Prognostic significance of CCND1 (cyclin D1) overexpression in primary resected non-small-cell lung cancer

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Summary  Amplification of the CCND1 gene encoding cyclin D1 was examined by Southern blotting and multiplex polymerase chain reaction (PCR) and occurred in 8 of 53 patients (15%) with primary resected non-small-cell lung cancer (NSCLC). These tumours and 17 additional tumours with a normal gene copy number showed overexpression of cyclin D1 (25/53, 47%), as assessed by immunostaining using a monoclonal antibody. In 23/53 cases, cyclin D1 was localised in the cytoplasm, but some (7/25) had simultaneous nuclear staining. This result is in marked contrast to that reported in breast, hepatocellular and colorectal carcinoma studies where immunostaining was invariably nuclear. Examination of a restriction fragment length polymorphic (RFLP) site within the 3' untranslated region of the cDNA following reverse transcriptase (RT)-PCR (29/53 informative cases) showed a strong association between overexpression and a balance in allele-specific message levels. Cyclin D1 overexpression was associated with a poorly differentiated histology ($P = 0.04$), less lymphocytic infiltration of the tumour ($P = 0.02$) and a reduction in local relapse rate ($P = 0.01$). The relative risk of local relapse was 9.1 in tumours without cyclin D1 overexpression ($P = 0.01$, Cox regression analysis). We conclude that genetic alteration of cyclin D1 is a key abnormality in lung carcinogenesis and may have diagnostic and prognostic importance in the treatment of resectable NSCLC.

Keywords: non-small-cell lung carcinoma; CCND1; cyclin D1; prognostic factor; local relapse; cell cycle regulation

Advances in molecular biology paved the way for the identification of proteins responsible for the regulation of cell proliferation. One of these molecules, cyclin D1, is expressed during the early phase of the cell cycle, before the restriction point (START), beyond which cells are committed to enter S phase. This cyclin may act as an initiator of the cell cycle, and perhaps as a growth factor sensor (Xiong et al., 1991; Motokura and Arnold, 1993a,b; Sherr, 1993; Hunter and Pires, 1994; Marx, 1994). The ability of cyclin D1 to immortalise cells in vitro established its oncogenic activity (Hinds et al., 1994; Lovec et al., 1994). The cyclin D1 gene (CCND1) is one of the most frequently amplified chromosomal regions (11q13) in human carcinomas (Motokura and Arnold, 1993b). In laryngeal (Jares et al., 1994) and head and neck carcinomas (Müller et al., 1994; Michalides et al., 1995), its overexpression has been shown to be associated with advanced local invasion and presence of lymph node metastases. Cyclin D1 may therefore play a key role in cell growth regulation and tumorigenesis.

Lung cancer is a worldwide problem and in many countries it is the most lethal malignancy. As relapse is frequent after resection of early stage non-small-cell lung cancer (NSCLC) (Ginsberg et al., 1993), there is an urgent need to define prognostic factors. These would help in the choice of the best therapeutic procedure (widescale surgical resection, (neo-)adjuvant chemo- and/or radiotherapy). In this study we will provide evidence that, surprisingly, cyclin D1 overexpression is associated with lower relapse risk in resectable NSCLC, despite also being associated with poor tumour differentiation, known to be a negative indicator.

Patients and methods

Patients’ characteristics and tumour specimens

Tumour samples were obtained from 53 consecutive patients [49 men, four women, median age 64 years (45–79)] who underwent resection of staged resectable NSCLC at the University Hospital of Berne, Switzerland. They had received no therapy before surgery [pneumonectomy ($n = 15$) or lobectomy ($n = 38$)]. Twenty-nine patients were in stage 1, five in stage 2 and 19 in stage 3 (UICC, 1987). All tumours were classified according to the standard criteria of the WHO (1981), by two pathologists, independently (HJA, PSH): there were 35 squamous carcinomas, 11 adenocarcinomas, four undifferentiated NSCLC, two large-cell carcinomas and one carcinoid. Tumour size was measured by the pathologists on the fresh specimen. The lymphocytic infiltration of the tumour, the amount of necrosis and vascular infiltration were determined histologically. Thirty-three of 53 patients are alive with a median follow-up of 16 months (range 2–42). In order to examine the possibility whether the immunostaining result was due to fixation artifacts we analysed also 33 NSCLC tumour sections prepared in the Wythenshawe Hospital for cyclin D1 overexpression.

DNA and RNA extraction

DNA and RNA were isolated from frozen tissue samples. High molecular weight DNA was prepared according to standard protocols (Blin et al., 1976) and RNA was extracted using Dynabeads (Dynal, Oslo, Norway) (Jakobsen et al., 1990) before being reverse transcribed to cDNA (Superscript Preamplification System, Life Technologies, Paisley, UK).

Southern blot analysis

TagI digests of 5 μg of genomic DNA were blotted onto Hybond-N filter (Amersham, Buckinghamshire, England). Purified DNA probes (CCND1, nt: 1100–1888 and adenosine deaminase, ADA, assigned to chromosome 20q12–13, nt: 34275–34785) were labelled with α-32P-dCTP (Amersham) using a random-primed DNA labelling kit (Boehringer, Mannheim, Germany) and hybridised overnight to the immobilised DNA. Filters were subsequently washed for 30 min, once with 2 x SSC and once with 0.5 x SSC at 65°C and exposed to Kodak X-OMAT film at 70°C for several days. The CCND1 gene was considered amplified when the
intensity of the ratio CCND1/ADA signal (assessed by Phosphor Imager analysis, 425S, Molecular Dynamics) was increased at least 3-fold relative to the ADA gene signal, which served as an internal control for DNA loading and was found to be amplified in lung tumours. Digested DNA isolated from normal lung tissue from each patient was used as a control.

PCR

The PCR multiplex was performed as described (Edwards and Gibbs, 1994). The CCND1 gene (chromosome 11q13) was compared with the adenosine deaminase (ADA) gene (chromosome 20q12–13) and the progesterone receptor (PR) gene (chromosome 11q23). The following primers were used: CCND1: 5’ATATCCGATTAGTATGTTAAC 3’, 5’TGTCACTATTCTGTCTTC 3’; ADA: 5’CCGGGTGAACTCAATGGTTC 3’, 5’CAACCTGAAGAGATTGTCAG 3’. PR: 5’GGTTGGTTCTCCTACATATAGC 3’, 5’TAGGACCTCAAGGTAG 3’. Genomic DNA (2 μg) was added to 98 μl of a PCR mixture containing dNTP at 250 μM, 0.5 μg of each CCND1 and 0.05 μg ADA primers at 117 μM PR primers (the ADA and PR primer concentrations were reduced in order to obtain an equal amplification compared with CCND1 in normal lung tissue), 1 × PCR buffer (Boehringer), and 2 Taq polymerase (Boehringer). PCR cycles (30) were run in an automated thermocycler: denaturation 94°C, 1 min, annealing 57°C, 1 min, and elongation temperature 74°C, 1 min. PCR products were visualised on 2% agarose gels. For the RFLP analysis of the 3’UTR of the cyclin D1 genomic and cDNA the following primers were used: primary PCR (30 cycles), only for cDNA: 5’ACAACCTCTCTGTCCTACTACGC3’, 5’ATAGTACGG-TATCTGATTAGG 3’ and secondary PCR (genomic primary, 30 cycles) cDNA analyses (1 μl of a 1:100 dilution of primary PCR amplification, 28 cycles) 5’CTCTGGTTACAGTACGGTAGC3’, 5’ATHTATAGG-ACTTGGCAG-GT-3’. The PCR products were digested with the restriction enzyme HaeIII and visualised on 2% gels. Allelic imbalance in DNA/cDNA derived products was determined visually with reference to normal control DNA samples.

Immunohistochemistry

The immunohistochemistry was performed as described previously (Gillett et al., 1994). Briefly, formalin-fixed paraffin sections (4 μm) from the 53 primary lung tumours prepared in Bern in Manchester and 14 breast cancers were air dried on 2% 3-aminopropyltriethoxysilane (APS) (Sigma, Poole, UK)-coated slides. After dewaxing in xylene, the sections were treated for 15 min with 300 ml methanol and 10 ml hydrogen peroxide in order to block endogenous peroxidase and rinsed thoroughly in water. They were then placed in citrate buffer, boiled twice in a microwave, washed with water and placed in TRIS buffer (pH 7.6). After incubation in 1: 100 goat serum for 20 min at room temperature, they were placed in 1% bovine serum albumin (BSA) and 1:100 monoclonal cyclin D1 antibody (Novocastra, Newcastle, England) overnight at room temperature. After two washes with TRIS buffer they were incubated in 1:100 biotinylated goat anti-mouse/rabbit IGI(Dako, Glostrop, Denmark) for 30 min, washed subsequently twice with TRIS, incubated in 1:100 solution of streptavidin – biotin complex for 30 min at room temperature, rewarshed in TRIS buffer and placed in dianinobenzene (10 mg 10 ml−1) for 10 min, counterstained with haematoxylin, washed in water, cleared, dehydrated and mounted. Cyclin D1 staining was examined according to the intensity of the majority of cells. The slides were assessed blind, and the pathologists did not know the results of the molecular analyses. Positive and negative controls were performed for each tumour section. Two categories of staining were used: nil – weak, and moderate – strong. A tumour was considered positive in the case of a moderate – strong staining. In some cases of negative cyclin D1 tumour staining we repeated the analyses in several regions of the tumour.

Statistical analyses

Patients were placed into two groups according to their cyclin D1 expression. Associations of group membership with other patient and tumour characteristics were made with chi-square tests for categorical features and Mann – Whitney U-tests for continuous ones. Disease end points analysed were overall survival and event-free survival (EFS) (defined as the time to the first of: (i) local relapse with or without metastases; (ii) metastases alone; (iii) death from an unrelated cause). Kaplan – Meier survivor function estimates were used and simple comparisons between the two groups were made with the log-rank test. EFS is a mixing of three types of event (Kay et al., 1983) and the type-specific cumulative event rates were estimated with the Nelson estimator (Nelson, 1969). Cox regression (Cox, 1972) was used to investigate if the group difference with respect to the local relapse rate remained after adjustment for age, T-stage, N-stage and tumour differentiation.

Results

Amplification of the CCND1 gene in NSCLC

Fifty-three patients with operable NSCLC were investigated for CCND1 gene amplification by Southern blotting and multiplex PCR analyses. In 8/53 (15%) of the NSCLC a 3- to 20-fold amplification of the CCND1 gene was identified (5/35 squamous cell, 2/11 adenoc and 1/2 large cell carcinoma, Figure 1). This result was confirmed by a PCR multiplex containing CCND1 amplification with the amplification of the adenine deaminase gene (chromosome 20q12–13), and numerical differences in chromosome 11 copy number were excluded by a PCR multiplex, comparing CCND1 with the progesterone receptor gene located on the same arm of chromosome 11 (Figure 1). Five patients with amplification were informative for the cyclin D1 gene polymorphism (Heighway, 1991); allelic imbalance was observed in each case supporting the amplification data obtained by Southern blotting and PCR multiplex.

Cyclin D1 expression analyses by immunostaining

Tumour sections from the 53 patients were stained using a cyclin D1 monoclonal antibody. Following the protocol kindly provided by C. Gillett and D Barnes (Gillett et al., 1994) we found cyclin D1 overexpression in 6 out of 14

Figure 1 (a) Southern blot: Tumour (T) and adjacent normal lung tissue DNA blot hybridised with the CCND1 and adenosine deaminase (control) probes. The lanes were loaded with a total of 5 μg of Taq-digested DNA. All tumours demonstrate a 3-to 20-fold increase in CCND1 copy number compared with adjacent non-tumour tissue. (b) PCR multiplex analyses: PCR amplification of CCND1 and progesterone receptor gene (chromosome 11q23) showed increase of CCND1 copy number compared with control genes, confirming the Southern analysis and excluding an aberrant copy number of chromosome 11.
breast tumours, which was mainly confined to the nucleus. In our 53 lung tumours moderate–strong staining occurred at a similar overall frequency (25/53, 47%). All tumours with amplification of the CCND1 gene demonstrated overexpression of cyclin D1. Additionally, 17 tumours (32%) with an apparently normal gene copy number also showed increased cyclin D1 protein levels. However, in contrast to breast tumours, the cyclin D1 intracellular distribution varied. Simultaneous nuclear and cytoplasmic staining was observed in 7/53 (13%) cases, and specific cytoplasmic or nuclear staining was seen in 15/53 (28%) and 3/53 (6%) cases respectively (Figure 2). Tumours with CCND1 amplification showed nuclear (n = 3), cytoplasmic (n = 3) or both, nuclear and cytoplasmic staining (n = 2). In order to exclude a fixation artifact we assessed the cyclin D1 overexpression in 33 NSCLC tumours fixed in the Wythenshawe Hospital. A very similar result (nuclear staining 2/33, cytoplasmic 6/33 and nuclear and cytoplasmic 3/33) was obtained in those patients, rendering this explanation rather unlikely. The cause and significance of this altered subcellular localisation, however, is not clear and cyclin D1 expression was further investigated using RT–PCR.

Allele-specific cyclin D1 expression

A polymorphism of cyclin D1 located in the 3' untranslated region allows a PCR-based determination of allele-specific expression within RNA samples by RFLP analysis (Heighway, 1991). Twenty-nine out of the 53 tumour samples were informative for this polymorphism. All informative patients with cyclin D1 overexpression (n = 14) had an imbalance in allele-specific expression levels suggesting up-regulation of one parental gene, consistent with the immunostaining results (Figure 3). However, in the case of the three tumours with both CCND1 amplification and cytoplasmic cyclin D1 staining, the allele imbalance was present but paradoxically, the amplified allele appeared to show reduced expression relative to the unamplified allele. As it is reasonable to assume that the amplified allele is in fact overexpressed, these results suggest that the majority of CCND1 mRNA encoded from this allele may be spliced in a way precluding PCR amplification of the cDNA with the primers necessary to visualise the RFLP.

Association of histopathological findings and clinical outcome

Tumours with cyclin D1 overexpression were less differentiated (P = 0.04) and showed less lymphocytic infiltration (P = 0.02). There was no difference in stage and tumour size at time of surgery. Sex, age distribution and histological type of tumour had no influence on cyclin D1 expression (Table I). Overall survival did not differ significantly in the two groups (P = 0.5). Similarly EFS did not differ significantly (P = 0.26). However, examination of the type-specific event rates (Figure 4) revealed a significantly increased risk of local relapse in the cyclin D1-negative group (P = 0.01), with very similar rates of metastases alone and a decreased death rate from other causes, although not significantly (P = 0.09). The increased risk of local relapse was maintained after adjustment for age, T-stage, N-stage and tumour differentiation (P = 0.01, Table II). The estimated relative risk of local relapse was 9.1 in the cyclin D1-negative groups compared with the positive group with approximately 95% CI (1.5,554).

Discussion

G1 cyclins such as cyclin D1 and their cyclin-dependent kinases (CDKs) are proving to be integrators of growth factor-mediated signals driving cells through the restriction point (START), early in the G1 phase of the cell cycle (Pines, 1993; Sherr, 1993, 1994). Growth factors act by binding to specific cell-surface receptors, which in turn trigger signalling cascades that ultimately govern the transcription of genes important in cell growth. The cyclin D1 gene. CCND1 was originally identified as the proto-oncogene (PRAD1), clonally rearranged with the parathyroid hormone gene, in parathyroid adenomas (Motokura et al., 1991). Its ability to rescue G1 cyclin-defective yeast (Lew et al., 1991; Xiong et al., 1991) suggested that this protein was a cell-cycle regulator. In addition, the murine homologue of cyclin D1, CYL1, isolated from mouse macrophages was induced by colony-stimulating factor 1 (CSF-1) (Matsushima et al., 1991). In human cell lines, cyclin D1 is stimulated by mitogens during G1, and, upon persistent growth factor stimulation, it is continuously synthesised throughout the cell cycle (Sewing et al., 1993). However, it excludes its critical function during G1, as it is not amplified in G1 phase, as cells cross the first cell cycle restriction point (for review see Motokura and Arnold, 1993b; Hunter and Pines, 1994; Sherr, 1995).

Several lines of evidence suggest that cyclin D1 might play a crucial role in carcinogenesis. Firstly, in addition to its involvement in parathyroid adenomas, in centrocytic B cell lymphomas (mantle cell lymphomas) CCND1 expression is up-regulated under the influence of the immunoglobulin heavy-chain gene enhancer, as a result of a reciprocal chromosomal translocation at the BCLI breakpoint, t(11;14)(q13;q32) (Withers et al., 1991; Seto et al., 1992; de Boer et al., 1993). Overexpression of cyclin D1 has been demonstrated in almost all lymphoproliferative disorders carrying this translocation (Bosch et al., 1994; Lukas et al., 1994; de Boer et al., 1995; Delmer et al., 1995). Secondly, the CCND1 gene has been shown to be frequently amplified and overexpressed in many human tumour types (Wyllie et al., 1991; Schuring et al., 1992; Gillette et al., 1994), head and neck (Williams et al., 1993; Callender et al., 1994; Müller et al., 1994), laryngeal (Jares et al., 1994), oesophageal (Jiang et al., 1992; Jiang et al., 1993; Kanda et al., 1994; Adélaïde et al., 1995), hepatocellular (Zhang et al., 1993; Nishida et al., 1994) and lung (Shapiro et al., 1995) carcinomas.

The role of cyclin D1 in carcinogenesis has been strengthened by in vitro studies demonstrating its oncogenic potential. Constitutive overexpression in rodent cells can shorten G1 phase (Quelle et al., 1993). Similarly, in breast cancer cells arrested in G1, cyclin D1 induction is sufficient to complete a cell cycle and shortens G1/S phase (Musgrove et al., 1994). Conversely, microinjection of cyclin D1 antibodies or antisense plasmid into dividing cells blocks them in the G1 phase (Baldin et al., 1993; Quelle et al., 1993). Transfection of CCND1 expression constructs into non-tumour fibroblasts stimulates proliferation indicating the function of the G1/S transition. Consistent with this, by complementing a defective adenovirus E1A oncogene, cyclin D1 can immortalise cells (Hinds et al., 1994), and finally when transfected into normal fibroblasts with activated HRAS, it promotes the formation of fibrosarcomas in nude mice (Lovec et al., 1994). In conclusion, there is no doubt that cyclin D1 may act as an oncogene.

The mechanism of action of cyclin D1 is not fully established and is still a field of intensive research. Nevertheless, one clue to its function comes from the fact that the retinoblastoma protein (pRb) is down-regulated in an undulating fashion in late G1 (Wiman, 1993), and that the pRb inactivation (permitting cell proliferation) occurs predominantly by phosphorylation of its threonine and serine residues. Interactions between cyclin D1 and other cell-cycle regulators, such as cyclin-dependent kinases (CDK4, CDK6) and the interaction of cyclin D1/CDK4 complexes with p16 (CDK4) have recently been suggested to play an important role in lung tumorigenesis (Shapiro et al., 1995). There are now accumulating data, showing MTS1 (the gene encoding p16) missense mutations and deletions with probable consequent loss of p16 function in some tumour types. In oesophageal carcinomas the mutation rate was 21–30% (Mori et al., 1994; Morita et al., 1994; Yoshida et al., 1994). In NSCLC 10–30% (Hayashi et al., 1994). In several lung cancer cell lines and tumour specimens, the presence of p16 protein is inversely correlated with detectable pRb and cyclin D1 proteins (Serrano et al., 1993; Shapiro et al., 1995). In
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particular, cells lacking functional pRb have no cyclin D1/CDK4 complexes (Bates et al., 1994), and in NSCLC cell lines, p16 was absent when pRb function was normal and present when pRb was mutated (Oterson et al., 1994). These results highlight the importance of the cyclin D1–CDK4–p16 pathway in tumorigenesis.

A monoclonal cyclin D1 antibody allows the detection of cyclin D1 by immunohistochemistry, enabling a clear distinction to be made between different levels of expression. Gillet et al. (1994) have found an excellent correlation between CCND1 gene amplification and cyclin D1 overexpression with this antibody. Immunostaining of sections from the eight lung tumours with CCND1 amplification showed that all overexpressed the protein. High-level expression was also seen in a further 17 cases in which amplification of the gene could not be demonstrated. In contrast to previous studies on other malignancies (Jiang et al., 1993; Zhang et al., 1993; Bartkova et al., 1994, 1995; Gillett et al., 1994)) in which cyclin D1 was confined to the nucleus, in our study, cyclin D1 protein was frequently observed at high levels, and sometimes exclusively, within the cytoplasm of the tumour cells. This finding is in agreement with the very recently published report (Lukas et al., 1995) on cyclin D1 and D2 in in vitro growing U-2-OS cells showing subcellular cytoplasmic localisation, during the G1/S phase while nuclear was observed during the G2 phase. The authors suggest a possible change of solubility of the protein dependent on the cell-cycle phase. Others have also reported cytoplasmic or cytoplasmic–nuclear cyclin D1 staining in mantle cell lymphoma (Banno et al., 1994; Nakamura et al., 1994). Different contrasting functions (positive regulators of growth in combination with CDK4 and CDK6, but an

Figure 2 Cyclin D1 immunostaining of NSCLC with monoclonal antibody (Novocastra, Newcastle, UK). (a) Nuclear staining. (b) Cytoplasmic staining. (c) Simultaneous nuclear and cytoplasmic staining.

Table 1 Association of cyclin D1 overexpression and patient characteristics

| Cyclin D1 not overexpressed | Cyclin D1 overexpressed | P-value |
|-----------------------------|-------------------------|---------|
| Number of patients          | 28                      | 25      | 0.15 |
| Sex                         | Male                    | 24      | 25      |       |
|                             | Female                  | 4       | 0       |       |
| Age (years)                 | 48–78                   | 45–79   | 0.94 |
| Median                      | 63.5                    | 65      |       |
| Mean ± SD                   | 64.1 ± 7.1              | 63.3 ± 9.0 |       |
| Histology                   | Squamous carcinoma      | 19      | 16      | 0.99 |
|                             | Adenocarcinoma          | 4       | 7       |       |
|                             | Large cell              | 0       | 2       |       |
|                             | Carcinoid               | 1       | 0       |       |
|                             | NSCLC                   | 4       | 0       |       |
| Stage                       | 1                       | 18      | 11      | 0.33 |
|                             | 2                       | 2       | 3       |       |
|                             | 3                       | 8       | 11      |       |
| Surgical intervention       | Lobectomy               | 19      | 19      | 0.73 |
|                             | Pneumonectomy           | 9       | 6       |       |
| Tumour size (cm)            | 1–9.3                   | 2–8.6   | 0.68 |
| Median                      | 4.0                     | 4.0     |       |
| Mean ± SD                   | 4.3 ± 2.1               | 4.4 ± 1.7 |       |
| Tumour differentiation      | Good                    | 10      | 2       | 0.04 |
|                             | Intermediate–poor       | 18      | 23      |       |
|                             | Lymphocytic infiltration| Poor    | 10      | 0.02 |
|                             | Moderate to important   | 18      | 7       |       |

Figure 3 RFLP analysis of CCND1 PCR products from three patients. Normal lung DNA from patient (N) (lanes 1 and 4), NSCLC DNA (T) (lanes 2, 5, 7) and cDNA (T*) (lanes 3, 6, 8): Lanes 1–3, 4–6, the amplified allele is overexpressed (both tumours showed nuclear cyclin D1 staining), lanes 7–8, tumour DNA shows no allelic imbalance but transcript levels are clearly imbalanced (cytoplasmic cyclin D1 overexpression); lane 9, phiX174 HaeIII marker (Promega).
inhibitor of CDK2) have been reported recently and might explain why some functional experiments have produced paradoxical results (Peters, 1994). In particular, cyclin D1 overexpression may lead in post-mitotic neurons to apoptosis (Freeman et al., 1994), and in human diploid fibroblasts it might induce senescence (Dulic et al., 1993; Lucibello et al., 1993).

Allele-specific expression analysis was carried out in the tumours studied. In all RFLP informative cases, in which elevated protein levels were demonstrated, an imbalance in allele-specific relative transcript abundance was seen. Conversely, no tumours scoring negative for cyclin D1 expression by immunoblotting displayed an imbalance in transcript levels (except for one tumour with a loss of heterozygosity at the CCND1 locus). These data suggest that overexpression of CCND1 was caused by specific genetic events affecting just one parental allele. Thus, the elevated expression seen in the tumour is not simply a result of non-specific up-regulation of a gene involved in rapid cell growth but is the result of genetic alterations directly affecting CCND1. In several cases the transcript imbalance seen in cDNA favoured the non-amplified allele. As the polymorphic restriction site is located distant to the coding sequence, within the long 3'UTR, the most likely explanation for this finding is that the mRNA encoded by the amplified allele is preferentially spliced, precluding amplification of the RFLP site.

The results show an association between cyclin D1 overexpression in the primary tumour and a low degree of differentiation, little lymphocytic infiltration of the tumour, and a low incidence of local relapse. Consistent with this result, overexpression of other oncogene products such as the BCL2 protein (Pezzella et al., 1993), which is known to inhibit programmed cell death and the epidermal growth factor receptor (EGFR) (Lee et al., 1989) that mediates the effects of the EGF and transforming growth factor (TGF)-α have been associated with a better patient survival. However, because of the relatively small number of patients in this study, the confidence interval for the local relapse hazard ratio was wide, indicating that these data must be interpreted with caution and require larger studies to confirm the usefulness of cyclin D1 overexpression as a prognostic factor.

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