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Cdc2 as prognostic marker in stage UICC II colon carcinomas

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ABSTRACT

Purpose: Cyclin-dependent kinase 2 (cdc2) controls the G2–M checkpoint and, therefore, the entrance of cells into mitosis. It might play a crucial role during tumour progression in colon carcinomas (CCA). Thus, the prognostic value of cdc2 expression and connected markers relevant for proliferation and apoptosis has to be evaluated.

Experimental design: Punch biopsies from the tumour centre and the invasion front of 0.6 mm diameter from 392 CCA stage UICC II–IV were integrated in 14 recipient paraffin blocks. After immunohistochemical staining for cdc2, p53, caspase 3 and ki-67, a present (+) and absent (–) scoring was performed in the tissue arrays. The logrank test was used to compare distant metastasis and cancer-related survival. Multivariate Cox regression analysis was done to identify independent prognostic factors for parameters with significant influence on cancer-related survival (CRS) and distant metastasis (DM).

Results: The pT-category ($p = 0.007$), nodal status ($p < 0.001$), extramural venous infiltration ($p < 0.001$) and lymphatic vessel invasion ($p = 0.003$) were identified as independent histological parameters for CRS. Univariate analysis relating to stage UICC II–IV CCA showed caspase 3 in the tumour centre ($p = 0.047$) to be a prognostic marker for CRS. In stage UICC II cdc2 ($p = 0.041$) and caspase 3 in the invasion front ($p = 0.026$) could be identified as independent prognostic factors for CRS and DM by multivariate analysis.

Conclusions: Cdc2 and caspase 3 could be identified as independent prognostic markers in stage UICC II CCA. They might be of value to select patients who should receive adjuvant treatment.

1. Introduction

The balance between mitosis and apoptosis is a crucial point in the control of cancer progression. Before entering mitosis, each cell is subjected to a number of checkpoints during the cell cycle, where the integrity of the genome is controlled. Here, we focused particularly on the transition point (G2–M phase), concerning mutations in eminent regulator proteins at this stage of the cell cycle in colon carcinoma. Chronological course of the cell cycle is determined by a variant concentration of cyclins, which are able to activate cyclin-dependent kinases (cdks) that play an important role in the progression of mitosis. Phosphatase cdc25b is phosphorylated by cyclin-dependent kinase 2 (cdc2), and thus enabled to activate cdc2-cyclin B1 complex by dephosphorylation.[6,23] Cdc2-cyclin B1 complex (also referred to as mitosis-promoting factor,

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MPF) held in its inactive state by kinases Wee1 and Myt1 prevents the cell from entering mitosis. As a consequence, via cdc25b dephosphorylated MPF releases the cell into division.[18,25] In case of DNA damages, p53 in its function as a gatekeeper gene is phosphorylated leading to a dissociation of MDM2 from the former inactive MDM2-p53 complex and assembling of p53 protein within the cytoplasm, which exerts an inhibitory effect on cdc2-cyclin B-complex, i.e. cell cycle arrest. Further p53-mediated induction of diverse initiator caspases (e.g. caspase 2) results finally in activation of different effector caspases (e.g. caspase 3), representing the actual tools of the apoptosis cascade.[13,16] So far, it has been shown that an increased expression of cdc2 is associated with uncontrolled proliferation in a range of tumour species.[19,27] On the other hand, alterations in the apoptotic cascade inhibit regular self destruction of genome deficient cells, immortalising them and promoting cancerogenesis in this way. Our current study's purpose was to examine the prognostic value of the specific markers involved in cell cycle regulation and apoptosis in colon carcinomas stage UICC II–IV by immunohistochemistry. Especially in stage UICC II, which is a heterogeneous collective with partly high risk cases but no general recommendation for adjuvant therapy, markers in our selected panel seem to be of prognostic value.

2. Material and methods

2.1. Patients

Tumour samples of 235 (59.9%) male and 157 (40.1%) female patients with a mean age of 64 years (range: 28–91), which were curatively resected for the reason of colon carcinoma were included in the study. Each patient's samples were preserved, fixed in formalin solution and subsequently embedded in paraffin (donor) blocks. Only patients meeting the following criteria were included: first manifestation of a solitary colon carcinoma (except appendiceal carcinoma) stage UICC II–IV, radical surgery including standardised regional lymph node dissection during the years 1991–2001, no preoperative chemotherapy or radiation and post-operative R0 – status, confirmed by histology. Within the observed follow-up period (range: 0–189 months, mean: 93; two patients were lost to follow-up), a number of 159 (40.6%) patients died, of which 90 (23.0%) were classified as cancer-related deaths. Over the course of this study, 105 (26.8%) patients developed distant metastases. Patients suffering from inflammatory bowel disease were excluded, as well as were patients with any other malignant tumour, synchronously or according to history. Patients suffering from stage UICC III and IV received a 5-FU/FA-based chemotherapy regimen. Detailed information concerning patient and tumour characteristics is shown in Table 1.

Table 1 – Tumour characteristics: TNM-category, stage UICC, grading, extramural venous invasion and lymphatic vessel invasion.

	n	%
Total	392	100.0
pT-category		
pT2	27	6.9
pT3	314	80.1
pT4	51	13.0
pN-category		
pN0	207	52.8
pN1	110	28.1
pN2	75	19.1
M-category		
M0	356	90.8
M1	36	9.2
Stage UICC		
II	197	50.3
III	159	40.6
IV	36	9.2
Tumour grading		
Low grade	320	81.6
High grade	72	18.4
Extramural venous invasion		
Negative	344	87.8
Positive	46	11.8
Not available	2	0.5
Lymphatic vessel invasion		
Negative	146	37.2
Positive	246	62.8

2.2. Tissue array

Punch biopsies of 392 tumour specimens were integrated in 14 recipient paraffin array blocks, which were then prepared with coordinates for further correct identification of the single tumour samples. The punches with a 0.6 mm diameter were placed in 0.4 mm holes of the recipient array block to ensure best adhesion of the specimen cores to the array block. Three cores of tonsil tissues were attached on the upper left side to each recipient block in order to facilitate orientation and also to serve as controls for later immunohistochemical staining. Array blocks were finally incubated for 30 min. at 37 °C, in turn to improve core-paraffin-adhesion, cut with a standard microtome (Reichert-Jung Autocut 2040, today: Leica Microsystems, Wetzlar, Germany) and were fixed on glass slides for immunohistochemical staining. To obtain a representative image a total of six cores were taken from each of these 392 tumour specimens and positioned in the 14 recipient array blocks as described above, consisting of three cores of tumour centre and three cores of tumour invasion front. After staining and scoring, separate analyses were performed for tumour centre samples and tumour invasion frontline samples.

2.3. Immunohistochemistry

Two micro metres sections from the 14 array blocks were fixed on glass slides and left to dry overnight. They were then deparaffinised in xylene and rehydrated with ascending ethanol series. Antigen retrieval was performed using a microwave oven at 120–85° for 45 min, while the sections were placed in citrate buffer. Antibodies to cdc2 (monoclonal mouse anti-human, Clone [POH-1] ab8040, dilution 1:200, Abcam, Cambridge, UK), p53 (monoclonal mouse anti-human,

Table 2 – Prognostic value of histological criteria, immunohistochemical markers and emergency surgery on 5-year cancer-related survival (CRS) in stage UICC II–IV colon carcinomas. 95% CI: 95% confidence interval; –: negative scoring for immunohistochemical marker; +: positive scoring for immunohistochemical staining.

	n	Cancer-related 5-year survival (%)	95% CI	*p*-Value
Total	392	81.1	77.7–85.2	
T-category				
T2	27	96.3	89.2–100.0	0.007
T3	314	82.2	77.9–86.5	
T4	51	65.5	52.2–8.8	
N-category				
N0	207	89.7	85.4–94.0	<0.001
N1	110	86.3	79.8–92.8	
N2	75	49.3	37.7–60.9	
M-category				
M0	356	83.5	79.6–87.4	<0.001
M1	36	57.3	40.8–73.8	
Stage UICC				
II	197	91.3	87.2–95.4	<0.001
III	159	74.3	67.4–81.2	
IV	36	57.3	40.8–73.8	
Tumour grading				
Low grade	320	82.4	78.1–86.7	0.18
High grade	72	75.2	65.0–85.4	
Extramural venous infiltration				
Negative	344	85.7	82.0–89.4	<0.001
Positive	46	47.7	32.8–62.6	
Lymphatic vessel invasion				
Negative	146	88.2	82.7–93.7	0.003
Positive	256	77.1	71.8–82.4	
Emergency operation				
No	348	83.3	79.4–87.2	<0.001
Yes	44	59.0	43.5–74.5	
Cdc2 tumour centre				
–	29	79.0	64.1–93.9	0.536
+	334	81.0	76.7–85.3	
Cdc2 invasion front				
–	48	76.2	63.9–88.5	0.390
+	259	79.6	74.5–84.7	
p53 tumour centre				
–	120	79.4	72.1–86.7	0.887
+	245	82.4	77.5–87.3	
p53 invasion front				
–	111	80.2	72.6–87.8	0.402
+	205	80.2	74.7–85.7	
Caspase 3 tumour centre				
–	123	77.4	70.0–84.8	0.047
+	230	83.6	78.7–88.5	
Caspase 3 invasion front				
–	120	77.6	70.0–85.2	0.530
+	172	82.3	76.4–88.2	
Ki-67 tumour centre				
–	44	79.9	67.4–92.4	0.614
+	348	81.2	77.1–85.3	
Ki-67 invasion front				
–	99	81.2	73.4–89.0	0.581
+	292	81.0	76.5–85.5	

Table 3 – Prognostic factors (histology, immunohistochemistry and emergency surgery) for 5-year cancer-related survival (CRS) identified during univariate analysis in stage UICC II colon carcinomas. 95% CI: 95% confidence interval.

	n	Cancer-related 5-year survival (%)	95% CI	*p*-Value
Total	197	88.3	83.6–93.0	
T-category				
pT3	174	87.3	82.0–92.6	0.256
pT4	23	95.2	86.2–100.0	
Tumour grading				
Low grade	169	87.5	82.2–92.8	0.486
High grade	28	92.7	82.9–100.0	
Extramural venous invasion				
Negative	185	89.9	85.2–94.4	0.008
Positive	12	72.7	45.9–98.5	
Lymphatic vessel invasion				
Negative	102	92.2	86.9–97.5	0.610
Positive	95	89.9	83.6–96.2	
Emergency operation				
No	180	92.2	88.1–96.3	0.055
Yes	17	80.8	61.2–100.0	
Cdc2 tumour centre				
–	19	83.5	66.4–100.0	0.104
+	162	92.7	88.6–96.8	
Cdc2 invasion front				
–	23	77.4	60.0–94.8	0.04
+	123	92.1	87.2–97.0	
p53 tumour centre				
–	62	94.9	89.2–100.0	0.221
+	122	90.2	84.7–95.7	
p53 invasion front				
–	54	93.9	87.2–100.0	0.227
+	99	88.1	81.4–94.8	
Caspase 3 tumour centre				
–	58	89.1	80.9–97.3	0.065
+	119	92.7	87.8–97.6	
Caspase 3 invasion front				
–	56	82.9	72.7–93.1	0.003
+	84	96.0	91.7–100.0	
Ki-67 tumour centre				
–	22	84.3	67.8–100.0	0.036
+	175	92.1	88.0–96.2	
Ki-67 invasion front				
–	55	88.5	79.9–97.1	0.933
+	142	92.4	87.9–96.9	

Clone [DO-7] M 7001, dilution 1:50, DakoCytomation, Glostrup, Denmark), ki-67 (monoclonal mouse anti-human, Clone [MIB-1] M7240, dilution 1:100, DakoCytomation, Glostrup, Denmark) and caspase 3 (polyclonal rabbit anti-human, Cleaved Caspase 3 [Asp 175] # 9661, dilution 1:200, Cell Signalling Technology Inc., Danvers MA, USA) were used. All slides were then processed manually. After the application of these primary antibodies, the sections were incubated overnight at room temperature. Cdc2, p53, ki-67 and caspase 3 were further processed using biotin–streptavidin–peroxidase (DAKO Hamburg, Germany) detection technique with subsequent coupled alkaline phosphatase (DAKO, Hamburg, Germany) and Fast Red (Sigma-Aldrich, Munich, Germany) detection technique for signal amplification.

2.4. *Scoring*

All analyses were performed with a light-optical microscope (Leica Microsystems, Wetzlar, Germany) under 40-fold magnifications. Two categories were introduced to assess staining intensity: positive (+) or negative (–). The arithmetic means were generated separately for both the tumour centre and the tumour invasion front per patient. In cases where no tumour tissue was included in the punch biopsy or the punch

Table 4 – Multivariate analysis of prognostic histological and immunohistochemical markers for cancer-related 5-year survival identified in stage UICC II colon carcinoma. +: positive scoring for immunohistochemical staining; –: negative for immunohistochemical staining; 95%CI: confidence interval.

	n	Relative risk	95% CI	*p*-Value
Extramural venous invasion				
Negative	127	1.0	0.7–10.6	0.129
Positive	8	2.8		
Cdc2 invasion front				
+	114	1.0	1.1–11.5	0.041
–	21	3.5		
Caspase 3 invasion front				
+	83	1.0	1.2–12.4	0.026
–	52	3.8		
Ki-67 tumour centre				
+	122	1.0	0.9–10.0	0.083
–	13	2.9		

biopsy was damaged, samples were excluded from evaluation.

2.5. Statistical analysis

The Kaplan–Meier method was used to calculate cancer-related survival (CRS) and distant metastasis (DM). The prognostic value of the TNM stage, tumour grading, lymphatic vessel invasion, extramural venous invasion, elective/emergency surgery, adjuvant chemotherapy in UICC III, and immunohistochemical markers was investigated by explorative univariate analysis. The 95% confidence intervals (CI) were calculated as proposed by Greenwood and a *p*-value of less than 0.05 was considered to be significant. The logrank test was used to compare distant metastasis and cancer-related survival. Multivariate Cox regression analysis was done to identify independent prognostic factors for parameters with significant influence on cancer-related survival in univariate analysis. Chi square test was utilised to compare frequencies. All analyses were performed using the statistic software SPSS for Windows Version 14 (SPSS Inc., Chicago, USA).

3. Results

3.1. Tumour characteristics and patient treatment

In 27 cases T2 (6.9%), in 314 cases T3 (80.1%) and in 51 cases (13.0%) T4 tumours were identified by histology. 185 tumours (47.1%) were lymph node positive, and 207 tumours (52.8%) were lymph node negative. Thirty-six (9.2%) patients suffered from distant metastasis while in 356 (90.8%) patients no distant metastasis was present at the time point of surgery. Tumours were characterised as low grade in 320 cases (81.6%) and as high grade in 72 cases (18.4%). Extra venous invasion occurred in 46 tumours (11.8%), but no venous invasion was detected in 344 cases (87.8%). Lymphatic vessel invasion was positive in 246 tumours (62.8%) and negative in 146 tumours (37.2%). One hundred and eighty-nine tumours (48.2%) were localised at the sigmoid colon, 58 (14.8%) at the ascending colon, 40 (10.2%) at the Cecum, 39 (9.9%) at the transverse colon, 26 (6.6%) at the hepatic flexure, 23 (5.9%) at the splenic flexure and 17 (4.3%) at the descending colon. Sigmoid resection was performed in 154 patients (39.3%), right hemicolectomy in 97 patients (24.7%), subtotal colectomy in 48 patients (12.2%), left hemicolectomy in 45 patients (11.5%), extended right hemicolectomy in 37 patients (9.4%), extended left hemicolectomy in 10 patients (2.6%) and segmental resection of the colon in one case (0.3%). Emergency surgery for the reason of bowel perforation, bleeding or obstruction was necessary in 44 patients (11.2%). Post-operative radiation was performed in 8 cases (2.0%) and adjuvant chemotherapy in 66 cases (16.8%). For the reason of hepatic metastasis in 10 patients (2.5%), neoadjuvant chemotherapy was performed prior to liver resection (Table 1).

3.2. Immunohistochemistry

For Cdc2, 334 tumours (85.2%) were scored as positive, and 29 (7.4%) were scored as negative in the centre of the tumour. In the invasion front, 259 tumours (66.1%) were diagnosed as positive and 48 cases (12.2%) as negative for cdc2. P53 in the tumour centre was identified as positive in 245 cases (62.5%) and negative in 120 tumours (30.6%). For 205 tumours (52.3%) in the invasion front p53 was scored as positive and in 111 cases (28.3%) as negative. Caspase 3 in the tumour centre was positive in 230 cases (56.7%) and negative in 123 tumours (31.4%). In the invasion front, 172 tumours (43.9%) were found to be positive for caspase 3, and 120 cases (30.6%) were found to be negative. Ki-67 was scored positive in 348 tumours (88.8%) and negative in 44 cases (11.2%) in the tumour centre. In 292 tumours (74.5%), ki-67 was positive and in 99 cases (25.3%) it was negative at the invasion front (Table 2). A UICC stage dependent, significantly differential expression of immunohistochemical makers, could be identified for caspase 3 ($p = 0.025$) and cdc2 ($p = 0.001$) in the invasion front in UICC stage II patients (Table 3).

3.3. Prognostic factors

As independent histological parameters for 5-year cancer-related survival, the pT-category ($p = 0.007$), nodal status ($p < 0.001$), the extramural venous infiltration ($p < 0.001$) and the lymphatic vessel invasion ($p = 0.003$) were identified. Furthermore, emergency operations were significantly ($p < 0.001$)

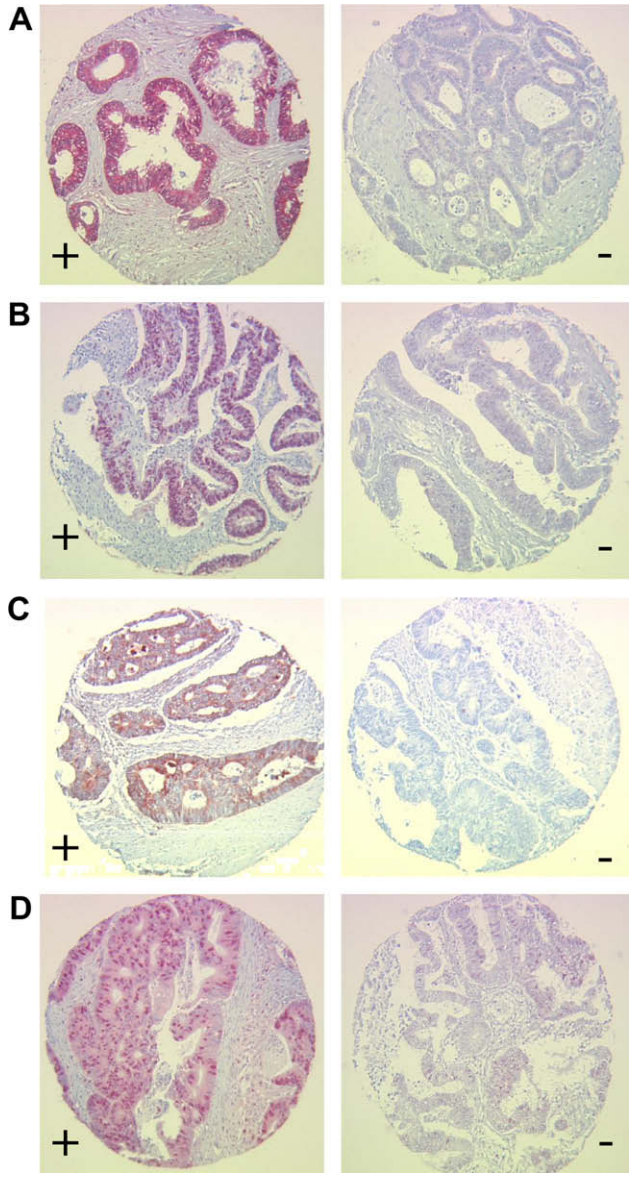

Fig. 1 – Samples for immunohistochemical staining in tissue arrays: (A) cdc2, (B) p53, (C) caspase 3 and (D) ki-67. +: positive scoring; –: negative scoring.

associated with poor 5-year outcome. In a combined analysis including stage UICC II–IV colon carcinomas only caspase 3 in the tumour centre ($p = 0.047$) was identified as independent prognostic marker for cancer-related survival (Table 2). For stage UICC II extramural venous infiltration ($p = 0.008$), cdc2 in the invasion front ($p = 0.04$), caspase 3 in the invasion front ($p = 0.003$) and ki-67 in the tumour centre were identified as significant predictors for survival by univariate analysis (Table 3). By multivariate analysis, only cdc2 in the invasion front ($p = 0.041$) and caspase 3 in the invasion front ($p = 0.026$) could be established as independent prognostic markers for cancer-related survival in stage UICC II colon carcinoma (Table 4, Fig. 1, Fig. 2a). Consequently, cdc2 in the invasion front ($p = 0.002$) and caspase 3 in the invasion front ($p = 0.014$) were significantly associated with five-year distant metastasis in stage UICC II colon carcinoma (Fig. 2b).

4. Discussion

Current staging of colon carcinomas is based on histopathological criteria regarding the TNM system.[10–12] In metastatic colon carcinomas (stage UICC III and IV) adjuvant chemotherapy is a recommended and established therapy regimen.[20–22] In stage UICC II, post-operative treatment is still a matter of discussion. There is strong evidence, that special high risk cases do exist, which would even benefit from adjuvant chemotherapy.[17] In our study, no common histopathological feature but the immunohistochemical markers cdc2 and caspase 3 could be identified as independent prognostic parameters for distant metastases and cancer-related survival in stage UICC II. Therefore, these markers represent an ideal tool to evaluate high risk cases in this category. They may be useful to select patients who should receive adjuvant chemotherapy. The different expression of our investigated molecular markers within the tumour centre and the invasion front reflects the heterogeneous expression of molecules in colon carcinoma throughout all stages.[1,15] One important feature of cancer is the uncontrolled tumour growth. The integrity of the DNA is essential to keep up correct cell function and control of proliferation. In cancer, these mechanisms are out of range and cells are not under regular growth control.[26] There are several checkpoints within the cell cycle to guarantee that the genome remains intact. If these checkpoints are not in place, the subsequent result is unrestricted proliferation. Cyclin-dependent kinases are essential factors for this process, showing a variable expression throughout the cell cycle.[7,18] Cdc2 (cdk1) regulates the transition between the G2- and M-phase in eukaryotic cells and, therefore, acts as a gatekeeper for entering mitosis.[7,8] Down regulation of cdc2 results in cell cycle arrest in the G2-phase allowing DNA repair before entering mitosis. But activation of cdc2 does not only result in mitosis. Under several circumstances depending on the kind of DNA damage it can either induce mitosis or apoptosis.[8,24] The overexpression of cdc2 in the invasion front in stage UICC II colon carcinomas reflects the increased turnover of cells in this region. Surprisingly, it did not correlate with the proliferation marker ki-67 but with the apoptosis indicator caspase 3. For this reason, our findings support the observations that cdc2 may even induce apoptosis. The expression of p53, an activator of apoptotic death caused by DNA damage, did not correlate with the apoptosis in the invasion front in stage UICC II. In cell cultures (HL60) with no expression of p53, an unscheduled activation of cdc2 was described as an early event of apoptosis.[24] Otherwise p53 mutations with deficient function are described in various tumour types including colon carcinoma.[2–4,9,14] Absence of p53 function decreases apoptosis and leads to uncontrolled proliferation. But in tumour tissue mechanisms of cellular proliferation do not follow regular mechanisms as they can be observed in normal cells and are not completely understood in detail. Nevertheless, ki-67 and p53 could not be identified as independent prognostic factors in stage UICC II or in any other stages. These findings are in congruence with the recent studies, where p53 and ki-67 had no influence on the disease outcome in colorectal cancer.[5]

In summary, we identified cdc2 and caspase 3 as independent prognostic markers for outcome in stage UICC II colon

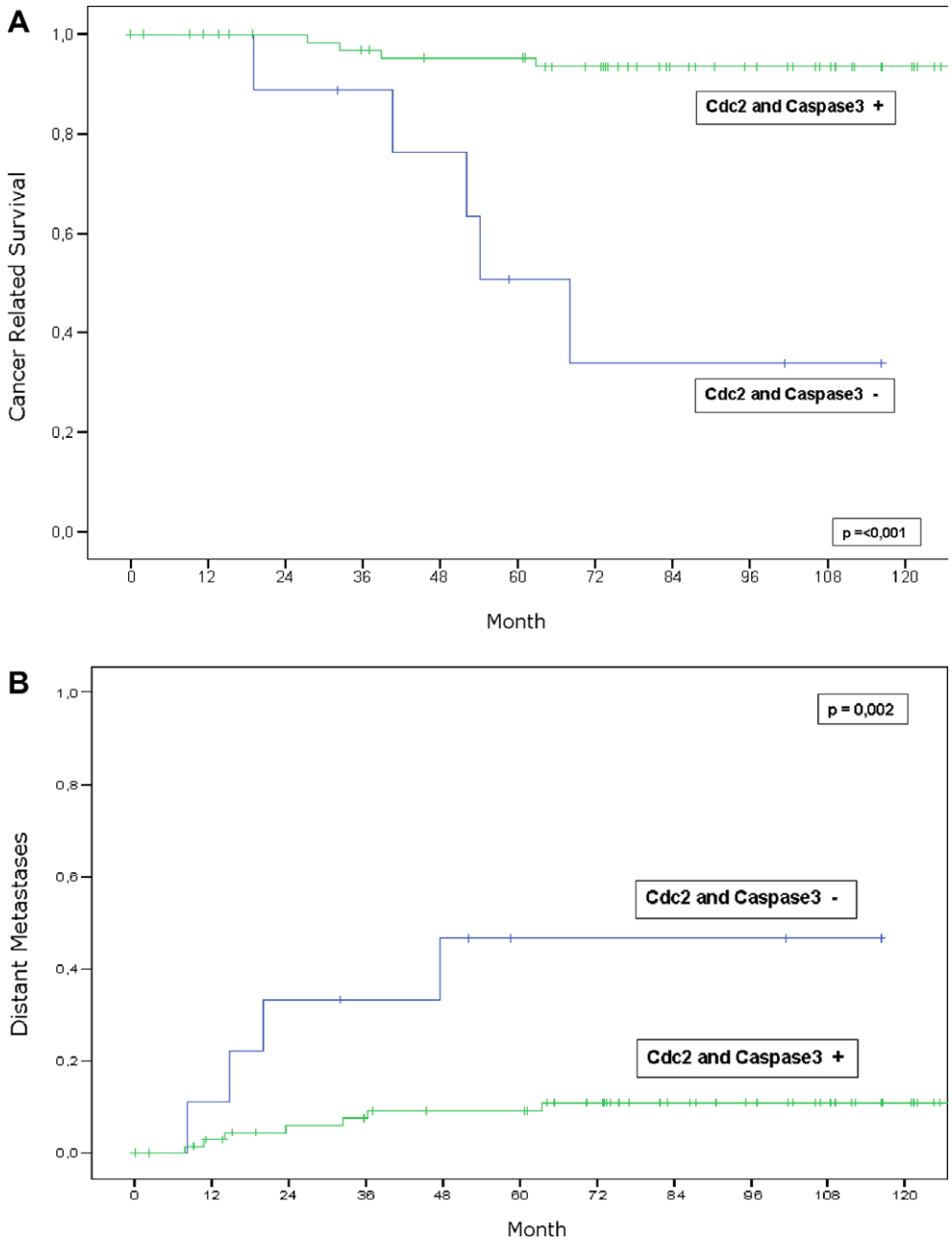

Fig. 2 – (A) Cancer-related survival in cyclin-dependent kinase 2 (cdc2) and caspase 3 positive (+) or negative (–) samples in the invasion front of stage UICC II colon carcinomas. (B) Distant metastases in cdc2 and caspase 3 positive (+) or negative (–) samples in the invasion front of stage UICC II colon carcinomas.

carcinomas. These markers may be useful to select patients for adjuvant treatment. The co-expression of cdc2 and caspase 3 underlines the recent findings that postulate an involvement of cdc2 not only as regulator of mitosis but also in the processes of apoptosis.

Conflict of interest statement

The authors of this manuscript have nothing to disclose. There are no financial, personal or other relationships with other people or organisations within which could inappropriately influence the published data.

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