



**HAL**  
open science

## **HER2 molecular subtype is a dominant subtype of mammary Paget's cells. An immunohistochemical study.**

Piotr Jerzy Sek, Antoni Zawrocki, Wojciech Biernat, Janusz Henryk Piekarski

### ► **To cite this version:**

Piotr Jerzy Sek, Antoni Zawrocki, Wojciech Biernat, Janusz Henryk Piekarski. HER2 molecular subtype is a dominant subtype of mammary Paget's cells. An immunohistochemical study.. *Histopathology*, 2010, 57 (4), pp.564. 10.1111/j.1365-2559.2010.03665.x . hal-00586993

**HAL Id: hal-00586993**

**<https://hal.science/hal-00586993>**

Submitted on 19 Apr 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**HER2 molecular subtype is a dominant subtype of mammary Paget's cells. An immunohistochemical study.**

Journal:	<i>Histopathology</i>
Manuscript ID:	HISTOP-08-09-0470.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	09-Jan-2010
Complete List of Authors:	Sek, Piotr; Medical University of Lodz, Department of Surgical Oncology Zawrocki, Antoni; Medical University of Gdansk, Department of Neuropathology and Molecular Pathology Biernat, Wojciech; Medical University of Lodz, Department of Molecular Pathology and Neuropathology; Medical University of Gdansk, Department of Neuropathology and Molecular Pathology Piekarski, Janusz; Medical University of Lodz, Poland, Department of Surgical Oncology
Keywords:	Mammary Paget's disease, tissue microarrays, molecular subtype, CK5/6, HER2



**HER2 molecular subtype is a dominant subtype of mammary Paget's cells. [An immunohistochemical study.](#)**

Piotr Sek<sup>1</sup>, Antoni Zawrocki<sup>2</sup>, Wojciech Biernat<sup>2,3</sup>, Janusz H. Piekarski<sup>1</sup>

Grant support: J.H. Piekarski was supported by a research grant from Medical University of Lodz, Poland. Grant 501-11-743

<sup>1</sup>Department of Surgical Oncology, Medical University of Lodz, Poland

<sup>2</sup>Department of Neuropathology and Molecular Pathology, Medical University of Gdansk, Poland

<sup>3</sup>Department of Molecular Pathology and Neuropathology, Medical University of Lodz, Poland

**Requests for reprints:**

Janusz H. Piekarski

Department of Surgical Oncology

Ul. Paderewskiego 4

93-509 Lodz, Poland

Phone: +4842 6895441

Fax: +4842 6895422

E-mail: [janusz.piekarski@umed.lodz.pl](mailto:janusz.piekarski@umed.lodz.pl)

**Running title:** subtypes of Paget's cells

**Key words:** Mammary Paget's disease, tissue microarrays, molecular subtype, CK5/6, HER2

**Abstract**

**Aims:** We hypothesise that similarity of the molecular subtypes of Paget's cells to molecular subtypes of underlying breast carcinomas would put in favour the epidermotropic theory of Paget's cells origin.

**Methods and Results:** We analysed immunohistochemical expression of markers that define particular molecular subtypes of breast carcinomas. The whole analysis was performed by means of tissue microarray in mammary Paget's disease and in the underlying breast carcinoma(s). We found that HER2-overexpression subtype (ER-; HER2+) was a dominant molecular subtype of Paget's cells (37 out of 43 analyzed cases; 86%). Luminal B (ER+; HER2+) and luminal A (ER+; HER-) subtypes were identified in 12% and 2% of cases, respectively. None of the analysed tumours presented basal-like subtype phenotype. Similar distribution of molecular subtypes was identified in the underlying *in situ* breast carcinomas (HER2 subtype, 82%; luminal A, 6%; luminal B, 6%; basal-like, 6% of cases) and in the invasive component (HER2 subtype, 84%; luminal A, 8%; luminal B, 8%; basal-like, 0% of cases).

**Conclusion:** HER2 molecular subtype was a dominant, but not the sole subtype presented by Paget's cells of the nipple. Similar distribution of molecular subtypes in Paget's cells and in the underlying carcinomas strongly suggest their common origin.

Deleted: the

Deleted: in 43 cases of

Deleted: /

## Introduction

Numerous studies proved that the clinical outcome in the invasive breast carcinoma is significantly associated with its genetic subtype<sup>1-6</sup>. Recently, the distinction of the specific molecular subtypes was also applied for *in situ* breast carcinomas<sup>7-9</sup>. The criteria for molecular classification remained the same as in case of invasive carcinomas. The markers used for defining molecular subtypes were the following: estrogen receptor (ER), human epidermal growth factor receptor type 2 (HER2), epidermal growth factor receptor (EGFR) and cytokeratin 5/6 (CK5/6). This classic panel of molecular profiling markers was proposed by Nielsen et al. for invasive breast carcinomas<sup>10</sup> and later extended to classify carcinomas *in situ*. Although numerous studies were performed on the molecular subtypes of breast carcinoma, mammary Paget's cells are still a set of specific neoplastic cells that remains to be defined on the molecular grounds. The association between the lesions located in the nipple and the underlying breast carcinoma was described originally in 1874 by Sir James Paget<sup>11</sup>. Nowadays, the coexistence of parenchymal breast carcinoma is observed in 67%-100% of patients with Paget's disease of the nipple<sup>12-14</sup>. On the other hand, Paget's disease of the nipple is diagnosed in 0.5%–5% of all breast carcinoma patients<sup>15-19</sup>. The expanding oozing and eczematous appearance of the nipple is the most common clinical presentation of the Paget's disease of nipple. The diagnosis of the disease is based on the identification of intraepidermal large, atypical cells with pale cytoplasm and these cells are referred to as 'the Paget's cells'<sup>20</sup>. They are dispersed among the basal layer but may be also identified in the upper portions of the epidermis ('pagetoid spread'). Their origin is not clear. There are two hypotheses explaining their derivation. 'Epidermotropic theory' suggests that they derive from the underlying breast carcinoma as they migrate along the mammary duct system into the nipple. The other hypothesis, 'intraepidermal transformation theory', states that the Paget's cells develop in the epidermis as a result of the morphological (and biological) change of the epidermal keratinocytes<sup>21-23</sup>. The more popular, epidermotropic theory is supported by the observation of frequent coexistence of the invasive breast carcinoma with Paget's disease of the nipple<sup>13,24</sup>. On the other hand, the minority of cases without diagnosable breast cancer

seem to support the intraepidermal transformation theory. However, the inability of detection of intraparenchymal breast cancer coexisting with the Paget disease may depend from the insufficient sensitivity of the diagnostic methods<sup>13</sup>. The third theory trying to combine both theories suggests that Paget's cells may develop on either way depending on the local circumstances<sup>25</sup>.

We hypothesise that similarity of the molecular subtypes of Paget's cells to molecular subtypes of underlying breast carcinomas would put in favour the epidermotropic theory. Our analysis of the medical literature concerning Paget disease of the nipple revealed that Paget's cells were not specifically classified in terms of molecular subtyping. To the best of our knowledge there was only one study defining the molecular subtype of breast carcinomas associated with the Paget's disease of the nipple<sup>26</sup>. However, the authors did not determine the molecular profile of the Paget's cells. Therefore, we decided to analyse the expression of the markers defining molecular subtypes of breast carcinomas: ER, HER2, EGFR, and CK5/6. The phenotypic profile was established both in the Paget's cells and in the underlying breast carcinoma. The molecular subtypes were defined as: luminal A (ER+, HER2-, EGFR+/-, CK5/6+/-), luminal B (ER+, HER2+, EGFR+/-, CK5/6+/-), HER2 positive (ER-, HER2+, EGFR+/-, CK5/6+/-), and basal-like (ER-, HER2-, EGFR+ or CK5/6+). Cases negative for all four analysed markers were referred to as 'unclassified'. In order to make our analysis of protein expression in Paget's cells more complete, we also included progesterone receptor (PR) and cytokeratin 17 (CK17). The whole analysis was performed by means of tissue microarray. We tried to determine whether this technique will appear suitable for such a demanding histologic material as the breast nipple with Paget cells. To the best of our knowledge, it is the first attempt of using tissue microarray technique for study on Paget disease of the breast.

## Material and methods

### Study design

The search of the database of the Department of Surgical Oncology and the Department of Pathology, Medical University of Lodz, Poland, identified 44 patients with Paget's disease of the nipple who were treated surgically from January 1977 to December 2005. Similarly, we looked for patients with Paget's disease of the nipple who were diagnosed in the Department of Neuropathology and Molecular Pathology, Medical University of Gdansk, Poland, identifying 32 patients in the period from January 1998 to December 2006. Inclusion criteria for the present study were as follows: (i) histologically confirmed Paget's disease of the nipple, (ii) no neoadjuvant chemotherapy applied, (iii) the amount of biological material from both parenchymal carcinoma and Paget disease was sufficient for immunohistochemical analysis, (iv) female gender of the patients. Sixty-four cases fulfilled these inclusion criteria.

The analysis was performed on the tissues obtained from the surgery; they were fixed in 10% buffered formalin and embedded in the paraffin blocks. For each patient we retrieved formalin-fixed, paraffin-embedded tissues from the nipple (with Paget's cells) and/or underlying parenchymal DCIS and/or underlying invasive carcinoma. From selected tissue blocks a single section was cut and stained with haematoxylin and eosin. The stained section was used to assess the quality of tissue material and to determine the site of the punch biopsy for the microarray study. Due to poor quality of the archival tissue, a number of cases was rejected from the study. Good quality archival tissue was found in 49 tissue blocks containing Paget's cells, 28 tissue blocks containing associated DCIS and 15 tissue blocks containing associated invasive carcinoma. In these 'good quality' archival tissues, complete immunophenotyping was performed. All these tissue blocks were derived from 57 patients. These patients composed the study group.

**Deleted:** Finally, 57 cases were included into the study group.

**Deleted:** In this group,

**Deleted:**

**Deleted:** for 49 cases of Paget's disease, 28 cases of DCIS and 15 cases of invasive carcinoma.

### Characteristics of study group

In 13 out of 57 cases the Paget disease of the nipple was the sole manifestation of the disease (23%). Paget's disease of the nipple coexisted with an underlying DCIS and invasive ductal carcinoma in 23 (23/57; 40%) and 14 cases (14/57; 25%), respectively. In 7 patients with Paget disease, simultaneous DCIS and invasive ductal carcinoma were identified (7/57; 12%). Altogether, DCIS was diagnosed in 28 cases (28/57; 49%). In this 28 DCIS cases, nuclear grade 3 was diagnosed in 22 cases; nuclear grade 2 was diagnosed in 6 cases. The presence of necrosis was found in 16 DCIS cases. ~~Altogether, invasive ductal carcinoma was diagnosed in 21 cases (21/57; 37%). In these 21 invasive ductal carcinoma cases, histologic grade of carcinoma was determined by means of Nottingham scale<sup>27</sup>. Grade 3 was diagnosed in 16 cases; grade 2 was found in the remaining 5 cases.~~

Deleted: Altogether,

Deleted: i

### ***Immunohistochemical staining***

The sections stained with haematoxylin and eosin (H&E) were used to identify morphologically representative area of interest within the blocks. The original tissue blocks formed the "donor" blocks for creating the Tissue Microarray (TMA). The tissue cores were removed from the "donor" and inserted into a "recipient" paraffin blocks using a Manual Tissue Arrayer MTA-I (Beecher Instruments) with 0,6 mm diameter needles (punches) and 1 mm space between the cores. From every representative area of interest within the block we sampled 4 cores. Therefore, we sampled up to 12 cores from one case (4 cores of Paget's cells, 4 cores of *in situ* carcinoma and 4 cores of invasive carcinoma). In total, we made four TMAs including tissue cores from the study blocks and cores from the control tissues. As the internal controls we used fragments of the breast carcinomas with known receptor profile (ER+, PR+, HER2+) and the skin biopsies. Sections from TMA blocks were 4 µm thick and were placed onto the silanised slides (Super Frost). The first slide from each TMAs was routinely stained with H&E as a control of morphology. The following cuts were used for immunohistochemical stainings which was performed as follows. After deparaffinization and rehydration the slides containing TMA sections were rinsed and immersed in Target Retrieval Solution (pH 6,0; DakoCytomation, Denmark). They were heated for 8 minutes (2 minutes of



max pressure) in a pressure cooker and then cooled slowly for 30 minutes. For detection of immunostaining we used Novolink Polymer Detection System (Novocastra Ltd., UK). After rinsing, the slides were incubated in 3% hydrogen peroxide for 5 minutes to inactivate endogenous peroxidase activity and were incubated in protein blocking agent for 5 minutes to stop nonspecific binding of the antibodies. The next step included incubation with the primary antibodies to ER, PR, EGFR, CK5/6 and CK17 (Table 1). Incubation lasted 90 minutes in the room temperature. Next, the post-primary blocker agent was applied for 30 minutes and then the slides were treated with Polymer, a polyvalent secondary antibody, for 30 minutes. That was followed by incubation with cerium-diaminobenzidine (DAB) solution for maximum 4 minutes and counterstaining with haematoxylin. The last step included dehydration and sealing of the slides. Every assay included tissue samples marked as positive and negative control. For HER2 assessment (Figure 1) we used the HercepTest (DakoCytomation, Denmark) and the whole test was performed according to the instructions of the manufacturer.

#### ***Evaluation of immunohistochemical staining***

Expression of CK 5/6 and CK 17 were considered positive if any degree of cytoplasmic staining was present in the tumour cells. EGFR were considered positive if any degree of distinct membranous staining was present. Expression of HER2 was considered positive, if strong complete membrane staining was identified in at least 30% of Paget's cells or breast carcinoma cells. Expression of ER and PR was regarded positive if the nuclear staining was present in at least 10% of Paget's cells or breast carcinoma cells.

#### ***Criteria of classification of Paget's cells and underlying carcinomas to specific molecular subtypes***

The molecular subtypes were defined as: luminal A (ER+, HER2-, EGFR+/-, CK5/6+/-), luminal B (ER+, HER2+, EGFR+/-, CK5/6+/-), HER2 positive (ER-, HER2+, EGFR+/-, CK5/6+/-), and basal-like (ER-, HER2-, EGFR+ or CK5/6+). Cases negative for all four

markers were referred to as 'unclassified'. The distribution of molecular subtypes in Paget's cells and in the underlying *in situ* and invasive breast carcinomas were compared with use of Fisher exact test with Freeman-Halton correction. *P*-value <0.05 was considered significant.

## Results

### *Immunohistochemical stainings*

Due to some loss of studied material during preparation of tissue microarray, final

Deleted: F

evaluation of immunohistochemical stainings of Paget's cells was feasible in 43 to 47 out of the initial 49 sampled cases depending on the antibody used. Evaluation of immunohistochemical stainings of DCIS was possible in 18-20 out of 28 initially sampled cases. Evaluation of immunohistochemical stainings of invasive ductal carcinoma was made in 12 to 14 out of 15 initially sampled cases. All results of immunohistochemical stainings are presented in Table 2.

### *Classification of Paget's cells and underlying carcinomas to specific molecular subtypes*

#### *Paget's cells*

Complete set of results of stainings (ER, HER2, EGFR, CK5/6) necessary for classification to specific molecular subtypes was achieved in 43 cases. HER2 subtype (ER-; HER2+; EGFR+/-;CK5/6+/-) was a dominant molecular subtype of Paget's cells. The diagnostic criteria for this subtype were met in 37 out of 43 cases (86%). In 5 out of 43 cases (12%) the phenotypic profile was indicative of luminal B subtype (ER+; HER2+; EGFR+/-; CK5/6+/-). One case (1/43; 2%) was classified as luminal A subtype (ER+; HER-; EGFR+/-; CK5/6+/-). None of the studied cases could be classified as basal-like nor an unclassified subtype (negative for all four defining markers).

#### *Ductal carcinoma in situ*

Complete set of results of stainings necessary for classification of cases to specific molecular subtypes was achieved in 17 cases. HER2 subtype was a dominant molecular subtype of ductal carcinoma *in situ* occurring in 14 out of 17 (82%) cases. Two cases were classified as luminal subtypes: one (1/17; 6%) was classified as luminal A subtype, the other (1/17; 6%) was classified as luminal B subtype. One case (1/17; 6%) was classified as basal-like subtype (ER-; HER-; EGFR+ and or CK5/6+).

#### *Invasive ductal carcinoma*

Complete set of results of stainings necessary for classification of cases to specific molecular subtypes was achieved in 12 cases. HER2-overexpression subtype was a dominant molecular subtype of invasive ductal carcinoma. The criteria for diagnosis of that subtype were met in 10 out of 12 cases (84%). Two cases were classified as luminal subtypes: one each (1/12; 8%) was classified as luminal A and luminal B subtype.

#### *Comparison of distributions of molecular subtypes*

The distributions of molecular subtypes in Paget's cells, in the underlying *in situ* and invasive breast carcinomas were similar (Fisher exact test with Freeman-Halton correction,  $P=0.93$ ).

#### **Discussion**

Paget's cells of the nipple presented predominantly with HER2 molecular subtype. This subtype was identified in 86% of cases. However, the molecular profiles of studied cases were not homogenous in this respect. Luminal B (in 12% of cases) and luminal A (in 2% of cases) were also identified, albeit rarely, and neither basal-like molecular subtype nor unclassified cases were diagnosed. Interestingly, the distribution of molecular subtypes in Paget's cells was very similar to those presented by the underlying *in situ* and invasive breast carcinomas. In studied groups of carcinoma cells, HER2-positive subtype was a dominant one (86%, 82% and 84% of cases, respectively). Luminal A subtype was

diagnosed in 2%, 6% and 7% of cases, respectively. Luminal B subtype was diagnosed in 12%, 6% and 7% of cases, respectively. Basal-like subtype was diagnosed in only one case (6%) of underlying *in situ* carcinoma. Very similar distribution of molecular subtypes in Paget's cells and in the underlying *in situ* and invasive breast carcinomas strongly support common origin of Paget's disease and coexisting invasive and *in situ* carcinomas. This indicates that Paget's cells are epidermotropic cellular counterpart of parenchymal malignancy. There is a small portion of cases that show discrepant phenotype, that may support independent origin of Paget's disease from underlying breast carcinoma. This observation is supported by another analytical approach. The molecular study of matched mammary Paget's disease and underlying breast carcinoma using loss of heterozygosity and mitochondrial DNA displacement loop sequence analysis revealed differences of molecular alterations in two out of 10 analysed pairs<sup>28</sup>.

Molecular subtypes in the breast carcinoma were distinguished basing on genomic profiling of mRNA expression, whereas our methodology relies on the identification of protein expression within the tumour cells. We found that the dominant immunohistochemical profile of Paget's cells was characterised by HER2 overexpression and lack of expression of ER, PR, EGFR, CK5/6 and CK17. Overexpression of HER2 in Paget's cells has been already reported in other studies that showed its frequency exceeding 80%<sup>29-33</sup>. In our study, the percentage of cases with overexpression of HER2 in Paget's cells was even higher and amounted to the level of 96%. In this respect, our results are concordant with those of other authors. Expression of steroid receptors (ER, PR) and EGFR in our study was also similar to results reported by other authors. The expression of ER in Paget's cells was described in 10-28.6% of studied cases<sup>30,32,33</sup>, and the expression of PR was described in 0-28.6%<sup>32,33</sup>. We have identified the frequency of these receptors in 14% and 7% of cases of Paget disease, respectively. EGFR expression in Paget's cells was studied by Schelfhout et al.<sup>31</sup> and revealed in 13% of cases. This generally remains in the similar rank in our study (9%). We did not find the results of any analysis of basal cytokeratin expression in the mammary Paget's cells. Strangely enough, Lester et al.<sup>26</sup> who made such immunostainings did not

present them in their manuscript. Therefore, we were not able to compare our results with those of others.

There is only one report concerning the molecular subtypes of underlying breast carcinomas in patients with Paget's disease of the nipple as defined by protein expression. Lester et al.<sup>26</sup> performed an extensive study of expression of ER, PR, HER2, EGFR, androgen receptor and five types of cytokeratins (CK5/6, CK14, CK17, CK8, CK18). The study was performed in the group of 28 cases (DCIS, 18 cases; invasive carcinoma, 10 cases). They found that in patients with Paget's disease of the nipple, distribution of the molecular profile of underlying DCIS is significantly different from that of invasive carcinomas. In 88% of underlying DCIS HER2 subtype was identified, whereas only 40% of underlying invasive carcinomas presented that profile. Luminal B subtype constituted 6% of DCIS cases and 30% of invasive carcinoma cases in that study. Lester et al. concluded that different molecular subtypes of parenchymal breast carcinomas were most predictive of mammary Paget's disease. Our observations are similar to that by Lester et al. in regard to the molecular profile of DCIS underlying Paget's disease of the nipple. We found that 82% of underlying DCIS belonged to the HER2 subtype whereas other molecular subtypes (luminal A, luminal B and basal-like) were uncommon (6% of cases). However, we found the molecular subtype of the underlying invasive carcinoma similar to that of DCIS coexisting with Paget's disease. In fact, in both studies the common feature was high frequency of HER2 overexpression as determined by immunohistochemistry in DCIS and invasive carcinoma. The discrepancy in molecular profile of these lesions between these studies stems in vast extent from the difference in estrogen receptor expression in the invasive carcinoma component. As the phenotypic profile (ER/EGFR/HER2/CK) identified by immunohistochemistry is 'translated' into molecularly-based classification, expression of HER2 and ER are basic determinants of designation a specific tumour into one of three subtypes: luminal A (ER+, HER2-), luminal B (ER+, HER2+) or HER2-positive (ER-, HER2+). In the study by Lester et al., ER-positivity was found in 30% of invasive carcinomas whereas in our study only 14% of cases expressed ER. The frequency of HER2 expression in both

studies was 80% and 93%. In our view, the main weakness of both studies comparing Paget's disease and underlying invasive carcinoma is low number of analysed cases (10 and 12). As the criteria of assessment of ER and HER2 expression are identical in our study and that by Lester et al., we suspect that this discrepancy in results have incidental character. These results should be verified on the larger group of cases with invasive breast carcinomas coexisting with Paget's disease of the nipple.

Deleted: , respectively, and this added less to the discrepant results

Other authors who undertook immunohistochemical studies on Paget's cells of the nipple, generally analysed smaller number of cases (from 5 to 30) than we did<sup>20,29-33</sup>. We performed our study in almost fifty cases. This number is similar to number of cases analysed by Liegl et al.<sup>33</sup>, however, they confined their analysis to Paget's cells whereas we also studied underlying carcinomas. Although other authors studied the expression of ER, PR, EGFR and HER2 in their studies, the panel of molecular subtype defining markers: ER, HER2, EGFR and CK5/6 was not used in studies of Paget's cells of the nipple. To study the expression of proteins in Paget's cells we chose tissue microarray analysis (TMA). The TMA technique has high (90-98%) concordance with the results of classic immunohistochemistry<sup>34-38</sup>. During the construction of the TMAs we took four cores from each selected portion of the tumour tissue and used 0,6 mm punches. According to several authors three cores per sample are fully representative of the whole tumour<sup>34,37,39,40</sup>. Taking into account random localization and scarcity of Paget's cells in the epidermis we decided to perform four cores from each case. Likewise, we considered using larger size of punches (1.5 mm), but as described by Kallioniemi et al. multiple 0.6 mm cores are sufficient to acquire an adequate amount of tissue<sup>41</sup>. Before we started the study we were fully aware of difficulties which could be encountered during construction of TMA in such a peculiar material as that occurring in Paget's disease. Indeed, precise sampling of clusters of Paget's cells from nipple epidermis was a difficult part of our study. However, despite difficulties, we were able to study expression of all the analysed proteins in 43 to 47 out of 49 initially sampled cases. Surprisingly, the major problems were encountered when foci of DCIS were sampled. It appeared that a substantial number of DCIS foci were too small to obtain the

Deleted: <sup>45</sup>

Deleted: biopsies

Deleted: tissue<sup>46</sup>

meaningful material for TMA. This was the reason of relatively inadequate gain of appreciable DCIS cases for immunohistochemical analysis. In our opinion we proved that technique of tissue microarrays can be efficiently used for study of Paget's disease, however some loss of studied material seems to be inevitable. Fortunately, in each of almost fifty sampled cases, the number of Paget's cells in specimens was sufficient to use proposed thresholds of positive cells criteria for evaluation of ER, PR and HER2. This aspect is very important in our study, because we were able to use the same criteria for evaluation of Paget's cells as those for underlying *in situ* breast carcinoma and invasive carcinoma. In consequence, we could make direct comparisons of protein expression and distribution of molecular subtypes between Paget's cells and breast carcinomas. For the remaining proteins the criteria of positivity were less demanding, as any degree of immunoreactivity was regarded as positive in regard to basal cytokeratins (CK5/6 and CK17) and EGFR. These criteria were shared by other authors<sup>7,9,10</sup>. Although a single cell with cytoplasmic staining was sufficient for diagnosis of cytokeratine-positive case, we found only one such case in the whole series. We found only one CK5/6-positive case of DCIS. There was no CK17-positive cases. However, we are convinced about good quality of our IHC staining as CK5/6 (Figure 2) and CK17 were demonstrated in the cells of the nipple epidermis.

In conclusion, our study revealed that HER2-positive molecular subtype was a dominant, but not the sole subtype of Paget's cells of the nipple. The distribution of molecular subtypes in Paget's cells was very similar to distribution of molecular subtypes of underlying *in situ* and invasive breast carcinomas. Similar distribution of molecular subtypes in Paget's cells and in underlying *in situ* and invasive breast carcinomas strongly suggest common origin of Paget's cells and underlying carcinomas.

**References**

1. Perou CM, Sorcie T, Eisen MB *et al.* Molecular portraits of human breast tumors. *Nature* 2000;**406**:747-752.
2. Sørlie T, Perou CM, Tibshirani R *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implication. *Proc. Natl. Acad. Sci. USA* 2001;**98**:10869-10874.
3. Sørlie T, Tibshirani R, Parker J *et al.* Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc. Natl. Acad. Sci. USA* 2003;**100**:8418-8423.
4. van de Vijver MJ, He YD, van't Veer LJ *et al.* A gene expression signature as a predictor of survival in breast cancer. *N. Engl. J. Med.* 2002;**347**:1999-2009.
5. Huang E, Cheng SH, Dressman H *et al.* Gene expression predictors of breast cancer outcomes. *Lancet* 2003;**361**:1590-1596.
6. Sortiriou C, Neo SY, McShane LM *et al.* Breast cancer classification and prognosis based on gene expression profiles from a population based study. *Proc. Natl. Acad. Sci. USA* 2003;**100**:10393-10398.
7. Livasy CA, Perou CM, Karaca G *et al.* Identification of a basal-like subtype of breast carcinoma in situ. *Hum. Pathol.* 2007;**38**:197-204.
8. Bryan BB, Schnitt SJ, Collins LC. Ductal carcinoma in situ with a basal-like phenotype: a possible precursor to invasive basal-like breast cancer. *Mod. Pathol.* 2006;**19**:617-621.
9. Tamimi RM, Baer HJ, Marotti J *et al.* Comparison of molecular phenotypes of ductal carcinoma in situ and invasive breast cancer. *Breast Cancer Res.* 2008;**10**:R67.
10. Nielsen TO, Hsu FD, Jensen K *et al.* Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin. Cancer Res.* 2004;**10**:5367-5374.
11. Paget J. On the disease of the mammary areola preceding cancer of the mammary gland. *St. Bartholomew Hosp. Rep.* 1874;**10**:87-89.
12. Stomper P, Penetrante R, Carson W. Sensitivity of mammography on patients with Paget's disease of the nipple. *Breast Dis.* 1995;**8**:173-178.



13. Kothari AS, Beechey-Newman N, Hamed H *et al*. Paget disease of the nipple: a multifocal manifestation of higher-risk disease. *Cancer* 2002;**95**:1-7.
14. Sheen-Chen SM, Chen HS, Chen WJ, Eng HL, Sheen CW, ChouFF. Paget disease of the breast – an easily overlooked disease? *J. Surg. Oncol.* 2001;**76**:261-265.
15. Sakorafas GH, Blanchard K, Sarr MG, Farley DR. Paget's disease of the breast. *Cancer Treat. Rev.* 2001;**27**:9-18.
16. Kollmorgen DR, Varanasi JS, Edge SB, Carson WE 3rd. Paget's disease of the breast: a 33-year experience. *J. Am. Coll. Surg.* 1998;**187**:171-177.
17. Dixon AR, Galea MH, Ellis IO, Elston CW, Blamey RW. Paget's disease of the nipple. *Br. J. Surg.* 1991;**78**:722-723.
18. Ashikari R, Park K, Huvos AG, Urban JA. Paget's disease of the breast. *Cancer* 1970;**2**:680-685.
19. Nance FC, DeLoach DH, Welsh RA, Becker WF. Paget's disease of the breast. *Ann. Surg.* 1970;**171**:864-872.
20. Chaudary MA, Millis RR, Lane EB, Miller NA. Paget's disease of the nipple: a ten year review including clinical, pathological and immunohistochemical findings. *Breast. Cancer. Res. Treat.* 1986;**8**:139-146.
21. Paone J, Baker R. Pathogenesis and treatment of Paget's disease of the breast. *Cancer* 1981;**48**:825-829.
22. Mai KT. Morphological evidence for field effect as mechanism of tumour spread in mammary Paget disease. *Histopathology* 1999;**35**:567-576.
23. Mai KT, Yazdi HM, Perkins DG. Mammary Paget's disease: evidence of diverse origin of the disease with subgroup of Paget's disease developing from the superficial portion of lactiferous duct and a discontinuous pattern of tumor spread. *Pathol. Int.* 1999;**49**:956-961.
24. Jamali FR, Ricci A Jr, Deckers PJ. Paget disease of the nipple-areola complex. *Surg. Clin. North. Am.* 1996;**76**:365-381.
25. Lloyd J, Flanagan AM. Mammary and extramammary Paget's disease. *J. Clin. Pathol.* 2000;**53**:742-749.

26. Lester T, Wang J, Bourne P, Yang Q, Fu L, Tang P. Different panels of markers should be used to predict mammary Paget's disease associated with in situ or invasive ductal carcinoma of the breast. *Ann. Clin. Lab. Sci.* 2009;**39**:17-24.

27. [Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. \*Histopathology\* 1991;\*\*19\*\*:403-410.](#)

28. [Morandi L, Pession A, Marucci GL et al. Intraepidermal cells of Paget's carcinoma of the breast can be genetically different from those of the underlying carcinoma. \*Hum. Pathol.\* 2003;\*\*34\*\*:1321-1330.](#)

**Deleted:** Morandi L, Pession A, Marucci GL et al. Intraepidermal cells of Paget's carcinoma of the breast can be genetically different from those of the underlying carcinoma. *Hum. Pathol.* 2003;**34**:1321-1330.

29. Brummer O, Steger HE, Böhmer G, Kühnle H, Perty HU. HER-2/neu expression in Paget disease of the vulve and the female breast. *Gynecol. Oncol.* 2004;**95**:336-340.

30. Anderson JM, Ariga R, Govil H et al. Assessment of HER-2/neu status by immunohistochemistry and fluorescence in situ hybridization in mammary Paget disease and underlying carcinoma. *Appl. Immunohistochem. Mol. Morphol.* 2003;**11**:120-124.

**Deleted:** Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991;**19**:403-410.

31. Schelfhout VRJ, Coene ED, Delaey B, Thys S, Page DL, De Potter CR. Pathogenesis of Paget's disease: epidermal heregulin- $\alpha$ , motility factor, and the HER receptor family. *J. Natl. Cancer. Inst.* 2000;**92**:622-628.

32. Fu W, Lobocki CA, Silberberg BK, Chelladurai M, Young SC. Molecular markers in Paget disease of the breast. *J. Surg. Oncol.* 2001;**77**:171-178.

33. Liegl B, Horn LC, Moinfar F. Androgen receptors are frequently expressed in mammary and extramammary Paget's disease. *Mod. Pathol.* 2005;**18**:1283-1288.

34. Ginestier C, Charafe-Jauffret E, Bertucci F et al. Distinct and complementary information provided by use of tissue and DNA microarrays in the study of breast tumor markers. *Am. J. Pathol.* 2002;**161**:1223-1233.

35. Bucher C, Torhorst J, Bubendorf L et al. Tissue microarrays ('tissue chips') for high-throughput cancer genetics: linking of molecular changes to clinical endpoints [abstract]. *Am J. Hum. Genet.* 1999; **65**(suppl.):43.

36. Gillett CE, Springall RJ, Barnes DM, Hanby AM. Multiple tissue core arrays in histopathology research: a validation study. *J. Pathol.* 2000;**192**:549-553.
37. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. *Lab. Invest.* 2000;**80**:1943-1949.
38. Zhang D, Salto-Tellez M, Putti TC, Do E, Koay ES. Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. *Mod. Pathol.* 2003;**16**:79-84.
39. Hoos A, Cordon-Cardo C. Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations. *Lab. Invest.* 2001;**81**:1331-1338.
40. Nocito A, Kononen J, Kallioniemi OP, Sauter G. Tissue microarrays (TMAs) for high-throughput molecular pathology research. *Int. J. Cancer* 2001;**94**:1-5.
41. Kallioniemi OP, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum. Mol. Genet.* 2001;**10**:657-662.

**Deleted:** 41. Rimm DL, Camp RL, Charette LA, Olsen DA, Provost E. Amplification of tissue by construction of tissue microarrays. *Exp. Mol. Pathol.* 2001;**70**:255-264.¶

42. Hoos A, Urist MJ, Stojadinovic A *et al.* Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. *Am. J. Pathol.* 2001;**158**:1245-1251. ¶

43. Torhorst J, Bucher C, Kononen J *et al.* Tissue microarrays for rapid linking of molecular changes to clinical endpoints. *Am. J. Pathol.* 2001;**159**:2249-2256.¶

44. Rubin MA, Dunn R, Strawderman M, Pienta KJ. Tissue microarray sampling strategy for prostate cancer biomarker analysis. *Am. J. Surg. Pathol.* 2002;**26**:312-319.¶

45. Rubin MA, Zhou M, Dhanasekaran SM *et al.* alpha-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. *J.A.M.A.* 2002;**287**:1662-1670.¶

**Deleted:** 6

**List of tables and figures**

Table 1. Antibodies used for immunoassays

Table 2. The results of the immunohistochemical analysis of Paget's disease and underlying *in situ* and invasive breast carcinomas

Figure 1. Overexpression of HER2 protein in Paget's cells in nipple epidermis. Magnification x200

Figure 2. Lack of expression of cytokeratin 5/6 in Paget's cells in nipple epidermis. Magnification x200

For Peer Review

Table 1. Antibodies used for immunoassays

<b>Antibody</b>	<b>Source</b>	<b>Dilution</b>	<b>Positive control</b>
Anti - estrogen receptor (mouse) clone 1D5	DakoCytomation (Denmark)	1:100	Estrogen receptor positive breast carcinoma
Anti – progesterone receptor (mouse) clone PgR 636	DakoCytomation, (Denmark)	1:100	Progesterone receptor positive breast carcinoma
Anti - epidermal growth factor receptor (mouse) clone EGFR.113	Novocastra (United Kingdom)	1:20	Skin
Cytokeratin 5/6 (mouse) clone D5/16B4	DakoCytomation (Denmark)	1:50	Skin
Cytokeratin 17 (mouse) clone E3	DakoCytomation (Denmark)	1:80	Skin
Human Epidermal Growth Factor Receptor 2 (rabbit) HercepTest	DakoCytomation (Denmark)	Solution prepared by manufacturer	Human epidermal growth factor receptor 2 - positive breast carcinoma

Deleted: studied

Table 2. The results of the immunohistochemical analysis of Paget's disease and underlying *in situ* and invasive breast carcinomas

	Number of cases with staining / number of <u>finally evaluated</u> cases (%)		
	Paget's cells in nipple epidermis	Underlying <i>in situ</i> breast carcinoma	Underlying invasive breast carcinoma
Estrogen receptor	6/43 (14%)	2/18 (11%)	2/14 (14%)
Progesterone receptor	3/44 (7%)	1/20 (5%)	1/13 (8%)
Human epidermal growth factor receptor 2	45/47 (96%)	18/20 (90%)	13/14 (93%)
Epidermal growth factor receptor	4/45 (9%)	3/18 (17%)	3/13 (23%)
Cytokeratin 5/6	1/46 (2%)	0/19 (0%)	0/12 (0%)
Cytokeratin 17	0/44 (0%)	0/19 (0%)	0/12 (0%)

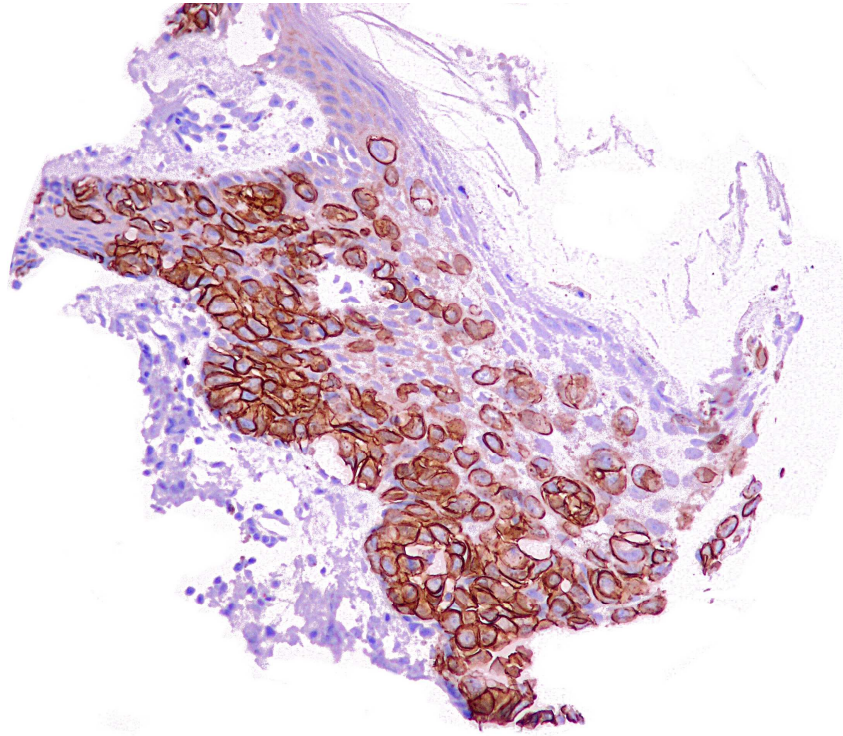


Figure 1. Overexpression of HER2 protein in Paget's cells in nipple epidermis. Magnification x200  
914x685mm (72 x 72 DPI)

Review

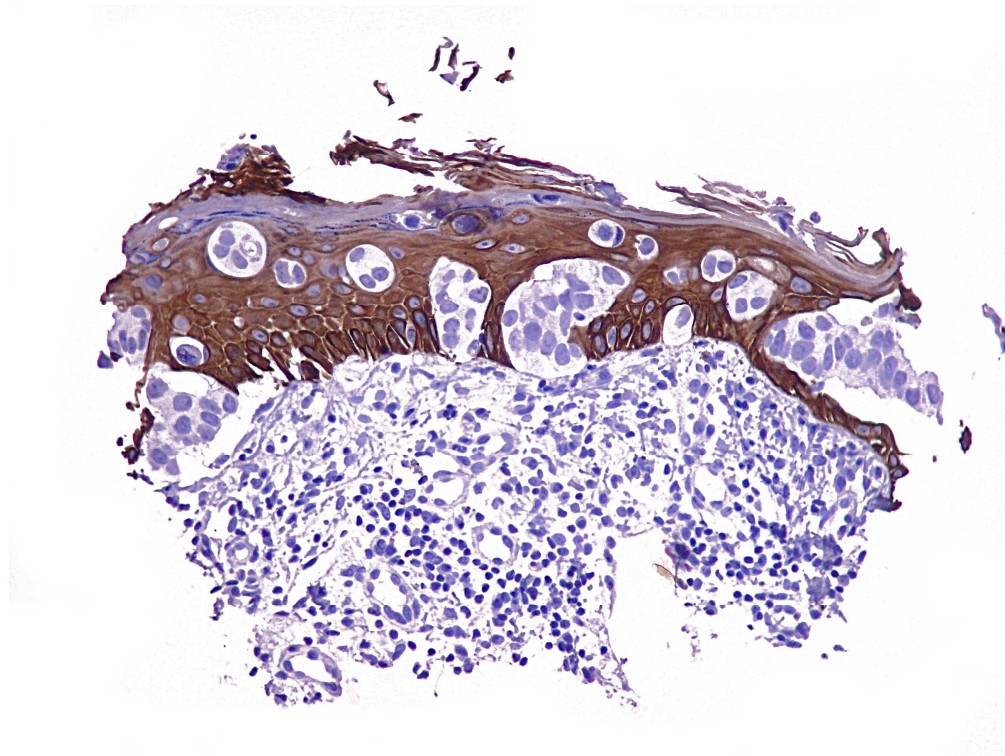


Figure 2. Lack of expression of cytokeratin 5/6 in Paget's cells in nipple epidermis. Magnification  
x200  
914x685mm (72 x 72 DPI)