Cyclin D1 amplification and expression in human breast carcinoma: correlation with histological prognostic markers and oestrogen receptor expression

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Abstract

Aims—To study the amplification of the Cyclin D1 gene (CCND1) in human breast carcinoma; to relate this to Cyclin D1 protein expression; to relate these parameters to recognised pathological prognostic factors, including oestrogen receptor (ER) status.

Methods—DNA extracted from frozen sections of breast tumours (n = 36) was used for Southern blotting. Probes for CCND1, *c-myc* and the immunoglobulin heavy chain locus (IgH) were hybridised to tumour DNA. Immunocytochemical expression of Cyclin D1 protein and ER was studied in paraffin wax sections from the same tumours.

Results—Amplification of CCND1 was observed in 11% (four of 36) of tumours studied. Over expression of Cyclin D1 protein was observed in 73% (30/41) of tumours. There was no correlation between recognised histological prognostic markers and either gene amplification or expression. However, a weak association was seen between Cyclin D1 expression and ER status.

Conclusions—A disparity exists between locus amplification and over expression of Cyclin D1, suggesting the existence of another mechanism for raised protein expression. No significant correlation was detected between either Cyclin D1 amplification or over expression and established prognostic markers.

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There is a continual search for new prognostic markers in human breast cancer which may refine the selection of patients for particular treatment regimes.

Cyclins are proteins which are involved in the control of the cell cycle.¹ One such protein, Cyclin D1, has a role in the control of the transition between G1 and S phase in the cell cycle.² High expression of Cyclin D1 is observed during G1 followed by low expression during S phase.^{3,4} Deregulated expression of Cyclin D1 causes abnormalities in growth control, resulting in a decrease in the duration of G1.^{5,6}

The gene encoding Cyclin D1, termed CCND1, D11S287 or PRAD1, is located on band q13 of chromosome 11,⁷ and has potential as an oncogene.⁸⁻¹⁰ Accumulating evidence suggests that CCND1 is the *bcl-1* gene.^{11,12} Structural abnormalities affecting this band have been reported in a variety of human tumours, including parathyroid neoplasia,⁷ centrocytic lymphomas,¹³ oesophageal carcinomas,^{14,15} and squamous and breast carcinomas.^{16,17} A number of different types of abnormalities have been reported, including DNA deletions,¹⁸ translocations^{19,20} and amplifications.²¹ Amplification occurs in 10–20% of primary breast tumours^{8,22-24} and has been associated with a subgroup of primary breast tumours that have an unexpectedly poor prognosis.^{23,25-27} Although other genes are frequently co-amplified with CCND1—for example, *int-2*,^{28,29} *HST1*,³⁰ *EMS1*,³¹ and *Gst pi*,³² it has been suggested that amplification of a gene close to *int-2* and *HST1* may result in a subset of ER positive tumours with poor prognosis. Amplification of CCND1 may be the selective force on the 11q13 amplicon that is actually providing a growth advantage in breast tumours.^{8,21}

Expression of the Cyclin D1 protein has been studied in breast tumours only recently. One study suggests that over expression of this protein may occur in the absence of gene amplification.³³

In this study, we have related gene amplification and protein expression to recognised prognostic markers, including tumour size and grade, axillary lymph node status and ER status. It is hoped that this may assist in the elucidation of the role of this gene in the pathogenesis of breast cancer.

Methods

Samples from 41 women treated surgically for primary breast carcinoma were included in the study. Their mean age was 60 years (range 35–80 years). None of the patients had received preoperative chemotherapy.

Tumour samples were obtained both from mastectomy and excision biopsy specimens which had been sent fresh to the histopathology laboratory. On receipt, areas of tumour were identified macroscopically by a pathologist and a representative 10–15 mm sample selected for molecular biology studies. This was then snap frozen in liquid nitrogen and stored at –80°C. The remainder of the tumour was processed

for histopathological examination according to routine laboratory protocols. Preoperative blood samples (10 ml) were available for 10 patients.

MOLECULAR BIOLOGY

Tumour samples were removed from the -80°C freezer and cut in a cryostat at -20°C . Haematoxylin and eosin stained sections cut at three levels were examined to confirm the presence and amount of tumour in each sample. To qualify for inclusion in the study a minimum of 70% of the tissue volume had to consist of tumour cells.

Sixty serial $10\text{ }\mu\text{m}$ sections were cut from each tumour sample. Following the addition of lysis buffer, each was incubated with 50 units proteinase K at 37°C for one hour. DNA was extracted using a standard phenol chloroform protocol on an automated nucleic acid purification system (341 Applied Biosystems, Warrington, UK).

Digestion of DNA from frozen sections of tumour with the restriction endonuclease *Pst*I was followed by Southern blotting.³⁴ Digests of DNA extracted from peripheral blood were used as controls.

DNA on the blot was then hybridised to probes labelled by the random priming method.³⁵ The CCND1 probe was a 0.8 kilobase *Eco*R1-*Xho*I genomic DNA fragment derived from the probe designated "B" by Ar-

nold *et al*⁷ (a gift from the Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London). The *c-myc* probe was a 1.4 kilobase *Cla*I-*Eco*R1 fragment covering exon 3 at the 3' end of the *c-myc* gene. A probe for immunoglobulin JH chain was used as a control for DNA loading.

Autoradiographs were produced by exposure to a piece of pre-flashed Hyperfilm-TM (Amersham, Little Chalfont, UK) for two to seven days at 80°C . The optical density readings of each probe binding to lymphocyte DNA from patient blood was measured. From this, an average ratio of CCND1 and *c-myc* to IgJH chain probe was calculated. The normal range was defined as the average ratio $\pm 2\text{SD}$. Values above this range were regarded as representative of gene amplification.

IMMUNOCYTOCHEMISTRY

Representative sections selected from each tumour were mounted on APES (2% 3'-aminopropyltriethoxysilane) coated slides and high pressure antigen retrieval was used.³⁶

A monoclonal antibody directed against Cyclin D1 (a gift from Dr Jiri Bartek, Danish Cancer Society) was used at a dilution of 1 in 2000 in phosphate buffered saline at pH 7.4 overnight.³³ A commercially available monoclonal antibody directed against human oestrogen receptor (Dako-ER1D5; Dako, High Wycombe, UK) was used at a dilution of 1 in 100 in Tris buffered saline at pH 7.2 for one hour. The indirect avidin-biotin immunoperoxidase technique was used to demonstrate antibody binding sites. Negative (primary antibody excluded) and positive controls (test slides supplied with antibody) were included with each batch of slides stained.

All cases were assessed independently by two pathologists and any difference of opinion on initial evaluation was resolved by subsequent discussion.

HISTOPATHOLOGY

Histopathological assessment of haematoxylin and eosin stained tumour sections included the following parameters: tumour type, grade and size, the presence or absence of vascular invasion, and the degree of lymphocytic infiltration. Haematoxylin and eosin stained sections of lymph nodes received with tumour samples were also examined for metastases.

Results

MOLECULAR BIOLOGY

Samples taken from 36 of tumours in the study contained sufficient malignant cells for Southern blotting. Amplification of CCND1 was demonstrated in four (11%) of these, with a two- to fivefold increase in gene copy number for these tumours (figs 1 and 2).

The number of tumours showing *c-myc* amplification in this study was 33% (12/36). Fisher's exact test ($p>0.4$) shows that this value is in agreement with other studies.³⁷

Figure 1 CCND1 amplification in primary breast carcinoma. The ratio of optical density (OD) readings for hybridisation of the CCND1 and IgH probe is shown for each tumour. Four tumours show amplification with values above the normal range.

Figure 2 Autoradiograph of a Southern blot hybridised with the probe for CCND1 and then stripped and probed with the IgH probe. Amplification of CCND1 is seen in DNA from tumour numbers T11 and T14.

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Pressure cooker antigen retrieval resulted in even, reproducible staining with good morphological preservation. No inappropriate staining was observed in negative controls and there was appropriate staining in positive controls.

Cyclin D1 staining was predominantly nuclear and varied in intensity. Although cytoplasmic staining was observed in some cases, this was rarely found without associated nuclear staining.

On initial evaluation, 28/41 (68%) tumours in the study strongly expressed Cyclin D1.

Tumours with very weak staining, with only occasional positive nuclei or with a perinuclear pattern of staining, were classified as negative (five/41, 12%). An intermediate level of staining was observed in eight/41 (20%) tumours. After further evaluation, two of these cases were classified as positive and six as negative.

In total, therefore, 30/41 (73%) tumours were considered to have significantly high expression of Cyclin D1 protein and so were classified as positive. Fisher's exact test ($p > 0.4$) shows that this number of positive tumours is in agreement with other studies.^{33,38} Representative staining patterns are illustrated in fig 3.

Figure 3 Immunocytochemical demonstration of Cyclin D1 and ER in breast tumour tissue. A, Strong nuclear expression of Cyclin D1 ($\times 100$); B, strong nuclear expression of ER in a section from the same tumour ($\times 150$); C, intermediate expression of Cyclin D1 subsequently classified as positive ($\times 100$); D, weak cytoplasmic expression of Cyclin D1 classified as negative ($\times 100$).

Tumour stage and grade

Tumour type	Number of tumours
Ductal	
grade 1	4
grade 2	14
grade 3	4
Lobular	4
Special type	1

OESTROGEN RECEPTOR EXPRESSION

Positive staining for ER was observed in 24/41 (59%) tumours (fig 3B) indicating that tumour sampling was representative of a normal tumour cell population (Fisher's exact test $p > 0.48$).³⁹

HISTOPATHOLOGY

The tumours ranged in size from 0.7 to 10 cm (mean 3.5 cm). The table summarises tumour type and grade. Vascular invasion was identified in 13 cases and nodal metastases in 20.

RELATION OF CCND1 AMPLIFICATION AND CYCLIN D1 EXPRESSION TO PROGNOSTIC FACTORS

No significant relation between either CCND1 amplification or Cyclin D1 expression and histopathological prognostic markers was identified.

RELATION OF CCND1 AMPLIFICATION AND CYCLIN D1 EXPRESSION AND ER STATUS

Three of the four tumours showing CCND1 amplification also showed high ER expression. Cyclin D1 expression showed a weak correlation with ER status (correlation coefficient +0.3).

Discussion

This study has shown that there is a disparity between amplification of CCND1 and expression of its protein product, Cyclin D1. Immunocytochemical staining has revealed cases where there is high expression of Cyclin D1 in the absence of any measurable change in DNA copy number; in fact only 6% of the tumours over expressing Cyclin D1 have associated gene amplification. This disparity between gene amplification and expression is in agreement with other recent reports.^{33,38} We conclude that an unidentified mechanism is operating in some tumours to cause increased protein expression. Possible mechanisms include alterations to the gene promoter region or altered expression of a regulatory transcription factor, such as *c-jun*.⁴⁰ A longer mRNA half-life would result in the production of more active protein without the necessity for an increased gene copy number or increased transcription. A report on the existence of a truncated Cyclin D1 gene encoding a stable 1.1–1.3 kilobase mRNA in a human breast cancer cell line suggests that this short message is more stable than the normally dominant 4.2 kilobase transcript.⁴¹ Whatever the mechanism

may be, deregulated expression of this cell cycle controlling protein is likely to provide a selective growth advantage in tumours.

In common with other immunocytochemical studies some subjectivity exists in the interpretation of Cyclin D1 staining. This is compounded by the lack of agreement as to how different staining patterns should be classified. Gillett *et al*³³ subdivided Cyclin D1 staining into four categories: weak (57%), moderate (13%), strong (17%), and very strong (13%). Zhang *et al*³⁸ used three staining categories: positive (28%), moderate (53%), or negative (19%). Staining for Cyclin D1 is in its infancy and more work is needed to clarify the significance of intermediate staining patterns.

In this study neither CCND1 amplification nor protein expression seemed to be related to known histological prognostic markers. This may be because of the small number of tumour samples studied. There was, however, a weak association between Cyclin D1 expression and high ER expression. This supports the hypothesis that over expression of Cyclin D1 may define a subgroup of ER positive tumours that have an unexpectedly poor prognosis.²³

In the future the classification of tumours on the basis of their pattern of genetic alterations may add to currently established markers and enable refinement of existing classifications. This would have an immediate impact on patient care and may also lead to the use of treatments whose design is based on the biological consequences of gene mutations. An important step towards this goal is the development of a fuller understanding of how genetic alterations and the expression of gene products relate to the development of the malignant phenotype.

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