Practice for Pathology, Frankfurt am Main, Germany) were retrieved from the archives. Diagnosis of MPD was validated by 2 pathologists (D.L.W. and J.D.S.) specialized in breast pathology. Relevant hematoxylin-eosin–stained tissue regions were identified for construction of a tissue microarray. The tissue microarray consisted of 2-mm tissue-donor cores embedded in paraffin blocks, which were then used to cut 1-μm sections for antibody detection of antigens with a fully automated immunohistochemistry system (Benchmark XT System, Ventana Medical Systems, Oro Valley, Arizona).
Arizona). Immunohistochemistry was performed with the following antibodies: Ki-67 (Dako clone MIB1, dilution 1:100, Agilent Technologies, Waldbronn, Germany), estrogen receptor (ER; clone SP1, F. Hoffmann-La Roche, Basel, Switzerland), progesterone receptor (PR; clone 16, dilution 1:200, Novocastra, Leica Biosystems, Wetzlar, Germany), and HER2 (dilution 1:200, Dako). All stains were then evaluated by 3 of the authors (D.L.W., P.W.W., and J.D.S.). For Ki-67, ER, and PR, the percentage of tumor cells with stained nuclei was documented. Regarding the ER and PR stains, only cases with nuclear positivity in more than 1% of tumor cells were considered positive. Regarding Ki-67, the number of positively stained nuclei within 1 high-power field was divided by the number of total tumor cells. Depending on the cellularity of the different cases, the total number of tumor cells ranged between 30 and more than 100 per high-power field. Regarding HER2 signals, the percentage of membranous positive tumor cells and the staining intensity (1+, weak; 2+, moderate; or 3+, strong) was determined. Finally, for all cases, the HER2 amplification status was ascertained with the aid of chromogenic in situ hybridization (CISH), following the manufacturer’s instructions (ZytoDot 2C SPEC HER2/CEN17, ZytoVision GmbH, Bremerhaven, Germany). For the HER2 CISH analysis, paraffin slides were deparaffinized with xylene and ethanol in decreasing concentrations (100%, 96%, and 70%) and then incubated in H2O2. Slides were incubated in EDTA, followed by pepsin proteolysis and dehybridization with ethanol in increasing concentrations. After that, slides were hybridized over night with digoxigenin-labeled HER2 and dinitrophenyl-labeled centromere 17 probes. The next day, slides were rinsed in a saline-sodium citrate wash buffer and then incubated with mouse anti-digoxigenin and mouse anti-dinitrophenyl. After washing the slides in Tris-buffered saline, an incubation step was performed with anti-mouse horseradish peroxidase/alkaline phosphatase/alkaline phosphatase polymer. After a wash step, an alkaline phosphatase-red solution and horseradish peroxidase–green solution was applied, and slides were counterstained with hematoxylin. After dehydration with ethanol in increasing concentrations and xylene, slides were covered with a cover slip. With this kit, HER2 signals were green, and CEN17 signals were red. The number of HER2 and CEN17 signals was counted in at least 25 tumor cells. A HER2 to CEN17 ratio greater than 2 was considered amplified.

Molecular subtypes were grouped as follows: luminal A subtype (ER+ or PR+, HER2– and Ki-67 index [percentage of Ki-67+ tumor cells] < 15%); luminal B subtype (ER+ or PR+ and HER2+; or Ki-67 index > 15%); HER2 subtype (ER- and PR-, HER2+), basal-like subtype (ER-, PR-, and HER2-). Regarding HER2 status, results of CISH were decisive.

Varying numbers of total cases in the results section resulted from technical problems (eg, inadequate number of tumor cells, missing cores in the tissue microarray after sectioning, among others).

After identification of eligible MPD cases, we searched those files for an association with DCIS and ICB. Molecular subtypes of ICB were defined as for the MPD cases above. The HER2 status in the archived ICB was determined during routine clinical workup by immunohistochemistry and CISH. Cases with a HER2 score of 0 or 1+ by immunohistochemistry (eg, no staining or weak, incomplete membranous staining) were considered HER2– cases, with a HER2 score of 2+ by immunohistochemistry (eg, moderate circular membranous staining of >10% of tumor cells) were further tested using HER2 CISH, and cases with a HER2 score of 3+ (eg, strong circular staining of >10% of tumor cells) were considered HER2+.

Cases with a HER2 to CEN17 ratio of more than 2 by CISH were considered HER2+.

RESULTS

Forty-eight cases of MPD were included in this study. Of those, 47 (97.9%) were women, and one patient was a man. The mean patient age at the time of diagnosis was 64.3 years for women (range, 27–90 years), and the single male patient was 63 years old. Twenty-two tumors (45.8%) were located for women (range, 27–90 years), and the single male patient was 63 years old. Twenty-two tumors (45.8%) were located in the right breast (including that of the male patient), 23 tumors (47.9%) were in the left breast. In 3 cases (6.2%), the location could not be retrieved from the documents.

Immunohistochemistry and CISH of MPD

The results of immunohistochemistry and CISH are summarized in Figure 1; 12 of 44 tumors (27.3%) expressed ER, and 3 of 44 tumors (6.8%) expressed PR. The Ki-67 index was 20% or greater in 95.3% (41 of 43) of the cases. In 1 case (2.3%), the Ki-67 index was 10% (in that case, the PR and HER2 stain could not be evaluated).

In 35 of 44 cases (79.5%), the Paget cells were noted as immunohistochemically moderately to strongly positive for HER2, and 9 cases (20.5%) showed weak HER2 expression. No immunohistochemically negative cases for HER2 were found. There were 40 cases (83%) available for CISH; 90% (36 of 40) of the tumors showed high-level HER2 amplification (HER2 to CEN17 ratio > 5 with clusters of HER2 signals), and 4 of 40 cases (10%) were not amplified. Seven of 9 cases (77.8%) that had HER2 scores of 1+ by immunohistochemistry were available for CISH, where 4 tumors (57.1%) demonstrated HER2 amplification, and 1 of 9 tumors (11.1%) with immunohistochemistry scores of 1+ for HER2 and amplification of HER2 by CISH analysis was also negative for ER and PR. This resulted in a change of the molecular subtype from basal-like (from the immunohistochemistry result) to HER2 (from the CISH result). All cases that showed strong (3+) membranous HER2 expression immunohistochemically also demonstrated HER2 amplification by CISH. Of the 5 cases with HER2 scores of 2+, 1 case (2.3%) was CISH negative and CISH positive in 1 case (2.3%). Of those, 9 (81.8%) demonstrated HER2 amplification, as determined by CISH. Two of 38 patients (5.3%) showed a triple-negative molecular subtype (basal-like; Figure 5, A through E). The luminal A subtype was not identified in this present study (the molecular subtype of the only case with an associated low-grade DCIS could not be determined because of missing ER and PR stains; the proliferative index in that case was 40%).

Associated Lesions

In the routine histopathologic reports of the cases included in this study, information regarding the absence or presence of DCIS and the absence or presence of invasive carcinoma was available in 40 cases (83.3%) and 43 cases (89.6%), respectively. In 37 cases (92.5%), an associated DCIS was documented, 34 (91.9%) of which were graded in the report (1 case [2.9%] of low-grade DCIS, 1 case [2.9%] of intermediate-grade DCIS, and 32 cases [94.1%] of high-grade DCIS). In the 3 remaining patients (8.1%, including the male patient), the grade of the associated DCIS was unknown. In 22 of 43 cases (51.2%), ICB was present. Of
those, 20 patients (90.9%) were documented as having invasive carcinoma of no special type, and 2 patients (9%) had special types of carcinomas. The molecular subtype of the ICB could be determined retrospectively in 20 cases (90.9%). Of those, 10 (50%) had the luminal B subtype, 8 (40%) had the HER2 subtype, and 2 (10%) were triple negative. No luminal A type ICB was found in this collective.

Correlation of Molecular Subtypes

In 14 of the 48 cases (29.2%) the molecular subtypes of MPD and the associated ICB could be determined; 10 of the 14 cases (71.4%) showed congruent molecular subtypes (6 cases of HER2 subtype [60%], 4 cases of luminal B subtype [40%]). In 4 cases (28.6%), the molecular subtypes differed between MPD and invasive carcinoma (MPD/invasive carcinoma: HER2/luminal B [3 cases], triple-negative/ luminal B [1 case]).

DISCUSSION

Our study shows that the molecular subtypes of ICB can be deduced from the subtype of the associated MPD in many cases. From a technical point of view, immunohistochemical determination of the molecular subtype of MPD is preferable to molecular analysis because Paget cells are often scattered within the basal epithelium, a specific growth that might confound molecular examination without laser microdissection.

Molecular subtypes of invasive carcinoma associate with different risk factors, differing prognoses, and differing responses to therapies and, therefore, are of clinical importance. The distribution of the molecular subtypes in ICB in Western Europe is relatively constant. Approximately 70% of all carcinomas represent the luminal subtype, approximately 15% show HER2 amplification (a combination of HER2 subtypes and luminal B subtypes), and 15% are triple-negative; the latter 2 groups demonstrate aggressive clinical behavior.9 Regarding precursor lesions, the luminal A subtype and HER2 amplification are overrepresented in DCIS compared with ICB.10,11 Triple-negative cases, in contrast, are underrepresented in DCIS. That discrepancy could be explained by assuming that luminal A DCIS or HER2-amplified DCIS cases need longer to develop into ICB.

In our cohort of MPD patients, HER2 positivity/amplification was even more prevalent than it was in DCIS, as corroborated by recent literature.12 That overrepresentation of HER2 amplification is also found in associated ICB, explaining the more-aggressive behavior of MPD-associated ICB. Most of the cases in our study showed congruent molecular subtypes of MPD and ICB, further underpinning the theory of DCIS-MPD as a precursor lesion to ICB.

In 2 cases (4.2%), we found a triple-negative phenotype of MPD. In the only other large MPD study that identified MPD molecular subtypes, by Sek et al12, no triple-negative cases were found. However, Sek et al12 described the HER2 subtype in 86% of cases. Taken together 98% of their cases showed positivity for HER2, but CISH was not used to validate the immunohistochemical results. In our study, 90% showed HER2 amplification by CISH, and 4 cases (8.3%) that had scores of 1+ in immunohistochemistry showed HER2 amplification, underscoring the greater sensitivity of HER2 CISH.

In 1 of our 2 triple-negative MPD cases, information regarding the associated invasive carcinoma was present.
The invasive carcinoma was diagnosed as a luminal-B carcinoma. In 4 cases, the results of the molecular subtypes between MPD and ICB were discrepant. Apart from technical difficulties, that could be explained by collision tumors of MPD and ICB. It is also conceivable that the discrepancy might be the result of tumor heterogeneity: MPD could be composed of tumor cells with different molecular characteristics, and only 1 of those tumor cell clones becomes invasive. In addition, it could be that HER2 amplification in some tumor cells facilitates the pagetoid spread into the epidermis.

Because of the many associated DCIS (in our study 92.5% [37 of 40]) and ICB (in our study, 51.2% [22 of 43]), a thorough examination of the breast is recommended in cases with a diagnosis of MPD. Our results also suggest that in the case of a HER2⁺ ICB with coexisting DCIS, the probability of an associated MPD is relatively high. Because the different molecular subtypes of ICB have different risk factors, it is likely that, for the development of MPD, there are also specific risk factors; however, currently, those factors remain unknown.

Figure 2. Distribution of molecular subtypes of mammary Paget disease (MPD) and associated invasive carcinoma of the breast (ICB) and invasive carcinoma of the breast in the literature according to Lakhani et al.⁹

Figure 3. A through E, Examples of HER2-type mammary Paget disease with immunohistochemistry results showing expression of HER2 and negativity for estrogen and progesterone receptors (hematoxylin-eosin, original magnification ×20 [A]; estrogen receptor, original magnification ×20 [B]; progesterone receptor, original magnification ×20 [C]; Ki-67, original magnification ×20 [D]; HER2, original magnification ×20 objective [E]).
The determination of molecular subtypes by immunohis-tochemistry is feasible, but the differentiation between luminal-A and luminal-B carcinomas can be difficult. Only about 30% of luminal B carcinomas show amplification of HER2; the other 70% must be identified by different means. The growing number of available RNA expression tests for this indication (eg, Oncotype DX [Genomic Health, Redwood City, California] or Prosigna [NanoString Technologies, Seattle, Washington]) shows that the distinction of luminal-A and luminal-B carcinomas is of great clinical significance. Of central importance is the protein Ki-67, which is expressed during the cell cycle and which is also used in RNA expression assays. Cheang et al showed that, using a cutoff of more than 14% Ki-67+ tumor cells (Ki-67 index), a relatively good discrimination of luminal-A and luminal-B subtypes is possible. There are only 3 studies, to our knowledge, that examined the Ki-67 index in MPD. The proliferative index of the Paget cells in those studies was between 10% and 30%. In our study, we found similar proliferative indices (>20% in 41 of 43 patients [95%]). Determination of the exact proliferation index can be difficult when only few Paget cells can be seen. The high proportion of morphologic, high-grade DCIS, and the high proliferative index in ER+ cases suggest that many, if not all, ER+ cases represent luminal-B subtypes. If low-grade DCIS is diagnosed in a patient, an accompanying MPD is highly unlikely.

The triple-negative MPD cases described here can pose diagnostic difficulties for pathologists, especially in the differential diagnosis of Morbus Bowen or melanoma in situ. Melanoma in situ can be ruled out with melanoma markers such as HMB-45 or Melan-A. Morbus Bowen is usually negative when stained with antibodies against low-molecular cytokeratins, such as CK7, and positive for markers of squamous differentiation (p63, p40).

In summary, this study shows that molecular subtypes, characteristic of ICB, are also a feature of MPD. In MPD, the frequency of molecular subtypes differs compared with ICB, with HER2-amplified cases being overrepresented. The often-concomitant ICB also shows overrepresentation of HER2+ cases. Diagnosis of MPD should lead to a thorough examination of the breast because MPD is associated with DCIS and/or ICB in most of the cases. Diagnosis of HER2+ ICB with DCIS should lead to a thorough exclusion of an accompanying MPD to avoid incomplete resection of the in situ neoplasia in the skin. Triple-negative cases of MPD exist but are very rare.

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