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Review Article

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Cytological Differentiation of Ductal Carcinoma *in Situ* and Invasive Ductal Carcinoma of the Breast: A Personal View and a Literature Review

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Abstract

Although some find it controversial, it is possible to differentiate breast ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) using cytology only, with certain limitations. Invasiveness is the consequence of specific biological, i.e. aggressiveness potential of malignant cells, which is different with respect to the pre-existent DCIS, consequentially with different morphology. During the invasion, malignant cells go through multiple morphological changes, losing their epithelial and acquiring mesenchymal features in the fantastic process of epithelial-mesenchymal transition, which explains their morphology in cohabitation with the environment, includes the disruption of intercellular junctions, the increase of mobility and the release of the original epithelium. This mesenchymal-like phenotype supports the migration and invasion of cells, i.e. thus epithelial-mesenchymal transition ensures the tumor dissemination and metastasizing. Therefore, invasiveness can cytologically be "measured" by detecting morphological signs of increase of biological aggressiveness of malignant cells – through the change of their appearance (cytoplasm elongation in malignant squamous cells, i.e. in adenocarcinoma intracytoplasmic lumina, atypical nucleoli, coarsely clumped chromatin, eu-/parachromatin), but also with stromal parameters (disruption of the intercellular matrix, elastin fragments, capillaries endothelium) presented by tumour diathesis, fibroblast proliferation, fragments of elastoid stroma, invasion of connective and/or adipose tissue by groups and individual malignant cells. For the invasion are also very predictive tubular malignant structures, irregular angulated clusters of reduced cohesiveness, absence of benign naked nuclei, polymorph single tumour cells, less myoepithelial cells on tumour groups, fewer microcalcifications and foamy macrophages. Opposite morphological findings suggest DCIS. Even though cytologically we do not see and cannot see the basement membrane, highly likely we can predict the invasion – necessarily and always with the triple-diagnostic approach or clinical-radiological-morphological correlation to every breast lesion, in the representative well cellular sample and with good knowledge of patohistology and cytology.

Keywords: Breast, Ductal Carcinoma In Situ, Invasive Ductal Carcinoma, Cytological Diagnosis, Cytological Differentiation

Introduction

It has been shown by several authors that it is possible to differentiate cytologically and with very high diagnostic reliability ductal carcinoma *in situ* (DCIS) from invasive ductal carcinoma (IDC), using well known, multiple times described and clearly defined cytological criteria known for years and, of course, respecting certain rules. Both - the cytological differentiation of DCIS from IDC and the cytological diagnosis of DCIS itself are still the subject of heated debates and conflicting views geographically and in some scientific circles. I would like to point out that, unfortunately, this issue is completely unknown in Croatia as well, so it is neither discussed nor written about. According to personal point of view and experience, breast diagnostics is somewhat neglected, what - at least in part, contributes to a still very high morbidity and mortality rate of breast cancer, together with the uncoordination or poor organization of actors of all relevant diagnostic factors [1, 2]. It is never enough or too much to emphasize the value of the so-called triple diagnostic approach or triple diagnostic evaluation to each, but in particular to the doubtful breast lesion. More, especially Scandinavian authors, who particularly stand out in this field, repeatedly insist on its importance in all the papers on this subject [3-9]. Such comprehensive and systematic clinical-radiological-morphological correlation minimizes the possibility of a diagnostic error, implying that all three links in this

"chain" are very familiar with their laws, capabilities, limitations, interdependence, the necessity of team collaboration and that this issue is dealt with on a daily basis and within specially organized units, for example as "Breast Diagnostic Center", "Breast Unit", "One Day Clinic", or similar. At the same time, the findings of all three diagnostic methods - clinical presentation, imaging (mammography/MMG and ultrasound/US) and cytology - must be in agreement, i.e. they must match perfectly well and without ambiguities. In other words, they must be quite clear in favor of benignity or, on the other hand, of the malignancy of the lesion in order to define it with a very high probability. However, if they are not correlated or if any of them is doubtful, unclear or suspicious we must repeat one of the methods or more often complete the treatment with further diagnostics - primarily tissue biopsy.

The Biological Basis of Invasion and Epithelial-Mesenchymal Transition

In support of the whole text and to some still controversial view that using only cytology (with some limitations) DCIS of the breast can be diagnosed and differentiated from the invasive carcinoma, I emphasize that first cervical squamous carcinoma *in situ* (CIS) and then adenocarcinoma *in situ* have been described, isolated and accepted as separate cytomorphological entities long ago [10, 11]. Because the morphological criteria are a direct reflection of the tumor biology, i.e. since the biological properties of each tumor determine its histological, cytological and radiological image - cytology of benign, malignant and *in situ* tumors directly mirrors their histology and implies logical reliable criteria. Histological finding of malignant epithelial cells beneath the basement membrane in the underlying lamina propria as clear evidence of carcinoma invasiveness into other tissue type and as a necessary precondition for distant dissemination if they invade the blood/lymphatic capillaries endothelium or perineurally, has somewhat of its counterpart in cytology (which is going to be discussed later) - again as a consequence of the biologically different potential of the malignant cells at a certain stage of tumor growth and progression, including the different potential of their aggressiveness, which therefore directly determines their different morphological appearance in each of these stages. It has been known for some time that when invading, malignant cells repeatedly and multiple times change in shape and appearance, that is morphologically; at initial invasion of the basement membrane, laminae propriae and connective tissue lose their epithelial and acquire mesenchymal features in an extremely interesting and fascinating process known as epithelial-to-mesenchymal transition (EMT), which especially contributes to the understanding of tumor cell cytomorphology and their environment [12, 13]. Tumor growth itself is also evidence that changes in its architecture occur through this specific tissue adaptation [12]. EMT also occurs in physiological processes of growth and development when epithelial cells exhibit reduced adhesiveness but increased motility, and acquire mesenchymal, fibroblast-like features [13]. The separation of intercellular junctions and the increase of cell motility are required to release the cells from the original epithelium. Such a mesenchymal-like phenotype also supports migration, invasion, dissemination and metastasis of malignant cells, apropos in this way EMT - as a critical factor in malignant transformation and progression - provides the initial tumor with invasive and metastatic features [12, 13]. Therefore, invasiveness can be cytologically "measured" by detecting morphological signs indicating an increase in the biological aggressiveness of tumor cells upon invasion, through alteration of the shape and appearance of their cytoplasms and nuclei - for example, as elongation of dense keratinized cytoplasms at the initial invasion of malignant squamous cells, or in already invading adenocarcinoma cells as so-called intracytoplasmic lumina or vacuoles (previously considered typical of invasive lobular carcinoma – ILC but also found in IDC), more denser and intensively basophilic (Romanowsky-type stainings) cytoplasms, changes in shape and thickness of nuclear membranes, prominent atypical angulated nucleoli, coarser and irregular chromatin clumping and/or eu-/parachromatin. However, invasiveness is as well manifested by "stromal parameters" - destruction of the intercellular matrix, surrounding elastin fragments and endothelium of blood/lymphatic capillaries which is morphologically presented by tumor diathesis, but also by the clear invasion of surrounded connective and/or adipose tissue that cytology reliably "depicts" as clusters or individual malignant cells within fragments of these tissues (Figures 1 a, b, c and d).

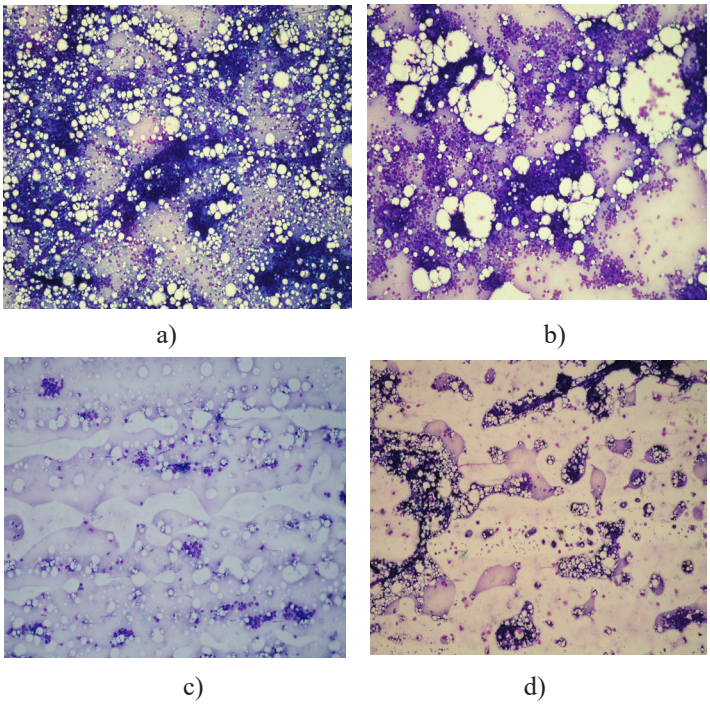

Figure 1: Cytological picture of IDC at low magnification; a) and b) large, partly dense 3D, partly monolayer and loose, but markedly poorly cohesive clusters or bundles with numerous single, relatively uniform malignant cells within the fat tissue strips, MGGx100, c) and d) smaller, irregular, poorly cohesive and loose groups as well as frequent single malignant cells, with fair amount of background foamy fats, MGGx100, and in d) inside strips of adipose tissue, MGGx100

In histology too, there are sometimes problems with the interpretation of tumor cell invasion in retraction cracks artifacts (if so?!) or tumor findings within blood or lymphatic capillaries that are not true tumor emboli but a superposed tumor tissue contaminants "from elsewhere".

Although cytology does not see and cannot see the basement membrane, it can very likely predict invasion with three necessary prerequisites - a triple-diagnostic approach to each lesion in the breast, well-sampled material and a good knowledge of cytological invasiveness criteria that has been described many times over the last two-three decades and discussed extensively in the following text. As an introduction, it should be emphasized that cytology must always but especially here, use architectural and not exclusively cellular criteria which is often not the case in practice, so architectural criteria are completely ignored or negated, while even mild forms of cellular/nuclear "atypia" (apocrine edge cell degeneration in clusters of otherwise normal ductal epithelium, mild anisonucleosis in fibroadenomas or in usual type of ductal hyperplasia) are extremely often overestimated, indicating unnecessary biopsies and excisions.

Cytological DCIS or IDC?

Both high- and low-grade breast DCIS have well-described cytomorphological features, with the mandatory use of rules of triple-diagnostic approach and of equal importance of architectural and cellular criteria [5-9, 14-17]. Considering the triple approach, radiologically (MMG and/or US) there is no clearly formed tumor mass or associated formation in the breast DCIS [5, 7, 17]; those are mostly (heterogeneous) zones of grouped, fine but irregular, pleomorphic, hyperechoic microcalcifications, sometimes with linear branching, that - by today's machines, high-resolution probes (12 MHz or more) and with an experienced eye of the sonographer can be visualized very well [17, 18]. However, if it is a radiologically clear tumor with microcalcifications, it is almost certainly an invasive carcinoma [5]. When cytologically observing such lesions, first - at lower magnifications of the objective lens (40x, 100x) we look at the "amount" of cellularity and architecture of the tumor groups, that is cellular organization in terms of size, shape, appearance, layering, density, cohesiveness and borders - in small or large, monolayer or 3D, thick or slight, cohesive or disintegrated, looking solid, cribriform, micropapillary or true papillary (with fibrovascular core), certainly observing the proportion of individual malignant cells, then obligatory absence or presence as well as location of myoepithelial cells - at or within cell groups, on the periphery of the same or in the background, semi-quantitatively assessing their proportion. Last but not least, we look very closely at the background of the smear, namely the absence or presence of necrosis - usually comedo type, then the presence, amount and appearance of microcalcifications, possibly the presence of fibrocytes/fibroblasts, cellular debris, fragments of elastoid stroma and adipose and connective tissue. Only then, at higher magnifications (400x, 600x or 1000x) we closely observe and describe the morphology of the cells - the size, shape, appearance of nuclei and cytoplasms, their mutual relationship as nuclear/cytoplasmic ratio (N/C ratio) as well as the mutual similarity between the cells - monomorphism or degree of pleomorphism (Figures 2 a, b, c, d, e, f and g).

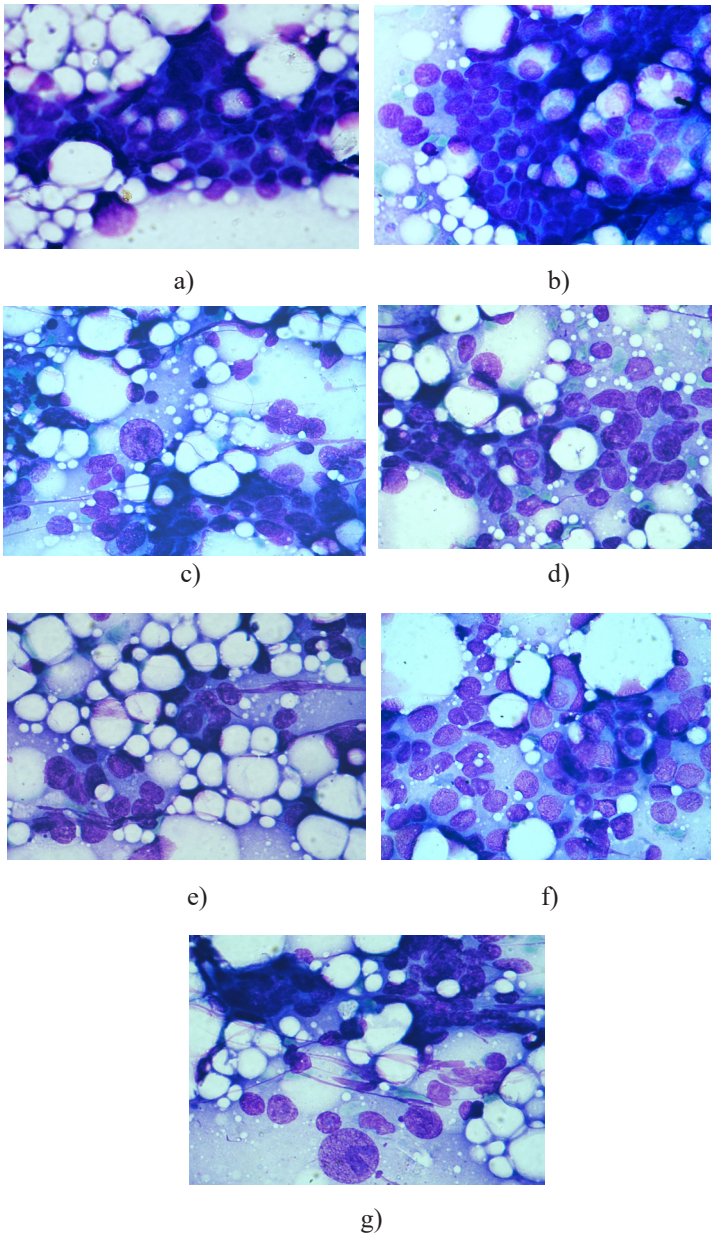

Figure 2: Cytomorphology of IDC at the highest magnification; a) well-defined, dense, 3D tubular structure, MGGx1000, and in b), c), d), e), f) i g) irregular - partly dense, partly loose and poorly cohesive clusters or bundles of anisomacronuclear, polymorphic malignant cells, frequent single tumor cells or their naked nuclei within the fat tissue fragments and with quite a lot of surrounding extracellular fat, MGGx1000

Each of these criteria is equally important, considered separately and in relation to the others, so that we can conclude with high likelihood what type of lesion it is – high- or low-grade DCIS or IDC (except ductal which is by far the most common, other histological types of breast carcinoma are not a subject of interest here). Sauer et al. have observed the described cytological criteria and their groups categorizing them into specific cytological diagnoses, which is presented clearly and in detail in Table 1 [7].

Table 1: Diagnostic categories and criteria according to Sauer et al.

Cytological diagnostic categories	Cytological diagnostic criteria
Unsatisfactory / No diagnosis / Inadequate	No / too few epithelial groups present
Benign, not otherwise specified (NOS)	Sheets / groups of benign apocrine and/or ductal epithelial cells and bare nuclei
Equivocal	Epithelial cells with nuclear changes of uncertain significance
Suspicious for carcinoma	Cells with some, but not sufficient, diagnostic features of carcinoma; scant cell material
Consistent with ductal carcinoma *in situ* (DCIS), low nuclear grade / atypical ductal hyperplasia (ADH)	Often large, more cohesive sheets and aggregates, balls or papillary fragments; variable, but usually rather mild atypia; some debris, often calcified; macrophages
Probable papillary carcinoma, cannot evaluate infiltration	Highly cellular smears; papillary aggregates, sometimes with a central fibrovascular core; complex folded sheets; columnar cells in rows, palisades and single; variable pleomorphism and atypia; bare bipolar nuclei absent; macrophages and epithelial cells with cytoplasmic vacuoles, some debris
Ductal carcinoma in situ, high nuclear grade, cannot evaluate infiltration	Large, pleomorphic cells showing obvious malignant features; irregular aggregates and single cells; comedo type necrotic debris with recognizable, necrotic tumour cells, often calcified
Invasive carcinoma	Variable, but most often a high cell yield; single population of atypical epithelial cells in irregular and angular clusters; reduced cohesiveness, variable nuclear enlargement and irregularity; single cells with intact cytoplasm; absence of bare, benign nuclei

Architectural and Cellular Invasion Criteria

IDC includes a number of general criteria of malignancy, but as elsewhere in cytology - individually none of them is necessarily an indicator of malignancy, and only a complete "pattern of the smear" correlated with clinical and radiological (MMG and/or US) findings provides a definitive diagnosis. IDC is a highly heterogeneous lesion in terms of growth patterns, appearance of the tumor groups and cells itself, degree of nuclear atypia, mitotic activity, stromal reaction types, tumor edges and presence or absence of additional *in situ* component, which is certainly reflected in the morphological appearance of the entire smear. Some of these features are the basis of cytological grading of breast carcinoma. One of the most commonly used grading system is according to Robinson et al., which is showed in my other paper ("Croatia's first experiences in cytological diagnosis of high-grade ductal carcinoma *in situ* of the breast: case reports and review of the literature") through Table 1 [19]. Punctate cellularity is usually moderate to abundant, although a scirrhous carcinoma may be less cellular; the well-sampled material contains large or even huge, often dense and 3D clusters or bundles as well as smaller irregular groups of mostly poorly cohesive or already disintegrated cells, but very frequent single malignant cells too [5]. Mixed carcinomas are common, that is IDCs may include other histological subtypes, so in the same punctate or smear we can find a papillary, micropapillary, mucinous or another component [5]. Sometimes smears are dominated by single tumor cells and/or naked malignant nuclei that may be predominantly ovoid and can be confused with bare bipolar benign nuclei of myoepithelial origin. Loss of cohesion, cell separation and abundant dispersed cells are not necessarily invasive attributes; many *in situ* lesions - especially high-grade DCIS include numerous single cells, as well as benign lesions may exhibit cellular disintegration [5, 6, 17]. Dissociation of malignant cells in breast carcinoma is evident before invasion and it is not typical feature of invasiveness [6]. It can also represent an artifact resulting from a mechanically strong smearing process. IDCs mostly show the absence of a biphasic epithelial/myoepithelial cell pattern and the absence of myoepithelial cells on the periphery of groups and in the background of smears, but some low-grade IDCs and tubular carcinomas that may contain few or some myoepithelial cells are the exceptions [5]. The presence of bare myoepithelial cell nuclei in a smear was previously considered as benign or "non-malignant" characteristic of lesion, however Sauer et al. noted them on epithelial aggregates in about half of non-high grade DCISs and in about a quarter of high-grade DCISs and concluded that myoepithelial cells do not disappear completely in DCIS but their number gradually decreases, so usually, but not always, they are no longer present in invasive carcinomas [6]. Careful observation of the cells at higher magnifications of the objective lens reveals important nuclear details that are quite typical of invasion - irregularly clumped and distributed chromatin,

appearance of eu- and parachromatin, in higher-grade carcinomas extracellular or satellite chromatin; often and pathological mitoses that are more common in high-grade carcinomas too; irregularities of the nuclear membranes (folds, grooves, notches, buds, clefts) and cubic or angulated nuclei; the size, shape, number and inter-individual polymorphism of atypical nucleoli as a direct sign of pathologically intense cell activity, with its clearly irregular to bizarre shapes and sharp edges (otherwise, benign epithelial cells in the breast aspirate usually have neither expressed nor multiple nucleoli except for apocrine metaplastic and secretory cells in pregnancy and lactation) [5, 19]. Most low-grade carcinomas have small or indistinct nucleoli, while expressed or abnormal nucleoli are features of mid- and high-grade carcinomas [5, 19]. In May-Grünwald-Giemsa (MGG) stained smears the nuclear details including chromatin changes are more discreet, but also noticeable or sometimes characteristic - e.g if its coarsely rope-like clumping is prominent it can give the impression of small holes in the nuclei [5]. The size of nuclei in breast carcinomas varies greatly - from 1.5 to >5x the size of red blood cells (RBCs) as well as the degree of their pleomorphism; in low-grade carcinomas the nuclei may be small and very uniform or monotonous ("monoclonal") which may lead to misdiagnosis of benignity or malignancy may be overlooked [5, 6, 19]. Most low-grade carcinomas show a nuclear size of 2-3xRBC [20]. In breast cytology the N/C ratio is not always helpful, since normal breast ductal cells have very scanty cytoplasms (high N/C), while carcinoma cells showing apocrine differentiation may have extremely abundant cytoplasms (low N/C) [5]. Regarding specific features of the cytoplasms, malignant cells in IDC mostly show increase in density and stronger basophilia as a signs of clear malignancy (due to increased protein synthesis in malignant cells) - sometimes with more or less fine pink to reddish granules (MGG staining), as well as the loss of a sharp distinct borders between cells or a syncytial appearance. The invasion is indicated by the already mentioned intracytoplasmic lumina that can be found in both the most common types of breast cancers (IDC and ILC) and they are extremely rare in benign breast epithelium. The smear background can also be typical for some carcinoma types - primarily comedo necrosis which is precisely significative for high-grade DCIS, can sometimes or less frequently be found in low-grade DCIS, uncommonly in high-grade IDC, is by no means a pathognomonic invasive feature and is more suggestive of DCIS [5-7, 9, 14-17]. Microcalcifications are quite rare in benign breast lesions, pretty common in invasive carcinomas, but we can found it in almost all DCISs - in 96% of non-high and 84% of high-grade DCIS [5-7, 9, 16, 17]. Described cytological criteria separately are not narrowly specific for invasion and can be found in both - DCIS and IDC, but in combination and in the presence of clear radiological (MMG/US) and clinical tumor mass, invasiveness is much more likely than *in situ* lesion [5]. However, high-grade DCIS can also be clinically presented with palpable, in that case often inflamed formation - due to a stronger desmoplastic reaction of stroma surrounding dilated ducts and lobules full of malignant cells, necrosis and calcifications (otherwise desmoplasia is much more common in invasion), while cytoplasmic lumina can be found in *in situ* carcinomas too, although they are much more common in invasive ones [5, 21, 22].

Cytological Invasion Criteria in the Relevant Literature

In a retrospective study of 300 non-palpable breast carcinomas (199 invasive and 101 DCIS), Bondeson and Lindholm identified 4 statistically significant out of 11 observed cytological criteria that could predict invasion: tubular structures of malignant cells, cytoplasmic lumina or vacuoles in malignant cells, fibroblast proliferation as a sign of tumor-induced stromal reaction and fragments of fine fibrillated elastoid stroma found in as many as 99% of invasive carcinomas (with one exception - in DCIS within a radial scar) and concluded that a combination of any two or more of these 4 criteria in a smear already diagnosed as a malignant, makes a positive predictive value (PPV) of an invasiveness of 96% [21]. Sauer et al. re-evaluated the cytological invasion criteria and their usefulness in estimating the invasive component within DCIS, on a 331 sample of cytologically suspected or clear DCIS and resolved histologically as DCIS or IDC [22]. They observed the infiltration of adipose and connective tissue bundles by tumor cells, fibroblast proliferation, cell poor elastoid stromal fragments, tubular structures and intracytoplasmic vacuoles, and found that all of these criteria except intracytoplasmic vacuoles correlate with invasion, but none is exclusively related to the invasive lesions. Pseudoinvasion of connective or adipose tissue fragments was found in 8 histologically pure DCISs, while one DCIS showed two or more invasive features. They point out that using established invasion criteria, virtually no pure DCIS will be cytologically diagnosed as invasive carcinoma, but only a part of those involving the invasive component will be recognized [22].

According to Bonzanini et al., the association of high cohesiveness of atypical cells and the absence of tubular aggregates showed good sensitivity and specificity for the diagnosis of DCIS versus IDC [23]. Sauer et al. also successfully cytologically distinguished invasive from *in situ* breast carcinomas in 294 of 320 cases; of 320 histologically released invasive carcinomas, as many as 294 (91.8%) were cytologically diagnosed as such, with a PPV of invasive carcinoma of 97% [7]. Key features in predicting invasion were the high cellularity of smears, irregular and angulated groups of reduced cohesiveness, single malignant cells with anisonucleosis and polymorphism, and the absence of benign bare nuclei. They concluded that definitive cytological diagnosis of invasive carcinoma was possible in >90% of the representative or diagnostic smears, which enabled the primary and final surgical treatment in these women. However, they do emphasize caution with smears with high-grade DCIS features that can also be represented by extensive dissociation of single malignant cells, which is one characteristic of malignancy but not necessarily of invasiveness [7].

Klijanienko et al. have retrospectively analyzed 223 non-palpable breast carcinomas and out of 10 observed cytological parameters to distinguish *in situ* from invasive, stromal infiltration was identified as the most potent predictor of invasion [24]. According to them, stromal infiltration by malignant cells was significantly more frequent in invasive than in *in situ* carcinomas (88% vs 11%), while cribriform pattern and necrosis were much more common in *in situ* than in invasive carcinomas (for the first feature 36% vs 16% and for the second 59% vs 19%). They concluded that the combination of stromal infiltration, cribriform growth pattern and necrosis may constitute a kind of "predictive index" useful for distinguishing *in situ* from invasive carcinomas [24]. In a study on 80 breast carcinomas (14 *in situ* and 66 invasive) and out of a total of 17

features, McKee et al. found that 6 were statistically significant for cytological differentiation; in invasive carcinomas connective or fat tissue infiltration by tumor cells is much more common (in 72% invasive / none in situ), intracytoplasmic vacuoles (in 50% invasive / 21% in situ) and tubular structures (in 30% invasive / 7% in situ), while myoepithelial cells in tumor groups were rarely seen (in 7% of invasive / 86% in situ) as well as much fewer microcalcifications (in 15% of invasive / 71% in situ) and foamy macrophages (in 16% of invasive / 64% in situ) [25]. They point out that invasion can be suggested in smears with clearly present fat or connective tissue infiltration by tumor cells, but cannot be ruled out in the presence of fragments of these tissues that are not infiltrated [25]. Signs of invasion may not always be found in cytological smears of invasive carcinomas, but their absence in an otherwise clearly malignant smear does not necessarily mean DCIS and does not exclude invasion [8]. However, since we always have to triple-diagnose each lesion in the breast, precisely in such ambiguous cases the clinical-radiological-morphological correlation is extremely helpful. Re-evaluating smears of 133 breast tumors and observing a total of 15 criteria, Bofin et al. have identified nuclear pleomorphism, the presence or absence of myoepithelial cells, signs of invasion and degree of cellular dissociation as key prognostic factors for distinguishing benign epithelial proliferative lesions, atypical ductal hyperplasia, DCIS and IDC [8]. In relation to the previously described studies, Guo et al. revealed similar findings in the observed 142 DCIS and 1978 IDC cases; background macrophages and extensive necrosis were significantly more frequent in DCISs than in IDCs, while finding of lymphocytes in conjunction and around malignant cells, stromal fragments associated with tumor cells and tubular tumor structures were significantly more frequent (lymphocytes) or found exclusively (the last two features) in IDCs [26].

However, for such a thorough and detailed approach to cytopathological diagnostics and to affirm its immense potential, it is necessary to have much more well-educated cytopathologists and cytotechnologists than there already are [27]. It could then expertly address this interesting and important topic, as cytology can and must be involved more and better in complex breast diagnostics. The same applies to the cytological profession - it needs to control itself more successfully and continuously. Therefore, in order to ensure the reliability and reproducibility of our results, quality control and standardization of all procedures - from sampling, through the entire process of technical processing of diagnostic material to interpretation and giving of results - is extremely important [27].

Conclusion

The cytological criteria that significantly suggest invasion are: fibroblast proliferation, fragments of poorly cellular elastoid stroma, findings of groups and individual malignant cells within the strips of adipose and/or connective tissue, intracytoplasmic vacuoles and tubular structures, while necrosis and calcifications are not typical or common in invasive lesions. The histological finding of malignant cells beneath the basement membrane has somewhat its cytological counterpart; although we do not see and cannot see a basement membrane we can predict invasion with a high probability, but exclusively and always with a triple-diagnostic approach to each lesion in the breast, well-sampled material, good knowledge of pathohistology and cytology and with also extremely important - continuous education and quality control at all levels of profession that need to become imperative.

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