Cell cycle regulators and their abnormalities in breast cancer

P L Fernández, P Jares, M J Rey, E Campo, A Cardesa

Abstract
One of the main properties of cancer cells is their increased and deregulated proliferative activity. It is now well known that abnormalities in many positive and negative modulators of the cell cycle are frequent in many cancer types, including breast carcinomas. Abnormalities such as defective function of the retinoblastoma gene and cyclin-dependent kinase inhibitors (for example, p16, p21, and p27), as well as upregulation of cyclins, are often seen in breast tumours. These abnormalities are sometimes coincidental, and newly described interplays between them suggest the existence of a complex regulatory web in the cell cycle.
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Malignant cells, like their normal counterparts, progress through a life cycle that encompasses several consecutive phases, each of which must be completed before entering the next one. These phases are G1, in which the cells prepare their machinery for replication; S phase in which duplication of genomic information occurs; G2, an intervening phase; and M phase, in which the actual division (and therefore proliferation) takes place. Cells thus generated can either start a new cycle or remain in a state of quiescence, known as G0 phase. Although this scheme seems to be common to both normal and tumour cells, one of the most important characteristics of the latter is their increased proliferative capability, most likely resulting from impaired control of the regulatory elements of the cell cycle. Indeed, cell cycle regulators are subject to strict control in normal cells and their activities fluctuate according to external stimuli, whereas in neoplastic cells a variable degree of independence from such stimuli seems to emerge.

Regulation of the cell cycle
The cell cycle has several checkpoints that are controlled by an increasingly understood complex system of modulators, among which the retinoblastoma gene product (pRB), cyclins, cyclin dependent kinases (CDKs), and CDK inhibitors (CDKIs) are key members. The orderly progression through the cell cycle requires sequential activation and inactivation of these modulators. So far, one of the most well studied pathways of cell cycle regulation is that involving pRB, a negative regulator of the G1 to S transition, whose inactivation by different mechanisms, including phosphorylation by upstream elements such as cyclin–CDK complexes leads to cell cycle progression and proliferation (fig 1). Alterations in most of the above elements leading to increased proliferative activity have been observed in many different cancer models, including breast carcinomas, both in vivo and in vitro.[1]

Retinoblastoma protein
The RB gene was the first tumour supressor gene to be discovered and its alteration has been observed in many tumour types. The RB gene can be inactivated by mutation, viral insertion, or deletion and the inactivation of pRB can be achieved by phosphorylation.[2][3] In breast cancers, RB loss of heterozygosity (LOH) has been seen in ~ 25% of informative cases, but this abnormality does not always correlate with decreased protein synthesis.[4] Therefore, immunohistochemistry seems to be a better tool to define the concentration (although not the phosphorylation status) of the protein product of this gene. Indeed, we and others have observed an absence or substantial decrease in pRB synthesis in 10–45% of infiltrating breast carcinomas, and this downregulation seems to be important for neoplastic progression because it is associated with increased proliferation of tumour cells.[5–8] Unfortunately, it has not been settled definitively whether the immunohistochemical evaluation of pRB has prognostic implications in breast carcinomas, because of the differences in methodology and criteria used for assessing pRB abrogation. The evaluation of the phosphorylation status of the protein has also been proposed as a prognostic tool,[9] because pRB controls an important regulatory pathway in G1 in those cases without genetic abnormality, and it is subjected directly to upstream regulators, such as cyclins and CDKs.

Cyclins D1 and E
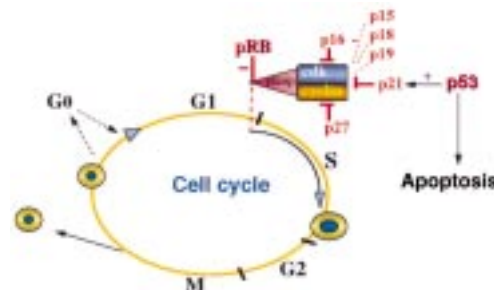Cyclins are proteins that regulate cell cycle specific kinases by direct binding, and are

Department of Anatomical Pathology, Hospital Clínic of Barcelona, Institut d'Investigacions Biomèdiques "August Pi i Sunyer" (IDIBAPS), University of Barcelona, Barcelona 08036, Spain
P L Fernández
P Jares
M J Rey
E Campo
A Cardesa

Correspondence to:
Dr Fernandez.
email:
fernandez@medicina.ub.es

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Figure 1 Scheme of cell cycle regulation through the retinoblastoma gene product pathway.

activated sequentially (cyclin D1 in early G1, cyclin E afterwards), therefore modulating CDK activity and thus stimulating pRB phosphorylation (inactivation) leading to cell cycle progression.[10] Among the different cyclins, D1 and E are probably the most extensively studied in most cancer systems, including breast tumours.[11]

Cyclin D1 is considered to be a weak proto-oncogene because of its capacity to transform fibroblasts together with activated H-Ras.[12] In addition, transgenic mice that overexpress the gene encoding this cyclin develop mammary hyperplasia and adenocarcinomas.[13] Cyclin D1 activation was first observed in parathyroid adenomas as a result of chromosomal inversion involving its locus at 11q13 and the parathyroid hormone locus at 11p15,[14] and subsequently by chromosomal translocation in some types of lymphomas.[15] However, in solid tumours, the most frequent mechanisms of cyclin D1 activation are gene amplification and mRNA/protein overexpression.

Alterations involving cyclin D1 in breast cancers include gene amplification in ~ 15–20% of cases,[16–21] and mRNA and/or protein overexpression in > 50% of cases,[6 17 21–23] indicating that abnormalities involving this cell cycle regulator can arise at different molecular levels. Interestingly, an association between overexpression of the cyclin D1 gene and hormone receptor expression has been observed consistently in breast tumours[6 21 24–27] and, moreover, we have observed an association between oestrogen receptor negativity and decreased cyclin D1 mRNA in tumours that do not overexpress cyclin D1.[6] This is consistent with in vitro experiments that show cyclin D1 upregulation by oestrogens[28–30] as well as downregulation by anti-oestrogens.[31 32] These observations support the hypothesis that one of the mechanisms by which steroids stimulate breast cancer cell proliferation might be through cyclin D1 induction. Unexpectedly, cyclin D1 has been reported recently to activate the oestrogen receptor by physically binding to it, thus upregulating oestrogen mediated transcription of a potentially wide range of targets.[33] An unexplained issue is why the upregulation of cyclin D1 does not usually correlate with increased proliferation of tumour cells.[6 24] Some authors have postulated that excessive amounts of this product could be toxic to the cell.[17] Alternatively, it might be hypothesised that upregulation of cyclin D1 occurs in the early steps of tumour development, being then clonally conserved without affecting tumour proliferation, which could then be controlled by other modulators. In this sense, cyclin D1 overexpression has been seen in up to 50–87% of ductal carcinomas in situ and in cases of atypical ductal hyperplasia.[27 34 35] A more likely explanation is that the great complexity and variety of modulators of the cell cycle precludes a simplistic assignment of direct proliferating or antiproliferating capability to only one of them.

Because of the technical feasibility of cyclin D1 immunohistochemical evaluation, several groups have attempted to use this assessment as a prognostic tool, and have produced contradictory information.[25 26 36 37] Given the likely proliferative effect of oestrogen on breast tumour cells through cyclin D1 stimulation, analysis of the effectiveness of anti-oestrogen treatment in cyclin D1 overexpressing tumours might prove fruitful, and it could be hypothesised that cases in which such upregulation is caused by gene amplification might be less responsive to this type of treatment because of its hormone independent activation. So far, in vitro studies have shown that the cytostatic effect of anti-oestrogens is not prevented by cyclin D1 overexpression in breast carcinoma cell lines.[38]

Cyclin E is a regulatory subunit of CDK2 and, like cyclin D1, seems also to modulate the G1 to S phase transition through phosphorylation of pRB as a possible redundant mechanism.[7 39] However, recent data suggest that the inactivation of pRB requires the sequential and complementary action of at least the cyclin D1–CDK4–6 and cyclin E–CDK2 complexes.[40] Cyclin E activity appears to be regulated in normal cells but its regulation seems to be impaired in breast tumour cells because of the existence of isoforms, which together with CDK2, can form a kinase complex capable of remaining active throughout the cell cycle.[41] Cyclin E is overexpressed in a subset of breast carcinomas and this expression usually correlates with low cyclin D1 and oestrogen receptor negativity.[7 42] Although long term studies and large series of breast tumours need to be analysed, potentially, the immunohistochemical demonstration of cyclin E overexpression could be more useful as a prognostic marker than cyclin D1.[42]

CDKIs

Apart from cyclin–CDK complexes, the progression from G1 to S phase is regulated by an increasingly recognised number of low molecular weight CDKIs. Currently, CDKIs are grouped into two families: the p16[INK4]–p15[INK4B] and the p21[WAF1]–p27[KIP1] families.[1 43] Because of their inhibitory activity on cell cycle progression, CDKIs are considered to be potential tumour supressor genes. The first CDKI that was implicated in carcinogenesis was p16, as a result of the existence of frequent gene alterations in different cancer cell lines, but these are rare in primary breast carcinomas.[44–49] So far, little is known about its expression in vivo in primary breast tumours because immunohistochemical results have been inconsistent, although abnormally low or absent expression of this product is frequent according to some authors.[50] In this respect, methylation of the gene might account for such types of abnormalities, and this phenomenon seems to occur frequently in breast tumours and other types of cancers.[51 52] Far from clarifying the subject, another recent report finds frequent p16 hypomethylation of primary and metastatic tumours when compared with normal breast tissue,[53] therefore making the regulation of this tumour supressor gene in normal and cancer mammary cells very controversial.
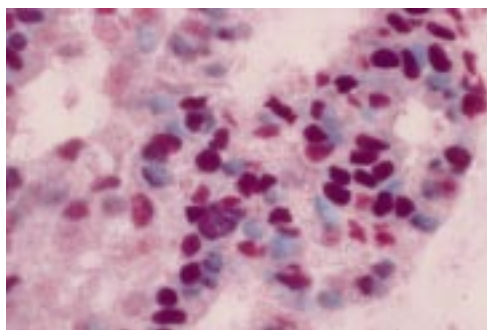
Figure 2   Double immunohistochemical staining for p53 (red) and p21 (blue) in a case of infiltrating ductal carcinoma. Coincidental staining for both markers is seen in the nuclei of several tumour cells (violet). Haematoxylin/alkaline phosphatase–fast red/beta galactoside–X-gal.[59]
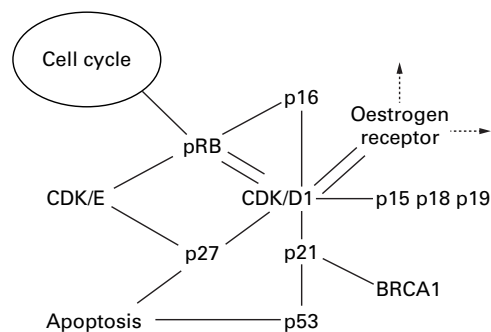

Figure 3   Scheme of some proposed interplays between cell cycle regulators. Double lines indicate regulatory loops and feedback mechanisms. Single lines can indicate either a stimulatory or an inhibitory association.

p21 is a CDKI with a wide spectrum of CDK substrates that has been implicated in the mechanisms of cell cycle arrest that allow cell DNA repair.[54] In this sense, p21 is responsive to wild-type but not mutant p53.[55] In addition, p21 seems to be related to differentiation in several tumour models including larynx and colon carcinomas.[56 57] Although p21 gene mutations are rare, p21 immunohistochemical overexpression is seen frequently in breast carcinomas in which it is associated consistently with high tumour grade, and it is also detected in early stages such as in situ lesions.[58–60]. Interestingly, when p53 protein status is concomitantly analysed, the expression of p21 seems to be independent of the former, because intense p53 (putatively inactive) and p21 nuclear expression can coexist (fig 2). Contrarily, p21 positive cases frequently overexpress the cyclin D1 gene,[59] suggesting that, as proposed by Chen *et al*,[61] this CDKI could be involved in cyclin D1 modulation in vivo. The potential prognostic use of p21 expression is not yet clear, because both high and low concentrations of the protein have been related to short survival.[58 60] Similarly to other cell cycle regulators, such as cyclin D1, and other cancer models, the predicted association between the deregulation of p21 with proliferation has not been demonstrated so far in primary tumours, because p21 downregulation does not correlate with increased proliferation.[58 59]

p27 is one of the latest cell cycle related proteins to come into the spotlight because of its potential strength to predict outcome in several types of tumours, such as stomach and breast carcinomas. This protein effectively induces cell cycle arrest and decreases cyclin–CDK activity in breast cancer cell lines.[62] In addition, and like other members of the CIP/KIP family including p21 and p57, p27 has a role in assembling and targeting CDK4 and cyclin D1 to the nucleus.[63] p27 is underexpressed frequently in breast carcinomas, and such underexpression seems to be associated with poor prognosis and a more aggressive phenotype.[64–66] How this gene is downregulated in these neoplasms is not clear, but p27 genomic alterations seem to be rare events in human neoplasms,[67 68] and post-transcriptional mechanisms have been proposed.[66 69 70] A relation between hormone stimuli and the expression of the gene was shown in breast carcinoma cell lines in which anti-oestrogen administration not only decreased expression of the cyclin D1 gene, but also the increased expression of the genes encoding p27 and p21,[32] which could possibly explain some of the therapeutic effects of hormone treatment.

Interplay between cell cycle regulators
From all the above, it is clear that the regulatory pathways of the progression through the cell cycle of both normal and tumour cells are by no means simple.[71] The discovery of new modulators (p15, p18, p19, p57, CDKs, etc.), whose inclusion in this review would require extensive data, causes the scheme to be redrawn continuously in a more complicated manner. In addition, it is not only the great number of modulators, but also the existence of increasingly more complex interplays among them, which further precludes a simplistic approach. For example, it is now known that pRB can regulate expression of the genes encoding cyclin D1 and p16 in cell lines, thus creating complex autoregulatory loops.[72–74] Studies in primary breast tumours have shown that there is an inverse relation between pRB and p16 synthesis.[5] Yet, it can be speculated that tumours lacking pRB function will have no advantage in concomitant alterations in other regulators like cyclin D1 and p16, whereas such abnormalities could interact to enhance tumour proliferation provided a normal pRB function.[75]

As mentioned before, cyclin D1 is not only involved in direct cell cycle activation , but it has also been proposed to activate the oestrogen receptor by physically binding to it, thus upregulating oestrogen mediated transcription.[33] Therefore, cyclin D1 may be regulated by a positive feedback loop. Another perplexing proposal is the possibility that p53, through p21, can induce cyclin D1,[61] thus linking the apoptotic pathway to cell cycle regulation (fig 3), a link strengthened by the possible apoptosis promoting activity of p27 in cancer cell lines.[76] The possible upregulation of the gene encoding p27 by cyclin E[77] helps to create complex regulatory feedbacks mechanisms involving CDKIs. Finally, it has been reported recently that the hereditary breast and ovarian cancer related tumour supressor

gene BRCA1 might contribute to cell cycle arrest and growth suppression through the induction of p21.[78]

Therefore, it seems that a kind of "regulatory web" with complex regulatory positive and negative feedbacks mechanisms and interconnections regulates cell cycle progression, and probably other cell functions (fig 3). Such complexity will most likely call into question the future value of the assessment of individual tumour markers for prognostic and therapeutic purposes, and it could be hypothesised that the assessment of the integrity of specific entwined pathways will be required.

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