

ORIGINAL ARTICLE

Expression and gene amplification of primary (A, B1, D1, D3, and E) and secondary (C and H) cyclins in colon adenocarcinomas and correlation with patient outcome

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Background/Aims: Deregulation of cell cycle control is a hallmark of cancer. The primary cyclins (A, B1, D1, D3, and E) are crucial for cell cycle progression. Secondary cyclins (C and H) have putative indirect effects on cell cycle progression and have not previously been evaluated in colon cancer. This study examined cyclin protein expression and gene amplification in colon adenocarcinoma and the correlation with patient outcome.

Methods: Immunohistochemistry and real time quantitative polymerase chain reaction were used to determine cyclin expression and gene amplification in 219 tumours. The results were compared with clinical variables and patient outcomes.

Results: Cyclin H was overexpressed in all tumours, cyclin C in 88%, cyclin B1 in 58%, cyclin A in 83%, cyclin D3 in 36%, cyclin E in 25%, and cyclin D1 in 11% of the tumours. Extra gene copies of cyclin A were seen in 6.2% of the tumours, cyclin B1 in 9%, cyclin C in 26.9%, cyclin D1 in 55%, cyclin D3 in 20.5%, cyclin E in 19.1%, and cyclin H in 5.1%. A significant correlation between protein overexpression and gene amplification was seen for cyclin C only. High expression of cyclin A was independently associated with improved survival. Amplification of cyclin C was independently associated with an unfavourable prognosis.

Conclusions: Amplification of the cyclin C gene was related to an unfavourable prognosis and high protein expression of cyclin A was associated with a better outcome in colon adenocarcinoma.

Deregulation of cell cycle control is a hallmark of cancer.¹ The control of the cell cycle depends upon cyclin dependent kinases (Cdks),² which function only after complex formation with cyclins.³ The G1 phase of the cell cycle is regulated by cyclin D and E associated kinases.⁴ Cyclin A–Cdk2 complexes take over at the G1/S transition, allowing S phase entry and replication. Mitosis is triggered by Cdk1 (Cdc2), which is activated by a multistep process beginning with the binding of cyclin B1.⁵

“There has been a limited number of reports concerning the secondary cyclins C and H in clinical samples”

Cyclin C has a putative phase G1 cyclin function.^{6,7} However, cyclin C can also regulate both cell cycle progression and gene transcription: cyclin C forms complexes with Cdk8 and induces the transcription of Cdc2 (Cdk1). The Cdk8–cyclin C complex is a component of the RNA polymerase II holoenzyme, where it functions as a kinase that phosphorylates the C-terminal domain,⁸ and can also repress transcription by phosphorylating the Cdk7–cyclin H subunits of the general transcription initiator factor IIH.⁹ The Cdk7–cyclin H complex can activate many other cyclin–Cdk complexes directly involved in cell cycle progression, such as Cdk2, Cdk4, and Cdc2.¹⁰ There has been a limited number of reports concerning the secondary cyclins C and H in clinical samples. The Cdk8 gene has been linked to lymphoblastic leukaemia,¹¹ and the cyclin C protein has been associated with the pathogenesis of Alzheimer's disease.¹²

The primary cyclins (A, B1, D1, D3, and E) have been studied in various malignancies, including colon cancer,^{13–19} but only one or a few cyclins have been included. Previous reports are contradictory regarding the prognostic role of

cyclin A in colon cancer.^{16,20} The amplification of cyclins in colorectal tumours and cell lines has been studied,^{21,22} and reports of specific gene amplifications with prognostic value have emerged.^{23–26}

We have mapped the protein expression and gene amplification status of the cyclins with a potential role in deregulation of the cell cycle (cyclins A, B1, C, D1, D3, E, and H) in colon adenocarcinoma and their prognostic value in patients undergoing surgery for the disease.

METHODS

Patient and tumour material

The study comprised tumour samples from a consecutive series of 219 patients with colonic adenocarcinoma (proximal to the rectum) and adjacent normal mucosa, removed surgically from patients at the Akershus University Hospital, Norway during the years 1988 to 2000. According to the Dukes classification,²⁷ 10 patients had Dukes A tumours, 107 had Dukes B, 59 Dukes C, and 43 Dukes D. Eleven patients had well differentiated tumours, 184 had moderately differentiated tumours, and 23 had poorly differentiated tumours. One tumour was anaplastic.

The patients (114 women, 105 men) had a mean age at surgery of 70.0 years (range, 39.6–93.5) and were followed postoperatively according to the Norwegian guidelines for patients with colorectal cancer. This includes clinical examination and measurement of carcinoembryonic antigen every third month for five years, ultrasound examination of the liver every three months for three years and every six months for the subsequent two years, and chest radiograph every six

Abbreviations: Cdk, cyclin dependent kinase; CI, confidence interval; HR, hazards ratio; HSA, human serum albumin; PCR, polymerase chain reaction

Table 1 Immunostaining protocol

Antibody	Source	Dilution	Pre-treatment (microwave oven, 850 W)
Cyclin A	Novocastra Laboratories (Newcastle upon Tyne, UK)	1/75	2×5 min, 1mM EDTA (pH 8)
Cyclin B1	BioSource International (Camarillo, California, USA)	1/200	5 min + 15 min 350 W, 10mM citrate buffer (pH 6)
Cyclin C	Transduction Laboratories (Lexington, Kentucky, USA)	1/50	5 min + 15 min 350 W, 10mM citrate buffer (pH 6)
Cyclin D1	Oncogene Research (San Diego, California, USA)	1/100	2×10 min, 1mM citrate buffer (pH 6)
Cyclin D3	Dako (Carpinteria, California, USA)	1/50	4×5 min, 1mM EDTA (pH 8)
Cyclin E	Novocastra Laboratories	1/75	4×5 min, 1mM EDTA (pH 8)
Cyclin H	Santa Cruz Biotechnology (Santa Cruz, California, USA)	1/300	5 min + 15 min 350 W, Tris/EDTA (pH 9)

months during the five year follow up. Follow up data were collected from the last clinical appointment or death registration and the Norwegian Cancer Registry. Mean survival was 5.6 years. Forty two of the 176 patients with Dukes stages A–C tumours at the time of surgery developed distant metastases during follow up. One hundred and twenty eight (58.4%) of the patients died, 61 (27.9%) from cancer (metastatic disease). Of the 61 patients who died from cancer, 27 underwent surgery for Dukes stages B and C and 34 for Dukes stage D.

Immunohistochemistry

Sections (4–6 µm thick) from formalin fixed, paraffin wax embedded tumour tissue were applied to coated slides. After antigen retrieval by microwaving, immunostaining was performed in an Optimax plus automated cell stainer (model 1.5; BioGenex, San Ramon, California, USA), using the biotin–streptavidin–peroxidase method (Supersensitive Immunodetection System, LP-UL; Biogenex). The slides were incubated at room temperature for 30 minutes and counterstained with Mayer's haematoxylin for visualisation of tissue structures. Table 1 shows the immunostaining protocol. All series included positive and negative staining controls.

A semiquantitative score for immunoreactivity was used¹⁸ as follows: grade 0, < 5% cells positive; +1, 5–30% positive cells; +2, 30–60% positive cells; and +3, > 60% positive cells. An internal control from the normal tissue was available for most of the samples. The cutoff value for cyclins in the tumour tissue was set at the level of expression in the normal

tissue (5% for cyclins A, B1, D1, D3, and E; 30% for cyclins C and H). Only cells with convincing nuclear staining were scored as positive, except for cyclin B1 that was considered positive also in the cytoplasm.²⁸ For all samples, at least 100 (usually more than 1000) cells were analysed in high power microscopic fields from all parts of the tumour tissue in each slide.

Gene amplification analysis

Genomic DNA was extracted from thin sections of the tumour samples, in a GenoMTM-48 Robotic Workstation (GenoVision, Oslo, Norway), as described by the manufacturer (GenoMTM-48, Automated DNA Isolation from Tissue Handbook, February 2001, GenoVision). The DNA concentration was determined by spectrophotometry (GeneQuantproTM; Amersham Pharmacia Biotech, Buckinghamshire, UK). All paraffin wax embedded sections contained > 75% (often > 90%) tumour tissue, confirmed by light microscopy of a haematoxylin and eosin stained slide made from the adjacent section of the paraffin wax block.

Primers and probes for the cyclin genes and the human serum albumin (HSA) gene were chosen with the assistance of the Primer Express 2.0 software program (Applied Biosystems, Foster City, California, USA). Primers were purchased from Invitrogen (Carlsbad, California, USA) and probes from Applied Biosystems. Table 2 shows the nucleotide sequences of the probes and primers used.

Real time quantitative polymerase chain reaction (PCR)²⁹ was performed using an ABI Prism 7900 sequence detection

Table 2 Primer and probe sequences used in real time quantitative polymerase chain reaction

Gene	Primer sequence (5'–3')	Hybridisation probe sequence (5'–3')
CCNA		
Sense	AAGAAGCCAGCTGAATCTCAA	AAAAGCCAGGGCATCTTCACGCTCTATT
Antisense	GGTCCAGGTAACAACTAATGGCTGAA	
CCNB1		
Sense	CCCTGCTGCAACCTCCAA	CCCGGACTGAGGCCAAGAACAGC
Antisense	TGTTCACTGACTTTGTACCAATGTC	
CCNC		
Sense	GCCGGCTGGTCTTTTTTA	TGCCATGGAACACAGCTTGCCCT
Antisense	TGGGAGCTCTGCCAAAAGTT	
CCND1		
Sense	CCGTCCATGCGGAAGATC	CCTCCAGCATCCAGGTGGCGA
Antisense	AACAAGTTGACAGGAAGTCTTAAGA	
CCND3		
Sense	CTGTCTCTCCCGCCAGTT	CACCCCCGACACGTATTGTCT CCC
Antisense	CTGATATCTCAAGCTTTCCTTTTCT	
CCNE		
Sense	CCCCGCTGCTGACTGA	TCAGTGCCGACTCTGCCACATGG
Antisense	AGCATGGAGTAAGAGACCTGGAA	
CCNH		
Sense	TATCCTTACCACACTTTTTCTCT	TCTACCAGGTCGTCATCAGTCCATTCTCT
Antisense	AGAAATCAACTCAAATGGTTAGAGAGA	
HSA		
Sense	TGTTGCATGAGAAAACGCCA	AAGTGACAGAGTCACCAAAT GCTGCACAG
Antisense	GTCGCCTGTTACCAAGGAT	

All probes were labelled at the 5' end with FAM as reporter and at the 3' end with TAMRA as quencher.
HSA, human serum albumin.

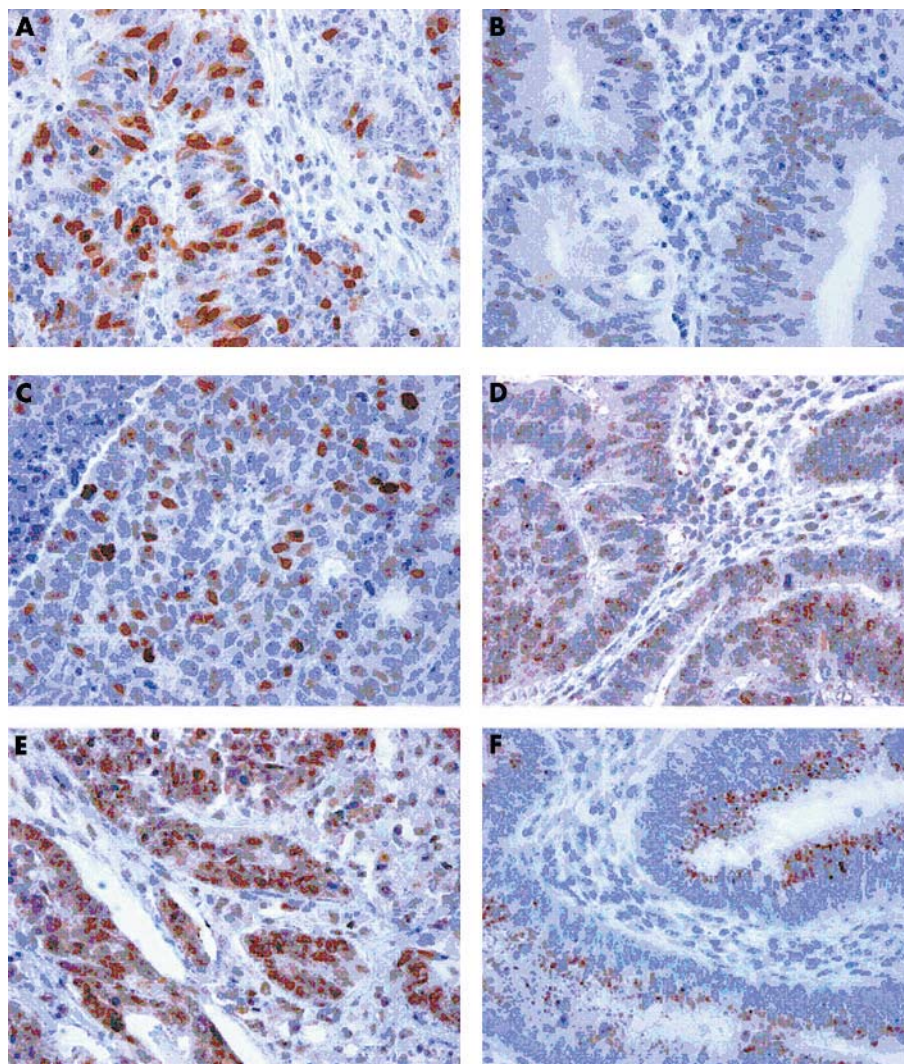


Figure 1 Representative examples of immunostaining (original magnification, $\times 400$) for (A) cyclin A, (B) cyclin D3, (C) cyclin E, (D) cyclin C, (E) and cyclin H. (F) An example of cytoplasmic immunopositivity for cyclin B1.

system (Perkin-Elmer Applied Biosystems, Foster City, California, USA) with the software program SDS 2.0 (Applied Biosystems). Each assay, run on a 96 well plate, included patient samples, normal controls (normal colon mucosa), and no template controls (distilled water). Each target sample and the controls were run in triplicate for both cyclin and HSA. The PCR mix consisted of: TaqMan Universal PCR Master Mix, 600nM primers (HSA primers, 700nM),

200nM probe, and 2 μ l DNA (2–50 ng/ μ l) (or H₂O) in a final volume of 25 μ l. Default cycling conditions were used.

Relative quantitation of the cyclin genes was performed by normalising the results to those of HSA. The normalised amount of each of the genes present in the tumour sample was determined by designating the normal tissue as a calibrator using a comparative C_t method ($2^{-\Delta\Delta C_t}$ method) according to the PE Applied Biosystems protocol (KJ Livak. *Comparative Ct method*. User Bulletin No. 2, 1997, PE Applied Biosystems). To verify that biochemical interactions or the PCR conditions per se did not provide PCR products, we performed real time quantitative PCR on different combinations of cyclin primers and probes, and only the appropriate cyclin specific combinations of primers and probe gave rise to a PCR product (data not shown).

An absolute number of the amount of amplification present in the tumour sample compared with the normal control was calculated as a $2^{-\Delta\Delta C_t}$ value in the software program Excel for Microsoft Office 2000, after importing the crude C_t values from SDS 2.0. The cutoff value for amplification was a $2^{-\Delta\Delta C_t}$ value > 2 .

Table 3 Cyclin immunoreactivity in colon adenocarcinomas

Cyclin	N (total)	Grade			
		0	+	++	+++
A	219	16	87	93	23
B1	200	84	60	34	22
C	201	25	27	84	65
D1	219	195	22	2	0
D3	218	139	48	23	8
E	217	162	30	24	1
H	201	0	0	29	172

Grade: 0, $< 5\%$ positive cells; +, 5–30% positive cells; ++, 30–60% positive cells; +++, $> 60\%$ positive cells. The cutoff value for secondary cyclins was set at 30% immunoreactive cells.

Statistical analysis

Survival analysis was performed by the Kaplan–Meier log rank test and was adjusted for covariates in a Cox regression

Table 4 Amplification of cyclin genes in primary human colon adenocarcinoma

Gene	N (total)	Amplification level*			
		<0.5	0.5–1.9	2.0–4.9	≥5
CCNA	210	1	196	13	0
CCNB1	188	2	169	16	1
CCNC	208	1	151	39	17
CCND1	209	0	94	96	19
CCND3	204	6	155	39	3
CCNE	204	1	164	36	3
CCNH	197	8	179	10	0

*N-fold difference from the normal control. Cutoff level, >2. Amplification was measured by real time quantitative polymerase chain reaction and levels were determined by the comparative C_t method ($2^{-\Delta\Delta C_t}$ method).

analysis that included only the parameters with significance in the univariate (log rank) test. Correlation analyses were performed by Kendall's τ test.

Multiple comparison post hoc tests (Bonferroni and Tukey) showed a significant difference between the immunohistochemical grades 0/+1 and grades +2/+3 for cyclin A with regard to cancer death. Hence, in prognostic analysis, grade +2 served as the cutoff value: cyclin A expression was regarded as low when the immunohistochemical score was grade 0 or grade +1 and high when the score was grade +2 or grade +3.

Statistical analyses were carried out using SPSS version 11.0 for Windows 2000, except for estimation of the proportional hazard for each covariate in the Cox model (software program S+ for Windows version 6.1). Significance was set at $p < 0.05$.

RESULTS

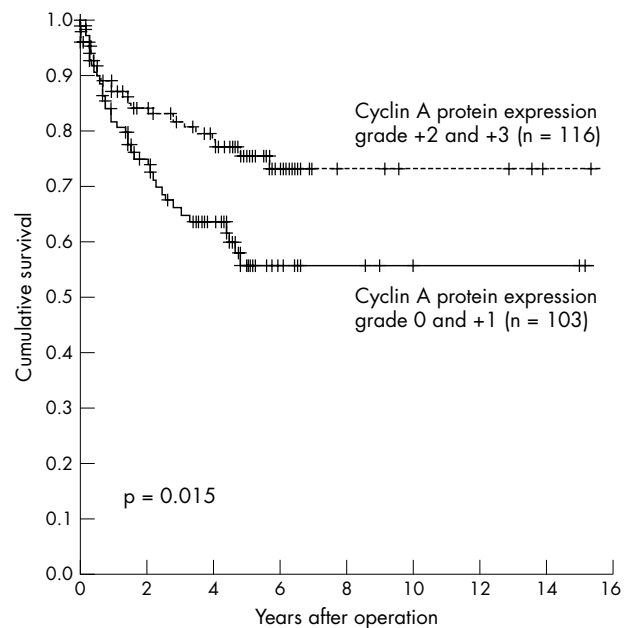
Tables 3 and 4 show the immunohistochemical scores and gene copy numbers, respectively. Figure 1 shows examples of immunostaining and a photograph of cyclin D1 immunostaining has been published previously.³⁰ Concurrent protein overexpression and gene amplification for cyclin A was seen in 12 patients, for cyclin B1 in 10 patients, for cyclin C in 51 patients, for cyclin D1 in 23 patients, for cyclin D3 in 11 patients, for cyclin E in 10 patients, and for cyclin H in 10 patients. A significant correlation between protein overexpression and gene amplification was seen only between cyclin C and CCNC (Kendall's $\tau = 0.144$; $p = 0.030$).

The expression of cyclin A correlated with that of cyclins D3 and E (Kendall's $\tau = 0.213$; $p < 0.001$ and Kendall's $\tau = 0.158$; $p = 0.011$, respectively). The amplification levels for the different cyclin genes correlated with each other, except for the amplification of CCND1, which correlated with none of the other cyclin genes (table 5).

Table 5 Correlation coefficients* between the amplification levels of the cyclin genes in 219 primary human colon adenocarcinomas

	CCNB1	CCNC	CCND1	CCND3	CCNE	CCNH
CCNA	0.481	0.352	-0.127	0.313	0.356	0.381
CCNB1	-	0.319	-0.007	0.386	0.393	0.456
CCNC	-	-	0.032	0.446	0.535	0.237
CCND1	-	-	-	0.012	-0.133	-0.011
CCND3	-	-	-	-	0.348	0.178
CCNE	-	-	-	-	-	0.205

*Kendall's τ non-parametric correlation. All correlations are significant the <0.01 level, except for CCND1 amplification, which correlated with none of the other cyclins.

**Figure 2** Cyclin A protein expression and survival. The Kaplan-Meier curves show the probability of overall survival in colon adenocarcinoma according to cyclin A protein expression determined by immunohistochemistry. The survival curves were analysed by the log rank test; +, censored data.

Of the cyclin proteins and genes, only cyclin A protein expression showed a significant correlation with Dukes stage and tumour differentiation grade (Kendall's $\tau = -0.137$; $p = 0.042$ and Kendall's $\tau = -0.140$; $p = 0.039$, respectively).

When analysing the expression of the different cyclin proteins individually with regard to survival in a univariate (log rank) test, only low expression of cyclin A showed a significant association with an unfavourable prognosis ($p = 0.015$). Figure 2 shows the cumulative survival curves in the groups with low expression of cyclin A (grades 0 and +1) and high expression of cyclin A (grades +2 and +3). Gene amplification was also analysed with regard to survival and only CCNC amplification was significantly associated with poor survival (log rank $p = 0.019$). Figure 3 shows the cumulative survival curves in the groups with relative CCNC gene copy numbers ≥ 2 and < 2 , respectively.

In a multivariate (Cox regression) analysis adjusted for age, including Dukes stage, tumour differentiation grade, cyclin A expression, and CCNC amplification, significance was maintained for all the parameters except for tumour differentiation grade (Dukes stage: $p < 0.001$; hazards ratio (HR), 4.89; 95% confidence interval (CI), 3.39 to 7.10; cyclin A expression: $p = 0.044$; HR, 0.57; 95% CI, 0.33 to 0.98; CCNC amplification: $p = 0.049$; HR, 1.72; 95% CI, 1.00 to 2.94; table 6).

DISCUSSION

In our present study, we analysed cyclin A, B1, C, D1, D3, E, and H protein expression and gene amplification levels in primary colon adenocarcinomas and found that amplification of CCNC correlated independently with an unfavourable outcome, whereas cyclin A protein overexpression correlated with a better prognosis.

The cyclins have previously been evaluated in colorectal carcinomas and cell lines.^{21, 22} We found higher levels of amplification in our study, possibly reflecting the sensitivity of real time quantitative PCR. Similar findings have been

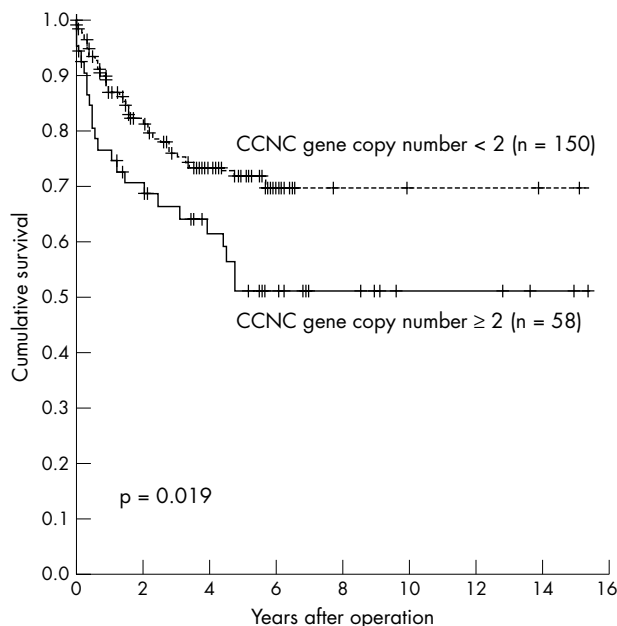


Figure 3 CCNC amplification status and survival. The Kaplan–Meier curves show the probability of overall survival in colon adenocarcinoma according to CCNC amplification determined by real time polymerase chain reaction. The survival curves were analysed by the log rank test; +, censored data.

reported in breast cancer using real time PCR, revealing a higher degree of gene amplification than is seen with Southern blotting,³¹ mainly in cases with low extra copy numbers (N from 2 to 2.9).

Of the proteins examined, only cyclin A showed independent prognostic value. In other cancers, cyclin A overexpression has been reported to predict a poor prognosis.^{18 32 33} The relation between cyclin A and prognosis in colon cancer is yet to be established. Our results disagree with the findings of a study on colorectal cancer by Handa *et al*,¹⁶ but are in agreement with a more recent study by Li *et al*.²⁰ Handa *et al* examined a limited number of patients (73), only seven of whom had late stage cancer (stage IV), and also included rectal cancers. Li and colleagues evaluated cyclin A in 132 patients with colon cancer and showed that cyclin A overexpression was reduced in carcinomas invading local tissues. They concluded that the loss of cyclin A overexpression may lead to a strong invasive capability and increased aggressiveness. In our study, cyclin A expression correlated inversely with Dukes stage, implying lower expression of cyclin A in the advanced stages of the disease.

“Concurrent amplification and overexpression of the respective cyclins varied in our study, supporting previous findings that protein overexpression is not necessarily caused by gene amplification”

The association between the amplification of CCNC and impaired survival seems to be independent of its gene product. However, expression of the cyclin C protein showed a borderline association with disease specific survival (p = 0.069; table 6). This could represent a biological association with disease severity, which did not reach significance in our study.

Amplifications occur more readily when p53 is inactivated,³⁴ and an amplicon could survive more readily in the absence of normal p53 and thereby accumulate additional amplicons after further cellular divisions.³⁵ Cyclin A

Table 6 Relation between various clinicopathological parameters and survival

	Log rank test	Cox regression test*		
	p Value	p Value	HR	95% CI
Cyclin A	0.015	0.044	0.57	0.33 to 0.98
Cyclin B1	0.557			
Cyclin C	0.069			
Cyclin D1	0.420			
Cyclin D3	0.162			
Cyclin E	0.070			
Cyclin H	†			
CCNA amplification	0.440			
CCNB1 amplification	0.623			
CCNC amplification	0.019	0.049	1.72	1.00 to 2.94
CCND1 amplification	0.280			
CCND3 amplification	0.971			
CCNE amplification	0.611			
CCNH amplification	0.468			
Tumour differentiation	0.044	0.062	0.51	0.25 to 1.04
Dukes stage	<0.001	<0.001	4.89	3.39 to 7.10

*Including only parameters with significance in the univariate test and adjusted for age; †all tumours were immunopositive for cyclin H.

overexpression can stimulate re-replication.³⁶ DNA amplification involves a stretch of DNA much larger than the selected gene that is amplified, and coamplification can occur.³⁷ However, the cyclin genes are located on different chromosomes so that this last mechanism cannot explain our findings. A non-discriminating mechanism for amplification accumulation, such as cyclin A overexpression stimulated re-replication, could represent a possible underlying mechanism for the enhanced gene copy numbers detected in our study. The probe based homogenous assay used in our study is meant to provide specific amplification products only, because hybridisation of both the primers and the probe is necessary to generate a signal. To rule out the possibility that the correlations between the amplification levels of the different cyclins were an artefact of the methodology used, we performed real time quantitative PCR using combinations of primers for one of the cyclins and probes for another cyclin and with appropriate combinations as controls in the same run. Only appropriate combinations of primers and probes gave a PCR product.

Concurrent amplification and overexpression of the respective cyclins varied in our study, supporting previous findings that protein overexpression is not necessarily caused by gene amplification.^{38–40}

Colon adenocarcinomas typically lose control of cyclin expression and high expression of cyclin A might be a marker of improved prognosis. The secondary cyclins C and H are highly overexpressed in colon cancer, but did not provide additional prognostic value. Expression of the cyclin C protein correlates with the amplification of its encoding gene

Take home messages

- Varying degrees of cyclin gene amplification and cyclin protein overexpression were seen in all colon adenocarcinomas
- Amplification of the cyclin C gene was related to an unfavourable prognosis
- High expression of the cyclin A protein was associated with a better outcome
- Further studies are needed to elucidate the role of the secondary cyclins C and H in colon carcinogenesis

and amplification of the CCNC gene was independently associated with an unfavourable prognosis. Further studies are needed to elucidate the role of secondary cyclins C and H in colon carcinogenesis.

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