# Internodal *HER2* heterogeneity of axillary lymph node metastases in breast cancer patients

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## ABSTRACT

Determination of human epidermal growth factor receptor 2 (HER2) status is important for adequate treatment of breast cancer (BC) patients. The novel HER2 gene protein assay (GPA) is particularly convenient, as it allows the simultaneous assessment of HER2 protein expression and gene amplification at individual cell level. Here we investigated the frequency of internodal HER2 heterogeneity in axillary lymph node macrometastases of BC patients and compared HER2 status between primary breast tumor and its metastases. We included a total of 41 female patients operated between 2014 and 2015 for primary BC with axillary lymph node macrometastases. Representative paraffin blocks of metastatic lymph nodes were sectioned and the slides were stained using the GPA in 38 BC cases. GPA results were assessed according to the ASCO/CAP 2013 criteria. We analyzed 12586 individual tumor cells, 120 cells per section of each metastatic lymph node. HER2 status differed between the primary tumor and its metastases in 5/38 cases (13.2%). In patients with at least two metastatic nodes, the HER2 status of lymph node metastases was only slightly different in 4/23 cases (17.4%). Our results indicate rare but substantial differences in HER2 status between primary breast tumor and its axillary lymph node metastases that may direct the choice and outcomes of targeted therapy in BC patients. The impact of the rare and subtle internodal HER2 heterogeneity evidenced in this study remains uncertain. Determining the HER2 status of lymph node metastases in BC seems to be rational, but assessing a limited number of metastatic nodes may be sufficient.

## INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed malignant tumor in women (non-melanoma skin cancers excluded), in 140 out of 180 countries around the world [1]. Molecular targeted therapy represents a major advancement in the treatment of BC, being most successful if directed toward human epidermal growth factor receptor 2 (*HER2*-targeted therapy) [2]. *HER2* is overexpressed in about 15% to 20% of BC cases [3]. *HER2*-positive tumors have a worse prognosis compared to *HER2*-negative tumors, indicating the importance of adequate and timely *HER2* assessment in BC patients.

The novel *HER2* gene protein assay (GPA) [4] allows the pathologist to simultaneously assess the expression of *HER2*

protein and amplification of the *HER2* gene at the individual cell level and in the context of cellular morphology. The GPA is suitable for formalin-fixed paraffin-embedded (FFPE) material, it enables brightfield detection of *HER2* protein and *HER2* gene expression and, because the colors do not fade over time, the assessment of slides is permanent. These advantageous characteristics make the GPA very useful in routine practice, especially in equivocal cases.

*HER2* intratumoral heterogeneity is defined as the presence of *HER2*-positive and *HER2*-negative tumor cell subpopulations within the same tumor [5]. *HER2* intertumoral heterogeneity, on the other hand, refers to discordance in *HER2* status between simultaneous ipsilateral invasive tumor foci, a primary tumor and its metastases and/or between metastatic deposits within the same individual. These factors make it difficult to treat BC patients with monotherapy [6]. Using a clinically relevant model of intratumoral *HER2* heterogeneity in immune competent mice Song et al. showed a correlation between the effectiveness of anti-*HER2* antibody monotherapy and the degree of *HER2* heterogeneity in BC [7]. Studies

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on human patients also indicated a prognostic and therapeutic value of *HER2* heterogeneity in BC [8]. While in general tumor heterogeneity is considered to be one of the main obstacles to efficient cancer treatment, the importance of internodal *HER2* heterogeneity of lymph node metastases in BC is still not clear.

The aim of our study was to investigate the frequency of internodal *HER2* heterogeneity in axillary lymph node macrometastases of BC patients. We also compared *HER2* status between primary breast tumor and its metastases.

## MATERIALS AND METHODS

### Patients

This study included a series of 41 female patients operated between 2014 and 2015 for primary BC with axillary lymph node macrometastases and diagnosed at the Department of Pathology and Cytology Dalarna of the County Hospital Falun in Sweden. The two criteria for inclusion into the study group were diagnosis of primary invasive BC with axillary lymph node macrometastases and determined hormone receptor status for primary breast tumor. Macrometastasis was defined as at least one metastatic deposit greater than 2 mm within a lymph node [9]. We excluded patients with recurrent disease and those having only micrometastasis (0.2 mm in size) or isolated tumor cells (<0.2 mm) in the examined lymph node(s). Patient age and the localization of tumor within the breast was registered from the patient's medical records. All patients provided informed consent to participate in the study. The study protocol was approved by the Ethical Committee of the Uppsala – Örebro Region in Sweden, EPN Dnr 2010/461.

The primary tumors were documented in large-format histology slides and the subgross growth pattern of the invasive tumor component (unifocal, multifocal, or diffuse) was determined using previously published criteria [10]. The histological type of breast tumor was assessed according to the World Health Organization (WHO) criteria [11], and the tumors were graded according to the Elston and Ellis grading system [12].

Representative paraffin blocks of metastatic lymph nodes were selected from the archived material to be stained using the GPA, as previously described by Nitta et al. [4]. The GPA method has three steps: 1) immunohistochemical detection of *HER2* protein using the *HER2*/neu Rabbit monoclonal primary antibody (clone 4B5; Ventana, Tucson, Arizona); 2) silver *in situ* hybridization (SISH) for visualizing the copies of the *HER2* gene; and 3) chromogenic red *in situ* hybridization (Red ISH) for visualizing the centromere of chromosome 17 (CEN17). The slides were counterstained with Hematoxylin II and Bluing Reagent™ (Ventana). The procedure was performed on the BenchMark® XT platform (Ventana Medical Systems, Inc., Tucson, Arizona).

After staining, the slides were microphotographed using an Olympus XC50 camera. At least three separate distant foci of tumor cells in a single metastatic lymph node were captured. *HER2* status was assessed in 120 tumor cells (40 cells per focus) or in all tumor cells if the total number of cells in a focus was less than 40. Image enhancement software (Microsoft Office Picture Manager 2010) was used to enhance microphotography images, after that *HER2* (black dots) and CEN17 (red dots) signals were counted.

The following parameters were evaluated for GPA results:
- Membranous expression of HER2 protein, graded as 0, 1+, 2+, or 3+ according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2013 guidelines [13].
- *HER2* gene and CEN17 copy number in all assessed individual tumor cells.
- Average *HER2* and CEN17 copy number calculated first separately for each microscopic field and then for all assessed tumor cells within a lymph node.
- The *HER2*/CEN17 ratio.

The *HER2* gene amplification status was evaluated according to the ASCO/CAP 2013 criteria [13] as follows:
- *HER2* gene amplified, if the *HER2*/CEP17 ratio was greater than 2.2 or the average number of copies of the *HER2* gene was greater than 6.0;
- Equivocal *HER2* amplification status, if the *HER2*/CEP17 ratio was 1.8–2.2 or the average number of copies of the *HER2* gene was 4.0–6.0;
- Negative *HER2* amplification status, if the *HER2*/CEP17 ratio was less than 1.8 or the average number of copies of the *HER2* gene was less than 4.0.

The *HER2* status of primary breast tumors was assessed during the routine diagnostic work-up in all cases. In addition to *HER2* status, estrogen receptor (ER) and progesterone receptor (PR) status (prediluted clone SP1 and clone 1E2, respectively; Ventana Medical Systems, Tucson, Arizona) was determined in primary tumors by immunohistochemistry (IHC) and 10% of tumor cells were considered as a cut-off. According to these IHC results and the ASCO/CAP 2013 guidelines [13], primary breast tumors were classified into luminal A-like, luminal B-like, *HER2* type, or triple negative BC (TNBC).

Intertumoral *HER2* heterogeneity was defined as differences in *HER2* status between primary breast tumor and its metastases. We used the aggregate *HER2* status of the axilla, meaning that the presence of a single *HER2*-positive lymph node qualified the case as *HER2*-positive in axillary lymph node metastasis, irrespective of the status of other nodes. Internodal *HER2* heterogeneity was defined as differences in *HER2* status between individual lymph node metastases within the axilla.

## Statistical analysis

Data were summarized using descriptive statistics, including the measures of central tendency (arithmetic mean), dispersion (standard deviation) as well as relative numbers in work-up of the results. The $\chi^2$ test was used for estimating the significance of the differences. The value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp., Armonk, NY).

## RESULTS

### Characteristics of the cohort

This study included 41 female patients with an age range of 42 to 92 years (mean age 69.54 ± 10.99 years, median 70 years). The tumor was localized in the right breast in 23/41 (56.1%) patients. It was localized in the upper medial quadrant of the breast in 8/41 (19.5%) patients, upper lateral quadrant in 20/41 (48.8%), lower medial quadrant in 4/41 (9.8%), lower lateral quadrant in 6/41 (14.6%), and centrally in 3/41 (7.3%) patients. The tumor was unifocal in 18/41 (43.9%), multifocal in 18/41 (43.9%), and diffuse in 5/41 (12.2%) cases. Most of the tumors were of histological grade II (23/41, 56.1%) and of no special type (30/41, 73.2%). The frequency of four molecular subtypes of BC was as follows: luminal A-like in 12 (29.3%) patients, luminal B-like in 21 (51.2%), *HER2* type in 3 (7.3%), and TNBC in 5 (12.2%) patients. Table 1 shows clinical and pathological characteristics of BC patients.

### GPA results

The median number of lymph nodes was eight nodes per case with a range of 1 to 29 lymph nodes, while the median number of lymph nodes with macrometastasis was two with a range of 0 to 17 lymph nodes. We analyzed 108 metastatic lymph nodes and 120 cells per metastasis (with four exceptions where only 28, 40, 53, and 105 cells could be analyzed per microscopic field). This gives a sum of 12586 tumor cells assessed.

The GPA proved to be a robust method with easily measurable gene and centromere signals as well as assessable characteristics of the membranous protein expression at a single cell level. Figure 1 shows representative cases of lymph node metastasis with negative, equivocal, and positive *HER2* status.

Axillary lymph node macrometastasis was present in 92.7% patients (38/41), while in the remaining three cases the metastatic focus was too small for an adequate assessment of *HER2* status using the GPA. Table 2 shows the concordance of *HER2* protein overexpression and gene amplification status in 38 BC cases [aggregate status of all the lymph nodes per case] ($p < 0.001$).

Among 33 BC cases negative for *HER2* protein overexpression (0/1+), four had equivocal *HER2* gene amplification status. None of those 33 cases were positive for *HER2* gene amplification. All BC cases positive for *HER2* gene amplification had *HER2* protein expression 3+. A single case with four metastatic lymph nodes and the overall *HER2* protein expression 0/1+ had a small focus of *HER2*-positive tumor cells (3+, cluster amplification) in less than 3% of tumor cells (Figure 2).

## Comparison of HER2 status between primary breast tumor and its metastases

Table 3 shows a comparison of *HER2* protein expression status between primary breast tumor and its lymph node metastases (aggregate status of the lymph nodes) in 38 BC patients ($p < 0.001$). Four out of 38 cases (10.5%) had equivocal *HER2* protein expression status in the primary tumor but negative *HER2* status in the metastases. A loss of *HER2* protein expression from 3+ to 0/1+ between primary tumor and lymph node metastases was observed in 2/38 BC cases (5.3%) and from 2+ to 0/1+ in 4/38 cases (10.5%).

**TABLE 1.** Clinical and pathological characteristics of patients with breast cancer (n=41)

| Parameter | Patients (n) | (%) |
|---|---|---|
| Laterality | | |
| Right | 23 | 56.1 |
| Left | 18 | 43.9 |
| Intra-mammary localization | | |
| Upper medial | 8 | 19.5 |
| Upper lateral | 20 | 48.8 |
| Lower medial | 4 | 9.8 |
| Lower lateral | 6 | 14.6 |
| Central | 3 | 7.3 |
| Subgross lesion distribution | | |
| Unifocal | 18 | 43.9 |
| Multifocal | 18 | 43.9 |
| Diffuse | 5 | 12.2 |
| Histological grade | | |
| I | 6 | 14.6 |
| II | 23 | 56.1 |
| III | 12 | 29.3 |
| Histological tumor type | | |
| Invasive carcinoma, NST | 30 | 73.2 |
| Invasive lobular carcinoma | 7 | 17.1 |
| Tubular carcinoma | 2 | 4.9 |
| Invasive apocrine carcinoma | 1 | 2.4 |
| Invasive micropapillary carcinoma | 1 | 2.4 |
| Molecular subtypes | | |
| Luminal A | 12 | 29.3 |
| Luminal B | 21 | 51.2 |
| HER2 type | 3 | 7.3 |
| TNBC | 5 | 12.2 |
| Total | 41 | 100.0 |

NOS: Not otherwise specified; HER2: Human epidermal growth factor receptor 2; TNBC: Triple negative breast cancer

A comparison of the *HER2* gene amplification status between primary breast tumor and its lymph node metastases in 38 BC cases (*p* < 0.001) is shown in Table 4. The *HER2* gene amplification status differed between the primary tumor and its metastases in 5/38 cases (13.2%). In one case, the *HER2* gene was amplified in the primary tumor but not in the metastatic lymph nodes. Two cases with *HER2*-amplified primary tumors and two with *HER2*–non-amplified primary tumors had equivocal *HER2* gene amplification status in the metastatic lymph nodes.

Overall, a single case of *HER2*-positive primary breast tumor had *HER2*-negative lymph node metastases. Two additional cases with *HER2*-positive primary tumor had metastases with equivocal *HER2* gene amplification status and negative for *HER2* protein overexpression. Two cases with *HER2*-negative primary tumors also had metastases with equivocal *HER2* gene amplification status and negative for *HER2* protein overexpression.

## Internodal HER2 heterogeneity

Internodal *HER2* heterogeneity between lymph node metastases of the same patient was analyzed in 23/38 patients (56.1%) who had more than one metastatic lymph node. Internodal *HER2* heterogeneity was found in 4/23 cases (17.4%) and resulted from the presence of metastatic lymph
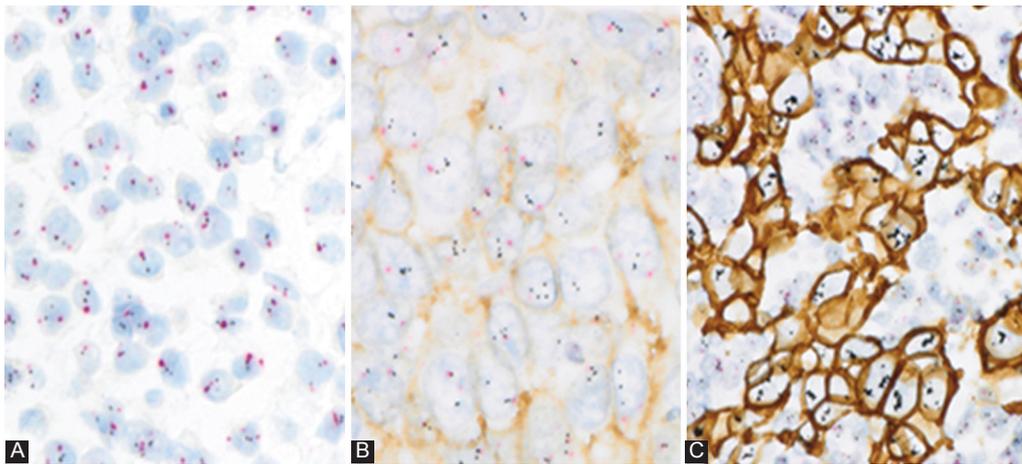


**FIGURE 1.** Representative breast cancer cases with HER2-negative (A), HER2-equivocal (B), and HER2-positive (C) lymph node metastases, stained using HER2 gene protein assay. HER2: Human epidermal growth factor receptor 2.
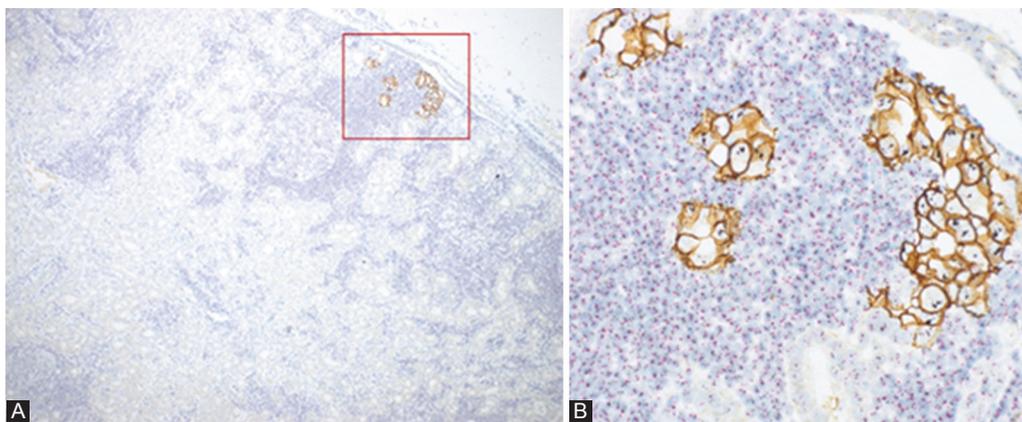


**FIGURE 2.** Breast cancer lymph node metastasis showing intratumoral heterogeneity (HER2 gene protein assay). A) low magnification image; B) high magnification image of the area marked with the rectangle in A. HER2: Human epidermal growth factor receptor 2.

**TABLE 2.** Comparison of aggregate *HER2* protein expression and gene amplification status of lymph node metastases in breast cancer patients (n=38)

| *HER2* protein expression status of lymph nodes | *HER2* gene amplification status of lymph nodes | | | |
|---|---|---|---|---|
| | Non-amplified | Equivocal | Amplified | Total |
| Negative (0/1+) | 76.3% (29/38) | 10.5% (4/38) | 0.0% (0/38) | 86.8% (33/38) |
| Equivocal (2+) | 0.0% (0/38) | 0.0% (0/38) | 0.0% (0/38) | 0.0% (0/38) |
| Positive (3+) | 0.0% (0/38) | 0.0% (0/38) | 13.2% (5/38) | 13.2% (5/38) |
| Total | 76.3% (29/38) | 10.5% (4/38) | 13.2% (5/38) | 100% (38/38) |

HER2: Human epidermal growth factor receptor 2

**TABLE 3.** Comparison of *HER2* protein expression status of primary breast tumors and aggregate *HER2* protein expression status of corresponding lymph node metastases

| HER2 protein expression status of lymph nodes | HER2 protein expression status of primary breast tumors ( n=38) | | | |
|---|---|---|---|---|
| | Negative (0/1+) | Equivocal (2+) | Positive (3+) | Total |
| Negative (0/1+) | 71.1% (27/38) | 10.5% (4/38) | 5.3% (2/38) | 86.8% (33/38) |
| Equivocal (2+) | 0.0% (0/38) | 0.0% (0/38) | 0.0% (0/38) | 0.0% (0/38) |
| Positive (3+) | 0.0% (0/38) | 0.0% (0/38) | 13.2% (5/38) | 13.2% (5/38) |
| Total | 71.1% (27/38) | 10.5% (4/38) | 18.4% (7/38) | 100% (38/38) |

*HER2*: Human epidermal growth factor receptor 2

**TABLE 4.** Comparison of *HER2* gene amplification status of primary breast tumors and aggregate *HER2* gene amplification status of corresponding lymph node metastases

| *HER2* gene amplification status of lymph nodes | *HER2* gene amplification status of primary breast tumors (n=38) | | | |
|---|---|---|---|---|
| | Non-amplified | Equivocal | Amplified | Total |
| Non-amplified | 73.7% (28/38) | 0.0% (0/38) | 2.6% (1/38) | 76.3% (29/38) |
| Equivocal | 5.3% (2/38) | 0.0% (0/38) | 5.3% (2/38) | 10.5% (4/38) |
| Amplified | 0.0% (0/38) | 0.0% (0/38) | 13.2% (5/38) | 13.2% (5/38) |
| Total | 78.9% (30/38) | 0.0% (0/38) | 21.1% (8/38) | 100% (38/38) |

*HER2*: Human epidermal growth factor receptor 2

**TABLE 5.** Internodal heterogeneity in *HER2* gene amplification status of axillary lymph node metastases

| Patients (n=4) | Number of lymph nodes | *HER2* non-amplified | *HER2* equivocal | *HER2* amplified |
|---|---|---|---|---|
| 1 | 2 | 1 | 1 | 0 |
| 2 | 3 | 2 | 1 | 0 |
| 3 | 4 | 3 | 1 | 0 |
| 4 | 4 | 1 | 3 | 0 |

*HER2*: Human epidermal growth factor receptor 2

nodes with both *HER2*-negative and *HER2*-equivocal gene amplification status in the same axilla. None of those metastases were positive for *HER2* gene amplification (Table 5).

In three out of the four patients with internodal *HER2* heterogeneity, *HER2* protein expression was negative in the metastases (0 or 1+). The patient number 3 in Table 5 had four metastatic lymph nodes, all with *HER2* protein expression level 0/1+. However, in one of those metastatic lymph nodes, a small area of clearly *HER2*-positive tumor cells was observed. In contrast to the regions with *HER2* protein expression 0/1+ and non-amplified *HER2* gene, these cells showed a strong and complete membranous positivity and cluster amplification of the *HER2* gene (Figure 2). However, because the *HER2*-positive focus was very limited in size (<3% of the tumor cells), this lymph node metastasis was categorized as *HER2*-negative.

None of 23 BC cases with more than one metastatic lymph nodes showed the presence of *HER2*-negative and *HER2*-positive metastases in the same axilla.

Thus, internodal *HER2* heterogeneity was limited to the four equivocal cases presented in Table 5. In most of these cases, we observed no discordance in *HER2* status. In one case, 17 metastatic lymph nodes were analyzed in the same axilla: 6120 individual tumor cells in 51 microscopic fields were negative for *HER2* protein overexpression (0), none of these cells had >6 *HER2* gene copies, and there was little variation in the *HER2* gene/centromere 17 ratio (0.9322–1.2407 per

microscopic field) between the cells. Another example is a case with 11 metastatic lymph nodes within the same axilla, all with negative *HER2* status (0/1+ *HER2* protein expression, the *HER2* gene/centromere 17 ratio around 1.000, and no tumor cells with >6 *HER2* gene copies among 1320 examined tumor cells).

Similarly, five *HER2*-positive metastatic cases had 3+ *HER2* protein expression and showed *HER2* gene amplification invariably in all examined lymph nodes across all examined microscopic fields.

## DISCUSSION

*HER2* heterogeneity is common in BC. Generally, tumoral heterogeneity in BC can undergo spatial and temporal evolution [14]. Spatial *HER2* heterogeneity may manifest as intratumoral heterogeneity, characterized by the presence of *HER2*-positive and *HER2*-negative tumor cell clones within the same invasive tumor focus, or as intertumoral heterogeneity, where separate *HER2*-positive and *HER2*-negative tumor foci (primary and/or metastatic) coexist in the same patient. The temporal tumoral heterogeneity, on the other hand, means that recurrent lesions differ from the primary tumor in their *HER2* status, or that the *HER2* status of the tumor changed during the natural history of the disease or under the effect of anticancer therapy [14]. Intratumoral *HER2* heterogeneity is observed in up to 40% of BC cases and represents the

main obstacle in treating early, nonmetastatic *HER2*-positive disease [5-8]. An additional obstacle is the frequent multifocality of the invasive component of BC and the fact that these simultaneous ipsilateral invasive tumor foci differ in their *HER2* status in about 10% of cases [15].

In the present study, we focused on internodal *HER2* heterogeneity of the axillary lymph node metastases of BC and the differences in *HER2* status between primary breast tumor and its metastases. Molecular differences between primary versus metastatic disease in BC are well studied and have been demonstrated using both IHC [16] and ISH [17] methods. Aurilio et al. [18] performed a meta-analysis of studies investigating the discordance in ER, PR and *HER2* status between primary breast tumor and corresponding relapse. Thirty-one studies including almost 3000 patients were evaluated for *HER2* discordance. The proportion of discordant cases for *HER2* varied between 0% and 24%, with the average being 8% [18]. Our results fall into the same range, with 15.8% of discordant cases for *HER2* protein expression and 13.2% for *HER2* gene amplification. One case with *HER2*-positive primary tumor had *HER2*-negative metastases, additional cases with *HER2*-positive primary tumor had metastases with equivocal *HER2* amplification status and without *HER2* protein overexpression, and two cases with *HER2*-negative primary tumor had metastases with equivocal *HER2* amplification status and without *HER2* protein overexpression. The therapeutic decision based on the *HER2* status of the primary tumor would differ from the decision made based on the *HER2* status of the metastases in at least three out of five cases, or more precisely in at least three out of 38 cases analyzed in our study.

In contrast to the above-discussed types of *HER2* heterogeneity that are well studied, there appears to be a lack of data on internodal *HER2* heterogeneity in BC, according to our search of relevant studies published in the English language. In this study, we analyzed the *HER2* status of lymph node metastases in 23 BC patients that had more than one axillary lymph node macrometastasis. Somewhat unexpected, we found that the *HER2* status of the metastastatic lymph nodes was concordant in most cases even between 17 or 11 metastatic lymph nodes of the same axilla. The *HER2*-positive metastatic cases retained *HER2* positivity in all examined lymph nodes and across all analyzed microscopic fields. Subtle differences were found in the form of intratumoral *HER2* heterogeneity in one case and in the form of equivocal *HER2* amplification status in four cases with *HER2*-negative primary tumors. We accepted the equivalent *HER2* status of metastases as deviating from the negative *HER2* status of the primary tumors. This was done due to reports indicating that *HER2* equivalent cases may benefit from targeted anti-*HER2* therapy [19].

Our study investigated the advantages of the novel *HER2* GPA allowing simultaneous analysis of *HER2* gene and protein expression in individual cells. We confirm that the method is robust and results are easy to assess, as suggested previously [20]. Digital microphotographs of the representative areas allowed repeatable analysis of the results. The disadvantages of our study are the relatively small number of BC cases, limited number of analyzed microscopic fields, and lack of follow-up. Multiple axillary lymph node metastases were present only in 23 out of 41 BC patients in our cohort obviously representing a limitation of the study, despite the large number of the individual cancer cells analyzed. Larger and prospective studies are needed to confirm our observations.

## CONCLUSION

Our results indicate relatively rare but substantial differences in *HER2* status between primary breast tumor and its axillary lymph node metastases that may direct the choice and outcomes of targeted therapy in these patients. On the other hand, the impact of rare and subtle internodal *HER2* heterogeneity evidenced in this study remains uncertain. Determining the *HER2* status of lymph node metastases in BC seems to be rational, but assessing a limited number of metastatic nodes may be sufficient. Larger and prospective studies are needed to confirm these observations.

## DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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