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PRECLINICAL STUDY

Is there 'progression through grade' in ductal invasive breast cancer?

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Abstract Recent molecular data pointed towards the possibility of a stepwise dedifferentiation in a subgroup of invasive breast cancer (BC) cases. It was hypothesized that oestrogen receptor positive (ER+) grade 3 (G3) ductal invasive BCs are the end stage of a dedifferentiation process of luminal BC. A progression of luminal A towards luminal B BCs associated with a 'progression through grade' and an increased cell proliferation seemed the obvious explanation. In order to verify this hypothesis on a morphological and immunohistochemical level, we investigated 865 invasive BC cases. All cases were reviewed for the presence of intratumoural heterogeneity in grade of the invasive cancer and the presence of associated ductal carcinoma in situ (DCIS). With the use of tissue microarrays, the molecular subtype was determined and correlated with clinico-pathological features. In addition, all cases were stained for p21, p27, Ki-67, Cyclin D1, bcl-2, p53, and p16 and the results subjected to a biomathematical dependency analysis. The frequency of ER-positivity decreased with tumour size. The frequency of luminal A BC decreased as well, whereas the number of luminal B BCs remained constant. A gradual increase of the frequency of basal-like, HER2-driven and non-expressor BCs with tumour size was seen. In only 1 out of 865 BC cases, both a G1 and a G3 invasive cancer component was seen within the same BC. In two cases, a ductal invasive G1 carcinoma was associated with a poorly-differentiated DCIS. The frequency of columnar cell lesions was evenly distributed over ER+ and ER− ductal invasive G3 carcinomas. The biomathematical analysis gave striking hints against an obligate progression of BC trough grade. In conclusion, our results show that a morphological recognizable striking 'progression through grade' at least in its extreme form from G1 towards G3 is a very rare event in the natural course of invasive BC, including luminal BC.

Keywords Breast cancer · Breast cancer progression · Estrogen receptor

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Introduction

The progression of invasive breast cancer (BC) has a multitude of facets, all associated with important clinical aspects. This includes especially progression from a pre-invasive state towards potentially life-threatening disease due to local spread and formation of distant metastases. From a pathomorphological and genetic point of view, the progression of BC has been defined for many decades by different morphologically recognizable stages associated with a stepwise acquisition of genetic alterations, often associated with a gain of tumour mass/size. Since the differentiation of invasive BC has mostly been defined by the

tumour grades (G1–3) [1], a progression through grade was commonly accepted [2]. However, this hypothesis could not be sustained with the description of a multitude of parallel, 'low-grade' and 'high-grade' pathways in breast carcinogenesis [3–6]. Whereas the latter is characterised by a variety of distinct subtypes, including HER2+ and basal-like BCs, the most outstanding molecular hallmarks of the low-grade cancers are expression of the estrogen receptor (ER) and loss of the long arm of chromosome 16 (16q) [7]. Noteworthy, a significant subset of poorly-differentiated ductal invasive BCs display ER-positivity and show the loss of 16q, making these tumours strong candidates for a subtype of ductal invasive G3 carcinomas originating from well-differentiated BCs [8, 9]. However, up-to-date no hypothesis has been proposed when this putative 'progression through grade,' if present, in fact occurs. A variety of studies within the last years have also shown that the biology of BC may be influenced or even determined by the cell of origin, pointing towards important steps in breast carcinogenesis, taking place far away from the invasive tumour state [10]. Transferring this knowledge to the morphological level requires focus on preinvasive tumour lesions, such as ductal carcinoma in situ (DCIS) or other suspected precursor lesions of BC.

In order to clarify at least some of these open questions, we investigated a series of 865 primary, ductal invasive BC cases, including 274 G3 carcinomas, by morphological, immunohistochemical, and biomathematical means. Our results strengthen the idea that 'progression through grade' in invasive BC is a very rare event. However, if this progression might underlie BC, this step rather takes place on the DCIS level.

Materials and methods

Patient material

A total of 865 invasive BC cases were retrieved from the archives of the Institute of Pathology, Husener Strasse in Paderborn. The clinico-pathological data were collected from the pathology and hospital information systems. All cases were diagnosed and treated in the years 1997–2003. All cases were primary BCs, comprising almost all subtypes of invasive BC. The average age of patients with primary BC was 61.1 years (22–91 years). In 713 cases, the diagnosis of a ductal invasive BC was made. All invasive carcinomas were graded according to Elston and Ellis [1]. Four hundred and eighty one patients were lymph node negative (N0). Further details are listed in Table 1.

In accordance to the work-up strategies for BC specimens of the Institute of Pathology, Paderborn, for all tumours at least three representative blocks from a tumour were taken and evaluated. In detail, all tumours with a diameter with 15 mm and less were completely embedded. However, for the actual review of the tumour blocks for the presence of cylinder cell lesions only 1–2 tumour blocks were available.

DCIS classification was done using the recommendations of the WHO classification [11].

New haematoxylin and eosin stained slides were prepared. All cases of ductal invasive G3 carcinomas were reviewed for the presence of synchronous columnar cell lesions [12].

A tissue array containing all 865 BC cases was constructed according to standard protocols using a dedicated TMA instrument (Beecher Instruments, Silver Spring, Maryland, USA) as previously described [13]. Two cores of 0.6 mm in diameter were punched out of the donor block and placed at a distance of 0.2 mm in the recipient block.

Immunohistochemistry

Staining procedures were done according to standard protocols on the above mentioned tissue arrays (4 μm sections). Immunohistochemistry was performed for ER, progesterone receptor (PR), the epidermal growth factor receptor (EGFR), HER2, cytokeratin 5/14 (CK 14), cytokeratin 5/6 (CK 5/6), cytokeratin 17 (CK 17), p21, p27, p53, p16, Cyclin D1, bcl-2, and Ki-67 (Table 1). The pretreatment conditions, the source, and the dilution of the commercially available primary antibodies are shown in Table 2. Immunohistochemistry was performed using an autostainer (Dako AutostainerPlus) and a detection kit (DakoReal^{TM} Detection Kit; Peroxidase/AEC). EGFR staining was done manually.

According to the literature and our own experience, the scoring of the IHC results was done in a semi-quantitative and with the exception of HER2 (Dako score). Expression levels were divided into three groups: no reactivity (–), faint expression in less than 10 % of the cells (±), moderate expression in 10–50 % of the cells (+) and strong expression in more than 50 % of the cells (++).

The determination of molecular subtypes was done using two different approaches, since no real consensus exists. First, molecular subtypes were determined as previously described [14]. In a second approach BCs were classified as luminal A (ER+ and/or PR+, low proliferation, HER2−), luminal B (ER+ and/or PR+, high proliferation and/or HER2+), HER2-driven (HER2+, ER− and PR−), basal-like (ER−, PR−, HER2−, positive for high molecular weight cytokeratins, such as CK 5/6, CK 5/14, CK 17, or EGFR), or non-expressor/triple-negative/unclassifiable (negative for all markers).

Table 1 Overview about the clinico-pathological details of 865 primary invasive BC cases

Histological type	*n*	Tumour grade	*n*	N-category	*n*	T-category	*n*	Molecular subtype	*n*
Ductal	713	G1	131	N0	481	T1a	5	Luminal A	498
Lobular	95								
Mucinous	22	G2	442	N1	218	T1b	49	Luminal B	114
Tubular	15								
Medullary	6	G3	292	N2	77	T1c	359	HER2	49
Tubulo-lobular	3								
Cribriform	3			N3	89	T2	362	Basal	57
Metaplastic	2								
Papillary	1					T3	35	Non-expressor	65
Micropapillary	1								
Apocrine	1					T4	65		
Adenoid-cystic	1								
Tubular-mixed	1								
Signet-ring cell	1								

Table 2 List of the used antibodies including the respective clone, the pre-treatment conditions and the respective dilutions

Antibody	Source	Clone	Dilution	Antigen retrieval
ER	Novocastra	6F11/2	1:800	EDTA pH 8
PR	Dako	PgR636	1:200	EDTA pH 8
HER2	Dako	-Kit-		Citrate buffer pH 6
Ki-67	Dako	Mib-1	1:100	EDTA pH 8
CK 5/14	DCS	XM26HL002	1:50	Citrate buffer pH 6
CK 5/6	Dako	D15/16B4	1:50	EDTA pH 8
CK 17	Dako	E3	1:50	Citrate buffer pH 6
p21	Calbiochem	EA10	1:500	Citrate buffer pH 6
p27	Pharmingen		1:1000	Citrate buffer pH 6
p53	Dako	Do-7	1:100	EDTA pH 8
bcl-2	Dako	124	1:100	Citrate buffer pH 6
Cyclin D1	Novocastra	NCL-L-Cyclin D1-GM	1:20	EDTA pH 8
EGFR	Dako	Pharm DX-Kit		/
p16	mtm	Kit		Citrate buffer pH 6

In an alternative approach, all BC cases were classified as HER2+ with a Dako score 3+, irrespectively of the expression of other markers. Invasive BCs were classified as basal, if at least one proposed marker for 'basalness' (CK 5/6, CK 5/14, CK 17, or EGFR) was expressed in at least 1 % of the cells, irrespectively of the ER, PR and HER2-status. BCs were regarded as ER+ if at least 1 % of all BC cells revealed expression of ER.

Biomathematical analysis of immunohistochemical data

The pseudo categorical nature of the initial raw data reflects the limitations of the observer and established procedures. Counting cells here is a surrogate marker for a ratio scale phenomenon. Expression in its nature has a ratio scale and so we proceed this way accepting that we might introduce some bias using this coarsely granular data. The collected protein expression data of all cases and molecular factors were checked for missing data (6.8 %). The number of the missing values was low and did not hamper further analysis. The distribution of the missing values in the data collection followed a random scheme. Due to the nature of the downstream analysis, missing values were replaced by their factor specific median values. In the resulting data matrix columns represent molecular factors while rows denote samples. The measurements of the matrix are the protein expression signals.

In the case of the defined artificial class factors which are used in Fig. 3a (luminal A, luminal B, basal etc.) several molecular factors were joint together. This was done by superimposing more than one factor by applying certain threshold rules. The resulting artificial class factors were introduced to simplify complex relations and to focus

on certain observations/classifications discussed in related publications (rules see third paragraph of the previous section).

Determining the optimal factor order defining the protein dependency pattern

To figure out which molecular factors might act synergistic, antagonistic, or indifferent concerning a defined set of molecular reference factors or set of class factors we applied a combinatorial procedure which in our case exhaustively analyse the given dataset to find the optimal dependency structure. Optimal in our case means that by the given measurements, we find the best dependency structure covered by the data. The interpretation of this solution is performed by analysing the resulting set of regression graphs of one analysis approach.

In detail two groups of factors will be selected from the complete dataset comprising all factors. The first group the so-called reference set is holding well known molecular factors defining physiological trigger or marker of the analysed tumour cells. The second group comprise factors (the so-called test set) which should define the expression environment in accordance to the reference factors. Reference and test group will show no overlap except in one case. This test set describes with its resulting order the physiological differences between each of the included reference situations. Three basic situations can be distinguished. No differential involvement between one reference and all test factors (denoted by a horizontal regression line or at least by those test factors with a correlation coefficient close to zero). Synergistic respectively antagonistic behaviour of the test factors to one reference factor denoted by a regression line with some significant slope. Test factors below zero denote antagonistic and above zero synergistic behaviour. Test factors at zero as mentioned above show no differential involvement in the defined physiological situation. The result is specific for the selected set of reference situations. Other reference situations might show a different physiological 'working point'. The qualitative result can directly be compared with observations described in the literature.

Technically, the enumeration of permutations of all test factors will be analysed concerning a minimal global sum of squares of all linear regression of the selected reference situations. This minimum defines the physiological 'working point' in the given constellation of factors i.e. the dependency pattern between the included factors. The permutation procedure is based on a cross-tabulation of all Pearson correlation values between the reference and the test group. The linear regression with the minimal sum of squares value in all permutations is shown in Fig. 3. All reference situations together are forming one panel and one set of results showing differences or similarities between the reference factors. The result is giving an estimate how closely related the reference factors act in the physiological situation of interest—in our case invasive BC.

Results

The clinico-pathological data and the results of the immunohistochemical stainings are listed in Tables 1 and 3. The frequencies of molecular subtypes are shown in Table 1. A decreasing incidence of luminal A BCs was seen with increasing tumour diameter. Frequency of luminal B BCs was not associated with tumour size (Fig. 1a). In contrast, HER2, basal-like, non-expressor/triple-negative cancers were seen in increasing frequencies with larger tumour diameter. Similar results could be demonstrated for ER+, HER2+, and basal-like BCs according to the alternative approach (Fig. 1b).

The review of all cases for the presence of an intratumoural heterogeneity revealed that only in one case a ductal invasive G1 carcinoma could be detected in combination with a ductal invasive G3 carcinoma. In this case, all three parameters for the definition of tumour grade (number of mitotic figures, nuclear atypia and tubule formation) differed significantly (Fig. 2c–e). In one ductal invasive G2 case, two morphologically different components, not differing in tumour grade, were detectable (Fig. 2a, b).

In 58 % of all invasive BC cases, an associated DCIS could be detected. The exact frequencies in relationship to the different BC subtypes and differentiation grade are shown in Table 4. In two cases, a ductal invasive G1 carcinoma was associated with a G3 DCIS. One case of a luminal B BC, a G1 cancer (ER+/Ki-67 high) was associated with a G1 DCIS (HER2−, Ki-67 low).

No G3 luminal B BC and no G3 ductal invasive carcinoma was associated with a G1 DCIS. Only small subgroups of these cancers (4 % and less than 1 % in G3 luminal B cancers and G3 ductal invasive cancers, respectively) were associated with DCIS with an intermediated differentiation grade. Differences with regard to DCIS grade between ER+ and ER− ductal invasive cancer could therefore not be detected. Also no difference was seen in ER+ and ER− ductal invasive BCs with regard to the presence of G2 DCIS.

No difference existed in the frequency of columnar cell lesions between ER+ and ER− ductal invasive G3 cancers.

The biomathematical analysis was done using 3 different approaches. In a first step, the expression of the seven candidate genes, involved in cell cycle regulation and tumour proliferation was correlated with the molecular subtype. The analysis revealed three different patterns for

Table 3 Results of the immunohistochemical staining

	0	1	2	3
CK 5/6	753/803 (93 %)	40/803 (5 %)	5/803 (<1 %)	3/803 (<1 %)
CK 5/14	698/756 (93 %)	34/756 (4 %)	12/756 (2 %)	10/756 (1 %)
CK 17	777/812 (96 %)	28/803 (3 %)	5/803 (<1 %)	1/803 (<1 %)
HER2	384/768 (50 %)	140/768 (18 %)	145/768 (19 %)	96/768 (12 %)
ER	207/781 (27 %)	114/781 (15 %)	152/781 (19 %)	306/781 (39 %)
PR	324/799 (40 %)	146/799 (18 %)	142/799 (18 %)	185/799 (24 %)
EGFR	762/803 (95 %)	20/803 (3 %)	10/803 (1 %)	9/803 (1 %)
Mib-1	371/782 (47 %)	275/782 (35 %)	110/782 (15 %)	24/782 (3 %)
p21	279/749 (37 %)	295/749 (39 %)	120/749 (16 %)	53/749 (8 %)
p27	109/758 (14 %)	154/758 (20 %)	270/758 (36 %)	223/758 (30 %)
p53	600/780 (77 %)	76/780 (10 %)	58/780 (7 %)	44/780 (6 %)
bcl-2	200/770 (26 %)	232/770 (30 %)	269/770 (35 %)	67/770 (9 %)
Cyclin D1	457/762 (60 %)	228/762 (30 %)	62/762 (8 %)	13/762 (2 %)
p16	677/781 (87 %)	80/781 (10 %)	19/781 (2 %)	3/781 (<1 %)

The absolute numbers and the respective percentages are given

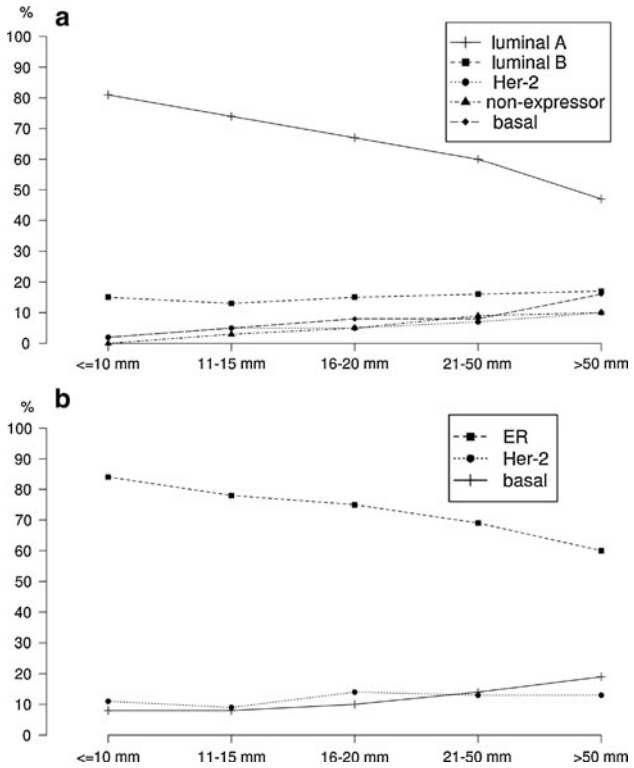


Fig. 1 a) *Diagram* showing the frequencies of distinct molecular subtypes in invasive BC in correlation to tumour diameter. A decrease in the frequency of luminal A BCs is seen. The frequency of luminal B BCs remained constant. In contrast, increasing frequencies for HER2-driven, basal and non-expressor BC cases were seen. **b** Diagram showing the frequencies of ER+, basal, and HER2+ BC cases. The frequency of ER-positivity decreased with increasing tumour diameter. The rate of HER2− BC cases was independent of tumour size, whereas the frequency of basal carcinomas increased

luminal A, luminal B, and HER2/basal/non-expressor BC cases, respectively (Fig. 3a). The molecular subtypes were significantly associated with tumour grade.

In a second step the expression of the candidate genes was correlated with tumour grade irrespectively of the ER-status. The results revealed almost identical expression patterns for G1 and G2 ductal invasive BCs in contrast to ductal invasive 3 ductal invasive carcinomas (Fig. 3b).

In a final step, the analysis was done investigating ductal invasive carcinomas with a positive ER-status, only. Again, identical expression patterns for the seven candidate genes were seen in ER+ ductal invasive G1 and G2 carcinomas in contrast to ER+ ductal invasive G3 carcinomas. Noteworthy, the expression patterns of ER+, ductal invasive G3 carcinomas were identical to luminal B BCs (Fig. 3c).

Discussion

Based on the distribution of chromosomal 16q-losses in in situ and invasive BC, morphological, immunohistochemical and cell-biological findings [3], it became evident that the evolution of BC follows multiple, parallel pathways. A 'progression through grade' in in situ and invasive BC in general seemed rather unlikely [7, 15]. However, a more detailed look into distinct subtypes of invasive BC [16] and observations from mammography detected BC cases gave hints that modifications within the concept of merely parallel 'low' and 'high-grade' pathways might be necessary [17]. In detail, conventional and array-CGH studies revealed a high frequency of 16q-losses in ER+, ductal invasive G3 carcinomas [9, 18]. Based on these genetic alteration patterns, it seemed logical to propose that these tumours originate from low-grade BCs [18] or, in translation to other nomenclature, a regular progression from invasive luminal A towards invasive luminal B BCs with an increase of tumour proliferation seemed obvious [8]. However, since this hypothesis is merely based on the

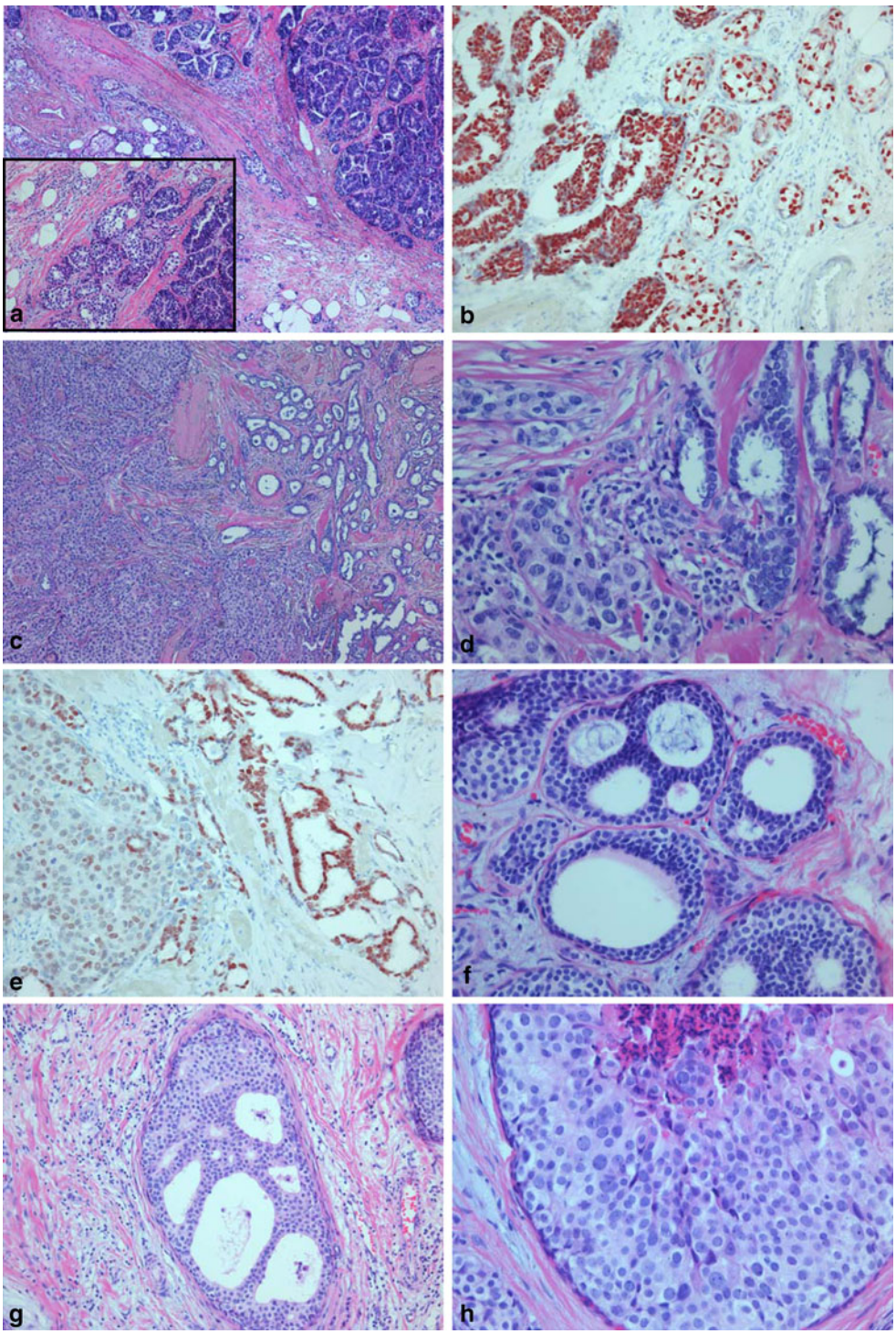


Fig. 2 **a**, **b** Example of a ductal invasive G2 carcinoma with a striking change in tumour cell morphology. Tumour cells with hyperchromatic nuclei and a slightly eosinophilic cytoplasm are mingled with tumour cells showing a clear cytoplasm. Both tumour cell components expressed ER. **c**–**e** Example of a ductal invasive BC case with a striking intratumoural heterogeneity. Invasive tumour components with features of ductal invasive G1 and G3 BCs are present and seem to intermingle. Obvious differences in the size of tumour cell nuclei, tubular formation and tumour cell proliferation are present. No difference in the expression of ER existed. **f**–**h** Case of a ductal invasive G2 carcinoma with associated DCIS. The DCIS component revealed a well and an intermediately differentiated DCIS and displayed in some tumour cells also features of a poorly-differentiated DCIS

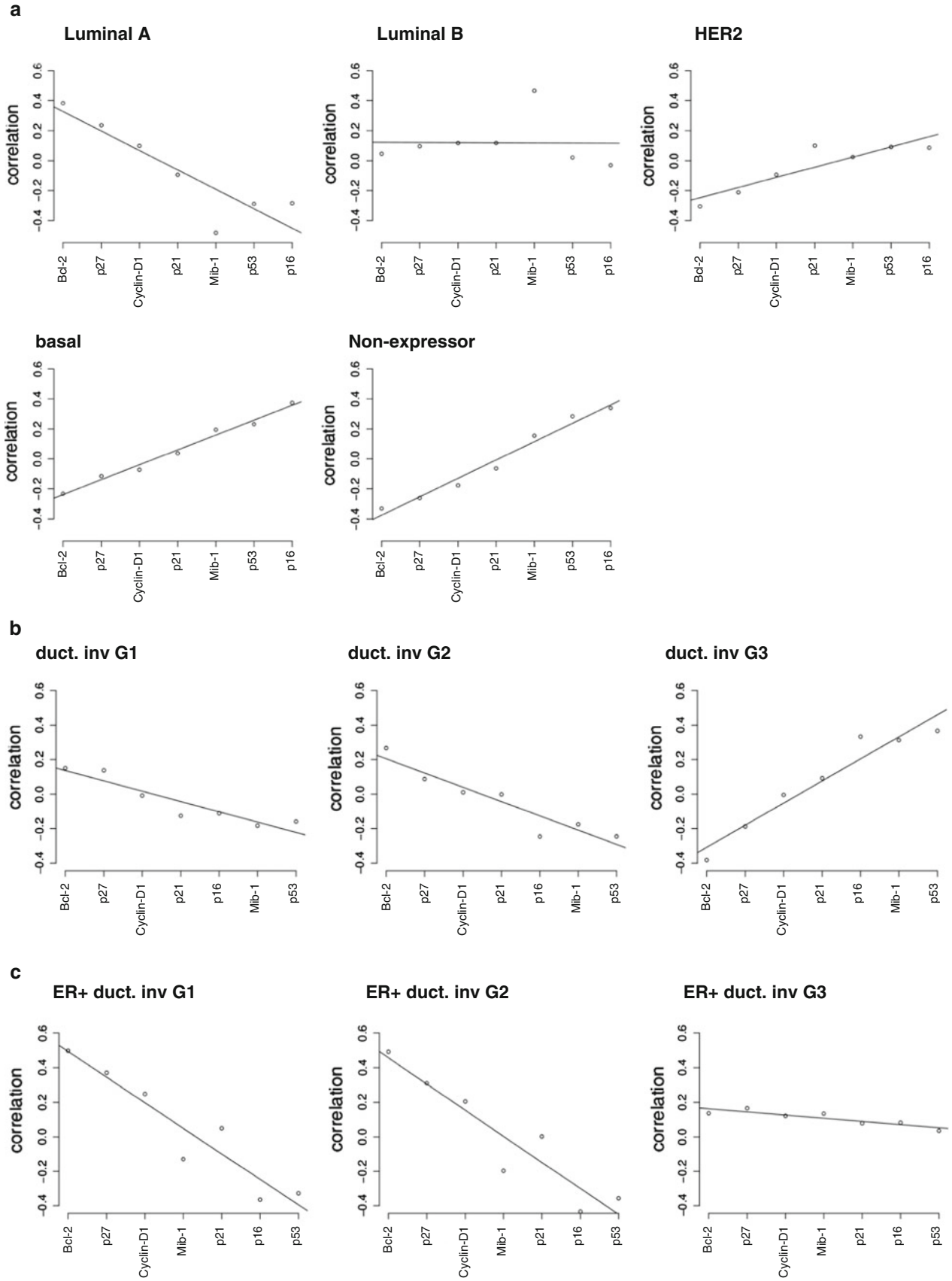
Table 4 The frequencies of tumour grade in DCIS in correlation to the respective invasive BC subtypes are shown

	DCIS		
	G1	G2	G3
All invasive	60/865 (7 %)	223/865 (26 %)	225/865 (26 %)
Invasive G1	49/131 (37 %)	46/131 (35 %)	2/131 (1.5 %)
Invasive G2	11/442 (2 %)	160/442 (36 %)	69/442 (16 %)
Invasive G3	0/292 (0 %)	7/292 (2 %)	154/292 (53 %)
Luminal A	52/498 (10 %)	163/498 (32 %)	95/498 (19 %)
Luminal B	1/114 (<1 %)	22/114 (19 %)	51/114 (44 %)
HER2+/proliferation low	1/36 (3 %)	6/36 (17 %)	17/36 (47 %)
HER2−/proliferation high	0/65 (0 %)	15/65 (23 %)	24/65 (37 %)
HER2+/proliferation high	0/10 (0 %)	1/10 (10 %)	8/10 (80 %)
Luminal B G1	1/4 (25 %)	0/4 (0 %)	1/4 (25 %)
Luminal B G2	0/60 (0 %)	20/60 (33 %)	18/60 (30 %)
Luminal B G3	0/47 (0 %)	2/47 (4 %)	30/47 (63 %)
Ductal invasive G3			
Ductal invasive G3 ER+	1/118 (<1 %)	2/118 (1 %)	81/118 (68 %)
Ductal invasive G3 ER−	0/136 (0 %)	1/136 (<1 %)	63/136 (46 %)
HER2	0/49 (0 %)	3/49 (6 %)	34/49 (69 %)
Basal	0/57 (0 %)	1/57 (2 %)	12/57 (22 %)
Non-expressor	2/79 (3 %)	9/79 (11 %)	22/79 (28 %)

synchronous finding of one distinct genetic alteration, more hints towards a progression of luminal A/ductal invasive G1/G2 carcinomas towards luminal B/ductal invasive G3 BCs should be found.

Our actual data do not support the idea of an obligate 'progression through grade' in invasive BC in its extreme form from G1 towards G3 invasive BC. Interestingly, the frequency of invasive luminal A BCs decreased with tumour size, the frequency of luminal B BCs remained constant, whereas the frequency of the other molecular subtypes increased with tumour size. Under the assumption that tumour progression is among other parameters associated with gain of tumour size and consequently by a change of tumour grade, reflected in distinct subtypes—such as luminal B, HER2, basal or triple-negative BCs—a progression of luminal A carcinomas towards these above-mentioned breast carcinomas would be logical. However, the striking differences between the genetic profiles of luminal A BCs and HER2-driven, basal BCs [3] and non-expressor BCs (personal data, not shown here) make such a relationship unlikely. Luminal B BCs represent a group of heterogeneous ER+ BCs with high proliferation and/or HER2-positivity, depending on the classification system. It has been recently shown that in the group of HER2+ BCs, ER+, and ER− tumours did not differ much, especially the low frequency of 16q-losses was similar in HER2+/ER+ and HER2+/ER− carcinomas [19]. In analogy to the above-mentioned subtypes, HER2-driven invasive luminal B BCs are also improbable candidates in originating from invasive luminal A breast carcinomas. Therefore, only a transition from invasive luminal A towards invasive luminal B/HER2−/Ki-67 high BCs would be supported by the recently available genetic data. However, the transfer of these data towards absolute numbers illustrates that a linear progression between luminals A and B invasive BCs is a rather rare event. Taking into account that tumour morphology reflects underlying genetic alterations, one should await at least in a significant percentage of ER+ G3 carcinomas and/or luminal B invasive BC, morphological hints towards their origin. However, our data show that the only in one case (<1 % of all cases) an invasive G3 and G1 component within the same tumour could be observed. Interestingly, this case revealed a maximum tumour diameter of 30 mm and exemplifies that an extreme tumour heterogeneity can be maintained during tumour progression, defined by the gain of tumour size. The rate of intratumoural heterogeneity might definitely be increased using lower thresholds or focussing on single parameters of tumour differentiation such as nuclear pleomorphism. Therefore, it has to be stated that the chosen morphological approach does not clarify a possible progression from G1/G2 or G2/G3 cancer unless associated with a change of histological type. Therefore, in order to circumvent the problems raised by different thresholds for the definition of morphological tumour heterogeneity, two further approaches were applied.

Even though not impressing in absolute numbers, the combination of an in situ and invasive component within the same patient, widely differing in their respective


a
Luminal A
Luminal B
HER2
basal
Non-expressor
b
duct. inv G1
duct. inv G2
duct. inv G3
c
ER+ duct. inv G1
ER+ duct. inv G2
ER+ duct. inv G3
correlation
Bcl-2
p27
Cyclin-D1
p21
Mib-1
p53
p16

◀**Fig. 3** The regression graphs show the synchronicity of protein expression according to the depicted breast tumour entities. Differences in the *regression lines* indicate different expression patterns behind this entity. Three basic situations were examined. Overall, the molecular subtypes were significantly associated with tumour grade. **a** The luminal A group, the luminal B group, the HER2-group, the basal group and the 'non-expressor' class were analysed in reference to one apoptosis and several cell cycle specific markers. Mib-1/Ki-67 was included as a positive control to test for the validity of the observations depicted by the *regression line*. Mib-1 is here non-informative because of its usage in the reference definition. It can be seen that luminals A and B are quite different between themselves, but also to the HER2, 'basal' and 'non-expressor' classes (indicated by the *slope* and the identical order of the factors on the *x* scale). **b** Here three groups were defined. Ductal invasive BC with G1–3, irrespective of the ER-status were analysed in reference to the same apoptosis and cell cycle specific markers. A striking difference between G1 and G2 on one side and G3, ductal invasive BCs on the other side could be observed. **c** The three classes from **b** were taken for analysis but only the ER+ ductal invasive BC cases were included. The response of the apoptosis and several cell cycle specific markers to the classes gets even stronger in the case of G1/2 but disappears in the case of G3. As can be seen in the slightly changed order on the *x* axis the Mib-1/Ki-67 expression lost its impact on this pattern

histological grade, could be more frequently observed, but this did not account for ER+ or ER− ductal invasive G3 carcinomas. Sixteen out of 24, ER+, ductal invasive G3 carcinomas with a maximum tumour diameter of 15 mm and less were associated with a DCIS. In only one case, the adjacent DCIS was of intermediate nuclear grade; the other cases were poorly-differentiated DCIS. It is also important to note that the frequency of columnar cell lesions with and without atypia in this series was also evenly distributed in ER+ and ER− ductal invasive BC cases as described before [20]. Since these lesions have been proposed as putative precursor lesions for carcinomas of the low-grade pathway [21, 22], an evolutionary progression line between ER+, G3, ductal invasive breast carcinomas, and this pathway could also not be drawn on this basis. Limiting this statement, however, is the fact that for most of the tumours only one or two blocks were available for review, and the definite role of these lesions as true precursor lesions is still under debate [23, 24].

We therefore rather conclude that ductal invasive, ER+ G3 carcinomas are a distinct subgroup in the heterogeneous melting pot of poorly-differentiated invasive BC. This hypothesis gets its strongest support from the biomathematical approach with clearly differing expression patterns in luminals A and B, as well as ductal invasive BC cases irrespective of the ER-status. Under the assumption that a progression through grade is a general finding and is also reflected by changes in tumour proliferation, one should await a gradual change in proliferation patterns from G1 towards G2 towards ductal invasive G3 carcinomas. Noteworthy, in contrast to this hypothesis, G1 and G2 ductal invasive carcinomas displayed almost identical expression patterns in contrast to ductal invasive G3 carcinomas. Similar results were observed for ER+ ductal invasive carcinomas with the respective tumour grades. Again G1 and G2 ER+ ductal invasive BCs revealed completely different expression patterns compared to G3 ER+ ductal invasive BCs. The idea of a stepwise dedifferentiation is definitely not supported by these observations. However, if any kind of 'progression through grade' should underlie the pathogenesis of these tumours, we have up-to-date no evidence that this progression predominantly takes place at the level of invasive BC. We rather conclude that the explanation of these findings has to be searched on the level of suspected precursor lesions. Molecular studies revealed that DCIS on a cytogenetic level has identical alteration patterns as invasive BC [25]. A multitude of studies on basal BCs and other BC subtypes revealed that the respective DCIS precursors have similar protein expression patterns [25–28]. However, the heterogeneity of DCIS seems to be higher compared to invasive BCs since striking differences in the molecular subtypes between DCIS and invasive BC have been demonstrated repeatedly [29–31]. Therefore, it seems logical to propose that a pronounced, intratumoural heterogeneity on the DCIS level as shown in Fig. 2f–h might serve as a basis to explain the above-mentioned findings. Even though speculative at the present state, multiple genetic, independent or related subclones might exist in DCIS [32] or other suspected precursor lesions—one of them finally becoming invasive and determining the further fate. The postulation of a 'genetic field' might be in this regard a valuable hypothetical construct in order to explain this pronounced heterogeneity in preinvasive breast tumours [33].

In summary, our results show that the concept of 'progression through grade' in invasive BC is very rare and not as straightforward as recently proposed. The mere concentration on genetic data in order to reveal progression pathways, without inclusion of morphological and immunohistochemical observations obviously runs the risk of a misleading dead end.

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Conflict of interest The authors declare no conflict of interest.

Ethical standards Data were analysed anonymously. Nonetheless, we have performed the study according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the Aerztekammer Westfalen-Lippe, Muenster, Germany. We have acquired tissue samples only with the informed consent of the patients or patients' next of kin with the understanding by all parties that it may well be used for research.

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