CIP2A Influences Survival in Colon Cancer and Is Critical for Maintaining Myc Expression

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Abstract

The cancerous inhibitor of protein phosphatase 2A (CIP2A) is an oncogenic factor that stabilises the c-Myc protein. CIP2A is overexpressed in several tumours, and expression levels are an independent marker for long-term outcome. To determine whether CIP2A expression is elevated in colon cancer and whether it might serve as a prognostic marker for survival, we analysed CIP2A mRNA expression by real-time PCR in 104 colon cancer samples. CIP2A mRNA was overexpressed in colon cancer samples and CIP2A expression levels correlated significantly with tumour stage. We found that CIP2A serves as an independent prognostic marker for disease-free and overall survival. Further, we investigated CIP2A-dependent effects on levels of c-Myc, Akt and on cell proliferation in three colon cancer cell lines by silencing CIP2A using small interfering (si) and short hairpin (sh) RNAs. Depletion of CIP2A substantially inhibited growth of colon cell lines and reduced c-Myc levels without affecting expression or function of the upstream regulatory kinase, Akt. Expression of CIP2A was found to be dependent on MAPK activity, linking elevated c-Myc expression to deregulated signal transduction in colon cancer.

Introduction

Colorectal cancer (CRC) is the most common gastrointestinal malignancy. There are approximately 664,000 new cases each year worldwide. Half of these patients will die from this carcinoma [1]. Currently, standard treatment is primary surgery and, depending on the tumour stage, additional chemotherapy [2]. Due to the high recurrence rate, new potential targets and prognostic markers are needed to identify patients that are likely to benefit from additional therapy.

In 2007, Junttila and Westermarck identified the cancerous inhibitor of protein phosphatase 2A (CIP2A) as a human oncoprotein. CIP2A is overexpressed in head and neck squamous cell carcinomas and in colon carcinomas [3]. CIP2A inhibits the protein phosphatase 2A (PP2A). PP2A in turn has a critical role in turnover of the c-Myc oncoprotein, since PP2A dephosphorylates c-Myc at serine-62 (S62). Dephosphorylation at S62 is required for ubiquitination of c-Myc by the ubiquitin ligase Fbw7 and therefore, initiates degradation of c-Myc [4]. Overexpression of CIP2A inhibits PP2A activity and thereby stabilizes c-Myc. Consequently, this induces immortalisation and malignant transformation of human cells [3]. c-Myc itself remains the most important oncogenic driver in colorectal cancer [5].

Recent studies have shown that CIP2A is overexpressed in several human malignancies. CIP2A expression levels correlate with overall survival (OS) and disease free survival (DFS) in gastric carcinomas, in serous ovarian cancer, in renal cell carcinoma and in breast cancer [6;7;8;9]. In chronic myeloid leukemia (CML), CIP2A expression at the time point of diagnosis is a prognostic marker for the development of a blast crisis later on [10]. Furthermore, some oncogenic factors, including helicobacter pylori and papilloma virus 16 E7, upregulate expression of CIP2A and this may be critical for their oncogenic activity [11;12].

Recently, two groups reported conflicting results regarding the prognostic impact of CIP2A in colorectal cancer [13,14]. Bockelman et al. studied 752 patients, but could not determine any prognostic significance; in contrast Teng et al. studied 167 patients and identified CIP2A expression as a prognostic factor for colon carcinoma.

The aim of the present study was to address the relevance of CIP2A expression in colon cancer. First, we analysed expression of CIP2A in a cohort of 104 colon cancer patients with documented follow-up and confirmed its overexpression. Secondly, we investigated the association between CIP2A mRNA expression and clinical-pathological variables; lastly, we aimed to determine the molecular pathways that regulate CIP2A expression and, are regulated by CIP2A. Our data support the notion that deregulated expression of CIP2A is a critical oncogenic event in colon carcinoma.


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Patients and Methods

Patient Samples

One hundred and four patients with colorectal cancer were included in the study. All patients underwent surgery at the University Hospital of Wuerzburg, Germany, between 2003 and 2012. None of the patients had received chemotherapy or radiotherapy before surgery. Treatments after surgery were performed according to guidelines for treating colorectal cancer. The diagnosis was confirmed by histopathological examination of the specimens. After surgery, tumour specimens were collected and stored in liquid nitrogen. Clinical data of all the patients were collected from hospital records and subsequent records were collected via the Comprehensive Cancer Centre Mainfranken.

Ethics Statement

Ethical approval for this research was obtained from the Human Research Ethics Committee of the University of Wuerzburg. All patients that provided tumor tissue and normal colon tissue samples for this research signed a consent form prior to surgical removal of the intestinal cancer.

Cell Lines and Cell Culture

Caco2, HCT116, and SW620 cells were purchased from American Type Culture Collection. All cells were cultured in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

Real-time Quantitative Reverse Transcription-PCR Analysis

Gene expression of CIP2A was analysed using real-time PCR. Total cellular RNA was extracted from tumour samples and cell lines with RNeasy Mini kit (Qiagen; Hilden, Germany) according to the manufacturer’s instructions. Primer sets (Qiagen) that targeted CIP2A RNA were designed by Biomers (Ulm, Germany). Matched human colon cDNA (Pharmingen; Heidelberg, Germany) served as a positive control, standardized to baseline. The housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-2 microglobulin (b2MG) were used as internal standards for relative quantification and cDNA quality control. All PCR reactions were carried out with a DNA Engine Opticon 2 System (MJ Research, Biozym; Oldendorf, Germany). Relative quantification, based on the fold difference, was calculated with the threshold cycle (Ct) method, expressed as $2^{-\Delta\Delta Ct}$ (Primer sequence in Table S1).

Immunoblot Analysis

 Cultured cells were rinsed three times with ice-cold PBS, harvested, and lysed directly in RIPA sample buffer for Immunoblot analysis. Cell debris was removed by centrifugation at 12,000 g for 10 min at 4°C, and the supernatant was used as total protein lysate. For each sample, 10 μg of total protein lysate was subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-Page), followed by Immunoblot analysis. Immunoblots were probed with antibodies against CIP2A (A301-
454A; Bethyl Laboratories), c-Myc C33 (C33, #42; Santa Cruz), beta-actin (AC-15/A5441; Sigma), vinculin (V9131; Sigma), AKT, pAKT473 (cs-9271), gsk3 (cs-9315), pGSK Serine-9 (cs-9336), s6 (cs-2212), ps6 (cs-2215); pmTOR (cs-2917), and mTOR (cs-2972). All antibodies were from Cell Signalling and were used according to the manufacturer’s instructions. The blots were visualised with secondary antibodies (GE Healthcare) against mouse (NA9310) or rabbit (NA9340) primary antibodies.

### Table 1. Univariate 1, 3, 5-year overall survival of CRC patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1-Year (n = 74)</th>
<th>3-Year (n = 58)</th>
<th>5-Year (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total overall survival</td>
<td>p-value</td>
<td>Total overall survival</td>
</tr>
<tr>
<td>T stage</td>
<td>T1,2</td>
<td>9</td>
<td>100%</td>
</tr>
<tr>
<td>T1,4</td>
<td>64</td>
<td>75%</td>
<td>53</td>
</tr>
<tr>
<td>N Stage</td>
<td>N0</td>
<td>32</td>
<td>93.8%</td>
</tr>
<tr>
<td>N1,2</td>
<td>41</td>
<td>65.9%</td>
<td>34</td>
</tr>
<tr>
<td>M stage</td>
<td>M0</td>
<td>50</td>
<td>100%</td>
</tr>
<tr>
<td>M1</td>
<td>23</td>
<td>30.4%</td>
<td>21</td>
</tr>
<tr>
<td>UICC stage</td>
<td>I-II</td>
<td>29</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>III-IV</td>
<td>44</td>
<td>63.6%</td>
</tr>
<tr>
<td>Histological grade</td>
<td>G2</td>
<td>56</td>
<td>83.9%</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>17</td>
<td>58.8%</td>
</tr>
<tr>
<td>CIP2A expression</td>
<td>&lt; median</td>
<td>32</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>&gt; median</td>
<td>41</td>
<td>61.0%</td>
</tr>
</tbody>
</table>

Figure 2. Patients with CIP2A high mRNA expression have an overall lower survival rate than patients with CIP2A low mRNA expression. The graphs show Kaplan–Meier curves of OS according to CIP2A mRNA expression. (red: CIP2A mRNA expression below median fold expression value of 10,5 above normal tissue), green: CIP2A mRNA expression above median fold expression value of 10,5 above normal tissue) (A & B) All patients with respect to CIP2A mRNA expression normalized to housekeeping gene (n = 75) (A: b2MG; B: GAPDH) (C) Patients in Stage UICC II with respect to CIP2A mRNA expression normalized to housekeeping gene GAPDH (n = 29) (D) Patients in Stage UICC III with respect to CIP2A mRNA expression normalized to housekeeping gene GAPDH (n = 21).

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**Immunohistochemistry**

The CIP2A antibody was the same as for Immunoblot analysis, isotype control antibody was purchased from eBioscience (San Diego, USA). The secondary antibody was a Cy3-conjugated AffiniPure anti-rabbit IgG at a 1:200 dilution. CIP2A staining was performed on cryostat sections of snap-frozen colon cancer specimens expressing different CIP2A mRNA levels with neighbouring normal colon tissue and 10 normal colon specimens. Cryostat sections (5 μm) were incubated with the primary antibody or control antibody followed by incubation with the secondary Cy3-conjugated antibody. Slides were counterstained with DAPI (4',6-Diamidino-2-phenylindoldihydrochlorid) (Sigma-Aldrich, Steinheim, Germany) and covered with polyvinyl-alcohol mounting medium (DABCO, Sigma-Aldrich) and analysed using a Zeiss camera (Oberkochen, Germany).

**siRNA Transfection**

To silence gene expression, cells were transfected with small interfering RNAs (siRNAs). On-target plus SMART pool (Dharmacon) siRNA to target CIP2A (L-014135-01-005) and a control siRNA (D-001810-10-05) were used. The siRNA pools (final concentration 100 nM) were transfected into the cells with the RNAmax kit according to the manufacturer’s protocol. Cells were harvested 72 h later; expression of proteins was determined by Immunoblot analysis.

**shRNA and Lentivirus**

To silence CIP2A mRNA expression with short hairpin RNA (shRNA) sequences, targeting CIP2A were cloned into a lentivirus vector (piko1 puro), according to the manufacturer’s protocol. The vector was transiently transfected into HEK293t cells together with package plasmids. After 48 and 72 h, supernatants containing the virus were collected and filtered. Colon cancer cell lines were infected with the CIP2A lentivirus and 24 h later, infected cells were selected with puromycin. Experiments were performed with the selected cells.

**Colony Formation Assay**

2.5 × 10^5 cells infected with shRNA CIP2A or Scr. were plated on six well plates. Colonies were stained with 0.5% crystal violet in 20% ethanol. Photos were taken and relative density determined with ImageJ.

**Statistical Analysis**

Univariate analyses to determine association with survival were evaluated with Kaplan–Meier curves, and comparisons were performed by Mann-Whitney U test. Multivariate analyses were performed with a Cox proportional hazards regression model. P-values <0.05 were considered statistically significant.

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**Table 2. Cox regression analysis in predicting the overall survival of CRC patients.**

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UICC stage</td>
<td>2.38</td>
<td>1.38–4.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Histological grade</td>
<td>1.03</td>
<td>0.49–2.16</td>
<td>n.s.</td>
</tr>
<tr>
<td>CIP2A expression (above median vs. below median)</td>
<td>3.06</td>
<td>1.27–7.37</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CIP2A expression (continues factor)</td>
<td>1.02</td>
<td>1.0–1.05</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

(HR Hazard ratio).

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**Figure 3.** CIP2A protein levels in colon cancer correlate with CIP2A mRNA expression.

The panels show representative examples of immunofluorescence staining, showing CIP2A protein expression in cancer cells of patients with low (A+B) or high CIP2A (C+D) mRNA expression (A+C x100, B+D x200 magnification).

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Figure 4. Depletion of CIP2A downregulates c-Myc protein expression in colon cancer cells. (A) Immunoblot analysis of CIP2A and c-Myc protein expression in Caco2, HCT116 and SW620 cells transfected with siRNA targeting CIP2A or control siRNA. Cells were harvested 72 h after transfection (n = 3 for each cell line). (B) Real-time PCR analysis of CIP2A and c-Myc mRNA expression in HCT116 cells transfected with siRNA targeting CIP2A or control siRNA (n = 3). (C) Depletion of CIP2A does not change activation status of AKT or its downstream targets. The panels show immunoblots of the indicated proteins and phosphorylated proteins (p) in Caco2, HCT116 and SW620 cells transfected with siRNA targeting CIP2A or control siRNA as before (n = 3 for each cell line).

doi:10.1371/journal.pone.0075292.g004
Results

Expression of CIP2A and Clinicopathological Variables of Colon Cancer Patients

Expression of CIP2A mRNA was initially assessed using expression data sets derived from a published microarray analysis, which was originally performed to identify prognostic markers for colorectal carcinomas according to the lymph node metastasis status [15]. Complete data sets (DFS, Tumor stages), comparing CIP2A mRNA expression in carcinoma to that in matched adjacent tissues, were available for 226 carcinomas. CIP2A expression was tested using two independent probes present on this array. We found no correlation between CIP2A expression and age at diagnosis, or gender in this data set, but CIP2A mRNA was expressed at higher levels in colorectal cancer tissues than in the matched adjacent tissues in both probe sets (data not shown).

Intriguingly, high CIP2A mRNA expression correlates significantly with reduced disease-free survival (DFS) (Fig. S1A, B). In separate analyses of patients in Union for International Cancer Control (UICC) stage I (tumour infiltration to muscularis propria), II...
(tumour infiltration beyond muscularis propria) or III (lymph node metastasis), only patients in stage UICC III showed a significant correlation between CIP2A expression and DFS. In comparison, patients in stage UICC I and II showed only a slight correlation between low CIP2A expression and prolonged survival that did not reach statistical significance. This may be due to the small number of samples and fewer relapse events in comparison to patients in stage UICC III (Fig. S2A-C). As DFS could not be reached in stage UICC IV (distant metastases), it was not possible to analyse the correlation of CIP2A expression to the survival of patients in stage UICC IV with this data set.

We further analysed CIP2A mRNA levels in an independent set of 104 colorectal cancer tissue samples using an RT-QPCR approach. The expression of CIP2A mRNA was determined relative to two housekeeping genes, beta-2 microglobulin (b2MG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression levels were compared to the median CIP2A mRNA expression in normal mucosal tissue. CIP2A expression relative to b2MG or relative to GAPDH showed a high correlation (R² = 0.86) (Fig. S3). Consistent with previous findings on CIP2A expression in cancer, we found CIP2A mRNA expression in 93 of 104 (89.4%) cancer samples to be at least four times higher than in normal colon mucosal tissues. Furthermore, CIP2A expression level was significantly correlated to the UICC stage, lymph node metastasis, distant metastasis, and histological tumour grading (Fig. 1). We found no association between CIP2A expression and patient age, gender or cancer location (right vs. left colon) (Table S2).

### CIP2A mRNA Expression and Postoperative Survival of Colon Cancer Patients

Overall survival (OS) was used for survival analysis. Overall survival was defined as the time interval between surgery and tumour-associated death. Univariate 1-, 3-, and 5-year OS analyses revealed that patients with an advanced primary tumour stage, lymph node metastasis, distant metastasis, advanced UICC-stage, and high histological grades had worst outcomes (Table 1). For further analysis, patients were divided into two groups, with CIP2A expression above (high) or below (low) median value. Patients with high CIP2A mRNA expression had significantly lower OS than those with low CIP2A mRNA expression (Fig. 2A, B). Comparing patients in UICC stages I-IV separately with high and low CIP2A mRNA expression, only in the UICC III stage, patients with high CIP2A levels had a reduced OS (Fig. 2C, D). In addition, a multivariate analysis indicated that an advanced UICC-stage and high CIP2A expression (CIP2A above median and CIP2A expression used as a continuous marker) were independent prognostic factors for poor outcome in patients with colon cancer (Table 2).

To test whether CIP2A mRNA expression is correlated with CIP2A protein expression in CRC, ten normal mucosa tissue sections, four CRC tissue samples expressing low CIP2A mRNA levels and five tissue samples expressing high CIP2A mRNA levels were stained for CIP2A protein expression. In normal mucosa tissue sections, no or only a very weak staining for CIP2A could be detected (data not shown). Cancers expressing low levels of CIP2A mRNA showed a weak staining for CIP2A protein, whereas cancers expressing high CIP2A mRNA levels showed a strong staining for CIP2A protein. This results show that CIP2A protein and mRNA levels correlate closely in vivo. (Fig. 3).
First, previous work showed that CIP2A expression is positively correlated with EGFR expression [13]. The MAPK pathway is one of the major targets of EGFR signalling. Second, we showed that CIP2A is downregulated after inhibiting the MAPK pathway in three colon carcinoma cell lines; downregulation in cell lines harbouring a KRAS mutation (HCT116, SW620) is more pronounced than in Caco2 not harbouring a MAPK-pathway mutation. Induction of CIP2A may, therefore, be a critical mediator that links deregulated mitogenic signalling to enhanced c-Myc expression in CRC.

In conclusion, the results of this study show that CIP2A is associated with colorectal cancer related survival. High expression of CIP2A mRNA is correlated with reduced DFS and OS. Therefore, CIP2A represents a new prognostic factor in the diagnosis of colorectal cancer. In addition, CIP2A may be a promising therapeutic target in the development of therapies for colorectal cancer.

Supporting Information

Figure S1 Kaplan–Meier curves of disease free survival of all patients (n = 226) with respect to CIP2A mRNA expression status in published microarray analysis. (A & B) DFS of all patients according to the microarray probe set. (EPS)

Figure S2 Disease free survival of patients with colon cancer in UICC I-III stage, grouped according to CIP2A mRNA expression status. (A) UICC I; (B) UICC II; (C) UICC III. (EPS)

Figure S3 Linear regression analysis of relative CIP2A mRNA expression normalized to two housekeeping genes, b2MG and GAPDH (n = 104; R² = 0.86). The linear regression is highly significant (p<0.001). (PSD)

Table S1 Primer sequences. (DOCX)

Table S2 Characteristics of patients with CRC and association between CIP2A expression and clinicopathologic variables (lymphovascular invasion and location was documented for 100 patients). (DOCX)

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Author Contributions

Conceived and designed the experiments: AW ME CTG. Performed the experiments: AW CP FWU LR UM AMWG MG. Analyzed the data: AW CO FWU CP LR UM AMWG ME CTG MG. Contributed reagents/materials/analysis tools: AW MG AMWG ME CTG. Wrote the paper: AW CO AMWG ME CTG.

References


