

## Concerted overexpression of the genes encoding p21 and cyclin D1 is associated with growth inhibition and differentiation in various carcinomas

J S de Jong, P J van Diest, R J A M Michalides, J P A Baak

### Abstract

**Aims**—To investigate the expression of the genes encoding cyclin D1 and p21 in proliferative and non-proliferative cells, as demonstrated by the Ki67 antibody, and to correlate these findings with differentiation.

**Methods**—Immunohistochemistry and immunofluorescence double staining were performed on three breast cancers, two squamous cell cancers of the head and neck, and one ovarium cystadenocarcinoma. In addition, the in vitro effect of cyclin D1 on p21 gene expression in MCF7 breast cancer cells was evaluated.

**Results**—Immunofluorescence double staining showed a differentiation related gradient in the detection of the Ki67 antigen, cyclin D1, and p21 in squamous cell cancers of the head and neck: Ki67 was detected in the basal layers of the tumour and the cyclin D1 and p21 genes were coexpressed in the higher, more differentiated layers of the tumour. The breast and ovarian cancers often had cells that coexpressed the p21 and cyclin D1 genes, whereas coexpression of cyclin D1 and Ki67 did not occur. Western blot analysis of the MCF7 breast cancer cells showed an upregulation of p21 production when cyclin D1 gene expression was induced.

**Conclusion**—Overexpression of the cyclin D1 gene seems to lead to growth arrest in a variety of human cancers, possibly through the induction of p21 by cyclin D1. In squamous cell cancer, concerted overexpression of the genes encoding cyclin D1 and p21 might also induce differentiation. (J Clin Pathol: Mol Pathol 1999;52:78-83)

Keywords: cyclin D1; p21; proliferation; differentiation

Cell cycle progression in eukaryotes is controlled by a series of proteins named cyclins. At least eight cyclin genes have been identified in mammalian cells. They are classified into three groups: G1 cyclins, an A-type cyclin, and two B-type cyclins. The genes encoding the G1 cyclins, cyclins D1-3 and cyclin E, are expressed maximally during the G1 phase of the cell cycle and regulate the transition of the cell cycle from G1 into the S phase.<sup>1,2</sup> Cyclin A is mainly expressed in the late G1 and early S phase and presumably enhances entry into and transition through the S phase.<sup>3</sup> The B-type

cyclins regulate entry into and exit from the mitotic phase.<sup>4</sup> The cyclins act by binding to and stimulating the activities of a series of proteins named the cyclin dependent kinases (CDKs). At least six CDKs (CDK 1-6) have been identified.<sup>5</sup> CDK4 and CDK6 can associate with the D-type cyclins. The CDK4-cyclin D1 complex plays a role in the transition from the G1 to S phase by phosphorylation of the retinoblastoma protein (pRb), thereby releasing the transcription factor E2F. The discovery of CDK kinase inhibitory (CKI) proteins has provided a new paradigm for the control of cell growth, which links the biochemical events surrounding cell cycle arrest with various physiological processes, including the cellular response to DNA damage, response to growth inhibitory signals, suppression of tumorigenesis, senescence, and differentiation.<sup>6,7</sup> Two families of CKIs have been described in mammalian cells. The p16 family, also known as the INK4 family (inhibitor of CDK4) can only inhibit CDK4-cyclin D and CDK6-cyclin D complexes.<sup>8</sup> The other family, the p21 family, which includes p21<sup>cip1/waf1</sup>,<sup>9</sup> p27<sup>kip1</sup>,<sup>10</sup> and p57<sup>kip2</sup>,<sup>11</sup> can inhibit the activity of a variety of CDK-cyclin kinase complexes. p21<sup>cip1/waf1</sup> is known to inhibit the kinase activity of the CDK4-cyclin D1 complex.<sup>12</sup> p21 regulates cyclin-CDK activity by the number of bound p21 molecules: active complexes contain a single p21 molecule, whereas inactive complexes contain multiple p21 molecules.<sup>13</sup> p21 gene expression can be regulated directly by the p53 tumour suppressor gene: the promoter region of p21 contains two consensus p53 binding sites.<sup>14</sup> Introduction of p21 into malignant cells can result in G1 phase arrest, altered morphology, and cell differentiation.<sup>15</sup>

There is accumulating evidence for a dual role of cyclin D1 in cell cycle control. Cyclin D1 can promote progression through the G1 phase by associating with CDK4, which results in phosphorylation of pRb, thereby releasing E2F from the pRb-E2F complex. Different studies showed a shortened G1 phase in mouse fibroblasts,<sup>16</sup> rat cells,<sup>17</sup> and breast cancer cells,<sup>18</sup> as a result of overexpression of the cyclin D1 gene. One study on human breast cancer, however, showed an association between overexpression of the cyclin D1 gene and high telomerase activity without an increase in tumour cell proliferation.<sup>19</sup> In other studies, cyclin D1 gene overexpression in HBL100 breast tumour cells could prolong the S phase, which resulted in increased expression of

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$\beta$ -casein in these cells,<sup>20</sup> and inhibited DNA replication,<sup>21</sup> pointing to a growth restricting function for cyclin D1. The latter observations fit well with our previous finding that overexpression of the cyclin D1 gene in invasive breast cancer correlated negatively with proliferation, as expressed by the number of mitotic figures.<sup>22</sup> In addition to the association between overexpression of the cyclin D1 gene and a low proliferation rate, cyclin D1 seemed to be related to tumour differentiation because well differentiated tumour types more often overexpress the cyclin D1 gene than do poorly differentiated tumours.<sup>22-25</sup> Alterations of the cyclin D1 gene (also known as PRAD1, bcl-1), located at chromosome 11q13, leading to increased synthesis of the cyclin D1 protein, have been found in many different tumour types, such as carcinomas of the breast, oesophagus, bladder, liver, and stomach, squamous carcinomas of the head and neck,<sup>26-28</sup> parathyroid adenomas,<sup>29</sup> and mantle cell lymphomas.<sup>30-32</sup> To investigate the association between cyclin D1 and proliferation and differentiation, we examined different tumours for their expression patterns of cyclin D1, p21, and the proliferation associated protein Ki67. In addition, we evaluated the *in vitro* effect of cyclin D1 on p21 gene expression in MCF7 breast cancer cells.

## Material and methods

### TUMOUR SPECIMENS

Fresh operation specimens from three invasive breast cancers, two squamous cell cancers of the head and neck, and one ovarium cystadenocarcinoma were cut into slices of ~ 0.5 cm thickness. These cancers were selected for their cyclin D1 positivity. Tissues were fixed for at least 24 hours in neutral buffered 10% formaldehyde. After paraffin wax embedding, 4  $\mu$ m thick sections were cut for routine staining with haematoxylin and eosin, immunohistochemistry, and immunofluorescence.

### IMMUNOHISTOCHEMISTRY

For immunohistochemistry, the sections were mounted on 3-aminopropyltriethoxy silane (APES; Sigma, Zayndrecht, The Netherlands) coated slides. An avidin-biotin peroxidase technique was used for staining the cyclin D1, p21, p53, and Ki67 antigens. After the slides were dewaxed, endogenous peroxidase activity was blocked by incubation in 0.3% (vol/vol) hydrogen peroxide in methanol for 30 minutes. The slides were heated at 100°C in a 0.01 M citrate buffer (pH 6.0) for 15 minutes for antigen retrieval. Thereafter, the slides were preincubated with normal rabbit serum (1/20) for 10 minutes. Subsequently, the slides were incubated for 16 hours at 4°C with mouse monoclonal antibodies against cyclin D1 (clone DCS-6, IgG2a; Novocastra, Newcastle, UK; 1/40 dilution), p21 (clone 6B6, IgG1; PharMingen, Los Angeles, California, USA; 1/200 dilution), p53 (clone DO-7; Dako, Glostrup, Denmark; 1/500 dilution), and Ki67 (Mib1, IgG1; Immunotech SA, Marseille, France; 1/40 dilution). The slides were then incubated with a biotinylated rabbit antimouse

antibody (1/500) for 30 minutes. The slides were subsequently incubated with avidin-biotinyl peroxidase complex (1/200) for one hour. 3,3'-diaminobenzidine tetrahydrochloride (Sigma) was used as a chromogen. Between steps, the slides were rinsed for 10 minutes in phosphate buffered saline (PBS) three times. All sections were lightly counterstained with haematoxylin.

### IMMUNOFLUORESCENCE DOUBLE STAINING

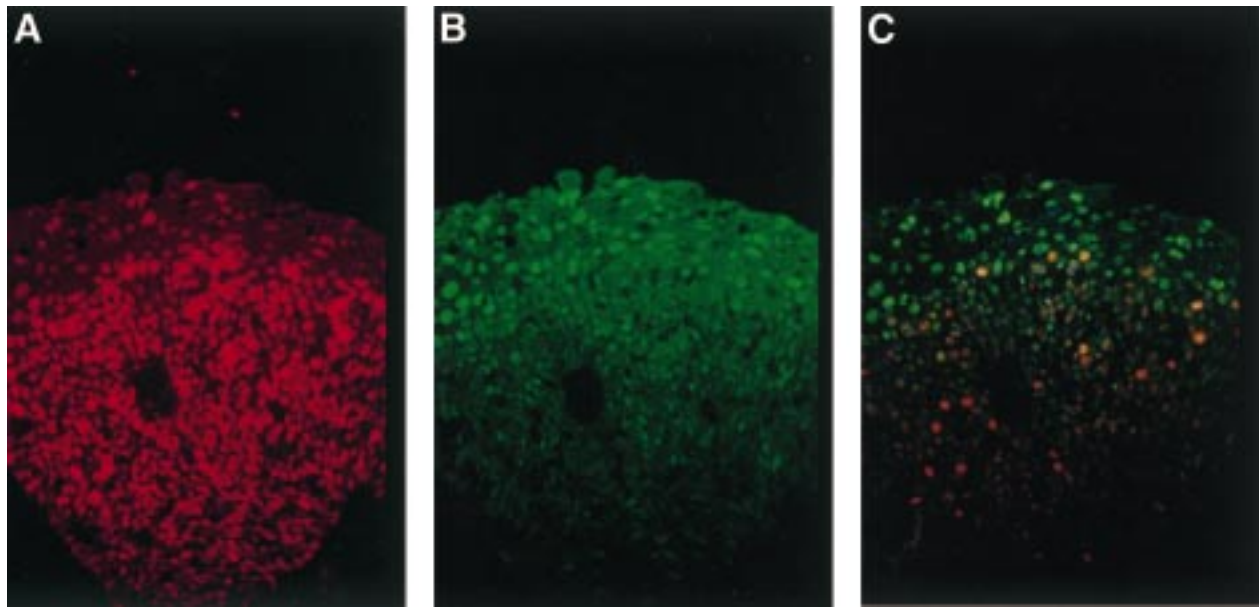
For double staining of cyclin D1/Ki67 and cyclin D1/p21, the sections were pretreated as for single staining (see above). Thereafter, sections were preincubated with normal goat serum (1/20) for 10 minutes. Subsequently, sections were incubated with a mixture of cyclin D1 and Mib1 (both antibodies at a dilution of 1/40) or cyclin D1 and p21 (1/40 and 1/200 dilutions, respectively) for 16 hours at 4°C. Slides were reacted with isotype specific secondary antibodies by incubation with a mixture of horseradish peroxidase (HRP) labelled goat antimouse IgG2a (1/50 dilution; Southern Biotechnology, Birmingham, USA) and biotin labelled goat antimouse IgG1 (1/50 dilution; Southern Biotechnology) for 30 minutes. Sections were incubated with fluorescein isothiocyanate (FITC) labelled tyramine (1/10 dilution) for 20 minutes<sup>33</sup> directed against the goat antimouse IgG2a-HRP, resulting in a green signal for cyclin D1 positive cells. Thereafter, sections were incubated for one hour with streptavidine-Cy3 (1/150 dilution; Jackson, West Grove, USA) directed against the goat antimouse IgG1-biotin, resulting in a red signal for Ki67 and p21 positive cells. Sections were counterstained with 4'6-diamidino-2-phenylindol-2-HCl in Tris, pH 7.5 (DAPI; Partec, Münster, Germany) for two minutes.

### IMMUNOFLUORESCENCE MICROSCOPY

For visualising the Cy-3 and FITC immunofluorescence signals a Leica DMRB immunofluorescence microscope was used. A standard Cy-3 filter (Leica filter cube N 2.1) was used for visualising the Cy-3 signal and a standard FITC filter (Leica filter cube L 4) was used for visualising the FITC signal. A DAPI/FITC/Cy-3 triple filter (Filter 61000; Chroma Technology, Vermont, USA) was used for visualising the Cy-3 (red), FITC (green), and DAPI (blue) signals and the coexpression of Cy-3 and FITC (yellow) in one view.

### CELL CULTURE AND INDUCTION OF OVEREXPRESSION OF THE CYCLIN D1 GENE

Human epithelial MCF7 breast tumour cells and the cyclin D1 transfected MCF7 clone 3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. The cyclin D1 transfected MCF7 clone 3 cells contained the pUhd15-1 and pSV2 neotetracycline sensitive transactivator, the tetracycline D1 plasmid, and the thymidine kinase-hygromycin plasmid, as described previously.<sup>34</sup> These cells were cultured in the presence of 10  $\mu$ g/ml tetracycline to suppress the production



**Figure 1** Immunofluorescence staining for cyclin D1 and Ki67 in a squamous cell carcinoma. (A) A gradient is seen for Ki67 positive cells (red), with positive cells being found frequently in the basal layers of the epithelium and few being detected in the higher layers; (B) the opposite is seen for cyclin D1 (green). Faint basal staining is probably attributable to crossover from the blue DAPI staining. (C) In double staining experiments, only very few cells stained for both Ki67 and cyclin D1 (yellow), with low (probably physiological) concentrations of cyclin D1 being detected.

of ectopic cyclin D1, whereas tetracycline was omitted when overproduction of ectopic cyclin D1 was wanted.

#### WESTERN BLOT ANALYSIS

MCF7 cells were cultured in medium with 10% FCS for 60 hours in the presence or absence of tetracycline. Total cell extracts were prepared by lysis of cell monolayers with Laemmli sample buffer without bromophenol blue. The protein concentration was measured by the method of Lowry *et al.*<sup>35</sup> Equal amounts of protein (50 µg) were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and blotted on to a cellulose nitrate membrane (Schleicher and Schuell).

Immunoblot analysis was performed on different strips of the membrane with an anti-pRb (IF-8; Santa Cruz Biotechnology, Santa Cruz, California, USA), anticyclin D1 (DCS-6; Progen, Newcastle, UK), anti-p21 (187; Santa Cruz Biotechnology), and an antitubulin monoclonal antibody (a kind gift of Dr S Feltkamp, the Netherlands Cancer Institute) as a control. Immunodetection was performed with the enhanced chemiluminescence ECL system (Amersham, Little Chalfont, UK).

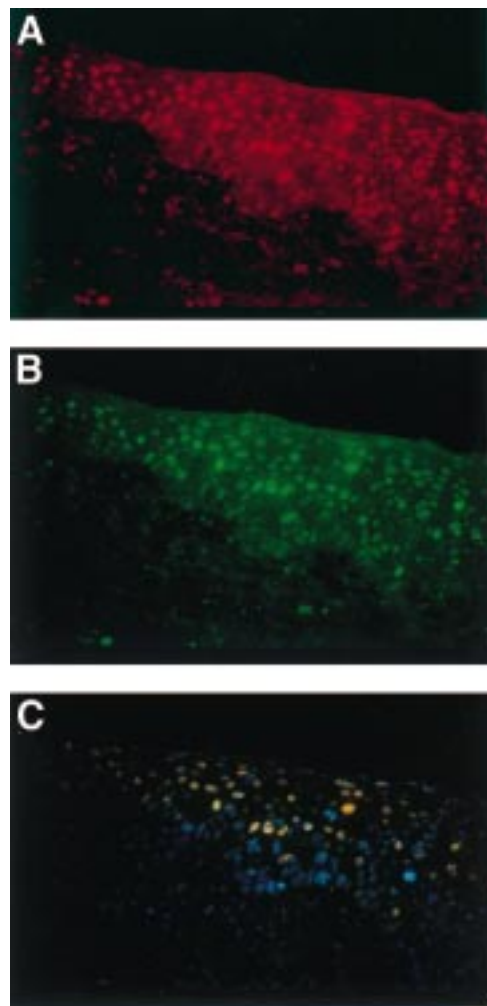
#### Results

The single stained sections of the different tumour types showed the following results. In squamous cell cancers, Ki67 was found in the basal layers, whereas cyclin D1 and p21 were seen exclusively in the higher layers of the tumours. Immunofluorescence double staining showed only occasional, low coexpression of cyclin D1 and Ki67 in the basal layers (probably at physiological levels). There was a clear gradient in the detection of cyclin D1, p21, and Ki67. The p21 and cyclin D1 strongly positive cells were located in the higher layers of the tumour, whereas the Ki67 positive cells were in the basal layers of the tumour (fig 1).

Cyclin D1 and p21 genes were coexpressed in many cells (fig 2). In addition, cells within the breast and ovarian cancers often coexpressed p21 and cyclin D1, whereas coexpression of cyclin D1 and Ki67 did not occur.

The squamous cell cancers, ovarium cystadenocarcinoma, and one of the breast cancers were positive for p53. The other two breast cancers did not express p53.

Western blot analysis of the MCF7 breast cancer cells showed an upregulation of p21 when cyclin D1 synthesis was induced (fig 3). Expression of the cyclin D1 gene was induced in MCF7 clone 3 cells by means of the tetracycline regulated transcriptional activation system,<sup>36</sup> as described previously.<sup>34</sup> The advantage of using the tetracycline responsive expression system is that results are not influenced by clonal variation. Similar results were obtained with another inducible clone of cyclin D1 in MCF7 clone 8 cells. The concentration of cyclin D1 seen in cells cultured in the presence of tetracycline reflected the endogenous concentration of cyclin D1, whereas omission of tetracycline from the medium resulted in a five to sixfold overproduction of cyclin D1 (fig 3). The extent of phosphorylation of pRb hardly differed when the cells were cultured in 10% FCS in the presence or absence of tetracycline. The control, tubulin, showed bands of a similar intensity in the presence and absence of tetracycline. However, p21 production changed drastically in MCF7 clone 3 cells, with overproduction of ectopic cyclin D1 in comparison with cells with a normal concentration of endogenous cyclin D1. The raised p21 concentration in MCF7 clone 3 cells did not result in an altered cell cycle distribution, because we did not see any pronounced differences in the cell cycle distribution of cells overexpressing the cyclin D1 gene compared with those not overexpressing



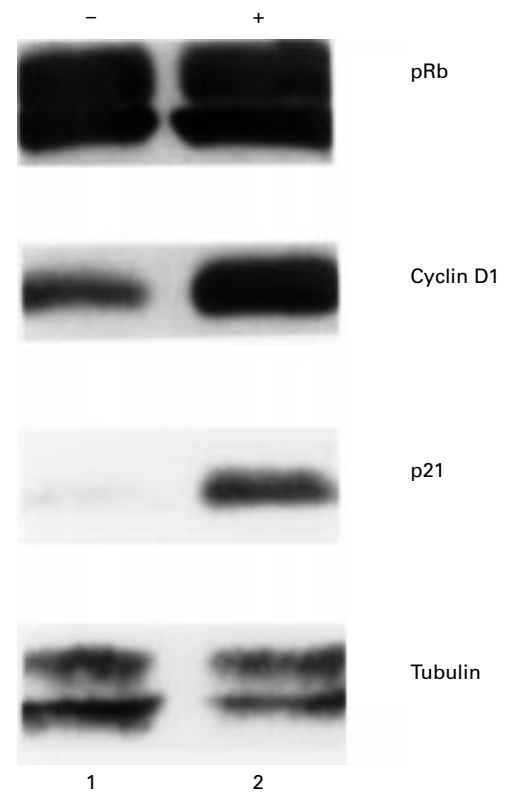
**Figure 2** Immunofluorescence double staining of cyclin D1 and p21 in a squamous cell carcinoma. (A) p21 positive cells (red) and (B) cyclin D1 positive cells (green) are seen mainly in the higher, more differentiated layers of the epithelium. Faint basal staining is probably attributable to crossover from the blue DAPI staining. (C) Double staining experiments show that many cells coexpress the cyclin D1 and p21 genes (yellow).

this gene (data not shown), nor in the growth curves between these two types of cell.<sup>34</sup>

### Discussion

In our study, we investigated the expression of the cyclin D1 and p21 genes in proliferative and non-proliferative cells, as demonstrated by the Ki67 antibody. This study was stimulated by the finding in previous studies of invasive breast cancer that cyclin D1 seemed to be associated with decreased proliferation and with enhanced differentiation.<sup>22-25</sup> These results pointed to a more complex role for cyclin D1 in human tumours than is suggested by other *in vitro* and *in vivo* studies, showing that high concentrations of cyclin D1 render cell growth independent of growth factors.<sup>34 37-39</sup>

In a previous study, we found raised concentrations of cyclin D1 in 59% of breast carcinomas, as detected by immunohistochemistry. Most of these tumours were well differentiated, characterised by oestrogen receptor positivity, a high degree of tubule formation, small nuclei, and a low proliferation rate, whereas the most



**Figure 3** Expression of the p21 gene after cyclin D1 induction in MCF7 clone 3 cells. Clone 3 cells were cultured for 60 hours with (+) or without (-) induction of exogenous cyclin D1 gene expression. Equal amounts of cell lysate were separated on denaturing gels and subjected to western blotting with antibodies against pRb, cyclin D1, p21, and tubulin as described. The broad pRb band indicates the position of hypophosphorylated and hyperphosphorylated pRb. Note the upregulation of the p21 gene after induction of cyclin D1.

poorly differentiated tumours of the medullary type, characterised by oestrogen receptor negativity, solid growth, large atypical nuclei, and a high proliferation rate, were consistently cyclin D1 negative.<sup>22</sup> In our present study, on the single cell level, we found almost no cyclin D1 in proliferating cells as marked by Ki67 staining. However, we did find a notable coexpression of the cyclin D1 and p21 genes in different tumour types. Furthermore, in squamous cell cancers, we found a gradient through the epithelium in the expression of Ki67, cyclin D1, and p21, whereby the Ki67 positive proliferating cells were localised in the basal layers of the epithelium, and the cyclin D1 and p21 genes were coexpressed in the higher layers of the epithelium. The localisation of cells overexpressing the cyclin D1 gene in higher epithelial layers in squamous cell cancers has been reported by others.<sup>40 41</sup> Because cells overexpressing the cyclin D1 gene were almost all negative for Ki67 in our study, high cellular concentrations of cyclin D1, as demonstrated by immunohistochemistry, might not be associated with proliferation. Rather, these cells seem to have undergone proliferation arrest. In these cells, high concentrations of cyclin D1 may be related to differentiation, as was shown in squamous cell cancers, where cyclin D1 is produced mostly in the higher, non-proliferating, differentiated

layers of the epithelium. In the adenocarcinomas, this could not be studied because the differentiation gradient as seen in squamous cell cancers is lacking. This could be more closely studied in raft cultures by transfecting cyclin D1 with a controllable promoter. Nevertheless, breast cancers with many cells overexpressing cyclin D1 are generally well differentiated as described above.

The cell cycle arrest and induction of differentiation in cells overexpressing the cyclin D1 gene might be a result of the induction of p21. Coexpression of the cyclin D1 and p21 genes was often seen in tumours positive for cyclin D1. In these tumour cells, p21 might cause proliferation arrest, thereby providing the opportunity for cells to differentiate. The involvement of p21 in the process of differentiation has been shown in different types of cells.<sup>42-45</sup> The results of the western blot analysis of the MCF7 cells show an upregulation of the p21 gene after induction of cyclin D1. This result, and the results of others,<sup>46</sup> indicate that cyclin D1 might upregulate p21 expression. However, the increased concentrations of p21 in cyclin D1 overexpressing cells does not always lead to growth arrest in vitro,<sup>34 46</sup> although high concentrations of cyclin D1 may be "toxic".<sup>16</sup> In human cancers, this seems to be different. We suggest that a balance between a positive regulator of the cell cycle, cyclin D1, and a negative regulator, p21, is generated by a feedback mechanism, where overexpression of the cyclin D1 gene results in induction of p21. By this mechanism, the effects of overexpression of the cyclin D1 gene might be neutralised, and the resulting proliferation arrest may allow cells to differentiate. How overexpression of the cyclin D1 gene is induced apart from amplification or translocation is as yet unknown, although growth factors have been suggested to play a role.<sup>47 48</sup> pRb deserves attention in this respect because a previous study showed that pRb stimulated by expression of the p21 gene may induce cyclin D1.<sup>49</sup>

In the tumours studied there were p21 positive cells that were cyclin D1 negative. This can be explained by the fact that p21 may also be induced by other proteins such as wild-type p53. However, the ovarian cancer, the two squamous cell cancers, and one of the breast cancers that showed cells with strong coexpression of the p21 and cyclin D1 genes showed strong nuclear staining for p53, which is highly indicative of a mutation in the p53 gene. Because mutant p53 is not able to induce p21,<sup>12 50</sup> the described mechanism seems to be independent of p53. The expression of the p21 gene might also be upregulated through the mitogen activated protein (MAP) kinase pathway.<sup>51</sup>

Mutually exclusive expression of Ki67 and p21 has also been reported for colonic epithelium.<sup>42</sup> The accumulation of p21 in suprabasal layers in squamous cell head and neck cancer was also reported by Erber *et al*,<sup>52</sup> and did not coincide with Ki67 positive cells. Remarkably, in that study, overexpression of the p21 gene was reported to be indicative of poor prognosis. Such an association with poor

prognosis was also found for overexpression of the cyclin D1 gene in squamous cell head and neck cancers,<sup>53 54</sup> suggesting that the concerted overproduction of two opposing regulators of the cell cycle, cyclin D1 and p21, may be indicative of poor prognosis in squamous cell cancer of the head and neck. In our study we looked at the single cell level and do not want to speculate about the impact of the described phenomena on the behaviour of tumours.

In conclusion, overexpression of the cyclin D1 gene seems to lead to growth arrest in a variety of human carcinomas, possibly through the induction of p21 by cyclin D1. In squamous cell cancer, a concerted overexpression of the genes encoding cyclin D1 and p21 might also induce differentiation. However, the exact mechanisms of cyclin D1 overexpression and p21 induction require further study.

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## Concerted overexpression of the genes encoding p21 and cyclin D1 is associated with growth inhibition and differentiation in various carcinomas.

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