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*Report*

# Differentiation between cancerous and normal hyperplastic lobules in breast lesions

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**Summary**

Determining the risk that a particular area of hyperplastic breast tissue will progress to cancer is difficult and is currently expressed only as a general risk factor within the population. Using an antibody against the apoptotic purinergic receptor $P2X_7$, we examined 40 cases each of the following histological categories: normal, moderate, florid and atypical hyperplasia, lobular carcinoma *in situ*, ductal carcinoma *in situ*, invasive lobular and invasive ductal carcinoma. These were previously diagnosed by H&E and supplied by clinical laboratories as tissue sections. Normal and mildly hyperplastic epithelium was devoid of the cytolytic $P2X_7$ receptors whereas all epithelial cells in all cases of *in situ* or invasive lobular or ductal carcinoma labelled intensely. The lobular and ductal *in situ* cases labelled intracellularly while the invasive epithelial cancer cells showed intense cell surface label indicating an attempt was being made to induce apoptosis. All these receptors however are non-functional and thus unable to induce apoptosis. Approximately 10% of all hyperplastic lobules examined in the biopsied tissue, regardless of H&E classification, labelled for $P2X_7$, which is suggestive of early metabolic cancerous change. The acini within lobules were either completely labelled with $P2X_7$ or were completely devoid of the receptor. A potential advantage of this method lies in identifying early cancerous change in hyperplastic lobules and in establishing the true extent of cancerous spread in infiltrating lesions, thus facilitating the task of reporting clear surgical margins.

## Introduction

Histological and cytological features as demonstrated by haematoxylin and eosin (H&E) staining of tissue sections currently form the basis for the classification of breast cancer. Normal acini are composed of a layer of myoepithelial cells adjacent to the basement membrane that interact with the underlying stroma and an epithelial layer with secretory and absorptive functions on the ductal aspect [1].

Epithelial hyperplasia, sometimes called papillomatosis or epitheliosis, is a relatively common benign microscopic lesion in the breast in women over 30 years of age. The condition involves an increase in the number of layers of epithelial cells through increased proliferation or reduced apoptosis. These cells are heterogeneous and proliferate in layers that extend to ultimately fill the duct lumen. Mild epithelial hyperplasia is defined as consisting of fewer than four cell layers. The terms moderate to usual epithelial hyperplasia describe an increasing number of layers. Atypical lobular hyperplasia refers to hyperplastic cells that resemble lobular carcinoma *in situ* (LCIS) in acinal organisation. Hyperplasia ultimately takes the form of a solid mass of heterogeneous cells that fill and distort the acinus and duct lumen.

It is not possible to determine if lobules containing moderate, usual or atypical hyperplasia will progress to cancer based solely on H&E stained slides. Several studies have shown an unacceptably high level of observer variability in estimating the type and degree of epithelial hyperplasia [2]. Possibly as a result of

this uncertainty, only estimations of general risk to the population are used. Moderate or florid hyperplasia without atypia is considered to carry a slight (1.5–2.0-fold) increase in risk of later developing cancer, while in atypical hyperplasia the risk is given as 4–5-fold that of the general population. Ductal carcinoma *in situ* (DCIS) and LCIS carry an 11-fold increased risk for breast carcinoma [3, 4]. It would be clearly advantageous to be able to differentiate between cells forming low-risk tumours and those that demonstrate the potential for becoming invasive.

The neoplastic transformation of normal breast cells is thought to result in part from a loss of the normal regulation of cell numbers, resulting in hyperplasia. Breast cancer is thought to develop from non-invasive precursor lesions, although the earliest steps of neoplastic transformation are still undefined. Genetic instability in clonal populations of cells is common in atypical hyperplasia. Progression to cancer often includes the increased expression of oncogenes, decreased expression of tumour-suppressor genes, loss of cell adhesion and disruption to cellular biochemistry. Alterations in cellular morphology occur later as a result of these initial changes. In established cancer, changes include the expression of angiogenic factors and proteases that facilitate tissue invasion. These malignant phenotypes are thought to be due to an accumulation of the above changes, rather than an orderly progression between stages [5–7].

As a result, most human breast cancers appear to develop over long periods from pre-existing benign lesions although the early biochemical changes involved in this evolution are not fully understood [8]. Morphological analyses of H&E-stained breast cancer cells suggest that this is a multistep process. Apparently benign lesions progress to various categories of hyperplasia, which represent the initial stages of uncontrolled and possible neoplastic growth. The next stages are carcinoma *in situ*, invasive carcinoma, and ultimately metastasis. Such processes have been documented in many other malignancies. This view suggests that usual ductal hyperplasia (UDH) may be a precursor of atypical ductal hyperplasia (ADH). Conversely, another prevalent view is that UDH is not part of the neoplastic progression but represents a benign proliferation of ductal epithelial cells. ADH does however represent the first step in a clonal neoplastic expansion [9].

The prognostic significance of histological change as demonstrated by H&E stain has been extensively studied and forms the basis of current diagnostic criteria. More recently, other markers of early cancerous change (i.e., unrelated to the expression of the cytolytic $P2X_7$ receptors described in this study) have been discovered. A loss of the genes 16q and 17p may also play an early role in breast carcinogenesis [10]. Altered protein expression from several proliferation and apoptosis-related genes are a known factor in the development of breast cancer and are found in UDH [11]. The biggest change in expression of proliferation and apoptosis related proteins appear to occur during the transition from UDH to DCIS [12]. This implies that 'benign' hyperplasia may indeed be a first event in breast oncogenesis. In a study of 66 human breast carcinomas and adjacent peritumoural tissues, tumour markers human erythrocyte glucose transporter 1 (GLUT1) and fatty acid synthase (FAS) were observed in non-neoplastic tissues that were adjacent to mammary carcinomas [13]. Hypermethylation of the 14-3-3-sigma gene occurs at an early stage in the progression of breast cancer, and has been noted in apparently normal epithelium adjacent to breast cancer. A loss of expression of 14-3-3 sigma is therefore possibly another early event in neoplastic transformation [14].

The clonal nature of neoplastic lesions such as *in situ* and invasive breast cancer has been widely proven by several proliferative, genetic or other malignancy-associated markers. The breast is organised into distinct stem cell-derived monoclonal patches and the normal terminal ductal lobular units (TDLU) are monoclonal in origin. Any proliferative lesion arising within such a pre-existing clonal patch should therefore be clonal, irrespective of whether it originates from one or more patch cells [15]. Loss of heterozygosity (LOH), a genetic change frequently detected in cancer, has been noted in 'benign' epithelial foci in the breast. LOH also occurs frequently in fibrocystic change, which suggests that foci of apocrine metaplasia can share a genetically altered precursor cell with an associated carcinoma [16].

In previous studies on the early neoplastic biochemical changes in another epithelial cell (prostate) cancer [17], we established that the expression of several purinergic receptors precedes any visible markers of cancer as visualised by H&E stain by up to 6 years. More recently, we discovered that the apoptotic receptor $P2X_7$ is expressed in all melanoma cases investigated, while remaining absent in normal skin epithelium [18]. This receptor has since been confirmed as being non-functional, entirely unable to induce apoptosis in the cells expressing surface

receptor/channels [19]. In the current study, we labelled previously diagnosed (by H&E stain) breast biopsies identified as normal, hyperplastic (mild, florid and atypical), *in situ* and invasive carcinoma with an antibody to the apoptotic calcium channel receptor $P2X_7$ to identify the early biochemical changes of breast cancer. These $P2X_7$ receptors expressed on breast cells have similarly been found to be non-functional.

## Materials and methods

Each of the following histological categories ($n = 40$) was investigated: normal, usual, and atypical hyperplasia, LCIS, DCIS, invasive lobular and invasive ductal carcinoma. Each case was previously diagnosed and supplied as tissue sections by clinical laboratories on a random basis accompanied by full case notes and an H&E-stained slide for correlative microscopy. One of the difficulties in this study was that, although each case had an overall diagnosis, the tissue within each block (up to 30 blocks per case) varied markedly. Examples of a variety of histological classifications were present not only in each block, but between blocks from the same case. Consequently, the classification state of each lobule had to be verified by a histopathologist before being included in the study. Despite this precaution, expert opinions varied as to the classification of some hyperplastic lobules.

As this was an initial study to establish the $P2X_7$ labelling characteristics compared with the established H&E diagnostic criteria, it was not appropriate to use a blind study. Each case was selected randomly from a list of available patients within each of the listed categories and diagnosed by a qualified pathologist to establish the cancer stage present, using current diagnostic H&E-stain criteria. The affinity-purified $P2X_7$ antibody was prepared as previously described [20]. Each tissue section was immunolabelled as previously described [21]. Identical labelling was observed using the antibody to non-functional receptors [19] verifying the suspicion that the $P2X_7$ apoptotic pathway is blocked in breast cancer. Each slide was de-waxed in two changes of fresh Histoclear for 10 min each and then rehydrated. All sections were immersed in 1% hydrogen peroxide for 5 min, washed and then incubated in anti-$P2X_7$ primary antibody at a concentration of 0.25 μg/mL IgG in PBS for 30 min. Thereafter, slides were washed three times in PBS for 5 min each, followed by a total 30 min incubation with anti-rabbit secondary (Dako). All slides were then washed in PBS for 5 min and visualised using a 0.05% solution of diamino benzidine (DAB) for 5 min, washed, dried and mounted in Entellan mounting medium (Merck). Approximately serial sections were stained with a standard H&E protocol. Peptide epitopes completely blocked labelling in pre-absorption controls. For practical, ethical and legal reasons, only formalin-fixed, paraffin-embedded and previously diagnosed biopsies were available for this study.

## Results

Figure 1(a) is a micrograph of a normal TDLU stained by H&E. The acini are composed of two cellular layers, an epithelial layer on the lumenal aspect that has secretory and absorptive functions, and a myoepithelial layer adjacent to the acinal basement membrane. The ducts draining the acini are lined with stratified epithelium. The interlobular stroma is stained magenta by the eosin component of the stain. In serial sections of lobules with this H&E appearance (Figure 1(a)), no labelling for the cytolytic receptor $P2X_7$ was seen (Figure 1(b)).

Approximately 10% of all hyperplastic lobules labelled strongly for $P2X_7$ regardless of the H&E classification, in all biopsied tissue. This label was 'all or nothing' in every acinus within a given lobule, suggesting that the phenomenon spread to neighbouring cells within the individual duct. This finding has also been noted in the prostate [21]. In usual hyperplasia, the proliferating epithelium takes the form of solid masses of cells that distort the acinal structure and obscure the duct lumen (Figures 2 and 3). Within this classification, 88% of lobules ($n = 3987$) displayed no neoplastic-associated biochemical changes as determined by cytolytic $P2X_7$ receptor labelling (Figures 2(a and b)) while the other 12% demonstrated a positive $P2X_7$ receptor label throughout all epithelial cells in the lobule (Figures 3(a and b)). It should be noted however that the receptor expression was intracellular. The receptors had not been transported to the cell membrane where they could be assembled to act as functional apoptotic pores following agonist activation [19].

Tissue sections often contained different types of lobule as classified by H&E. Often, however the tissue sections would contain lobules that were indistinguishable from one another by H&E stain but were either strongly positive or transparent (negative)

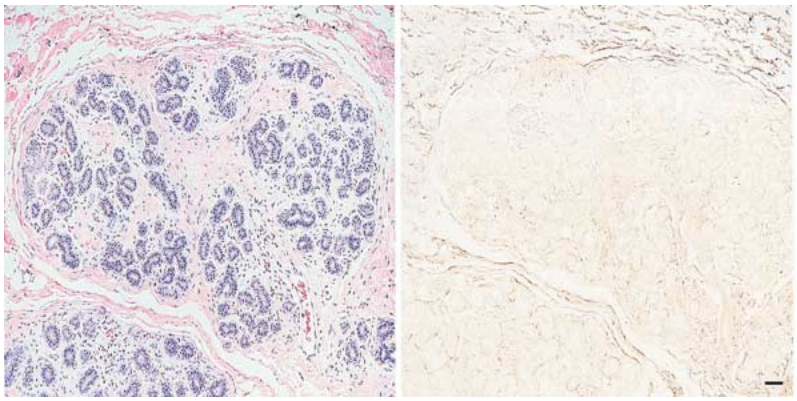


*Figure 1.* (a) A micrograph of a terminal duct lobular unit stained by H&E. (b) A serial section of the same lobule as (a) labelled for the cytolytic receptor anti-$P2X_7$. There is no positive label for this receptor in normal tissue. Bar = 100 μm.

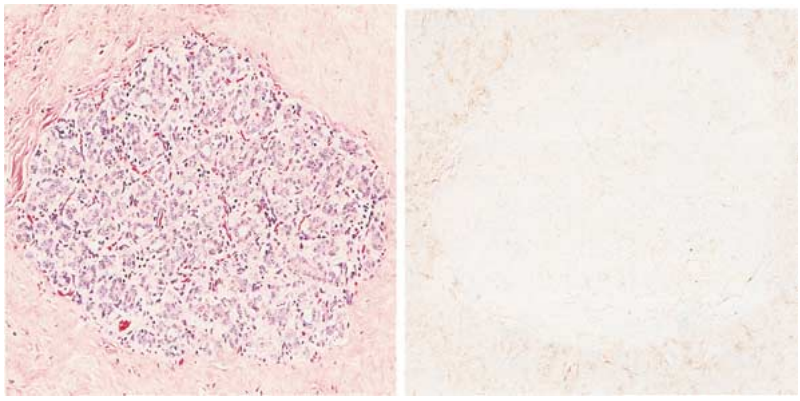


*Figure 2.* (a) An H&E stained section of a hyperplastic lobule. The proliferating epithelium has taken the form of solid masses of cells that obscure the duct lumen. (b) Serial section of (a) that has been labelled for $P2X_7$ showing no label (i.e., normal) in the cytoplasm and apical epithelium (arrow). Bar = 100 μm.

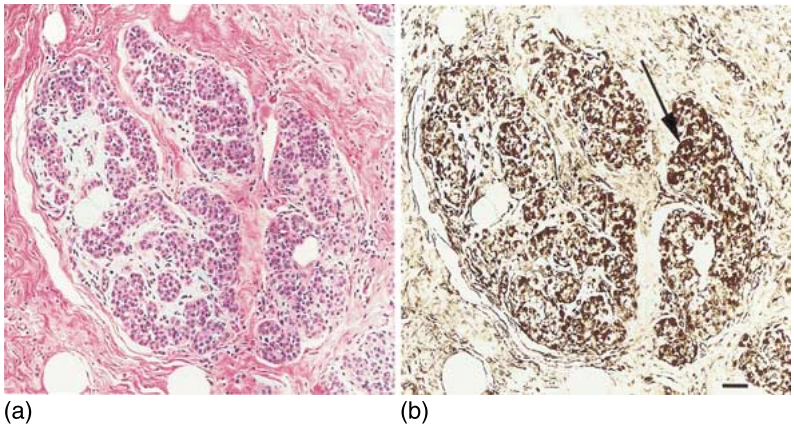


*Figure 3.* (a) An H&E stained section of hyperplastic lobules. The proliferating epithelium has taken the form of solid masses of cells that obscure the duct lumen. (b) A serial section of Figure 2(a) that has been labelled for $P2X_7$ showing an intense label in the cytoplasm and beneath the apical epithelium (arrow). Bar = 100 μm.

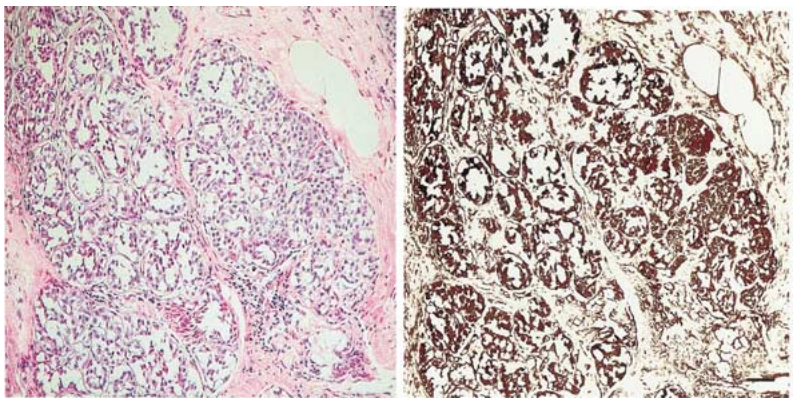


*Figure 4.* (a) An H&E stained section that shows a lobule diagnosed as atypical hyperplasia. (b) A serial section that shows intense intracellular $P2X_7$ receptor label in this lobule, suggesting the presence of early neoplastic change. Bar = 50 μm.

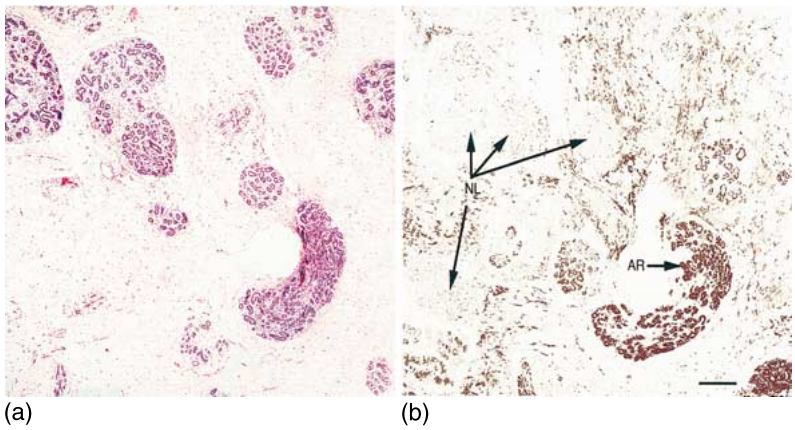


*Figure 5.* (a) A low power H&E stained section of an area of tissue diagnosed as mixed normal and atypical hyperplasia. (b) An approximate serial section of the same area labelled for $P2X_7$. Using this marker, some lobules are normal (NL-arrows) and some show positive neoplastic changes in the form of intracellular $P2X_7$ receptors (AR-arrow). Note that this observation is an 'all or nothing' phenomenon at the lobular level. Individual acini conform to the labelling state of their lobule. Bar = 500 μm.

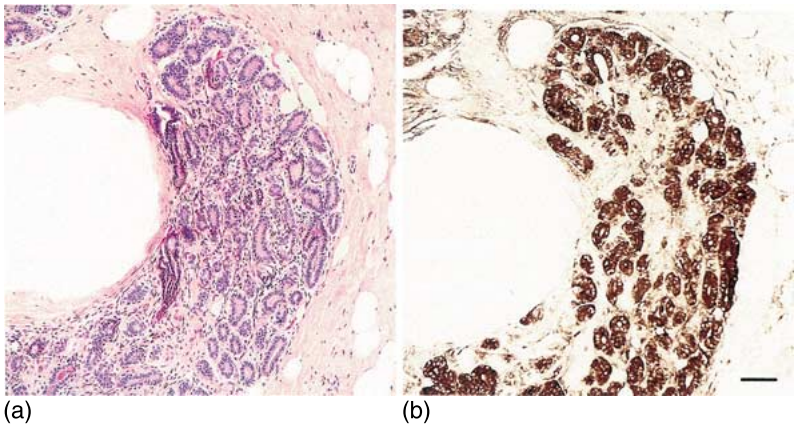


*Figure 6.* (a) A low power H&E stained section of the lobule labelled 'AR-arrow' in Figure 4(b). While some acini appear normal, others do not. (b) A serial section that shows an intense intracellular $P2X_7$ receptor label in the lobule shown in Figure 4(c). Each acinus within the lobule, regardless of H&E morphology, is labelled for the $P2X_7$ receptor. Bar = 20 μm.

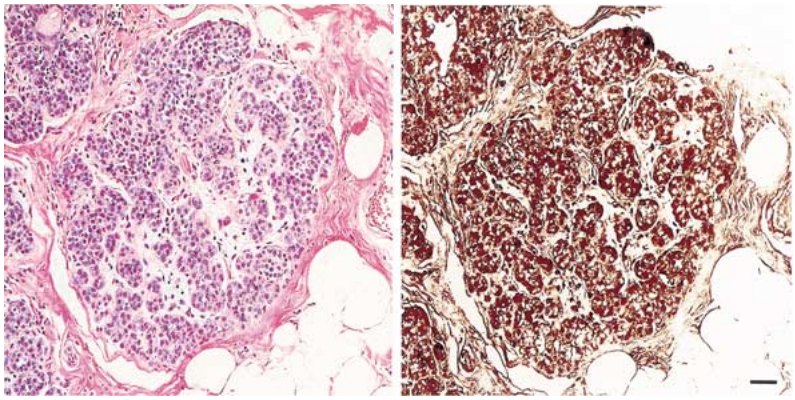


*Figure 7.* (a) An H&E-stained section previously diagnosed as LCIS. (b) A serial section labelled for the $P2X_7$ cytolytic receptor. All LCIS lobules had an intense intracellular $P2X_7$ label. Bar = 50 μm.

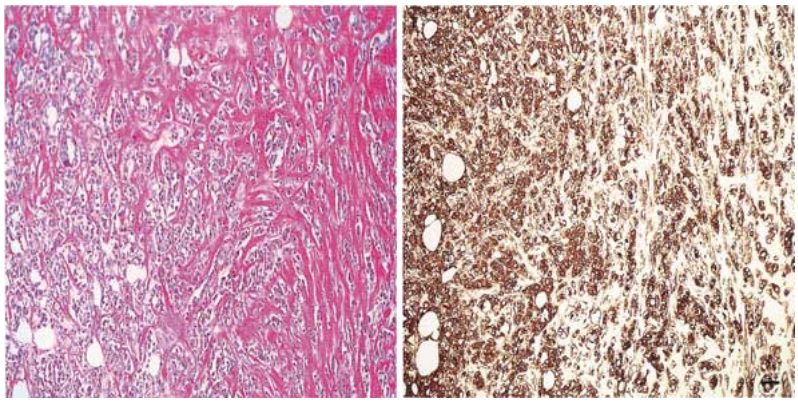


*Figure 8.* (a) An H&E-stained tissue section previously diagnosed as invasive carcinoma. (b) An approximate serial section of Figure 6(a). Each cell shows an intense cell surface label for $P2X_7$ despite histological degradation. Bar = 50 μm.

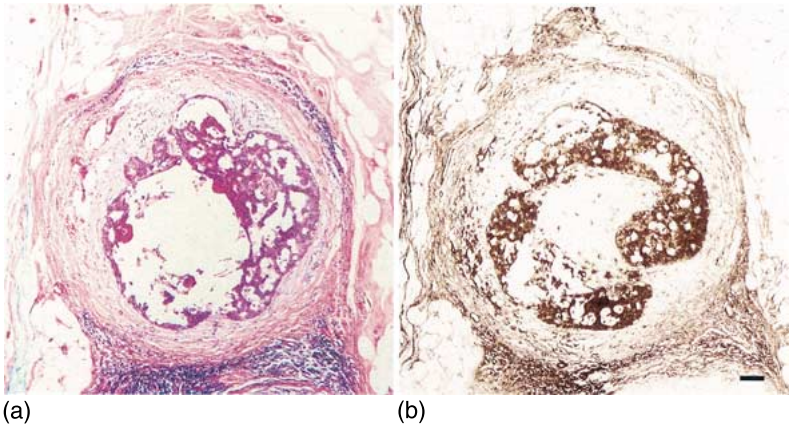


*Figure 9.* (a) An H&E-stained section of a DCIS of the comedo type. (b) A serial section of Figure 7(a). Despite some histological degradation, an intense intracellular label for $P2X_7$ is visible in all cells. Bar = 100 μm.

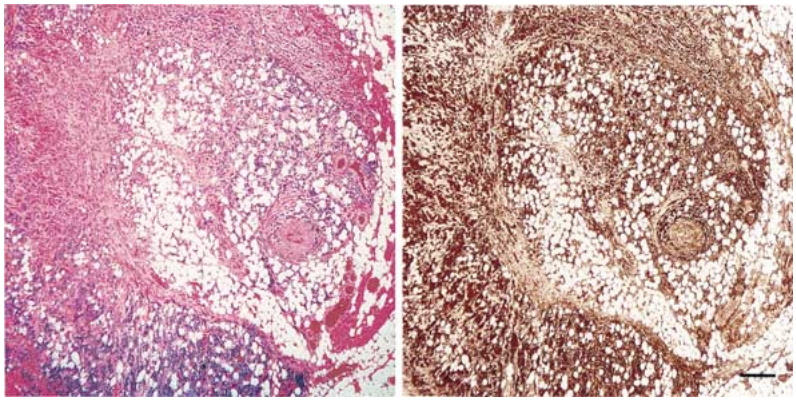


*Figure 10.* (a) An H&E-stained section of invasive ductal carcinoma. (b) Each epithelial cell surface is intensely labelled for $P2X_7$. Bar = 30 μm.

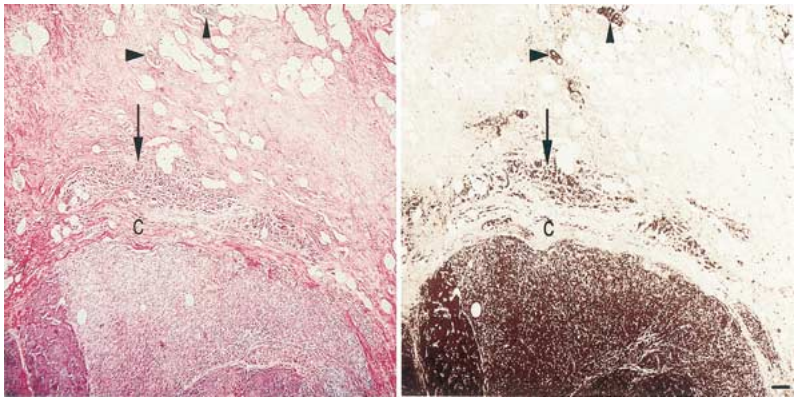


*Figure 11.* (a) An H&E stained-section of a large area of carcinoma *in situ*. A capsule (C) surrounds the cancerous area. (b) Areas of intracellular $P2X_7$ labelled cells (Figure 9(a and b)-arrows) can be seen at least 1.2 cm from the primary tumour on the upper margin, while all the other margins were found to be entirely clear of label. Cells within the tumour show mostly cell surface label. Bar = 500 μm.

when labelled for the $P2X_7$ receptor. Figure 4(a) shows a lobule clinically diagnosed with atypical hyperplasia. In atypical hyperplasia a total of 90.4% of all lobules were unlabelled, suggesting that they had not been subject to early neoplastic biochemical change ($n = 4328$). However, the remaining 9.6% were strongly positive, indicating possible early potential neoplastic change. Once again, the receptors were found to be intracellular. As transport to the cell membrane had not yet occurred there was not yet even the potential for apoptosis to be induced through stimulation of these receptors. Figure 4(b) demonstrates an intense $P2X_7$ receptor label in a serial section. Both nuclei and cytoplasm in all epithelial cells are labelled.

Figure 5(a) is a low power H&E stained section of an area of tissue diagnosed as comprising mixed normal and atypical hyperplastic lobules. Figure 5(b) is an approximate serial section of the same area labelled for $P2X_7$ showing some lobules are normal (NL-arrows) while some are positively labelled (AR-arrow). The 'all or nothing' nature of this phenomenon is clear in this micrograph. Figures 6(a and b) are high power micrographs of the lobule labelled 'AR' in Figure 5(b). All receptors are found to be intracellular in these tissues.

In tissue that was diagnosed as clearly cancerous by H&E stain, all epithelial cells labelled strongly for $P2X_7$. Figure 7(a) is an H&E-stained section previously diagnosed as LCIS while Figure 7(b) is a serial section labelled for $P2X_7$. All LCIS lobules were strongly labelled with the $P2X_7$ antibodies (Figure 5(b)). While the number of receptors appeared

to be substantially increased in these tissues over the levels observed in moderate and atypical hyperplasia, they remained intracellular.

Figure 8(a) is an H&E-stained tissue section previously diagnosed as invasive lobular carcinoma. All the epithelial cells demonstrate an intense $P2X_7$ label (Figure 8(b)). However, the receptors are mostly deployed on the cell surface where they have the potential to induce apoptosis. They are unable to do so as the pores remain closed as revealed by the binding of the antibody to the non-functional receptors.

Figure 9(a) is an H&E-stained section of a DCIS of the comedo type. Once again an intense label for $P2X_7$ is visible (Figure 9(b)) in all epithelial cells. These cells also show the receptors are almost entirely intracellular.

Figure 10(a) is an H&E-stained section diagnosed as invasive ductal carcinoma. Figure 10(b) shows that all the epithelial cells in the field are intensely labelled for $P2X_7$ as expected. All cells identified as cancerous by H&E were labelled with the $P2X_7$ antibody. Essentially all the receptors in these cells also have been deployed on the cell membrane in an apparently unsuccessful attempt to induce apoptosis.

This simple immunohistochemistry technique also proves useful for determining the extent of tissue invasion from a primary cancer. Figure 11(a) is an H&E-stained section of invasive ductal carcinoma. Areas of $P2X_7$ labelled cells (Figure 11(b)-arrows) can be seen at least 1.2 cm from the primary tumour on the upper margin, while all the other margins were found to be clear of label. These labelled acini otherwise appear unremarkable by H&E. Most of the receptors in these otherwise unremarkable cells are found to be intracellular so that apoptosis cannot yet be induced by this pathway.

## Discussion

In this study, $P2X_7$ receptors were not found in either normal or in approximately 90% of hyperplastic lobules. In all forms of lobular, ductal, *in situ* and invasive carcinomas however, labelling was intense but only invasive carcinoma cells displayed surface receptor. These findings were completely independent of hormonal cyclic changes and are consistent with recent studies that show that apoptosis is increased with increased proliferation and is associated with breast cancer [22]. However, the fact that the $P2X_7$ receptors expressed on the cell surface in invasive cancer were non-functional indicates that this potential apoptotic pathway is not available in breast cancer. This simple immunohistochemistry method has more value in identifying early cancerous change in hyperplastic lobules rather than established cancer, as the latter is clearly identifiable by both H&E and cell surface $P2X_7$ staining. Furthermore, cell surface expression of the receptors can be used to verify the invasive state of affected cells.

Mild hyperplasia displayed no $P2X_7$ label in the affected epithelial cells. Usual and atypical hyperplasias had distinct $P2X_7$ receptor labelling patterns that could clearly differentiate between normal and potentially cancerous lobules. These differences were not visible by H&E stain. In usual hyperplastic epithelium, 88% of lobules ($n = 3984$) showed no label while 12% were strongly positive. These receptors remained intracellular. Although they were upregulated, they were not yet transported to the cell surface where they have the potential to induce apoptosis. This observation was essentially an 'all-or nothing' lobular phenomenon. Each cell of an affected acinus was labelled whereas in adjacent normal lobules, no acinal cells were labelled. This result suggests that a fundamental neoplastic change had occurred in 12% of the lobules and that the expression of $P2X_7$ receptors was possibly a protective response to that change, with an attempt to up-regulate apoptosis. The affected lobules may also be connected in the same affected duct.

Atypical lobular and ductal hyperplasia is thought to have a malignancy progression risk that is 4–5 times greater than that of the general population [3, 4, 23]. In the current study, 90.4% of all lobules in tissue clinically diagnosed as atypical hyperplasia did not label for $P2X_7$ cytolytic receptor, suggesting that they had not been subject to the early biochemical changes associated with neoplasia. However, the remaining 9.6% of the total lobules (Figure 5(b) AR-arrow) were strongly and uniformly labelled with intracellular receptor, possibly indicating the presence of early *in situ* cancer somewhere within this ductal unit. Moreover, the connectivity between the labelling of a minority of lobules in UDH and the likely progression of these affected lobules to ADH and then DCIS appears to be well established from the results shown here by means of the upregulation of the intracellular $P2X_7$ receptors.

All cases of diagnosed *in situ* and invasive cancer exhibited an intense nuclear and cytoplasmic $P2X_7$ label in all epithelial cells with the invasive cancers progressing to the stage of deploying the receptors on the cell surface. Figure 11, from a case of invasive

ductal carcinoma, demonstrates that receptor labelling can be used to determine the extent of neoplastic invasion. Figure 11(b) shows that pre-cancerous acini are present at least 1.2 cm from the primary tumour on this margin with all other margins having been shown to be clear of label, suggesting that not all affected tissue was excised.

DCIS represents approximately half of all breast carcinomas detected by mammographic screening. The lesion is divided into several architectural subtypes as defined by H&E stain. As a whole though, DCIS consists of a malignant population of cells that lack the capacity to invade through the basement membrane. They may however spread throughout the ductal system thereby involving an entire sector of the breast. DCIS has several morphological variants [24]. LCIS involves proliferation of a monomorphic population of cells in the terminal ducts or acini. The cells are larger than normal and often signet ring cells containing mucin are present. Compared with ductal carcinoma, lobular carcinoma is found less frequently, representing only 1–6% of all breast carcinomas. Invasive carcinoma develops from LCIS in only 25–35% of cases [25]. In the current study, all cases of DCIS and LCIS as well as invasive carcinoma, showed an intense up-regulation of $P2X_7$ receptors in all affected epithelial cells, but significantly the more dangerous invasive cells could be differentiated from the *in situ* tumours by means of the surface expression of the receptors observed only in the invasive cells.

We have shown that labelling for $P2X_7$ can distinguish between cells that have been transformed biochemically from those that are biochemically normal, an observation not possible using H&E staining. Precise identification of early biochemical changes associated with signs of preneoplasia in usual and atypical hyperplastic cells is therefore clear and unambiguous. All normal cells are devoid of the receptor while all cancer cells and those cells that are turning cancerous are uniformly and heavily labelled for the apoptotic receptor. Only the invasive cells show surface deployment of the receptors. Thus there is the potential to discriminate between cells forming low-risk tumours and cells that have more invasive potential. As we have previously shown in the prostate, this method allows early and appropriate intervention where the H&E findings might be ambiguous. Conversely, anxious patients with non-cancerous lesions or those with no sign of invasive cancer can be confidently reassured. This method also identifies the true extent of precancerous spread in infiltrating lesions, to facilitate the accurate reporting of clear surgical margins. A multiple-time point prospective study is necessary to establish these findings as an independent predictor of risk.

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