EXPRESSION OF THE APOPTOSIS REPRESSOR WITH CASPASE RECRUITMENT DOMAIN (ARC) IN LIVER METASTASIS OF COLORECTAL CANCER AND ITS CORRELATION WITH CLINICAL DATA AND OTHER PROGNOSTIC AND PREDICTIVE PROTEINS

Ph.D. thesis

Csaba Tóth, M.D.

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„Az igazi tudós kész arra, hogy elviselje a megpróbáltatásokat, akár még az éhezést is, csak hogy senki ne szabja meg neki, hogy milyen irányba folytassa a munkáját.“

Szent-Györgyi Albert

„The real scientist is ready to bear privation and, if need be, starvation rather than let anyone dictate to him which direction his work must take.“

Szent-Györgyi Albert

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LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. **Tóth C**, Meinrath J, Herpel E, Derix J, Fries J, Buettner R, Schirmacher P, Heikaus S: **Expression of the apoptosis repressor with caspase recruitment domain (ARC) in liver metastasis of colorectal cancer and its correlation with DNA mismatch repair proteins and p53.** J Cancer Res Clin Oncol. 2016 May;142(5):927-35. doi: 10.1007/s00432-015-2102-3. **[IF: 3.1]**

II. **Tóth C**, Sukosd, F, Valicsek, E, Herpel, E, Schirmacher, P, Renner, M, Mader, C, Tiszlavicz, L and Kriegsmann, J: **Expression of ERCC1, RRM1, TUBB3 in correlation with apoptosis repressor ARC, DNA mismatch repair proteins and p53 in liver metastasis of colorectal cancer.** Int J Mol Med, 2017. **40**(5): p. 1457-1465. **[IF: 2.3]**

III. **Tóth, C.**, Sükösd, F., Valicsek, E., Herpel, E., Schirmacher, P., Tiszlavicz, L.: **Loss of CDX2 gene expression is associated with DNA repair proteins and is a crucial member of the Wnt signaling pathway in liver metastasis of colorectal cancer.** Oncology Letters 15, no. 3 (2018): 3586-3593. https://doi.org/10.3892/ol.2018.7756. **[IF: 1.3]**

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I. **Toth C**, Funke S, Nitsche V, Liverts A, Zlachevska V, Gasis M, Wiek C, Hanenberg H, Mahotka C, Schirmacher P, Heikaus S: **The role of apoptosis repressor with a CARD domain (ARC) in the therapeutic resistance of renal cell carcinoma (RCC): the crucial role of ARC in the inhibition of extrinsic and intrinsic apoptotic signalling.** Cell Commun Signal, 2017. 15(1): p. 16. **[IF: 3.6]**

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V. Michael Hoffmeister, Lina Jansen, Anja Rudolph, **Csaba Toth**, Matthias Kloor, Wilfried Roth, Hendrik Bläker, Jenny Chang-Claude, Hermann Brenner: **Statin Use and Survival After Colorectal Cancer: The Importance of Comprehensive Confounder Adjustment.** JNCI: Journal of the National Cancer Institute, Volume 107, Issue 6, 1 June 2015, djv045, https://doi.org/10.1093/jnci/djv045 **[IF: 11.3]**

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XIV. **Toth, C.: Tracheopathia osteoplastica – Ein 100-jähriges Mysterium** [Tracheopathia osteoplastica. A 100-year-old mystery]. Pathologe. 2012 Mar;33(2):129-34. [Article in German] **[IF: 0.6]**

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
APC	adenomatous polyposis coli
ARC	apoptosis repressor with caspase recruitment domain
ATM	atelectasia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
Bad	Bcl-2-associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-X_L	B-cell lymphoma-extra large
Bid	BH3 interacting-domain death agonist
CDA	cytidine deaminase
cdc2	cyclin-dependent protein kinase Cdk1/Cdc2
Cdc25	cyclin-dependent kinase 25
CDX2	caudal type homeobox 2
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CRC	colorectal cancer
dFdC	2',2'-difluorodeoxycytidine (gemcitabine)
dFdCTP	gemcitabine triphosphate
DISC	death-inducing signaling complex
ERCC1	excision repair cross-complementing 1
FADD	fas-associated death domain
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
HE stain	haematoxylin and eosin stain
hENT	human equilibrative nucleotide transporter
HIF1α	hypoxia-inducible factor 1-alpha
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HuR	hypoxic upregulation antigene
MAP	microtubule associated protein
mdm2	Mouse double minute 2 homolog
MGMT	methylguanine-DNA methyltransferase
MLH1	MutL homolog 1
MMR	mismatch repair
MSH2	MutS protein homolog 2
MSH6	MutS protein homolog 6
MT	Microtubule
NER	nucleotide excision repair
NSAID	non-steroidal anti-inflammatory drugs
NSCLC	Non-Small Cell Lung Cancer
PanIN	pancreatic intraepithelial neoplasm
PDAC	pancreatic ductal adenocarcinoma
PTEN	phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
RAS	rat sarcoma protein
RIP1	receptor interacting protein 1
RNR	ribonucleotide reductase
RR	ribonucleotide reductase
RRM1	ribonucleoside-diphosphate reductase 1
TBA	tubulin binding agent
TGFβ	transforming growth factor β
TMA	tissue microarray
TNFR1	tumor necrosis factor receptor 1
TNFα	tumor necrosis factor
TUBB3	β(III)-tubulin
UICC	Union internationale contre le cancer
VEGFR	vascular endothelial growth factor receptor

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SUMMARY

Liver metastasis in colorectal cancer is still common and the primary treatment is chemotherapy. Until now there is no routinely used test in the clinical practice to predict effectiveness of conventional chemotherapy. Therefore, biomarkers with predictive value also for conventional chemotherapy would be of considerable benefit in the treatment planning.

Apoptotic signaling is one of the most important processes in the measurement of chemotherapeutic effectiveness. In apoptotic machinery various pathways and proteins are involved (i.e. mismatch repair proteins, p53 etc.). One of the regulatory proteins is ARC, which can inhibit not only the extrinsic but also the intrinsic apoptotic signaling. In this study we investigated the expression levels of ARC in colorectal liver metastasis and compared them with the expression of mismatch repair proteins and p53. Furthermore, we investigated ARC expression level depending on sex, age, tumor grade, mucin production, tumor size and number of liver metastasis. ARC expression level in colorectal cancer liver metastasis was independent from clinical data (i.e. age, gender, tumor size, tumor number or mucin production) but strongly correlated with MSH2 and MSH6 expression, which further supported the evidence for the regulatory role of MSH2 and MSH6 in apoptosis: i.e. in case of sufficient MSH2 and MSH6 expression significantly higher ARC level is required to suppress the apoptosis. A regulatory interaction between ARC and p53 has been described, but we found no correlation between p53 expression levels and ARC levels.

In the second phase we analysed three proteins (ERCC1, RRM1 and TUBB3) in colorectal cancer liver metastasis. We used tissue microarray slides with hundred and one liver metastasis; stained for ERCC1, RRM1 and TUBB3 and established scoring systems (fitted for tissue microarray) for each protein. In statistical analysis we compared the expression of ERCC1, RRM1 and TUBB3 to mismatch proteins (MLH1, MSH2, MSH6 and PMS2), p53 and to apoptosis repressor protein (ARC).

Statistically significant correlations were found between ERCC1, TUBB3 and MLH1, MSH2 and RRM1 and MSH2, MSH6. Noteworthy, our analysis declares strong significant correlation between cytoplasmic ARC expression and RRM1, TUBB3 ($p=0.000$ and $p=0.001$, respectively), implying additional role of TUBB3 and RRM1 not only in therapy resistance but also in the apoptotic machinery. Our data strengthens the importance of ERCC1, TUBB3 and RRM1 in

prediction of chemotherapy effectiveness and suggest new functional connections in DNA repair, microtubule network and apoptotic signaling (i.e. ARC protein).

CDX2 is well-established as a diagnostic marker for colorectal cancer, but less is known about its regulation, especially about its possible interactions with DNA repair proteins, APC and β-catenin in non-transcriptional manner. In this study we analysed the protein expression of CDX2 depending on the expression of DNA repair proteins (mismatch repair proteins, MGMT and ERCC1) and crucial member of Wnt signalling. CDX2 loss of expression was found in 38.5% of our cases of colorectal cancer liver metastasis. We found statistically significant association between CDX2 and each of the investigated mismatch repair protein: MLH1 ($p<0.01$), MSH2 ($p<0.01$), MSH6 ($p<0.01$), and PMS2 ($p=0.040$). Furthermore, loss of MGMT and ERCC1 was also associated with CDX2 loss ($p=0.039$, and $p<0.01$, respectively). In addition, CDX2 and ERCC1 were inversely associated with metastatic tumor size ($p=0.038$, and $p=0.027$, respectively). Sustained CDX2 expression was associated with higher expression of cytoplasmic/membranous β-catenin and with nuclear APC expression ($p=0.042$, and $p<0.01$, respectively). In conclusion, CDX2 expression loss is not a rare event in liver metastasis of colorectal cancer and our results suggest that CDX2 is involved in mechanisms resulting in loss of DNA repair protein expression (i.e. methylation) and may be a part of this mechanism; however, its exact function in this context remains to be investigated further.

We showed the importance and need of predictive biomarkers in metastasized colorectal cancer and pointed out the relevance not only of single predictive markers but also of their interactions with other known and newly explored relations between different signaling pathways. In conclusion, we can state that further studies are needed to define the exact role of ARC in apoptotic signaling and thus its role in chemoresistence and survival of tumor cells. In this study, we were able to describe the expression manner of ARC protein and could demonstrate an important link between the nuclear and cytoplasmic expression of ARC to MMR proteins and ERCC1, TUBB3 and RRM1 in colorectal cancer liver metastasis, which has not been shown before.

INTRODUCTION

Colorectal cancer (CRC) is still a leading cause of cancer-associated deaths worldwide with an incidence of over one million newly diagnosed cases per year and a mortality rate of approximately 40-50% [1]. Colorectal cancer (CRC) is the third most common type of cancer in the industrialised world having a cumulative 9.4% cancer risk. The highest incidence occurs in North America, Australia and Europe, while the lowest incidence is found in Africa and Asia [2]. Almost 1 million new cases are diagnosed every year. The rive-year disease-free survival rate in UICC stage I cancers is about 90%, whereas in UICC stage III carcinomas reduced to 63% [3]. In Germany, colorectal cancer is the most common cancer with approximately 5% lifetime risk in both genders. About 70,000 new cases are diagnosed in the country and the five-year survival rate is only about 40% [4]. Despite intensive research and therapeutic efforts, the mortality rate of CRC is still approximately 40-50% [1]. Furthermore, the rate of metastatic cases is still common [5]. Early detection of colorectal cancer is crucial for a successful therapy, but despite screening programs more than two thirds of colorectal cancer cases are diagnosed at an already advanced stage (UICC stage III/IV). The incidence is 19.4 in men and 15.3 in women per 100,000 persons [6].

Furthermore, there is an age-related exponential increase in colorectal cancer occurrence [7], which can also (explain the higher incidence in the more developed world. Early onset of colorectal cancer (in patients younger than 45 years of age) assumes not only acquired genetic changes, but also hereditary factors (i.e. HNPCC syndrome)[8].

Environmental and life style factors such as meat and alcohol consumption, smoking, obesity can increase the risk of colorectal cancer [9]. On the other hand dietary fibres, vegetables, non-steroidal anti-inflammatory drugs (NSAID) and hormone replacement therapy (for example, estrogen) seems to be protective [10]. However, recent studies did not verify the inverse correlation between high-fiber diet and colorectal cancer [10, 11].

Pre-existing conditions such as inflammatory bowel disease (i.e. ulcerative colitis) can increase the risk of colorectal cancer (up to 8.2-fold). Ulcerative colitis tends to be a bona fide premalignant condition, thus any patient should be closer screened when diagnosed [12].

Drug resistance is responsible for poor prognosis in many cancer types [13]. Thus, to find proteins, which may have predictive value is of pretty importance not only in metastasized colorectal cancer, but also in other advanced epithelial cancers. In this regard, the deregulation of

DNA damage repair systems (i.e. mismatch repair, NER) represents an important aspect, since it contributes to the resistance of cancer cells to conventional chemotherapy.

1. MISMATCH REPAIR SYSTEM IN CANCER

Despite intensive screening efforts to diagnose colorectal cancer at an early stage, metastases into other organs like liver or lung are still common [5]. Several studies showed that DNA mismatch repair proteins (MMR) take part not only in the DNA repair machinery, but also in the regulation of cell cycle check points and in the apoptotic machinery – deficiency in one of the MMR proteins is responsible for resistance to various chemotherapeutic drugs and subsequently for resistance to apoptosis. MLH1, MSH2, and MSH6 are the main proteins involved in chemotherapeutic resistance. MLH1 and MSH2 are responsible for drug resistence (i.e. cisplatin, 6-thioguanine or methylating agents) in the treatment of colorectal cancer [14]. Furthermore, the MMR system has an important role in apoptosis by activation of cell cycle check points (i.e. G2/M check point). A defective MMR system cannot recognise the DNA damage caused by cisplatin, which leads to continuous proliferation [15]. In addition, the over-expression of MSH2 and MLH1 is toxic to the tumor cells by triggering apoptosis [16].

2. NUCLEOTIDE EXCISION REPAIR AND ERCC1 PROTEIN IN CANCER

One further repair protein is ERCC1 (excision repair cross-complementing 1), which is also implicated in therapy resistance. ERCC1 is a structure specific DNA repair endonuclease responsible for 5' incision (5'-endonuclease), a key enzyme in nucleotide excision repair (NER) pathway and is essential for repair of platinum-DNA adducts, thus associated with therapy resistance to platinum-containing compounds [13, 17]. NER is responsible for repair of DNA damages caused by oxidative and alkylating agents [13]. ERCC1 was suggested as a promising marker in colorectal cancer [17]. ERCC1 overexpression in cancer cells is thought to be more resistant to platinum-based chemotherapy. Increased ERCC1 mRNA levels were found to be associated with resistance to platinum-based chemotherapy (i.e.: ovarian, gastric, cervical, colorectal and non-small cell lung cancer) suggesting that platinum-paclitaxel chemotherapy would be more effective in ERCC1-negative cancer [13]. It is known, that ERCC1 protein expression, estimated by immunohistochemistry, is an independent prognostic factor for progression-free and overall survival in NSCLC patients treated with platinum-based

chemotherapy [18]. Similar data could be achieved in colorectal cancer [19]. In several trials on colorectal cancer, ERCC1 expression level has been proposed as a candidate marker for predicting efficiacy of oxaliplatin therapy for metastatic patients. In stage III colon cancer, ERCC1 expression is strongly predictive in the selection of patients which will benefit from additional oxaliplatin to 5-fluorouracil (5-FU) therapy [20].

3. ROLE OF DNA SYNTHESIS AND RIBONUCLEOSIDE-DIPHOSPHATE REDUCTASE IN CANCER

RRM1 (ribonucleoside-diphosphate reductase 1) gene encodes the regulatory subunit of ribonucleotide reductase enzyme. Ribonucleotide reductase, composed of regulatory subunit RRM1 and the catalytic subunit RRM2, is a crucial enzyme in new DNA synthesis, catalysing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides [21]. RRM1 is a key molecule for gemcitabine efficiacy and is also involved in tumor progression. High RRM1 expression in tumor tissue predict significantly better prognosis while only patients with low RRM1 benefit from gemcitabine therapy. In turn, overexpression of RRM1 protein is strongly associated with gemcitabine resistance [21]. RRM1 expression was also reported to correlate with the tumorigenic and metastatic potential in lung cancer [21].

RRM1 major functions: (a) RRM1 overexpression induces PTEN overexpression and reduces FAK phosphorylation, (b) PTEN is required for the RRM1 induced motility inhibition, (c) RRM1 overexpression inhibits spontaneous metastasis formation, (d) RRM1 overexpression increases survival. Overexpression of RRM1 inhibits in vivo metastasis formation, which requires increased expression of the tumor suppressor gene PTEN. The mechanism by which RRM1 induces expression of PTEN is unclear. One possible connection between RRM1 and PTEN is through TGFβ [22].

RRM1, aside from its role in the generation of deoxyribonucleotidediphosphates, which are essential for DNA synthesis and repair, has an essential role in cellular motility through increased PTEN expression [22]. RRM1 is a key molecule for gemcitabine efficiacy and is also involved in tumor progression. High RRM1 expression in tumor tissue predict significantly better prognosis whereas only patients with low RRM1 benefit from gemcitabine therapy. Overexpression of RRM1 protein is strongly associated with gemcitabine resistance [21]. RRM1 expression was also reported to correlate with the tumorigenic and metastatic potential in lung cancer [21].

Gemcitabine, a nucleoside analogue and antimetabolite with similar structure to the nucleoside cytidine (dFdC). Gemcitabine has benefit for only 25% of the patients with median survival less than six months [23]. Drugs resistance is a common feature in patients with PDAC treated with gemcitabine. Combination therapy (i.e. gemcitabine with abraxane (albumin-binded formulation of paclitaxel)) has shown improved overall and progression-free survival in metastatic PDAC [24]. FOLFIRINOX protocol (5-FU, leucovorin, irinotecan and oxaliplatin) has significantly prolonged median overall survival compared to gemcitabine monotherapy in metastatic PDAC [25]. Gemcitabine is actively transported inside the cells through hENT (human equilibrative nucleotide transporter) and transformed into its active form dFdCTP by deoxycytidine kinase (dCK). Gemcitabine gets inactivated through deamination of cytidine and deoxycytidine to form uridine and deoxyuridine by cytidine deaminase (CDA) [26]. The main mechanism of action of dFdCTP is the inhibition of DNA synthesis: dFdCTP competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA, leading to termination of DNA duplication [27].

In addition, gemcitabine binds and irreversibly inactivates ribonucleotide reductase (RNR or RR) large subunit 1 (RRM1)[28, 29]. RR is the rate-limiting step of DNA duplication [30], as this enzyme exclusively catalysis the conversion of ribonucleotides into 2`-deoxyribonucleotides required for new DNA synthesis and repair [31]. RNR consists of two heterodimers, each of them composed of two subunits: a large regulatory subunit RRM1 and a small catalytic subunit RRM2 [32]. RRM1 can also bind to P53R2, a subunit dependent on p53 activity [30]. Two subunits (RRM1 and RRM2) associate, forming an active holoenzyme [32]. Levels of RRM1 protein are constant during the cell cycle; RRM2 levels oscillate during the cell cycle with a peak in S phase [33]. High expression of RRM1 suppressed formation of metastasis by inducing the expression of tumor suppressor gene PTEN and prolonged survival in lung cancer [22].

4. THE ROLE OF β(III)-TUBULIN (TUBB3) IN CYTOSKELETON AND IN CANCER

Cell cytoskeleton is built up from microtubules, microfilaments and intermediate filaments. Various changes of the microtubule network have been identified in a wide range of cancers, i.e. altered expression of tubulin isotypes, alterations in tubulin posttranslational modifications and changes in the expression of MAPs (MAP – microtubule associated protein)[34]. TUBB3 is one of the main microtubule (MT) proteins and is primarily expressed in neurons and Sertoli cells in the testis [35, 36]. In lung cancer TUBB3 protein expression level has no correlation with age,

gender, smoking status or recurrence pattern or response rate to chemotherapy. The response rate in TUBB3 positive cases was 18%, while in negative cases was 27% (no significant differences could be detected)[18]. High TUBB3 expression levels are associated with poor prognosis in many epithelial cancers. Additionally, TUBB3 is been suggested to take a part in disease aggressiveness by acting as a survival factor for cancer cells [37]. In colorectal adenomas TUBB3 expression can be detected up to 100% in high-grade dysplasia. Expression of TUBB3 has no association with grade of dysplasia or other clinical data in preneoplastic lesions of colorectal cancer but associated with Dukes' stages [38]. TUBB3 overexpression in colon cancer cells may contribute to a higher stability of microtubular network which may explain the lower activity of anti-microtubule agents [39]. In addition, high TUBB3 expression levels was localized to invasive edge in colorectal cancer: positive TUBB3 staining was observed in all cases, the was most prominent at the invasion front with presence of tumor budding [37]. This preferential localization of TUBB3 at invasive margin arises the possibility that changes in tubulin isotypes can modulate the invading activity of cancer cells. Microtubules are indispensable for the directional migration of cells. Tubulins, the major constituent protein of microtubules, build up from heterodimers of α and β subunits [37]. It is believed that tumor buds consists of migrating cells and TUBB3 expression in these cells is linked to their motility. Furthermore, TUBB3 is expressed in a variety of tumors, particularly in those that are aggressive and likely to metastasize, and were found to be more resistant to several chemotherapy regimens (i.e. estramustine, taxol, paclitaxel, docetaxel)[37].

5. APOPTOSIS REPRESSOR WITH CASPASE RECRUITMENT DOMAIN (ARC) AND THE APOPTOTIC SIGNALLING

Apoptosis can be induced by diverse stimuli (i.e. DNA damage, chemotherapeutic drugs, oxidative stress etc.). The exact role of apoptosis in CRC metastases and chemotherapeutic resistance is not fully understood. This is of interest, especially regarding therapeutic interventions in metastatic cases. One attractive potential therapeutic target is ARC (apoptosis repressor with CARD (caspase recruitment domain)).

ARC is a protein that, over the last years, has become of special interest as a potent inhibitor of both central apoptotic pathways and is expressed throughout all cell types of cells especially in neurons, skeletal and cardiac myocytes [40], as well as in carcinomas of different origins, like

ovarian cancer, colon cancer or cervical cancer [41]. Different expression levels of ARC have already been observed in different cell lines (MCF-7 - breast cancer, A-549 - non-small lung cancer, HT-29 - colon cancer, PC-3 prostate cancer, A-498 - kidney cancer). The ARC levels were different not only in different cancer cell types, but also between cells of the same cancer types [42]. Interestingly, immortalised epithelial cell lines from normal breast tissue showed a low level of ARC [42]. Furthermore, in reduction-mammoplasty specimens the ARC expression was restricted only to the nuclei. Mercier et al. found cytoplasmic ARC positivity in invasive breast cancer, normal breast tissue and in reduction-mammoplasty (89%, 21% and 10%, respectively) [42]. ARC has been thought to be mainly cytoplasmic and mitochondria-associated and its anti-apoptotic activity has been linked to those locations [42, 43]. For this reason, nuclear ARC expression was unexpected, but was presented in MCF-7 breast cancer cells, ductal carcinoma in situ and also in cardiomyocytes. In breast cancer cell lines, high levels of cytoplasmic ARC were linked with treatment resistence: in MCF-7 cells high ARC level protected the cells from doxorubicin and γ-radiation induced cell death [42]. ARC is known to be induced by Ras and repressed by p53 signaling [44] and is involved in the inactivation of extrinsic as well as intrinsic apoptosis pathways, by interacting with pro-apoptotic proteins like p53, Bcl2, Bax, Bad, Puma, MSH2, MSH6, and others [45].

In summary, abundant expression of ARC in cancer cells or in its premalignant lesions can promote cell survival and protect cancer cells from cell death thus providing a benefit to these cells [42]. Upstream regulatory mechanisms of cytoplasmic and nuclear ARC expression are still unknown. Regarding the different ARC abundance in normal tissue and cancer cells, it was suggested that increased cytoplasmic ARC expression is not only a result of redistribution of nuclear ARC, but also augmented by increased production of ARC [42].

6. CDX2 AND WNT SIGNALING IN COLORECTAL CANCER

At the diagnosis, a quarter of the patients with primary CRC have synchronous hepatic metastasis, and more than 50% of the patients with CRC will develop liver metastases in the course. Almost half of the patients undergoing resection for primary CRC eventually develop metachronous liver metastasis. Survival in metastatic cases is rarely longer than three years [46]. Interestingly, although CDX2 is widely used in the daily routine diagnostic, there are less than

sixty publications in the last sixty years performed on human tissue investigating the role of CDX2 [47].

The Cdx family of transcription factors contributes also to the CRC phenotype, but a mechanism by which CDX2 expression is lost or downregulated in colorectal tumors is currently not clear. The caudal-related homeobox transcription factor 2 (CDX2) is necessary for the proper development of the intestinal tract and is crucial for development and homeostasis of the intestinal epithelium throughout life [48]. The role of Cdx2 in colorectal carcinogenesis is multi-sided. The CDX2 expression is reduced in colorectal cancer and its expression is inversely correlated to tumor grade, tumor stage and lymph node metastasis [49]. Loss of CDX2 expression can strongly predict high level CpG island methylation phenotype (CIMP-H) independently from microsatellite status of colorectal cancers. Thus Cdx2 was proposed as a surrogate marker for CIMP-H [50]. In addition, CDX2 was attributed to play a regulatory role in apoptosis and DNA repair. Colon epithelium with decreased CDX2 expression lead to impaired apoptosis potential after γ-irradiation, thus resulting in higher resistance to genotoxic stress. Besides, the effect of CDX2 in DNA repair activity can contribute to its attributed tumor suppressor function [51].

DNA methylation of tumor suppressor genes resulting in its transcriptional inactivation and has been identified as an important mechanism. CIMP characterized by the extensive hypermethylation of multiple CpG islands, and belongs to one of the major mechanisms in the colorectal carcinogenesis [52]. O6-methylguanine DNA methyltransferase (MGMT), a surrogate marker for CIMP, gene promoter methylation plays an important role in colorectal carcinogenesis. Loss of MGMT expression, which is secondary to gene promoter methylation, occurs in about 30%-40% of metastatic colorectal cancer. In addition, loss of MGMT expression results in high response to alkylating agents (i.e. dacarbazine or temozolomide)[53]. Thus, MGMT is believed to have predictive potential for therapy.

A further level of DNA damage defence mechanism is represented by the mismatch repair system (MMR), which take part not only in the DNA repair processes, but also in the regulation of cell cycle check-points and apoptosis [14]. Deficiency of MMR proteins (i.e. MLH1 and MSH2) is responsible for resistance to various chemotherapeutic drugs and subsequently for resistance to apoptosis [14]. Interestingly, loss of MGMT expression is more frequent in CRC with microsatellite instability, suggesting that methylated MGMT selects cellular clones with MMR deficient status [53]. Moreover, mismatch repair deficiency is also correlated with loss of

CDX2 [54]. ERCC1 (excision repair cross-complementing 1) is a structure specific DNA repair endonuclease responsible for 5' incision (5'-endonuclease), a key enzyme in nucleotide excision repair (NER) pathway and is essential for repair of platinum-DNA adducts, thus associated with therapy resistance to platinum-containing compounds (i.e. cisplatin)[13, 17].

Aberrant β-catenin expression and disturbed Wnt signaling is recognized as an important event in the genesis of several malignancies, especially in colorectal cancer. β-catenin mutations or loss-of-function mutations of the APC tumor suppressor gene appear to be crucial steps in the progression of this disease [55]. APC and β-catenin were found to traffic independently from each other into and out of the nucleus in response to internal and external signals. This fact has prompted debate about the previously proposed role of APC as a β-catenin chaperone [56]. Germline mutations in the APC gene cause familial adenomatous polyposis (FAP), and over 80% of colorectal cancers (both inherited and sporadic) carry truncating mutations that inactivate the APC protein. Most of these mutations occur in the so-called 'mutation cluster region' of the APC gene, accounting for a truncated protein incapable of binding regulatory proteins (i.e. Axin) or associating with microtubules. The relevance of truncating mutations for β-catenin is enormous: mutated APC cannot stimulate its degradation (because of its failure to bind Axin), although APC still can bind to β-catenin (albeit less efficiently)[56, 57]. β-catenin has been observed to accumulate in the nuclei of colon cancer cells, which results from the inability of APC to promote β-catenin degradation, rather than a lack of export function, leading to nuclear accumulation of β-catenin in APC-mutant tumor cells [56]. There are only few studies that focused on interactions between CDX2 and Wnt signalling in colon cancer. It has been demonstrated that CDX2 can inhibit the transcriptional activity of β-catenin/TCF lines in a non- transcriptional way [49].

AIMS AND OBJECTIVES OF THE DISSERTATION

At first we would like to investigate the expression manner of ARC protein in colorectal cancer liver metastasis. Furthermore, we would like to detect correlations between MMR proteins and the expression of apoptosis repressor protein ARC. We would like to prove the known relationship between p53 protein and ARC at protein expression level.

It is known that (over-)expression of ERCC1, RRM1 and TUBB3 is linked to therapeutic resistance against therapeutic regime, which are also given in advanced (stage IV) colorectal cancer [58]. Thus, in the second phase, we would like to investigate the

expression manner of ERCC1, RRM1 and TUBB3 proteins and their correlation to ARC protein expression, which is known to be upregulated in colorectal cancer and associated to therapeutic resistance inhibiting both extrinsic and intrinsic apoptotic signaling.

Expression of CDX2 in association with DNA repair proteins and members of Wnt signaling pathway has not been studied previously in liver metastasis of colorectal cancer. In the third paper, we analysed the expression distribution of CDX2 in matters of expression status of DNA repair proteins (MMR proteins, MGMT and ERCC1), APC, and β-catenin. Furthermore, we correlated CDX2 protein expression with clinical data.

MATERIALS AND METHODS

1. TISSUE SAMPLES

Paraffin-embedded operation specimens of liver metastasis of colorectal cancer were selected from the archives of the Institute of Pathology at the University Hospital of Heidelberg. Hundred-one patients (64 male, 37 female; mean age 62 years) were included. None of the patients had received neo-adjuvant chemotherapy. Tissue samples were fixed in neutral-buffered formalin and embedded in paraffin. Paraffin sections were cut at 4 μm and examined on coated slide glas for immunohistochemistry. Further data, such as: age, gender, size and number of metastases were collected from histological reports. Tissue samples were provided by the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the Ethics Committee of Heidelberg University according to ethical standards formulated in the Declaration of Helsinki 1975 (revised in 1983).

2. TISSUE MICROARRAY

Tissue microarray (TMA) blocks were obtained from paraffin-embedded human liver specimens with a tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA). From one case, two cores of tumor tissue with a diameter size of 1.6 mm were punched and for orientation of the TMA slides two muscle cores were used. Muscle punches served also as positive controls for ARC immunostaining.

2.1. Design and processing of tissue microarray slides

Tumour and normal tissue blocks were taken together and one routine HE stain was made from each block. On the HE slides, the tumour and normal colorectal mucosa was encircled by a

pathologist (C.T.). Under the microscope gland-rich areas were chosen and necrotic tissue or stroma rich areas were avoided. Tumor area was marked with waterproof pen (**Figure 1**). These histological slides were used as template for paraffin blocks to decide which area has to be taken for the TMA block. HE slide was placed on the paraffin block to identify the area that has to be sampled. From one patient, we took two cores of tumour tissue (if enough tissue was available). The cores were embedded into the recipient block in a formerly defined manner. The general

organisation and design of TMA blocks can be seen on a figure below (**Figure 2**). The distance between sample cores was 1.0 mm and we made greater separation between the left and right side of TMA block. The relatively high distance provides much less tense in the TMA block. Furthermore, a so-called protection wall would be planned around the cores to avoid staining artefacts (i.e. different antibody titer at the borders) during immunohistochemistry. The protection wall in TMAs was first described by Hoos [59]. For orientation and to avoid confusions, we used the gap technique: in the last column the last two cores on each slide were not filled (**Figure 3**). Others use orientation cores in distinct portions outside or inside of TMA cores. These cores can also be used as negative or positive on slide controls.

Figure 1 Exemplary paraffin slide for identification of tumour tissue for punching.

Multi- Tissue- Array

Metastase Colon 1 Feb 12 AD

MSH 2

		0	2,6	5,2	7,8		10,9	13,5	16,1	18,7	
		1	2	3	4		5	6	7	8	
0	A	Muskel	Muskel	1	1		Muskel	Muskel	E/1990/004422	E/1995/030643	A
2,6	B	1	0	1	1		E/1995/032308	E/1995/032514	E/1995/035304	E/1995/035305	B
5,2	C	1	0	1	1		E/1995/036047	E/1995/036233	E/1995/036654	E/1995/036845	C
7,8	D	1	1	1	1		E/1995/036873	E/1995/038153	E/1995/039006	E/1995/040748	D
10,4	E	0	x	1	1		E/1995/044815	E/1995/044978	E/1995/050198	E/1995/052115	E
13	F	1	1	0			E/1995/052120	E/1995/053635	E/1996/003922		F
		1	2	3	4		5	6	7	8	

Stanze: 1,6 mm

Figure 2 Design of TMA blocks and the arrangement of tumor cores. On each mapping plan the number of array block (Metastase Colon 1), the histological number and study identification number are denoted. On the horizontal and vertical axis, the localisation of tissue cores is also shown (in mm). Core diameter is also denoted (1.6 mm).

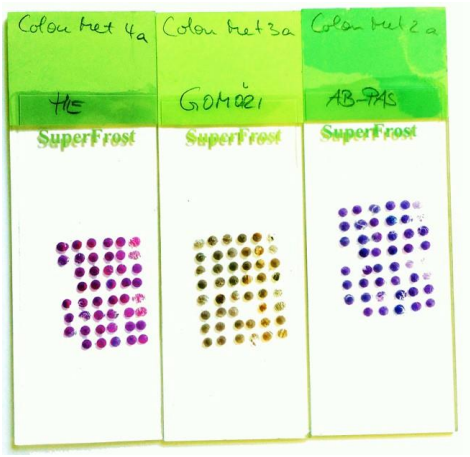


Figure 3 Core arrangements on TMA slides and display the gap orientation technique.

2.2. Tissue microarray equipment

Manual tissue arrayer from Beecher Instruments (Woodland, USA) was used to make the TMA blocks (**Figure 4**). The Beecher arrayer works with two punches. One is to take a tissue core from the donor block and he second one is to make a hole for tissue core in the recipient TMA block. The needles for tissue cores are usually minimally bigger in diameter, than the needles for empty hole punching to enable the best fitting for tissue cores in the recipient block. The recipient blocks were empty paraffin blocks made with conventional metal moulds for diagnostic purpose. The needles can be positioned with high accuracy along two axes over the recipient block. The depth of the hole can be set with a depth stop. With magnet hold and moveable bridge, the position of the donor and recipient blocks can be changed. At first, the hole for tissue core has to be punched out in the appropriate position. After that, the donor core of tissue has to be extracted and has to be moved through the bridge over the recipient block. Now the tissue core can be inserted in its position with the help of the steel stylet. For the next core the needles have to be positioned along the two axes to its appropiate position. Each recipient block was arrayed three times (a-c), but we used only block (a) for our analysis.

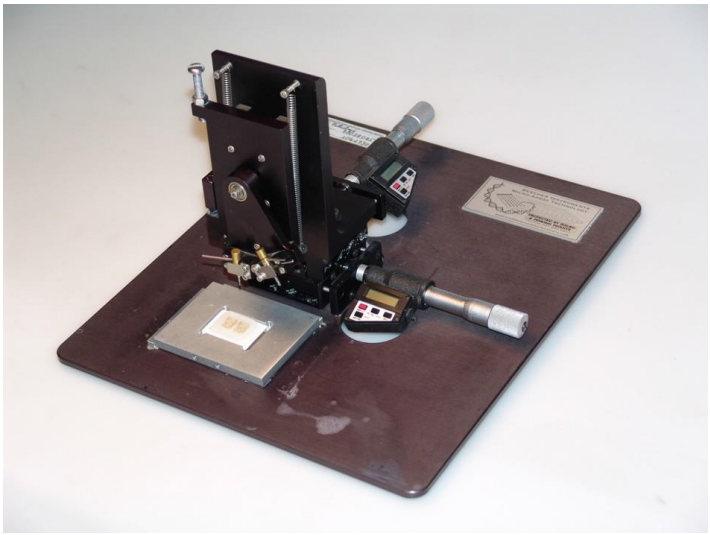


Figure 4 Manual tissue microarrayer from Beecher Instruments (Woodland, USA).

3. IMMUNOHISTOCHEMISTRY

0.4 µm thick slides were obtained from TMA blocks. Slides were then deparaffinised according to standard protocol by xylene, and dehydrated with 95-96 % ethanol, 70 % ethanol and distilled water. All slides were stained simultaneously using a computer-controlled autostainer (Dako TechMate 500 cytomation and Dako EnVision-Sytem (Dako) and pretreated with 3 % Hydrogen Peroxide prior to antibody incubation. MLH1, MSH6, PMS2 (all BD Pharmingen), MSH2 (Calbiochem), p53, ARC (Santa Cruz) antibodies were used, ranging in dilutions from 1:50 to 1:500. Secondary antibody binding (all Dako, 1:200) was visualised using a streptavidin ABC- kit (Dako), followed by 3,3'-diaminobenzidine (Vector, Peterborough, UK). For MMR proteins, p53 and ARC the staining methods were performed as published before [60].

In the second phase, the slides were stained simultaneously using a computer-controlled autostainer (Ventana BenchMark Ultra, Ventana Medical Systems Inc.). Then primary antibodies were used: ERCC1 (8F1, Neomarkers; dilution: 1:100), RRM1 (Protein Tech Europe; dilution 1:200) and TUBB3 (Tuj-1/TubIII/4G3, Covalab; dilution 1:2000). Primary antibodies were incubated according to routine staining protocols for diagnostic purpose. To detect immunoreactions Ultraview Universal DAB dectection kit (Ventana Medical Systems Inc.) and

3,3´-diaminobenzidine were used. A counterstain was done with hematoxylin and blueing reagent and all slides were covered.

A counterstain was done with hematoxylin and all slides were covered with Aquatex (Merck, Darmstadt, Germany). Control immunostaining was also performed with blocking peptide showing absence of ARC signal.

In the third phase the following antibodies were used: MGMT (MT-23.2, Thermo Fischer; 1:20) and CDX2 (EPR2764Y, Thermo Fischer; 1:200) antibodies were used. Secondary antibody binding (all Dako, 1:200) was visualised using a streptavidin ABC-kit (Dako), followed by 3,3'- diaminobenzidine (Vector, Peterborough, UK). For ERCC1 (8F1, Neomarkers; dilution: 1:100) and β-catenin (14, RTU, Ventana, Roche Diagnostics) slides were stained by a computer- controlled autostainer (Ventana BenchMark Ultra, Ventana Medical Systems Inc.). Polyclonal rabbit anti-APC antibody (DP2.5 1:200 Fa. Acris) were used for APC staining. Staining was performed using ChemMate Detection Kit (Dako, Hamburg, Deutschland) according to recommendations of the manufacturer. The antibodies were incubated overnight at 4 °C followed by avidin-biotin complex peroxidease technique using aminoethylcarbazole for visualization and hematoxylin for nuclear counterstaining. All slides were covered with Aquatex (Merck, Darmstadt, Germany).

4. EVALUATION OF IMMUNOHISTOCHEMISTRY

The immunostained tissue microarray sections were evaluated and scored under a light microscope independently by two pathologists in a blinded fashion. Discordant cases were reviewed and re-evaluated based on a consensus opinion. For semi-quantitative assessment of staining intensity we adjusted a previously published scoring system for each proteins and fitted to TMA dots [61-63]. As baseline expression level normal colorectal mucosa was set. Cytoplasmic and nuclear ARC staining was separately scored. For cytoplasmic ARC staining the reference score (score 1) was the normal colorectal mucosa, which was also used as external control for immunohistochemistry. For nuclear ARC staining a four-graded scale was used: *score 0*: no or weaker staining in lesser than 10% of tumor cells and weaker staining compared to normal colonic mucosa; *score 1*: equivalent cytoplasmic staining compared to normal colorectal mucosa, perceptible staining intensity at 4X; *score 2*: slightly stronger staining compared to normal mucosa and *score 3*: diffuse and strong cytoplasmic staining. For nuclear ARC staining a three-graded scale was used: *score 0* – no nuclear staining or weak staining in

lesser than 10% of the nuclei; *score 1* – 10-75% of the nuclei positive with moderate intensity and *score 2* – more than 75% of the nuclei are positive with high staining intensity.

For MSI proteins the staining was evaluated according to Bethesda guidelines [64]. Immunostaining for p53 were scored in a three-graded scale: *score 0* – weakly staining in less than 10% of the tumor cells, *score 1* – moderate staining in up to 75% of the tumor cells and *score 2* – strong nuclear staining in more than 75% of the tumor cells.

ERCC1 and RRM1 immunostainings were scored in a three-graded scale: *score 0* – no expression detectable or faint partial expression in less than 10% of the tumor cells; *score 1* – weak to moderate expression of the entire tumor tissue; *score 2* – strong positivity in the entire tumor tissue.

For TUBB3 a modified three-graded score were established: *score 0* – no expression detectable or faint partial expression in less than 10% of the tumor cells; *score 1* – diffuse and strong positive staining associated to invasion front and tumor budding, central tumor regions negative or with weaker intensity than at the invasive front; *score 2* – strong positivity in the entire tumor tissue.

Immunostaining for CDX2 and nuclear β-catenin was scored in a three-graded scale: *score 0* – weak staining in less than 10% of the tumor cells, *score 1* – moderate staining in up to 75% of the tumor cells and *score 2* – strong nuclear staining in more than 75% of the tumor cells.

For cytoplasmic β-catenin staining a two-graded scale was used: *score 0:* no or weak staining in less than 10% of tumor cells and weaker staining compared to normal colonic mucosa; *score 1:* nuclear staining in more than 10% of the tumor cells. Cytoplasmic and nuclear APC staining was separately scored. For nuclear APC staining a two-graded scale was used: *score 0:* no or weaker staining in less than 10% of tumor cells and weaker staining compared to normal colonic mucosa; *score 1:* nuclear staining in more than 10% of the tumor cells. For cytoplasmic APC staining a three-graded scale was used: *score 0* – no cytoplasmic staining or weak staining in less than 10% of tumor cells; *score 1* – 10-75% of the tumor cells with moderate intensity and *score 2* – more than 75% of the tumor cells are positive with high staining intensity. Normal colorectal mucosa was set as baseline expression level for APC (score 2).

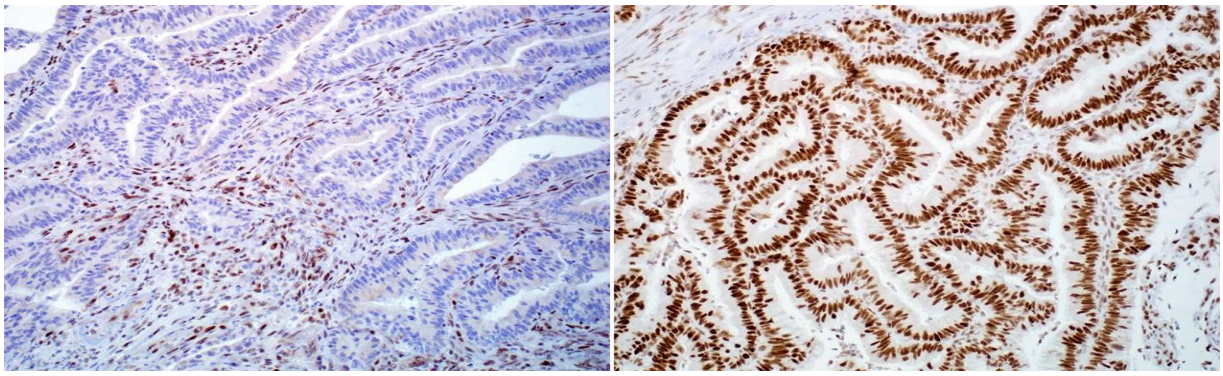
5. STATISTICAL ANALYSIS

The statistical analyses were performed with SAS software (SAS institute, Cary, NC, USA). Spearman-Rho test was used to evaluate the relationship between clinical data, ARC, MLH1, MSH2, MSH6, PMS2 and p53. Also Spearman-Rho test was used to evaluate the relationship between clinical data, CDX2, MLH1, MSH2, MSH6, PMS2, MGMT, ERCC1, APC and β- catenin. Associations between clinical data, ARC, MMR proteins, ERCC1, TUBB3 and RRM1 were estimated by Pearson's correlation and linear regression test. The statistical significance was set at $p<0.05$ and $p<0.01$

RESULTS

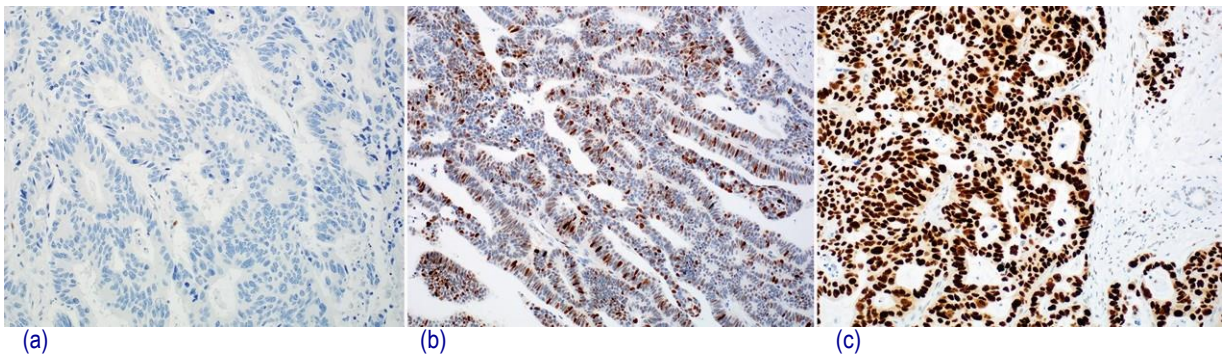
1. EXPRESSION OF MMR PROTEINS AND P53

The staining for MSI proteins shows loss of expression in 4.2% to 26% of the cases (MLH1 4.2%, MSH2 26%, MSH6 24% and PMS2 9.5%) (**Figure 5**). For p53 n=102 valid immunohistochemical results were used for evaluation, for which in 23% of the cases (n=23/102) p53 was negative (score 0) (**Figure 6**). Positive stainings were subdivided into two groups: moderate positivity or so-called restrictive overexpression with a score of 1 (46 %, n=47/102); and strong positivity or so-called strong overexpression with a score of 2 (31 %, n=32/102). Regarding only the strong overexpression (score 2) as positive, we observed, p53 positivity in 31% (n=32/102) while 69% (n=70/102) of the cases were negative. Regarding all positive cases (score 1 and 2), we could detect in 77% (n=79/102) nuclear positivity and so 23% (n=23/102) of the cases were negative for p53.



(a) (b)

Figure 5 (a-b) Representative examples of the **MMR protein** expression in colorectal liver metastasis. a Score 0: negative staining – less than 10% of the nuclei are stained; b Score 1: more than 10% of the nuclei are positive. Note that in negative cases the cells of the stroma are still positive.



(a) (b) (c)

Figure 6 (a-c) Representative examples of the expression of **p53** protein in colorectal liver metastasis. **a** Score 0: negative staining – less than 10% of the nuclei are stained; **b** Score 1: moderate overexpression in less than 75% of the nuclei; **c** Score 3: strong overexpression in almost all tumor cells, magnification ×400.

2.

3. ARC EXPRESSION

In normal colonic mucosa ARC expression is limited to the upper two-third of the colonic crypt. Normal colonic mucosa showed a slightly cytoplasmic and nuclear ARC staining with higher level in the upper two-thirds of the colonic crypts. At the bottom of the crypts there was almost no staining activity detected (**Figure 7**). Ancillary studies detected a similarly low or absent ARC expression in the proliferation zone of the colonic crypts, where the apoptotic activity is low [63, 65]. In some other studies no or a very low level of nuclear ARC was detected. This can be due to the use of different antibody: ARC (F-11) antibody covers the whole ARC protein and also Nop30, a splicing variant of NOL3 locus. ARC staining pattern is similar for Bcl-2 and Bcl-w, which are also absent in the proliferation zone of the colonic crypts whereas overexpressed in colorectal cancer [66].

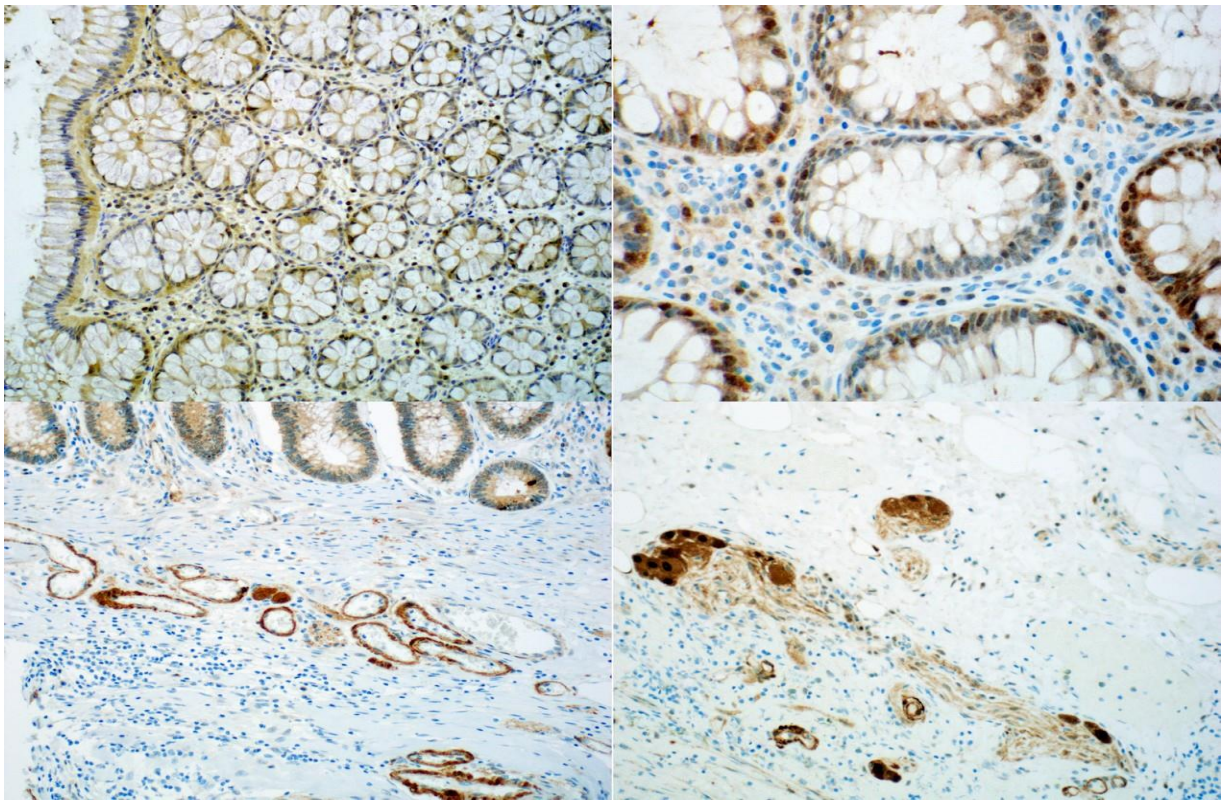


Figure 7 Normal colorectal mucosa and submucosa showing **cytoplasmic and nuclear ARC** staining with higher level in upper two-thirds of the colonic crypts. At the bottom of the crypts there was almost no staining activity detected. Further note the smooth muscle in the vessels and the ganglionic cells of the plexus submucosus as a positive control.

3.1. Cytoplasmic ARC expression in liver metastasis of colorectal cancer

Cytoplasmic results for ARC staining are subdivided into three groups: score 0 for no cytoplasmic staining, score 1 for staining equivalent to normal mucosa, score 2 for moderate overexpression and score 3 for strong overexpression (**Table 1**). The different staining intensities are described and depicted (**Figure 8**).

3.2. Nuclear ARC expression in liver metastasis of colorectal cancer

Additionally to cytoplasmic staining of ARC, nuclear staining was evaluated in a three-graded score. Score 0 represents no nuclear staining and score 1 and 2 demonstrate moderate and strong staining for nuclear ARC respectively (**Table 2**). The different staining intensities are described and depicted (**Figure 8**).

Score	Valid cases (n)	% of valid cases
0	14	14
1	25	25
2	21	21
3	40	40
∑	**100**	**100**

Table 1 Valid cytoplasmic immunhistochemical stainings for ARC. Subdivisions of valid cytoplasmic stainings for ARC in three scores and the number of cases (n) and %.

Score	Valid cases (n)	% of valid cases
0	2	2
1	38	38
2	60	60
∑	**100**	**100**

Table 2 Valid nuclear immunohistochemical stainings for ARC. Subdivisions of valid nuclear stainings for ARC in three scores and the number of cases (n) and %.

3.3. ARC protein expression in association with histopathological parameters

Neither cytoplasmic nor nuclear ARC expression has statistically significant correlation with clinical parameters. Concerning clinical parameters like: age, gender of the patients, grading of the tumor and the number and size of metastases, there was no significant correlation to nuclear or cytoplasmic ARC expression. Regarding the patients` age, ranging

from 33 years to 82 years, there was neither a correlation for nuclear ARC expression (p=0.622) nor with cytoplasmic expression (p=0.548).

There was no association between gender and nuclear or cytoplasmic staining (nuclear p=0.602; cytoplasmic p=0.697). We found only one case without nuclear staining. From all patients there was only one case with a G1 graded tumor with a strong nuclear staining for ARC but weak cytoplasmic positivity. No correlation was found regarding the grade (for nuclear ARC

expression p=0.858, whereas cytoplasmic was p=0.692). No association between number of metastases and nuclear (p=0.941) or cytoplasmic (p=0.612) ARC expression could be detected. Patients in the study group had metastases from 5 to 160 mm in diameter, 63 of them with a diameter of 63 mm and bigger. Here the ARC expression also showed no association to the size (nuclear p=0.996; cytoplasmic p=0.520).

3.4. Correlation between ARC and p53 protein expression

Cellular ARC expression levels are independent from p53 staining status (**Table 3**). Furthermore, no correlation could be detected between p53 expression status and expression level of nuclear or cytoplasmic ARC (p=0.465 and p=0.491, respectively), even if only the strong p53 overexpression were classified as pathologic (p=0.256 for nuclear and p=0.388 for cytoplasmic ARC expression versus p53).

3.5. ARC protein expression and MMR proteins

Correlations between MSH2, MSH6 and ARC are shown in **Table 3**. Surprisingly, cytoplasmic ARC expression had a strong positive correlation with MSH2 (p=0.003) besides a strong positive correlation between nuclear ARC expression and MSH6 protein status (p=0.006). Moreover, MSH2 expression status shows an almost significant positive relation to nuclear ARC expression (p=0.063). MLH1 and PMS2 had no significant correlation with ARC expression.

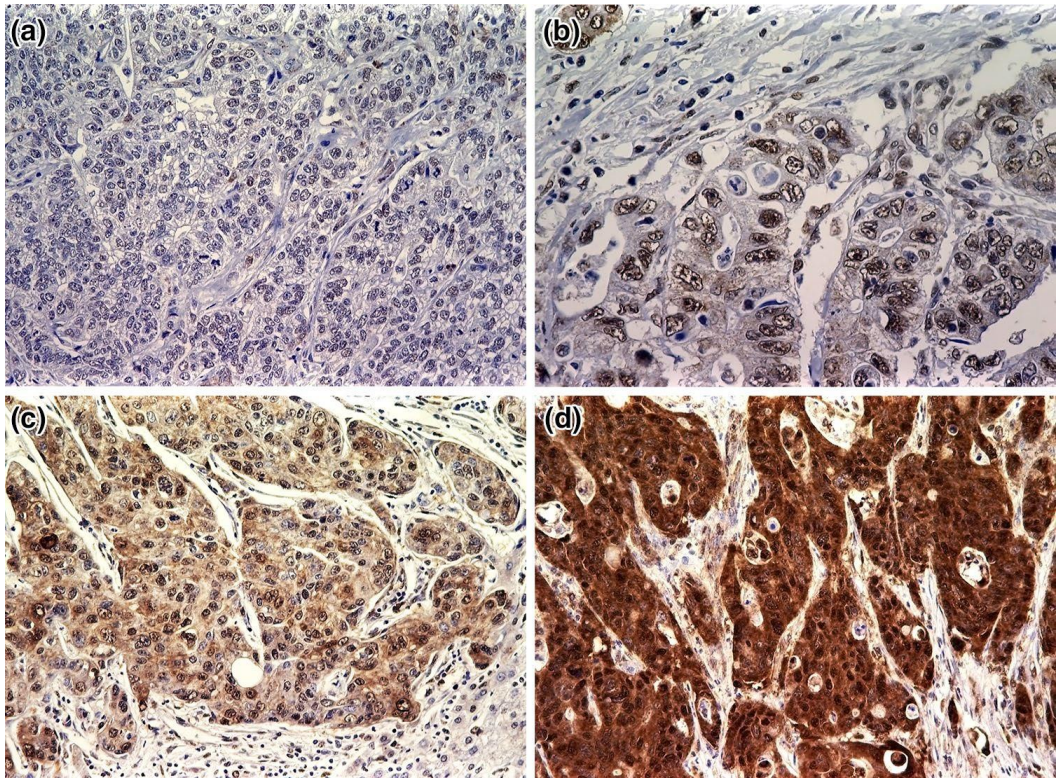


Figure 8 (a-d) Representative images of **ARC** immunohistochemical staining in colorectal cancer liver metastasis. **a** No cytoplasmic with weak nuclear positivity lesser than 10% of the tumor cells, magnification x400. **b** Weak cytoplasmic positivity with similar intensity as in normal colon mucosa (score 1 for cytoplasmic staining) next to moderate nuclear staining (score 1 for nuclear staining) magnification x400. **c** Moderate cytoplasmic staining intensity, stronger than normal colorectal mucosa (score 2 for cytoplasmic intensity) and moderate staining in tumor cell nuclei (score 1 for nuclear staining), magnification x400. **d** Strong positivity in cytoplasm and strong nuclear positivity in almost all tumor cells (score 3 for cytoplasmic and score 2 for nuclear staining), magnification x400.

		MLH1	MSH2	MSH6	PMS2	p53
ARC cytoplasm	Correlation coefficient	-0.078	**0.299****	0.160	0.145	0.070
	Significance (2-sided)	0.504	**0.003**	0.114	0.152	0.491
	Number of valid cases	75	99	99	99	98
ARC nucleus	Correlation coefficient	0.197	0.187	**0.276****	0.093	0.075
	Significance (2-sided)	0.090	0.063	**0.006**	0.359	0.465
	Number of valid cases	75	99	99	99	98

** The correlation is significant at the level of 0,01 (2-sided)
* The correlation is significant at the level of 0,05 (2-sided)

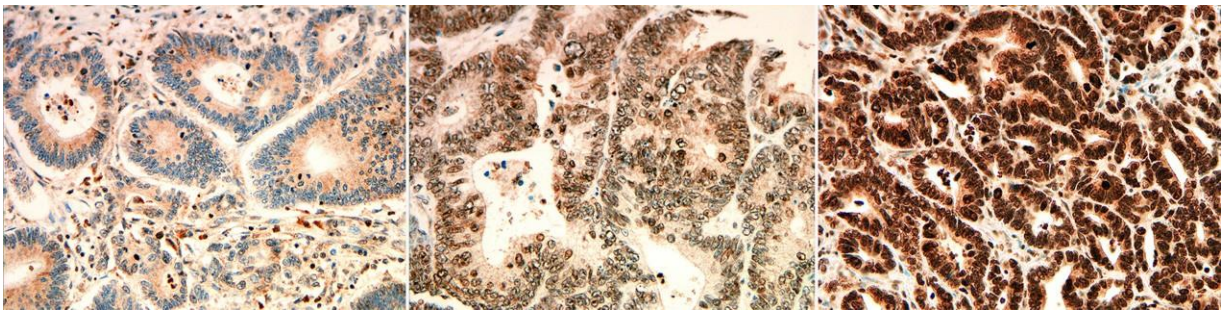
Table 3 Relationship between ARC expression, MMR proteins and p53.

4. DISTRIBUTION OF ERCC1, RRM1 AND TUBB3 PROTEIN EXPRESSION IN THE COLLECTIVE

The results of the immunohistochemistry for ERCC1, RRM1 and TUBB3 are listed in **Table 4**. For ERCC1 we found 29.8% of the cases negative (score 0). Positive ERCC1 staining could be in 70.2% of the cases detected (30.8% score 1 and 39.4% score 2). For RRM1 the distribution was different: only 11 cases out of 95 valid cases (11.6%) were found to be negative (score 0). 84 cases (88.4%) showed positive staining for RRM1, 51 cases showed even a high expression level (score 2 – 53.7%). TUBB3 staining showed an interesting distribution: the most of the cases showed pronounced positivity at the invasion margin (52%). 35 cases (35%) had negative staining and only 13% had a diffuse positive staining reaction for TUBB3. Representative pictures of the staining scores can be seen for ERCC1 in **Figure 9**, for RRM1 in **Figure 10** and for TUBB3 in **Figure 11**.

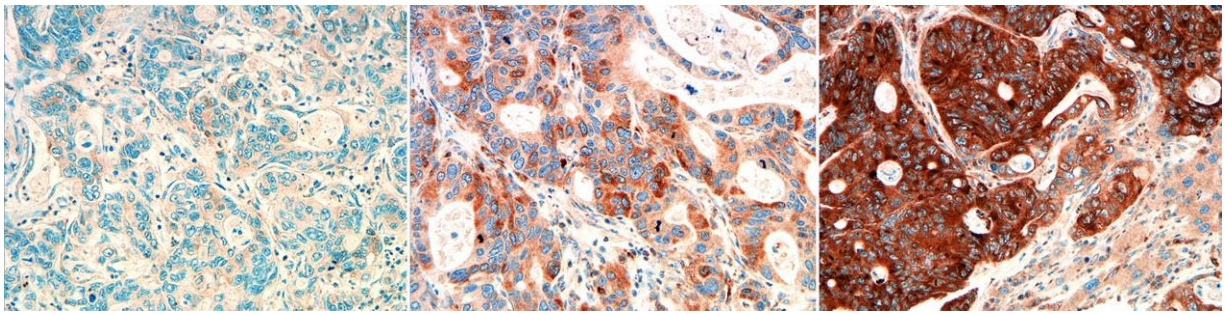
	Immunoreactive score 0	Immunoreactive score 1	Immunoreactive score 2	Valid cases
ERCC1	28 (29.8%)	29 (30.8%)	37 (39.4%)	94 (100%)
RRM1	11 (11.6%)	33 (34.7%)	51 (53.7%)	95 (100%)
TUBB3	35 (35%)	52 (52%)	13 (13%)	100 (100%)

Table 4 The results of the immunohistochemistry for ERCC1, RRM1 and TUBB3.



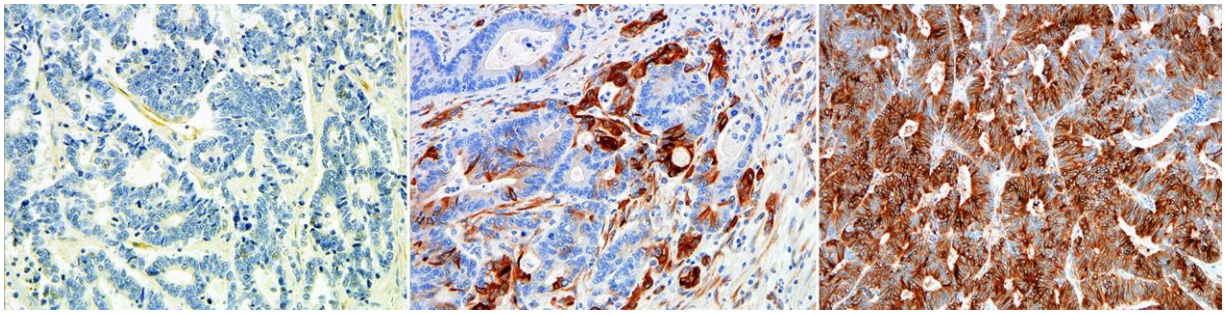
(a) (b) (c)

Figure 9 (a-c) Representative examples of the expression of **ERCC1** protein in colorectal liver metastasis. **a** *Score 0:* no expression detectable or faint partial expression in less than 10% of the tumor cells; **b** *Score 1:* weak to moderate expression of the entire tumor tissue; **c** *Score 2:* strong positivity in the entire tumor tissue, magnification ×400.



(a) (b) (c)

Figure 10 (a-c) Representative examples of the expression of **RRM1** protein in colorectal liver metastasis. **a** Score 0: no expression detectable or faint partial expression in less than 10% of the tumor cells; **b** Score 1: weak to moderate expression of the entire tumor tissue; **c** Score 2: strong positivity in the entire tumor tissue, magnification ×400.



(a) (b) (c)

Figure 11 (a-c) Representative examples of the expression of **TUBB3** protein in colorectal cancer liver metastasis. **a** Score 0: no expression detectable or faint partial expression in less than 10% of the tumor cells; **b** Score 1: diffuse and strong positive staining associated to invasion front and tumor budding, central tumor regions negative or with weaker intensity then at the invasive front; **c** Score 2: strong positivity in the entire tumor tissue, magnification ×400.

5. ERCC1, RRM1 AND TUBB3 IN CORRELATION WITH MMR PROTEINS AND P53

We found statistically significant correlation between ERCC1, RRM1, TUBB3 and MMR proteins, but not with p53. Regarding MMR proteins we found statistically significant correlations between MMR proteins and ERCC1, RRM1 and TUBB3. In turn, none of the three markers had correlation with p53 expression level. MLH1 and MSH2 proteins show positive, statistically significant correlation with ERCC1 ($p<0.000$ and $p=0.008$, respectively). This means in turn loss of MLH1 and MSH2 is associated with lower expression or loss of ERCC1 in colorectal liver metastasis. A similar correlation can be detected between MSH2, MSH6 and

RRM1 (p=0.005 for MSH2 and p=0.011 for MSH6). Higher RRM1 expression levels can be detected at intact expression of MSH2 and MSH6.

Interestingly, TUBB3 expression has strong positive correlation with MLH1 and MSH2 (p=0.019 and p=0.012, respectively). The detailed correlations are listed in **Table 5.**

		MLH1	MSH2	MSH6	PMS2	p53
ERCC1	Correlation coefficient	**0.541****	**0.273****	0.186	0.168	0.164
	Significance (2-sided)	**0.000**	**0.008**	0.072	0.106	0.116
	Number of valid cases	71	94	94	94	93
RRM1	Correlation coefficient	0.104	**0.283****	**0.261***	0.198	0.146
	Significance (2-sided)	0.387	**0.005**	**0.011**	0.055	0.160
	Number of valid cases	71	95	95	95	94
TUBB3	Correlation coefficient	**0.271***	**0.252***	0.025	0.033	0.035
	Significance (2-sided)	**0.019**	**0.012**	0.808	0.748	0.736
	Number of valid cases	75	99	99	99	98

** The correlation is significant at the level of 0.01 (2-sided)
* The correlation is significant at the level of 0.05 (2-sided)

Table 5 Statistical correlations between ERCC1, RRM1 and TUBB3 with MMR proteins and p53.

6. ARC PROTEIN EXPRESSION IN CORRELATION WITH TUBB3 AND RRM1 PROTEINS

Cytoplasmic ARC staining intensity is strongly correlated with TUBB3 and RRM1 expression levels. In negative RRM1 cases the ARC cytoplasmic expression was also low (score 0/1)(6/10 – 60%). Cases with moderate RRM1 expression (score 1) have in the majority also low level of ARC expression (19/33 – 57.6%). 14 of 33 cases (42.4%) with moderate RRM1 expression had high level of cytoplasmic ARC (score 2/3). In cancers expressing RRM1 at high levels (score 2 – 51 cases) show in majority of the cases elevated cytoplasmic ARC levels (low ARC level in only 12 cases (12/51 – 23.53%), high ARC level in 39 cases (39/51 – 76.47%). In conclusion, it can be stated, that high level of RRM1 expression in most of the cases occurs with elevated, high level cytoplasmic ARC protein expression (score 2/3 in 76.47% of the valid cases). Cytoplasmic

ARC protein expression showed a positive, statistically significant correlation with RRM1 expression levels (p<0.000). 98 cases were valid for both proteins (ARC cytoplasmic and TUBB3). In TUBB3 negative cases (Score 0) cytoplasmic expression of ARC were detected in 15 cases (15/35 – 42.85%). In TUBB3 score 1 cases it was even higher (35/51 – 68.62%). In strongly diffuse positive TUBB3 cases the highest cytoplasmic ARC expression was found (9/12 – 75%). We found a progressive staining intensity for cytoplasmic ARC regarding TUBB3 status. This association was also significant (p<0.001). The distribution of the statistical results is listed in **Table 6**.

7. CORRELATIONS BETWEEN ERCC1, RRM1, TUBB3 AND CLINICAL DATA

Concerning clinical parameters like: age, gender of the patients, grading of the tumor and the number and size of metastases, there was no significant correlation to ERCC1 or RRM1 expression (**Table 7**). Regarding the patients` age, there is a strong negative correlation with TUBB3 expression level (p=0.008). In addition TUBB3 expression level was also positively associated to tumor grade (p=0.047) and TUBB3 expression correlates also with RRM1 expression level (p=0.022).

		Cytoplasmic ARC expression	Nuclear ARC expression
ERCC1	Correlation coefficient	-0.053	-0.020
	Significance (2-sided)	0.613	0.851
	Number of valid cases	93	93
RRM1	Correlation coefficient	**0.378****	0.147
	Significance (2-sided)	**0.000**	0.156
	Number of valid cases	**94**	94
TUBB3	Correlation coefficient	**0.323****	-0.048
	Significance (2-sided)	**0.001**	0.641
	Number of valid cases	**98**	98

** The correlation is significant at the level of 0,01 (2-sided)
* The correlation is significant at the level of 0,05 (2-sided)

Table 6 Results of statistical analysis between apoptosis repressor ARC protein, ERCC1, RRM1 and TUBB3.

		Age	Sex	Tumor grade	Number of metastasis
ERCC1	Correlation coefficient	0.106	-0.205	-0.010	-0.030
	Significance (2-sided)	0.320	0.054	0.930	0.781
	Number of valid cases	90	89	82	86
RRM1	Correlation coefficient	0.004	0.001	0.151	0.027
	Significance (2-sided)	0.971	0.995	0.174	0.803
	Number of valid cases	91	90	83	87
TUBB3	Correlation coefficient	**-0.269****	-0.139	**0.213***	0.026
	Significance (2-sided)	**0.008**	0.178	**0.047**	0.807
	Number of valid cases	96	95	87	92

** The correlation is significant at the level of 0,01 (2-sided)
* The correlation is significant at the level of 0,05 (2-sided)

Table 7 Results of statistical analysis between ERCC1, RRM1 and TUBB3 and clinical data.

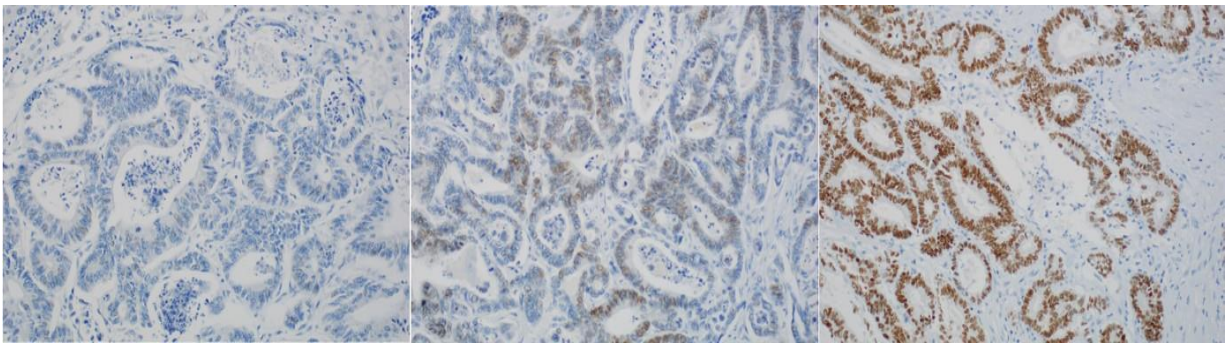
8. CDX2 EXPRESSION AND ITS CORRELATION WITH CLINICAL DATA

We could reach valid expression data for CDX2 (**Table 8**) in 83 of 101 cases. 32 cases (38.55%) show no nuclear expression. Positive stainings (61.45%, n=51/83) can be subdivided into two groups: moderate nuclear expression with score 1 (16.87% n=14); and strong positivity with score 2 (44.58% %, n=37). Representative photomicrographs of CDX2 immunohistochemistry are depicted in **Figure 12.**

Concerning clinical parameters like: age, gender of the patients, grading of the tumor and the number of metastases, there was no significant correlation to CDX2 expression. Regarding the size of the metastasis a strong negative correlation could be detected (p=0.038). In addition to CDX2, ERCC1 expression was also strongly correlated with the size of the metastases (p=0.027). Bigger metastasis size diameter was seen in cases with CDX2 and ERCC1 loss.

	Score 0	Score 1	Score 2	Nr. of valid cases
CDX2	32 (38.55%)	14 (16.87%)	37 (44.58%)	83 (100%)
nuclear APC	62 (61.38%)	39 (38.62%)	----	101 (100%)
cytoplasmic APC	13 (12.87%)	75 (74.26%)	13 (12.87%)	101 (100%)
cytoplasmic β -catenin	37 (38.14%)	60 (61.86%)	----	97 (100%)
nuclear β -catenin	60 (61.86%)	21 (21.65%)	16 (16.49%)	97 (100%)

Table 8 Distribution of immunostaining results of CDX2, APC and β-catenin.



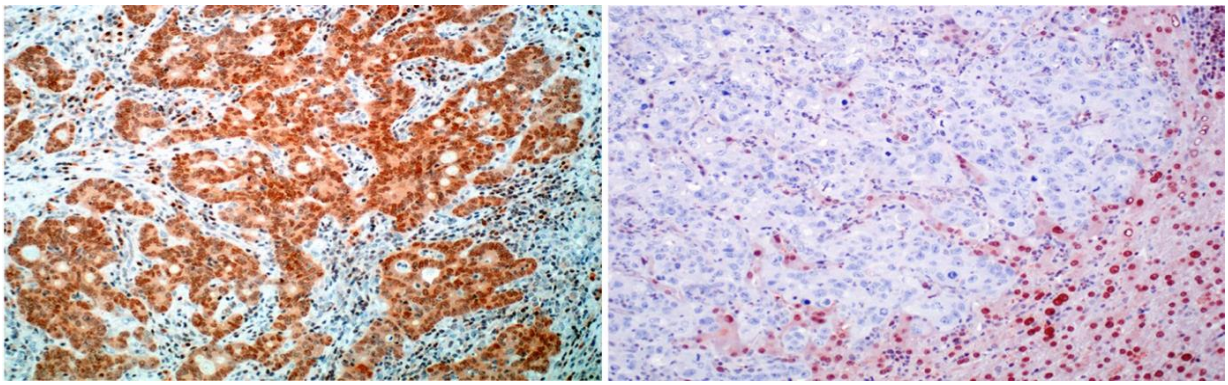
(a) (b) (c)

Figure 12 (a-c) Representative examples of the expression of **CDX2** protein in colorectal liver metastasis. **(a)** *Score 0:* negative staining – less than 10% of the nuclei are stained; **(b)** *Score 1*: moderate overexpression in less than 75% of the nuclei; **(c)** *Score 2:* strong overexpression in almost all tumor cells, magnification ×200.

9. EXPRESSION DISTRIBUTION OF DNA REPAIR PROTEINS AND PROTEINS OF WNT-SIGNALING

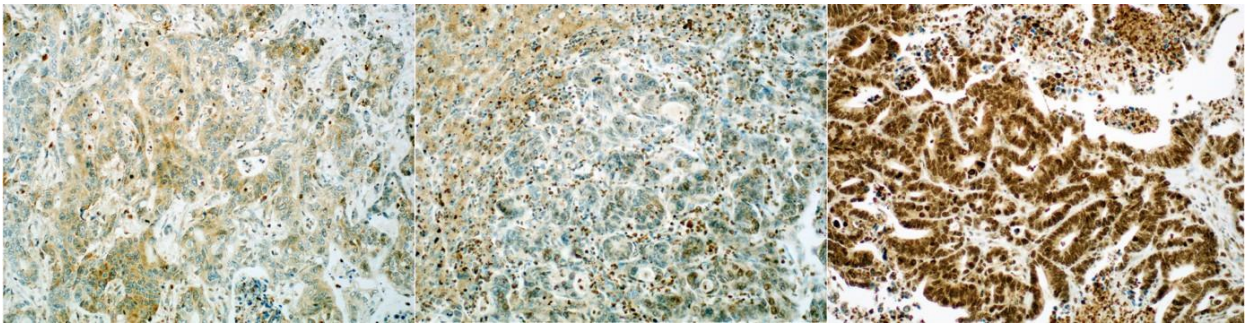
For MGMT 97 valid cases were obtained. Loss of MGMT expression was found in 24 cases (24.75%). Representative photomicrographs of MGMT immunohistochemistry are depicted in **Figure 13.** Nuclear positivity was sustained in 73 cases (75.25%). Out of 94 valid cases for ERCC1 we found 29.8% of the cases negative (score 0). Positive ERCC1 staining could be in 70.2% of the cases detected (30.8% score 1 and 39.4% score 2). Representative photomicrographs of ERCC1 immunohistochemistry are depicted in **Figure 14.** Both MGMT and ERCC1 loss is strongly associated with female gender ($p=0.011$, and $p=0.047$, respectively).

Regarding mismatch repair proteins, the following distribution was seen: loss of expression was detected in 4.2% to 26% of the cases (MLH1 4.2%, MSH2 26%, MSH6 24% and PMS2 9.5%, respectively) as published before [60]. Loss of PMS2 is associated with loss of MGMT ($p=0.014$) and loss of MLH1 and MSH2 were also associated with loss of ERCC1 ($p<0.01$, and $p<0.01$, respectively). Representative photomicrographs of MMR protein immunohistochemistry are depicted in **Figure 15.** Expression distribution of β-catenin, and APC proteins are depicted in **Table 8.**



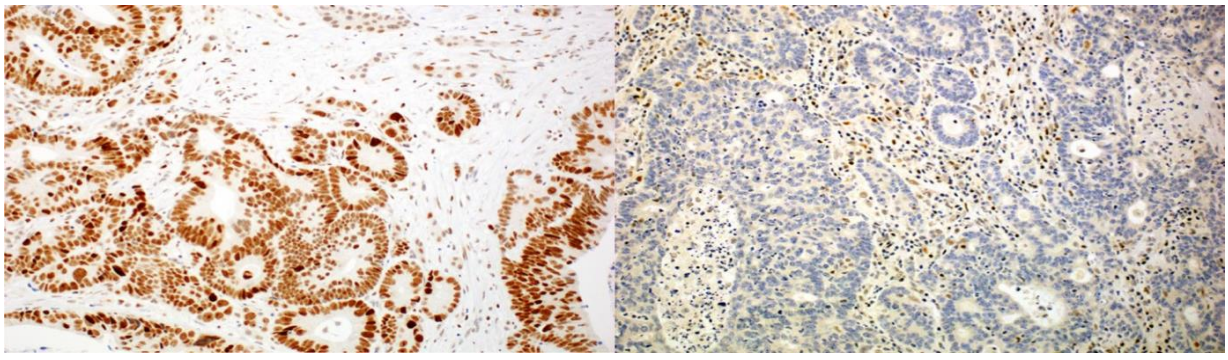
(a) (b)

Figure 13 (a-b) Representative examples of **MGMT** protein expression in colorectal liver metastasis. **(a)** Score 0: negative staining – less than 10% of the nuclei are stained; **(b)** Score 1: more than 10% percent of the nuclei are positive. Note that in negative cases stromal cells, lymphocytes and hepatocytes are still nuclear positive, magnification ×200.



(a) (b) (c)

Figure 14 (a-c) Representative examples of the expression of **ERCC1** protein in colorectal liver metastasis. **(a)** *Score 0:* no expression detectable or faint partial expression in less than 10% of the tumor cells; **(b)** *Score 1:* weak to moderate expression of the entire tumor tissue; **(c)** *Score 2:* strong positivity in the entire tumor tissue, magnification ×200.



(a) (b)

Figure 15 (a-b) Representative examples of MMR protein expression (MLH1 staining) in colorectal liver metastasis. **(a)** Score 0: negative staining – less than 10% of the nuclei are stained; **(b)** Score 1: more than 10% percent of the nuclei are positive. Note that in negative cases stromal cells are still positive, magnification ×200.

10. STATISTICAL CORRELATIONS BETWEEN CDX2 AND DNA REPAIR PROTEINS

We found statistically strong positive correlation between CDX2 and all of analysed DNA repair proteins (**Table 9**). These results mean that loss of CDX2 expression is strongly associated with loss of expression of DNA repair proteins (MMR proteins, MGMT and ERCC1).

		Tumor size (mm)	DNA repair proteins					
			MLH1	MSH2	MSH6	PMS2	MGMT	ERCC1
CDX2	Correlation coefficient	-0.247*	0.388**	0.334**	0.317**	0.228*	0.236*	0.574**
	Significance (2-sided)	0.038	<0.001	0.002	0.004	0.040	0.039	<0.001
	Number of valid	71	77	82	82	82	77	74

** The correlation is significant at the level of 0.01 (2-sided)
* The correlation is significant at the level of 0.05 (2-sided)

Table 9 Results of statistical analysis between CDX2 and tumor size and DNA repair proteins.

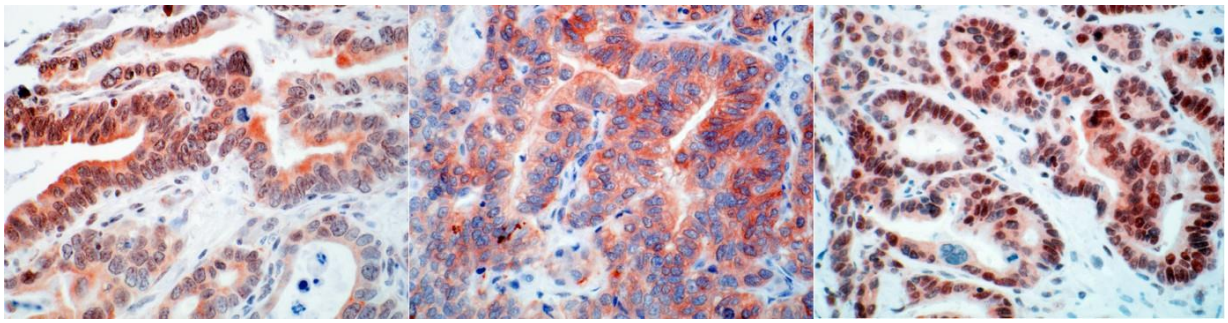
11. STATISTICAL CORRELATIONS BETWEEN CDX2, APC AND β-CATENIN

We analysed the possible statistical correlation between CDX2 and β-catenin, and APC (**Table 10**). Cytoplasmic, but not nuclear β-catenin expression is associated with sustained nuclear CDX2 expression (p=0.042). In addition, CDX2 is positively correlated with nuclear APC expression (p<0.01). Cytoplasmic and nuclear β-catenin is associated also positive with each other (p<0.01). Representative photomicrographs of APC and β-catenin immunohistochemistry are depicted in **Figure 16** and **Figure 17**, respectively.

		Membraneous/cytoplasmic β -catenin	Nuclear β -catenin	Cytoplasmic APC	Nuclear APC
CDX2	Correlation coefficient	0.231*	0.152	0.065	0.415**
	Significance (2-sided)	0.042	0.183	0.567	<0.001
	Number of valid cases	78	78	79	79

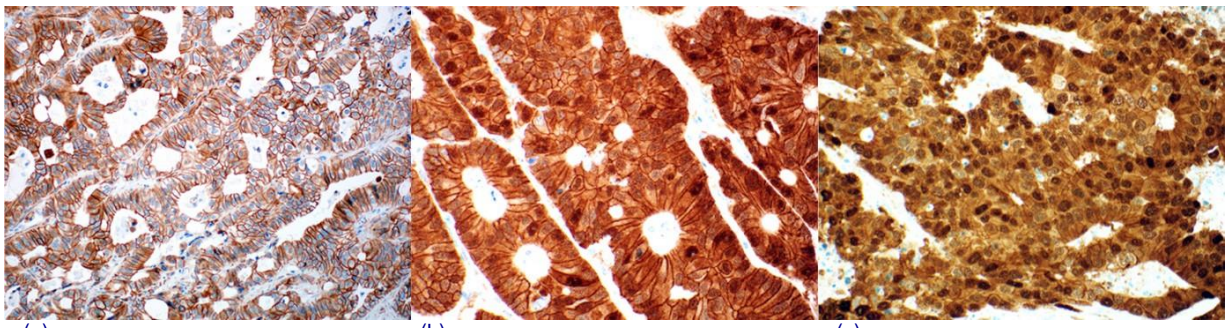
** The correlation is significant at the level of 0.01 (2-sided)
* The correlation is significant at the level of 0.05 (2-sided)

Table 10 Results of statistical analysis between CDX2, APC, and β-catenin.



(a) (b) (c)

Figure 16 (a-c) Representative examples of **APC** protein expression in colorectal liver metastasis. **(a)** cytoplasmic and nuclear expression of APC protein; **(b)** only cytoplasmic APC positivity in the tumor cells; and **(c)** strong nuclear positivity next to faint cytoplasmic stain, magnification ×400.



(a) (b) (c)

Figure 17 (a-c) Representative examples of β-catenin expression in colorectal liver metastasis. **(a)** only membranous staining are seen (score 0 for both cytoplasm and nucleus) **(b)** cytoplasmic expression of β-catenin with some positive nuclei (score 1 for cytoplasm and nuclei) **(c)** diffuse and strong cytoplasmic and nuclear staining (score 1 for cytoplasm and score 2 for nuclei), magnification ×200.

DISCUSSION

Apoptotic signaling is one of the most important processes in therapeutic resistance. Besides known regulatory proteins there are many others, which can influence the apoptotic process and some of them can thus enhance or inhibit the therapy effects. Beside well-known regulatory proteins many more can influence the apoptotic signalling and thus enhance or inhibit therapy effects. Dysregulation in apoptotic signaling is a common event in colorectal cancers and in its liver metastasis. Mechanisms involved in apoptosis are important therapeutic targets (i.e. Bcl-2 inhibitors) [67]. Besides the known classical apoptotic regulatory proteins many others exist which have influence on the effectiveness of apoptosis, such as mismatch repair (MMR) proteins and p53. Loss of MMR proteins has been associated with defected apoptotic signaling or therapy resistance [14]. It is known that cancer cells can suppress apoptosis decreasing the level of pro-apoptotic proteins and increasing the level of apoptosis inhibitors. Many caspases have a decreased level in lung, breast or colon cancer, whereas survivin or Bcl-2 and Bcl-X_L are increased in colon cancer and associated with a worse prognosis [63, 68].

1. ARC, p53 AND MMR PROTEIN EXPRESSION IN COLORECTAL CANCER LIVER METASTASIS

The role of apoptosis in CRC metastases and drug resistance is still unclear. One potential therapeutic target is apoptosis repressor with caspase recruitment domain (ARC). ARC protein is expressed in stable tissue, i.e. neurons, skeletal and cardiac muscle fibres [40], as well as in carcinomas of different origins, like ovarian cancer, colon cancer or cervical cancer [41]. ARC is known to be induced by Ras protein and repressed by p53 [44] and is involved in the inactivation of extrinsic as well as intrinsic apoptosis pathways, by interacting with pro-apoptotic proteins like p53, Bcl2, Bax, Bad, Puma, MSH2, MSH6, and others [69]. In breast cancer cell lines, high levels of cytoplasmic ARC were linked with treatment resistance (doxorubicin and γ-radiation induced cell death) [42]. Taken together, abundant expression of ARC in cancer cells can promote cell survival by protecting cancer cells from apoptosis and may play an important role in therapeutic resistance [42]. In our first study, ARC expression level in colorectal cancer liver metastasis was independent from clinical data (i.e. age, gender, tumor size, tumor number or mucin production) but strongly correlated with MSH2 and MSH6 expression, which further supported the evidence for the regulatory role of MSH2 and MSH6 in apoptosis [60].

In our study we demonstrate the expression pattern of ARC in colorectal cancer liver metastasis and its correlation with other known member of apoptosis regulation. This is the first study that analyses the subcellular localisation of ARC in colorectal liver metastasis. Furthermore, we were able to show a significant correlation between ARC and other, indirect regulators of the apoptotic signals, such as MSH2, MSH6. It is possible that cytoplasmic ARC is responsible for the inhibition of extrinsic and intrinsic apoptotic signaling interacting with other apoptotic proteins.

Some nuclear functions of ARC have been already discovered (**Figure 18** and **Figure 20**) but the exact role of nuclear ARC protein in colon cancer, and in all other cancer types, remains still unclear [70]. Furthermore, our findings that nuclear ARC expression is significantly associated with MSH2 and MSH6, but not with Mlh1 and Pms2 also have to be elucidated. One explanation could be that defected MSH2 or MSH6 protein lose their pro-apoptotic capability, thus a significantly lower level of nuclear ARC is needed to repress apoptosis. But there is still the question why nuclear ARC expression is associated with this phenomenon. This question cannot be explained by means of immunohistochemistry alone. Further studies are needed to explore the relation between MMR system and ARC regulation. This is important, because ARC is a potential therapeutic target and with MMR system together can be responsible for chemo- and radioresistence.

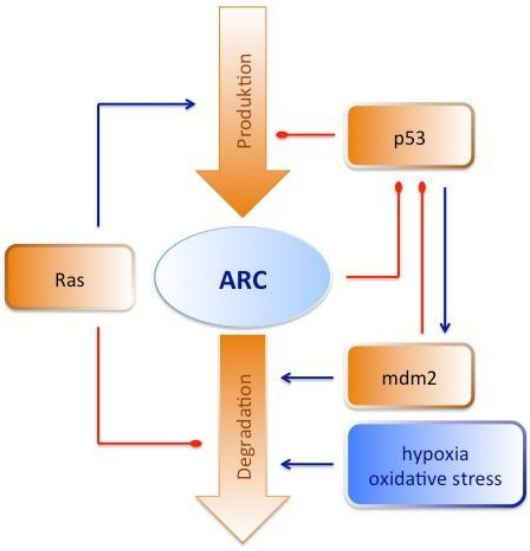


Figure 18 Regulation of ARC expression. ARC production is promoted by Ras signaling pathway and inhibited by p53 [69].

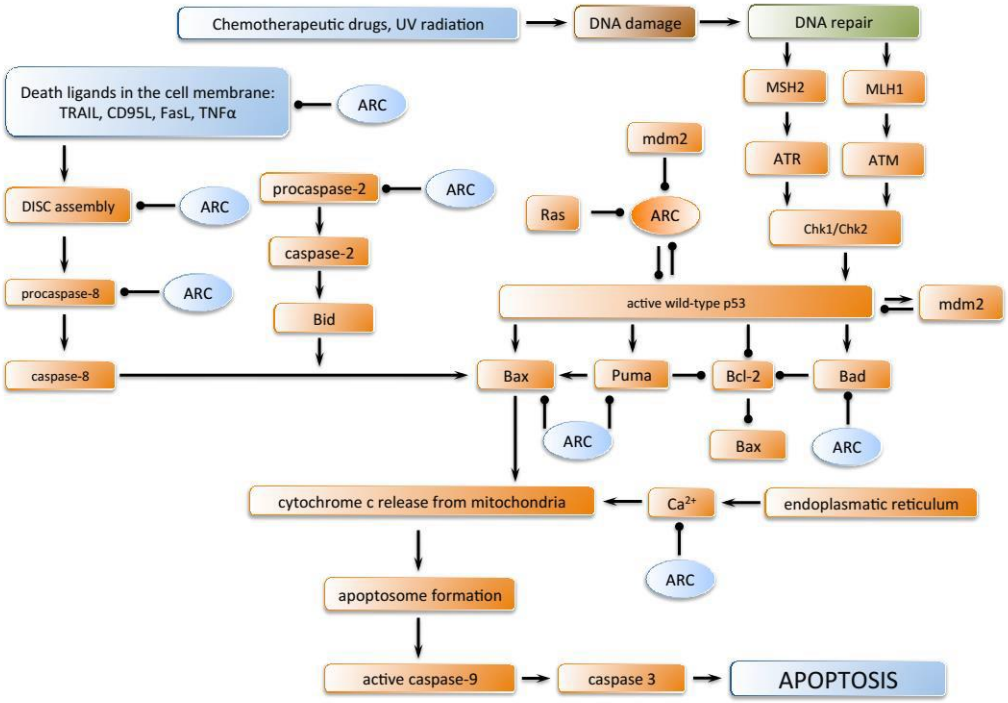


Figure 19 Simplified diagram of pathways involving ARC. It can be seen that ARC involved in various pathways lead to apoptosis: ARC inhibits plenty of proteins (i.e. DISC partners, procaspase-8 and -2, Bax, Puma and Bad) thus hindering apoptosis. Some regulatory mechanism also depicted: Mdm2 and p53 inhibit ARC functions, through transcriptional repression and enhancing its degradation. Furthermore, it is also known, that Ras protein promotes ARC production and hinder its degradation [14, 44, 69, 71, 72].

ARC is a unique protein that inhibits both the extrinsic (death receptor mediated) and intrinsic (mitochondrial/ER stress induced) apoptotic pathways. ARC binds the components of both apoptotic pathways interacting with them in a non-homotypic death-fold manner [43]. ARC is expressed normally in terminally differentiated, so-called stable tissues; such as cardiac and skeletal muscle or nervous tissue [42, 63]. ARC with its CARD binds to death receptors, Fas, FADD and pro-caspase-8, inhibiting the assembly of DISC, thus abrogating the extrinsic apoptotic signaling. ARC has two different ways to inhibit the intrinsic apoptotic mechanism: through direct binding Bax with its CARD (that inhibits the conformational activation and

mitochondrial translocation of Bax) or direct binding to p53 precluding the tetramerisation of p53, thereby inducing a nuclear transport signal which relocates p53 to the cytoplasm [63]. In case of ARC knockdown, assembly of death-inducing signaling complex (DISC) will be facilitated and spontaneous Bax activation will be triggered resulting in apoptosis [43, 73].

PUMA and Bad are up-regulated by p53 in response to anoxia or reactive oxygen species (ROS). In addition, ARC interacts with PUMA and Bad through its N terminus. This interaction displaces the association of PUMA or Bad with Bcl-2 [44]. Thus, p53 can repress ARC in a transcription dependent manner. If ARC is repressed by p53, it will lead to failures in counteraction of pro-apoptotic activity of PUMA and Bad [44].

Truncated p53 is not able to exert its transcriptional activity, but still has apoptotic functions and may participate in caspase activity [74]. Distinct pro-apoptotic activities of p53 are clearly established: in cellular stress cytoplasmic p53 translocates to mitochondria, where it interacts with different members of the Bcl-2 family resulting in robust mitochondrial outer membrane permeabilization, followed by release of caspase activation and chromatin degradation [74, 75]. We assume that this is the reason why we could not find statistically relevant significance between p53 and ARC expression levels. Overexpression of p53 in the nuclei, as an evidence for the mutated protein, may not be appropriate to detect its apoptotic activity.

ARC can inhibit apoptosis almost independently from the inducing cause, such as death receptor activation, hypoxia, hydrogen peroxide, oxidative stress, serum deprivation, ischemic reperfusion, doxorubicin or γ-radiation [40, 42, 43, 73, 76]. The fact that ARC inhibits both extrinsic and intrinsic apoptotic pathways can provide a growth advantage to cancer cells. High level of ARC protein in breast cancer cells is associated with chemo- and radioresistence [42, 73]. In addition, ARC has influence not only on extrinsic and intrinsic apoptotic pathways, but also on TNFα-induced regulated necrosis: ARC interacts with TNF receptor 1 (TNFR1) that interferes with recruitment of RIP1 suggesting a wider role of ARC in cell death [77]. ARC overexpression could be detected in a couple of cancer types and cancer cell lines and in colorectal cancer cells. Its expression level is correlated inversely with the apoptotic activity in response to chemotherapy [63]. One of these studies analysed the ARC expression in breast cancer and found not only cytoplasmic, as in normal stable tissues, but also nuclear translocation of ARC [42]. Mercier et al. found cytoplasmic ARC positivity in invasive breast cancer, normal breast tissue and in reduction-mammoplasty (89%, 21% and 10%, respectively) [42]. They could

not detect any correlation between ARC expression level and the histopathological grade of tumors. We used a similar scoring system for ARC, and we could not detect any correlation between ARC expression and histopathological grade of CRC liver metastasis either.

Colorectal cancer showed decreased apoptosis compared to normal colon mucosa [78]. ARC levels in colon cancer can be detected up to 5.8-fold higher [63]. Cytoplasmic ARC expression is more common than nuclear (94.7% and 31.6% respectively). In one study on colorectal cancer (including cell lines, normal mucosa and cancer of different grades) highly elevated cytoplasmic and moderately elevated nuclear ARC levels could be detected, compared to normal mucosa and normal colon cell lines. The cytoplasmic overexpression was associated with higher grades, whereas the nuclear ARC expression was the highest in cancers with moderate grade [63]. Kitsis et al. have found similar levels of ARC in all analysed colon cancers [63]. In contrast to Kitsis et al. we could detect at least three distinct levels of ARC expression in liver metastasis of colorectal cancer. However, we found no correlation between ARC expression levels and clinical data (i.e. age, gender, tumor size, tumor number or mucin production). In our study, cytoplasmic and nuclear ARC expression had even no association with histopathological grade (p=0,692 and p=0,858, respectively).

2. ERCC1, RRM1 AND TUBB3 EXPRESSION IN COLORECTAL CANCER LIVER METASTASIS

ERCC1, RRM1 and TUBB3 are known to have therapeutic predictive value in current therapy of metastasized colorectal cancer [13, 18, 19]. In this study we investigated the expression levels of ERCC1, RRM1 and TUBB3 in liver metastasis of colorectal cancer and analysed their associations to sex, age, tumor grade, mucin production, tumor size and number of metastasis. The primary interest of our study was to evaluate the expression manner of ERCC1, RRM1 and TUBB3. Furthermore, we investigated their correlation to MMR proteins, p53 and apoptosis repressor ARC. For patients with metastatic colorectal cancer, the majority of cytotoxic chemotherapy is given without any predictive biomarker analysis. However, a tremendous molecular diversity exists in colorectal cancer and the defining of tumor subgroups which are more or less likely to response to specific chemotherapy regimes is important. Thus, administration of a chemotherapeutic regime according to its expected patient benefit should be an integral part of future therapeutic decisions.

In our collective, ERCC1 protein loss was detected in one third of the cases (29.8%). A share of 70.2% showed positive reaction and score 2 (strong nuclear expression) was confirmed in 39.4% of all valid cases. In one another study, which investigated stage III colorectal cancer, ERCC1 and MSI levels it was found to be positive in 55% and 17%, respectively [20]. According to literature data the score 2 cases would not benefit from platinum-based chemotherapy. On the other hand, one third of the cases (loss of ERCC1) would have a better prognosis with platinum-based chemotherapy [58]. Cases with moderate ERCC1 expression (score 1), to our opinion, need further investigation: it has to be evaluated in functional studies whether a mild loss in ERCC1 expression is enough to let cancer cells sensitise to platinum-based chemotherapy. According to our scoring system, we can detect the certain negative and positive cases, thus allowing a prediction for platinum-based chemotherapy in two third of the patients after a single immunohistochemistry.

We found statistically significant positive correlation between ERCC1 and MLH1, MSH2 ($p=0.000$ and $p=0.008$, respectively). The frequent loss of ERCC1 and MLH1 both could be explained by methylation. Similar correlations were found in mesothelioma [79]. Microsatellite instability did not affect the resistance to oxaliplatin, which formed DNA adducts not recognizable by MMR machinery [80]. While MMR status had no relevant predictive value for oxaliplatin or 5-FU therapy used for stage III colorectal cancer, ERCC1 status is highly predictive which patient will benefit from addition of oxaliplatin to 5-FU in stage III colorectal cancer [20].

In NSCLC ERCC1 nuclear staining was in 45.59%, TUBB3 cytoplasmic staining was in 65.44% of the cases detected. ERCC1 and TUBB3 double negative had better therapeutic response to platinum-based therapy [13]. We found 11 cases (11.7% of valid cases) being double negative for ERCC1 and TUBB3. Analogous to other epithelial neoplasia, 11.7% of the metastatic cases could benefit much more from platinum-based therapy, than the others. Anyhow, literature data are even in well analysed lung cancer collectives still controverse: one study found that ERCC1 protein expression level has no correlation with age, gender, smoking status or recurrence pattern or response rate to chemotherapy. The response rate is in ERCC1 positive cases 20%, while in negative cases 28% (no significant differences could be detected)[18]. In contrary, another study found that ERCC1 and TUBB3 have predictive value in non-small cell lung cancer treated with platinum-based therapy. Negative expression of ERCC1 and TUBB3 are associated with significantly higher response rate, longer median progression-free survival and overall survival

after platinum-paclitaxel treatment [13]. One study found that the advanced colorectal cancer with high expression of ERCC1 should not accept oxaliplatin-based chemotherapy [61]. Whereas patients with low levels of ERCC1 expression have been reported to have an improved response and a longer overall-survival in metastatic colorectal cancer treated with FOLFOX combination [62]. In this study low level of ERCC1 was detected in 90% of the cases [62]. One study, in consistence with other study results, could demonstrate the potential utility of ERCC1 expression as prognostic and possibly predictive biomarker in metastasized colorectal cancer. It could identify a population with poor prognosis, as well as a population with remarkably high response rate to FOLFOX therapy combination [62]. The cases were evaluated according to a pre-established cut off for ERCC1 [61].

As seen, the expression levels of ERCC1 are not always consistent with sensitivity to platinum-based treatment. We assume that cut off levels were set too high and suggest to define more than only two groups (positive and negative). Therefore, we propose large prospective or retrospective studies with standard chemotherapy analyzing the expression of predictive markers, such as ERCC1, RRM1 or TUBB3 (and its interaction partners) to set a cut off for cases, which can certainly benefit from a therapy regime. Too high or too low cut of levels result in subsets of patients who will suffer from long-term toxicity with no benefit of treatment on one hand, and patient groups that do not receive the optimal therapy regime on the other.

RRM1 suppresses metastasis formation and influences cell survival through induction of PTEN expression [22]. PTEN is an inhibitor of cell proliferation and suppresses cell migration and invasion by reducing the phosphorylation of FAK [21]. Overexpression of RRM1 inhibits in vivo metastasis formation, which requires increased expression of the tumor suppressor gene PTEN. The mechanism by which RRM1 induces expression of PTEN is unclear. One possible connection between RRM1 and PTEN is through TGFβ [22].

RRM1 overexpression is associated with gemcitabine resistance. RRM1 is a key molecule for gemcitabine efficiacy and is also involved in tumor progression. High RRM1 expression in tumor tissue predicts significantly better prognosis, whereas only patients with low RRM1 benefit from gemcitabine therapy. In turn, overexpression of RRM1 protein is strongly associated with gemcitabine resistance [21]. RRM1 expression was also reported to correlate with the tumorigenic and metastatic potential in lung cancer [21]. High level of RRM1 expression was observed in 53.7% of the cases and 26.5% of the valid cases showed strong expression not only for RRM1 but also for ERCC1.

In pancreatic adenocarcinoma patients, cases with low RRM1 expression will benefit from gemcitabine therapy. In turn, it is expected that over 50% of colorectal cancer patients will show no therapeutic benefits and will only suffer from side effects. According to our data, only 11.6% of the cases with loss of RRM1 expression have the potential to bear positive response to gemcitabine. In other words, patients with high RRM1 protein expression in tumor tissue may be treated with other drugs as well, i.e. oxaliplatin, 5-FU and leucovorin (CONKO-003) instead of gemcitabine [21]. As mentioned above, the score 1 cases need further functional analysis and maybe the lower RRM1 activity will also be enough not to overcome gemcitabine-induced DNA damage leading to death in the tumor cells.

Loss of MSH2 and MSH6 expression are associated with lower levels of RRM1 protein ($p=0.005$ and $p=0.011$, respectively). For this association the following mechanisms could be responsible: (1) RRM1 is a key enzyme in synthesis of new DNA, thus defected MMR proteins, i.e. MSH2 and MSH6 lead to DNA damage which can downregulate the new DNA synthesis, leading to cell cycle arrest. One possible connection between RRM1 and MMR system is through the DNA damage: DNA damage leads to cell cycle arrest, which results in lower RRM1 expression level or (2) there is a possible connection through TGF-β: in normal cells TGF-β can activate MSH2 promoter (through Smad p53 dependent mechanism), whereas at posttranscriptional level, miR-21 induced by TGF-β targets MSH2 transcript and suppresses its expression. In contrast, in cancer cells p53 is inactivated and miR-21 is overexpressed, thus TGF-β fails to activate MSH2 promoter resulting in genomic instability [81].

RRM1 and ERCC1 expression levels had no significant differences in respect to age, sex, histopathological grade, tumor size, T stage or lymph node status [21]. Furthermore, in regard to disease-free survival, high ERCC1 expression was significantly associated with better outcome. In low and high RRM1 expression groups, no significant difference could be detected, but only patient with low RRM1 expression benefited from gemcitabine therapy [21]. In pancreatic cancer, the expression level of RRM1 and ERCC1 found to affect the clinical outcome similar to that described in non-small cell lung cancer [21].

Cytoplasmic ARC protein expression showed a positive, statistically significant correlation with RRM1 expression levels ($p<0.000$), which leads to higher RRM1 expression with ARC overexpression or in turn how ARC expression can induce RRM1 overexpression should be elucidated in functional studies, but it is known that RRM1 overexpressing cells have an

increased level of apoptosis [82], thus it is possible that a certain overexpression level can induce apoptosis signaling, which in turn induce ARC expression to suppress apoptosis induction. This possibility is further strengthened by positive correlation between RRM1 and TUBB3 found in our collective ($p=0.022$).

The main staining manner for TUBB3 was the expression at the invasion front, similar to primary colorectal cancer studied before [37]. No TUBB3 expression was detected in 35% of the valid cases – these cases are potential candidates for taxane-based chemotherapy with highly predicted response. In our collective we found statistically significant correlation between MLH1, MSH2, and TUBB3 ($p=0.019$ and $p=0.012$, respectively), which further strengthens the evidence for regulatory role of mismatch repair proteins in apoptosis. In case of sufficient MLH1 and MSH2 expression TUBB3 is significantly higher expressed to suppress the activities of MLH1 and MSH2.These results can mean that defected MMR system would induce TUBB3 overexpression leading to MT rearrangement, which can influence apoptosis (i.e. activating pro-apoptotic signaling proteins). Microtubules (MT) have an important role in apoptosis, i.e. surviving is believed to regulate apoptosis by controlling microtubule polymerization. Thus, the disruption of normal MT function (either increasing or decreasing MT length) may trigger apoptosis. MT system (and thus TUBB3) has an important role in the regulation of DNA damage-induced apoptosis. DNA damage (i.e. γ-radiation) induces α-, β-, and γ-tubulin production and polymerization and stimulates MT reorganization [83]. One explanation is that DNA damage through cyclin B1 and cdc2 kinase activation leads to tubulin polymerization and to release of apoptosis [83]. The possible connection between TUBB3, DNA damage and mismatch repair are depicted in **Figure 20**.

Furthermore, MLH1 and MSH2 are responsible for resistance to cisplatin or methylating agents. The defective MMR system cannot recognize the cisplatin-induced DNA damage resulting in cell survival and therapy resistence [14, 15]. Taken together, cases with defected MMR system (microsatellite instable cancer) and with high expression level of TUBB3 are potentially resistant not only for taxanes, but also for platinum-based therapy. Thus, we favorise in case of MSI the immunohistochemical testing also for TUBB3 to exclude taxane resistance. The interactions between DNA repair systems, microtubules and apoptotic proteins (i.e. ARC) should be further investigated to elucidate resistance mechanism of tumor cells and survival regulatory mechanism.

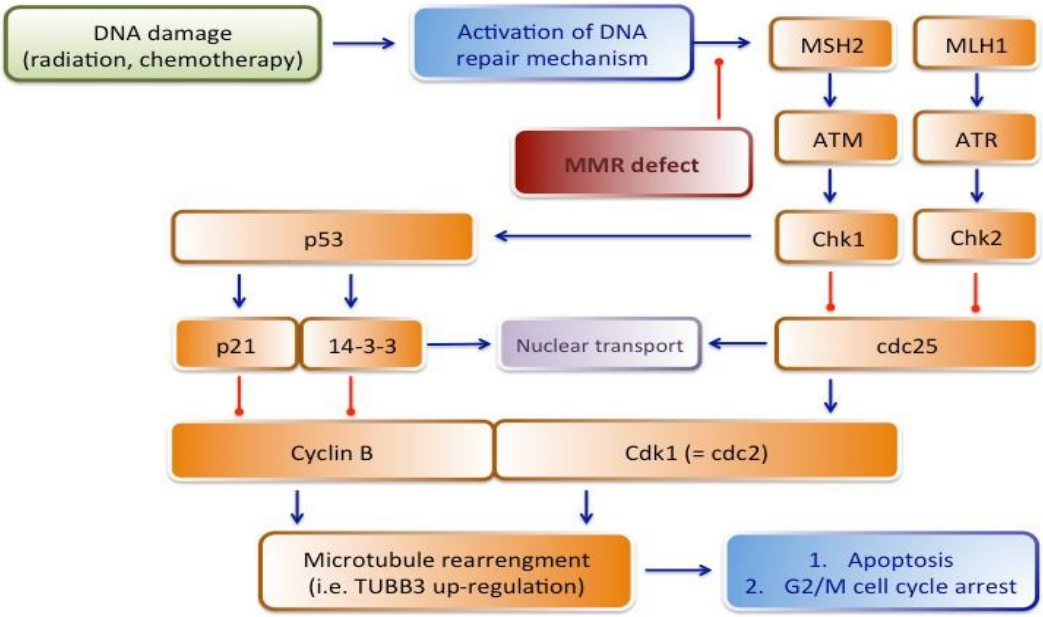


Figure 20 Schematic presentation of DNA damage induced apoptosis and the possible role of microtubule system. After DNA damage ATM/ATR signaling pathway is activated and phosphorylates (and activates) Chk1 and Chk2, which subsequently phosphorylates cdc25 [83]. Phosporylated cdc25 is sequestered in the cytoplasm by 14-3-3 proteins, which hinder the activation of cyclin B1/Cdk1 complex by cdc25 and results in G2/M cell cycle arrest. In case of MMR loss (i.e. in our cases, the loss of MLH1 and MSH2) the ATM/ATR system cannot be activated by MMR proteins and finally do not lead to cell cycle arrest. Consequently, microtubule rearrangement and TUBB3 upregulation is missed. This correlation between MLH1, MSH2 and TUBB3 is even statistically significant in our collective (p=0.019 and p=0.012, respectively).

In addition and alternatively to above mentioned mechanisms the statistically significant correlation between TUBB3 and cytoplasmic ARC expression (p=0.001) can be also explained: survival feedback mechanism can induce ARC expression to suppress the pro-apoptotic signaling, thus cancer cells can survive despite of DNA damage (i.e. microsatellite instability). In our previous study we found strong correlation between ARC expression level and MSH2 status [60]. TUBB3 overexpression can stabilize the MT system and make cancer cells resistant against anti-microtubuli agents. Direct interaction between tubulin with several members of the

Bcl-2 family has been described. Bcl-2, Bid and Bad inhibit the assembly, whereas Bak and Bax promote tubulin polymerization. Thereby, tubulin is localized not only in the cytoplasm, but also bounded to mitochondria (associated to VDAC in mitochondrial membrane). Both pro- and anti-apoptotic proteins bind to tubulin and those of lower affinity are more easily released following a conformational change induced by a ligand. Thus, Bcl-2, Bid and Bad might remain bound while Bax would be released changing the ratio of free pro- and anti-apoptotic proteins. Furthermore, in case of TUBB3 overexpression pro- and anti-apoptotic proteins stay bound, but tubulin ligands can change the affinity towards proteases. In addition, Bcl-2 protects against acetylation of tubulin and Bcl-2 is able to normalize the level of acetylated tubulin [84]. The interaction between TUBB3 and apoptotic proteins (especially between ARC and TUBB3) seems to be more complex. There are many common interaction partners of ARC and TUBB3 and we assume that it depends on intracellular circumstances, which protein effects will dominate. The known interactions between apoptosis proteins, including ARC, and TUBB3 are depicted in **Figure 21.**

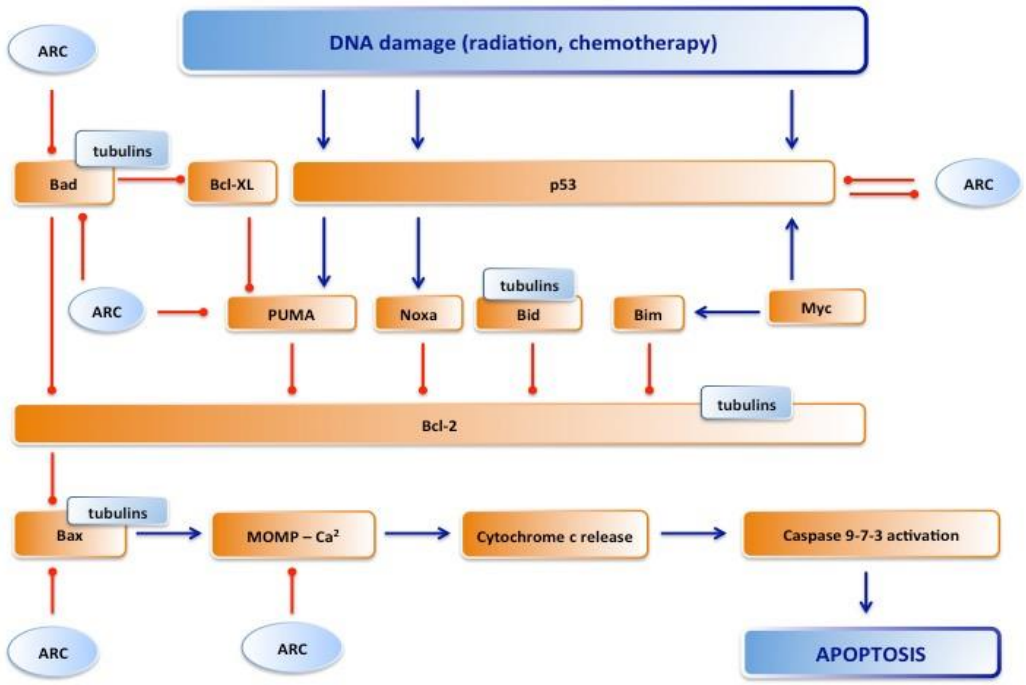


Figure 21 Interactions between tubulins and apoptotic signaling proteins including ARC. As seen, there are many common interaction partners of TUBB3 and ARC. We suggest that is a vulnerable balance influenced by TUBB3 and ARC. The actual cellular homeostasis will decide which mechanism (pro- or anti-apoptotic) overweights. In cancer cells genetic changes (activating mutations, epigenetic silencing etc.) can disturb this balance, leading to sustained suppress of apoptosis, which cannot be overcome even by overexpression of other proteins.

Despite the increasing number of studies that highlight the importance of TUBB3 in tumor cells, its mode of action still needs to be fully determined. It appears that the intrinsic apoptotic pathway was involved as evidenced by increased caspase 3/7 activity [85]. Evidence in other cell types suggests that TUBB3 may be part of a cell survival pathway. For instance, its expression level can be modulated by different types of cell stress, i.e. hypoxia (anti-VEGFR therapy) and nutrient deprivation [86, 87]. To declare the interaction between ARC and TUBB3 functional studies are needed.Testing the expression of ERCC1, RRM1 and TUBB3 is ineligible before treatment for gemcitabine, cisplatin and 5-fluorouracil. As known, MSI tumors will not benefit from 5-FU treatment and – to this analogy – the testing for ERCC1, RRM1 and TUBB3 before platinum- based, gemcitabine and taxane therapy, respectively.

Diagnostic tests are missing to determine which chemotherapy regimen offers the greatest chance for response in an individual patient [62]. For metastatic colorectal cancer, the current treatment paradigm consists of 5-FU based regimens in combination with either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI), potentially combined with therapy targeting either EGFR or VEGFR inhibitor [88]. Large, prospective clinical trials have shown that the response rates for either FOLFOX or FOLFIRI are only around 55% [58]. Thus, there is an urgent need for reliable predictive markers before therapeutic decision in metastasized colorectal cancer.

In gastric cancer, TUBB3 was strongly predictive in recurrent and metastatic gastric cancer cases receiving taxane-based first line palliative chemotherapy (taxane and cisplatin [89]). In contrast, ERCC1 has no clinical impact in these settings [89]. ERCC1 was a predictor of lack of benefit for platinum-based adjuvant chemotherapy [90]. TUBB3 and ERCC1 staining intensities were graded on a scale of 0 to 2 (0 = none, 1 = weak and 2 = strong) using the adjacent non-malignant cells for reference. Thus, showing 52,7% of the cases high level of expression for TUBB3 and 53,4% of the cases high level of expression for ERCC1 – TUBB3 and ERCC1 did not correlate with each other [89].

TUBB3 is highly expressed in pancreatic adenocarcinoma. Silencing of TUBB3 expression resulted in reduced cancer cell growth and tumorigenic potential in the absence and presence of chemotherapeutic drugs. Suppression of TUBB3 reduced tumor growth and metastasis formation in vivo [85]. Interestingly, aberrant TUBB3 expression in PDAC was associated with activation of KRAS and appeared to be progressively up-regulated in pancreatic intraepithelial neoplasm (PanIN) 1 to 3, the precursor lesions of PDAC [91]. Silencing TUBB3 in pancreatic cells lead to 1) decreased clonogenicity, 2) decreased anchorage-dependent and independent proliferation, 3) increased apoptosis and anoikis (= anchorage independent apoptosis), 4) increased sensitivity to chemotherapy drugs including gemcitabine, paclitaxel and vincristine. TUBB3 suppression in

pancreatic cancer cells induced marked increase in apoptosis in the absence of chemotherapy – this was echoed by significant increase in caspase 3/7 activity [85].

Despite the increasing number of studies that highlight the importance of TUBB3 in tumor cells, its mode of action has yet to be fully determined. It is appeared that the intrinsic apoptotic pathway was involved as evidenced by increased caspase 3/7 activity [85]. Evidences in other cell types suggest that TUBB3 may be part of a cell survival pathway. For instance, its expression level can be modulated by different types of cell stress, i.e. hypoxia (anti-VEGFR therapy) and nutrient deprivation [86, 87]. In addition, glycosylated and phosphorylated form of TUBB3 has been identified in mitochondria of cancer cells, thus it may be possible that TUBB3 is involved in modulating apoptosis via the mitochondria [92].

The decrease in anchorage-dependent cell growth was associated with increased anoikis (anchorage-dependent apoptosis), reinforcing the link between TUBB3 silencing and induction of apoptosis in pancreatic cancer cells. Indeed, tumor cells with metastatic potential have developed mechanisms of resistance to this form of apoptosis [93]. Taken together, these data demonstrate that TUBB3 is important in providing pancreatic cancer cells with a key survival advantage, thus allowing them to grow and metastasize [85].

TUBB3 protein can be induced or inhibited by drugs, i.e.: vinca alkaloids through AP-1 site on TUBB3 promoter can induce its expression (this would mean that CTX with vinca alkaloids predict poor response – may be depending on basal level of TUBB3). Beside therapeutic induction, hypoxia and hypoglycaemia in cancer cells also can induce TUBB3 expression through HIF1α and HuR (Hu – hypoxic upregulation antigene), but the peak of expression level depends on basal TUBB3 expression [34]. It has been observed that high TUBB3 expression can be detected in the close neighbourhood of necrotic tumor regions providing further support to the role of TUBB3 in hypoxic adaptation [94]. In induced cellular glucose stress, blocking glycosylation by tunicamycin or wortmannin, TUBB3 is also upregulated. Additionally, TUBB3 seems to have an important role in metabolic processes interacting with numerous enzymes (i.e.: pyruvate kinase, phosphofructokinase, aldolase, hexokinase, GAPDH, lactate dehydrogenase)[34].

The regulation of TUBB3 expression is not fully understood. Recently, it has been shown that hypoxia increases the expression of TUBB3 gene by binding with HIF1α [86]. Furthermore, it has been speculated about the role of epigenetic modulation (DNA methylation and chromatin acetylation) in aberrant expression of TUBB3 [95].

It is hypothesized that microtubule dynamic alterations are due to cyclin B1 and cdc2 serine threonine kinase. Cyclin B1 and cdc2 form a heterodimer responsible for the initiation of mitosis

through increased activity at the G2/M check-point and so role of tubulins in cell cycle and apoptosis has been hypothesized [96].

Interaction between microtubules and apoptotic signaling proteins is also suggested by Bcl-2 involvement in TBA-mediated cell death. On the one hand, Bcl-2 overexpression suppresses the TBA-mediated apoptotic response independently of G2/M arrest or other structural microtubule alterations. On the other hand, high Bcl-xL expression is protective against taxol-induced stress [34]. Direct interactions between Bcl-2 and tubulin have been already described [84, 97]: Bim (Bcl-2 interacting mediator of cell death) binds to dynein light chain, thereby preventing initiation of apoptotic signaling. Once, Bim released from microtubules and translocates to mitochondria, will interact with Bcl-2, Bcl-xL or Bax to promote apoptosis [98]. In addition, tubulin can interact with BH3-domain (of which Bim is a member)[84]. By interacting with apoptotic proteins, TUBB3 may have a pro-survival effect by reducing apoptotic potential of cancer cells. TBAs (tubulin-bindings agents – TBAs) are known to induce Bcl-2 phosphorylation, a state that inhibits the anti-apoptotic activity Bcl-2 [99]. This result suggests that Bcl-2 activity can depend on microtubule integrity [34]. The fact that Bcl-2 phosphorylation is elevated G2/M arrest may reflect the action of TBAs on the cell cycle checkpoint, rather than apoptotic signaling [100].

Immunostaining for ERCC1 and TUBB3 can provide predictive information crucial for planning personalized chemotherapy and is superior to quantitative real-time PCR as methodology for predictive value of ERCC1 and TUBB3 [18, 101]. TUBB3 and ERCC1 together influences the therapeutic response to taxane and paclitaxel combination, however the distinct mechanism is still unknown [34].

Platinum-based therapy regimes (i.e. cisplatin, carboplatin, oxaliplatin) forms intra-strand and inter-strand cross-links in the DNA double strands. The effective removal of these cisplatin-DNA adducts depends on the integrity of DNA repair mechanism, leading to cell death. Hence, enzymes involved in DNA repair, i.e.: ERCC1, are proposed to be good predictive marker for efficiency of chemotherapy [13]. Oxaliplatin is part of the standard adjuvant chemotherapy for stage III colorectal cancer, but many patients have a cumulative dose-dependent peripheral neuropathy and other serious side effects [20].

Taxanes (i.e. paclitaxel, docetaxel) are widely used chemotherapeutics, either as monotherapy or in combination with other platinum-based compounds (i.e. cisplatin, carboplatin). The main targets of taxanes are tubulin and microtubules. Taxanes bind to microtubules and induce hyperstabilization, causing cell cycle arrest and apoptosis [89]. Taxanes stabilize microtubulues and enhance their assembly [102]. At high concentrations, taxanes promote the polymerization

of purified tubulin in vitro and enhance the fraction of polymerized tubulin in cells [89]. Taxanes exert their growth inhibitory effect by binding to TUBB3 resulting in growth arrest of tumor cells at the G2/M phase through the inhibition of microtubule dynamics [17]. Thus, overexpression of TUBB3 implicates the taxane resistance of tumor cells, i.e. increased TUBB3 expression predicts poor prognosis and associated with taxane-resistance in advanced NSCLC (and in ovary, prostate, breast and pancreatic cancer) treated with paclitaxel-based treatment by reducing taxanes' ability to suppress microtubule dynamics [17, 18]. Although paclitaxel inhibits microtubule dynamics, it does not alter microtubule mass. On the one hand, this could mean that

TUBB3 expression level may not affected by the cytotoxic effect of chemotherapy. On the other hand, TUBB3 expression is associated to taxane-resistance in a wide range of tumors [13].

3. CORRELATIONS BETWEEN CDX2, DNA REPAIR PROTEINS AND MEMBERS OF WNT SIGNALING

In the third phase of our study, we have demonstrated significant correlations between CDX2, DNA repair proteins and crucial members of Wnt signaling. To our knowledge, this is the first report performed on human tissue of colorectal cancer liver metastasis presenting statistically significant correlations between expression of CDX2 referring to expression of mismatch repair proteins and key proteins of base and nuclear excision repair. Furthermore, we show, for the first time, significant correlation between CDX2, APC and β-catenin in liver metastasis of colorectal cancer.

Loss of CDX2 expression is seen in approximately 30% of human CRC and is associated with higher tumor grade [48]. We found loss of CDX2 expression in 38.55% of the cases. Loss of CDX2 expression was negatively correlated with tumor size, but no correlation with age, gender of the patients, grade of the tumor and the number of metastases. Interestingly, ERCC1 expression loss was also correlated with tumor size. Furthermore, loss of CDX2 is strongly correlated with loss of ERCC1. Thus, we can conclude, that loss of CDX2 or ERCC1 expression is strongly associated with bigger metastatic tumor size. Similar results for ERCC1 were found recently in breast cancer [103], but the exact mechanisms are still unclear.

We can demonstrate statistically significant correlations between CDX2 and DNA repair proteins: loss of CDX2 expression is associated with loss of mismatch repair proteins, MGMT, and ERCC1. These results are consistent with literature data from primary colorectal cancer: mismatch repair (MMR)-deficient or MSI high colorectal cancers have significant losses of CDX2 expression. In addition, loss of CDX2 is associated with CIMP-high, more aggressive histomorphological features, and unfavourable survival [104]. In a study on primary colorectal

cancer and its lymph node metastasis reduced expression of CDX2 were found to be as predictor of MMR-deficiency in colorectal cancer. Moreover, loss of CDX2 is a poor prognostic factor, even among patients with MMR-proficient cancers [105].

Mutations in DNA repair genes are rare in sporadic cancers with DNA repair deficiency. However, DNA repair deficiency occurs in a majority of sporadic cancers caused by epigenetic alterations that reduce or silence DNA repair gene expression. For example, a majority of primary colorectal cancers have reduced MGMT expression due to i.e. methylation of the MGMT promoter region (an epigenetic alteration)[106]. MGMT can be epigenetically depressed in many ways. Beside hypermethylation, MGMT can be depressed by di-methylation of lysine 9 of histone 3 [107] or by over-expression of a number of microRNAs including miR-181d, miR-767-3p and miR-603 [108].

Methylation of MGMT promoter region plays a significant role not only in carcinogenesis but also predictive for therapy response. In glioblastoma multiforme, the methylation state of the MGMT gene determined whether patients would be responsive to temozolomide therapy [109]. On a clinical level, this translates into a prolonged survival of glioblastoma patients with a methylated MGMT promoter. In addition, MGMT methylation can be used to predict patient survival in clinical prediction models [110].

Loss of MGMT and ERCC1 expression was associated with female sex in our study. Similar data were demonstrated in primary colorectal cancer for MGMT [111] and for ERCC1 in lung cancer [112], thus we can conclude that this phenomenon stay maintained in liver metastasis. For ERCC1 our study is the first demonstrating statistically significant correlation with female gender in colorectal cancer. ERCC1 is essential for a functional NER system and ERCC1 expression loss may contribute to impaired DNA repair capacity thus increasing cancer risk. Reduced expression or loss of ERCC1 and MGMT were reported in vast majority of colorectal cancers [113, 114], and ERCC1 promoter hypermethylation in 38% of gliomas, resulting in reduced mRNA and protein expression [115]. Disturbed ERCC1 protein expression appears to be an early event in colorectal carcinogenesis: reduced or loss of ERCC1 expression was detected in 40% of the colonic crypts within early field defects in colorectal mucosa [113]. Similarly to MGMT, ERCC1 silencing can be resulted not only from promoter methylation, but can also be evocated by miRNAs repressing its expression [116]. Whether epigenetic mechanisms reduce ERCC1 and MGMT protein expression in liver metastasis of CRC has to be determined in methylation studies. In general, the exact role of ERCC1 should be further elucidated because of its predictive role in chemotherapy. Pre-clinical studies have demonstrated its important role in determining cisplatin resistance [117].

In summary, loss of CDX2 is associated with each DNA repair protein, which we analysed and our results in liver metastasis are in accordance with the literature data originated from primary colorectal cancer [104, 105]. Loss of CDX2 has also been found to be an independent predictor of the CIMP-high phenotype [105]. We used MGMT as surrogate marker for CIMP phenotype, but it has been noted that studies about MGMT methylation and CIMP had inconsistent findings, thus tumors with loss of MGMT cannot be clearly classified as CIMP phenotype [118]. CIMP-high CRCs have been reported to have a different clinicopathological features than CIMP-low ones. CIMP-high phenotype is associated with older age, cigarette smoking, proximal tumor location, female gender, poorly differentiated or mucinous adenocarcinoma, MSI, and BRAF mutation. In addition, CIMP-high cancers regardless of microsatellite status show a poorer outcome [119]. We suggest that MGMT is an adequate marker to detect CIMP phenotype.

The Wnt–β-catenin pathway is a crucial signalling pathway in control of embryonic development and tissue homeostasis. Its deregulation is observed in many cancers (i.e. colorectal cancer, non-hepatitis-related hepatocellular cancers, cholangiocarcinoma, desmoid tumor, breast cancer, osteosarcoma etc)[120]. The pathway is over-activated in almost all colon cancer because of mutations of APC tumor suppressor gene, which actually represent the initiating event in colorectal carcinogenesis [121]. Nevertheless, the actual mechanisms, which regulate β-catenin still remain highly controversial. Furthermore, the exact role of APC in particular is unclear, and the consequences of the mutations found in cancer cells are still poorly defined [121]. Subcellular localisation of APC protein is differentially regulated in normal tissues and cell lines: in normal human colorectal epithelium, APC is located in the nuclei at basal segment of the crypts; in HT29 colon cancer cells, truncated APC translocated to the nucleus during early apoptosis [122], and cellular APC accumulates in the nucleus of sub-confluent cells but is partly excluded in super-confluent cells [56]. Although there is consensus in many areas in the field of nuclear APC localization and function, there have also been some conflicting results with no apparent resolution. Moreover, the specificity of several APC antibodies has been investigated, with no clear consensus about the “best” antibody to detect APC protein [123]. The nuclear transport of APC in tumor cells occurs independently of β-catenin translocation to the nucleus or plasma membrane [124].

Nuclear accumulation of β-catenin is also observed in cancers resulting from mutations in the β-catenin, adenomatous polyposis coli (APC) or Axin genes [57, 125]. The APC tumor suppressor binds to β-catenin and the scaffold protein Axin to form a complex promoting GSK-3β phosphorylation of β-catenin. However, overexpression of APC(1–1309), the most frequently occurring APC cancer mutant, translocates β-catenin from the nucleus to the cytoplasm. This

mutant therefore has the ability to bind and regulate localization but lacks the Axin binding sites required for β-catenin degradation. Therefore, it seems more likely that it is the inability of APC to promote β-catenin degradation, rather than a lack of export function, that causes the nuclear accumulation of β-catenin in APC-mutant tumor cells [56].

Little is known about the connections between CDX2 and Wnt signaling pathway. In a study on Caco-2 cells lower CDX2 expression is associated with endogenous downregulation of APC expression, but did not affect GSK3β expression [49]. Our analysis led to similar results: reduced expression or loss of CDX2 is associated with reduced nuclear APC expression ($p<0.01$). In our study, the cytoplasmic APC expression was not associated with CDX2 expression. We assume that although CDX2 induce APC expression, which is already proven [49], the truncated APC protein cannot be shifted to cytoplasm, but we could detect this truncated protein with our antibody. In conclusion, truncated APC can be detected with immunohistochemistry and has certainly not lost its full function and can still participate in β-catenin regulation. Thus, APC can still fulfil an unexpectedly large spectrum of APC function [121]. Furthermore, we found statistically significant correlation between CDX2 and cytoplasmic β-catenin. We think this correlation can be explained through the Mucdhl, a common interaction partner for β-catenin and CDX2. It has been shown that β-catenin interacts with a protocadherin Mucdhl, which is regulated by CDX2 in mice. Membrane-bound β-catenin is a consequence of interactions to membranous-expressed Mucdhl. Thus, Mucdhl can inhibit β-catenin translocation to the nucleus [49].

CONCLUSION

In conclusion, it could be shown that the ARC expression level in colorectal cancer liver metastasis is independent from clinical data (i.e. age, gender, tumor size, tumor number or mucin production), but is strongly associated with MSH2 and MSH6 expression. This could further support the evidence of the regulatory role of MSH2 and MSH6 in apoptosis: at sufficient MSH2 and MSH6 expression a significant higher ARC level is required to suppress the apoptosis. Although a regulatory mechanism between ARC and p53 is known, no correlation was found between p53 expression levels and ARC levels, which could mean that p53 immunohistochemistry is inappropriate to investigate the pro-apoptotic activity of p53 protein.
Further studies are needed to declare the exact role of ARC in apoptotic signaling and so its role in chemoresistence and survival of tumor cells.

Statistically significant correlations between MMR proteins and ERCC1, RRM1 and TUBB3 were detected. Furthermore, statistically significant correlation was found between the apoptosis repressor protein ARC and RRM1 and TUBB3. Taken together, regarding these proteins, there is a high therapeutic resistance potential in colorectal cancer metastasis. Thus it is proposed to test the known associated predictive proteins before any therapy option is offered. Further functional studies need to declare the exact regulatory mechanism between RRM1, TUBB3 and ARC, as exact relations among these proteins cannot be measured by means of immunohistochemistry alone. The assessment of the abovementioned markers may be a helpful tool to design chemotherapy protocols for colorectal cancer liver metastasis and to define patients who may expect a larger clinical benefit. Selection of chemotherapeutic drugs according to their predicted efficiacy should be a part of future therapeutic decisions and prospective studies. A prospective validation of these markers is warranted.
CDX2 is indeed expressed in all stages of colorectal cancer, little is known about its expression manner in association with other established prognostic or predictive proteins. In this report, we have directly demonstrated that CDX2 gene expression is strongly associated with DNA repair proteins and crucial members of Wnt signaling. Our results further strengthen the role of CDX2 in DNA repair and in regulation of APC and β-catenin expression. In fact, our analysis is restricted only for metastasis, our results strongly suggest potential (functional) interactions between the investigated proteins. To our knowledge, this is the first study to investigate CDX2 in this context on human liver metastasis of colorectal cancer. Although, CDX2 is a useful marker in routine diagnostics for colorectal cancer, its exact role in liver metastasis remains to be further elucidated. In further studies should be investigated if primary colorectal cancer differs from liver metastasis regarding CDX2 expression.

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Dr. Csaba Tóth

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APPENDIX

I. **Tóth C**, Meinrath J, Herpel E, Derix J, Fries J, Buettner R, Schirmacher P, Heikaus S: **Expression of the apoptosis repressor with caspase recruitment domain (ARC) in liver metastasis of colorectal cancer and its correlation with DNA mismatch repair proteins and p53.** J Cancer Res Clin Oncol. 2016 May;142(5):927-35. doi: 10.1007/s00432-015-2102-3. **[IF: 3.1]**

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ORIGINAL ARTICLE – CANCER RESEARCH

Expression of the apoptosis repressor with caspase recruitment domain (ARC) in liver metastasis of colorectal cancer and its correlation with DNA mismatch repair proteins and p53

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Abstract
Introduction Apoptotic signaling is one of the most important processes in the measurement of chemotherapeutic effectiveness. In apoptotic machinery, various pathways and proteins are involved (i.e., mismatch repair proteins, p53). One of the regulatory proteins is ARC, which can inhibit not only the extrinsic but also the intrinsic apoptotic signaling.
Materials and Methods In this study, we investigated the expression levels of ARC in colorectal liver metastasis and compared them with the expression of mismatch repair proteins and p53. Furthermore, we investigated ARC expression level depending on sex, age, tumor grade, mucin production, tumor size and number of liver metastasis.
Results ARC expression level in colorectal cancer liver metastasis was independent from clinical data (i.e., age, gender, tumor size, tumor number or mucin production) but strongly correlated with MSH2 and MSH6 expression, which further supported the evidence for the regulatory role of MSH2 and MSH6 in apoptosis; i.e., in case of sufficient MSH2 and MSH6 expression, significantly higher ARC level is required to suppress the apoptosis. A regulatory interaction between ARC and p53 has been described, but we found no correlation between p53 expression levels and ARC levels.
Conclusion Further studies are needed to define the exact role of ARC in apoptotic signaling and thus its role in chemoresistance and survival of tumor cells.

Keywords ARC · Apoptosis · Liver metastasis · Colorectal cancer · MMR proteins · p53

Introduction

Colorectal cancer (CRC) is still a leading cause of cancer-associated deaths worldwide with an incidence of over one million newly diagnosed cases per year and a mortality rate of approximately 40–50 % (Siegel et al. 2014). Despite intensive screening efforts to diagnose colorectal cancer at an early stage, metastases into other organs like liver or lung are still common (Binefa et al. 2014). Several studies showed that DNA mismatch repair (MMR) proteins take part not only in the DNA repair machinery but also in the regulation of cell-cycle checkpoints and in the apoptotic machinery—deficiency in one of the MMR proteins is responsible for resistance to various chemotherapeutic drugs and subsequently for resistance to apoptosis. MLH1, MSH2 and MSH6 are the main proteins involved in chemotherapeutic resistance. MLH1 and MSH2 are responsible for drug resistance (i.e., cisplatin, 6-thioguanine or methylating agents) in the treatment of colorectal cancer (Hassen et al. 2012). Furthermore, the MMR system has an important role in apoptosis by activation of cell-cycle checkpoints (i.e., G2/M checkpoint). A defective MMR system cannot recognize the DNA damage caused by cisplatin, which leads to continuous proliferation (Martin et al. 2008). In addition, the overexpression of MSH2 and MLH1 is toxic to the tumor cells by triggering apoptosis (Zhang et al. 1999).

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Apoptosis can be induced by diverse stimuli (i.e., DNA damage, chemotherapeutic drugs, oxidative stress). The role of apoptosis in CRC metastases and chemotherapeutic resistance is still unclear. This is of interest, especially regarding therapeutic interventions in metastatic cases. One attractive potential therapeutic target is ARC [apoptosis repressor with caspase recruitment domain (CARD)].

ARC is a protein that, over the last years, has become of special interest as a potent inhibitor of both central apoptotic pathways and is expressed throughout all cell types of cells especially in neurons, skeletal and cardiac myocytes (Koseki et al. 1998), as well as in carcinomas of different origins, like ovarian cancer, colon cancer or cervical cancer (Wu et al. 2010). Different expression levels of ARC have already been observed in different cell lines (MCF-7—breast cancer, A-549—non-small cell lung cancer, HT-29—colon cancer, PC-3—prostate cancer, A-498—kidney cancer). The ARC levels were different not only in different cancer cell types but also between cells of the same cancer types (Mercier et al. 2005). Interestingly, immortalized epithelial cell lines from normal breast tissue showed a low level of ARC (Mercier et al. 2005). Furthermore, in reduction mammoplasty specimens, the ARC expression was restricted only to the nuclei. Mercier et al. (2005) found cytoplasmic ARC positivity in invasive breast cancer, normal breast tissue and in reduction mammoplasty (89, 21 and 10 %, respectively). ARC has been thought to be mainly cytoplasmic and mitochondria-associated, and its anti-apoptotic activity has been linked to those locations (Mercier et al. 2005; Nam et al. 2004). For this reason, nuclear ARC expression was unexpected, but was presented in MCF-7 breast cancer cells, ductal carcinoma in situ and also cardiomyocytes. In breast cancer cell lines, high levels of cytoplasmic ARC were linked with treatment resistance: In MCF-7 cells, high ARC level protected the cells from doxorubicin and γ-radiation-induced cell death (Mercier et al. 2005). ARC is known to be induced by Ras and repressed by p53 signaling (Li et al. 2008) and is involved in the inactivation of extrinsic as well as intrinsic apoptosis pathways, by interacting with pro-apoptotic proteins like p53, Bcl-2, Bax, Bad, PUMA, MSH2, MSH6 and others (Ludwig-Galezowska et al. 2011).

In summary, abundant expression of ARC in cancer cells or in its premalignant lesions can promote cell survival and protect cancer cells from cell death, thus providing a benefit to these cells (Mercier et al. 2005). Upstream regulatory mechanisms of cytoplasmic and nuclear ARC expression are still unknown. Regarding the different ARC abundance in normal tissue and cancer cells, it was suggested that increased cytoplasmic ARC expression is not only a result of redistribution of nuclear ARC but also augmented by increased production of ARC (Mercier et al. 2005).

In this study, we were able to demonstrate an important link between the nuclear and cytoplasmic expression of ARC and MMR proteins in colorectal cancer liver metastasis, which has not been shown before.

Materials and methods

Tissue samples

Paraffin-embedded operation specimens of liver metastasis of colorectal cancer were selected from the archives of the Institute of Pathology at the University Hospital of Heidelberg. One hundred and one patients (64 males, 37 females; mean age 62 years) were included. Further data, such as age, gender, size and number of metastases, were collected from histological reports. Collection of material was performed according to ethical standards formulated in the Declaration of Helsinki 1975 (and revised in 1983) with approval by the Ethics Committee at the University Hospital, Heidelberg.

Tissue microarray

Tissue microarray (TMA) blocks were obtained from paraffin-embedded human liver specimens with a tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA). From one case, two cores of tumor tissue with a diameter size of 1.6 mm were punched and for orientation of the TMA slides two muscle cores were used. Muscle punches served also as positive controls for ARC immunostaining.

Immunohistochemistry

Slides of 0.4 µm thick were obtained from TMA. Slides were then deparaffinized according to standard protocol by xylene and dehydrated with 95–96 % ethanol, 70 % ethanol and distilled water. All slides were stained simultaneously using a computer-controlled autostainer (Dako TechMate 500 cytomation) and Dako EnVision System (Dako) and pretreated with 3 % hydrogen peroxide prior to antibody incubation. MLH1, MSH6, PMS2 (all BD Pharmingen), MSH2 (Calbiochem), p53, and ARC (Santa Cruz) antibodies were used, ranging in dilutions from 1:50 to 1:500. Secondary antibody binding (all Dako, 1:200) was visualized using a streptavidin ABC kit (Dako), followed by 3,3′-diaminobenzidine (Vector, Peterborough, UK). A counterstain was done with hematoxylin, and all slides were covered with Aquatex (Merck, Darmstadt, Germany). Control immunostaining was also performed with blocking peptide showing the absence of ARC signal.

Evaluation of immunohistochemistry

For semiquantitative assessment of ARC staining intensity, we adjusted a previously published scoring system and fitted to TMA dots (Mercier et al. 2008). As baseline expression level, normal colorectal mucosa was set. Cytoplasmic and nuclear ARC staining was separately scored. For cytoplasmic ARC staining, the reference score (score 1) was the normal colorectal mucosa, which was also used as external control for immunohistochemistry. For cytoplasmic ARC staining, a four-graded scale was used: score 0: no or weaker staining in lesser than 10 % of tumor cells and weaker staining compared to normal colonic mucosa; score 1: equivalent cytoplasmic staining compared to normal colorectal mucosa, perceptible staining intensity at 4×; score 2: slightly stronger staining compared to normal mucosa and score 3: diffuse and strong cytoplasmic staining. For nuclear ARC staining, a three-graded scale was used: score 0—no nuclear staining or weak staining in lesser than 10 % of the nuclei; score 1—10–75 % of the nuclei positive with moderate intensity and score 2—more than 75 % of the nuclei are positive with high staining intensity. Assessment of the immunohistochemical stainings was carried out by two independent pathologists (CT and JM).

For MSI proteins, the staining was evaluated according to Bethesda guidelines (Umar et al. 2004). Immunostaining for p53 was scored in a three-graded scale: score 0—weakly staining in less than 10 % of the tumor cells, score 1—moderate staining in up to 75 % of the tumor cells and score 2—strong nuclear staining in more than 75 % of the tumor cells.

Statistical analysis

The statistical analyses were performed with SAS software (SAS institute, Cary, NC, USA). Spearman rho test was used to evaluate the relationship between clinical data, ARC, MLH1, MSH2, MSH6, PMS2 and p53.

Results

MMR proteins and p53 expression

The staining for MSI proteins shows loss of expression in 4.2–26 % of the cases (MLH1 4.2 %, MSH2 26 %, MSH6 24 % and PMS2 9.5 %) (Fig. 1). For p53 $n = 102$, valid immunohistochemical results were used for evaluation, for which in 23 % of the cases ($n = 23/102$) p53 was negative (score 0) (Fig. 2a–c). Positive stainings were subdivided into two groups: moderate positivity or so-called restrictive overexpression with a score of 1 (46 %, $n = 47/102$) and strong positivity or so-called strong overexpression with a score of 2 (31 %, $n = 32/102$). Regarding only the strong overexpression (score 2) as positive, we observed p53 positivity in 31 % ($n = 32/102$), while 69 % ($n = 70/102$) of the cases were negative. Regarding all positive cases (scores 1 and 2), we could detect in 77 % ($n = 79/102$) nuclear positivity and so 23 % ($n = 23/102$) of the cases were negative for p53.

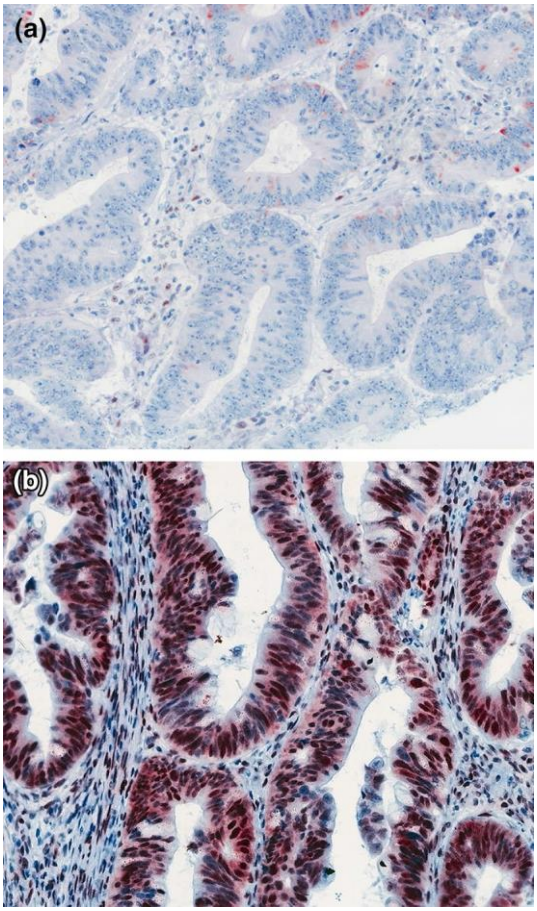

Fig. 1 Representative examples of the MMR protein expression in colorectal liver metastasis. **a** Score 0: negative staining—less than 10 % of the nuclei are stained; **b** score 1: more than 10 % percent of the nuclei are positive. Note that in negative cases the cells of the stroma are still positive

In normal colonic mucosa ARC expression is limited to the upper two-thirds of the colonic crypt

Normal colonic mucosa showed a slightly cytoplasmic and nuclear ARC staining with higher level in the upper two-thirds of the colonic crypts. At the bottom of the crypts, there was almost no staining activity detected (Fig. 3). Ancillary studies detected a similarly low or absent ARC expression in the proliferation zone of the colonic crypts, where the apoptotic activity is low (Mercier et al. 2008; Li et al. 2005). In some other studies, no or a very low level

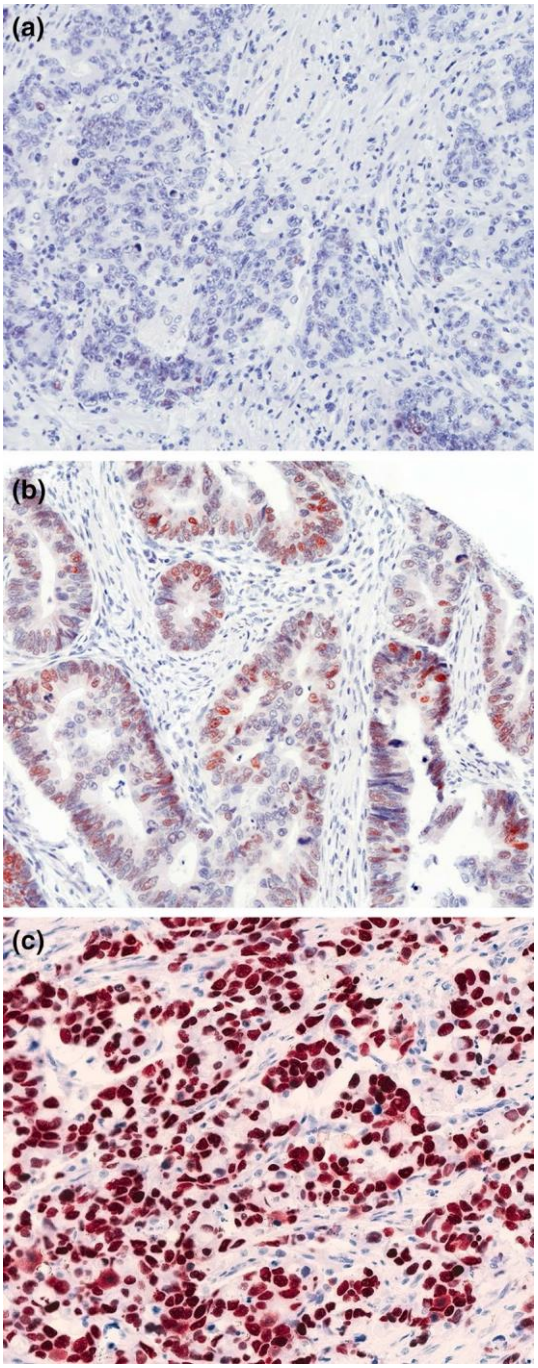

Fig. 2 Representative examples of the expression of p53 protein in colorectal liver metastasis. **a** Score 0: negative staining—less than 10 % of the nuclei are stained; **b** score 1: moderate overexpression in less than 75 % of the nuclei; **c** score 3: strong overexpression in almost all tumor cells, magnification ×400

of nuclear ARC was detected. This can be due to the use of different antibody: ARC (F-11) antibody covers the whole ARC protein and also Nop30, a splicing variant of

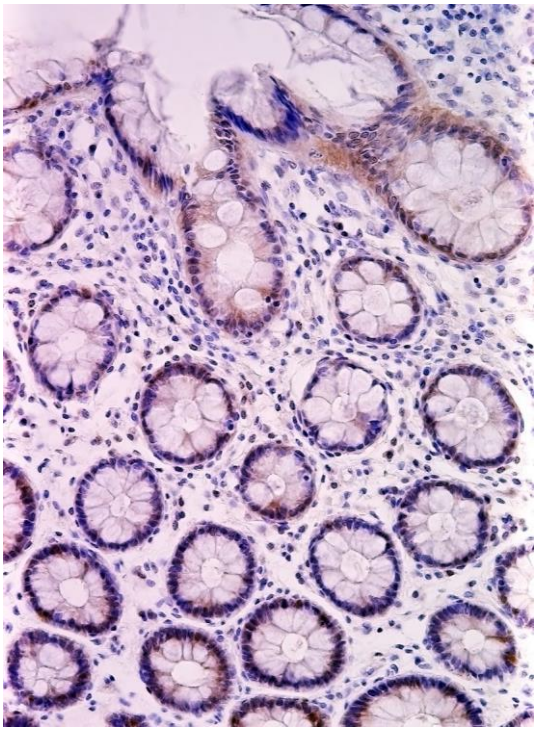

Fig. 3 Normal colonic mucosa showed a slightly cytoplasmic and nuclear ARC staining with higher level in upper two-thirds of the colonic crypts. At the bottom of the crypts, there was almost no staining activity detected

NOL3 locus. ARC staining pattern is similar to Bcl-2 and Bcl-w, which are also absent in the proliferation zone of the colonic crypts whereas overexpressed in colorectal cancer (Wilson et al. 2000).

Cytoplasmic ARC expression

Cytoplasmic results for ARC staining are subdivided into three groups: score 0 for no cytoplasmic staining, 1 for staining equivalent to normal mucosa, 2 for moderate overexpression and score 3 for strong overexpression (Table 1). The different staining intensities are described and depicted (Fig. 4a–d).

Nuclear ARC expression

In addition to cytoplasmic staining of ARC, nuclear staining was evaluated in a three-graded score. Score 0 represents no nuclear staining, and scores 1 and 2 demonstrate moderate and strong staining for nuclear ARC, respectively (Table 2). The different staining intensities are described and depicted (Fig. 4a–d).

Table 1 Valid cytoplasmic immunohistochemical stainings for ARC

Score	Valid cases (*n*)	% of valid cases
0	14	14
1	25	25
2	21	21
3	40	40
∑	100	100

Subdivision of valid cytoplasmic stainings for ARC in three scores and the number of cases (*n*) and %

Table 2 Valid nuclear immunohistochemical stainings for ARC

Score	Valid cases (*n*)	% of valid cases
0	2	2
1	38	38
2	60	60
∑	100	100

Subdivisions of valid nuclear stainings for ARC in three scores and the number of cases (*n*) and %

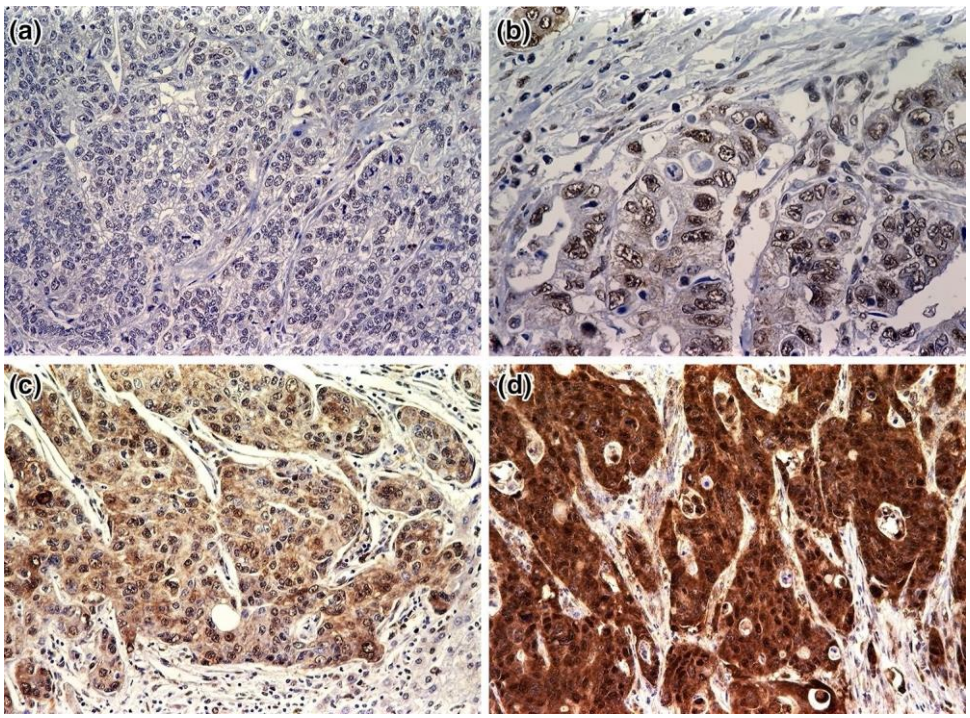

Fig. 4 Representative images of ARC immunohistochemical staining in colorectal cancer liver metastasis. **a** No cytoplasmic with weak nuclear positivity lesser than 10 % of the tumor cells, magnification ×400. **b** Weak cytoplasmic positivity with similar intensity as in normal colon mucosa (score 1 for cytoplasmic staining) next to moderate nuclear staining (score 1 for nuclear staining) magnification ×400. **c** Moderate cytoplasmic staining intensity, stronger than normal colorectal mucosa (score 2 for cytoplasmic intensity) and moderate staining in tumor cell nuclei (score 1 for nuclear staining), magnification ×400. **d** Strong positivity in cytoplasm and strong nuclear positivity in almost all tumor cells (score 3 for cytoplasmic and score 2 for nuclear staining), magnification ×400

Neither cytoplasmic nor nuclear ARC expression has statistically significant correlation with clinical parameters

Concerning clinical parameters like age, gender of the patients, grading of the tumor and the number and size of metastases, there was no significant correlation with nuclear or cytoplasmic ARC expression. Regarding the patients' age, ranging from 33 to 82 years, there was a correlation neither with nuclear ARC expression ($p = 0.622$) nor with cytoplasmic expression ($p = 0.548$).

There was no association between gender and nuclear or cytoplasmic staining (nuclear $p = 0.602$; cytoplasmic $p = 0.697$). We found only one case without nuclear staining. From all patients, there was only one case with a G1 graded tumor with a strong nuclear staining for ARC but weak cytoplasmic positivity. No correlation was found regarding the grade (for nuclear ARC expression, $p = 0.858$, whereas cytoplasmic was $p = 0.692$). No association between number of metastases and nuclear ($p = 0.941$) or cytoplasmic ($p = 0.612$) ARC expression could be detected.

Patients in the study group had metastases from 5 to 160 mm in diameter, 63 of them with a diameter of 63 mm

Table 3 Relationship between cytoplasmic and nuclear ARC expression, MMR proteins and p53

	MLH1	MSH2	MSH6	PMS2	p53
ARC cytoplasm					
Correlation coefficient	−0.078	0.299**	0.160	0.145	0.070
Significance (two-sided)	0.504	0.003	0.114	0.152	0.491
Number of valid cases	75	99	99	99	98
ARC nucleus					
Correlation coefficient	0.197	0.187	0.276**	0.093	0.075
Significance (two-sided)	0.090	0.063	0.006	0.359	0.465
Number of valid cases	75	99	99	99	98

** The correlation is significant at the level of 0.01 (two-sided)

and bigger. Here, the ARC expression also showed no association to the size (nuclear $p = 0.996$; cytoplasmic $p = 0.520$).

Cellular ARC expression levels are independent from p53 staining status (Table 3)

Furthermore, no correlation could be detected between p53 expression status and expression level of nuclear or cytoplasmic ARC ($p = 0.465$ and $p = 0.491$, respectively), even if only the strong p53 overexpression were classified as pathological ($p = 0.256$ for nuclear and $p = 0.388$ for cytoplasmic ARC expression versus p53).

MSH2 and MSH6 may have a role in regulation of cytoplasmic and nuclear ARC levels (Table 3)

Surprisingly, cytoplasmic ARC expression had a strong positive correlation with MSH2 ($p = 0.003$) besides a strong positive correlation between nuclear ARC expression and MSH6 protein status ($p = 0.006$). Moreover, MSH2 expression status shows an almost significant positive relation to nuclear ARC expression ($p = 0.063$). MLH1 and PMS2 had no significant correlation with ARC expression.

Discussion

Dysregulation in apoptotic signaling is a common event in colorectal cancers and so in its liver metastasis. Mechanisms involved in apoptosis are important therapeutic targets (i.e., Bcl-2 inhibitors) (Koehler et al. 2014). Besides the known classical apoptotic regulatory proteins, many others exist which have influence on the effectiveness of apoptosis, such as mismatch repair (MMR) proteins and p53. Loss of MMR proteins has been associated with defected apoptotic signaling or therapy resistance (Hassen et al. 2012).

It is known that cancer cells can suppress apoptosis decreasing the level of pro-apoptotic proteins and increasing the level of apoptosis inhibitors. Many caspases have a decreased level in lung, breast or colon cancer, whereas survivin or Bcl-2 and Bcl-X_L are increased in colon cancer and associated with a worse prognosis (Mercier et al. 2008; Sarela et al. 2000).

In our study, we demonstrate the expression pattern of ARC in colorectal cancer liver metastasis and its correlation with other known member of apoptosis regulation. This is the first study that analyze the subcellular localization of ARC in colorectal liver metastasis. Furthermore, we were able to show a significant correlation between ARC and other indirect regulators of the apoptotic signals, such as MSH2, MSH6. It is possible that cytoplasmic ARC is responsible for the inhibition of extrinsic and intrinsic apoptotic signaling interacting with other apoptotic proteins.

Some nuclear functions of ARC have been already discovered (Fig. 5), but the exact role of nuclear ARC protein in colon cancer, and in all other cancer types, remains still unclear (Foo et al. 2007). Furthermore, our findings that nuclear ARC expression is significantly associated with MSH2 and MSH6 but not with MLH1 and PMS2 also have to be elucidated. One explanation could be that defected MSH2 or MSH6 protein loses their pro-apoptotic capability; thus, a significantly lower level of nuclear ARC is needed to repress apoptosis. But there is still the question why nuclear ARC expression is associated with this phenomenon. This question cannot be explained by means of immunohistochemistry alone. Further studies are needed to explore the relation between MMR system and ARC regulation. This is of interest because ARC is a potential therapeutic target and with MMR system together can be responsible for chemo- and radioresistance.

ARC is a unique protein that inhibits both the extrinsic (death receptor mediated) and intrinsic (mitochondrial/ER stress induced) apoptotic pathways. ARC binds the components of both apoptotic pathways interacting with them in a non-homotypic death-fold manner (Nam et al. 2004). ARC is expressed normally in terminally differentiated, so-called stable tissues, such as cardiac and skeletal muscle or nervous tissue (Mercier et al. 2005, 2008). ARC with its CARD binds to death receptors, Fas, FADD and pro-caspase-8, and inhibits the assembly of DISC, thus abrogating the extrinsic apoptotic signaling. ARC has two different ways to inhibit the intrinsic apoptotic mechanism: through direct binding Bax with its CARD (that inhibits the conformational activation and mitochondrial translocation of Bax) or direct binding to p53 precluding the tetramerization of p53,

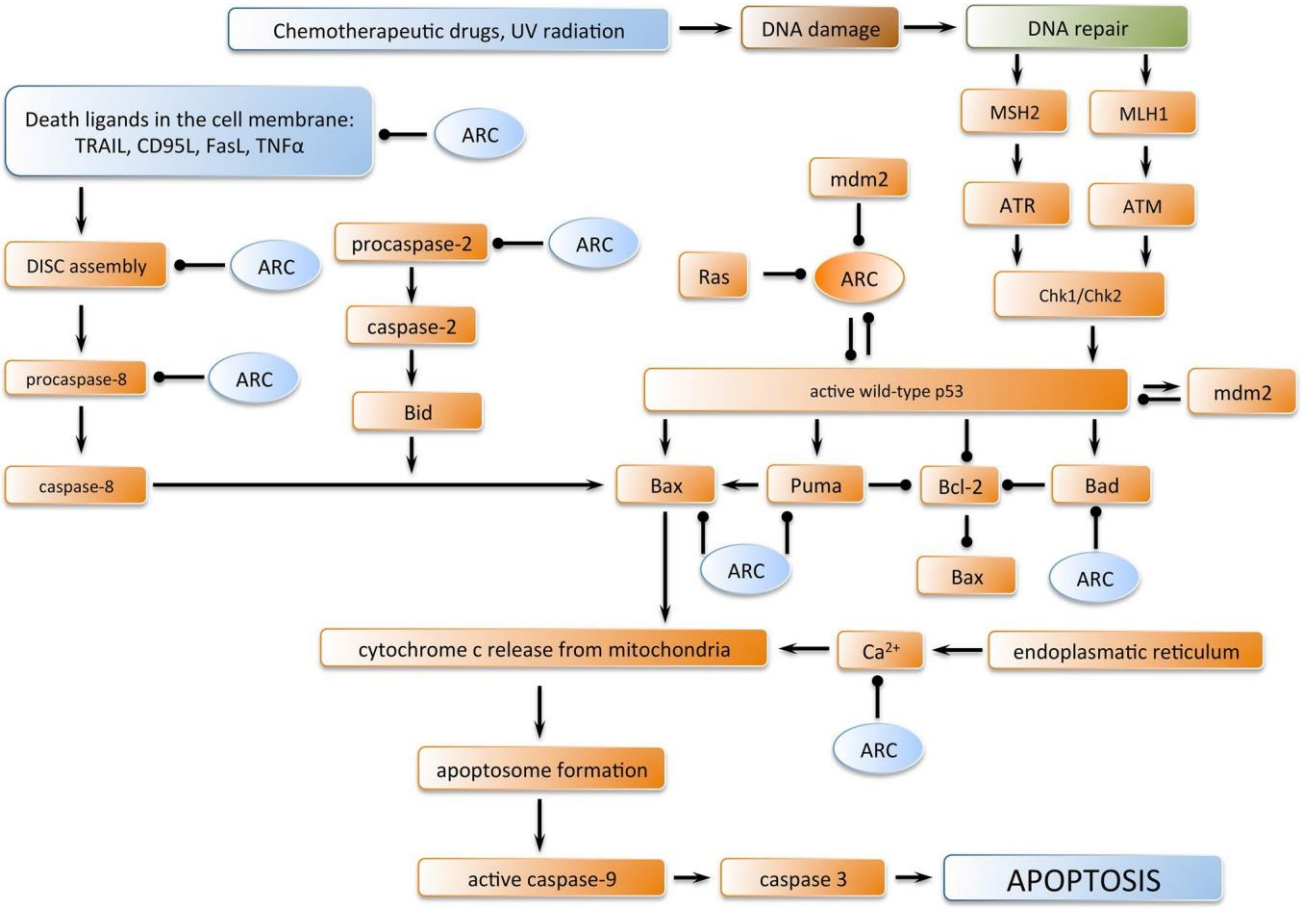

Fig. 5 Simplified diagram of pathways involving ARC. It can be seen that ARC involved in various pathways leads to apoptosis: ARC inhibits plenty of proteins (i.e., DISC partners, pro-caspase-8 and pro-caspase-2, Bax, PUMA and Bad), thus hindering apoptosis. Some regulatory mechanism also depicted: MDM2 and p53 inhibit ARC functions, through transcriptional repression and enhancing its degradation. Furthermore, it is also known that Ras protein promotes ARC production and hinders its degradation (Hassen et al. 2012; Li et al. 2008; Ludwig-Galezowska et al. 2011; Green and Kroemer 2009; Foo et al. 2007)

thereby inducing a nuclear transport signal which relocates p53 to the cytoplasm (Mercier et al. 2008). In case of ARC knockdown, assembly of death-inducing signaling complex (DISC) will be facilitated and spontaneous Bax activation will be triggered resulting in apoptosis (Nam et al. 2004; Wang et al. 2005).

p53 up-regulates PUMA and Bad in response to anoxia or reactive oxygen species (ROS). In addition, ARC interacts with PUMA and Bad through its N terminus. This interaction displaces the association of PUMA or Bad with Bcl-2 (Li et al. 2008). Thus, p53 can repress ARC in a transcription-dependent manner. If ARC is repressed by p53, then this will lead to failures in counteraction of pro-apoptotic activity of PUMA and Bad (Li et al. 2008).

Truncated p53 is not able to exert its transcriptional activity, but still has apoptotic functions and may participate in caspase activity (Vaseva and Moll 2009). Distinct pro-apoptotic activities of p53 are clearly established: in cellular stress, cytoplasmic p53 translocates to mitochondria, where it interacts with different members of the Bcl-2 family resulting in robust mitochondrial outer membrane permeabilization, followed by release of caspase activation and chromatin degradation (Vaseva and Moll 2009; Chipuk et al. 2005). We think this is the reason why we could not find statistically relevant significance between p53 and ARC expression levels. Overexpression of p53 in the nuclei, as an evidence for the mutated protein, may be not appropriate to detect its apoptotic activity.

ARC can inhibit apoptosis almost independently from the inducing cause, such as death receptor activation, hypoxia, hydrogen peroxide, oxidative stress, serum deprivation, ischemic reperfusion, doxorubicin or γ-radiation (Koseki et al. 1998; Mercier et al. 2005; Nam et al. 2004; Wang et al. 2005; Neuss et al. 2001). The fact that ARC inhibits both extrinsic and intrinsic apoptotic pathways can provide a growth advantage to cancer cells. High level of ARC protein in breast cancer cells is associated with chemo- and radioresistance (Mercier et al. 2005; Wang

et al. 2005). In addition, ARC has influence not only on extrinsic and intrinsic apoptotic pathways but also on TNF-α-induced regulated necrosis: ARC interacts with TNF receptor 1 (TNFR1) that interferes with recruitment of RIP1 suggesting a wider role of ARC in cell death (Kung et al. 2014). ARC overexpression could be detected in a couple of cancer types and cancer cell lines and in colorectal cancer cells. Its expression level is correlated inversely with the apoptotic activity in response to chemotherapy (Mercier et al. 2008). One of these studies analyzed the ARC expression in breast cancer and found not only cytoplasmic, as in normal stable tissues, but also nuclear translocation of ARC (Mercier et al. 2005). Mercier et al. (2005) found cytoplasmic ARC positivity in invasive breast cancer, normal breast tissue and reduction mammoplasty (89, 21 and 10 %, respectively). They could not detect any correlation between ARC expression level and the histopathological grade of tumors. We used a similar scoring system for ARC, and we could not detect any correlation between ARC expression and histopathological grade of CRC liver metastasis either.

Colorectal cancer showed decreased apoptosis compared to normal colon mucosa (Valentini et al. 1999). ARC levels in colon cancer can be detected up to 5.8-fold higher (Mercier et al. 2008). Cytoplasmic ARC expression is more common than nuclear (94.7 and 31.6 %, respectively). In one study on colorectal cancer (including cell lines, normal mucosa and cancer of different grades), highly elevated cytoplasmic and moderately elevated nuclear ARC levels could be detected, compared to normal mucosa and normal colon cell lines. Interestingly, the cytoplasmic overexpression was associated with higher grades, whereas the nuclear ARC expression was the highest in cancers with moderate grade (Mercier et al. 2008). Mercier et al. have found similar levels of ARC in all analyzed colon cancers. In contrast to Mercier et al., we could detect at least three distinct levels of ARC expression in liver metastasis of colorectal cancer (Mercier et al. 2008). However, we found no correlation between ARC expression levels and clinical data (i.e., age, gender, tumor size, tumor number or mucin production). In our study, cytoplasmic and nuclear ARC expression had even no association with histopathological grade ($p = 0.692$ and $p = 0.858$, respectively).

Conclusion

In conclusion, we could show that the ARC expression level in colorectal cancer liver metastasis is independent from clinical data (i.e., age, gender, tumor size, tumor number or mucin production) but is strongly associated with MSH2 and MSH6 expression. This could further support the evidence of the regulatory role of MSH2 and MSH6 in apoptosis: At sufficient MSH2 and MSH6 expression, a significant higher ARC level is required to suppress the apoptosis. Although a regulatory mechanism between ARC and p53 is known, we found no correlation between p53 expression levels and ARC levels, which could mean that p53 immunohistochemistry is inappropriate to investigate the pro-apoptotic activity of p53 protein.

Further studies are needed to declare the exact role of ARC in apoptotic signaling and so its role in chemoresistance and survival of tumor cells.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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II. **Tóth C**, Sukosd, F, Valicsek, E, Herpel, E, Schirmacher, P, Renner, M, Mader, C, Tiszlavicz, L and Kriegsmann, J: **Expression of ERCC1, RRM1, TUBB3 in correlation with apoptosis repressor ARC, DNA mismatch repair proteins and p53 in liver metastasis of colorectal cancer.** Int J Mol Med, 2017. **40**(5): p. 1457-1465. **[IF: 2.3]**

Expression of ERCC1, RRM1, TUBB3 in correlation with apoptosis repressor ARC, DNA mismatch repair proteins and p53 in liver metastasis of colorectal cancer

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Abstract. Liver metastasis in colorectal cancer is common and the primary treatment is chemotherapy. To date, there is no routinely used test in clinical practice to predict the effectiveness of conventional chemotherapy. Therefore, biomarkers with predictive value for conventional chemotherapy would be of considerable benefit in treatment planning. We analysed three proteins [excision repair cross-complementing 1 (ERCC1), ribonucleoside-diphosphate reductase 1 (RRM1) and class III β-tubulin (TUBB3)] in colorectal cancer liver metastasis. We used tissue microarray slides with 101 liver metastasis samples, stained for ERCC1, RRM1 and TUBB3 and established scoring systems (fitted for tissue microarray) for each protein. In statistical analysis, we compared the expression of ERCC1, RRM1 and TUBB3 to mismatch proteins (MLH1, MSH2, MSH6 and PMS2), p53 and to apoptosis repressor protein (ARC). Statistically significant correlations were found between ERCC1, TUBB3 and MLH1, MSH2 and RRM1 and MSH2, MSH6. Noteworthy, our analysis revealed a strong significant correlation between cytoplasmic ARC expression and RRM1, TUBB3 (p=0.000 and p=0.001, respectively), implying an additional role of TUBB3 and RRM1 not only in therapy resistance, but also in the apoptotic machinery. Our data strengthens the importance of ERCC1, TUBB3 and RRM1 in the prediction of chemotherapy effectiveness and suggest new functional connections in DNA repair, microtubule network and apoptotic signaling (i.e. ARC protein). In conclusion, we showed the importance and need of predictive biomarkers in metastasized colorectal cancer and pointed out the relevance not only of single predictive markers but also of their interactions with other known and newly explored relations between different signaling pathways.

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Key words: excision repair cross-complementing 1, class III β-tubulin, apoptosis repressor protein, ribonucleoside-diphosphate reductase 1, colorectal cancer, liver metastasis, MMR proteins

Introduction

Colorectal cancer (CRC) remains a leading cause of cancer-associated deaths worldwide with an incidence of over one million newly diagnosed cases per year. Despite intensive research and therapeutic efforts, the mortality rate of CRC is ~40-50% (1). Furthermore, the rate of metastatic cases is high (2). Drug resistance is responsible for poor prognosis in many cancer types (3). Thus, to identify proteins which may have predictive value is of importance not only in metastasized CRC, but also in other advanced epithelial cancers. In this regard, the deregulation of DNA damage repair systems (i.e. mismatch repair) represents an important aspect, since it contributes to the resistance of cancer cells to conventional chemotherapy.

One further repair protein is excision repair cross-complementing 1 (ERCC1), which is also implicated in therapy resistance. ERCC1 is a structure specific DNA repair endonuclease responsible for 5' incision (5'-endonuclease), a key enzyme in the nucleotide excision repair (NER) pathway and is essential for repair of platinum-DNA adducts, and is thus associated with therapy resistance to platinum-containing compounds (3,4). NER is responsible for repair of DNA damages caused by oxidative and alkylating agents (3). ERCC1 was suggested as a promising marker in CRC (4). ERCC1-overexpressing cancer cells are thought to be more resistant to platinum-based chemotherapy. Increased ERCC1 mRNA levels were found to be associated with resistance to platinum-based chemotherapy (i.e. ovarian, gastric, cervical, colorectal and non-small cell lung cancer) suggesting that platinum-paclitaxel chemotherapy would be more effective in ERCC1-negative cancer (3). It is known that ERCC1 protein expression, estimated by immunohistochemistry, is an independent prognostic factor for progression-free and overall

survival in NsCLC patients treated with platinum-based chemotherapy (5). Similar data could be achieved in CRC (6). In several trials on CRC, the ERCC1 expression level was proposed as a candidate marker for predicting the efficacy of oxaliplatin therapy for metastatic patients. In stage III colon cancer, ERCC1 expression is strongly predictive in the selection of patients which will benefit from additional oxaliplatin to 5-fluorouracil (5-FU) therapy (7).

Ribonucleoside-diphosphate reductase 1 (RRM1) gene encodes the regulatory subunit of ribonucleotide reductase enzyme. Ribonucleotide reductase, composed of regulatory subunit RRM1 and the catalytic subunit RRM2, is a crucial enzyme in new DNA synthesis, catalysing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides (8). RRM1 is a key molecule for gemcitabine efficacy and is also involved in tumor progression. High RRM1 expression in tumor tissue predicts significantly better prognosis while only patients with low RRM1 benefit from gemcitabine therapy. In turn, overexpression of RRM1 protein is strongly associated with gemcitabine resistance (8). RRM1 expression was also reported to correlate with the tumorigenic and metastatic potential in lung cancer (8).

The cell cytoskeleton is built up from microtubules, microfilaments and intermediate filaments. Various changes in the microtubule network have been identified in a wide range of cancers, i.e. altered expression of tubulin isotypes, alterations in tubulin posttranslational modifications and changes in the expression of microtubule-associated proteins (MAPs) (9). Class III β-tubulin (TUBB3) is one of the main microtubule (MT) proteins and is primarily expressed in neurons and Sertoli cells in the testis (10,11). In lung cancer, the TUBB3 protein expression level was found to have no correlation with age, gender, smoking status or recurrence pattern or response rate to chemotherapy. The response rate in TUBB3-positive cases was 18%, while the rate was 27% in negative cases (no significant differences could be detected) (5). High TUBB3 expression levels are associated with poor prognosis in many epithelial cancers. Additionally, TUBB3 has been suggested to take part in disease aggressiveness by acting as a survival factor for cancer cells (12). In colorectal adenomas, TUBB3 expression can be detected in up to 100% of high-grade dysplasia. Expression of TUBB3 was found to have no association with grade of dysplasia or other clinical data in preneoplastic lesions of CRC but was associated with Dukes' stage (13). TUBB3 overexpression in colon cancer cells may contribute to a higher stability of the microtubular network which may explain the lower activity of anti-microtubule agents (14). In addition, high TUBB3 expression levels were localized to the invasive edge in CRC; positive TUBB3 staining was observed in all cases, yet this was most prominent at the invasive front with the presence of tumor budding (12). This preferential localization of TUBB3 at the invasive margin raises the possibility that changes in tubulin isotypes can modulate the invasive activity of cancer cells. Microtubules are indispensable for the directional migration of cells. Tubulins, the major constituent protein of microtubules, are built up from heterodimers of α and β subunits (12). It is believed that tumor buds consist of migrating cells and TUBB3 expression in these cells is linked to their motility. Furthermore, TUBB3 is expressed in a variety of tumors, particularly in those that are aggressive and likely to metastasize, and were found to be more resistant to several chemotherapy regimens (i.e. estramustine, Taxol, paclitaxel and docetaxel) (12). As we previously demonstrated, MMR protein expression is correlated with the expression of the apoptosis repressor protein apoptosis repressor protein (ARC). It is known that overexpression of ERCC1, RRM1 and TUBB3 is linked to therapeutic resistance against therapeutic regimens, which is also found in advanced (stage IV) CRC (15). In this context, we investigated the expression trend of ERCC1, RRM1 and TUBB3 proteins and their correlation to ARC protein expression, which is known to be upregulated in CRC and associated with therapeutic resistance inhibiting both extrinsic and intrinsic apoptotic signaling.

Materials and methods

Tissue samples. Paraffin-embedded surgical specimens of liver metastasis of CRC were selected from the archives of the Institute of Pathology at the University Hospital of Heidelberg. One hundred patients (64 male, 37 female; mean age 62 years) were included. None of the patients had received neo-adjuvant chemotherapy. Tissue samples were fixed in neutral-buffered formalin and embedded in paraffin. Paraffin sections were cut (4 μm) and examined on coated slide glass for immunohistochemistry. Further data, such as age, sex, size and number of metastases were collected from histological studies. Tissue samples were provided by the Tissue Bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the Ethics Committee of Heidelberg University according to ethical standards formulated in the Declaration of Helsinki 1975 (revised in 1983).

Tissue microarray. Tissue microarray (TMA) blocks were obtained from paraffin-embedded human liver specimens with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). From each case, two cores of tumor tissue with a diameter size of 1.6 mm were punched and for orientation of the TMA slides two muscle cores were used. Muscle punches served also as positive controls for ARC immunostaining.

Immunohistochemistry. Four-micrometer-thick slides were obtained from the TMA. slides were then deparaffinised according to standard protocol by xylene, and dehydrated with 95-96% ethanol, 70% ethanol and distilled water. All slides were stained simultaneously using a computer-controlled autostainer (Ventana BenchMark Ultra, Ventana Medical Systems, Inc., Tucson, Az, USA). Then primary antibodies were used: ERCC1 (8F1, Neomarkers; dilution 1:100), RRM1 (Protein Tech Europe; dilution 1:200) and TUBB3 (Tuj-1/TubIII/4G3, Covalab; dilution 1:2,000). Primary antibodies were incubated according to routine staining protocols for diagnostic purpose. To detect immunoreactions, Ultraview Universal DAB dectection kit (Ventana Medical Systems, Inc.) and 3,3'-diaminobenzidine were used. A counterstain was performed with hematoxylin and bluing reagent and all slides were covered. For MMR proteins, p53 and ARC, the staining methods were performed as previously published (16).

Evaluation of immunohistochemistry. For semi-quantitative assessment of staining intensity, we adjusted a previously

Table I. Results of the immunohistochemistry for ERCC1, RRM1 and TUBB3.

Proteins	Immunoreactive score 0 n (%)	Immunoreactive score 1 n (%)	Immunoreactive score 2 n (%)	Valid cases n (%)
ERCC1	28 (29.8)	29 (30.8)	37 (39.4)	94 (100)
RRM1	11 (11.6)	33 (34.7)	51 (53.7)	95 (100)
TUBB3	35 (35)	52 (52)	13 (13)	100 (100)

ERCC1, excision repair cross-complementing 1; RRM1, ribonucleoside-diphosphate reductase 1; TUBB3, class III β-tubulin.

published scoring system for each protein and fitted to TMA dots (17,18). ERCC1 and RRM1 immunostainings were scored using a three-graded scale: score 0, no expression detectable or faint partial expression in <10% of the tumor cells; score 1, weak to moderate expression of the entire tumor tissue; score 2, strong positivity in the entire tumor tissue.

For TUBB3, a modified three-graded score was established: score 0, no expression detectable or faint partial expression in <10% of the tumor cells; score 1, diffuse and strong positive staining associated to invasive front and tumor budding, central tumor regions negative or with weaker intensity than at the invasive front; score 2, strong positivity in the entire tumor tissue.

For MSI proteins, the staining was evaluated according to Bethesda guidelines (19). Immunostaining for p53 was scored using a three-graded scale: score 0, weak staining in <10% of the tumor cells, score 1, moderate staining in up to 75% of the tumor cells and score 2, strong nuclear staining in >75% of the tumor cells. The results of MMR, p53 and ARC immunohistochemistry for this collective have already been published (16).

The immunostained tissue microarray sections were evaluated and scored under a light microscope independently by two pathologists in a blinded manner. Discordant cases were reviewed and re-evaluated based on a consensus opinion.

Statistical analysis. The statistical analyses were performed with SAS software (SAS Institute, Cary, NC, USA). Associations between clinical data, ARC, MMR proteins, ERCC1, TUBB3 and RRM1 were estimated by Pearson's correlation and linear regression test. The statistical significance was set at $p<0.05$ and $p<0.01$.

Results

Distribution of ERCC1, RRM1 and TUBB3 protein expression in the collective. The results of the immunohistochemistry for ERCC1, RRM1 and TUBB3 are listed in Table I. For ERCC1 we found 29.8% of the cases to be negative (score 0). Positive ERCC1 staining was detected in 70.2% of the cases (score 1, 30.8% and score 2, 39.4%). For RRM1, the distribution was different. Only 11 cases out of 95 valid cases (11.6%) were found to be negative (score 0). Eighty-four cases (88.4%) showed positive staining for RRM1, 51 cases showed a high expression level (score 2, 53.7%).

TUBB3 staining showed an interesting distribution. Most of the cases showed pronounced positivity at the invasive margin (52%). Thirty-five cases (35%) had negative staining and only 13% had a diffuse positive staining reaction for TUBB3. Representative images of the staining scores for ERCC1, RRM1 and TUBB3 are shown in Figs. 1-3.

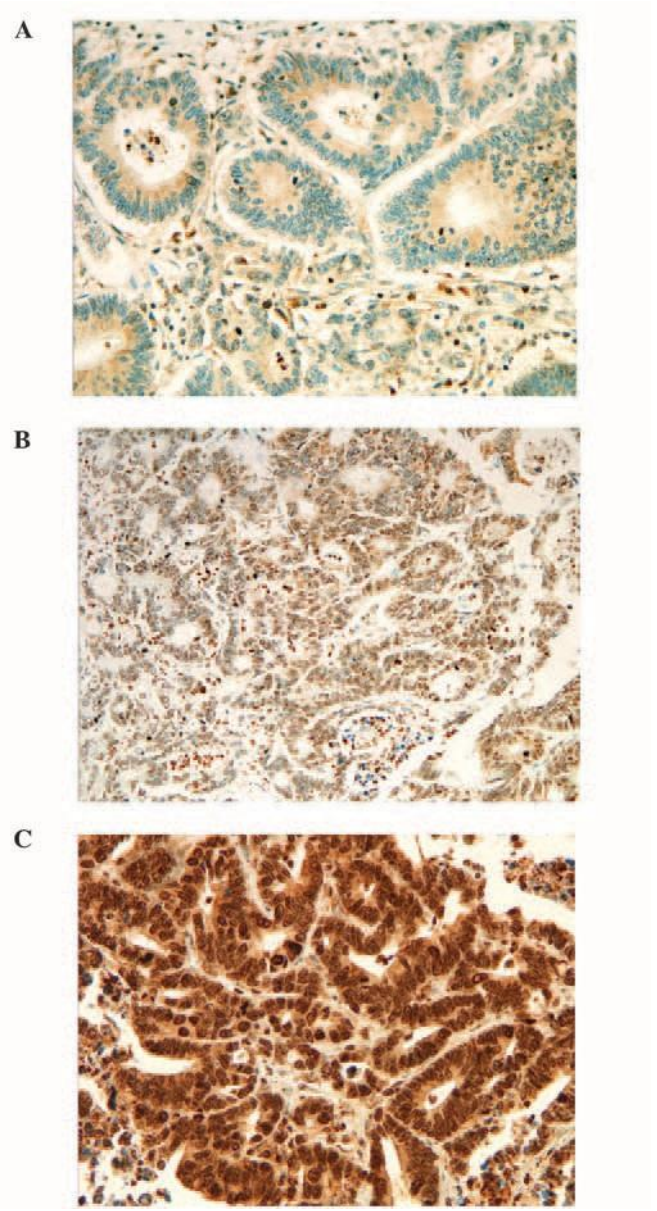

Figure 1. Representative examples of the expression of excision repair cross-complementing 1 (ERCC1) protein in colorectal liver metastasis. (A) Score 0, no expression detectable or faint partial expression in <10% of the tumor cells; (B) score 1, weak to moderate expression of the entire tumor tissue; (C) score 2, strong positivity in the entire tumor tissue; magnification, x400.

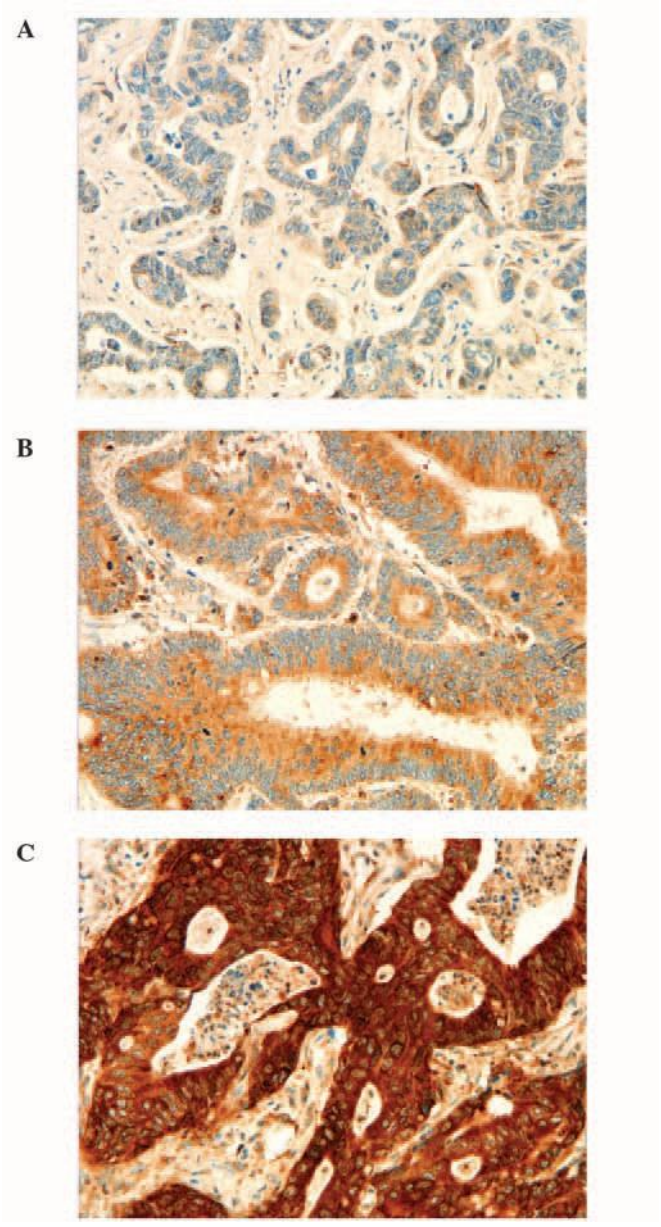

Figure 2. Representative examples of the expression of ribonucleoside-diphosphate reductase 1 (RRM1) protein in colorectal liver metastasis. (A) Score 0, no expression detectable or faint partial expression in <10% of the tumor cells; (B) score 1, weak to moderate expression of the entire tumor tissue; (C) score 2, strong positivity in the entire tumor tissue; magnification, x400.

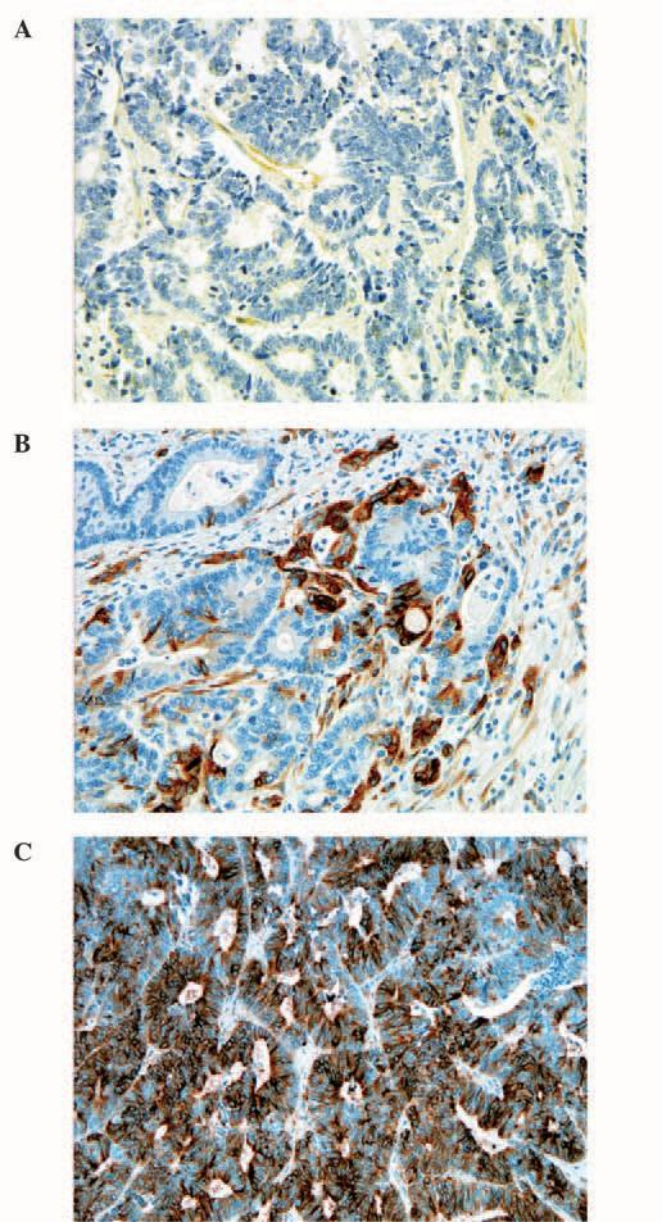

Figure 3. Representative examples of the expression of class III β-tubulin (TUBB3) protein in colorectal liver metastasis. (A) Score 0, no expression detectable or faint partial expression in <10% of the tumor cells; (B) score 1, diffuse and strong positive staining associated to invasive front and tumor budding, central tumor regions negative or with weaker intensity then at the invasive front; (C) score 2, strong positivity in the entire tumor tissue; magnification, x400.

Statistically significant correlation between ERCC1, RRM1, TUBB3 and MMR proteins, but not with p53. Regarding MMR proteins we found statistically significant correlations between MMR proteins and ERCC1, RRM1 and TUBB3. In turn, none of the three markers demonstrated a correlation with the p53 expression level. MLH1 and MSH2 proteins showed a positive statistically significant correlation with ERCC1 ($p<0.000$ and $p=0.008$, respectively). This means that loss of MLH1 and MSH2 is associated with lower expression or loss of ERCC1 in colorectal liver metastasis. A similar correlation was detected between MSH2, MSH6 and RRM1 ($p=0.005$ for MSH2 and $p=0.011$ for MSH6). Higher RRM1 expression levels were detected at intact expression of MSH2 and MSH6.

Notably, TUBB3 expression showed a strong positive correlation with MLH1 and MSH2 ($p=0.019$ and $p=0.012$, respectively). The detailed correlations are documented in Table II.

Cytoplasmic ARC staining intensity is strongly correlated with TUBB3 and RRM1 expression levels. In negative RRM1 cases, the ARC cytoplasmic expression was also low (score 0/1) (6/10, 60%). Cases with moderate RRM1 expression (score 1) also had in the majority of cases a low level of ARC expression (19/33, 57.6%). Fourteen of 33 cases (42.4%) with moderate RRM1 expression had a high level of cytoplasmic ARC (score 2/3). Cancers expressing RRM1 at high levels (score 2, 51 cases) showed, in the majority of cases, elevated cytoplasmic ARC levels [low ARC level in only 12 cases

Table II. Statistical correlations between ERCC1, RRM1 and TUBB3 with mismatch repair proteins and p53.

Protein	MLH1	MSH2	MSH6	PMS2	p53
ERCC1					
Correlation coefficient	**0.541**[a]	**0.273**[a]	0.186	0.168	0.164
Significance (2-sided)	**0.000**	**0.008**	0.072	0.106	0.116
No. of valid cases	71	94	94	94	93
RRM1					
Correlation coefficient	0.104	**0.283**[a]	**0.261**[b]	0.198	0.146
Significance (2-sided)	0.387	**0.005**	**0.011**	0.055	0.160
No. of valid cases	71	95	95	95	94
TUBB3					
Correlation coefficient	**0.271**[b]	**0.252**[b]	0.025	0.033	0.035
Significance (2-sided)	**0.019**	**0.012**	0.808	0.748	0.736
No. of valid cases	75	99	99	99	98

[a]The correlation is significant at the level of 0.01 (2-sided); [b]The correlation is significant at the level of 0.05 (2-sided). ERCC1, excision repair cross-complementing 1; RRM1, ribonucleoside-diphosphate reductase 1; TUBB3, class III β-tubulin.

Table III. Results of the statistical analysis between apoptosis repressor ARC protein and ERCC1, RRM1 and TUBB3.

Protein	Cytoplasmic ARC expression	Nuclear ARC expression
ERCC1		
Correlation coefficient	-0.053	-0.020
Significance (2-sided)	0.613	0.851
Number of valid cases	93	93
RRM1		
Correlation coefficient	**0.378**[a]	0.147
Significance (2-sided)	**0.000**	0.156
Number of valid cases	**94**	94
TUBB3		
Correlation coefficient	**0.323**[a]	-0.048
Significance (2-sided)	**0.001**	0.641
Number of valid cases	**98**	98

[a]The correlation is significant at the level of 0.01 (2-sided); [b]The correlation is significant at the level of 0.05 (2-sided). ERCC1, excision repair cross-complementing 1; RRM1, ribonucleoside-diphosphate reductase 1; TUBB3, class III β-tubulin.

Table IV. Results of the statistical analysis between ERCC1, RRM1 and TUBB3 and clinical data.

Protein	Age	Sex	Tumor grade	No. of metastases
ERCC1				
Correlation coefficient	0.106	-0.205	-0.010	-0.030
Significance (2-sided)	0.320	0.054	0.930	0.781
No. of valid cases	90	89	82	86
RRM1				
Correlation coefficient	0.004	0.001	0.151	0.027
Significance (2-sided)	0.971	0.995	0.174	0.803
No. of valid cases	91	90	83	87
TUBB3				
Correlation coefficient	**-0.269**[a]	-0.139	**0.213**[b]	0.026
Significance (2-sided)	0.008	0.178	0.047	0.807
No. of valid cases	96	95	87	92

[a]The correlation is significant at the level of 0.01 (2-sided); [b]The correlation is significant at the level of 0.05 (2-sided). ERCC1, excision repair cross-complementing 1; RRM1, ribonucleoside-diphosphate reductase 1; TUBB3, class III β-tubulin.

(12/51, 23.53%), high ARC level in 39 cases (39/51, 76.47%)]. In conclusion, a high level of RRM1 expression in most of the cases occurred simultaneously with elevated, high level cytoplasmic ARC protein expression (score 2/3 in 76.47% of the valid cases). Cytoplasmic ARC protein expression showed a positive, statistically significant correlation with RRM1 expression levels (p<0.000). Ninty-eight cases were valid for both proteins (ARC cytoplasmic and TUBB3). In TUBB3 negative cases (score 0), cytoplasmic expression of ARC was detected in 15 cases (15/35, 42.85%). In TUBB3 score 1 cases, it was even higher (35/51, 68.62%). In strongly diffuse positive TUBB3 cases, the highest cytoplasmic ARC expression was found (9/12, 75%). We found a progressive staining intensity for cytoplasmic ARC regarding TUBB3 status. This association was also significant (p<0.001). The distribution of the statistical results is listed in Table III.

Correlations between ERCC1, RRM1, TUBB3 and clinical data. Concerning clinical parameters such as age, sex of the patients, grade of the tumor and the number and size of metastases, there was no significant correlation with ERCC1 or RRM1 expression (Table IV). Regarding patient age, there

was a strong negative correlation with TUBB3 expression level (p=0.008). In addition, the TUBB3 expression level was also positively associated with tumor grade (p=0.047) and TUBB3 expression was also correlated with the RRM1 expression level (p=0.022).

Discussion

Apoptotic signaling is one of the most important processes in therapeutic resistance. In addition to known regulatory proteins, there are many others, which can influence the apoptotic process, and some can thus enhance or inhibit therapeutic effects. ERCC1, RRM1 and TUBB3 are known to have therapeutic predictive value in the current therapy of metastasized CRC (3,5,6). In the present study, we investigated the expression levels of ERCC1, RRM1 and TUBB3 in liver metastasis of CRC and analysed their associations to sex, age, tumor grade, mucin production, tumor size and number of metastases. Furthermore, we investigated their correlation to MMR proteins, p53 and apoptosis repressor ARC.

In our collective, ERCC1 protein loss was detected in one-third of the cases (29.8%). A proportion of 70.2% showed a positive reaction and score 2 (strong nuclear expression) was confirmed in 39.4% of all valid cases. In another study, which investigated stage III CRC, ERCC1 and MSI levels were found to be positive in 55 and 17%, respectively (7). According to literature data, score 2 cases do not benefit from platinum-based chemotherapy. On the other hand, one-third of cases (loss of ERCC1) have a better prognosis following platinum-based chemotherapy (15). Cases with moderate ERCC1 expression (score 1), in our opinion, require further investigation. It must be evaluated in functional studies, whether a mild loss in ERCC1 expression is enough to sensitize cancer cells to platinum-based chemotherapy. According to our scoring system, we detected certain negative and positive cases, thus allowing a prediction for platinum-based chemotherapy in two-thirds of the patients after a single immunohistochemistry.

We found a statistically significant positive correlation between ERCC1 and MLH1, MSH2 (p=0.000 and p=0.008, respectively). The frequent loss of ERCC1 and MLH1 both could be explained by methylation. similar correlations were found in mesothelioma (20). In NSCLC, ERCC1 nuclear staining was noted in 45.59% of the cases, and TUBB3 cytoplasmic staining was noted in 65.44% of the cases detected. ERCC1 and TUBB3 double-negative cases exhibited a better therapeutic response to platinum-based therapy (3). We found 11 cases (11.7% of valid cases) as double-negative for ERCC1 and TUBB3. Analogous to other epithelial neoplasia, 11.7% of the metastatic cases could benefit much more from platinum-based therapy, than the others. Negative expression of ERCC1 and TUBB3 was found to be associated with a significantly higher response rate, longer median progression-free survival and overall survival after platinum-paclitaxel treatment (3). One study found that patients with advanced CRC with high expression of ERCC1 are not indicated for oxaliplatin-based chemotherapy (17), whereas patients with low levels of ERCC1 expression have been reported to have an improved response and a longer overall survival in metastatic CRC treated with FOLFOx combination (18). In this study, a low level of ERCC1 was detected in 90% of the cases (18). One study, in accordance with other study results, demonstrated the potential utility of ERCC1 expression as a prognostic and possibly predictive biomarker in metastasized CRC. It was able to identify a population with poor prognosis, as well as a population with a markedly high response rate to FOLFOx combination therapy (18). The cases were evaluated according to a pre-established cut-off for ERCC1 (17). As noted, the expression levels of ERCC1 are not always consistent with sensitivity to platinum-based treatment. We assume that the cut-off level was set too high and suggest to define more than only two groups (positive and negative). Therefore, we propose the use of large prospective or retrospective studies with standard chemotherapy to analyse the expression of predictive markers, such as ERCC1, RRM1 or TUBB3 (and its interaction partners) to set a cut-off for cases, which can benefit from a therapeutic regimen. Too high or too low cut-off levels result in subsets of patients, who may suffer from long-term toxicity with no benefit of treatment, and patient groups that do not receive the optimal therapy regimen.

RRM1 overexpression is associated with gemcitabine resistance. RRM1 is a key molecule for gemcitabine efficacy and is also involved in tumor progression. High RRM1 expression in tumor tissue predicts significantly better prognosis, whereas only patients with low RRM1 benefit from gemcitabine therapy. In turn, overexpression of RRM1 protein is strongly associated with gemcitabine resistance (8). RRM1 expression was also reported to correlate with tumorigenic and metastatic potential in lung cancer (8). According to our data, only 11.6% of the cases with loss of RRM1 expression had a positive response to gemcitabine. In other words, patients with high RRM1 protein expression in tumor tissue should be treated with alternative drugs, i.e. oxaliplatin, 5-FU and leucovorin (CONkO-003) instead of gemcitabine (8). As mentioned above, score 1 cases need further functional analysis and lower RRM1 activity may also be sufficient not to overcome gemcitabine-induced DNA damage leading to the death of tumor cells.

Loss of MSH2 and MSH6 expression is associated with lower levels of RRM1 protein (p=0.005 and p=0.011, respectively). For this association the following mechanisms could be responsible: i) RRM1 is a key enzyme in the synthesis of new DNA, thus defected MMR proteins, i.e. MSH2 and MSH6 lead to DNA damage which can downregulate the new DNA synthesis, leading to cell cycle arrest. One possible connection between RRM1 and the MMR system is through DNA damage. It leads to cell cycle arrest, which results in lower RRM1 expression level or ii) there is a possible connection through transforming growth factor-β (TGF-β); in normal cells TGF-β can activate the MSH2 promoter (through Smad p53 dependent mechanism), whereas at the posttranscriptional level, miR-21 induced by TgF-β targets MSH2 transcript and suppresses its expression. In contrast, in cancer cells p53 is inactivated and miR-21 is overexpressed, thus TgF-β fails to activate the MSH2 promoter resulting in genomic instability (21).

Cytoplasmic ARC protein expression showed a positive, statistically significant correlation with RRM1 expression levels (p<0.000). Which mechanism leads to higher RRM1 expression with ARC overexpression or in turn how ARC expression can induce RRM1 overexpression should be elucidated in functional studies, but it is known that RRM1-overexpressing cells have an increased level of apoptosis (22),

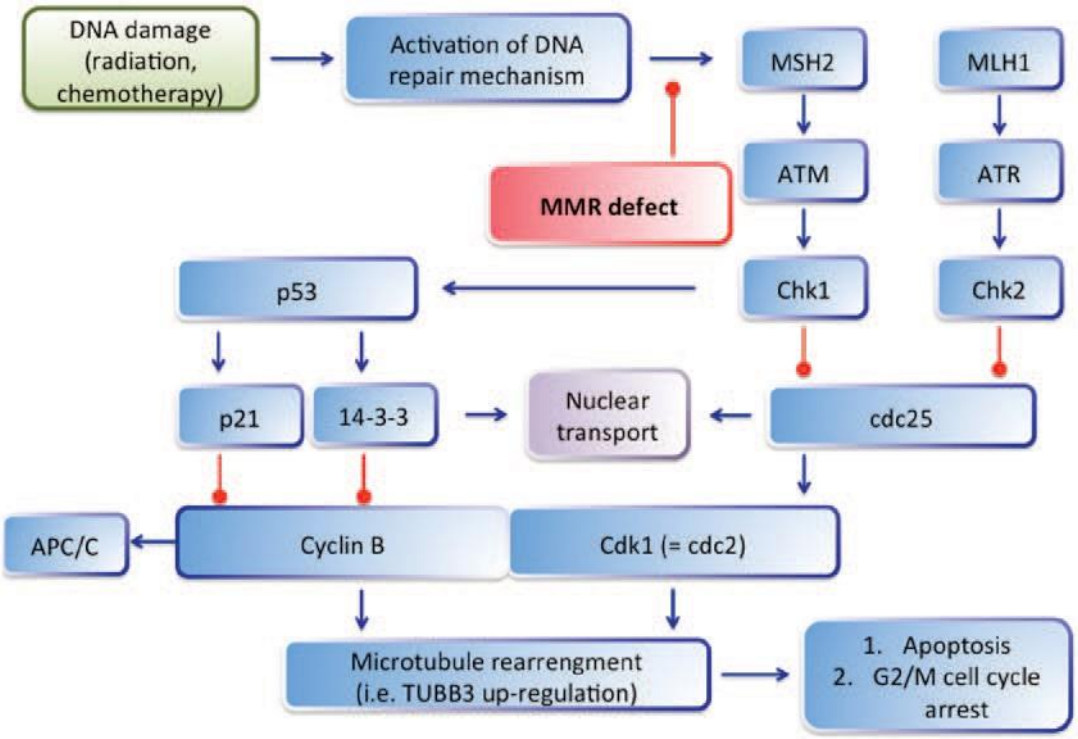

Figure 4. Schematic presentation of DNA damage-induced apoptosis and the possible role of the microtubule system.

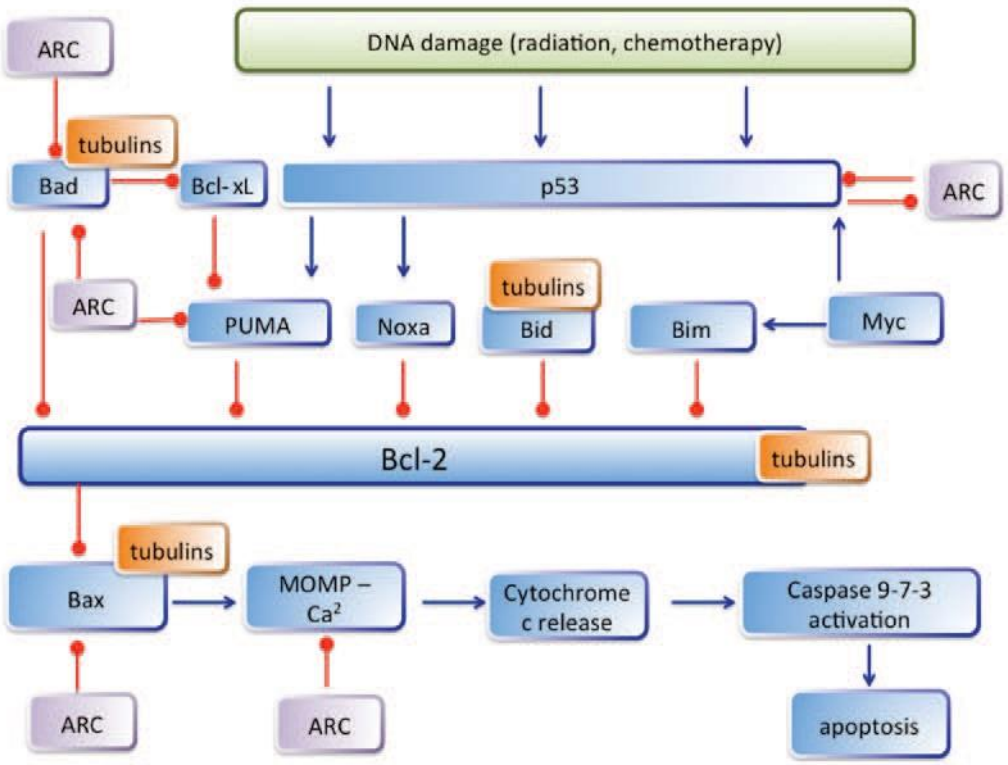

Figure 5. Interactions between tubulins and apoptotic signaling proteins including apoptosis repressor protein (ARC).

thus it is possible that a certain overexpression level can induce apoptotic signaling, which in turn induces ARC expression to suppress apoptosis induction. This possibility is further strengthened by the positive correlation between RRM1 and TUBB3 found in our collective (p=0.022).

The main staining pattern for TUBB3 was expression at the invasive front, similar to primary CRC studied previously (12). TUBB3 expression was not detected in 35% of the valid cases. These cases are potential candidates for taxane-based chemotherapy with highly predicted response. In our

collective, we found a statistically significant correlation between MLH1, MSH2 and TUBB3 (p=0.019 and p=0.012, respectively), which further strengthens the evidence of the regulatory role of mismatch repair proteins in apoptosis. In the case of sufficient MLH1 and MSH2 expression, TUBB3 is significantly highly expressed to suppress the activities of MLH1 and MSH2.

These results can indicate that a defected MMR system would induce TUBB3 overexpression leading to MT rearrangement, which can influence apoptosis (i.e. activating pro-apoptotic signaling proteins). Microtubules (MTs) have an important role in apoptosis, i.e. survivin is believed to regulate apoptosis by controlling microtubule polymerization. Thus, the disruption of normal MT function (either increasing or decreasing MT length) may trigger apoptosis. MT system (and thus TUBB3) has an important role in the regulation of DNA damage-induced apoptosis. DNA damage (i.e. γ-radiation) induces α-, β- and γ-tubulin production and polymerization, and stimulates MT reorganization (23). One explanation is that DNA damage through cyclin B1 and cdc2 kinase activation leads to tubulin polymerization and to release of apoptosis (23). The possible connection between TUBB3, DNA damage and mismatch repair are depicted in Fig. 4. After DNA damage, the ATM/ATR signaling pathway is activated and phosphorylates (and activates) Chk1 and Chk2, which subsequently phosphorylate cdc25 (23). Phosporylated cdc25 is sequestered in the cytoplasm by 14-3-3 proteins, which hinder the activation of cyclin B1/Cdk1 complex by cdc25 resulting in G2/M cell cycle arrest. In the case of MMR loss (i.e. in our cases, the loss of MLH1 and MSH2) the ATM/ATR system cannot be activated by MMR proteins and finally do not lead to cell cycle arrest. Consequently, microtubule rearrangement and TUBB3 upregulation is lacking. This correlation between MLH1/MSH2 and TUBB3 was statistically significant in our collective (p=0.019 and p=0.012, respectively).

Furthermore, MLH1 and MSH2 are responsible for resistance to cisplatin or methylating agents. The defective MMR system cannot recognize the cisplatin-induced DNA damage resulting in cell survival and therapeutic resistance (24,25). Taken together, cases with defected MMR system (microsatellite instable cancer) and with a high expression level of TUBB3 are potentially resistant not only to taxanes, but also to platinum-based therapy. Thus, we favor, in the case of MsI, immunohistochemical testing also for TUBB3 to exclude taxane resistance. The interactions between DNA repair systems, MT and apoptotic proteins (i.e. ARC) should be further investigated to elucidate the resistance mechanism of tumor cells and the survival regulatory mechanism.

In addition and alternatively to the above mentioned mechanisms, the statistically significant correlation between TUBB3 and cytoplasmic ARC expression (p=0.001) can also be explained. survival feedback mechanism can induce ARC expression to suppress pro-apoptotic signaling, thus cancer cells can survive despite of DNA damage (i.e. microsatellite instability). In our previous study, we found a strong correlation between ARC expression level and MSH2 status (16). TUBB3 overexpression can stabilize the MT system and make cancer cells resistant against anti-microtubule agents. Direct interaction between tubulin with several members of the Bcl-2 family has been described. Bcl-2, Bid and Bad were found to inhibit the assembly, whereas Bak and Bax promote tubulin polymerization. Thereby, tubulin is localized not only in the cytoplasm, but also binds to mitochondria (associated with VDAC in mitochondrial membrane). Both pro- and anti-apoptotic proteins bind to tubulin and those of lower affinity are more easily released following a conformational change induced by a ligand. Thus, Bcl-2, Bid and Bad may remain bound, while Bax would be released changing the ratio of free pro- and anti-apoptotic proteins. Furthermore, in the case of TUBB3 overexpression pro- and anti-apoptotic proteins stay bound, but tubulin ligands can change the affinity towards proteases. In addition, Bcl-2 protects against acetylation of tubulin and Bcl-2 is able to normalize the level of acetylated tubulin (26). The interaction between TUBB3 and apoptotic proteins (especially between ARC and TUBB3) seems to be more complex. There are many common interaction partners of ARC and TUBB3 and which protein effects will dominate depends on intracellular circumstances. The known interactions between apoptotic proteins, including ARC and TUBB3 are depicted in Fig. 5. Despite the increasing number of studies that highlight the importance of TUBB3 in tumor cells, its mode of action still needs to be fully determined. It appears that the intrinsic apoptotic pathway is involved as evidenced by increased caspase-3/7 activity (27). Evidence in other cell types suggests that TUBB3 may be part of a cell survival pathway. For instance, its expression level can be modulated by different types of cell stress, i.e. hypoxia (anti-VEGFR therapy) and nutrient deprivation (28,29). To confirm the interaction between ARC and TUBB3, functional studies are needed.

Testing the expression of ERCC1, RRM1 and TUBB3 is crucial and necessary before treatment for gemcitabine, cisplatin and 5-FU. As known, MSI tumors will not benefit from 5-FU treatment and, to this analogy, the testing for ERCC1, RRM1 and TUBB3 before platinum-based, gemcitabine and taxane therapy, respectively. There are a lack of diagnostic tests to determine which chemotherapy regimen offers the greatest chance for response in an individual patient (18). For metastatic CRC, the current treatment paradigm consists of 5-FU-based regimens in combination with either oxaliplatin (FOLFOx) or irinotecan (FOLFIRI), potentially combined with therapy targeting either EGFR or VEGFR inhibitor (30). Large, prospective clinical trials have shown that the response rates for either FOLFOx or FOLFIRI are only approximately 55% (15). Thus, there is an urgent need for reliable predictive markers before therapeutic decision in metastasized CRC.

In conclusion, we found statistically significant correlations between MMR proteins and ERCC1, RRM1 and TUBB3. Furthermore, we found a statistically significant correlation between the apoptosis repressor protein ARC and RRM1 and TUBB3. Taken together, regarding these proteins, there is a high therapeutic resistance potential in CRC metastasis. Thus we propose to test the known associated predictive proteins, before any therapeutic option is offered. Further functional studies need to declare the exact regulatory mechanism between RRM1, TUBB3 and ARC, as exact relations among these proteins cannot be measured by means of immunohistochemistry alone. The assessment of the abovementioned markers may be a helpful tool to design chemotherapy protocols for CRC liver metastasis and to define patients who may expect a greater clinical benefit. Selection of chemotherapeutic

drugs according to their predicted efficacy should be a part of future therapeutic decisions and prospective studies. A prospective validation of these markers is warranted.

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Loss of CDX2 gene expression is associated with DNA repair proteins and is a crucial member of the Wnt signaling pathway in liver metastasis of colorectal cancer

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Abstract. Caudal type homeobox 2 (CDX2) has been well-established as a diagnostic marker for colorectal cancer (CRC); however, less is known about its regulation, particularly its potential interactions with the DNA repair proteins, adenomatous polyposis coli (APC) and β-catenin, in a non-transcriptional manner. In the present study, the protein expression of CDX2 was analyzed, depending on the expression of the DNA repair proteins, mismatch repair (MMR), O6-methylguanine DNA methyltransferase (MGMT) and excision repair cross-complementing 1 (ERCC1), and its importance in Wnt signaling was also determined. A total of 101 liver metastases were punched into tissue microarray (TMA) blocks and serial sections were cut for immunohistochemistry. For each protein, an immunoreactive score was generated according to literature data and the scores were fitted to TMA. Subsequently, statistical analysis was performed to compare the levels of expression with each other and with clinical data. CDX2 loss of expression was observed in 38.5% of the CRC liver metastasis cases. A statistically significant association between CDX2 and each of the investigated MMRs was observed: MutL Homolog 1 (P<0.01), MutS protein Homolog (MSH) 2 (P<0.01), MSH6 (P<0.01), and postmeiotic segregation increased 2 (P=0.040). Furthermore, loss of MGMT and ERCC1 was also associated with CDX2 loss (P=0.039 and P<0.01, respectively). In addition, CDX2 and ERCC1 were inversely associated with metastatic tumor size (P=0.038 and P=0.027, respectively). Sustained CDX2 expression was associated with a higher expression of cytoplasmic/membranous β-catenin and with nuclear APC expression (P=0.042 and P<0.01, respectively). In conclusion, CDX2 loss of expression was not a rare event in liver metastasis of CRC and the results suggested that CDX2 may be involved in mechanisms resulting in the loss of DNA repair protein expression, and in turn methylation; however, its exact function in this context remains to be elucidated.

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Key words: caudal type homeobox 2, colorectal cancer, liver metastasis, DNA repair proteins

Introduction

Colorectal cancer (CRC) is the third most deadly cancer worldwide accounting for more than 600,000 deaths annually (1). At the diagnosis, a quarter of the patients with primary CRC have synchronous hepatic metastasis, and more than 50% of the patients with CRC will develop liver metastases in the course. Almost half of the patients undergoing resection for primary CRC eventually develop metachronous liver metastasis. Survival in metastatic cases is rarely longer than three years (2). Interestingly, although caudal type homeobox 2 (CDX2) is widely used in the daily routine diagnostic, there are less than sixty publications in the last sixty years performed on human tissue investigating the role of CDX2 (3).

The Cdx family of transcription factors contributes also to the CRC phenotype, but a mechanism by which CDX2 expression is lost or downregulated in colorectal tumors is currently not clear. The CDX2 is necessary for the proper development of the intestinal tract and is crucial for development and homeostasis of the intestinal epithelium throughout life (1). The role of Cdx2 in colorectal carcinogenesis is multi-sided. The CDX2 expression is reduced in CRC and its expression is inversely correlated to tumor grade, tumor stage and lymph node metastasis (4). Loss of CDX2 expression can strongly predict high level CpG island methylation phenotype (CIMP-H) independently from microsatellite status of CRCs. Thus Cdx2 was proposed as a surrogate marker for CIMP-H (5). In addition, CDX2 was attributed to play a regulatory role in apoptosis and DNA repair. Colon epithelium with decreased CDX2 expression lead to impaired apoptosis potential after γ-irradiation,

thus resulting in higher resistance to genotoxic stress. Besides, the effect of CDX2 in DNA repair activity can contribute to its attributed tumor suppressor function (6).

DNA methylation of tumor suppressor genes resulting in its transcriptional inactivation and has been identified as an important mechanism. CIMP characterized by the extensive hypermethylation of multiple CpG islands, and belongs to one of the major mechanisms in the colorectal carcinogenesis (7). O6-methylguanine DNA methyltransferase (MGMT), a surrogate marker for CIMP, gene promoter methylation plays an important role in colorectal carcinogenesis. Loss of MGMT expression, which is secondary to gene promoter methylation, occurs in approximately 30-40% of metastatic CRC. In addition, loss of MGMT expression results in high response to alkylating agents (i.e., dacarbazine or temozolomide) (8). Thus, MGMT is believed to have predictive potential for therapy.

A further level of DNA damage defence mechanism is represented by the mismatch repair (MMR) system, which take part not only in the DNA repair processes, but also in the regulation of cell cycle check-points and apoptosis (9). Deficiency of MMR proteins (i.e., MLH1 and MSH2) is responsible for resistance to various chemotherapeutic drugs and subsequently for resistance to apoptosis (9). Interestingly, loss of MGMT expression is more frequent in CRC with microsatellite instability, suggesting that methylated MGMT selects cellular clones with MMR deficient status (8). Moreover, MMR deficiency is also correlated with loss of CDX2 (10).

Excision repair cross-complementing 1 (ERCC1) is a structure specific DNA repair endonuclease responsible for 5' incision (5'-endonuclease), a key enzyme in nucleotide excision repair (NER) pathway and is essential for repair of platinum-DNA adducts, thus associated with therapy resistance to platinum-containing compounds (i.e., cisplatin) (11,12).

Aberrant β-catenin expression and disturbed Wnt signaling is recognized as an important event in the genesis of several malignancies, especially in CRC. β-catenin mutations or loss-of-function mutations of the adenomatous polyposis coli (APC) tumor suppressor gene appear to be crucial steps in the progression of this disease (13). APC and β-catenin were found to traffic independently from each other into and out of the nucleus in response to internal and external signals. This fact has prompted debate about the previously proposed role of APC as a β-catenin chaperone (14). Germline mutations in the APC gene cause familial adenomatous polyposis (FAP), and over 80% of CRCs (both inherited and sporadic) carry truncating mutations that inactivate the APC protein. Most of these mutations occur in the so-called 'mutation cluster region' of the APC gene, accounting for a truncated protein incapable of binding regulatory proteins (i.e., Axin) or associating with microtubules. The relevance of truncating mutations for β-catenin is enormous: Mutated APC cannot stimulate its degradation (because of its failure to bind Axin), although APC still can bind to β-catenin (albeit less efficiently) (14,15). β-catenin has been observed to accumulate in the nuclei of colon cancer cells, which results from the inability of APC to promote β-catenin degradation, rather than a lack of export function, leading to nuclear accumulation of β-catenin in APC-mutant tumor cells (14). There are only few studies that focused on interactions between CDX2 and Wnt signalling in colon cancer. It has been demonstrated that CDX2 can inhibit the transcriptional activity of β-catenin/TCF lines in a non-transcriptional way (4).

Expression of CDX2 in association with DNA repair proteins and members of Wnt signaling pathway has not been studied previously in liver metastasis of CRC. In this study, we analysed the expression distribution of CDX2 in matters of expression status of DNA repair proteins (MMR proteins, MGMT and ERCC1), APC, and β-catenin. Furthermore, we correlated CDX2 protein expression with clinical data.

Materials and methods

Tissue samples. Formalin-fixed paraffin-embedded surgical specimens of liver metastasis of CRC were selected from the archives of the Institute of Pathology at the University Hospital of Heidelberg. Hundred and one patients without neo-adjuvant chemotherapy (64 male, 37 female; mean age 62 years) were included. Tumor size was between 5 mm and 16 cm in diameter. 12 cases showed mucinous adenocarcinoma histology and 89 cases showed histology of adenocarcinoma NOS. We had only two cases with grade 1 adenocarcinoma, 83 cases had grade 2 and 12 cases grade 3 histology. Serial paraffin sections were cut at 4 μm for immunohistochemistry. Important clinical data, such as: Age, gender, size and number of metastases were collected from histological reports. Tissue samples were provided by the tissue bank of the National Centre for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the ethics committee of Heidelberg University according to ethical standards formulated in the Declaration of Helsinki 1975 (revised in 1983).

Tissue microarray (TMA). TMA blocks were punched from paraffin-embedded human liver specimens with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). From each case, two cores of tumor tissue were punched with a diameter size of 1.6 mm and two muscle cores were used for orientation of the TMA slides. Therefore serial sections were cut from the TMA block. So far, there is no standardised operating protocol or universal agreement for sampling and staining of TMA blocks and slides. The general consensus is that at least two 0.6 mm cores adequately represent for immunohistochemical changes (16,17).

Immunohistochemistry. 4 μm thick slides were obtained from TMA blocks. Slides were then deparaffinised according to standard protocol by xylene, and dehydrated with 95-96% ethanol, 70% ethanol and distilled water. All slides were stained simultaneously using a computer-controlled autostainer (Dako TechMate 500 cytomation) and Dako EnVision-Sytem (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and pretreated with 3% Hydrogen Peroxide prior to antibody incubation. MLH1 [M1, ready-to-use (RTU), Ventana Medical Systems, Inc.; Roche Diagnostics, Basel, Switzerland], MSH6 (44, RTU; Ventana Medical Systems, Inc.; Roche Diagnostics), PMS2 (EPR3947, RTU; Ventana Medical Systems, Inc.; Roche Diagnostics), MSH2 (G219-1129, RTU; Ventana Medical Systems, Inc.; Roche Diagnostics), MGMT (MT-23.2; Thermo Fisher Scientific, Inc., Waltham, MA, USA; 1:20) and CDX2 (EPR2764Y; Thermo Fisher Scientific, Inc.; 1:200) antibodies were used. Secondary antibody binding (all Dako, 1:200) was

visualised using a streptavidin ABC-kit (Dako), followed by 3,3'-diaminobenzidine (Vector, Peterborough, UK). For ERCC1 (8F1, Neomarkers; dilution: 1:100) and β-catenin (14, RTU; Ventana Medical Systems, Inc.; Roche Diagnostics) slides were stained by a computer-controlled autostainer (Ventana BenchMark Ultra; Ventana Medical Systems, Inc.; Roche Diagnostics). Polyclonal rabbit anti-APC antibody (DP2.5 1:200 Fa; Acris Antibodies; OriGene Technologies, Inc., Rockville, MD, USA) were used for APC staining. Staining was performed using ChemMate Detection kit (Dako) according to recommendations of the manufacturer. The antibodies were incubated overnight at 4˚C followed by avidin-biotin complex peroxidease technique using amino-ethylcarbazole for visualization and hematoxylin for nuclear counterstaining. All slides were covered with Aquatex (Merck KGaA, Darmstadt, Germany).

Evaluation of immunohistochemistry. For the semi-quantitative assessment of staining intensity, we adjusted a previously published scoring system for each protein and fitted to TMA dots. For MSI proteins and for MGMT the staining was evaluated according to Bethesda guidelines (18): score 1, more than 10% of tumor cell nuclei are positive; score 0, less than 10% of tumor cell nuclei positive (but: positive internal control, i.e., stromal cells and lymphocytes). The immunostained TMA sections were evaluated and scored under a light microscope independently by two pathologists in a blinded fashion. Discordant cases were reviewed and re-evaluated based on a consensus opinion.

Immunostaining for CDX2, ERCC1 and nuclear β-catenin was scored in a three-graded scale: score 0, weak staining in less than 10% of the tumor cells; score 1, moderate staining in up to 75% of the tumor cells; and score 2, strong nuclear staining in more than 75% of the tumor cells. For cytoplasmic β-catenin staining a two-graded scale was used: score 0, no or weak staining in less than 10% of tumor cells and weaker staining compared to normal colonic mucosa; score 1, nuclear staining in more than 10% of the tumor cells.

Cytoplasmic and nuclear APC staining was separately scored. For nuclear APC staining a two-graded scale was used: score 0, No or weaker staining in less than 10% of tumor cells and weaker staining compared to normal colonic mucosa; score 1, nuclear staining in more than 10% of the tumor cells. For cytoplasmic APC staining a three-graded scale was used: score 0, no cytoplasmic staining or weak staining in less than 10% of tumor cells; score 1, 10-75% of the tumor cells with moderate intensity; and score 2, more than 75% of the tumor cells are positive with high staining intensity. Normal colorectal mucosa was set as baseline expression level for APC (score 2).

Statistical analysis. The statistical analyses were performed with SAS software (SAS Institute, Inc., Cary, NC, USA). Spearman-Rho test was used to evaluate the relationship between clinical data, CDX2, MLH1, MSH2, MSH6, PMS2, MGMT, ERCC1, APC and β-catenin.

Results

CDX2 expression and its correlation with clinical data. We could reach valid expression data for CDX2 (Table I) in 83 of 101 cases. 32 cases (38.55%) show no nuclear expression. Positive stainings (61.45%, n=51/83) can be subdivided into two groups: Moderate nuclear expression with score 1 (16.87% n=14); and strong positivity with score 2 (44.58% %, n=37). Representative photomicrographs of CDX2 immunohisto-chemistry are depicted in Fig. 1.

Concerning clinical parameters like: Age, gender of the patients, grading of the tumor and the number of metastases, there was no significant correlation to CDX2 expression. Regarding the size of the metastasis a strong negative correlation could be detected (P=0.038). In addition to CDX2, ERCC1 expression was also strongly correlated with the size of the metastases (P=0.027). Bigger metastasis size diameter was seen in cases with CDX2 and ERCC1 loss.

Expression distribution of DNA repair proteins and proteins involved in Wnt-signaling. For MGMT 97 valid cases were obtained. Loss of MGMT expression was found in 24 cases (24.75%). Representative photomicrographs of MGMT immunohistochemistry are depicted in Fig. 2. Nuclear positivity was sustained in 73 cases (75.25%). Out of 94 valid cases for ERCC1 we found 29.8% of the cases negative (score 0). Positive ERCC1 staining could be in 70.2% of the cases detected (30.8% score 1 and 39.4% score 2). Representative photomicrographs of ERCC1 immunohistochemistry are depicted in Fig. 3. Both MGMT and ERCC1 loss is strongly associated with female gender (P=0.011, and P=0.047, respectively).

Regarding MMR proteins, the following distribution was seen: Loss of expression was detected in 4.2 to 26% of the cases (MLH1 4.2%, MSH2 26%, MSH6 24% and PMS2 9.5%, respectively) as published before (19). Loss of PMS2 is associated with loss of MGMT (P=0.014) and loss of MLH1 and MSH2 were also associated with loss of ERCC1 (P<0.01, and P<0.01, respectively). Representative photomicrographs of MMR protein immunohistochemistry are depicted in Fig. 4. Expression distribution of β-catenin, and APC proteins are depicted in Table I.

Statistical correlations between CDX2 and DNA repair proteins. We found statistically strong positive correlation between CDX2 and all of analysed DNA repair proteins (Table II). These results mean that loss of CDX2 expression is strongly associated with loss of expression of DNA repair proteins (MMR proteins, MGMT and ERCC1).

Statistical correlations between CDX2, APC and β-catenin. We analysed the possible statistical correlation between CDX2 and β-catenin, and APC (Table III). Cytoplasmic, but not nuclear β-catenin expression is associated with sustained nuclear CDX2 expression (P=0.042). In addition, CDX2 is positively correlated with nuclear APC expression (P<0.01). Cytoplasmic and nuclear β-catenin is associated also positive with each other (P<0.01). Representative photomicrographs of APC and β-catenin immunohistochemistry are depicted in Figs. 5 and 6, respectively.

Discussion

In this study, we have demonstrated significant correlations between CDX2, DNA repair proteins and crucial members of Wnt signaling. To our knowledge, this is the first report

Table I. Distribution of immunostaining results of CDX2, APC and β-catenin.

Protein	Score 0 (%)	Score 1 (%)	Score 2 (%)	No. of valid cases (%)
CDX2	32 (38.55)	14 (16.87)	37 (44.58)	83 (100)
Nuclear APC	62 (61.38)	39 (38.62)	-	101 (100)
Cytoplasmic APC	13 (12.87)	75 (74.26)	13 (12.87)	101 (100)
Cytoplasmic β-catenin	37 (38.14)	60 (61.86)	-	97 (100)
Nuclear β-catenin	60 (61.86)	21 (21.65)	16 (16.49)	97 (100)

CDX2, caudal type homeobox 2; APC, adenomatous polyposis coli.

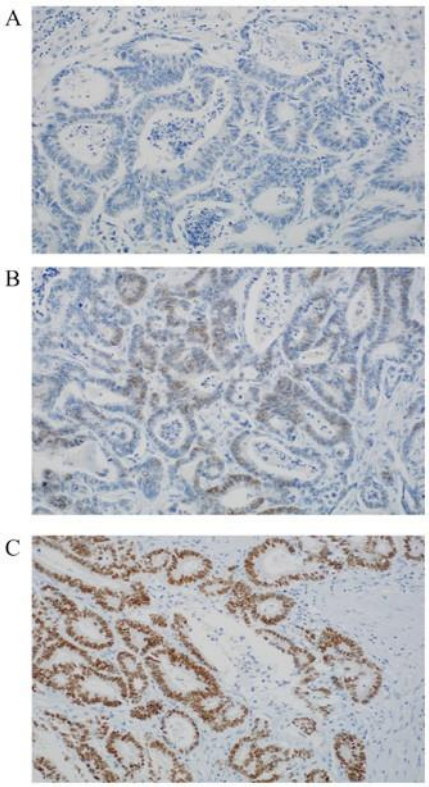

Figure 1. Representative examples of caudal type homeobox 2 protein expression in colorectal liver metastasis. (A) Score 0, negative staining- <10% of the nuclei are stained. (B) Score 1, moderate overexpression in <75% of the nuclei. (C) Score 2, strong overexpression in almost all tumor cells. Magnification, x200.

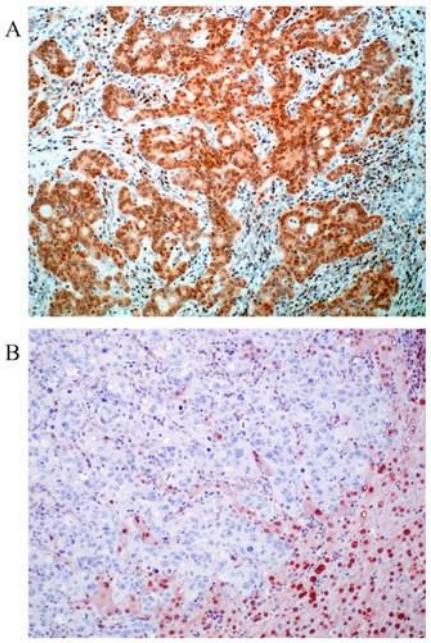

Figure 2. Representative examples of O6-methylguanine DNA methyltransferase protein expression in colorectal liver metastasis. (A) Score 0, negative staining- <10% of the nuclei are stained. (B) Score 1, >10% percent of the nuclei are positive. Note that in negative cases, stromal cells, lymphocytes and hepatocytes are still nuclear positive. Magnification, x200.

performed on human tissue of CRC liver metastasis presenting statistically significant correlations between expression of CDX2 referring to expression of MMR proteins and key proteins of base and nuclear excision repair. Furthermore, we show, for the first time, significant correlation between CDX2, APC and β-catenin in liver metastasis of CRC.

Loss of CDX2 expression is seen in approximately 30% of human CRC and is associated with higher tumor grade (1). We found loss of CDX2 expression in 38.55% of the cases. Loss of CDX2 expression was negatively correlated with tumor size, but no correlation with age, gender of the patients, grade of the tumor and the number of metastases. Interestingly, ERCC1 expression loss was also correlated with tumor size. Furthermore, loss of CDX2 is strongly correlated with loss of ERCC1. Thus, we can conclude, that loss of CDX2 or ERCC1 expression is strongly associated with bigger metastatic tumor size. Similar results for ERCC1 were found recently in breast cancer (20), but the exact mechanisms are still unclear.

We can demonstrate statistically significant correlations between CDX2 and DNA repair proteins: Loss of CDX2 expression is associated with loss of MMR proteins, MGMT, and ERCC1. These results are consistent with literature data from primary CRC: MMR-deficient or MSI high CRCs have significant losses of CDX2 expression. In addition, loss of CDX2 is associated with CIMP-high, more aggressive histomorphological features, and unfavourable survival (21). In a study on primary CRC and its lymph node metastasis reduced expression of CDX2 were found to be as predictor of MMR-deficiency in CRC. Moreover, loss of CDX2 is a poor prognostic factor, even among patients with MMR-proficient cancers (22).

Mutations in DNA repair genes are rare in sporadic cancers with DNA repair deficiency. However, DNA repair deficiency occurs in a majority of sporadic cancers caused

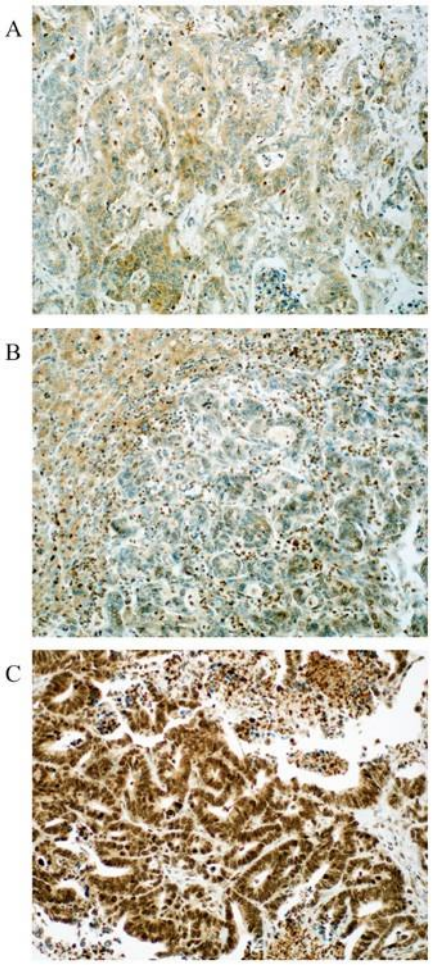

Figure 3. Representative examples of excision repair cross-complementing 1 protein expression in colorectal liver metastasis. (A) Score 0, no expression detectable or faint partial expression in <10% of the tumor cells. (B) Score 1, weak to moderate expression of the entire tumor tissue. (C) Score 2, strong positivity in the entire tumor tissue. Magnification, x200.

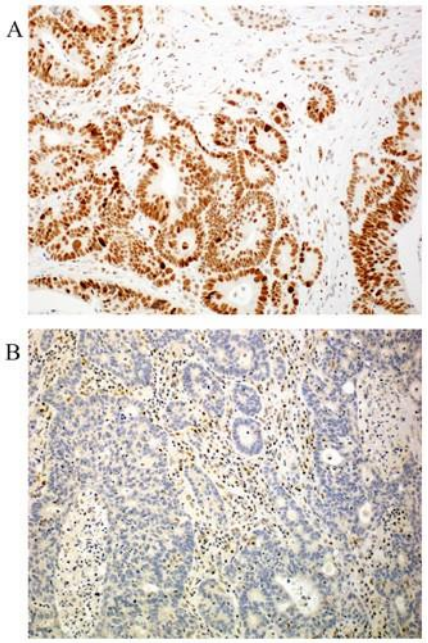

Figure 4. Representative examples of mismatch repair system protein expression (MutL Homolog 1 staining) in colorectal liver metastasis. (A) Score 0, negative staining- <10% of the nuclei are stained. (B) Score 1, >10% percent of the nuclei are positive. Note that in negative cases, stromal cells are still positive. Magnification, x200.

by epigenetic alterations that reduce or silence DNA repair gene expression. For example, a majority of primary CRCs have reduced MGMT expression due to i.e., methylation of the MGMT promoter region (an epigenetic alteration) (23). MGMT can be epigenetically depressed in many ways. Beside hypermethylation, MGMT can be depressed by di-methylation of lysine 9 of histone 3 (24) or by over-expression of a number of microRNAs including miR-181d, miR-767-3p and miR-603 (25).

Methylation of MGMT promoter region plays a significant role not only in carcinogenesis but also predictive for therapy response. In glioblastoma multiforme, the methylation state of the MGMT gene determined whether patients would be responsive to temozolomide therapy (26). On a clinical level, this translates into a prolonged survival of glioblastoma patients with a methylated MGMT promoter. In addition, MGMT methylation can be used to predict patient survival in clinical prediction models (27).

Loss of MGMT and ERCC1 expression was associated with female sex in our study. Similar data were demonstrated in primary CRC for MGMT (28) and for ERCC1 in lung cancer (29), thus we can conclude that this phenomenon stay maintained in liver metastasis. For ERCC1 our study is the first demonstrating statistically significant correlation with female gender in CRC. ERCC1 is essential for a functional NER system and ERCC1 expression loss may contribute to impaired DNA repair capacity thus increasing cancer risk. Reduced expression or loss of ERCC1 and MGMT were reported in vast majority of CRCs (30,31), and ERCC1 promoter hypermethylation in 38% of gliomas, resulting in reduced mRNA and protein expression (32). Disturbed ERCC1 protein expression appears to be an early event in colorectal carcinogenesis: reduced or loss of ERCC1 expression was detected in 40% of the colonic crypts within early field defects in colorectal mucosa (30). Similarly to MGMT, ERCC1 silencing can be resulted not only from promoter methylation, but can also be evocated by miRNAs repressing its expression (33). Whether epigenetic mechanisms reduce ERCC1 and MGMT protein expression in liver metastasis of CRC has to be determined in methylation studies. In general, the exact role of ERCC1 should be further elucidated because of its predictive role in chemotherapy. Pre-clinical studies have demonstrated its important role in determining cisplatin resistance (34).

In summary, loss of CDX2 is associated with each DNA repair protein, which we analysed and our results in liver metastasis are in accordance with the literature data originated from primary CRC (21,22). Loss of CDX2 has also been found to be an independent predictor of the CIMP-high phenotype (22). We used MGMT as surrogate marker for CIMP phenotype, but it has been noted that studies about MGMT methylation and CIMP had inconsistent findings, thus tumors with loss of MGMT cannot be clearly classified as CIMP phenotype (35). CIMP-high CRCs have been reported to have a different clinicopathological features than CIMP-low ones. CIMP-high phenotype is associated with older age, cigarette smoking, proximal tumor location, female gender, poorly differentiated

Table II. Results of statistical analysis between CDX2, DNA repair proteins and tumor size.

			DNA repair proteins					
Gene	Analysis	Tumor size (mm)	MLH1	MSH2	MSH6	PMS2	MGMT	ERCC1
CDX2	Correlation coefficient	-0.247[a]	0.388[b]	0.334[b]	0.317[b]	0.228[a]	0.236[a]	0.574[b]
	Significance (2-sided)	0.038	<0.001	0.002	0.004	0.040	0.039	<0.001
	Number of valid cases	71	77	82	82	82	77	74

[a]P<0.05 and [b]P<0.01. CDX2, caudal type homeobox 2; MLH1, MutL Homolog 1; MSH, MutS protein Homolog; PMS2, postmeiotic segregation increased 2; MGMT, O6-methylguanine DNA methyltransferase; ERCC1, excision repair cross-complementing 1.

Table III. Results of statistical analysis between CDX2, APC and β-catenin.

Gene	Analysis	Membranous/cytoplasmic β-catenin	Nuclear β-catenin	Cytoplasmic APC	Nuclear APC
CDX2	Correlation coefficient	0.231[a]	0.152	0.065	0.415[b]
	Significance (2-sided)	0.042	0.183	0.567	<0.001
	Number of valid cases	78	78	79	79

[a]P<0.05 and [b]P<0.01. CDX2, caudal type homeobox 2; APC, adenomatous polyposis coli.

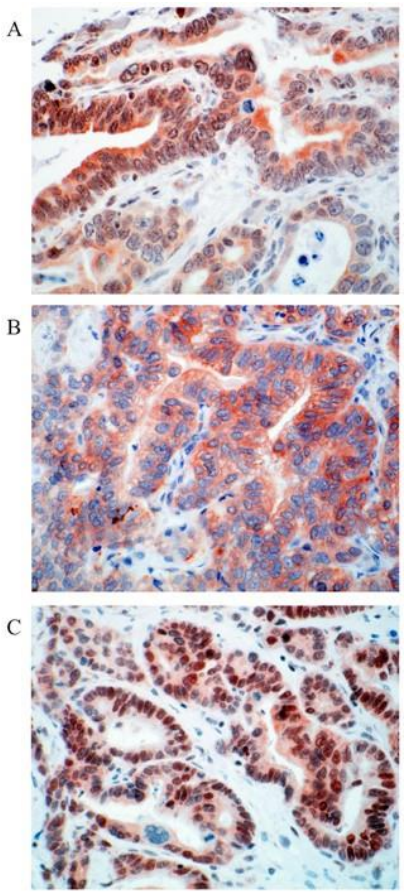

Figure 5. Representative examples of APC protein expression in colorectal liver metastasis. (A) Cytoplasmic and nuclear expression of APC protein. (B) Only cytoplasmic APC positivity in the tumor cells was observed. (C) Strong nuclear positivity next to faint cytoplasmic staining. Magnification, x400. APC, adenomatous polyposis coli.

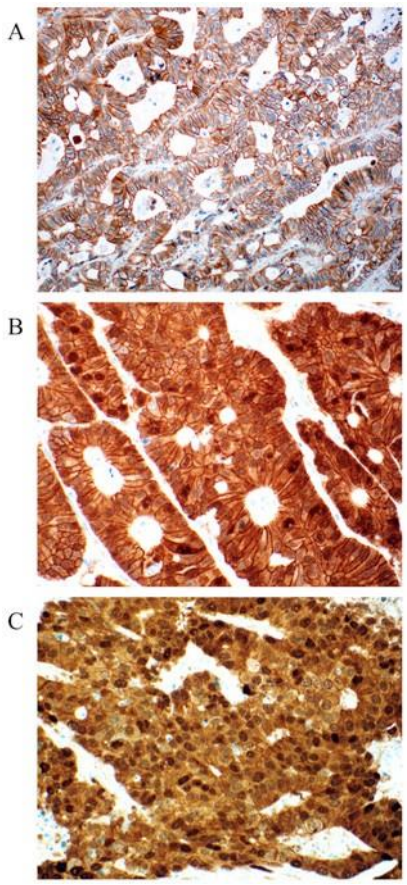

Figure 6. Representative examples of β-catenin expression in colorectal liver metastasis. (A) Only membranous staining was observed (score 0 for cytoplasm and nucleus). (B) Cytoplasmic expression of β-catenin with some positive nuclei (score 1 for cytoplasm and nuclei). (C) Diffuse and strong cytoplasmic and nuclear staining (score 1 for cytoplasm, and score 2 for nuclei). Magnification, x200.

or mucinous adenocarcinoma, MSI, and BRAF mutation. In addition, CIMP-high cancers regardless of microsatellite status show a poorer outcome (36). We suggest that MGMT is an adequate marker to detect CIMP phenotype.

The Wnt-β-catenin pathway is a crucial signalling pathway in control of embryonic development and tissue homeostasis. Its deregulation is observed in many cancers (i.e., CRC, non-hepatitis-related hepatocellular cancers, cholangiocarcinoma, desmoid tumor, breast cancer, osteosarcoma etc) (37). The pathway is over-activated in almost all colon cancer because of mutations of APC tumor suppressor gene, which actually represent the initiating event in colorectal carcinogenesis (38). Nevertheless, the actual mechanisms, which regulate β-catenin still remain highly controversial. Furthermore, the exact role of APC in particular is unclear, and the consequences of the mutations found in cancer cells are still poorly defined (38). Subcellular localisation of APC protein is differentially regulated in normal tissues and cell lines: in normal human colorectal epithelium, APC is located in the nuclei at basal segment of the crypts; in HT29 colon cancer cells, truncated APC translocated to the nucleus during early apoptosis (39), and cellular APC accumulates in the nucleus of sub-confluent cells but is partly excluded in super-confluent cells (14). Although there is consensus in many areas in the field of nuclear APC localization and function, there have also been some conflicting results with no apparent resolution. Moreover, the specificity of several APC antibodies has been investigated, with no clear consensus about the 'best' antibody to detect APC protein (40). The nuclear transport of APC in tumor cells occurs independently of β-catenin translocation to the nucleus or plasma membrane (41).

Nuclear accumulation of β-catenin is also observed in cancers resulting from mutations in the β-catenin, APC or Axin genes (15,42). The APC tumor suppressor binds to β-catenin and the scaffold protein Axin to form a complex promoting GSK-3β phosphorylation of β-catenin. However, overexpression of APC (1-1309), the most frequently occurring APC cancer mutant, translocates β-catenin from the nucleus to the cytoplasm. This mutant therefore has the ability to bind and regulate localization but lacks the Axin binding sites required for β-catenin degradation. Therefore, it seems more likely that it is the inability of APC to promote β-catenin degradation, rather than a lack of export function, that causes the nuclear accumulation of β-catenin in APC-mutant tumor cells (14).

Little is known about the connections between CDX2 and Wnt signaling pathway. In a study on Caco-2 cells lower CDX2 expression is associated with endogenous downregulation of APC expression, but did not affect GSK3β expression (4). Our analysis led to similar results: Reduced expression or loss of CDX2 is associated with reduced nuclear APC expression ($P<0.01$). In our study, the cytoplasmic APC expression was not associated with CDX2 expression. We assume that although CDX2 induce APC expression, which is already proven (4), the truncated APC protein cannot be shifted to cytoplasm, but we could detect this truncated protein with our antibody. In conclusion, truncated APC can be detected with immunohistochemistry and has certainly not lost its full function and can still participate in β-catenin regulation. Thus, APC can still fulfil an unexpectedly large spectrum of APC function (38). Furthermore, we found statistically significant correlation between CDX2 and cytoplasmic β-catenin. We think this correlation can be explained through the Mucdhl, a common interaction partner for β-catenin and CDX2. It has been shown that β-catenin interacts with a protocadherin Mucdhl, which is regulated by CDX2 in mice. Membrane-bound β-catenin is a consequence of interactions to membranous-expressed Mucdhl. Thus, Mucdhl can inhibit β-catenin translocation to the nucleus (4).

CDX2 is indeed expressed in all stages of CRC, little is known about its expression manner in association with other established prognostic or predictive proteins. In this report, we have directly demonstrated that CDX2 gene expression is strongly associated with DNA repair proteins and crucial members of Wnt signaling. Our results further strengthen the role of CDX2 in DNA repair and in regulation of APC and β-catenin expression. In fact, our analysis is restricted only for metastasis, our results strongly suggest potential (functional) interactions between the investigated proteins. To our knowledge, this is the first study to investigate CDX2 in this context on human liver metastasis of CRC. Although, CDX2 is a useful marker in routine diagnostics for CRC, its exact role in liver metastasis remains to be further elucidated. In further studies should be investigated if primary CRC differs from liver metastasis regarding CDX2 expression.

Acknowledgements

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