Comparison of core oestrogen receptor (ER) assay with excised tumour: intratumoral distribution of ER in breast carcinoma

A G Douglas-Jones, N Collett, J M Morgan, B Jasani

Abstract

***Aims*—The use of the H score (involving the assessment of intensity and distribution of positivity) on sections stained for the oestrogen receptor (ER) by immunocytochemistry (ICC) allows different samples to be compared and detailed correlations to be made between hormone receptor expression and morphology. This study assessed the reliability of core biopsy in predicting ER expression in the same tumour excised later at treatment. The distribution of ER within excised tumours was investigated.**

***Methods*—The distribution of ER positivity was investigated in 51 diagnostic core biopsies and across the diameter of 51 subsequently excised tumours in a field by field (magnification, ×40; field diameter, 0.4 mm) assessment using the semiquantitive H scoring system.**

***Results*—The ER H score in diagnostic core biopsy was significantly higher (p = 0.05, paired rank test; overall mean, 130; n = 51) than the mean in the corresponding excised tumour (mean, 110; n = 51). There was a significant downward trend in ER positivity from the periphery of tumours towards the centre (p = 0.001). The reduction of ER positivity was 6 H score units (2%)/mm. If core biopsies were orientated with the tumour edge at one end no change in ER positivity with field number along the length of the core could be demonstrated.**

***Conclusions*—ER estimation in core biopsies correlated well with expression in tumours but ER expression was higher in the core biopsies than in the excised tumours. ER expression was higher at the periphery of tumours than at the centre. The higher ER expression in cores may reflect the higher chance of sampling the peripheral part of a tumour using a needle core.**

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Traditionally, the biochemical assessment of the oestrogen receptor (ER) status of a breast tumour using the dextran charcoal method has involved obtaining a piece of fresh tumour from the gross specimen and homogenising this tissue for the assay. The ER content was expressed as a proportion of the cytosolic protein extracted, but there were major uncertainties as to the proportion of tumour to stroma and the inclusion of other elements, particularly normal breast tissue, within the material.[1–3] Recently, immunocytochemistry (ICC) on formalin fixed paraffin wax embedded material has allowed correlations to be made between immunopositivity and morphological features.[4] The assessment of biological markers including ER on fine needle aspirate material or tumour imprints using an immunocytochemical assay correlates well with the measurement of ER on the excised tumour by ICC or the dextran charcoal method.[5–11] The increasing use of needle core biopsy for the preoperative assessment of breast lesions allows the preoperative determination of ER status by ICC on the core.[12] Therefore, it is possible to compare the ER status of a tumour, as determined by a preoperative needle core biopsy, with the ICC ER positivity of the subsequently excised tumour.

In our study, ER expression was assessed semiquantitatively in the core and compared with expression in the subsequently excised tumour. The distribution of ER within the excised tumour was examined and reduced ER positivity in the centre of the tumour was shown. The distribution of ER positivity in the cores was investigated to determine whether this trend in oestrogen positivity was the result of delayed fixation in the centre relative to the periphery of the tumour or a biological difference between the centre and the periphery of breast tumours.

Materials and methods

CASE MATERIAL

Fifty one pairs of core biopsies and subsequently resected tumours were identified from the files of the University Hospital of Wales. In all cases, the core and tumour had routine ER ICC performed on both the core and the tumour sections. Because the cores and tumours had been assayed at different times it was possible that any difference was the result of variations in assay sensitivity on the two separate occasions. To investigate this, fresh sections were cut from the core and the subsequently excised tumour mass, and these were placed on the same slide and assayed for ER together.

ER assay ICC was performed using the 6F11 antibody and an automated immunostainer (Optimax plus; Menarini, Wokingham, UK). Bound antibody was detected using the Vector Elite link and label secondary detection system (Vector, Peterborough, UK). The laboratory is a participant in the UK National External Quality Assurance Scheme (NEQAS) ER ICC

scheme and the assay has been validated against UK NEQAS standards.[13]

SEMIQUANTITATIVE ANALYSIS OF ER POSITIVITY

The H score system was used by a single observer (NC) (to eliminate interobserver variation) examining the tumour with a ×40 objective (field diameter, 0.4 mm). In each field, the proportion of cells staining positively was assessed and then within that positive population the percentage staining strongly (readily visible at low power), the percentage staining weakly (visible clearly only at high power), and the percentage with intermediate positivity was assessed. The sum of these categories equates to the overall percentage positivity. A "staining" H score was then calculated as follows: score (out of a maximum of 300) = 1 × percentage of weak (+); 2 × percentage of moderate (++); 3 × percentage of strong (+++).

For excised tumours, the edge of the tumour was taken as the reference point for all analyses. Fields were examined sequentially from the edge of the tumour through the centre and to the other edge of the tumour. The mean number of fields examined was 18 (range, 11–27)/core and 16.5 (range 9–25) for excised tumours.

If a field contained no tumour this was noted and no H score reading was available. For larger tumours, not all of the tumour was represented in the block being analysed. In these cases, the edge was identified and fields sequentially analysed to the deepest edge of the tumour. In a subgroup of core biopsies (n = 11), the edge of the tumour could be identified by the presence of fat containing normal breast lobules. In these cases, the edge of the tumour was taken as the reference point and H scores were assessed from the edge of the tumour towards the centre in sequence. In core biopsies (n = 40) in which the edge of the tumour could not be identified, fields were assessed from one end of the core to the other.

ANALYSIS

In analysing the data, the edge of the tumour was taken as the reference point. For complete tumour diameters, fields were arranged sequentially from the edge to the centre of the tumour from both directions, so that sequential fields from the periphery towards the centre were analysed together. Where only part of the tumour was available for assessment, the fields were arranged from the periphery towards the centre. Because the size of tumours varied, the numbers of observations reduced with increasing distance from the reference edge of the tumour.

INTRAOBSERVER VARIATION OF H SCORE

All H score assessments were performed by a single observer (NC) to eliminate interobserver variation. The intraobserver variation in H score assessment (for NC) from one reading to another was investigated by re-examination of 10 cases (five core biopsies, 57 fields; five excised tumours, 93 fields) on separate occasions at least two weeks apart without knowledge of the previous assessment.

STATISTICAL METHODS

The null hypothesis was that the ER positivity was uniform throughout the tumours and cores and this was tested in two ways.

H scores for ER staining were paired with the corresponding microscopic field so that there were multiple values of H for each field number regression analysis performed. The preconditions for applying a regression analysis were examined by noting that the variance of H scores for each field number was homogeneous across the group of all field numbers. However, the ER data sets (H scores) for whole tumours and core samples were not normally distributed, as determined by normal plots of the standard normal deviate and the application of D'Agtostino's test. Therefore, regression analysis was performed both on raw data and data transformed using Anscombe's transform, $y = (H + 0.375)$, which normalised the data. Both analyses produced identical results. The null hypothesis that the regression coefficient is zero was then tested with a t test. The regression coefficients for whole tumours and core samples were also compared using a t test.

Dunnet's test was used on transformed data to compare the H scores of a control mean (tumour edge field; that is, field 1) with each of the other field means in sequence.

Paired data for cores and resections were compared using the Wilcoxon paired rank test.

Results

INTRAOBSERVER VARIATION OF H SCORE

The reassessed mean H scores were less than 3% different to the first reading (range, 0–9.64 H score units) for excised tumours, and less than 3% (range, 0–9.4 H score units) for core biopsies (data not shown).

DISTRIBUTION OF ER POSITIVITY WITHIN WHOLE TUMOURS

Figure 1 shows the H score readings through the diameter of an individual tumour; reduced ER positivity is seen towards the centre. Figure 2 shows a scattergram of H scores for all excised tumours, together with the linear regression line. The null hypothesis was that the H score would not vary with field number and it predicted that the slope of this regression line would be zero (indicating a horizontal trend line through the data). The real value of the regression coefficient was −2.428 (95% confidence interval (CI), −1.71 to −3.15; SE, 0.368; for 659 data pairs of H score and field number). The t test showed a significant

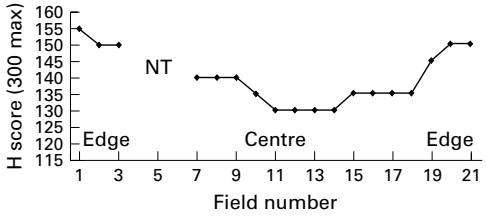

Figure 1 Plot of field by field H scores across the diameter of a single whole excised tumour showing reduced immunopositivity for oestrogen receptors (ER) in the centre. No tumour was present for assessment in fields 4, 5, and 6 (NT, no tumour).

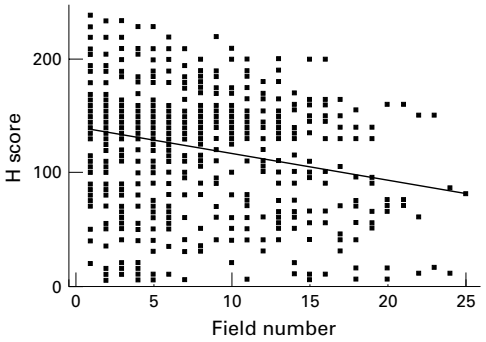

Figure 2 Plot of all H scores from all excised tumours plotted from tumour edge (field 1) to tumour centre for regression analysis. There is a significant downward trend in oestrogen receptor (ER) positivity as the field number increases (p = 0.001).

downward trend of the value of the H score as the field number increased ($t = -6.6$; p = 0.001). The equation that predicted the H score against field number was: H = −2.428n + 140.9 (where n is the field number). Figure 2 illustrates that for every successive microscopic field, from the edge of a tumour towards the centre, the H score is predicted to decrease by 2.4 units.

The H scores for the 11 core samples in which the edge of the tumour could be determined were treated similarly. Figure 3 shows the scattergram and fitted regression line for data pairs of H values and corresponding field numbers for the core data (n = 147). The value of the regression coefficient was −0.00757 (SD, 0.045). This regression coefficient was not significant ($t = -0.3827$), indicating no significant downward trend of the H value as the field number increases.

Dunnet's test was used as an additional statistical test to detect a trend in the H scores versus field number. In this application, the H score at the tumour edge (field 1) was compared with each other mean sequentially, with the aim of finding a trend in the q values (which are a measure of the degree of difference between the mean H scores and the control mean). For the whole tumour data, Dunnet's test showed higher q values associated with the higher field numbers, with two q values for fields 18 and 20 exceeding the value

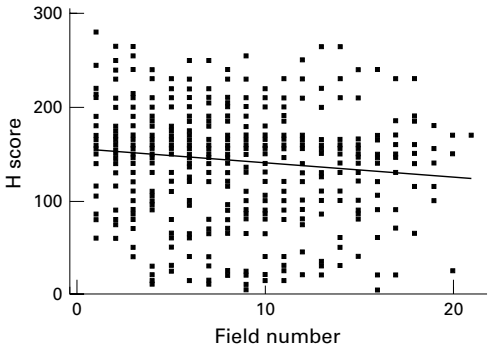

Figure 3 Plot of H scores from 10 core biopsy specimens in which the edge could be determined (orientated cores) plotted from tumour edge (field 1) towards tumour centre for regression analysis. For cores there is a no significant downward trend in oestrogen receptor (ER) positivity as the field number increases.

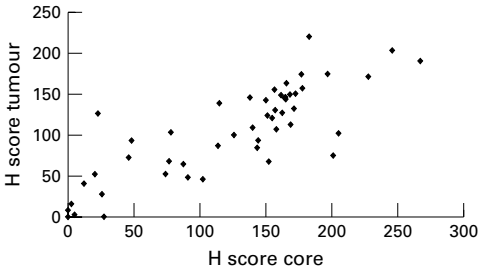

Figure 4 Plot of mean of all H scores from core specimens against mean of all H scores from subsequently excised tumours. There is a significant correlation between oestrogen receptor (ER) positivity in cores and subsequently excised tumours (correlation coefficient = 0.876; p = 0.001).

for significance, confirming the results from regression analysis for whole tumour data.

CORRELATION OF ER ESTIMATION BETWEEN CORE AND EXCISED SPECIMENS

Figure 4 shows the core ER (mean of all fields examined) plotted against excised tumour ER. There is a good correlation between H score in cores and excised tumours ($r^2 = 0.768$; correlation coefficient $r = 0.876$).

The mean of all available H scores for each core was paired with the mean H score of the corresponding excised tumour in a paired rank test. The H score for cores was significantly higher from resections (p = 0.05, paired rank test). To illustrate the higher ER in core samples the mean of all core samples (n = 51; mean, 130) is plotted with the mean of all excised specimens (n = 51; mean, 110) in fig 5.

In the first analysis, ICC on the core samples and resected tumours was performed at different times. Thus, it was possible that the difference in H score staining between the two specimens was related to interspecimen technical variation. To investigate this possibility, the core and tumour were cut on to the same slide and immunostained together in the same assay in a sample of 10 cases. Knowing that the H score from the periphery to the centre of the tumour varied, immunostaining was assessed at the edge of the tumour, the middle of the tumour, and a point halfway between for the resection specimens. For cores, assessment was performed at each end of the core and in the

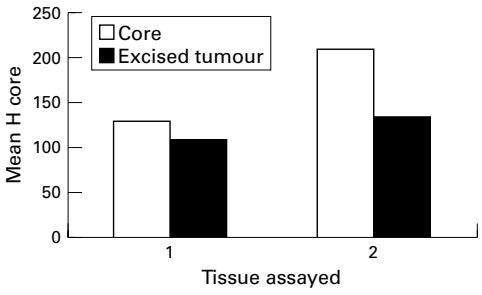

Figure 5 1: Plot of mean of H scores from all core specimens (n = 51) against mean of H scores from all subsequently excised tumours (n = 51) when oestrogen receptor (ER) immunocytochemistry (ICC) was performed on different slides in different assays. 2: Plot of mean of H scores from a sample of core specimens (n = 10) against mean of H scores from subsequently excised tumours (n = 10) when ER ICC was performed on the same slide in one assay. ER positivity is significantly higher in the cores (p = 0.05, paired rank test) using both approaches.

centre field. A paired rank test showed a significant difference between the cores and the resection specimens, confirming that more immunocytochemically detectable ER is seen in core samples than in subsequently excised tumours. Again, the mean H score for cores (n = 10; mean, 210) was higher than the mean of excised tumours (n = 10; mean, 135) (fig 5).

Discussion

The analysis of a large number of clinical trials has established that the benefit of hormone based treatment is directly related to the degree of ER expression by breast tumours. There is evidence that those patients who have ER negative tumours gain no benefit from hormone based treatment.[14 15] The assessment of ER status has become important in routine clinical practice to identify those patients with ER negative breast tumours for whom hormone based treatment is likely to be ineffective, so that chemotherapy can be offered as adjuvant treatment in its place. In recent years, there has been a rapid change from the use of fine needle aspiration cytology of breast cancers for preoperative diagnosis to the use of core biopsy.[12] This has allowed ER analysis to be performed on core biopsies and a previous study has shown a high degree of correlation (97%) between needle core biopsy and subsequent open excision for ER assessed as positive or negative (not using the H score assessment).[16] In our present study, a significant correlation between ER expression in the core and subsequent resection has been demonstrated using semiquantitative H scoring, which takes into account the intensity and distribution of positivity (correlation coefficient = 0.876; p = 0.001). The H score for cores was significantly higher than in the subsequent resection and this difference was also seen when the core and subsequent resection specimens were immunostained together on the same slide. This indicates that the difference is not the result of variation in ICC sensitivity from assay to assay but is the result of more immunocytochemically detectable ER in the cores. Possible reasons for this are rapid fixation of the core compared with the whole tumour or core samples being more often obtained from the periphery of the tumour rather than the centre. Alternatively, cores spend a shorter time in fixative before being processed because a rapid diagnosis is required, which might reduce ER positivity, whereas whole tumours may remain in fixative for longer periods.

A further consideration is the problem of the so called edge effect in histochemistry, where tissue at the periphery may show enhanced immunopositivity. The edge effect usually appears as a non-specific brown positivity of all structures at the periphery of the tissue. However, in H score estimation for ER, only nuclear positivity is assessed and non-specific positivity of cell membranes and cytoplasm is ignored. In the analysis of whole tumours, this is unlikely to be an important confounding factor because in most instances there was breast fatty tissue around the tumour edge, so that tumour tissue was not present at the edge of the section. In core biopsies, tumour is present at the edge of the tissue and it is possible that the increased positivity seen in cores is a result of the edge effect. However, this was not the case in our study because the use of automated immunostaining machines has eliminated this artefact.

In the past, there has been discussion on the heterogeneous expression of ER in breast carcinoma. Many authors have not defined with any precision what is meant by the term tumour heterogeneity. The use of immunocytochemical analysis has allowed the detailed correlation of ER positivity with morphology, overcoming the problems associated with the biochemical methods and allowing a reassessment of heterogeneity. At the individual cell level, it is not uncommon to find within a microscopic field of 0.4 mm diameter a proportion of nuclei that are negative for ER, whereas adjacent nuclei show a range of positivity. The tumour may be said to be heterogeneous at the cellular level. The H scoring methodology allows for this range within one field. The overall percentage of positive nuclei is estimated at the start of the analysis of the field, with subsequent weighting given for intensity of positive staining. When the ER positivity of the tumour is analysed on a field by field basis across the diameter of a tumour at intervals of 0.4 mm, as in our study, the H score is fairly constant, indicating little intratumoral variation in ER content at this level (fig 1). Some tumours show strong ER expression in some areas and weakly positive or negative expression in others at low power (×2). In our experience, this type of heterogeneity, which amounts to two adjacent tumour clones (one positive and one negative), is uncommon and was seen in five of 980 (0.5%) breast tumours. Early studies in which samples of fresh tumour were taken from the centre, mid zone, and periphery of large breast tumours showed differences in the ER measurements by dextran charcoal assay across the diameter of the tumour, which is in accordance with the observations presented here.[2 17] In these studies, large amounts of tissue were required for the assay, so that only large steps of 5 mm across the diameter of the tumour were possible. It was known that the tumour cell to stromal ratio could vary across the tumour diameter, which could artificially change the measured ER values. Some authors think that the results of the dextran charcoal assay should only be interpreted in association with measurements of the stroma tumour ratio of the material assayed.[1 2 18] In our present study, we have performed serial estimation of ER expression at a much finer level, using steps of 0.4 mm from the edge of excised tumours towards the centre. The H scoring system is independent of the tumour stroma ratio and showed a downward trend in ER positivity of approximately 2%/mm from the periphery of the tumour towards the centre. Within an individual tumour, the H score system is not sensitive enough to document changes as small as this on a field by field analysis, but in a large

tumour fields from the periphery and the centre may show detectable differences in immunopositivity. Thus, for a tumour 20 mm in diameter (radius, 10 mm), our observations would predict a fall in ER positivity of approximately 20% from the periphery to the centre. For a tumour with an H score of 200 at the periphery this would predict an H score of 160 at the centre. This reduction in tumour receptor positivity is unlikely to change the designation of a tumour from ER positive to ER negative on the basis of sampling, unless the tumour is weakly positive with an H score of 60 at the periphery of a 20 mm tumour. Because core ER expression has been shown to be higher than excised tumour ER expression, it is possible that some cases negative on ICC analysis of the excised tumour would show ER positivity on ICC of corresponding core biopsies. This possibility could be investigated by selecting a group of excised tumours that are ER negative on ICC and attempting to demonstrate ER positivity on the corresponding core biopsy. Such a study would provide an indication of the impact of the findings of our study on ER categorisation of tumours in clinical practice.

The variation of ER positivity depending on distance from the tumour surface raises the question as to whether this is a biological phenomenon associated with increased metabolic and mitotic activity and growth at the periphery or whether it is an artefact caused by the delayed fixation of cells in the centre of the tumour. Analysis of the H score along the length of the core biopsies was used to investigate this question. It can be assumed that the core biopsies were uniformly and rapidly fixed along their length immediately after excision. The lack of any detectable change in ER H score along the length of orientable cores suggests that the reduction in ER positivity in the centre of a tumour may be related to delay in fixation.

Conclusions

Our study has shown that there is more immunocytochemically detectable ER positivity at the periphery of a tumour than at the centre. No such decline could be measured from the edge of a tumour in a core biopsy. This result means that the reduction in ER positivity in the centre of tumours might result from differences in rates of fixation. There is more immunoreactivity for ER in a core biopsy than in the resected specimen and this may reflect the increased preservation of ER immunoreactivity by rapid fixation of the needle core or the higher chance of sampling the peripheral part of a tumour using a needle biopsy.

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