ROLE OF CIP2A IN CARCINOGENESIS

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ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Medicine of the University of Helsinki, in Haartman Institute, on 10 February, at 12 noon.

Helsinki 2012

Helsinki University Biomedical Dissertations No. 158

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ISBN: 978-952-10-7578-0 (paperback)
ISBN: 978-952-10-7579-7 (PDF)
ISSN: 1457-8433
http://ethesis.helsinki.fi
Unigrafia Oy
Helsinki 2012

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than you found it

Lord Robert Baden-Powell

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1. ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I Khanna A*, Böckelman C*, Hemmes A, Junttila MR, Wiksten J-P, Lundin M, Junnila S, Murphy DJ, Evan GI, Haglund C, Westermarck J**, Ristimäki A**: MYC-Dependent regulation and prognostic role of CIP2A in gastric cancer. *J Natl Cancer Inst.* 2009;101(11):793-805.

II Böckelman C, Lassus H, Hemmes A, Westermarck J, Leminen A, Haglund C, Bützow R, Ristimäki A: CIP2A is a marker of reduced survival in serous ovarian cancer patients. *Brit J Cancer* 2011;105(7):989-995.

III Böckelman C, Hagström J, Mäkinen LK, Keski-Säntti H, Häyry V, Lundin J, Atula T, Ristimäki A, Haglund C: High CIP2A immunoreactivity is an independent prognostic indicator in early-stage tongue cancer. *Brit J Cancer* 2011;104(12):1890-1895.

IV Böckelman C, Koskensalo S, Hagström J, Lundin M, Ristimäki A, Haglund C: CIP2A overexpression is associated with c-Myc expression in colorectal cancer. *Cancer Biol & Ther* 2012;13(6):Epub ahead of print.

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2. ABSTRACT

Worldwide and notably in the developed countries, cancer is an increasing cause of morbidity and mortality, being the second most common cause of death after ischemic heart disease. Now and in the future new cancer cases need to be diagnosed earlier. Prognostic factors may be helpful in recognizing and handling those patients who need more aggressive therapy, and it is also desirable to predict treatment response accurately. Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an oncoprotein predominantly expressed in malignant tissues and inhibiting protein phosphatase 2A (PP2A) activity; it is a promising target for cancer therapy. The aim of this thesis was to evaluate the prognostic role of CIP2A in solid cancers, and for this purpose to explore expression of CIP2A, and investigating regulation of CIP2A in order to gain insight into signalling pathways leading to alteration in prognosis.

Patients diagnosed with gastric, serous ovarian, tongue, or colorectal cancer at Helsinki University Central Hospital were included. Tumour tissue microarrays assembled from specimens from these patients were prepared and stained immunohistochemically for CIP2A protein expression. Associations with clinicopathologic parameters and other biomarkers were explored, and survival analyses were done according to the Kaplan-Meier method. Study of the role of CIP2A in intracellular signalling *in vitro* involved gastric, ovarian, and tongue cancer cell lines.

We found CIP2A to be highly expressed in gastric, ovarian, tongue, and colorectal cancer specimens. CIP2A was associated with clinicopathologic parameters characterizing an aggressive disease, namely advanced stage, high grade, p53 immunopositivity, and high proliferation index. CIP2A led to recognition of gastric, ovarian, and tongue cancer patients with poor prognosis, however, with a cancer type-specific cut-off level for prognostic significance. In tongue cancer, it served as an independent prognostic marker. In contrast, in colorectal cancer, CIP2A provided no prognostic value. In cancer cell lines, CIP2A was highly expressed at both protein and mRNA levels, and promoted cell proliferation and anchorage-independent growth. In gastric cancer, we demonstrated with a MYCER construct in mouse embryo fibroblasts that activation of MYC led to increased CIP2A mRNA expression, and hence we suggested that a positive feedback mechanism between CIP2A and MYC may potentiate and prolong the oncogenic activity of these proteins. We demonstrated in ovarian cancer an association between CIP2A and EGFR protein overexpression and *EGFR* gene amplification. In ovarian and tongue cancer cells we showed that depletion of EGFR downregulates CIP2A expression.

In conclusion, high CIP2A expression occurred frequently among patients with aggressive disease. CIP2A may serve as a prognostic marker in gastric, ovarian, and tongue cancer and thus may help in tailoring therapy for cancer patients. The positive feedback mechanism between CIP2A and MYC, as well as the positive regulation of CIP2A by EGFR, are a few signalling pathways regulating and regulated by CIP2A. These and other mechanisms need to be studied further, however. CIP2A is a potential target for therapy, and its potential role as predictive marker and as a tumour marker in serum requires exploration.

3. ABBREVIATIONS

AML	Acute myeloid leukaemia
AMPK	AMP (adenosine monophosphate) activated kinase
AJCC	American Joint Committee on Cancer
ATCC	American Type Culture Collection
cagA	Cytotoxin-associated protein A
CEA	Carcinoembryogenic antigen
CI	Confidence interval
CIP2A	Cancerous inhibitor of protein phosphatase 2A
DAPk	Death-associated protein kinase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSMZ	German collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen)
EGFR	Epidermal growth factor receptor
EGTM	The European Group on Tumour Markers
ERBB2	v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog (avian)
ERK	Extracellular signal-regulated kinase
H&E	Haematoxylin and eosin
HER-2	Human epidermal growth factor receptor 2
FOBT	Faecal occult blood testing
HCC	Hepatocellular carcinoma
H. pylori	*Helicobacter pylori*
HR	Hazard ratio
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
JNK	c-Jun N-terminal kinase
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein-serine/threonine kinase
MEK	*M*AP kinase/*E*RK *k*inase
MEFs	Mouse embryo fibroblasts
NA	Not applicable
PI3K	Phosphatidylinositide (PI) 3-kinase
PBS	Phosphate-buffered saline
PP2A	Protein phosphatase 2A

qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SEM	Standard error of mean
siRNA	Small interfering ribonucleic acid
SPF	S-phase fraction
TBS-NP40	Tris-buffered saline with NP40 (Igepal CA630, Sigma)
TCF	T-cell factor
TMA	Tissue microarray
TNM	Tumour, Node, Metastasis
TP53	Tumour protein p53
UICC	Union for Cancer Control (Union Internationale Contre le Cancer)
WB	Western blot
WHO	World Health Organization

4. INTRODUCTION

In 2007, over 26 000 persons were diagnosed with cancer in Finland and 41% of these died of their disease. Cancer accounts for a large proportion of morbidity and mortality and is the second most common cause of death after ischemic heart disease. In a national prospect, the Finnish Cancer Registry has estimated by 2020 an 29% increase in incidence rate for men and 24% for women, with 17 700 new cancer cases diagnosed in men and 16 100 in women, largely explained by the ageing population. The age-adjusted overall mortality rate is predicted to decrease by 10 to 20% until 2020, but with a 13 to 18% increase in the annual number of cancer deaths. Hence, cancer diseases are a major public health problem, demanding research in the field.

Cancer arises from malignantly transformed cells. The genetic information is stored in the genome in chromosomes, which are copied in the process of cell proliferation and differentiation. Cancer may arise from mistakes in this copying process as the result of an incorrect genome. Hanahan and Weinberg (2000, 2011) have proposed six hallmark capabilities of cancer: sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death.

Prognostic factors are defined as "variables that can account for some of the heterogeneity associated with the expected course and outcome of a disease" but in the epidemiological literature, the term *prognostic factor* is commonly reserved for "a probability of future event in patients who currently have a disease" (Gospodarowicz *et al.* 2001). The term *predictive factor* commonly refers to "prognosis for a measurable response" following a treatment intervention, whereas the term *prognostic factor* stands for a more narrow context with the end-points probability of cure or prolongation of survival (Gasparini *et al.* 1993, Henderson and Patek 1998). Today, the term *predictive marker* is used for biomarkers that provide information as to whether patients are likely to benefit from a specific therapy (Walgren *et al.* 2005, Duffy *et al.* 2011). This is in contrast to *prognostic markers*, which "allow the natural course of a specific disease to be predicted", or in other words differentiate between patients with a good versus a poor prognosis (Sawyers 2008, Duffy *et al.* 2011). In most cancers even today, tumour-stage classification is the prognostic factor most commonly used. Although numerous biomarkers have undergone evaluation for a prognostic role, only a few serve as predictive markers in clinical practice. Mackillop states, that "To be relevant to the clinical practice, prognostic factors must either have a significant impact on cancer outcome, or be used to select treatment methods" (Gospodarowicz *et al.* 2001). To improve the predictive value and clinical usefulness

of biomarkers, cancer-specific prediction models may prove useful, which include several predictive factors, together with common clinicopathologic parameters.

Tumour markers are divided into serum markers, tissue-based markers, and in colorectal cancer also stool-based markers (Duffy *et al.* 2007). Serum markers mainly serve in postoperative surveillance, whereas tissue-based markers may serve as potential prognostic or predictive markers. Stool-based markers have proven beneficial in trials screening for colorectal cancer, but their definitive role in clinical practice remains to be discussed.

To improve the prognosis of cancer patients, tumours must be diagnosed at an early stage. Tumour markers may allow detection of cancer in its early course, hence improving prognosis and survival, and possibly allowing selection of patients who would benefit from adjuvant treatment. TNM (Tumour Node Metastasis) -staging of tumours is commonly used in planning of tumour treatment and prognosis. Prognostication based on the TNM-stage classification by the World Health Organization (WHO) and the International Agency for Research Against Cancer (IARC) is today insufficient, however, and new prognostic markers are still needed.

When this thesis project began, the first publication addressing the role of the oncoprotein, called cancerous inhibitor of protein phosphatase 2A (CIP2A; PP2A), had just appeared (Junttila *et al.* 2007). Since then, several authors have addressed the functional and clinical role of CIP2A in cancer. To date, CIP2A has been studied mostly in carcinomas, but a few studies address its role also in haematological malignancies (Lucas *et al.* 2011, Wang *et al.* 2011) and non-malignant diseases (Lee *et al.* 2011). In general, CIP2A is expressed at low levels or not at all in normal tissues, whereas in malignant tissues, CIP2A expression frequency has been high.

This thesis addresses the prognostic role of cancerous inhibitor of protein phosphatase (CIP2A) in gastric, colorectal, tongue, and ovarian cancer. It also investigates associations between CIP2A expression and clinicopathologic factors, and the regulation of CIP2A expression in cancer cells.

5. REVIEW OF THE LITERATURE

5.1. Gastric cancer

5.1.1. Epidemiology and etiology

During the past five decades, gastric cancer incidence in Finland has declined; formerly one of the most common cancers, in 2007 it was the 11th most common cancer in men, and 14th in women, accounting for only 5% of all deaths from cancer (Finnish Cancer Registry 2009). Worldwide, however, gastric cancer is still the second most common cause of cancer deaths (Jemal *et al.* 2011). Its declining incidence in Finland and in the world follows primarily the declining incidence of *Helicobacter pylori (H. pylori)*, better hygiene, and less crowded living. Worldwide, the incidence is high in eastern Asia, and in central and eastern Europe, conversely, in North America and most African countries, incidence is low (Jemal *et al.* 2011). Gastric cancer is twice as common in men, and more common in the lower social classes (Weiderpass and Pukkala 2006, Nagel *et al.* 2007). In Finland in 2007, the age-adjusted incidence rate for men was 7.8/100 000 personyears and for women 4.6/100 000 personyears (Figure 1) (Finnish Cancer Registry 2009).

Gastric cancer includes the more common non-cardia cancer and cardia cancer; for these, etiology and clinical manifestation differ. Risk factors for are mainly environmental. *H. pylori* is, by the International Agency for Research on Cancer (IARC), classified as a class 1 carcinogen (The Eurogast Study Group, 1993, IARC 1994). Patients positive for *H. pylori* have a 3- to 6-fold greater risk for developing non-cardia gastric cancer, especially if positive for the *H. pylori* virulence factor cagA (IARC 1994, Blaser *et al.* 1995, Palli *et al.* 2007, Bornschein *et al.* 2009). In contrast, no association has been detectable between cardia cancer and *H. pylori.* Smoked and salted food have been considered risk factors (Tsugane and Sasazuki 2007), and high intake of fruit and vegetables, considered protective (Huang *et al.* 2000). Large prospective studies have, however, failed to confirm this association (Gonzalez *et al.* 2006, Freedman *et al.* 2008b, Key 2011). Results from studies conducted to assess the role of smoking and high alcohol intake vary (Dicken *et al.* 2005, Shang and Pena 2005). In contrast to non-cardia cancer, smoking and obesity are in cardia cancer established risk factors (Donohoe *et al.* 2010).

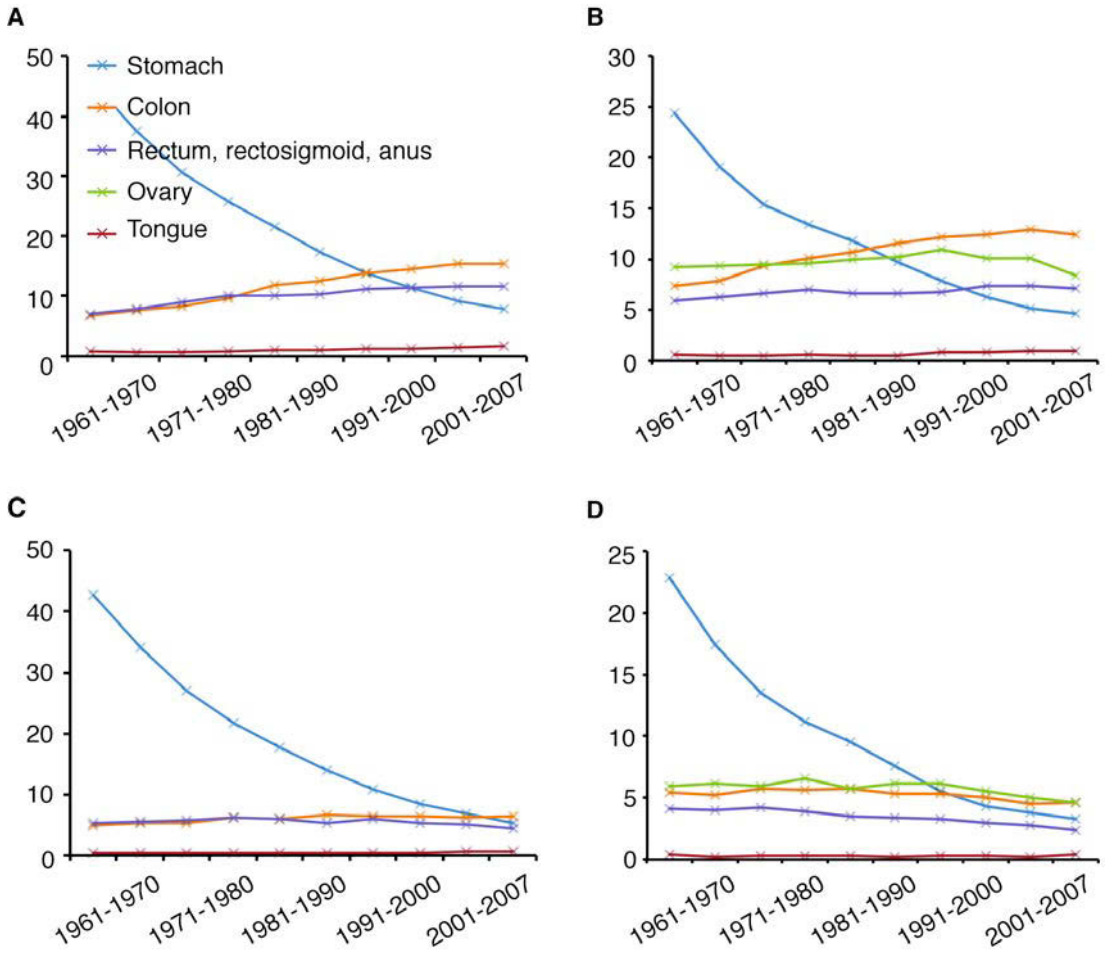


Figure 1. Cancer incidence and mortality in Finland. A) Age-adjusted incidence rates in 1961-2007 for men and B) women. C) Age-adjusted mortality rates in 1961-2007 for men and D) women. Adapted from the Finnish Cancer Registry, 2009.

5.1.2. Pathogenesis and diagnosis

Gastric cancer is believed to arise partly as a consequence of chronic atrophic gastritis induced by *H. pylori* infection. Chronic inflammation predisposes to development of atrophic gastritis, which may induce intestinal metaplasia, and later dysplasia (Correa and Houghton 2007). The less acidic environment arising in atrophic gastritis allows bacteria producing nitrites and carcinogenic N-nitroso compounds to colonize the gastric mucosa, predisposing to genetic alterations.

Of gastric tumours, 90% are adenocarcinomas, of which 40% are localized to the pylorus and antrum, 40% to the corpus and fundus, and the rest to the cardia area. Histologically, according to the WHO, the five main types of adenocarcinoma are tubular, papillary, mucinous, and poorly cohesive (including signet-ring cell type), and mixed carcinomas (Bosman *et al.* 2010). According to Laurén's classification, 45 to 60% are of intestinal type and usually associate with *H. pylori* positivity, distal location, and liver metastasis (Laurén 1965). The diffuse type gastric cancer, on the other hand, is believed to arise spontaneously without any predisposing chronic

Table 1. TNM classification of gastric tumours. Adapted from Bosman *et al.* (2010).

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial tumour without invasion of the lamina propria, high-grade dysplasia
T1	Tumour invades lamina propria, muscularis mucosae, or submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades subserosa
T4	Tumour perforates serosa or invades adjacent structures
T4a	Tumour perforates serosa (visceral peritoneum)
T4b	Tumour invades adjacent structures

Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-2 regional lymph nodes
N2	Metastasis in 3-6 regional lymph nodes
N3	Metastasis in more than 7 regional lymph nodes

Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage							
Stage 0	Tis	N0	M0	Stage IIIA	T4a	N1	M0
Stage IA	T1	N0	M0		T3	N2	M0
Stage IB	T2	N0	M0		T2	N3	M0
	T1	N1	M0	Stage IIIB	T4b	N0-1	M0
Stage IIA	T3	N0	M0		T4a	N2	M0
	T2	N1	M0		T3	N3	M0
	T1	N2	M0	Stage IIIC	T4a	N2	M0
Stage IIB	T4a	N0	M0		T4b	N2-3	M0
	T3	N1	M0	Stage IV	Any T	Any M	M1
	T2	N2	M0				
	T1	N3	M0				

atrophic gastritis and intestinal metaplasia, and is usually marked by a lack of tubular structure and by metastasis to lymphatic vessels and the lungs. TNM-stage classification by the WHO/IARC is still today the most important prognostic factor, because tumours limited to the serosa show lymphatic infiltration in only 18% of the cases, whereas tumours penetrating the serosa present with distant metastasis in 80% of the cases (Table 1).

The fact that symptoms are unspecific and relatively few leads to late diagnosis. Common but unspecific symptoms are weight loss (70 to 80% of the patients), epigastric pain (70%), and anaemia. Dysphagia arises in the advanced stage of cardia cancer when over 80% of the lumen is occluded. Symptoms of retention and nausea can be the result of tumours in the pyloric area. No single blood test for screening or diagnosis is available. Carcinoembyronic antigen (CEA) is positive in 18% of the gastric cancer patients, but 50% are false positives, whereas CA 19-9 is more specific, but usually implies metastasized disease (Roberts *et al.* 1989). Louhimo *et al.* (2004) found that human chorionic gonadotropin (hCGβ) and CA 72-4 serve as independent prognostic markers, whereas CA 19-9 and CA 242 are dependent prognostic factors. Usually diagnosis is reached by gastroscopy, for which sensitivity is high. Computed tomography is utilized for clinical staging prior to surgery; however, sometimes diagnostic laparoscopy is necessary to confirm the extent of the disease.

5.1.3. Treatment

The only curative treatment is radical surgery with subtotal or total gastrectomy (Heberer *et al.* 1988). In D1-lymph node dissection, perigastric lymph nodes are removed and in D2, the lymph node dissection is extended to the surrounding arteries. In D3, distant lymph nodes in areas surrounding the hepatic portal and the abdominal aorta undergo dissection in addition to the local lymph node dissection (Siewert *et al.* 1998, Bonenkamp *et al.* 1999, Cuschieri *et al.* 1999). The 15-year follow-up in the Dutch Gastric Cancer Trial (DGCT) showed that gastric-cancer-related death and locoregional recurrence were significantly greater in patients who underwent D1 lymphadenectomy than for those in the D2 group (Songun *et al.* 2010). Disadvantages with D2 resection were high postoperative mortality, complications, and reoperation rates; however, D2 resection with a spleen and pancreas-preserving technique has proven safer and recommendable (Wang *et al.* 2004, Yu *et al.* 2006). In Japan, where more radical surgery has been adopted, prognosis is better (Maruyama *et al.* 1996).

The INT0116 randomized controlled trial (Macdonald *et al.* 2001) showed that surgery together with postoperative chemoradiotherapy is beneficial when compared to surgery alone. Their result has been criticized, however, for inadequate lymph node dissection. Sakuramoto *et al.* (2007) later showed that for stage II/III patients after D2

dissection, the chemotherapeutic agent S-1 improves survival. Treatment modalities differ between Western and Asian centres, and Kurokawa and Sasako (2008) suggest that surgery with D2 lymph node resection alone is superior to D1 dissection in combination with chemoradiotherapy. The MAGIC (Medical Research Council Adjuvant Gastric Infusional Chemotherapy) trial demonstrated that perioperative chemotherapy, with epirubicin, cisplatin, and infused fluorouracil (ECF) administered in three preoperative and three postoperative cycles, is preferable to surgery only (Cunningham *et al.* 2006). Bang *et al.* (2010) reported recently that for patients with advanced gastric cancer positive for human epidermal growth factor receptor 2 (HER-2), trastuzumab, a monoclonal antibody against HER-2, in combination with chemotherapy significantly improved survival compared to survival with chemotherapy alone.

5.1.4. Prognosis

The age-standardized 5-year gastric cancer-specific relative survival ratio was, in 2005-2007, 25% for men and 28% for women (Finnish Cancer Registry 2009). In over half the cases, gastric cancer is found in its advanced stage (pT3-T4). Median survival for patients with T3 disease is 12 months and for those with T4 disease only 6 months (Victorzon 1996).

5.2. Ovarian cancer

5.2.1. Epidemiology and etiology

Ovarian cancer is the second most common gynaecological cancer, after endometrial cancer. In 2007, it was the 5th most common cancer in women in Finland with an incidence rate of 8.4/100 000 personyears (Figure 1) (Finnish Cancer Registry 2009). Ovarian tumours are classified as common epithelial tumours, which constitute the majority, as sex cord stromal tumours, and germ cell tumours. They originate from the embryonic Müllerian epithelium, which in addition gives rise to the Fallopian tubes, the epithelium of the corpus and cervix of the uterus, and the proximal part of the vagina. Hence, Dubeau (2008) has proposed that the Fallopian tubes represent the normal tissue that is equivalent to ovarian tumour tissue. The risk for developing ovarian cancer is greatest among those belonging to families with germline mutations in the *BRCA1*- or *BRCA2*-genes or having ovarian cancer cases among close relatives. Increased risk has been demonstratable among infertile and nulliparous women (Risch *et al.* 1994). According to the ovulation hypothesis proposed by Fathalla (1971), a greater number of ovulatory cycles (more than 40 years of ovulation, late menopause) leads to increased risk for ovarian cancer because ovarian surface epithelial cells are

more prone to develop mutations in the frequent repair of the ovarian surface. The gonadotropin stimulation theory, on the other hand, states that stimulation of the ovarian surface epithelium by fertility drugs may increase the risk in infertile women of developing epithelial ovarian cancer (reviewed in Landen *et al.* 2008). The greater incidence of ovarian cancer in Western countries is illustrated by a high living standard and more frequent obesity (Lane 2008, Bettochi *et al.* 1982).

5.2.2. Pathogenesis, screening, and diagnosis

Epithelial ovarian tumours are grouped into serous, mucinous, endometrioid, clear cell, transitional cell, mixed epithelial, and undifferentiated tumours. The abundance of different types lies in their mutual origin in the Müllerian system. Serous cystadenocarcinomas constitute the majority (40 to 50%) of ovarian cancers and endometrioid carcinomas, and mucinous cystadenocarcinomas are common (10 to 20% each). Ovarian tumorigenesis has been proposed to evolve through low- and high-grade pathways: type-I tumours are characterized by their low-grade serous, mucinous, endometrioid, and clear cell carcinoma type, whereas type-II tumours are characterized by high-grade serous and undifferentiated carcinomas (Shih and Kurman 2004, reviewed in Landen *et al.* 2008, Levanon *et al.* 2008). Low-grade type-I tumours are believed to arise through precursor lesions, with frequent mutations in *BRAF* and *KRAS* genes (Singer *et al.* 2003a, b). In contrast, high-grade type II tumours develop without precursor lesions and commonly overexpress ERBB2 (Ross *et al.* 1999, Lassus *et al.* 2006) and AKT (Cheng *et al.* 1992), and harbour mutations in the *TP53* gene (Singer *et al.* 2005).

Ovarian tumours rarely give rise to specific symptoms. The currently available methods for screening (clinical examination, intravaginal ultrasonography, and tumour markers) are unsuitable for population-based screening (Clarke-Pearson 2009, Daly *et al.* 2010, Schorge *et al.* 2010, reviewed in Cragun 2011). Diffuse abdominal symptoms, lack of appetite, and general fatigue are the initial symptoms. In a progressed disease, symptoms may appear in neighbouring organs, leading to frequent micturition, urinary urgency, constipation, and dyspareunia. Diagnosis is achieved by gynaecological examination, radiological findings, and tumour markers; final diagnosis is, however, achieved only by histological verification.

Staging is established by surgery together with cytological and histological examination (Table 2; AJCC 2002, IARC 2002, Tavassoli and Devilee 2003). Local spread in the abdominal area is common, although the disease can remain asymptomatic. Ovarian cancer metastasizes through the lymphatic vessels; haematogenic spread of the disease is uncommon, seen possibly in the late stage of the disease.

Table 2. TNM classification and FIGO (the International Federation of Gynecology and Obstetrics) staging of ovarian tumours. Adapted from Tavassoli and Devilee (2003).

FIGO			TNM
Stage I	Tumour limited to the ovaries		
	IA	Tumour limited to one ovary; capsule intact, no tumour on ovarian surface; no malignant cells in ascites or peritoneal washings	T1aN0M0
	IB	Tumour limited to both ovaries; capsule intact, no tumour on ovarian surface; no malignant cells in ascites or peritoneal washings	T1bN0M0
	IC	Tumour limited to one or both ovaries with any of the following: capsule ruptured, tumour on ovarian surface, malignant cells in ascites or peritoneal washings	T1cN0M0
Stage II	Tumour involves one or both ovaries with pelvic extension		
	IIA	Extension and/or implants on uterus and/or tube(s); no malignant cells in ascites or peritoneal washings	T2aN0M0
	IIB	Extension to other pelvic tissues; no malignant cells in ascites or peritoneal washings	T2bN0M0
	IIC	Pelvic extension (2a or 2b) with malignant cells in ascites or peritoneal washings	T2cN0M0
Stage III	Tumour involves one or both ovaries, with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis		
	IIIA	Tumour involves one or both ovaries with microscopically confirmed peritoneal metastasis beyond pelvis	T3aN0M0
	IIIB	Tumour involves one or both ovaries with macroscopic peritoneal metastasis beyond pelvis ≤ 2 cm in greatest dimension	T3bN0M0
	IIIC	Tumour involves one or both ovaries with peritoneal metastasis beyond pelvis > 2 cm in greatest dimension	T3cN0M0/ Any T, N1M0
Stage IV	Distant metastases outside the abdomen, malignant cells in pleural fluid, or parenchymal metastasis in the liver		Any T, Any N, M1

5.2.3. Treatment

Surgery is necessary not only for a radical cure, but also for appropriate staging of the disease. Cytological examination from ascites or peritoneal washings obtained from surgery is important for diagnosis. For radical surgery, debulking of the tumour accompanies resection of the female reproductive organs (hysterectomy, bilateral salpingo-oophorectomy). A second-look operation may be needed after postoperative

adjuvant treatment to evaluate treatment effects. In 1990, Young *et al.* concluded that adjuvant treatment with melphalan is of no benefit for patients with stage Ia or Ib disease with well-differentiated or moderately well-differentiated histological characteristics. A recent study targeted a study population of high-risk early-stage ovarian cancer patients (stage IA and IB grade 3 [or clear cell], stage IC, or stage II) (Mannel *et al.* 2011). The study-arm received intravenous paclitaxel and carboplatin together with a maintenance protocol with paclitaxel weekly for 24 weeks, and the control arm, intravenous paclitaxel and carboplatin only, without the maintenance paclitaxel. No improvement occurred in recurrence-free survival, but patients receiving the maintenance paclitaxel showed adverse toxicity. In advanced-stage ovarian cancer, intravenous paclitaxel and cisplatin has become the gold standard oncological treatment (McGuire *et al.* 1996, Bell *et al.* 2006, reviewed in DiSaia and Bloss 2003, Darcy and Birrer 2010). High-dose intraperitoneal cisplatin has shown additional benefits in combination with intravenous paclitaxel and carboplatin (Markman *et al.* 2001). This work was continued by Armstrong *et al.* (2006), who showed that intraperitoneal cisplatin and paclitaxel in combination with intravenous paclitaxel improves survival. The addition of a third cytotoxic agent to the standard paclitaxel and cisplatin regimen provided no improvement in survival (Bookman *et al.* 2009). Several on-going trials have been designed to evaluate more effective treatment modalities (Darcy and Birrer 2010). Radiotherapy cannot be targeted towards the local spread to the abdominal cavity without considerable side-effects, and hence is not a useful treatment for ovarian cancer.

5.2.4. Prognosis

Age-standardized ovarian cancer-specific 5-year survival in Finland was 49% in 2005-2007 (Finnish Cancer Registry 2009). This survival rate is relatively high for early-stage ovarian cancer patients, however. Because the majority are diagnosed at a late stage, overall survival remains poor (Jemal *et al.* 2008). Several clinicopathologic features are recognizable as prognostic factors. Among these are residual tumour size, histological subtype, stage (according to FIGO; the International Federation of Gynecology and Obstetrics), grade, tumour ploidy, and presence of ascites; however, probability models taking several factors into account seem most important (Friedlander 1998).

5.3. Tongue cancer

5.3.1. Epidemiology and etiology

Among the head and neck cancers, lip cancer, and cancers in the oral cavity (including tongue cancer) are the most common. In Finland, the most common location of cancers in the oral cavity is the tongue (Mäkitie *et al.* 2007). In 2007, the incidence rate of tongue cancer in Finland was for men 1.6/100 000 personyears, and for women 1.0/100 000 personyears (Figure 1) (Finnish Cancer Registry 2009). Smokers are at substantially increased risk for developing oral cancer, as are those with a high intake of alcohol. For smokers with a high intake, risk is even further increased (Blot *et al.* 1988). A significantly lower risk for oral cancer has been suggested among those with a relatively high fruit and vegetable intake (Boeing *et al.* 2006, Freedman *et al.* 2008a). This association, however, may relate to residual confounding with tobacco and alcohol (Key 2011). Leukoplakia, erytroplasia, and lichen ruber planus are precancerous conditions, which may develop into oral carcinoma if not recognized and treated early.

5.3.2. Pathogenesis and diagnosis

Oral cancer is thought to develop through epithelial dysplasia, where mild, moderate, or severe dysplasia may further evolve into carcinoma (Lippman *et al.* 2005). In the multifocal process, the concept of so-called “field cancerization” becomes important, implying that the entire area is exposed to the same carcinogens such as alcohol and tobacco, which increases the risk for accumulation of genetic alterations and hence, for malignant transformation (Slaughter *et al.* 1953, Califano *et al.* 1996, Braakhuis *et al.* 2003, Choi and Myers 2008).

The mobile, proximal two-thirds of the tongue in the oral cavity is commonly referred to as the oral tongue, whereas the posterior third belongs to the oropharynx. Squamous cell carcinomas constitute 90% of all malignant tumours in the oral cavity, which, according to WHO, are divided into verrucous, basaloid squamous cell, papillary squamous cell, spindle cell, acantolythic squamous cell, adenosquamous carcinomas, and carcinoma cuniculatum (Barnes *et al.* 2005). Other rare neoplasms are lymphoepithelial carcinomas, salivary gland tumours, soft tissue tumours, haematolymphoid tumours, and mucosal malignant melanoma. At first presentation, the only symptom may be a painless lesion which does not heal completely or a metastatic neck tumour without evidence of a primary tumour in the oral cavity. In Finland, however, the majority of oral tongue cancers are diagnosed at a relatively early stage (T1-T2) (Mäkitie *et al.* 2007). Results on survival of young tongue cancer patients are conflicting. Popovtzer *et al.* (2004) conclude that overall survival is

similar among patients younger than 45 years and among those over 45. Nevertheless, they suggest that two different patient groups can be recognized among the young: those with an aggressive disease and high mortality within two years, and others with an indolent disease. Atula *et al.* (1996), on the other hand, found no differences in the clinical course or prognosis of tongue cancer patients under 40.

Tongue cancer is generally regarded as a potentially aggressive disease, as even small tumours may metastasize (Keski-Säntti *et al.* 2007). Mäkitie *et al.* (2007) found in a Finnish cohort that 31% of the patients with an early-stage disease (I-II) presented with locoregional recurrence. The TNM classification of oral carcinomas is presented in Table 3.

5.3.3. Treatment

In Finland in 1995-1999, 97% of the oral tongue cancer patients underwent surgery, together with an ipsilateral neck dissection in 51% of the cases and a bilateral neck dissection in 4% (Mäkitie *et al.* 2007). Adjuvant treatment with radiotherapy was chosen for 58% of the cases, of which the majority had advanced-stage (III-IV) disease. Elective neck dissection has proven beneficial for early-stage tongue cancer patients (Keski-Säntti *et al.* 2006). Laramore *et al.* (1992) found no advantage of chemotherapy for patients with low risk for recurrence, whereas patients with high-risk disease characteristics (two or more positive regional nodes, extracapsular growth, positive margins of resection) may benefit from it (Forastiere *et al.* 2001). For patients with stage III or stage IV disease, recommended standard care is chemotherapy in combination with radiotherapy (Forastiere *et al.* 2001, Finnegan *et al.* 2009, Pederson *et al.* 2010).

5.3.4. Prognosis

In Finland, tongue cancer-specific 5-year survival was 64% in 1995-1999 (Mäkitie *et al.* 2007). Mean disease-specific survival for patients under 60 was 96 months, compared to 80 months for patients over 60. The role of tumour thickness and depth of infiltration as prognostic factors remains contradictory (Keski-Säntti *et al.* 2007, Woolgar 2006). pT-stage, however, demonstratably predicts local recurrence (Keski-Säntti *et al.* 2007).

Table 3. TNM classification of carcinomas of the oral cavity. Adapted from Barnes *et al.* (2005).

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour ≤ 2 cm in greatest dimension
T2	Tumour 2-4 cm in greatest dimension
T3	Tumour > 4 cm in greatest dimension
T4a	Tumour invades through cortical bone, into deep/extrinsic muscle of tongue, maxillary sinus, or skin of face
T4b	Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery

Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, ≤ 3 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, 3-6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, ≤ 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, ≤ 6 cm in greatest dimension
N3	Metastasis in a lymph node > 6 cm in greatest dimension

Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1-T2	N1	M0
	T3	N0-N1	M0
Stage IVA	T1-T3	N2	M0
	T4a	N0-N2	M0
Stage IVB	T1-T4	N3	M0
	T4b	N0-N3	M0
Stage IVC	T1-T4	N0-N3	M1

5.4. Colorectal cancer

5.4.1. Epidemiology and etiology

In Finland, colorectal cancer is the second most common cancer in men, and the third in women. Worldwide, incidence varies, being high in Western countries. In 2007, the incidence rate of colon cancer for men was 15.4/100 000 personyears, and for women 12.4/100 000 personyears (Finnish Cancer Registry 2009). The respective figures for rectal, rectosigmoid, and anal cancer was 11.6/100 000 for men and 7.1/100 000 for women (Figure 1). For colorectal cancer, a rising trend in incidence rate is observable in countries with a previously low incidence, such as Spain and Japan. A decreasing trend in incidence is evident in developed countries as a result of earlier diagnosis and improved treatment (Jemal *et al.* 2011, Center *et al.* 2009a, b). Risk factors for colorectal cancer relate mostly to life style. Dietary fibre has been suggested to reduce the risk for colorectal cancer, however, large prospective studies have shown that a high intake of fruits and vegetables reduces the risk only marginally (Key 2011). Recent meta-analyses have demonstrated a modest association between obesity and colorectal cancer (Donohoe *et al.* 2010, Moghaddam *et al.* 2007). Smoking is a well-known risk factor for adenomatous polyps, regarded as precursor lesions for colorectal cancer (Botteri *et al.* 2008b). In addition, smoking elevates significantly the risk for colorectal cancer, as well as the risk for death from colorectal cancer (Botteri *et al.* 2008a).

5.4.2. Pathogenesis, screening, and diagnosis

Colorectal cancer arises through benign neoplasms, adenomas, which can develop into dysplasias and later, into carcinomas (Vogelstein *et al.* 1988). In the beginning of colorectal carcinogenesis, adenomatous polyposis coli (*APC)* -gene inactivating mutations lead to deregulated WNT signalling, and β-catenin accumulates intracellularly. Hence, proliferating and undifferentiated cells at the bottom of colonic crypts fail to migrate upwards and may generate an adenomatous polyp. In addition, activating *KRAS* mutations may develop later in the adenoma-carcinoma sequence, causing cells to grow more aggressively (Figure 2) (Grady and Markowitz 2002, Pritchard and Grady 2011). In colorectal cancer, 18q loss of heterozygosity (LOH) is frequently altered and found to induce malignant transformation (Popat and Houlston 2005). Petrova *et al.* (2008) report, that PROX1, a transcription factor that is a target of the β–catenin/T-cell factor (TCF) pathway, is an important factor in progression to severe dysplasia, and thus its targeted silencing may prevent development of subsequent colorectal carcinoma. In carcinomas, several aberrations in signalling pathways have been detected (*PIK3CA, TP53, TGFBR2, SMAD4*), but few have

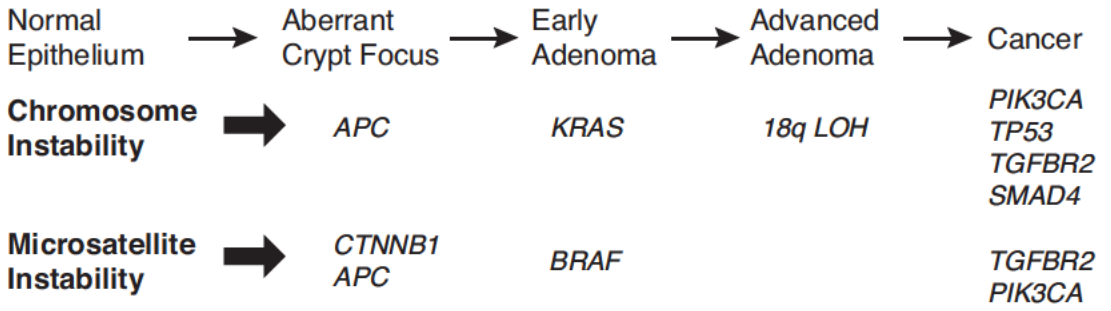


Figure 2. Proposal of two pathways for adenoma-carcinoma progression in colorectal carcinogenesis. The chromosome instability (CIN) pathway is characterized by tubular histology, whereas sessile serrated adenomas are typical for the microsatellite instable (MSI) tumours. Adapted from Pritchard and Grady (2011).

altered therapy (Pritchard and Grady 2011).

The European Group on Tumour Markers (EGTM) recommends centralized population-based screening for colorectal cancer biennially by an immunochemical, rather than a guanine-based, faecal occult blood test (FOBT) (Duffy *et al*. 2011). A recent multicentre randomised trial showed that a once-only flexible sigmoidoscopy between ages 55 and 64 reduced colorectal cancer incidence in the intervention group by 23% and their mortality by 31% in comparison to the control group with observation only (Atkin *et al*. 2010). A Dutch study compared screening methods and found biennal immunochemical FOBT superior in regards to participation and detection rates, compared to the guanine-based FOBT. Flexible sigmoidoscopy every five years was, however, more efficient in regards to higher detection rates of advanced neoplasia (Hol *et al*. 2010). In Finland, the colorectal cancer screening coverage in 2007 was one-third of the general population aged 60-69 (Malila *et al*. 2011).

Without screening, symptoms commonly arise late, with abdominal pain, constipation, anaemia, and melena being the most common. Diagnosis is commonly reached with colonoscopy or radiologic approaches, or both, and 60% of colorectal adenocarcinomas are located in the colon. The majority (60%) are moderately differentiated (grade 2). Tumours with abundant mucus formation are called mucinous and constitute 10% of all colorectal tumours. TNM stage is the most important prognostic factor and helps in planning primary treatment (Table 4). The formerly more commonly used modified Dukes classification A to D serves as a basis for several colorectal cancer staging systems. Dukes A tumours are limited to the bowel wall, Dukes B tumours spread beyond the muscularis propria into adjacent tissues or organs but do not involve lymph nodes, Dukes C tumours involve lymph nodes, and any cancer remaining locally or with widespread metastasis is classified as Dukes D (Bosman *et al*. 2010, Dukes 1932, Davis and Newland 1982).

Table 4. TNM classification of colorectal tumours. Adapted from Bosman *et al.* (2010).

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of the lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumour perforates visceral peritoneum and/or directly invades other organs or structures

Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes

Distant metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2-T3	N0	M0
Stage III	T4	N0	M0
	Any T	N1	M0
Stage IV	Any T	Any N	M1

5.4.3. Treatment

Radical surgery is necessary for a cure, typically with right- or left-sided hemicolectomy for colon cancers. Preoperative radiotherapy allows better local control, especially for tumours in the rectum. Anterior resection, together with colorectal anastomosis when possible, is usually chosen when rectal tumours are resected. When metastasis is evident at diagnosis, palliative surgery with colostomy

or endoscopic stents may be indicated to prevent gastrointestinal emergencies such as obstruction or perforation.

Adjuvant treatment with an oxaliplatin-based regimen has proven beneficial for stage III colon cancer patients in the MOSAIC (Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leukovorin in the Adjuvant Treatment of Colon Cancer) study (Andre *et al.* 2009) and in the NSABP (National Surgical Adjuvant Breast and Bowel Project) C-07 protocol (Kuebler *et al.* 2007, reviewed in Gangadhar *et al.* 2010, Lombardi *et al.* 2010, Silvestris *et al.* 2010). In a recent Cochrane systematic review, disease-free survival was significantly better for those stage-II colon cancer patients who received adjuvant treatment (Figueredo *et al.* 2008). In cases of metastasized disease, the *KRAS* mutation aids in recognizing patients who fail to benefit from treatment with epidermal growth factor receptor (EGFR) -targeted antibodies (Karapetis *et al.* 2008).

5.4.4. Prognosis

The age-standardized 5-year colon cancer-specific relative survival ratio was, in 2005-2007, according to the Finnish Cancer Registry, 80% for men and 63% for women, and the corresponding figures were 57% for men and 61% for women with rectosigmoid, rectal, and anal cancers (Finnish Cancer Registry 2009). The 5-year survival for patients with Dukes A disease was 90% at the Department of Surgery, Helsinki University Central Hospital in 1982-1998, for Dukes B 75%, for Dukes C 50%, and for Dukes D below 10% (Carpelan-Holmström 1996, Louhimo 2003).

5.5. Protein phosphatase 2A (PP2A)

Protein phosphatase 2A (PP2A) is a family of serine/threonine phosphatases that widely regulate phosphatase activity in cells. This enzyme consists of a 65 kDa scaffolding subunit, termed PR65 or the A subunit, and a catalytic subunit ($PP2A_C$) with a molecular mass of 36 kDa, together forming the core dimer ($PP2A_D$). In association with four different families of B regulatory subunits (B, B', B'', and B'''), PP2A binds to various target proteins. The diversity of subunit isoforms creates altogether at least 75 different PP2A holoenzyme compositions, all with different cellular and subcellular localization, as well as specific target proteins (Janssens and Goris 2001).

5.6. Cancerous inhibitor of PP2A (CIP2A)

CIP2A, cancerous inhibitor of protein 2A, encoded by the gene *KIAA1524* and located in the 3q13.13 human chromosome region, was first recognized as the p90 auto-antigen in hepatocellular carcinoma (Soo Hoo *et al.* 2002). Soo Hoo *et al.* demonstrated its localization to the perinuclear regions of the cytosol, which was later confirmed by Junttila *et al.* (2007). In this first study, the strongest expression of p90/CIP2A appeared in mouse embryonic liver specimens, but high expression was also noted in the brain, in muscle fibres, and in epidermal layers. Auto-antibodies against p90/CIP2A were detectable in sera from patients with hepatocellular carcinoma (22%, n = 95), and in a few samples from gastric (3%) and oesophageal cancer patients (5%), but not in colon cancer samples. In gastric cancer specimens, CIP2A was expressed predominantly in the cytoplasm (Soo Hoo *et al.* 2002).

In a subsequent study from the same laboratory, auto-antibodies to p90/CIP2A occurred in 31% of 133 prostate cancer patients, significantly more than in the previous study and high compared to rates in benign prostate hyperplasia patients (2%, n = 68) (Shi *et al.* 2005). Junttila *et al.* (2007) recognized p90 as an oncoprotein inhibiting PP2A-mediated dephosphorylation of MYC and renamed it cancerous inhibitor of PP2A. They showed that CIP2A targets the PR65 scaffolding subunit of protein phosphatase 2A, whereby the phosphatase activity of PP2A towards MYC serine 62 (S62) is inhibited. The phosphorylated active MYC stimulates cell proliferation and transformation (Figure 3). Depletion of CIP2A leads to downregulation of MYC both at mRNA and protein expression levels, which is unrelated to cell-cycle progression. Furthermore, downregulation of CIP2A reduces dense foci formation of HeLa cells in monolayers, as well as reduces anchorage-independent growth in soft agar. Interestingly, upregulation of CIP2A in head and neck squamous cell carcinoma cells and in mouse embryonal fibroblasts leads to increased tumour growth. In clinical samples, CIP2A mRNA is expressed at very low levels in non-malignant tissues, with the exception of bone marrow, prostate, testis, cerebellum, and brain. Overexpression of CIP2A protein can be noted in head and neck squamous cell carcinoma specimens, with predominant cytoplasmic localization and only weak nuclear expression, whereas in colon cancer, CIP2A mRNA is significantly overexpressed when compared to control specimens. Junttila *et al.* (2007) concluded that CIP2A is an oncoprotein important for maintaining the malignant cellular phenotype.

CIP2A functions as a downstream target in the RAS signalling pathway and may be induced, in combination with inhibition of the TGF-β tumour suppressor pathway, in premalignant head and neck squamous cell lesions (Junttila *et al.* 2007, Junttila and Westermarck 2007). In gastric cancer, Zhao *et al.* (2010) studied the role

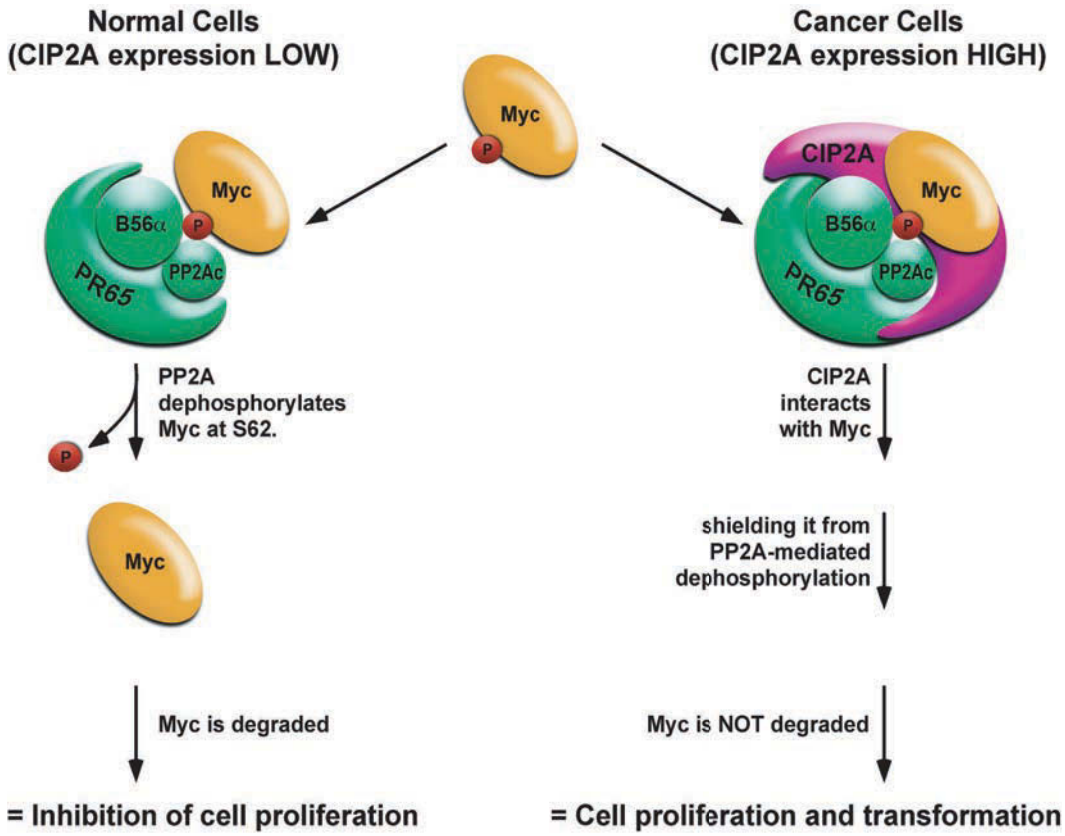


Figure 3. CIP2A-mediated stabilization of MYC. Reprinted with permission from Prof. J. Westermarck (Junttila and Westermarck 2007).

of *cagA*-positive *H. pylori* in CIP2A expression, and found that the CagA-induced upregulation of CIP2A is mediated through the MEK/ERK pathway. Furthermore, Khanna *et al.* (2011) showed MEK1/2 and EGFR inhibitors to deplete CIP2A expression, whereas activation of the MEK1/2-ERK signalling pathway stimulates CIP2A expression. They established the ETS1 transcription factor as the mediator of the EGFR-MEK1/2-ERK-induced positive regulation of CIP2A and concluded that CIP2A overexpression is dependent on the EGFR-MEK1/2-ETS1 signalling pathway.

CIP2A may promote malignant growth by reducing cell death-associated protein kinase (DAPk)-induced apoptosis via the UNC5H2-receptor (Gozuacik and Kimchi 2006, Guenebeaud *et al.* 2010). DAPk serves as a tumour suppressor protein by inducing apoptosis and cell death upon activation by dephosphorylation. Netrin-1 is a diffusible laminin-related protein, which serves in several cell-regulating mechanisms and functions as a ligand for the UNC5H2 (also called UNC5B) dependence receptor. In the absence of netrin-1, the UNC5H2-receptor recruits PP2A to dephosphorylate and hence activates DAPk and induces apoptosis. Guenebeaud *et al.* (2010) demonstrated that netrin-1 favours the recruitment of CIP2A to this UNC5H2-DAPk complex, whereby PP2A-mediated dephosphorylation of DAPk is inhibited which leads to increased cell survival.

In hepatocellular carcinoma (HCC), a role has been proposed for CIP2A in causing resistance to the chemotherapeutic drug bortezomib (Chen *et al.* 2010). In HCC cells with high CIP2A expression, the PP2A inhibition, which is mediated by CIP2A, inactivates AKT and hence induces apoptosis of HCC cells. Chen *et al.* further suggested that CIP2A may serve as a predictive factor for bortezomib resistance. In a subsequent study, the same group showed that a combination of bortezomib together with radiotherapy is even more efficient in reduction of tumour growth (Huang *et al.* 2011). They suggest that the enhancement of radiosensitivity by bortezomib in solid tumour cells is dependent on CIP2A expression. In lung cancer cells, Ma *et al.* (2011) demonstrated that rabdocoetsin B, a novel natural compound extracted from a Chinese medicinal herbal Rabdosia coetsa, inhibits CIP2A at mRNA level, resulting in downregulation of both CIP2A and pAKT proteins. They propose that the inhibition of the CIP2A-AKT pathway by rabdocoetsin B in lung cancer cells leads to reduced proliferation and induced apoptosis. Interestingly, Choi *et al.* (2011) demonstrated in breast and colon cancer cells that high CIP2A expression is, in addition, associated with doxorubicin resistance, suggesting that mutation of p53 inhibits the doxorubicin-mediated downregulation of CIP2A protein. Growth arrest and reduced clonogenic capabilities of tumour cells induced by CIP2A depletion, however, are independent of p53 and pRB signalling pathways (Li *et al.* 2008).

5.7. Clinical role of CIP2A

CIP2A is overexpressed in in several solid cancers. In gastric cancer, CIP2A mRNA has been expressed at a significantly higher level in 87% of 37 gastric cancer specimens compared with corresponding adjacent tissue (Li *et al.* 2008), and in breast cancer it is overexpressed in 159 specimens compared with normal breast tissue (Come *et al.* 2009). In non-small-cell lung cancer, CIP2A mRNA has been overexpressed in 83% and CIP2A protein in 72% (Dong *et al.* 2010), whereas Ma *et al.* (2011) found CIP2A mRNA to be overexpressed in 67% and high CIP2A immunoreactivity in 67% of the lung cancer specimens studied. In oral squamous cell cancer, Katz *et al.* (2010) demonstrated strong intensity of CIP2A protein in all 8 carcinoma specimens. Cytoplasmic CIP2A immunopositivity has occurred in 90% of 40 in oesophageal squamous cell (Qu *et al.* 2010) and in 97% of 59 prostate carcinoma specimens (Vaarala *et al.* 2010). In cervical cancer, of 72 specimens, 53% were immunopositive for CIP2A (Liu *et al.* 2011a).

In breast cancer, CIP2A is associated with aggressive disease characteristics (Come *et al.* 2009), whereas in non-small-cell lung cancer, CIP2A expression is associated with high proliferation index, but not with any other clinicopathologic

parameters (Dong *et al.* 2010). In renal cell cancer, immunopositive CIP2A is associated with advanced disease, high grade, and clear cell type (Ren *et al.* 2011). This is in contrast with the recent results by Liu *et al.* (2011a) in cervical cancer, which showed no association between CIP2A expression and proliferation index, tumour size, differentiation status, node metastasis, nor clinical stage. CIP2A is more frequently expressed in prostate cancer specimens than in benign prostate hyperplasia and associates with high Gleason score (> 7) (Vaarala *et al.* 2010). In dysplastic oral lesions, strong CIP2A expression is evident in the basal and parabasal cell layers (Katz *et al.* 2010). A prognostic role for CIP2A has been demonstrated in lung (Dong *et al.* 2010, Xu *et al.* 2011) and renal cell (Ren *et al.* 2011) cancer, in both of which CIP2A showed independent prognostic value. In breast cancer, on the contrary, no prognostic significance has been apparent (Come *et al.* 2009).

CIP2A is present in cervical intraepithelial neoplasia (CIN) III and cervical cancer, but not in normal tissues or CIN I and II, suggesting a potential role for CIP2A as a diagnostic marker to improve cytological diagnosis for cervical cancer (Liu *et al.* 2011a). Furthermore, CIP2A expression correlates with HPV16 E7 immunoreactivity, both in regards to location of the expression and of immunointensity. Liu *et al.* (2011a) demonstrated that depletion of HPV16 E7 in cervical cancer cells downregulates CIP2A expression both at mRNA and protein level, indicating that HPV16 E7 may regulate CIP2A expression in cervical cancer.

In neural tissue, CIP2A is expressed in the periventricular areas of the developing cerebrum, the region in which neural progenitor cells (NPCs) are located (Kerosuo *et al.* 2010). The proportion of self-renewing neural progenitor cells varies with CIP2A expression. Kerosuo *et al.* (2010) suggest that CIP2A plays a role in the self-renewal of neural progrenitor cells both during embryonic development and in adulthood, which at least to a certain degree is explained by the increased MYC signalling.

In rheumatoid arthritis, Lee *et al.* (2011) demonstrated overexpression of CIP2A in fibroblast-like synoviocytes and synovial tissues in comparison to that in ostheoarthritis. CIP2A mRNA expression correlates with the aggressive histopathological grade of synovial tissue, notably with synovial hyperplasia. CIP2A mRNA and protein overexpression has, similarly, been demonstrated in the bone marrow of acute myeloid leukaemia (AML) patients, where 77% of newly diagnosed AML and 71% of relapsed AML cases were positive for CIP2A (Wang *et al.* 2011). In HL60 human AML cells, CIP2A depletion induced partial differentiation into late promyelocytes, suggesting a potential therapeutic target (Li *et al.* 2008, Wang *et al.* 2011). In chronic myeloid leukaemia (CML), high CIP2A protein levels in

mononuclear cells taken at diagnosis predict progression into blast crisis (Lucas *et al.* 2011).

6. AIMS OF THE STUDY

The purpose of the study was to evaluate the prognostic role of cancerous inhibitor of protein phosphatase 2A (CIP2A) in solid cancers. The study was limited to gastric, serous ovarian, tongue, and colorectal carcinomas.

The specific aims of the study were to determine:

1. The significance of CIP2A in prognostic evaluation – for this purpose the expression of CIP2A in the cancer specimens was studied by immunohistochemistry and immunoreactivity evaluated
2. Association of CIP2A with other biomarkers, such as MYC, that could explain any alteration in prognosis
3. Regulation of CIP2A expression in order to gain insight in the signalling pathways that lead to alterations in prognoses

7. PATIENTS AND METHODS

Table 5. Overview of methods used in Studies I-IV.

Method	Study	Section
Patients	I-IV	7.1
Tumour tissue microarrays	I-IV	7.2
Immunohistochemistry	I-IV	7.3
Cells	I, (II, III)*	7.4
Small interfering RNA (siRNA experiments)	I, (II, III)	7.5
Small molecular inhibitor experiments	I, (II, III)	7.6
Protein extraction and Western blot analysis	I, (II, III)	7.7
RNA extraction and qRT-PCR	I, (III)	7.8
Cell viability assays	I	7.9
Cell proliferation assay	I	7.10
Soft agar assay	I	7.11
Cycloheximide pulse chase (protein stability) experiments	I	7.12
Preparation of mouse embryo fibroblasts	I	7.13
Cell cycle experiments	I	7.14
Statistical analysis	I-IV	7.15

* Unpublished data are referred to the related study in parenthesis.

7.1. Patients

Survival data and cause of death for Studies I to IV came from patient records, the Finnish Cancer Registry, and Statistics Finland. Approval of the studies came from the local Ethics Committee and National Supervisory Authority of Welfare and Health. An overview of patient materials in Studies I to IV is presented in Table 6.

7.1.1. Study I

Study I included 337 consecutive gastric cancer patients treated at the Department of Surgery, Helsinki University Central Hospital, from 1983 to 1999, of whom 141 (42%) had low-stage disease (I-II), and 196 (58%) high-stage (III-IV) disease. Lymph node metastases existed in 184 (55%) and distant metastases in 93 (28%); 176 (52%) patients underwent surgery for cure (total or partial gastrectomy with lymphadenectomy), whereas 143 (43%) underwent palliative surgery (partial gastrectomy, bypass, or laparotomy only). Extended lymphadenectomy (D2-4) was done for 34 (10%). No patient received neoadjuvant treatment, but 32 received post-

Table 6. Overview of the patient materials in Studies I-IV.

Study	Disease	Number of patients[1] (%)	Year	Inclusion criteria
I	Gastric cancer	223/337 (67)	1983-1999	All patients
II	Ovarian cancer	524/562 (93)	1964-2000	Serous ovarian cancers
III	Tongue cancer	71/73 (97)	1992-2002	T1N0M0 and T2N0M0
IV	Colorectal cancer[2]	540/643 (84)	1989-1998	All patients
IV	Colorectal cancer[3]	212/220 (96)	1998-2000	All patients

[1] The figures for successfully scored specimens for CIP2A immunoreactivity and total number of patients.
[2] Test dataset.
[3] Validation dataset.

operative adjuvant treatment (28 chemotherapy, 2 radiotherapy, and for 2, both). Median age was 66 (range 30-87). During follow-up, 210 (64%) patients died of gastric cancer, and median follow-up time was 12.7 years (range 4.7-20.8). The 5-year overall survival rate for the whole cohort was 35.1% (95% confidence interval (CI) 29.8-40.4%).

7.1.2. Study II

Study II comprised 562 consecutive patients treated for serous ovarian carcinoma at the Department of Obstetrics and Gynaecology of Helsinki University Central Hospital, from 1964 to 2000 (median 1994). At the end of the 1980s, radical surgery was adopted. Of 562 patients, 451 (80%) underwent total abdominal hysterectomy and bilateral salpingo-ophorectomy, along with surgical removal of tumour masses, together with pelvic or para-aortic lymphadenectomy or both for 175 of these. Of the 562, 54 (10%) underwent uni- or bilateral salpingo-ophorectomy, and in 57 (10%) only biopsies were done. Before 1990, all patients received chemotherapy according to current praxis. After 1990, all patients except those with stage Ia to Ib and grade 1-2 disease received chemotherapy (Young *et al*. 1990). Platinum-based chemotherapy served as part of first-line treatment in 404 (72%) cases combined with taxanes in 194 (35%). Median age at diagnosis was 60 (range 18-92) and median follow-up time of patients alive at study end was 8.8 years (range 0.1 to 41.3). The 5-year overall survival rate for the whole cohort was 41.2% (95% CI 37.1-45.7).

7.1.3. Study III

In Study III, we collected retrospectively 73 consecutive patients with early-stage oral squamous cell carcinoma of the tongue, preoperatively staged as T1N0M0 and

T2N0M0. All patients were treated with curative intent between 1992 and 2002 at the Helsinki University Central Hospital according to the guidelines of the tumour board meeting. Detailed patient characteristics have been described (Keski-Säntti *et al.* 2006, 2007). Preoperatively, 35 (48%) tumours were clinically classified as T1, and 38 (52%) as T2. Following resection for cure of that primary tumour, 31 patients received no further treatment, whereas 42 underwent elective neck treatment (neck dissection, 9; neck dissection and radiotherapy, 32; radiotherapy only, 1). In elective neck dissection, primary lymph node positivity (pN+) was evident in 14 cases. During follow-up, 10 developed neck recurrences apparently representing late lymph node metastases without a primary recurrence. Median age at diagnosis was 59 (range 23-95), and median follow-up of patients at study end was 7.9 years (range 0.3-17.2). 5-year overall survival for the whole cohort was 68.5% (95% CI 57.9-79.1).

7.1.4. Study IV

Between 1989 and 1998, 643 consecutive colorectal cancer patients treated at the Department of Surgery, Helsinki University Central Hospital, entered Study IV (referred to as the "test set"). Of these, 92 (15%) belonged to a Dukes A group, 224 (36%) to Dukes B, 162 (26%) to Dukes C, and 145 (23%) to Dukes D. Tumours were located mostly in the colon (55%; n = 341). Median age of the study population was 65. Median follow-up time at the end of follow-up was 4.7 years (range 0-24.7), with a 5-year disease-specific overall survival of 54.4% (95% CI 50.5-58.3). For validation, we used a later dataset, which comprised 220 colorectal cancer patients treated from 1998 to 2000. 33 (15%) were staged as Dukes A, 70 (32%) as Dukes B, 70 (32%) as Dukes C, and 45 (21%) as Dukes D. The majority of the tumours were located in the rectum: 129 (59%). Median age was 67, with a median follow-up time of 6.0 years (range 0-13.2) and a 5-year disease-specific overall survival of 64.8% (95% CI 58.1-71.5).

7.2. Tumour tissue specimens

Tumour samples were fixed in buffered 10%-formalin solution, embedded in paraffin, and stored at the Department of Pathology, Helsinki University Central Hospital. Experienced pathologists reviewed all tumour samples from H&E stainings and indicated representative areas for microarrays. In Study I, a technical assistant punched three 0.6-mm cores with a tissue microarray instrument (Manual Tissue Arrayer 1, Beecher Instruments, Silver Spring, MD USA) and arranged them into paraffin as tissue-array blocks (Linder *et al.* 2006). In Study II, four representative 0.8-mm cores, in Study III, six 1.0-mm cores, and in Study IV, three 1.0-mm cores

were retrieved (Kononen *et al.* 1998, Kallioniemi *et al.* 2001, Torhorst *et al.* 2001). In addition, in Study III, whole sections from seven tumour specimens of the nine cases in which all array cores were missing or included no tumour tissue were stained.

In Study II, three human and three mouse ovarian tissue specimens were collected as normal controls, whereas in Study III, 37 representative specimens came from normal oral mucosa, dysplastic mucosal lesions, and invasive oral carcinoma.

7.3. Immunohistochemistry

Prior to immunohistochemical staining, the tissue blocks were freshly cut into 4-μm thick sections, fixed on slides, and dried for 12 to 24 hours at 37°C. The sections underwent deparaffinization in xylene, followed by rehydration through graded ethanol and distilled water. The sections were kept in Tris-HCl (pH 8.5) or Tris-EDTA (pH 9.0) buffer for 20 minutes at 98°C in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) for antigen retrieval. We stained the sections in an Autostainer 480 (Lab Vision Corp.) with the Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse kit (Dako, Glostrup, Denmark). Sections were first treated for 5 minutes with 0.3% Dako REAL Peroxidase-Blocking Solution to block endogenous peroxidases. A rabbit polyclonal CIP2A antibody (Dako REAL Antibody Diluent) served for one hour (Soo Hoo *et al.* 2002) at room temperature as the primary antibody in all studies. Alternative primary antibodies were the mouse monoclonal MYC antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Studies I, III-IV) and rabbit polyclonal CIP2A antibody (Novus Biologicals, Littleton, CO, USA; Study II). Subsequently, a 30-minute incubation with peroxidase-conjugated Dako REAL EnVision/HRP, Rabbit/Mouse reagent followed, after which visualization was developed with Dako REAL DAB+ Chromogen for 10 minutes. Between each pair of steps in the staining procedure, sections were washed with PBS-0.04%-Tween20. Meyer's haematoxylin served for counterstaining, followed by a 10-minute wash in tap water and mounting in aqueous mounting medium (Aquamount, BDH, Poole, UK).

In Studies I, II, and IV, sections were stained for p53 with the mouse monoclonal DO-7 antibody, which recognizes both mutant and wild-type p53 proteins (1:300 in Study I, 1:100 in Study II, 1:50 in Study IV, Dako) as described (Victorzon *et al.* 1996a, Lassus *et al.* 2003, Böckelman *et al.* 2012). For Ki-67 (1:500), the rabbit polyclonal A0047 antibody served as the primary antibody (Dako) in Study I (Mrena *et al.* 2010), in Study II the dilution was 1:150 (Lassus *et al.* 2004), in Study III, 1:100 (Häyry *et al.* 2010), and in Study IV the mouse monoclonal MIB-1 antibody (Dako)

Table 7. Overview of antibodies in the studies.

Antigen	Study	Antibody	Antibody type[1]	Manufacturer	Dilution	Purpose[2]	Reference
Beta-actin	I, (II, III)	C-11/sc-1615	Goat pAb	Santa Cruz	1:1000	WB	Khanna *et al.* (2009)
Beta-tubulin	(II)	H-235/sc-9104	Rabbit pAb	Santa Cruz			–
CIP2A	I	–	Rabbit pAb	Dr. Chan	1:2000	IHC	Soo Hoo *et al.* (2002)
	II	–	Rabbit pAb	Dr. Chan	1:10000	IHC	Soo Hoo *et al.* (2002)
	III, IV	–	Rabbit pAb	Dr. Chan	1:3000	IHC	Soo Hoo *et al.* (2002)
	I, (II, III)	–	Rabbit pAb	Dr. Chan	1:5000	WB	Soo Hoo *et al.* (2002)
	II	NB100-74663	Rabbit pAb	Novus	1:500	IHC	–
	II, III	NB100-68264	Rabbit pAb	Novus	1:2000	WB	–
JUN	I		Rabbit mAb	Cell Signaling[3]	1:1000	WB	Khanna *et al.* (2009)
MYC	I	C-33/sc-42/9E10	Mouse mAb	Santa Cruz	1:500	IHC	Khanna *et al.* (2009)
	III	C-33/sc-42/9E10	Mouse mAb	Santa Cruz	1:400	IHC	Häyry *et al.* (2010)
	IV	C-33/sc-42/9E10	Mouse mAb	Santa Cruz	1:200	IHC	–
Ki-67	I	A0047	Rabbit pAb	Dako	1:500	IHC	Mrena *et al.* (2010)
	II	A0047	Rabbit pAb	Dako	1:150	IHC	Lassus *et al.* (2004)
	III	A0047	Rabbit pAb	Dako	1:100	IHC	Häyry *et al.* (2010)
	IV	MIB-1	Mouse mAb	Dako	1:100	IHC	–
Lamin A/C	(II)	#2032	Rabbit pAb	Cell Signaling[3]	1:1000	WB	–
p53	I	DO-7	Mouse mAb	Dako	1:300	IHC	Victorzon *et al.* (1996a)
	II	DO-7	Mouse mAb	Dako	1:100	IHC	Lassus *et al.* (2003)
	IV	DO-7	Mouse mAb	Dako	1:50	IHC	–
	I	DO-1/sc-126	Mouse mAb	Santa Cruz	1:500	WB	Khanna *et al.* (2009)
S62 phospho-MYC	I		Mouse mAb	BioAcademica[4]	1:500	WB	Khanna *et al.* (2009)
Total MYC	I		Mouse mAb	Nordic Biosite[5]	1:500	CHX	Khanna *et al.* (2009)

Table 7 cont. Overview of antibodies in the studies.

Secondary antibody	Study	Antibody	Antibody type[1]	Manufacturer	Dilution	Purpose[2]	Reference
Anti-goat	I	Sc-2020	Donkey pAb	Santa Cruz	1:2000	WB	Khanna *et al.* (2009)
Anti-mouse	I		Sheep pAb	Amersham[6]	1:2000	WB	Khanna *et al.* (2009)
Anti-rabbit	I, (II, III)	#1858415	Goat pAb	Pierce[7]	1:500	WB	Khanna *et al.* (2009)

[1] Abbreviations: pAb = polyclonal antibody, mAb = monoclonal antibody
[2] Abbreviations: WB = Western blot, IHC = immunohistochemistry, CHX = cycloheximide pulse chase experiment
[3] Cell Signaling Technology, Inc., Danvers, MA, USA.
[4] BioAcademica Inc., Osaka, Japan.
[5] Nordic Biosite, Täby, Sweden.
[6] Amersham Biosciences, Piscataway, NJ, USA.
[7] Pierce Biotechnology, Inc., Rockford, IL, USA.

was diluted 1:100. An overview of the antibodies used for immunohistochemistry and Western blotting is presented in Table 7.

7.3.1. Scoring of samples

Tumour specimens were scored independently by two researchers blinded to clinical status and outcome data. Cytoplasmic CIP2A immunopositivity was scored as 0 to 3 based on intensity of cancer-cell immunoreactivity, and the one with the highest intensity served for further analysis. Negative immunoreactivity was scored as 0, and diffuse weak cytoplasmic positivity as 1. Moderately positive or focally strongly positive intensity was scored as 2, and homogeneously strong intensity as 3. Specimens with discordant scores underwent re-evaluation with a multiheaded microscope, and the consensus score served for further analysis.

In Study II, CIP2A nuclear immunoreactivity was scored as negative (score 0) when <10% of the nuclei stained positive and as positive (score 1) when ≥10% of the nuclei were positive. In Study III, in addition to tissue microarray blocks, we collected histological specimens from normal oral mucosa, dysplastic lesions, and invasive carcinoma (n = 37). Cytoplasmic CIP2A immunoreactivity was scored from whole-tissue sections for hot-spot areas as described, and nuclear CIP2A immunoreactivity was evaluated for trend.

In Studies I and IV, MYC immunoreactivity was scored separately for cytoplasmic and nuclear expression. Cytoplasmic MYC immunoreactivity was scored for intensity in the same way as CIP2A expression. In Study I nuclear MYC immunoreactivity was scored as negative (<10%) or positive (≥ 10%), whereas in Study IV nuclear MYC expression was scored according to percentage of immunopositive nuclei (0%; 1-10%; 11-30%; 31-50%; 51-80%; >80%). In Study III, MYC immunoreactivity was scored separately for cytoplasma and nuclei as follows: score 0 represented immunonegativity, score 1 positivity in less than 30% of the cells, score 2 positivity in 30 to 50%, score 3 positivity in 50 to 80%, and score 4 positivity in more than 80% (Häyry *et al.* 2010).

For p53 in Study I, nuclear positivity was evaluated as low (negative or ≤20%) or high (>20%) expression (Victorzon *et al.* 1996a). In Study II, immunohistochemical staining for p53 was regarded as aberrant when tumour cells showed excessive p53 (homogeneous moderate or strong nuclear immunopositivity in over 50%) or were completely negative for p53 (no staining in any of the tumour cells). The p53 expression was considered to be normal when tumours showed – similar to the situation in normal fallopian and ovarian epithelium – weak p53 immunostaining (Lassus *et al.* 2003). In Study IV, nuclear p53 was evaluated by percentage of stained cells. No immunoreactivity was scored as 0, 1 to 10% as 1, 11

to 49% as 2, and more than 50% as 3, and in the final analysis grouped as ≤10% and >10% (Böckelman *et al.* 2012). Regarding the proliferation index Ki-67 in Study I, immunoreactivity evaluated as <10% was representative of negative expression and ≥10% of positive (Mrena *et al.* 2010). In Study II, nuclear Ki-67 was analysed as low (<10%), moderate (10 to 25%), or high (>25%) (Lassus *et al.* 2004), whereas in Study III we scored it as negative or very low (0 to 29%), low (30 to 49%), moderate (50 to 79%), or high (≥80%) expression (Häyry *et al.* 2010). In Study IV, Ki-67 was scored as 0, 1 to 10% as 1, 11 to 49% as 2, and more than 50% as 3, and analysed as ≤10% and >10% (Böckelman *et al.* 2012).

7.3.2. DNA flow cytometry

Ploidity was analysed in Studies I and II according to the protocol for DNA flow cytometry (Hedley *et al.* 1983, Victorzon *et al.* 1996b, Jahkola *et al.* 1998, Lassus *et al.* 2006). The lowest peak represented the reference with a DNA index value of 1.00. DNA index values between 1 and 1.20 represented diploidy. The S-phase fraction (SPF) was calculated with the Cellfit program of the FACScan flow cytometer (FACScan, Becton Dickinson, Mountain View, CA, USA) or manually by a modified rectilinear method, with the lower SPF chosen. The number of nuclei analysed from each specimen was at least 10 000. The median SPF of 7.6% served as the cut-off level for statistical analysis.

7.4. Cells

Gastric and ovarian cancer cell lines for Studies I to II (Table 8) were cultured in RPMI-1640 cell culture growth medium supplemented with 10% fetal calf serum (PromoCell GmbH, Heidelberg, Germany), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin, (Bio Whittaker Europe, Verviers, Belgium), and maintained at 37°C at 5% CO_2 in air. Squamous-cell carcinoma cell lines HSC-3 and SAS for Study III were cultured in 1:1 Dulbecco's modified Eagle Medium (glucose 4.5 g/l) together with Nutrient mixture F-12 Ham (Sigma-Aldrich Chemie, Steinham, Germany) supplemented with 10% fetal calf serum (PromoCell), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma), 0.4 ng/ml hydrocortisone (Sigma), 100 μg/ml streptomycin, 100 units/ml penicillin, and 250 ng/ml fungizone (Invitrogen, Carlsbad, CA, USA).

Table 8. Overview of the cell lines used in Studies I to III.

Cell line	Origin	Study	Source[1]	Reference
AGS	Gastric adenocarcinoma	I	ATCC	(Barranco *et al.* 1983)
CaOV3	Ovarian adenocarcinoma	(II)	ATCC	(Buick *et al.* 1985)
EFO-27	Mucinous papillary ovarian adenocarcinoma	(II)	DSMZ	(Emons *et al.* 1993)
ES-2	Ovarian clear cell carcinoma	(II)	ATCC	(Lau *et al.* 1989)
HSC-3	Squamous cell carcinoma from the tongue	(III)	T. Salo	(Momose *et al.* 1989)
KATOIII	Gastric adenocarcinoma, diffuse type	I	ATCC	(Yokozaki 2000)
MKN-28	Gastric adenocarcinoma, intestinal type	I	H. Yokozaki	(Yokozaki 2000)
MKN-45	Gastric adenocarcinoma, diffuse type	I	H. Yokozaki	(Yokozaki 2000)
OV-4	Ovarian adenocarcinoma	(II)	TJ. Eberlein	
OVCAR-3	Ovarian adenocarcinoma, from ascites	(II)	ATCC	(Hamilton *et al.* 1983)
SAS	Squamous cell carcinoma from the tongue	(III)	T. Salo	(Takahashi *et al.* 1989)
SKOV-3	Serous ovarian cyst-adenocarcinoma, from ascites	(II)	ATCC	(Hua *et al.* 1995)
TMK-1	Gastric adenocarcinoma, diffuse type	I	H. Yokozaki	(Yokozaki 2000)

[1] ATCC = American Type Culture Collection, Manassas, VA, USA; DSMZ = German collection of Microorganisms and Cell Cultures; Prof. T. Salo, University of Oulu, Oulu, Finland; Prof. H. Yokozaki, Hiroshima University School of Medicine, Hiroshima, Japan; and Dr. Timothy J. Eberlein, Harvard Medical School, Boston, MA, USA.

7.5. Small interfering RNA (siRNA) experiments

Gastric, ovarian, and tongue cancer cell lines were transfected with 20 to 50 nM siRNA by Lipofectamine 2000 Reagent (Invitrogen). The CIP2A and scrambled CIP2A siRNAs for Study I came from Eurofins MWG operon (Ebersberg, Germany): CIP2A scrambled: 5'- UAACAAUGAGAGCACGGCTT-3', CIP2A.1: 5'-CUGUGGUUGUGUUUGCACUTT-3', CIP2A.2: 5'-ACCAUUGAUAUCCUUAGAATT-3'. HP-validated siRNAs for human MYC (Hs_Myc_7, Hs_Myc_5) came from Qiagen Sciences (Germantown, MD, USA). Cells were harvested 24 h post-transfection for mRNA experiments, and 72 h post-transfection for protein expression experiments. In Studies II and III, the CIP2A (ON-TARGETplus SMART pool, human KIAA-1524, Cat. number L-014135-01), EGFR (ON-TARGETplus SMART pool, human EGFR, Cat. number L-003114-00), and control (ON-TARGET*plus* Control pool, Non-Targeting pool, Cat. number D-001810-10-05) siRNAs came from Thermo Scientific Dharmacon (Lafayette, CO, USA).

7.6. Small molecular inhibitor experiments

The following signalling pathways were targeted: EGFR (AG-1478, Calbiochem, Darmstadt, Germany), ERK (PD98059, Calbiochem), JNK (SP600125, Calbiochem), and Notch (GSI, Calbiochem). Dimethyl sulphoxide (DMSO; Sigma) served as the control. The inhibitor concentrations were 5 to 20 μM, and total proteins were extracted after 6 to 48 hours.

7.7. Protein extraction and Western blot analysis

Total proteins were extracted in Radio-Immunoassay Precipitation (RIPA) lysis buffer (150 mM NaCl; 1% NP40, Igepal CA-630, Sigma; 1% sodium deoxycholate, Sigma; 0.1% sodium dodecyl sulphate; 50 mM Trizma Base adjusted with HCl to pH 8.0; 1 mM EDTA adjusted with HCl to pH 8.0, Sigma; Complete Mini protease inhibitor cocktail tablet, Roche Diagnostics GmbH, Mannheim, Germany) or with hot Laemmli sample buffer (60 mM Tris-HCl pH 6.8; 2% sodium dodecyl sulphate; 10% glycerol, Sigma). Cytoplasmic and nuclear fractions were prepared with the NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology Inc., Rockford, IL, USA). Protein concentrations were measured with the Bio-Rad D_C Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) or with BCA Protein Assay Kit (Pierce). For Western blot analysis, 30 μg protein extracts were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS; 20 mM Trizma Base, Sigma; 150 mM NaCl dissolved in distilled water and adjusted with HCl to pH 7.5) containing 0.1%-NP40 (Igepal CA-630, Sigma), and subsequently incubated with primary antibodies as indicated in Table 7 (p. 40-41). After incubation with the secondary antibodies conjugated with horseradish peroxidase, proteins were visualized with the SuperSignal West Femto Maximum Sensitivity Substrate or with the Proteome Grasp ECL Kit (both from Pierce).

7.8. RNA extraction and qRT-PCR

RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA, USA) or the Nucleospin RNA/Protein extraction kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), and transcribed to cDNA with the M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant cDNA synthesis kit (Promega Corporation, Madison, WI, USA). Next, cDNAs were amplified with quantitative real-time polymerase chain reaction (qRT-PCR) with Light Cycler (Roche) and SYBR Green PCR Master Mix

kit (Roche Diagnostics, Indianapolis, IN, USA). We used the following primer sequences (Sigma-Proligo, St Louis, MO, USA): CIP2A forward: 5´-CTGGTGAGATAATCAGCAATTT-3´, and CIP2A reverse: 5´-CGAAACATTCATCAGACTTTTCA-3´. We used Jumonji, a protein that is also known as KDM3A (or formerly as JMJD1A) and is involved in chromatin regulation as a control for general transcription activity: Jumonji forward: 5´-CACCCTGTTGGCAATTCTTT -3´, and Jumonji reverse: 5´-GCCAACATTGGAGACCACTT -3´. TATA-binding protein (TBP) or β-actin expression levels served for normalization of transcript levels: TBP forward: 5´-GAATATAATCCCAAGCGGTTTG -3´, TBP reverse: 5´-ACTTCACATCACAGCTCCCC -3´, actin forward: 5´-CGAGCACAGAGCCTCGCCTTTGC-3´, actin reverse: 5´-CATAGGAATCCTTCTGACCCATG-3´.

7.9. Cell viability assay

Cells were plated (2 x 10^3 cells per well) on 96-well plates and subjected to siRNA transfection with Lipofectamine 2000 reagent (Invitrogen) as described (section 7.5). The resazurin-based CellTiter-Blue Assay (Promega) served for measuring the relative amount of viable cells after one to eight days at 544/590 nm in a FLUOstar OPTIMA Microplate Reader (BMG LABTECH, Inc., Durham, NC, USA).

7.10. Cell proliferation assay

Cells were plated (2 x 10^4 or 1 x 10^5 cells per well) on 6-well plates and subjected to siRNA transfection with Lipofectamine 2000 reagent (Invitrogen) as described (section 7.5). Six days post-transfection, cells were trypsinized and counted in a Bürker chamber or with the Beckman Coulter Counter Analyzer (Beckman Coulter, Fullerton, CA, USA).

7.11. Soft agar assay

Cells (1 x 10^4 per plate) were suspended 48 h after siRNA transfection in 1 ml of 0.25% agarose (GellyPhor, EuroClone Spa, Pero, Italy) supplemented with 2 ml of complete culture medium. Their suspension was added on top of 1 ml of a 0.25% base layer in 6-well plates. Cells were stained with Giemsa (Sigma) after 8 to 12 days in agarose. We took representative pictures with a Leica MZFLIII microscope (Leica,

Hicksville, NY, USA) and counted the colonies with the ImageJ Software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

7.12. Cycloheximide pulse chase (protein stability) experiment

In Study I, scrambled or CIP2A siRNA-treated gastric cancer cells were subjected to cycloheximide treatment (100 µg/ml) for up to 120 minutes 72 h after siRNA transfection. Total proteins were immunoblotted for serine-62-phosphorylated MYC (S62-p-MYC), total endogenous MYC (MYC), CIP2A, and β-actin, and quantitated with the Image Quant TL (Amersham Biosciences) image analysis software. We normalized the relative values for MYC, which had first been normalized to actin, to the levels in non-cycloheximide-treated cells. The relative MYC levels were plotted on a scatter graph with the best-fit exponential curve.

7.13. Preparation of mouse embryo fibroblasts

In Study I, mouse embryo fibroblasts (MEFs) were prepared from E13.5 R26-MYCER^{T2} mice by the standard technique. Embryos were separated from their yolk-sacs, decapitated, and their internal organs were removed; carcasses were minced with a fixed-head cell scraper and trypsinized for 30 minutes at 37°C. MEFs were collected by centrifugation, resuspended in culture media (DMEM) supplemented with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin), and plated on 150-mM dishes. Cells were harvested after confluency, and aliquoted for freezing. Cells were denoted as passage one when thawed and maintained on a 3T3 protocol. We used 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) at 100 nM to activate the MYC-ER protein.

7.14. Cell cycle experiments

In Study I, we synchronized AGS cells (1 x 10^5 per well) by serum starvation with 0.5% fetal calf serum in RPMI-1640 for 48 h. Subsequently, cells were stimulated to re-enter the cell cycle by addition of RPMI-1640 supplemented with 10% fetal calf serum. Cells were arrested in the S-phase by an established thymidine/aphidicolin protocol (Sillje *et al.* 1999) by treatment with 2 mM thymidine (Calbiochem) for 14 h, leaving them to rest in 10% FCS in RPMI-1640 for another 12 h, after which aphidicolin (1.6 µg/ml, Calbiochem) was added for 12 h. Finally, cells were treated either with the MYC inhibitor 10058-F4 (Sigma) for 6 h, or analysed for CIP2A and cell cycle marker expression levels.

7.15. Statistical analysis

Associations between CIP2A positivity and clinicopathologic variables or biomarkers were assessed by the chi-square and Fisher's exact tests, and the correlation between different CIP2A antibodies with Spearman's correlation test (IBM SPSS Statistics, versions 16.0-19.0 for Mac; SPSS, Inc., an IBM Company, Chicago, IL, USA). Overall survival was calculated from date of surgery to death, disease-specific overall survival from date of surgery to death from cancer, and disease-free (in Study II progression-free) survival from date of surgery to first recurrence. Patients who died of causes other than the disease were censored at the date of their death. Survival curves were constructed according to the Kaplan-Meier method and compared with the logrank test (IBM SPSS Statistics and StatView for Mac, version 5.0.1; SAS Institute, Inc., Cary, NC, USA) (Kaplan and Meier 1958, Mantel 1966). Multivariate survival analysis was performed with the Cox proportional hazard model according to the backward stepwise method. In Study I, statistical significance between different treatment groups in *in vitro* experiments was analysed by an unpaired nonparametric test (Mann-Whitney U) or with Student's t-test (SPSS). All statistical tests were two-sided.

8. RESULTS

8.1. Immunohistochemistry (I-IV)

CIP2A expression was in all studies scored 0 to 3 for cytoplasmic immunoreactivity (Table 9). Representative images of negative and positive CIP2A expression are presented in Figure 4. In gastric cancer (I), we were able to score 223 of 337 (66%) specimens for cytoplasmic CIP2A expression; in ovarian cancer (II), 524 of 562 (93%); in tongue cancer (III), 71 of 73 (97%); and in colorectal cancer (IV) 540 of 643 (84%) specimens in the test dataset, and 212 of 220 (96%) specimens in the validation dataset. In gastric cancer (I) 145 (65%) showed positive immunoreactivity (scores 1-3) for CIP2A and 78 (35%) remained negative. In ovarian cancer (II) 212 (40%) were strongly positive (scores 2-3), 222 (42%) weakly positive (score 1), and 90 (17%) were negative for CIP2A. In tongue cancer (III) we analysed 32 (45%) specimens as strongly positive (score 3) for CIP2A and 39 (55%) as negative, weakly, or moderately positive (scores 0-2).

In the test dataset in colorectal cancer (IV), 484 (90%) specimens were moderately and strongly positive (scores 2-3) for CIP2A and 56 (10%) negative or weakly positive (scores 0-1). In the validation dataset in colorectal cancer, 177 (83%) specimens were positive (scores 2-3) for CIP2A, and 35 (17%) were negative (scores 0-1).

In addition to cytoplasmic expression, nuclear CIP2A immunoreactivity was evaluated in ovarian cancer (II). We found positive nuclear CIP2A expression in 307 (59%) and negative nuclear CIP2A expression in 217 (41%) specimens (Study II: Figure 1D-F).

In order to validate the CIP2A antibody used in Studies I to IV (Soo Hoo *et al.* 2002), we analysed in Study II a subset with an alternative, commercial CIP2A anti-

Table 9. Score distribution of cytoplasmic CIP2A immunoreactivity in Studies I-IV.

Study	Cytoplasmic CIP2A expression, score, *n* (%)				Total
	0	1	2	3	
I. Gastric	78 (35)	107 (48)	36 (16)	2 (0.9)	223
II. Ovarian	90 (17)	22 (42)	167 (32)	45 (9)	524
III. Tongue	2 (3)	9 (13)	28 (39)	32 (45)	71
IV. Colorectal, test	4 (0.7)	52 (10)	283 (52)	201 (37)	540
IV. Colorectal, validation	1 (0.5)	34 (16)	97 (46)	80 (37)	212

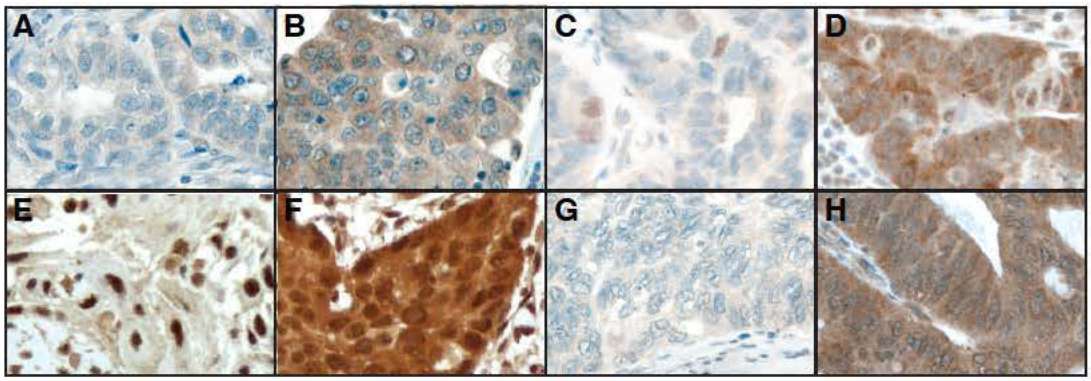


Figure 4. Representative images of cytoplasmic CIP2A immunoreactivity. A) Negative CIP2A and B) positive CIP2A in gastric cancer (I). C) Negative and D) positive CIP2A in ovarian cancer (II). E) Negative and F) positive CIP2A in tongue cancer (III). G) Negative and H) positive CIP2A in colorectal cancer (IV).

body (Novus Biologicals NB100-74663). We found that both CIP2A antibodies recognized cytoplasmic CIP2A immunoreactivity similarly ($r_S = 0.362$, $n = 95$, $p < 0.0001$, Spearman's correlation test). Regarding nuclear CIP2A expression, the original rabbit polyclonal anti-CIP2A antibody (Soo Hoo *et al*. 2002) recognized both cytoplasmic and nuclear CIP2A expression, whereas the commercial rabbit polyclonal (Novus NB100-74663) immunostained the cytoplasm only. In Study IV, we used both test and validation datasets to study CIP2A expression in colorectal cancer. We found that in both datasets the score distributions for CIP2A immunoreactivity were similar.

8.1.1. Characterization of CIP2A expression in normal tissue (unpublished data)

In Study II, we collected normal tissue from mouse and human female reproductive organs and stained it with Novus (NB100-74663) anti-CIP2A antibody. In normal mouse ovarian tissue, the granulosa cells of the graafian follicle expressed cytoplasmic CIP2A strongly (Figure 5A), whereas the ovarian surface epithelium showed only weak cytoplasmic expression (Figure 5B). Strong cytoplasmic positivity, however, was evident in the mouse tubal epithelium (Figure 5C). In human specimens, the follicular granulosa cells showed strong cytoplasmic positivity (Figure 5D), whereas the ovarian surface epithelium expressed CIP2A weakly (Figure 5E). The tubal surface epithelium and the epithelium of inclusion cysts showed strong positivity for cytoplasmic CIP2A (Figure 5F).

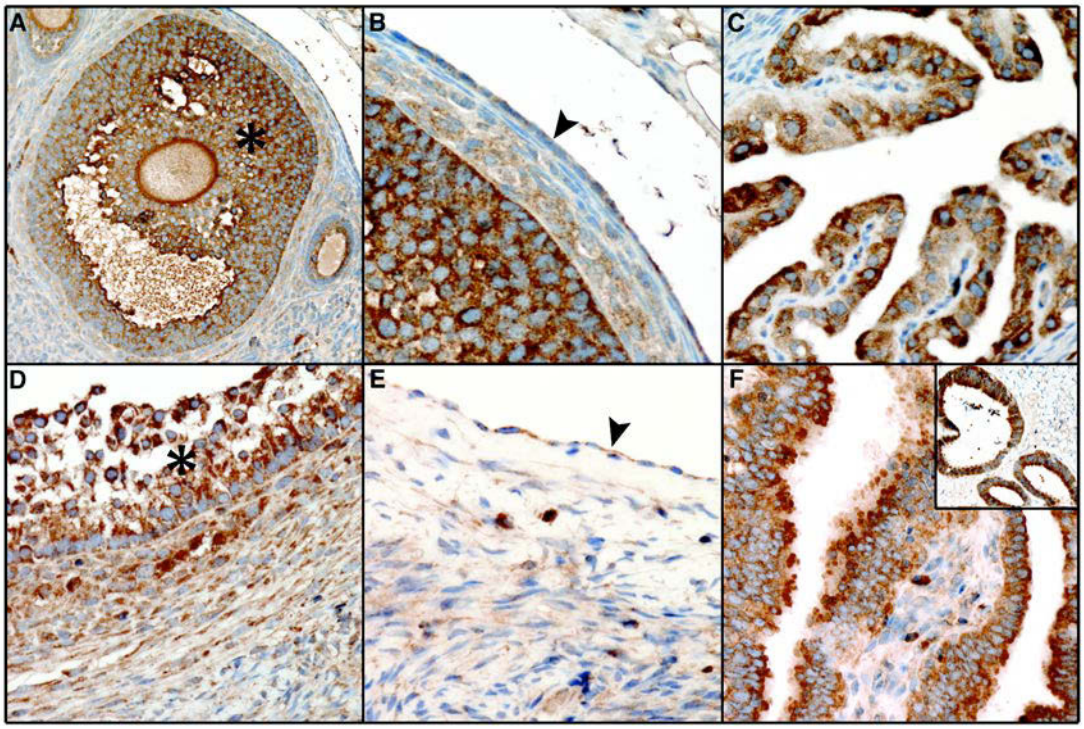


Figure 5. CIP2A expression in normal ovarian and tubal tissues by the commercial Novus anti-CIP2A antibody. CIP2A expression in A) mouse graafian follicle, B) at the ovarian surface epithelium, and C) in tubal tissues. CIP2A expression in D) human follicular granulosa cells, E) in the ovarian surface epithelium, and F) in tubal tissues as well as in the inclusion cysts (insert of F). Granulosa cells are indicated with asterisks and surface epithelial cells with arrowheads. Original magnification was 200x for A, 400x for C-D and F, and 630x for B and E.

8.1.2. Characterization of CIP2A expression in normal and dysplastic lesions (III)

In Study III, we collected specimens from 37 oral mucosal epithelial lesions and stained them with the original anti-CIP2A antibody (Soo Hoo *et al.* 2002). In normal oral mucosa, the basal cells showed negative or weakly positive cytoplasmic CIP2A expression, whereas nuclei showed it as homogenously positive (Study III: Figure 3). Cytoplasmic CIP2A expression tended to be higher in severe epithelial dysplasia than in mild dysplasia. Nuclear CIP2A expression, on the other hand, was low in severe dysplasia and was expressed to a greater extent in lesions with mild dysplasia. In invasive carcinomas, we noted, however, either low or high cytoplasmic CIP2A protein expression.

8.2. Association of CIP2A with clinicopathologic characteristics (I-IV)

The association of cytoplasmic CIP2A immunoreactivity with clinicopathologic characteristics (Tables 10 and 11, p. 50-51) and previously established biomarkers (Tables 12 and 13, p. 52-53) were analysed by the chi-square test. Cytoplasmic CIP2A immunopositivity was associated in gastric cancer (I) with high age (≥67

Table 10. Association of CIP2A expression with clinicopathologic variables in Studies I-III.

Clinicopathologic variable	Gastric cancer (I)			Ovarian cancer (II)			Tongue cancer (III)		
	n	CIP2A positive[1] (%)	*p*-value*	*n*	CIP2A positive[1] (%)	*p*-value*	*n*	CIP2A positive[1] (%)	*p*-value*
Age[2]									
< 60 years	95	56	0.013	260	81	0.314	36	47	0.712
≥ 60 years	128	72		264	84		35	43	
Gender									
Male	121	74	0.001	NA	NA		37	51	0.267
Female	102	54		NA	NA		34	39	
TNM stage									
I	36	72	0.668	81	68	< 0.001	31	33	0.075
II	29	59		62	92		13	32	
III	80	66		294	85		10	69	
IV	78	63		83	84		2	70	
Grade									
I	16	75	0.924	125	59	< 0.001	23	22	0.009
II	33	82		157	87		34	50	
III	53	79		242	93		14	71	
IV	7	86		NA	NA		NA	NA	
Lauréns classification[3]									
Intestinal type	107	84	< 0.001	NA	NA		NA	NA	
Diffuse type	116	47		NA	NA		NA	NA	

* Chi-square test
[1] CIP2A cytoplasmic immunopositivity (I: scores 1-3; II: scores 1-3; III: score 3)
[2] Age: I: <67 vs. ≥67
[3] Lauréns classification applicable only to Study I.

Table 11. Association of CIP2A expression with clinicopathologic variables in Study IV.

Clinicopathologic variabel	Colorectal cancer (IV), test dataset			Colorectal cancer (IV), validation dataset		
	n	CIP2A positive[1] (%)	*p-value**	*n*	CIP2A positive[1] (%)	*p-value**
Age						
< 65 years	231	90	0.785	97	79	0.139
≥ 65 years	309	89		115	87	
Gender						
Male	297	91	0.173	131	81	0.199
Female	243	88		81	88	
Dukes classification						
A	80	84	0.310	30	80	0.232
B	192	91		70	87	
C	135	90		68	87	
D	133	90		42	74	
Grade						
I	18	78	0.014	8	50	0.018
II	354	91		159	85	
III	145	91		37	87	
IV	22	73		4	50	
Location						
Colon	302	89	0.885	80	86	0.288
Rectum	235	90		123	81	

* Chi-square test
[1] CIP2A cytoplasmic immunopositivity (IV: scores 2-3)

Table 12. Association of CIP2A expression with biomarkers in Studies I-III.

Clinicopathological variable			Gastric cancer (I)			Ovarian cancer (II)			Tongue cancer (III)		
			n	CIP2A positive (%)	*p*-value*	*n*	CIP2A positive (%)	*p*-value*	*n*	CIP2A positive (%)	*p*-value*
(I)	(II)	(III)									
p53											
≤20%	Normal		143	64	0.562	168	67	< 0.001	NA	NA	
>20%	Aberrant		80	68		341	91		NA	NA	
Ki-67, %											
<10	<10	0-29	57	42	< 0.001	173	70	< 0.001	13	39	0.008
≥10	10-25	30-49	143	76		120	92		23	22	
	>25	50-79				91	95		14	57	
		≥ 80							16	75	
DNA-index											
Diploid			141	57	< 0.001	179	76	<0.001	NA	NA	
Aneuploid			66	82		173	90		NA	NA	
SPF											
<7.6			73	41	< 0.001	NA	NA		NA	NA	
≥7.6			115	79		NA	NA		NA	NA	
MYC, cytoplasmic											
Negative			129	57	< 0.001	NA	NA		NA	NA	
Weak positive			36	94		NA	NA		NA	NA	
Strong positive			10	80		NA	NA		NA	NA	

* Chi-square test

Table 13. Association of CIP2A expression with biomarkers in Study IV.

Clinicopathologic variabel	Colorectal cancer (IV), test dataset			Colorectal cancer (IV), validation dataset		
	n	CIP2A positive (%)	*p-value**	*n*	CIP2A positive (%)	*p-value**
p53						
≤20%	388	89	0.042	NA	NA	
>20%	70	97		NA	NA	
Ki-67						
<10%	271	89	0.488	NA	NA	
≥10%	162	91		NA	NA	
MYC, nuclear, %						
0-10	NA	NA		63	81	0.018
11-30	NA	NA		30	100	
31-50	NA	NA		37	76	
51-80	NA	NA		47	92	
>80	NA	NA		28	75	

* Chi-square test

years; $p = 0.013$), male gender ($p = 0.001$), and, according to Laurén's classification, intestinal histological type ($p < 0.001$, Table 10). In ovarian cancer (II), it was associated with low TNM stage (stages I-II; $p < 0.001$) and high grade ($p < 0.001$). In tongue cancer (III), CIP2A positivity was also associated with high grade ($p = 0.009$). In colorectal cancer (IV), CIP2A positivity was associated with differentiation grades II and III ($p = 0.014$ in the test dataset and $p = 0.018$ in the validation dataset, Table 11).

In ovarian cancer (II), CIP2A nuclear positivity was more frequent in young ($p = 0.015$) and low-stage patients ($p = 0.023$), as well as in those patients with low-grade disease ($p < 0.001$) or in ones free from ascites ($p = 0.049$, Table 1 in Study IV).

8.3. Association of CIP2A with other biomarkers (I-IV)

CIP2A was, in ovarian ($p < 0.001$) and colorectal cancer ($p = 0.042$), associated with strong p53 immunopositivity (Tables 12 and 13). We found an association with high proliferation index and CIP2A expression in gastric ($p < 0.001$), ovarian ($p < 0.001$), and tongue cancer ($p = 0.008$), but not in colorectal cancer ($p = 0.488$). Aneuploidy was associated with CIP2A positivity in gastric ($p < 0.001$) and ovarian cancer ($p < 0.001$). In gastric cancer, CIP2A was associated with high S-phase fraction ($p < 0.001$). We studied the association between CIP2A and MYC protein expression in gastric cancer and in the validation dataset of colorectal cancer. In gastric cancer, we found an association between cytoplasmic MYC immunopositivity and CIP2A immunopositivity ($p < 0.001$), whereas in colorectal cancer we noted an association between CIP2A positivity and nuclear MYC immunopositivity ($p = 0.018$).

8.4. Univariate survival analyses (I-IV)

For survival analyses, we constructed Kaplan-Meier curves and compared them with the logrank test. In gastric cancer (I), 5-year cumulative overall survival was 15.9% (95% CI 10.0-21.8) for CIP2A-positive patients and 23.1% (95% CI 13.7-32.5) for those CIP2A-negative ($p = 0.064$, Figure 6A). In ovarian cancer (II), we counted the survival disease-specifically. Patients with strong CIP2A positivity had a 5-year survival of 31.7% (95% CI 24.8-38.6), those weakly CIP2A-positive of 42.4% (95% CI 35.5-49.3), and CIP2A-negative patients of 63.0% (95% CI 52.6-73.4, $p < 0.001$, Figure 6B). For tongue cancer (III), the overall 5-year survival for CIP2A-strongly positive patients was 59.4% (95% CI 42.4-76.4) and 74.4% (95% CI 60.7-88.1) for negative, and weakly and moderately positive patients ($p = 0.038$, Figure 6C). In colo

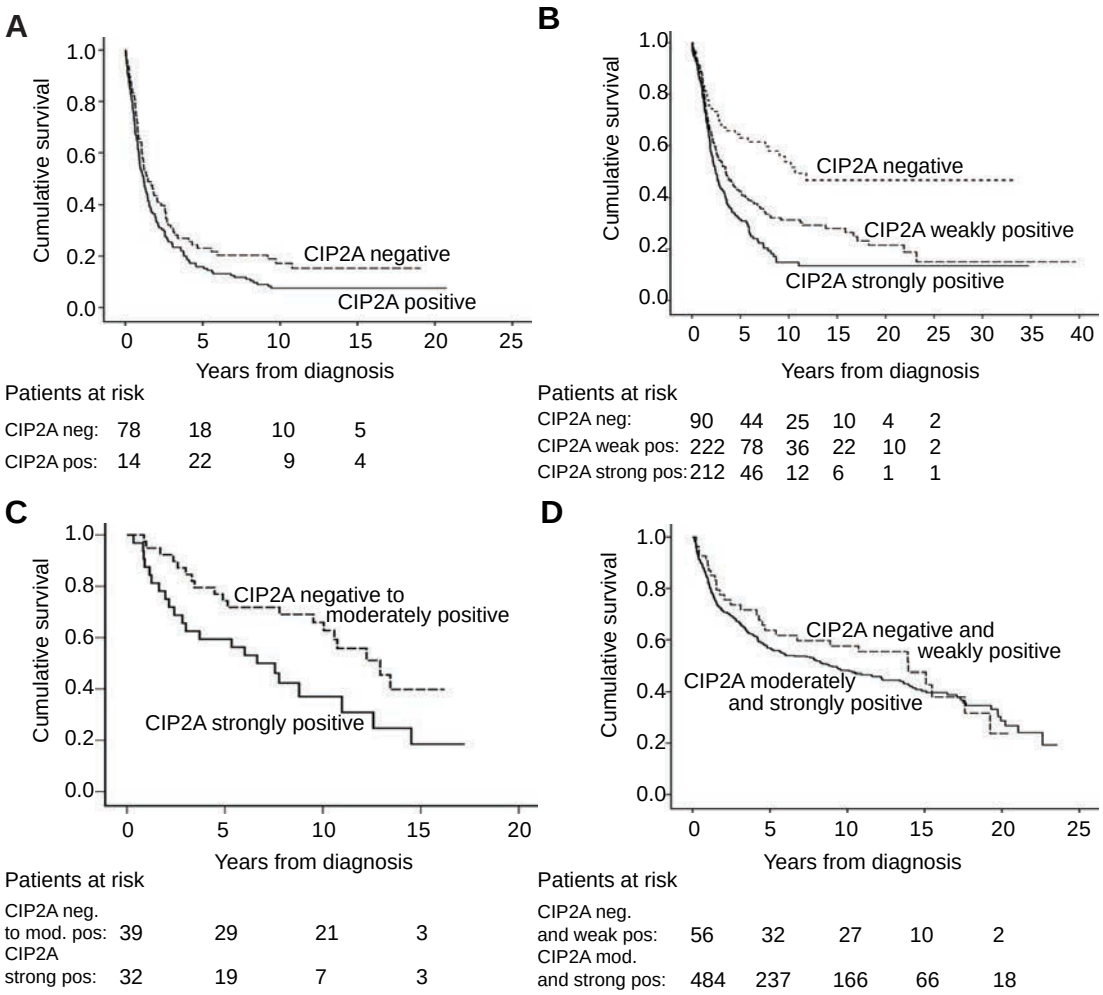
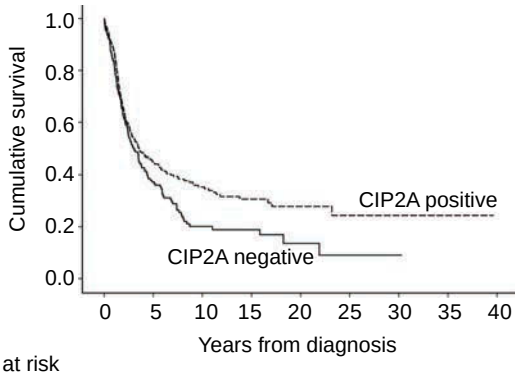


Figure 6. Overall survival according to cytoplasmic CIP2A expression. A) Overall survival in gastric cancer patients (p = 0.063). B) Disease-specific survival in serous ovarian cancer patients (p < 0.001). C) Overall survival in T1N0M0 and T2N0M0 tongue cancer patients (p = 0.027). D) Disease-specific survival in colorectal cancer patients (p = 0.270).

colorectal cancer (IV), the 5-year disease-specific overall survival was 56.8% (95% CI 52.1-61.5) for moderately and strongly positive patients and 63.7% (95% CI 50.6-76.8) for negative and CIP2A-weakly positive patients (p = 0.270, Figure 6D). In sum, cytoplasmic CIP2A immunopositivity predicted poor survival in ovarian and tongue cancer.



Patients at risk

CIP2A negative:	217	61	19	10	3	1
CIP2A positive:	307	107	54	28	13	4

Figure 7. Disease-specific survival in ovarian cancer patients according to nuclear CIP2A (p = 0.013).

In ovarian cancer, patients with negative nuclear CIP2A immunoreactivity had a 5-year disease-specific survival of 37.2% (95% CI 30.3-44.2), whereas it was 45.0% (95% CI 39.2-50.8) for those who showed nuclear positivity (p = 0.013, Figure 7).

8.4.1. Stratified survival analyses for clinical subgroups (I, II, and IV, unpublished data)

In gastric, ovarian, and colorectal cancer we did survival analyses for certain subgroups. In gastric cancer (I), CIP2A predicted poor survival in those patients with small tumours (≤ 5 cm) with a 5-year survival of 18.0% (95% CI 3.9-26.2) for those CIP2A-positive, compared to 47.0% (95% CI 30.9-63.5) for those negative for CIP2A (p = 0.001, Figure 8A). For gastric cancer patients with advanced disease (pT3-4), 5-year survival for those CIP2A-positive was 8.0% (95% CI 3.0-13.2) and 15.0% (95% CI 5.9-23.7) for those CIP2A-negative (p = 0.044, Figure 8B). Among p53-immunopositive gastric cancer patients, the 5-year survival was 7.0% (95% CI 0.4-14.4) for CIP2A-positive and 23.0% (95% CI 6.9-39.3) for CIP2A-negative tumours (p = 0.017, Figure 8C).

In stratified analysis for ovarian cancer patients (II) with optimal debulking surgery (residual tumour < 1 cm), 5-year survival for CIP2A-positive patients was 60.4% (95% CI 52.4-68.4) and 94.4% (95% CI 87.0-1.02) for those CIP2A-negative (p < 0.001, Figure 9A). For patients with a low-stage disease (stage I-II), the figures were 64.0% (95% CI 54.4-73.6) for those CIP2A-positive, and 96.6% (95% CI 89.9-1.03) for those CIP2A-negative (p < 0.001, Figure 9B). Among patients who received platinum-based chemotherapy combined with chemotherapeutics other than taxanes, the 5-year survival for CIP2A-strongly positive patients was 25.2% (95% CI 16.2-34.2), was 48.3% (95% CI 36.9-59.7) for patients with weak CIP2A positivity, and 64.0% (95% CI 45.2-82.8) for those CIP2A-negative (p < 0.001, Figure 9C). Among

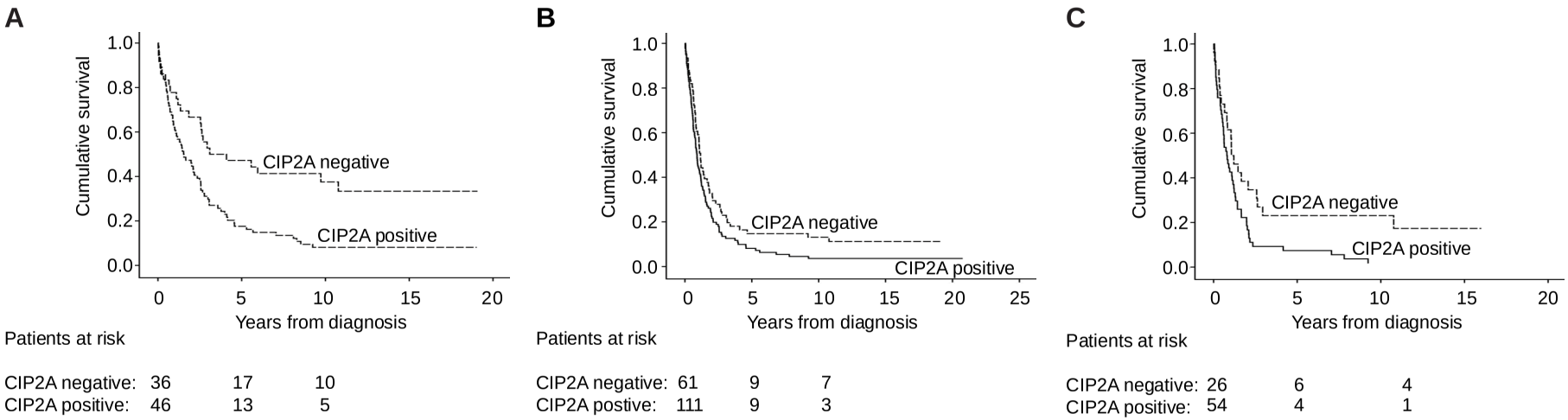


Figure 8. Overall survival in gastric cancer patients stratified by clinical subgroup. A) Overall survival in gastric cancer patients with small tumours (≤ 5 cm; p = 0.001), B) advanced disease (pT3-4; p = 0.044), and C) p53-immunopositive tumours (p = 0.017).

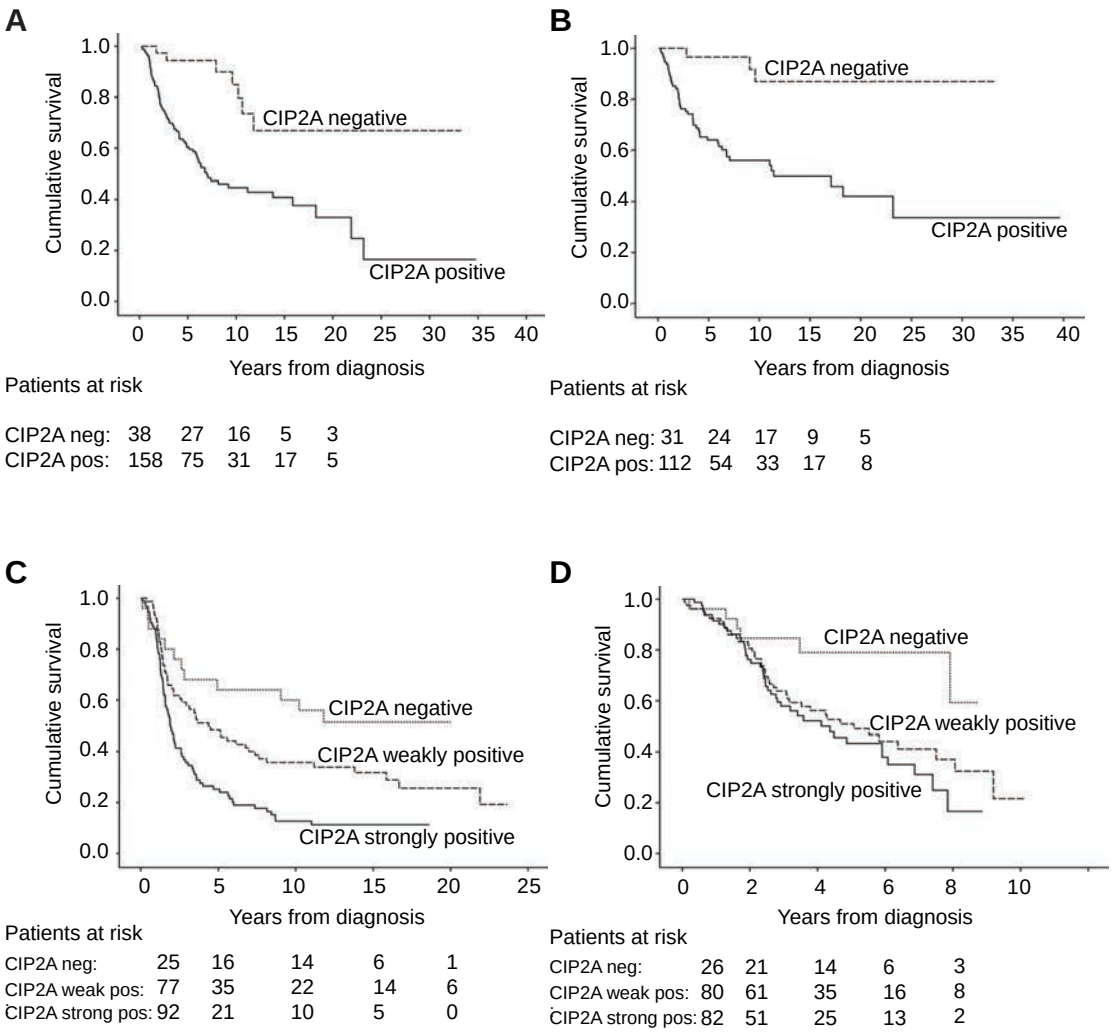


Figure 9. Overall disease-specific survival in serous ovarian cancer patients stratified by clinical subgroup. A) Disease-specific survival for ovarian cancer patients undergoing optimal debulking (residual tumour < 1 cm; $p = 0.001$), and B) with a low-stage disease (pT1-2; $p < 0.001$). C) Disease-specific survival for patients who received platinum-based chemotherapy together with chemotherapeutics other than taxanes ($p = 0.001$), and D) for patients who received platinum-based chemotherapy in combination with taxanes ($p = 0.024$).

patients who received platinum-based chemotherapy combined with taxanes, the 5-year survival for those CIP2A-strongly positive was 43.3% (95% CI 30.6-56.0), was 50.8% (95% CI 38.8-62.8) for those weakly positive, and 79.0% (95% CI, 62.1-95.9) for those immunonegative ($p = 0.024$, Figure 9D).

In the colorectal cancer test series (IV), CIP2A showed no prognostic value in the stratified analyses for colon, rectal, male, and female cancer patients. For colon cancer patients, 5-year survival was 58.4% (95% CI 52.3-64.5) for those CIP2A-moderately or strongly positive, compared to 60.5% (95% CI 43.1-77.9) for those who were negative or weakly positive for CIP2A ($p = 0.874$, Figure 10A). For rectal cancer patients, 5-year survival for those CIP2A-moderately or strongly positive was 55.0% (95% CI 47.9-62.1) and 68.3% (95% CI 48.9-87.7) for those CIP2A-negative

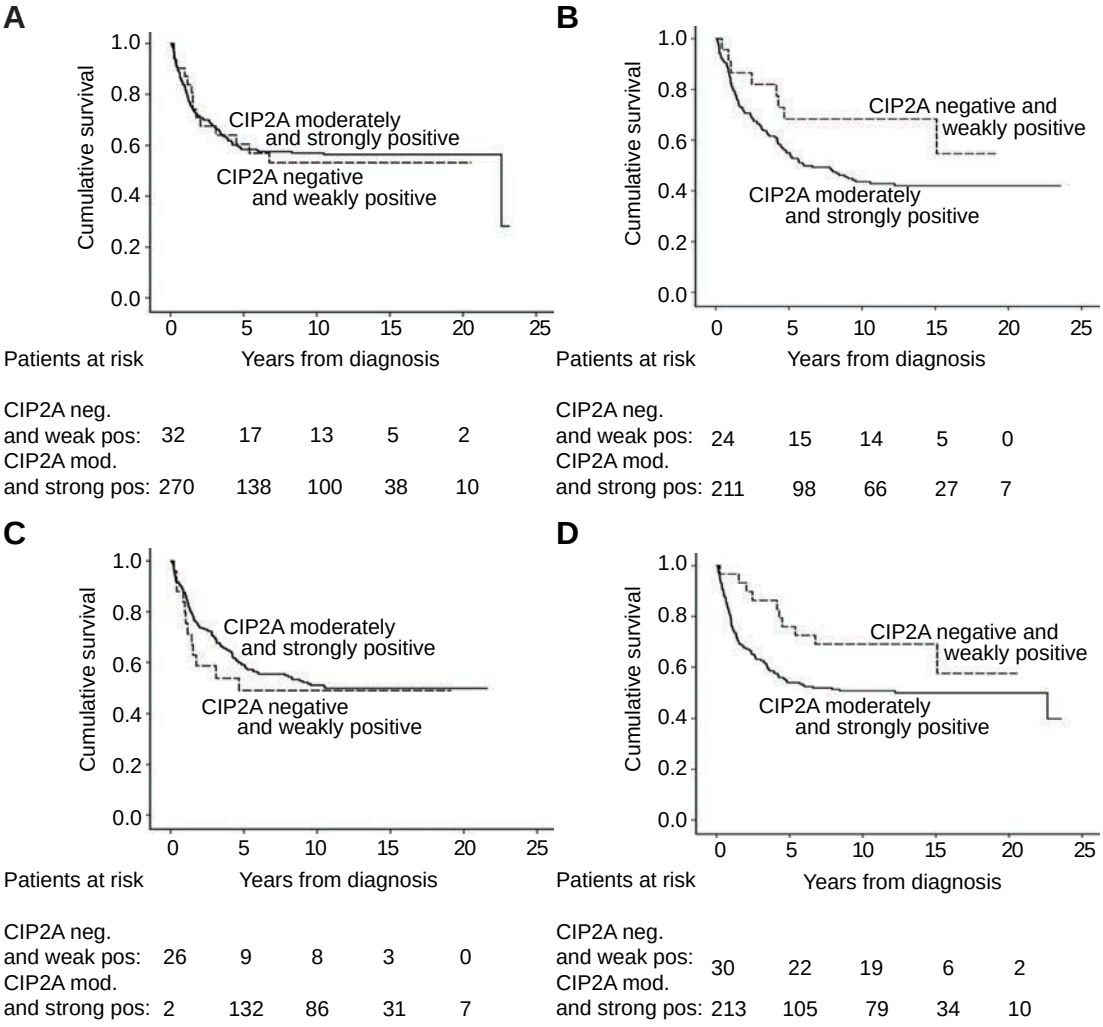


Figure 10. Overall disease-specific survival in colorectal cancer patients (test series) stratified by clinical subgroup. A) Disease-specific survival for colon cancer patients (p = 0.874), and B) rectal cancer patients (p = 0.071). C) Disease-specific survival for male (p = 0.54), and D) female (p = 0.067) colorectal cancer patients.

or weakly positive (p = 0.071, Figure 10B). Among male colorectal cancer patients, the 5-year survival was 59.0% (95% CI 52.7-65.3) for those CIP2A-moderately or strongly positive and 48.9% (95% CI 28.3-69.5) for those with CIP2A-negative or weakly positive tumours (p = 0.540, Figure 10C). For women, the figures were 53.9% (95% CI 47.0-60.8) for those with CIP2A-moderately or strongly positive, and 76.0% (95% CI 60.5-91.5) if CIP2A-negative or weakly positive (p = 0.067, Figure 10D).

8.4.2. Prognostic role of MYC (IV and unpublished data)

In gastric cancer, 5-year cumulative overall survival for patients with cytoplasmic MYC-immunopositive tumours was 36.2% (95% CI 22.7-49.7) and for the MYC-negative, 30.2% (95% CI 23.1-37.3, p = 0.55, Figure 11A). The 5-year survival for those nuclear MYC-positive was 41.9% (95% CI 20.7-63.1) compared to 30.3% (95% CI 23.6-37.0) for patients with tumours negative for nuclear MYC (p = 0.22, Figure

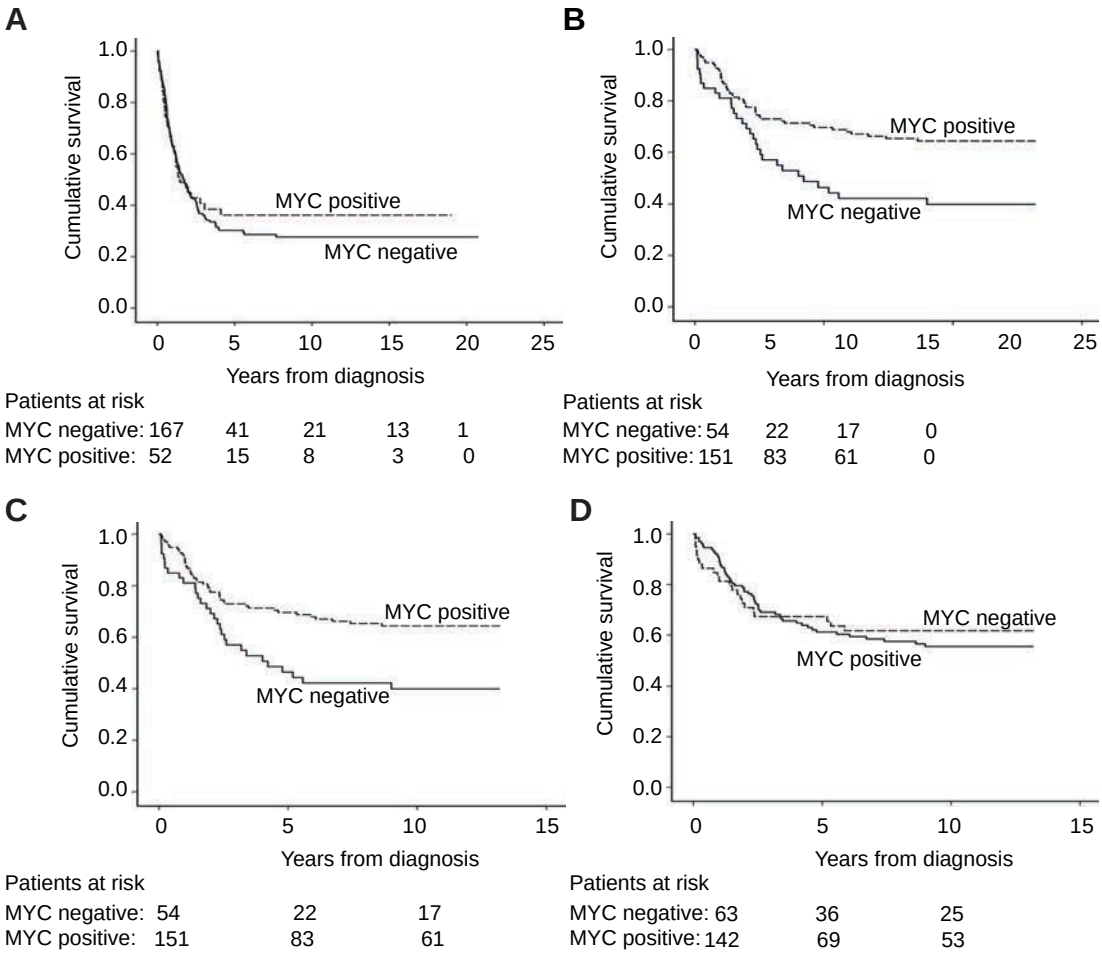


Figure 11. Overall survival according to MYC immunoreactivity. A) Overall survival in gastric cancer patients according to cytoplasmic MYC immunoreactivity (p = 0.55) and B) nuclear MYC immunoreactivity (p = 0.22). C) Disease-specific survival in colorectal cancer patients according to cytoplasmic MYC immunoreactivity (p = 0.003) and D) nuclear MYC immunoreactivity (p = 0.74).

11B). In the validation dataset of colorectal cancer, the 5-year disease-specific survival for patients with tumours negative for cytoplasmic MYC immunoreactivity was 44.4% (95% CI 30.5-58.3), compared to 69.6% (95% CI 61.8-77.4) for those with cytoplasmic positivity of MYC (p = 0.003, Figure 11C). Nuclear MYC immunoreactivity showed no prognostic significance. The 5-year cumulative disease-specific survival for patients with nuclear MYC immunopositive tumours was 61.2% (95% CI 52.6-69.8) and with MYC negative, 73.0% (95% CI 60.7-85.3, p = 0.74, Figure 11D).

8.5. Multivariate survival analyses (I-IV)

In gastric cancer (I), age, gender, stage, grade, Laurén's classification, CIP2A expression, p53 expression, proliferation index, DNA ploidy, and MYC expression were entered into the multivariate survival analysis. TNM stage and ploidy were independent prognostic factors. In ovarian cancer (II), age, TNM stage, grade,

residual tumour size, and p53 expression have previously been found to show independent prognostic value (Lassus *et al.* 2003). When CIP2A expression was included, it added no prognostic information. In tongue cancer (III), age, gender, tumour size (pT-classification), grade, tumour invasion depth, proliferation index, and CIP2A expression were included. Age, tumour size, and CIP2A expression remained as independent prognostic factors. Multivariate survival analysis was performed in colorectal cancer (IV) for the test dataset with the following variables: age, gender, Dukes classification, grade, histological type, tumour location (colon or rectum), p53 immunoreactivity, proliferation index, and CIP2A expression. Age and Dukes classification remained as independent prognostic factors (data not shown).

8.6. CIP2A mRNA and protein expression *in vitro* (I and unpublished data)

CIP2A protein was expressed at high levels in gastric cancer cell lines (AGS) of intestinal (MKN-28) and diffuse type cancers (MKN-45, TMK-1, and KATOIII), as well as in ovarian cancer cells (OV-4, OVCAR-3, CaOV3, ES-2, of which EFO-27 is

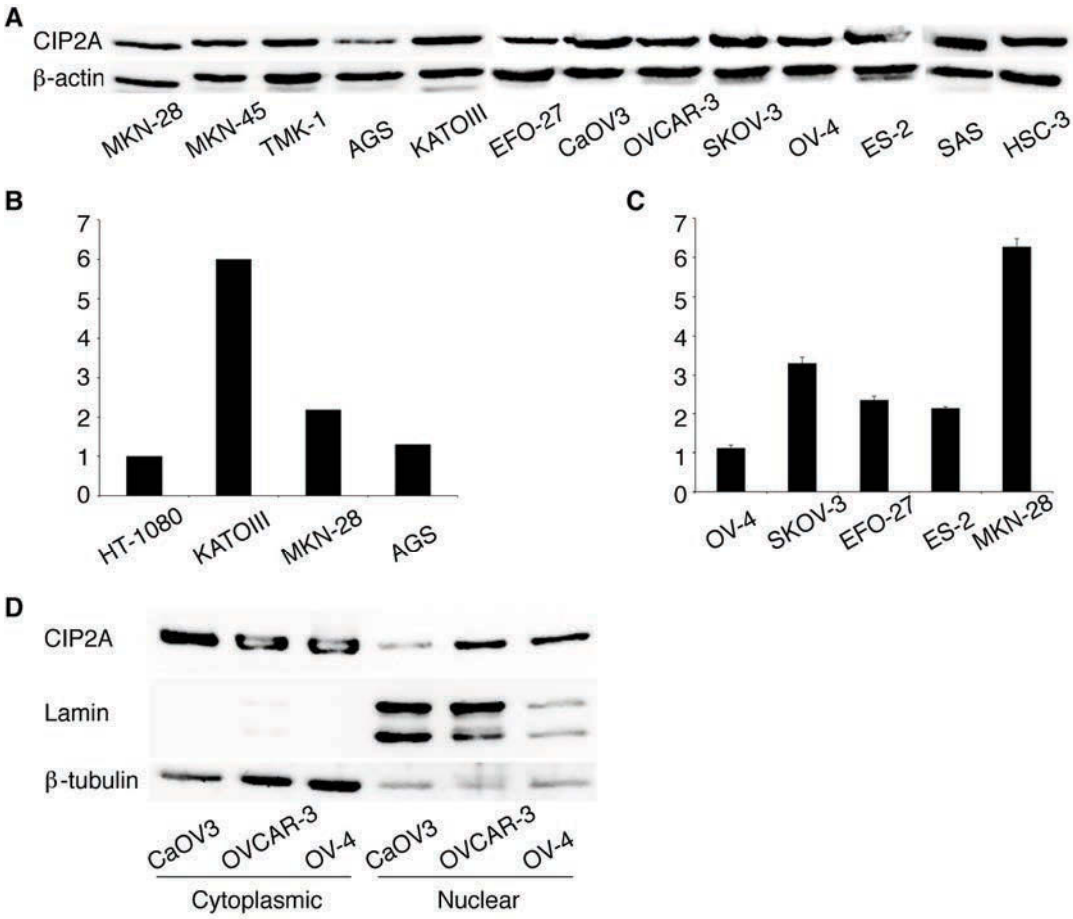


Figure 12. CIP2A mRNA and protein expression. A) CIP2A protein expression in gastric, ovarian, and tongue cancer cell lines. B) Relative CIP2A mRNA expression in gastric and C) ovarian cancer cell lines. D) CIP2A expression in cytoplasmic and nuclear protein fractions in ovarian adenocarcinoma cells. Lamin served as a nuclear-positive control and β-tubulin as cytoplasmic positive control.

of mucinous type, and SKOV-3 of serous type) and squamous cell carcinoma from the tongue (HSC-3 and SAS; Figure 12A). At mRNA level, CIP2A was expressed in gastric and ovarian cancer cell lines to a significantly higher extent than is HT-1080, a fibrosarcoma cell line with previously demonstrated high CIP2A mRNA expression levels (Figure 12B and 12C; Junttila *et al.* 2007). In ovarian cancer cells, CIP2A protein was evident in both cytoplasmic and nuclear protein fractions (Figure 12D).

8.7. Effect of CIP2A on proliferation (I)

In gastric cancer cells, we studied the effect of CIP2A depletion on cell proliferation and found a decreased proliferation of CIP2A siRNA-treated MKN-28 cells in an eight-day experiment (p = 0.004 at day 8, Mann-Whitney U-test, Figure 13A). The effect of CIP2A depletion on proliferation was evident also in KATOIII (p < 0.001) and AGS cells (p = 0.015, Mann-Whitney U-test, Figure 13B). We further studied the

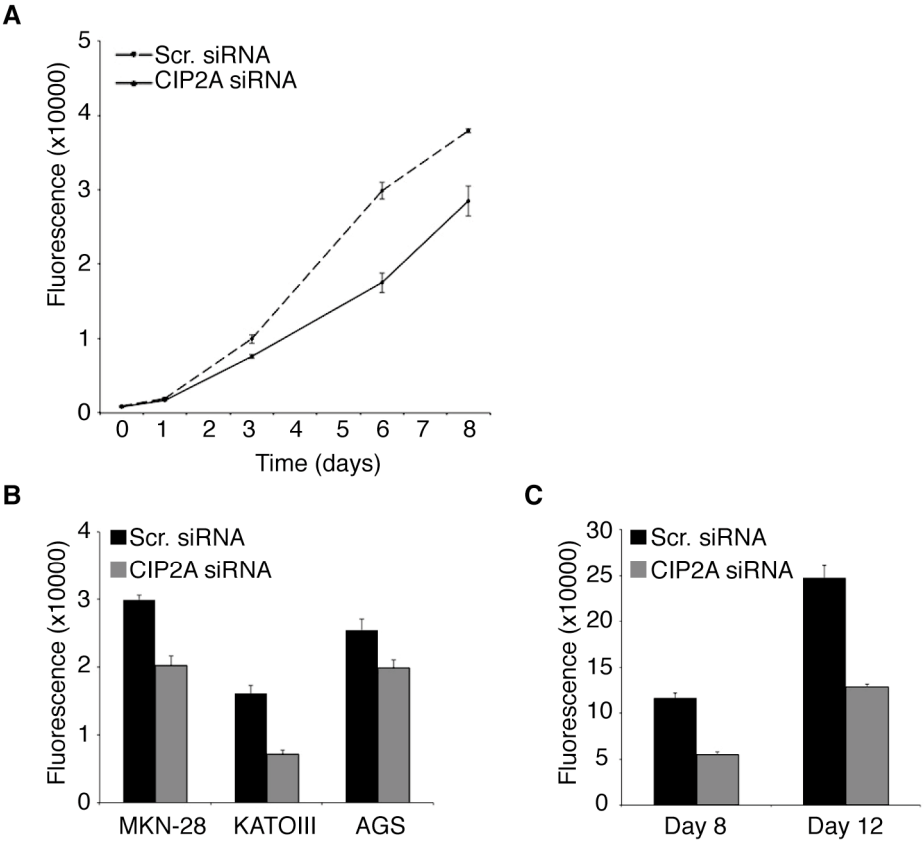


Figure 13. Effect of CIP2A on proliferation. A) Effect of CIP2A siRNA on proliferation of MKN-28 cells. Cells were transfected with CIP2A siRNA or scrambled siRNA. Proliferation was detected by a fluorometric cell viability assay. The mean from six samples with SEM is plotted. B) Effect of CIP2A siRNA on proliferation of MKN-28, KATOIII, and AGS cells. Proliferation at day 6 post-transfection measured by a fluorometric cell viability assay. The mean from 10-12 samples with SEM is shown. C) Effect of CIP2A siRNA on anchorage-independent growth in MKN-28. CIP2A or scrambled siRNA-treated cells were stained with Giemsa after 8 or 12 days of growth and colonies were counted. The mean of eight samples with SEM is shown.

role of CIP2A in malignantly transformed cells on semisolid agar and demonstrated that CIP2A depletion by siRNA inhibited anchorage-independent growth in MKN-28 cells (p = 0.017 at day 8, Mann-Whitney U-test, Figure 13C).

8.8. Effect of CIP2A on MYC stability (I)

In gastric cancer AGS cells, CIP2A depletion by siRNA significantly inhibited the steady-state MYC protein expression (Figure 14A). The active S62-phosphorylated steady-state MYC protein was also inhibited as a result of CIP2A depletion (Figure 14B). We studied the half-life of endogenous MYC protein by treating the cells with cycloheximide, a protein synthesis inhibitor, for 0 to 120 minutes 72 hours after CIP2A siRNA transfection. The half-life of MYC in non-transformed cells is typically 20 to 30 minutes (Junttila and Westermarck 2007) and was measured at the time at which 50% of the MYC protein was remaining. In scrambled siRNA-treated AGS cells, the half-life of MYC was over 100 minutes reflecting the increased MYC protein stability in these transformed gastric cancer cells, whereas in CIP2A-depleted cells it was less than 50 minutes, indicating that CIP2A is necessary for increased MYC protein stability in AGS cells (Figure 14C).

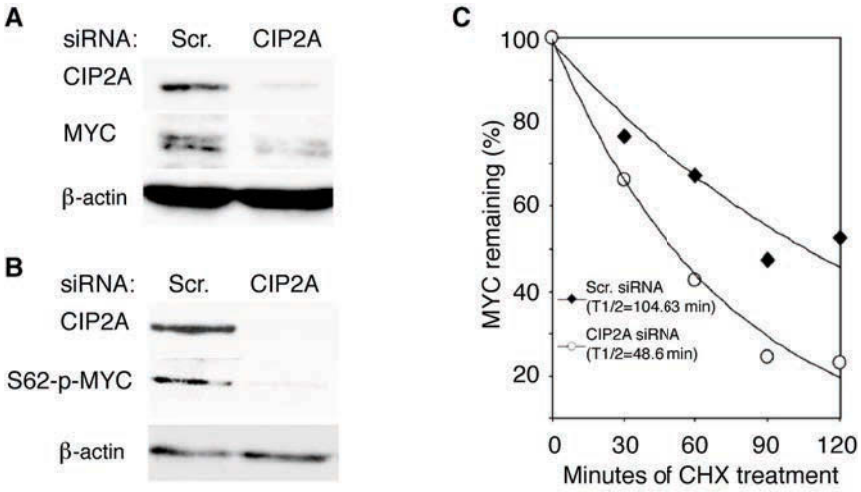


Figure 14. Effect of CIP2A on MYC protein stability. A-B) Effect of CIP2A siRNA on CIP2A, MYC, and S62-phospho-MYC protein levels in AGS gastric cancer cells. CIP2A or scrambled siRNA-treated cells were harvested 72 hours posttransfection. C) Effect of CIP2A on endogenous MYC protein stability. 72 hours after CIP2A or scrambled siRNA-transfection cycloheximide was added for 0-120 prior to protein lysis. The fraction of MYC protein present compared to, after normalization to actin levels, the level in untreated cells. Best-fit exponential curves were plotted.

8.9. Identification of a positive feedback mechanism between CIP2A and MYC (I)

We noted that in AGS gastric cancer cells, CIP2A protein expression was inhibited as a result of MYC siRNA-transfection (Figure 15A). The similar effect of MYC depletion on CIP2A protein expression was noted in MKN-28 gastric cancer and HT-1080 fibrosarcoma cells (Study I: Figure 4B and Supplementary Figure 3C). At mRNA level, the effect was similar with a significantly lower CIP2A mRNA expression after MYC depletion ($p < 0.001$, Mann-Whitney U-test, Figure 15B). This led us to study further the mechanism by which CIP2A and MYC regulate each other's expression, revealing increased CIP2A mRNA expression upon activation of MYC in a conditionally active MYCER construct in mouse embryo fibroblasts (Figure 15C).

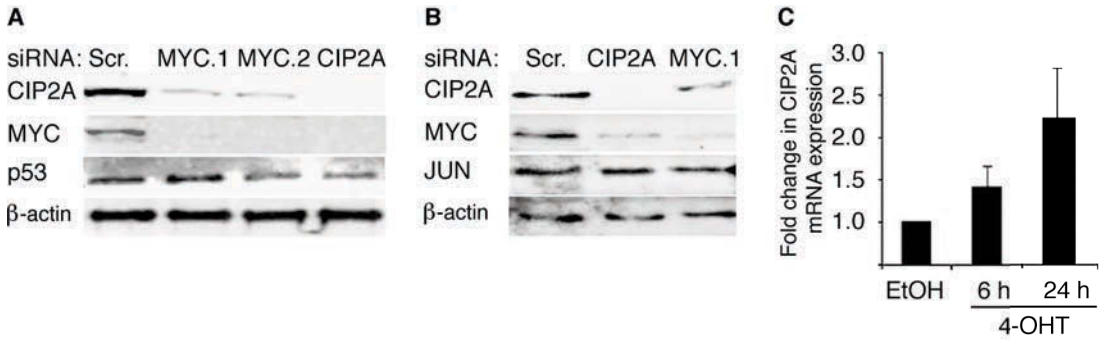


Figure 15. Identification of a positive feedback mechanism between MYC and CIP2A. A) Effect of MYC siRNA on CIP2A protein expression in AGS gastric cancer cells. Proteins were harvested 72 hours posttransfection with MYC.1, MYC.2, CIP2A, or scrambled siRNA. B) Effect of MYC siRNA on CIP2A mRNA expression in AGS cells. Relative mRNA expression 24 hours posttransfection with CIP2A, MYC, or scrambled siRNA. C) Effect of activating expression from a conditionally active MYC construct, MYCER, on CIP2A mRNA expression. Mouse embryo fibroblasts expressing MYCER were activated with 4-OHT treatment. Relative CIP2A mRNA levels are shown as means with 95% CI from three independent experiments.

8.10. Inhibition of signalling pathways (unpublished data)

In gastric cancer, we studied the effect of signalling-pathway inhibition on CIP2A expression in MKN-28 and KATOIII cells. Inhibition of the Notch signalling pathway by gamma-secretase inhibitor (GSI) did not affect CIP2A expression in MKN-28 and KATOIII cells (Figure 16). In KATOIII cells, inhibition of the epidermal growth factor receptor (EGFR) downregulated CIP2A protein at a modest level, however, an effect that could not be confirmed in MKN-28 cells. Inhibition of the c-Jun N-terminal kinase (JNK) –signalling pathway resulted in a downregulation of CIP2A protein expression already occurred at 5 μM of SP600125 in MKN-28 and KATOIII cells. In MKN-28 cells, we noted modestly decreased CIP2A protein expression after inhibition of the extracellular signal-regulated kinase (ERK) –signalling pathway. In KATOIII cells, however, this effect was not evident.

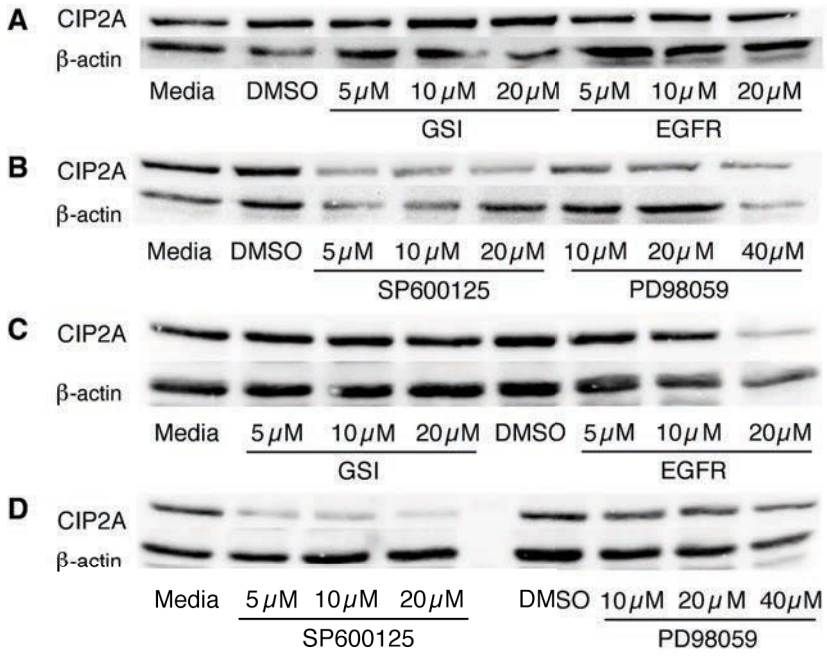


Figure 16. Effect of signalling pathway inhibition on CIP2A protein expression in MKN-28 and KATOIII gastric cancer cells. A) Effect of Notch (GSI), EGFR (AG-1478), B) JNK (SP600125), and ERK (PD98059) signalling pathway inhibitors on CIP2A protein expression in MKN-28 cells, and in C-D) KATOIII cells. Proteins were harvested after 24 hours.

In ovarian cancer, we noted a downregulation of CIP2A protein expression after inhibition of EGFR in EFO-27 and CaOV3 ovarian cancer cells (Figure 17A). By inhibiting EGFR in EFO-27 cells, CIP2A was already downregulated after 24 hours, and this effect was sustained for at least 72 hours, as shown in Figure 17B. Inhibition of EGFR in the tongue cancer squamous cell carcinoma cells HSC-3 and SAS confirmed the downregulation of CIP2A protein expression noted in ovarian cancer (Figure 17C-D). Further, we demonstrated that an alternative inhibition of EGFR by siRNA also downregulated CIP2A protein expression in these tongue cancer cells (Figure 17E).

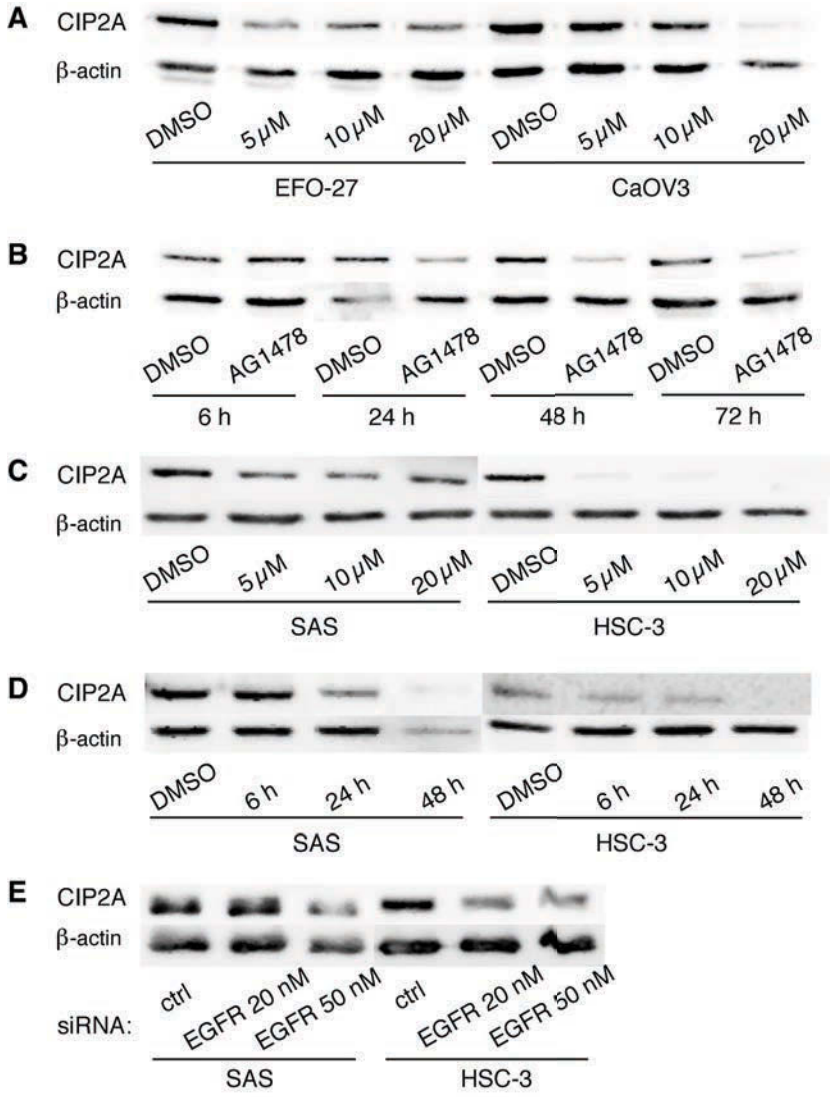


Figure 17. Effect of EGFR inhibition on CIP2A protein expression in ovarian and tongue cancer cells. A) Effect of EGFR inhibitor (AG-1478) on CIP2A protein expression in EFO-27 and CaOV3 ovarian cancer cell lines. Proteins were harvested after 48 hours. B) Time-dependent effect of the EGFR inhibitor on CIP2A protein expression in EFO-27 cells. EFO-27 cells were treated with 10 μM AG-1478 for 6 to 72 hours. C) Effect of AG-1478 on CIP2A protein expression in HSC-3 and SAS tongue cancer cell lines. Proteins were harvested after 24 hours. D) Time-dependent effect of the EGFR inhibitor on CIP2A protein expression in HSC-3 and SAS cells. Tongue cancer cells were treated with 10 μM AG-1478 for 6 to 24 hours. E) Effect of EGFR siRNA on CIP2A protein expression in tongue cancer cells. EGFR or control siRNA-treated cells were harvested 72 hours post-transfection.

9. DISCUSSION

9.1. Expression of cytoplasmic CIP2A in cancer

We found CIP2A immunopositivity to be detectable at high frequency levels in all cancers studied. In gastric cancer patients (*n* = 223), CIP2A was immunopositive (scores 1-3) in 65% and in ovarian cancer (*n* = 524) in 83%. In tongue cancer (*n* = 71) CIP2A was moderately or strongly positive (scores 2 and 3) in 85% and in colorectal cancer (*n* = 752) in 88%. This high frequency of CIP2A protein expression in cancer specimens is in line with previous findings on gastric, lung, oral, oesophageal, and prostate cancers, where the occurrence of high CIP2A mRNA or protein expression has been over 80% (Li *et al.* 2008, Dong *et al.* 2010, Katz *et al.* 2010, Qu *et al.* 2010, Vaarala *et al.* 2010). Cytoplasmic CIP2A expression was in Studies I to IV scored from 0 to 3 according to staining intensity. In further analyses, the cut-off for grouping the CIP2A expression was determined separately for each cancer type. In gastric cancer (I), we analysed CIP2A protein expression as negative (score 0) versus positive (scores 1 to 3), and in serous ovarian cancer (II) as negative (score 0), weakly positive (score 1), and strongly positive (scores 2 and 3). In tongue cancer (III), very few specimens were completely negative, with most of the specimens expressing CIP2A strongly, and thus we grouped CIP2A expression into low expression representing scores 0 to 2 and high expression as score 3. In colorectal cancer (IV), we divided CIP2A expression according to score distribution into approximately two equal groups with negative and weakly positive CIP2A (scores 0 and 1) versus moderately and strongly positive CIP2A (scores 2 and 3).

Grouping CIP2A expression individually for each cancer type was necessary, as CIP2A was expressed at such differing levels. Vaarala *et al.* (2010) analysed CIP2A immunopositivity (*n* = 59) in prostate cancer according to cytoplasmic intensity (scores 0-3) and grouped CIP2A as negative (scores 0-1) versus positive (scores 2-3) in further analyses, whereas Katz *et al.* (2010) found cytoplasmic CIP2A to be strongly positive (score 3) in all oral cancer specimens studied (*n* = 8). In short, CIP2A is generally expressed to a relatively high extent in cancers, but the optimal cut-off level must be determined as to specific cancer types. In future studies, the most convenient way of analysing CIP2A would be to analyse data as immunonegative versus immunopositive; this may, however, depending on CIP2A expression level, be unsuitable for obtaining biologically relevant results regarding clinicopathologic associations and prognosis.

9.2. Expression of cytoplasmic CIP2A in normal tissues

Relatively few studies have addressed CIP2A expression in normal tissues, and results are conflicting. In specimens from normal mouse and human female reproductive organs (II), we found that CIP2A was expressed strongly in the follicular granulosa cells and in the tubal surface epithelium, which is regarded as the normal equivalent of serous epithelium. The debate regarding tissue of origin of ovarian cancer is ongoing. It has been suggested that ovarian epithelial tumors arise from tissues that have their origin in the Müllerian ducts (Dubeau 2008). Interestingly, CIP2A was strongly expressed in inclusion cysts, which are regarded as one of the main origins of ovarian carcinomas.

In normal cervical tissue, in cervical intraepithelial neoplasia (CIN) I, and in CIN II lesions, CIP2A expression was negative, whereas in CIN III specimens CIP2A was positive in 12.5%, and in cervical cancer specimens in 52.8% (Liu *et al.* 2011a). In oesophageal cancer, of 40 specimens, CIP2A mRNA was overexpressed in the cytoplasm of 36 (90%) when compared to the non-cancerous adjacent tissue, where CIP2A was expressed in only 8 (20%) (Qu *et al.* 2010). Ren *et al.* (2011) demonstrated in renal cell cancer that CIP2A mRNA was expressed at higher levels in cancer tissues than in matched adjacent tissues and normal tissues. CIP2A showed immunopositivity in 70% of renal cell cancer specimens, in 32% matched adjacent tissues, and in 17% nonmalignant renal tissues obtained from renal hamartomas. In gastric mucosal specimens adjacent to malignant lesions (I), we found that non-malignant tissue is generally negative for CIP2A, with the exception of proliferating cells at the bottom of the gastric crypts (data not shown). Adjacent tissues from cancer patients are sometimes considered representative of normal tissue when other non-malignant tissues are difficult to obtain. These non-cancerous adjacent tissues, however, may express genetic mutations similar to those in malignant tissues, and hence may not reflect the expression level in normal tissues. Although comparing expression in malignant tissues with that in adjacent tissues is not optimal, adjacent tissues may prove valuable when healthy human tissues cannot be resected for pure research purposes. In normal, dysplastic, and carcinoma specimens from the oral cavity (III), we noted an increase in the cytoplasmic CIP2A expression with increasing invasive morphology, whereas nuclear CIP2A expression tended to decrease with increasing invasive morphology. Junttila *et al.* (2007) found in most normal tissues very low levels of CIP2A mRNA. Taken together, these studies suggest that CIP2A is expressed at undetectable or low levels in normal tissues; however, certain female cell types from reproductive organs showed strong CIP2A expression, suggesting that CIP2A may play a role in female reproduction.

9.3. Cytoplasmic CIP2A and clinicopathologic associations

In gastric cancer (I), cytoplasmic CIP2A immunopositivity was associated with an intestinal type of cancer according to Laurén's classification, which is usually associated with better prognosis than for diffuse tumours. In ovarian cancer (II), CIP2A positivity was associated with low TNM stage and high grade, and in tongue cancer (III), with high grade. In colorectal cancer (IV), CIP2A positivity was associated with differentiation grades II and III. In oesophageal cancer, no association between CIP2A immunopositivity and differentiation status has been demonstratable (Qu *et al.* 2010). In breast cancer, on the other hand, CIP2A is associated with high Scarff-Bloom-Richardson grade and lymph node positivity (Come *et al.* 2009), and in prostate cancer with high Gleason score (Gleason scores 7-10) (Vaarala *et al.* 2010).

In our study, high proliferation index (Ki-67) was associated with CIP2A expression in gastric, ovarian, and in tongue cancer, but not in colorectal cancer. Dong *et al.* (2010) have demonstrated in non-small-cell lung cancer an association between CIP2A and high proliferation index, but in cervical cancer, no such association, nor any association with tumour size, differentiation status, node metastasis, nor clinical stage emerged (Liu *et al.* 2011a). Another study on lung cancer showed that CIP2A is associated with smoking and squamous cell histological type, but not with stage (Ma *et al.* 2011). In ovarian (II) and colorectal cancer (IV), CIP2A was associated with p53 immunopositivity. In breast cancer, CIP2A has been associated with proliferation markers and p53 mutations (Come *et al.* 2009). They further demonstrated, that in a breast cancer mouse model, CIP2A is strongly expressed in epithelial cancer cells with mammary gland-specific depletion of p53 and either BRCA1 or BRCA2. In gastric cancer, we noted an association between CIP2A expression and high S-phase fraction. Based on these results, CIP2A is in several cancers associated with aggressive disease characteristics such as high grade, p53 immunopositivity, high proliferation index, and high S-phase fraction. In sum, cytoplasmic CIP2A immunopositivity may indicate an aggressive type of cancer that needs particular surveillance for recurrence or late metastasis.

9.4. Clinical association between CIP2A and MYC

Junttila *et al.* (2007) have shown that CIP2A inhibits protein phosphatase 2A and therefore keeps MYC phosphorylated and active. We found that CIP2A was associated with cytoplasmic MYC immunopositivity in gastric cancer (I) and with nuclear MYC immunopositivity in colorectal cancer (IV). In non-small-cell lung cancer, Dong *et al.* (2010) demonstrated an association between CIP2A mRNA expression and MYC mRNA expression. They also found a correlation between

CIP2A and MYC immunointensity. The stabilizing effect of CIP2A on the oncoprotein MYC, which is known to promote proliferation, may partly be the explanation of the aggressive behaviour of CIP2A-expressing cells. Furthermore, both oncoproteins are expressed during embryonal development in undifferentiated stem cells (Kerosuo *et al.* 2010). Cancer cells, as well as stem cells, express several genes important for cell proliferation, which may promote tumorigenesis. Kerosuo *et al.* (2010) demonstrate that CIP2A is involved in the maintenance of the self-renewing and proliferative identity of neural progrenitor cells, partly triggered by increased MYC signalling. Hence, one may conclude that the aggressive behaviour shown by cells expressing CIP2A is because of cancer stem cell-like characteristics.

9.5. Role of CIP2A as a prognostic marker

We found that cytoplasmic CIP2A immunopositivity was a marker of poor survival in serous ovarian (II) and early-stage tongue cancer patients (III). In tongue cancer, CIP2A was an independent prognostic marker, together with age and tumour size. This is in line with recent results on non-small-cell lung cancer, where CIP2A was demonstrated to serve as an independent prognostic marker (Dong *et al.* 2010). Interestingly, in ovarian cancer patients who underwent optimal debulking surgery or had low-stage disease (II), patients with CIP2A-positive tumours had a poor outcome, indicating that CIP2A may serve as a prognostic marker in these subgroups with otherwise favourable disease characteristics. In gastric cancer (I), we demonstrated that CIP2A serves as a prognostic marker in the subgroups of patients with small tumours, in advanced disease (pT3-4), and in p53-immunopositive tumours. Neither in colorectal cancer (IV) CIP2A showed no prognostic significance, nor did Come *et al.* (2009), regarding breast cancer, find any prognostic role for CIP2A. However, in female colorectal cancer patients and in the subgroup of patients with rectal tumours CIP2A-positivity was of borderline significancy for poor survival. Interestingly, Wangefjord *et al.* (2011) have proposed that in colorectal cancer, certain biomarkers may relate to sex hormone-status and found cyclin D1 to serve as a prognostic marker in men but not women.

In conclusion, cytoplasmic CIP2A positivity revealed patients with poor outcome in certain, but not in all, cancers. Hence, to find, on the one hand, the optimal cut-off for studying the prognostic value of CIP2A and, on the other hand, to address the role of CIP2A as a prognostic factor in cancers, the clinical role of CIP2A should be evaluated separately for different cancers in large clinical populations.

9.6. Nuclear CIP2A expression

The predominant cytoplasmic location of CIP2A that we noted has been demonstrated in several other studies (Soo Hoo *et al.* 2002, Junttila *et al.* 2007, Liu *et al.* 2011a). The clinical significance of nuclear CIP2A expression has been poorly addressed. In our studies, CIP2A protein was evident both in the cytoplasmic and in the nuclear compartments of cells. In ovarian cancer (II), the original anti-CIP2A antibody (Soo Hoo *et al.* 2002) recognized nuclear CIP2A in 59% of ovarian cancer specimens and cytoplasmic CIP2A in 83%, however, when we used the commercial CIP2A antibody (NB100-74663, Novus Biologicals), we found no nuclear CIP2A immunoreactivity. In contrast, Liu *et al.* (2011a) detected, by use of the same commercial antibody, CIP2A protein in normal cervix, in cervical intraepithelial neoplasia, and in cervical cancer specimens, in both cytoplasmic and nuclear compartments. Recently, several commercial CIP2A antibodies have become available. In ovarian cancer (II), positive nuclear CIP2A has been associated with low TNM stage and low grade, which contrasts with the positive cytoplasmic CIP2A that was associated with advanced TNM stage and high grade. We further demonstrated that negative nuclear CIP2A expression predicted poor outcome, suggesting that nuclear CIP2A expression may express different tumour behaviour than does cytoplasmic CIP2A. In ovarian cancer cells, we demonstrated CIP2A protein expression in both the cytoplasmic and nuclear compartments in all cell lines studied. The absence of CIP2A from the nuclear compartment may promote activation and transcription of pro-oncogenic genes such as *MYC* and hence, negative nuclear CIP2A may be associated with characteristics of an aggressive disease. In sum, nuclear CIP2A expression may play a role in tumour biology, a role that remains to be determined.

9.7. Positive feedback mechanism between CIP2A and MYC

CIP2A protein was expressed abundantly in all of our gastric, ovarian, and tongue cancer cell lines. CIP2A mRNA expression was also high in gastric and ovarian cancer cells. In Study I, we showed for the first time that depletion of CIP2A inhibits cell proliferation and anchorage-independent growth. This is in contrast with later findings by Ren *et al.* (2011) in renal cancer, where CIP2A depletion had no effect on proliferation. Inhibition of CIP2A reduced migration in a scratch migration assay and invasiveness in a Matrigel invasion assay (Ren *et al.* 2011). We further demonstrated that depletion of CIP2A downregulates MYC and, interestingly, that depletion of MYC downregulates CIP2A. When we further explored this mechanism between CIP2A and MYC, we noted that activation of MYC in a MYCER construct in mouse embryo fibroblasts resulted in increased CIP2A mRNA expression, and this led us to

propose a positive feedback regulation mechanism between MYC and CIP2A (Figure 18). The positive regulation of CIP2A by MYC that we demonstrated, and vice versa, may lead to a positive feedback loop ultimately enhancing MYC-mediated cellular responses and MYC's oncogenic activity both in duration and strength. In CIP2A- and MYC-driven cancers, the CIP2A- and MYC-positive feedback loop may be a useful therapeutic target.

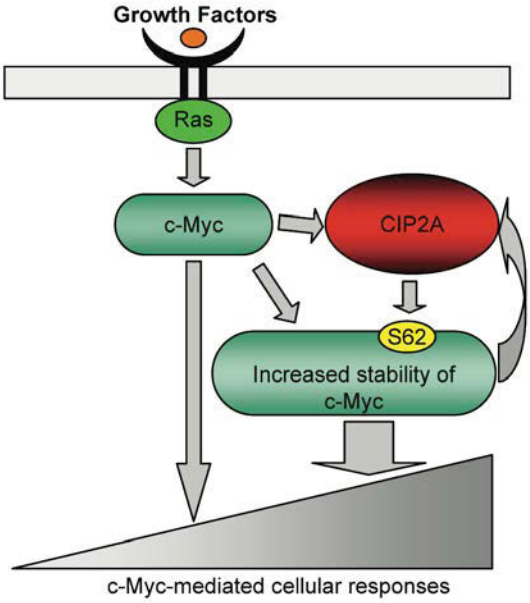


Figure 18. Positive feedback mechanism between CIP2A and MYC. MYC regulates, in addition to other target genes, CIP2A positively. When serine 62 (S62) dephosphorylation of MYC is prevented by CIP2A upregulation, MYC protein stability is enchanced. This leads in turn to an accumulation of the stable S62-phosphorylated MYC and hence, a further increase in CIP2A expression. The positive feedback between CIP2A and MYC may result in increased strength and duration of MYC -mediated cellular responses and oncogenic activity of MYC.

Intriguingly, Eckhardt *et al.* (1994) have shown that depletion of MYC in HL60 cells induces cell differentiation. After the finding of Wang *et al.* (2011) in acute myeloid leukaemia that CIP2A depletion induces differentiation into promyelocytes, a question arises. Does the CIP2A depletion itself or does the decreased MYC expression resulting from the CIP2A depletion and the positive feedback mechanism between these two proteins serve as the trigger for the induced differentiation of HL60 cells? This mechanism, in which CIP2A depletion induces differentiation, should be a potential target for exploration of acute myeloid leukaemia therapy, with efforts to transfer the findings to solid cancers. Furthermore, in chronic myeloid leukaemia, CIP2A serves as a predictive marker for progression into blast crisis; moreover, high CIP2A-levels prevent imatinib from JAK2-SET-mediated activation of PP2A, whereby MYC remains active, increasing the probability of subsequent genetic damage (Lucas *et al.* 2011). In hepatocellular carcinoma, CIP2A mediates AKT inactivation and apoptosis by bortezomib (Chen *et al.* 2010), an effect that enhances radiosensitivity and reduces tumour growth *in vivo* (Huang *et al.* 2011). That fact, together with the recent discovery of rabdocoetsin B, a natural compound functioning as the first small-molecular inhibitor of CIP2A that by inhibition of CIP2A and pAKT reduces apoptosis and promotes proliferation of lung cancer cells (Ma *et al.* 2011), the role of CIP2A-targeting therapies either alone or together with established chemoradiotherapeutic agents deserves more intense investigation.

9.8. Regulation of CIP2A and potential clinical applications

In order to investigate the oncogenic role of CIP2A and explore signal-transduction mechanisms possibly regulating CIP2A, we targeted four common signalling pathways. In our gastric cancer cells, inhibition of JNK in the MAPK signalling pathway resulted in a downregulation of CIP2A protein expression. Inhibition of Notch and ERK signalling pathways, however, gave inconsistent results regarding their effect on CIP2A protein expression. In gastric cancer cells, inhibition of EGFR resulted, however, in a modest depletion of CIP2A expression in KATOIII cells only, an effect that may be explained by a toxic concentration of the small-molecular inhibitor used. In EFO-27 and CaOV3 ovarian cancer cells, CIP2A protein at 24 hours after inhibition of EGFR, was already downregulated. The clearest effect was evident in tongue cancer cells, where CIP2A protein expression was reduced following EGFR inhibition by AG-1478; this was confirmed by an alternative inhibition by EGFR-targeting siRNA. Inhibition of the EGFR signalling pathway in tongue cancer cells, seemed to downregulate CIP2A expression at protein level. These results are in line with the findings of Zhao *et al.* (2010), who recently demonstrated that CagA-mediated overexpression of CIP2A is inhibited as a result of Src and MEK/ERK signalling-pathway inhibition. The phospho-CagA activates the MEK/ERK pathway and leads to an upregulation of CIP2A expression. In Study II, we demonstrated in ovarian cancer an association between CIP2A expression and EGFR protein overexpression and gene amplification. A partial co-localization of EGFR and CIP2A occurrs in oral squamous cell cancer tissues (Katz *et al.* 2010). Together with the finding that the ETS1 transcription factor mediates EGFR-MEK1/2-ERK-induced positive regulation of CIP2A (Khanna *et al.* 2011), our present and others' results suggest that EGFR signalling may be an important step in CIP2A regulation, and thus EGFR-targeted therapy may function partly via the CIP2A oncoprotein.

Recent findings show that CIP2A inhibits death-associated protein kinase (DAPk)-induced apoptosis and hence enhances cell survival (Guenebeaud *et al.* 2010). In the presence of CIP2A, PP2A cannot exert its effect on DAPk, which remains autophosphorylated and inactive, whereby no apoptosis is induced. In head and neck cancers, hypermethylation at the DAP-kinase gene promoter correlates with advanced disease and lymph-node metastases (Sanchez-Cespedes *et al.* 2000), whereas in gastric cancer (Chan *et al.* 2005) and hepatocellular carcinoma (Matsumoto *et al.* 2003) loss of DAPk expression is associated with advanced stage and poor prognosis. The apparent oncogenic role of CIP2A and tumour suppressor role of DAPk, together with the interaction of these proteins at the UNC5H2/DAPk complex, suggest a potential clinically relevant association between CIP2A and DAPk; future studies should be planned to explore the role of this protein complex as a therapeutic target.

9.9. Strengths and limitations of this work

Intra-tumoural heterogeneity may give rise to differences in staining intensity. This is a potential source of misinterpretation in the use of tissue array samples. However, results from tumour tissue microarrays obtained by accurate multiple sampling from histologically representative areas are in concordance with other biochemical and whole-section analysis (Kononen *et al.* 1998, Kallioniemi *et al.* 2001, Torhorst *et al.* 2001). Tumour tissue microarrays are especially useful for research involving evaluation of large numbers of specimens. The advantage with our immunohistochemistry protocol using an immunohistochemical autostainer is that we were able to stain the relatively large clinical materials on a few slides in one set. Use of whole-section slides for the corresponding clinical materials would have required several staining series, and hence could have produced significant inter-series variation. The scoring method based on cytoplasmic intensity was established based on the initial, preliminary stainings in Study I, after which pre-defined scoring categories served for further data analyses. In the final analyses, the highest score for any of the TMA cores per specimen was chosen as representative for that patient. One may argue that the mean score for a histological section would serve as a better indicator of the biological role of a tumour marker than would the highest intensity score. However, it may well be that the highest score reflects tumour aggressivness more accurately. The cut-off for CIP2A expression was decided separately for all studies based on the distribution of CIP2A expression, as mentioned.

Previous studies on ovarian cancer have suggested a distinct molecular pathogenesis and clinical manifestation for different histological types (Kobel *et al.* 2008), and hence we decided to limit Study II to the serous histological type. In ovarian cancer, we validated our data by staining a subgroup of our clinical specimens with another commercial CIP2A antibody (NB100-74663, Novus Biologicals), and found a positive correlation between the CIP2A expression recognized by the original (Soo Hoo *et al.* 2002) and the commercial antibodies. In colorectal cancer, an earlier dataset served as the test set, and a later one for validation. By the use of the original CIP2A antibody, results for associations between CIP2A and clinicopathologic characteristics, as well as survival analyses, were in both datasets similar. These validations served to indicate that our results were independent of the antibody used and were sustained despite the datasets' being from different time periods.

Our relatively large ovarian cancer population with a long follow-up time unfortunately implies the heterogeneous treatment modalities of past decades. Median year of diagnosis was 1994, indicating that a significant proportion of the patients received the currently used platinum-based therapy in combination with taxanes.

Among such patients, CIP2A was a marker of poor outcome, demonstrating that the prognostic role of CIP2A is independent of the chemotherapeutic regimen.

9.10. Concluding remarks

What is of the utmost necessity, are new biomarkers to monitor therapeutic responses or detect early disease progression. Tumour auto-antigens such as p62 and p90/CIP2A may serve these purposes (Liu *et al.* 2011b). New biomarkers must be properly validated in large, prospective clinical trials for their prognostic and predictive value before conclusions can be drawn regarding benefits in individualized cancer treatment (Gangadhar *et al.* 2010). Based on our findings in a retrospective setting, CIP2A may serve as a prognostic marker in certain subgroups of gastric cancer patients, in serous ovarian cancer patients, and in early tongue cancer patients, but not in colorectal cancer patients. CIP2A is in some, but not in all solid cancers, associated with markers known to indicate an aggressive disease. Its clinical significance thus needs evaluation separately for each cancer.

In future, the role of CIP2A as a predictive marker for recurrence and poor survival should be evaluated in order to select the patients for adjuvant treatment who need it most. CIP2A is found in haematological malignancies in mononuclear cells (Lucas *et al.* 2011), and efforts should be made to evaluate the role of CIP2A in solid cancers as a serum tumour marker. CIP2A is a potential therapeutic target, and antagonists of UNC5H/CIP2A or activators of the functional PP2A complex involved in mediated DAPk dephosphorylation may prove useful (Guenebeaud *et al.* 2010). Together with established chemoradiotherapeutic treatments such as bortezomib functioning via pAkt (Chan *et al.* 2010, Huang *et al.* 2011), inhibiton of CIP2A by the newly recognized rabdocoetsin B (Ma *et al.* 2011), may improve prognosis for CIP2A-positive cancer patients.

10. CONCLUSIONS

Based on the four studies and previously unpublished data also presented here, CIP2A could recognize those with poor outcome among ovarian and tongue cancer patients. The association with high grade, p53, and MYC was evident in some cancers; thus, the clinical implication of CIP2A positivity demands separate evaluation for each cancer.

1. CIP2A protein was expressed at high levels in gastric, serous ovarian, tongue, and colorectal cancer specimens. CIP2A served as a prognostic marker in certain subgroups of gastric cancer patients, in serous ovarian cancer, in early tongue cancer, but not in colorectal cancer, however, serving as an independent prognostic marker only for tongue cancer. The cut-off level for prognostic significance was cancer-type specific.
2. Cytoplasmic CIP2A immunopositivity was associated in ovarian and tongue cancer with high grade. In addition, it was associated with characteristics of an aggressive disease, namely high proliferation index and p53 positivity. CIP2A was also associated with MYC in gastric and colorectal cancer.
3. A positive feedback regulation mechanism between CIP2A and its target protein MYC in gastric cancer was recognized. Furthermore, depletion of EGFR by means of small interfering RNA and by the small-molecular inhibitor AG-1478 downregulated CIP2A protein expression.

11. ACKNOWLEDGMENTS

This work took place in the Research Program Units of Molecular and Cancer Biology, and Genome-Scale Biology at Biomedicum Helsinki, at the Department of Pathology, Haartman Institute, University of Helsinki, and at the Department of Surgery, Helsinki University Central Hospital during 2006-2011. I thank Professors Olli Jänne, Tomi Mäkelä, Lauri Aaltonen, Seppo Meri, and Veli-Pekka Lehto for excellent research facilities.

My supervisors Professor Ari Ristimäki and Docent Caj Haglund deserve my warmest appreciation for all their guidance and support. Ari accepted me into his lab even during my early undergraduate studies and taught me so many things. I am thankful for all his practical advice regarding research and academic writing all these years; without the inspiration that started during that work, this work would never have been accomplished. Caj always encouraged me by showing his belief in my postgraduate work and helped me to complete this thesis. I am grateful for how he relied on my independent work but still always had time for detailed discussions, both regarding research, the entire medical profession, and even leisure-time activities.

The members of my thesis committee Professor Leif Andersson and Docent Kirsi Sainio I warmly acknowledge for being an active part of the process and offering valuable suggestions for improvements. The reviewers of the thesis, Kirsi Sainio and Professor Timo Paavonen, deserve a thousand thanks for thorough and prompt work. Their constructive and encouraging comments and discussions were very helpful in finalizing the thesis.

The Helsinki Biomedical Graduate School receives my sincere acknowledgment for all the opportunities provided.

Dr. Carol Norris is warmly acknowledged for fast and thorough author-editing of the language. Through her suggestions, the language rose to a new level that indeed improved readability. Graphic designer Ea Söderberg is warmly thanked for implementing the cover layout according to my wishes.

Professor Jukka Westermarck deserve my warmest appreciation for contagious enthusiasm seldom seen and for sharing his detailed knowledge in the area of CIP2A research. I am deeply grateful for the smooth collaboration with him and with Dr. Anchit Khanna, and their laboratories on research combining preclinical and clinical findings, resulting in true translational results.

Docent Jaana Hagström is warmly acknowledged for the valuable precise advice that only an experienced researcher can give. I am grateful for all our research and far-from-research related discussions, especially those on life experience. Her inspiring approach encouraged me to follow my own way and to rely on my own intuition.

Docents Ralf Bützow, Arto Leminen, and Timo Atula, and Drs. Laura Mäkinen and Selja Koskensalo all deserve warm appreciation for providing clinical materials and close collaboration on clinical data analyses. Docent Johan Lundin and Drs. Heini Lassus, Mikael Lundin, and Jan-Patrik Wiksten I warmly thank for all of their help with statistics. All my collaborators deserve my greatest thanks for their help: Melissa Junttila, Siina Junnila, Daniel Murphy, Gerard Evan, Harri Keski-Säntti, and Valtteri Häyry.

Elina Aspiala generously provided practical advice and cheerful encouragement when most needed. Päivi Peltokangas and Tuire Koski I thank with all my heart for always prioritizing my projects and doing their best to help me finish projects on time.

Mira Heinonen and Alexandra Thiel deserve a thousand thanks for excellent interactive discussions and for sharing prosperities and adversities. I appreciate especially all their help and valuable expertise and patience in the lab, not to mention our small picnics in the summertime and long lunches that made the workdays more bearable. Annabrita Hemmes and Helena Ahola kindly helped me out in the lab when I was unable to take care of the lab work myself.

My deepest thanks go to my family for love and support: to my parents Marianne and Thomas, for always being there, sharing all joys and sorrows, and for believing in me; and to my brother Sebastian and his beloved Sallamaari for being so near to my heart and my home, just next door, always listening to me and sharing everything.

My fellow-students Diana Grénman, Inari Lepistö, Linda Stoor, and Martin Granlund I warmly thank for always showing me understanding when I have been constantly on the move, sighing and moaning about all the work to be done. Although kindly questioning my ambitions, they have still encouraged me to do what I believe in. Together we have shared the best parts of study life; without you, this process would have been much more difficult (and definitely more boring).

My dear friends I thank with all my heart for being there for me and for giving me all the support that only good friends can give. Johanna Eklund and Josefin Söderman deserve my deepest thanks for so many fruitful discussions.

I have been financially supported by the Helsinki Biomedical Graduate School, Finska Läkaresällskapet, the K. Albin Johansson Foundation, the Kurt and Doris Palander Foundation, the Waldemar von Frenckell Foundation, and the Weikko Wilhelm Peltonen Foundation; all are sincerely acknowledged.

Helsinki, January 2012

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