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Assessment of necrosis and hypoxia in ductal carcinoma in situ of the breast: basis for a new classification

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Abstract Modern classifications of ductal in situ carcinoma (DCIS) of the breast suffer from unsatisfactory reproducibility in inter-observer circulation analyses. Ducts in DCIS are markedly enlarged in the range of 360 µm in diameter. Since the diffusion of oxygen from peri-ductal vessels is limited to 100 µm, cells in the center of DCIS are poorly oxygenated and become either necrotic or remain hypoxic but viable. There is evidence that such alternative fate is dictated by the biological characteristics of the neoplastic cells. Therefore, determination of presence or absence of necrosis in ducts up to 360 µm in diameter might represent a simple, reproducible, and biologically sound criterion to classify DCIS. In the present work, following this criterion, we classified 32 cases of intra-ductal lesions as either "necrotic" or "hypoxic" and tested the reproducibility of such classification using K statistics. These cases had already been circulated among a group of European pathologists, who classified the lesions using five different classifications. The K statistics value obtained with the presently proposed system was extremely high (0.91). It remains to be established whether the classification "necrotic/hypoxic" withstands large inter-observer circulation analyses, whether it is predictive of the clinical evolution of DCIS, and whether it might constitute a reproducible basis for selecting appropriate treatments.

Keywords Ductal carcinomas in situ · Necrotic · Hypoxia

This work is dedicated to the memory of our colleague and friend Prof. J. Sloane

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Introduction

Classifications of ductal carcinomas in situ (DCIS) of the breast are based on a variety of histological and cytological parameters (structural patterns, nuclear size and pleomorphism, mitoses, cellular shape and cell polarization, presence or absence of necrosis) [12, 23, 26, 29, 30, 32]. Despite the fact that the different classifications tried to emphasize the most objective and easily assessable characteristics, none of them has reached the goal of full reproducibility, as evaluated in the external quality assessment (EQA) series [2, 31, 32, 33]

In DCIS, tumor cells proliferate inside the ducts to such an extent that some of them are found away from the peri-ductal vascular net, beyond the 100-µm range of effective diffusion of oxygen and other essential metabolites [10]. In some DCIS types, cell necrosis is an early event. In other DCIS types, neoplastic cells located 150 µm or more from the nearest blood vessels survive despite hypoxia [11, 21]. It is probable that the metabolism of these hypoxic but still viable cells is kept at a very low level. The nature and biological properties of such cells (and of related intra-ductal lesions) must be quite different from that of cells that constitute lesions with necrotic central areas. In fact, in comedo-type DCIS, the thickness of the viable cell layer, dictated by the range of oxygen diffusion, has been measured in the range of 100 µm [19].

Morphometric evaluations in DCIS cases already demonstrated that the size of the ducts correlates with the probability of necrosis, which becomes a frequent phenomenon in ducts greater than 500 µm in diameter. Below the threshold of 360 µm, necrosis has been observed in approximately 50% of neoplastic ducts [19].

The present study suggests that the presence of necrosis or the evidence of hypoxia in DCIS lesions up to 360-µm in size might constitute the basis for a simple, reproducible, and biologically meaningful classification of DCIS. This hypothesis has been tested on 32 cases of DCIS which had already been studied by a group of European pathologists with expertise on breast cancer and

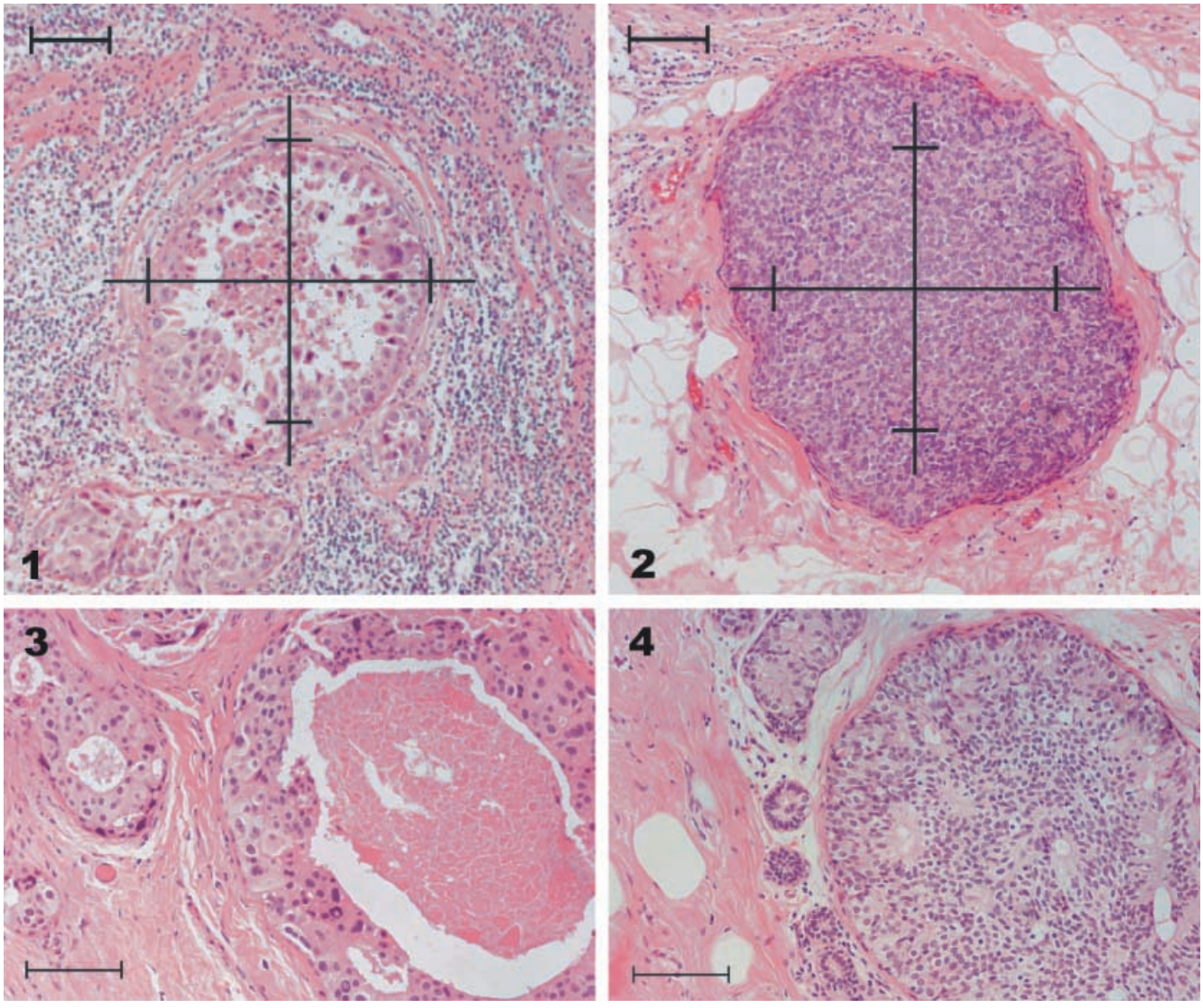

Fig. 1 Ductal in situ carcinoma of the breast, case no. 96/27, corresponding to the necrotic type. Transversal sections of the ductal lesions are plotted against a cross marking 360 µm on the diameter. The duct shows comedo-type central necrosis and a high nuclear grade. Hematoxylin and eosin; *bar* 100 µm

Fig. 2 Ductal in situ carcinoma of the breast, case no. 96/30, corresponding to the hypoxic type. Transversal sections of the ductal lesions are plotted against a cross marking 360 µm on the diameter. The duct, with a diameter of approximately 450 µm, shows a solid pattern and lacks necrosis. Hematoxylin and eosin; *bar* 100 µm

Fig. 3 Necrotic ductal in situ carcinoma (DCIS) showing necrosis even in small ducts. The thickness of the viable cell layer is less than 100 µm. This case was diagnosed as comedo-type DCIS by the European Commission Working Group on Breast Screening Pathology (ECWGBSP) (case no. 96/39). Hematoxylin and eosin; *bar* 100 µm

Fig. 4 Hypoxic ductal in situ carcinoma (DCIS). Centrally located cells appear viable despite their hypoxic state and are located more than 100 µm from vessels surrounding the basement membrane. This case was diagnosed by the European Commission Working Group on Breast Screening Pathology (ECWGBSP) as a low nuclear grade cribriform DCIS with cell polarization (case no. 96/25). Hematoxylin and eosin; *bar* 100 µm

categorized according to five different recently published classifications [31].

Materials and methods

This study was based on 32 cases of DCIS. These cases had been circulated among 23 pathologists in the European Commission Working Group on Breast Screening Pathology (ECWGBSP). The cases had been submitted to the co-ordinating center (University of Liverpool) by members of the ECWGBSP. One hematoxylin and eosin (H&E)-stained section from a selected tissue block was sent to each member of the working group. Each participant classified the case according to five different classifications of DCIS: (1) European pathologists' working group [12]; (2) one based on three categories of nuclear grade [32, 33]; (3) one based on two categories of nuclear grade [32]; (4) the Van Nuys system [28]; and (5) a two-category classification based on comedo necrosis [23, 32]. The agreement between participants on the categorization of cases using each classification, measured by calculating K statistics, was 0.42 or lower, as reported elsewhere [32]. One of the present authors (G.B.) is a member of the ECWGBSP and received permission from the group co-ordinator (Prof. J. Sloane) to use this material.

We examined the single slide of each of the 32 cases previously circulated among members of the ECWGBSP with a microscope (Leitz, Orthoplan) fitted with a Sony trinitron camera and display. The size of the ducts was measured at 100× magnification by projecting the image over the display on which a probe (a cross or a circle) with a 360-μm diameter had been drawn with a black marker (Fig. 1 and Fig. 2). The probe was obtained using the scale of an objective micrometer (Olympus OB-M-1/100) or using, as a reference, the circle inscribing a square of Bürke or Fuchs-Rosenthal hemochromocytometric chambers, whose sides were 250 μm and diagonal corners therefore 354 μm distant. In practice, the slide was examined under the microscope, and the smallest duct with central necrosis was plotted over the display against the probing cross or circle.

Each case was independently analyzed by seven pathologists, all from our department. Cases were classified as necrotic when central cellular necrosis, characterized by ghost cells and nuclear fragments, was detected in ducts measuring 360 μm or less in the minor diameter. Even a single duct with the above-reported features was sufficient to classify the lesion as "necrotic". The occasional presence of single scattered necrotic cells was not considered. Necrosis in ducts larger than 360 μm was not considered. Cases were classified as hypoxic when centrally located cells were still viable and no necrosis was observed in ducts up to 360 μm in size. Two rounds of circulation were made. In the first round, the slides were not marked in any way, no specific areas were selected, and no specific instructions were given on how to make the assessment. In the second round, we made a seminar on the definition of "central necrosis" and, whenever doubts were expressed on the necrotic or secretory nature of the intra-ductal material (in four cases), a decision was reached by agreement. The agreement between participants on the categorization of cases using the classification "necrotic versus hypoxic" was measured by calculating K statistics [15].

Results

Assessment of the size of the affected ducts proved an easy task for all pathologists. Some difficulty was encountered in categorizing low papillary lesions, since ducts were large but empty and the cell layer was thin (less than 100 μm). No necrosis was observed in such lesions, and these were accordingly classified as hypoxic. On transverse sections, the shape of most ducts appeared round. This indicated that the center of DCIS was at equal distance from the surrounding stroma. In the first circulation, consistency in the classification of the 32 cases as either necrotic or hypoxic proved substantial, with a K statistics value of 0.75. Wide variations were observed among participants.

The reason for the observed discrepancies was related to the interpretation of necrosis and its differentiation from intra-ductal accumulation of secretory proteins and amorphous debris. Before the second circulation (performed 2 months later), we focused on the definition of necrosis. In four cases, agreement on the presence or absence of necrosis was reached by common discussion. In the second circulation, a nearly perfect agreement was reached, with a K statistics value of 0.91 (range 0.95–0.75). A clear majority diagnosis was reached in all cases.

Of the 32 cases, 17 were defined as necrotic and the rest as hypoxic since no necrosis was detected in ducts up to 360 μm in size. Some cases were heterogeneous. In fact, in seven cases classified as necrotic large ducts (up to 500 μm in diameter) without necrosis were also detected. In two cases classified as hypoxic, necrosis was detected inside some ducts larger than 360 μm.

All of the nine cases, for which a majority diagnosis of typical or atypical hyperplasia had been expressed by the ECWGBSP group, fell into the hypoxic category. In three of these cases, all ducts were relatively small, i.e., about 200 μm. The agreement was still nearly perfect (K value of 0.85) if we considered only the 23 cases for which a majority diagnosis of DCIS had been reached by the ECWGBSP group.

Comparison of the present data with those previously obtained (on the same cases) by the ECWGBSP group was only possible for classifications involving two alternatives and in those cases for which a clear majority diagnosis (over two-thirds) was reached by the European experts. Comparison with the classification based on nuclear grade was made in ten cases for which the ECWGBSP group had reached a majority diagnosis by classifying lesions as either high (six cases) or low nuclear grade. Five cases classified as necrotic had a high nuclear grade, while among the five cases classified as hypoxic, four had low nuclear grade and one had high nuclear grade. Agreement with the comedo/non-comedo classification was reached in 15 cases and absent in 3 cases. Correlation with the criterion on presence/absence of polarization was present in 12 cases and absent in 4 cases. In general, cases classified as necrotic, following the present proposal, had high nuclear grade, lacked polarization, and corresponded to comedo carcinomas. Hypoxic cases were mostly of low nuclear grade, showed cell polarization, and were of the non-comedo type (Fig. 3 and Fig. 4).

Discussion

Intra-ductal carcinoma of the breast represents a major diagnostic and therapeutic problem [14, 22, 25, 34]. The adequacy of surgical excision, by evaluation of the margins, remains the most important determinant in predicting recurrence [27, 32, 35]. In addition, pathologists have been developing histological criteria to differentiate indolent DCIS cases from those more likely to recur and to proceed towards invasive cancer in order to provide clinicians with a reliable basis for alternative therapeutic regimens [30]. Recent developments in DCIS classification addressed criteria based on progressively greater details, from histological patterns [2, 3] to cell morphology and cell polarization, to nuclear morphology [8], hyperchromasia, and pleomorphism [12, 29, 32]. However, the clinical impact of such classifications might be hampered by their moderate inter-observer reproducibility [2, 32, 33]. Unfortunately, all classifications proposed so far showed low consistency in inter-observer circulations because they resulted in K statistics values in the range of 0.5 or lower [31, 32].

The present study represents a novel approach because it tends to reduce the bias due to interpretation of

cytological criteria by introducing a strictly morphometric assessment in order to determine presence or absence of necrosis in ducts of a given size. Intra-ductal neoplastic proliferation induces an enlargement of duct size widely exceeding the normal duct size, which is in the range of 90 µm [19]. Peri-ductal vessels (either pre-existing or induced) [11, 21] sustain the neoplastic proliferation by releasing nutritional factors and oxygen, whose diffusion cannot, however, exceed 100 µm [10].

The rationale of our approach is that intra-ductal necrosis is the result of two distinct factors: lack of oxygenation (related to the size of the ducts) and sensitivity of neoplastic cells. As a consequence of the first factor, necrosis can be ubiquitous in very large ducts (diameter 500 µm or more) and therefore present even in well-differentiated DCIS. On the contrary, in smaller ducts, presence or absence of necrosis only rests on the sensitivity of neoplastic cells to hypoxic conditions. Such sensitivity is likely to be related to the metabolic status of the neoplastic cells. We adopted the threshold of 360 µm in diameter, which is bound to leave a hypoxic area of 150 µm in the center of the ducts. Previous morphometric evaluations had detected necrosis inside ducts of 360 µm in size in 50% of the cases of DCIS [19]. We obtained a similar result and concluded that the use of this criterion allows us to divide DCIS into two groups.

We are well aware that the dichotomous classification presently adopted might turn out to be too rigid and that intermediate cases might have to be allocated into a separate category. In addition, we detected the presence of hypoxic ducts, larger than 500 µm, in 7 of the 17 cases classified as necrotic, an observation clearly related to the heterogeneity of cell patterns in individual cases of DCIS [14, 17].

All of the nine cases, which the majority of the EC-WGBSP group had classified as typical or atypical hyperplasia, fell into the hypoxic category. However, in three of these cases, the ducts were relatively small (approximately 200 µm in diameter) and therefore could not be properly considered as hypoxic.

The differentiation of necrotic material, characterized by ghost cells and nuclear debris from intra-ductal amorphous material of proteinaceous or mucoid nature, was in some case uncertain and a cause of disagreement, especially among non-experts. The use of specific histochemical stains may overcome such problems [9].

Through the comparison of our data with those obtained in the same cases by the ECWGBSP group, we reached supportive evidence that cases classified as necrotic correspond mostly to high nuclear grade, comedo-type lesions. On the contrary, cases here categorized as hypoxic correspond mostly to low nuclear grade DCIS with cell polarization.

Several studies confirmed that the biological properties of neoplastic cells featuring either the low grade, solid or cribriform carcinoma or the high grade comedo-type DCIS are quite different. In fact, the former show a proliferative activity, as revealed by Ki-67 staining, that is low at the periphery and absent in the center of ducts. In addition, cells are rich in bcl-2 expression, which indicates a lack of apoptotic activity. Comedo-type DCIS have a high proliferative activity, lack bcl-2, are rich in Bax, and are p53 positive [4, 5, 13, 16, 18, 20, 23, 24].

All of these data concur that the presence of necrosis or evidence of hypoxia at the center of ductal lesions up to 360 µm largely reflects a marked biological difference in the neoplastic cell nature and behavior. Moreover, it can be suggested that cases of DCIS with extensive hypoxia might have to be treated differently, since hypoxic cells are known to be less sensitive to radiation [1, 6, 36]. In addition, it has recently been shown by DeFatta et al. [7] that hypoxia in DCIS results in overexpression of the translation-initiation factor eIF4E, thus inducing vascularization and tumor cell proliferation.

The presently proposed classification system appears biologically sound and highly reproducible even for non-experts. Since the number of participants was relatively small and all came from the same institution, such conclusions await confirmation in the EQA series. In addition, retrospective studies on selected case series will have to be conducted in order to prove whether such classification is predictive of the clinical evolution of DCIS and whether it might form the basis for selecting appropriate treatments.

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