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.

University of Szeged
Faculty of Medicine Department of Pathology
Doctoral School of Interdisciplinary Medicine

Supervisor: Farkas Sükösd, M.D., Ph.D.

Szeged, Hungary

2018

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

Szent-Györgyi Albert

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 important 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.

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 [14]. 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 [15]. Interestingly, loss of MGMT expression is more frequent in CRC with microsatellite instability, suggesting that methylated MGMT selects cellular clones with MMR deficient status [16]. Moreover, mismatch repair deficiency is also correlated with loss of CDX2 [17].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 [18].

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)). 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 [19]. 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 [19].

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 [20]. 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-and-one patients (64 male, 37 female; mean age 62 years) were included. None of the patients had received neo-adjuvant chemotherapy. 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.

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.

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. 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.

4. 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. 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%). 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). 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.

2. ARC EXPRESSION

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**). 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**).

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 immunohistochemical 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 %.

2.1. ARC protein expression in association with histopathological parameters

Neither cytoplasmic nor nuclear ARC expression has statistically significant correlation with clinical parameters. Concerning clinical parameters such as 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 ($p=0.548$).

2.2. Correlation between ARC and p53 protein expression

Cellular ARC expression levels are independent from p53 staining status. 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).

2.3. ARC protein expression and MMR proteins

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.

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

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.

4. CDX2 EXPRESSION AND ITS CORRELATION WITH CLINICAL DATA

We could reach valid expression data for CDX2 (**Table 3**) 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). 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 3 Distribution of immunostaining results of CDX2, APC and β-catenin.

5. 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%). 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). 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 [21]. 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). Expression distribution of β-catenin, and APC proteins are depicted in **Table 3**.

6. STATISTICAL CORRELATIONS BETWEEN CDX2 AND DNA REPAIR PROTEINS

We found statistically strong positive correlation between CDX2 and all of analysed DNA repair proteins (**Table 4**). 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 cases	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 4 Results of statistical analysis between CDX2 and tumor size and DNA repair proteins.

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

We analysed the possible statistical correlation between CDX2 and β-catenin, and APC (**Table 5**). 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).

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

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) [22]. 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 [23]. 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 [24, 25].

1. ARC, p53 AND MMR 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 [26], as well as in carcinomas of different origins, like ovarian cancer, colon cancer or cervical cancer [27]. ARC is known to be induced by Ras protein and repressed by p53 [28] 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 [29]. In breast cancer cell lines, high levels of cytoplasmic ARC were linked with treatment resistance (doxorubicin and γ-radiation induced cell death) [19]. 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 [19]. 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 [21].

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 but the exact role of nuclear ARC protein in colon cancer, and in all other cancer types, remains still unclear [30]. 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.

2. ERCC1, RRM1 AND TUBB3 EXPRESSION IN LIVER METASTASIS OF CRC

ERCC1, RRM1 and TUBB3 are known to have therapeutic predictive value in current therapy of metastasized colorectal cancer [13, 31, 32]. 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.

The main staining manner for TUBB3 was the expression at the invasion front, similar to primary colorectal cancer studied before [33]. 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.

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 [32, 34]. TUBB3 and ERCC1 together influences the therapeutic response to taxane and paclitaxel combination, however the distinct mechanism is still unknown [35].

3. Correlations between CDX2, DNA repair proteins and crucial member of Wnt signaling

Loss of CDX2 expression is seen in approximately 30% of human CRC and is associated with higher tumor grade [36]. 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 [37], 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 [38]. 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 [39].

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 [18]. 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 [18], 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 [40]. 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 [18].

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.

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.

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, 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, MSH2, MSH6, and PMS2. Furthermore, loss of MGMT and ERCC1 was also associated with CDX2 loss . In addition, CDX2 and ERCC1 were inversely associated with metastatic tumor size. Sustained CDX2 expression was associated with higher expression of cytoplasmic/membranous β-catenin and with nuclear APC expression. 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.

ACKNOWLEDGEMENTS

First and foremost I wish to express my sincere gratitude to Prof. Dr. László Tiszlavicz and to my supervisor Dr. Farkas Sükösd for their ongoing support. I am thankful for their criticism, encouragement and numerous advices during my Ph.D. work. And I am obliged to all my colleagues for their support in everyday work.

I express my thanks to all my co-authors and all the members of the staff of the Department of Pathology in Szeged and in Heidelberg for their collaboration and help in this work.

I am deeply grateful to my family for their continuous support and encouragement that I have received during these years.

This work was supported by GINOP 2.3.2-15-2016-00020 project, which is co-financed by the European Union, European Regional Developmental Fund.

Szeged, 15th March 2018

Dr. Csaba Tóth

REFERENCES

1. Siegel, R., C. Desantis, and A. Jemal, *Colorectal cancer statistics, 2014.* CA Cancer J Clin, 2014. **64**(2): p. 104-17.
2. Parkin, D.M., P. Pisani, and J. Ferlay, *Global cancer statistics.* CA Cancer J Clin, 1999. **49**(1): p. 33-64, 1.
3. O'Connell, J.B., M.A. Maggard, and C.Y. Ko, *Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging.* J Natl Cancer Inst, 2004. **96**(19): p. 1420-5.
4. Brenner, H., et al., *Reduction of clinically manifest colorectal cancer by endoscopic screening: empirical evaluation and comparison of screening at various ages.* Eur J Cancer Prev, 2005. **14**(3): p. 231-7.
5. Binefa, G., et al., *Colorectal cancer: from prevention to personalized medicine.* World J Gastroenterol, 2014. **20**(22): p. 6786-808.
6. Parkin, D.M., *Global cancer statistics in the year 2000.* Lancet Oncol, 2001. **2**(9): p. 533-43.
7. DePinho, R.A., *The age of cancer.* Nature, 2000. **408**(6809): p. 248-54.
8. Turkiewicz, D., et al., *Young patients with colorectal cancer: how do they fare?* ANZ J Surg, 2001. **71**(12): p. 707-10.
9. Boyle, P. and J.S. Langman, *ABC of colorectal cancer: Epidemiology.* BMJ, 2000. **321**(7264): p. 805-8.
10. Potter, J.D., *Colorectal cancer: molecules and populations.* J Natl Cancer Inst, 1999. **91**(11): p. 916-32.
11. Willett, W.C., et al., *Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women.* N Engl J Med, 1990. **323**(24): p. 1664-72.
12. Daperno, M., et al., *The role of endoscopy in inflammatory bowel disease.* Eur Rev Med Pharmacol Sci, 2004. **8**(5): p. 209-14.
13. Li, Z., et al., *Predictive value of APE1, BRCA1, ERCC1 and TUBB3 expression in patients with advanced non-small cell lung cancer (NSCLC) receiving first-line platinum-paclitaxel chemotherapy.* Cancer Chemother Pharmacol, 2014. **74**(4): p. 777-86.
14. Misiakos, E.P., N.P. Karidis, and G. Kouraklis, *Current treatment for colorectal liver metastases.* World J Gastroenterol, 2011. **17**(36): p. 4067-75.
15. Olsen, J., et al., *The clinical perspectives of CDX2 expression in colorectal cancer: a qualitative systematic review.* Surg Oncol, 2014. **23**(3): p. 167-76.
16. Inno, A., et al., *Role of MGMT as biomarker in colorectal cancer.* World J Clin Cases, 2014. **2**(12): p. 835-9.
17. Sayar, I., et al., *Relationship among mismatch repair deficiency, CDX2 loss, p53 and E-cadherin in colon carcinoma and suitability of using a double panel of mismatch repair proteins by immunohistochemistry.* Pol J Pathol, 2015. **66**(3): p. 246-53.
18. Olsen, A.K., et al., *Regulation of APC and AXIN2 expression by intestinal tumor suppressor CDX2 in colon cancer cells.* Carcinogenesis, 2013. **34**(6): p. 1361-9.
19. Mercier, I., et al., *ARC, an apoptosis suppressor limited to terminally differentiated cells, is induced in human breast cancer and confers chemo- and radiation-resistance.* Cell Death Differ, 2005. **12**(6): p. 682-6.
20. Colucci, G., et al., *Phase III randomized trial of FOLFIRI versus FOLFOX4 in the treatment of advanced colorectal cancer: a multicenter study of the Gruppo Oncologico Dell'Italia Meridionale.* J Clin Oncol, 2005. **23**(22): p. 4866-75.
21. Toth, C., et al., *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, 2015.
22. Koehler, B.C., et al., *Pan-Bcl-2 inhibitor obatoclax delays cell cycle progression and blocks migration of colorectal cancer cells.* PLoS One, 2014. **9**(9): p. e106571.
23. Hassen, S., N. Ali, and P. Chowdhury, *Molecular signaling mechanisms of apoptosis in hereditary non-polyposis colorectal cancer.* World J Gastrointest Pathophysiol, 2012. **3**(3): p. 71-9.
24. Mercier, I., et al., *ARC (apoptosis repressor with caspase recruitment domain) is a novel marker of human colon cancer.* Cell Cycle, 2008. **7**(11): p. 1640-7.
25. Sarela, A.I., et al., *Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma.* Gut, 2000. **46**(5): p. 645-50.
26. Koseki, T., et al., *ARC, an inhibitor of apoptosis expressed in skeletal muscle and heart that interacts selectively with caspases.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 5156-60.
27. Wu, L., et al., *Induction of the apoptosis inhibitor ARC by Ras in human cancers.* J Biol Chem, 2010. **285**(25): p. 19235-45.

28. Li, Y.Z., et al., *p53 initiates apoptosis by transcriptionally targeting the antiapoptotic protein ARC.* Mol Cell Biol, 2008. **28**(2): p. 564-74.
29. Ludwig-Galezowska, A.H., L. Flanagan, and M. Rehm, *Apoptosis repressor with caspase recruitment domain, a multifunctional modulator of cell death.* J Cell Mol Med, 2011. **15**(5): p. 1044-53.
30. Foo, R.S., et al., *Regulation of p53 tetramerization and nuclear export by ARC.* Proc Natl Acad Sci U S A, 2007. **104**(52): p. 20826-31.
31. Shirota, Y., et al., *ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy.* J Clin Oncol, 2001. **19**(23): p. 4298-304.
32. Azuma, K., et al., *Expression of ERCC1 and class III beta-tubulin in non-small cell lung cancer patients treated with carboplatin and paclitaxel.* Lung Cancer, 2009. **64**(3): p. 326-33.
33. Portyanko, A., et al., *beta(III)-tubulin at the invasive margin of colorectal cancer: possible link to invasion.* Virchows Arch, 2009. **454**(5): p. 541-8.
34. Vilmar, A., et al., *RT-PCR versus immunohistochemistry for correlation and quantification of ERCC1, BRCA1, TUBB3 and RRM1 in NSCLC.* Lung Cancer, 2012. **75**(3): p. 306-12.
35. Parker, A.L., M. Kavallaris, and J.A. McCarroll, *Microtubules and their role in cellular stress in cancer.* Front Oncol, 2014. **4**: p. 153.
36. Hryniuk, A., et al., *Cdx1 and Cdx2 function as tumor suppressors.* J Biol Chem, 2014. **289**(48): p. 33343-54.
37. Gerhard, R., et al., *Clinicopathological significance of ERCC1 expression in breast cancer.* Pathol Res Pract, 2013. **209**(6): p. 331-6.
38. Dawson, H., et al., *Possible role of Cdx2 in the serrated pathway of colorectal cancer characterized by BRAF mutation, high-level CpG Island methylator phenotype and mismatch repair-deficiency.* Int J Cancer, 2014. **134**(10): p. 2342-51.
39. Dawson, H., et al., *Loss of Cdx2 Expression in Primary Tumors and Lymph Node Metastases is Specific for Mismatch Repair-Deficiency in Colorectal Cancer.* Front Oncol, 2013. **3**: p. 265.
40. Wang, L., et al., *Regulation of the phosphorylation and nuclear import and export of beta-catenin by APC and its cancer-related truncated form.* J Cell Sci, 2014. **127**(Pt 8): p. 1647-59.

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]**

LIST OF PUBLICATIONS NOT DIRECTLY RELATED TO THE THESIS

IV. **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]**

V. Amer, W., **Toth, C.,** Vassella, E., Meinrath, J., Koitzsch, U., Arens, A., Huang, J., Eischeid, H., Adam, A., Buettner, R., et al. (2017). **Evolution analysis of heterogeneous non-small cell lung carcinoma by ultra-deep sequencing of the mitochondrial genome.** Nature Scientific reports 7, 11069. **[IF: 4.2]**

VI. Sproll C, Freund AK, Hassel A, Hölbling M, Aust V, Storb SH, Handschel J, Teichmann C, Depprich R, Behrens B, Neves RP, Kübler NR, Kaiser P, Baldus SE, **Tóth C**, Kaisers W, Stoecklein NH: **Immunohistochemical detection of lymph node-DTCs in patients with node-negative HNSCC.** Int J Cancer, 2017. 140(9): p. 2112-2124. **[IF: 5.5]**

VII. **Tóth, C.**: **Clinical pathology of granulomatous inflammation.** Der Radiologe, 2016. 56(10): p. 856-865. **[IF: 0.4]**

VIII. 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]**

IX. **Toth, C.,** Lee, H-S., Sebastian Heikaus, S. (2014). **Rapidly growing mass in the pancreas: intraductal Candida infection in a chronic recurrent pancreatitis.** Case Reports in Clinical Pathology, 2014, Vol. 1, No. 2 DOI: 10.5430/crcp.v1n2p146 **[IF: 0.0]**

X. Prigge, E.S., **Toth, C.,** Dyckhoff, G., Wagner, S., Muller, F., Wittekindt, C., Freier, K., Plinkert, P., Hoffmann, J., Vinokurova, S., et al. (2014). **p16 /Ki-67 co-expression specifically identifies transformed cells in the head and neck region.** Int J Cancer. **[IF: 5.0]**

XI. Bickeboller, M., Tagscherer, K.E., Kloor, M., Jansen, L., Chang-Claude, J., Brenner, H., Hoffmeister, M., **Toth, C.,** Schirmacher, P., Roth, W., et al. (2014). **Functional characterization of the tumor-suppressor MARCKS in colorectal cancer and its association with survival.** Oncogene 0. **[IF: 8.4]**

XII. Weis, B., Schmidt, J., Maamar, H., Raj, A., Lin, H., **Toth, C.,** Riedmann, K., Raddatz, G., Seitz, H.K., Ho, A.D., et al. (2014). **Inhibition of intestinal tumor formation by deletion of the DNA methyltransferase 3a.** Oncogene 0. **[IF: 8.4]**

XIII. Hoffmeister, M., Blaker, H., Kloor, M., Roth, W., **Toth, C.,** Herpel, E., Frank, B., Schirmacher, P., Chang-Claude, J., and Brenner, H. (2013). **Body mass index and microsatellite instability in colorectal cancer: a population-based study.** Cancer Epidemiol Biomarkers Prev. **[IF: 4.1]**

XIV. Reuschenbach, M., Kansy, K., Garbe, K., Vinokurova, S., Flechtenmacher, C., **Toth, C.,** Prigge, E. S., Thiele, O. C., Reinert, S., Hoffmann, J., von Knebel Doeberitz, M., Freier, K.: **Lack of evidence of human papillomavirus-induced squamous cell carcinomas of the oral cavity in southern Germany.** Oral Oncol. 2013 Apr pii: S1368-8375(13)00535-6. **[IF: 3.0]**

XV. Rudolph, A., **Toth, C.,** Hoffmeister, M., Roth, W., Herpel, E., Schirmacher, P., Brenner, H., Chang-Claude, J.: **Colorectal cancer risk associated with hormone use varies by expression of estrogen receptor beta.** Cancer Res *73*, 3306-3315. **[IF: 9.2]**

XVI. Rudolph, A., **Toth, C.,** Hoffmeister, M., Roth, W., Herpel, E., Jansen, L., Marx, A., Brenner, H., Chang-Claude, J.: **Expression of oestrogen receptor beta and prognosis of colorectal cancer.** Br J Cancer. 2012 107(5):831-9. **[IF: 5.0]**

XVII. **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]**

XVIII. **Tóth, C.**: **Role of R classification in the interdisciplinary oncology** [Az R-klasszifikáció az interdiszciplináris onkológiában] Orv Hetil. (Hungarian Medical Journal) 2011 Dec 25; 152(52):2086-90. [Article in Hungarian] **[IF: 0.0]**

XIX. **Toth, C.**: **Obduktionen 2010. Quid(ne) mortui vivos docent?** *[Autopsies 2010. Is death still teaching the living?].* Pathologe, 2010. **31**(4): p. 297-302. **[IF: 0.5]**

XX. **Toth, C.: A boncolások szerepe a XXI. század medicinájában** (The role of autopsies in the 21st century medicine) Orvosi Hetilap (Hungarian Medical Journal) DOI10.1556/OH.2010.28837 **[IF: 0.0]**

Cumulative impact factor: 75.9

Szeged, 15th March 2018

C. Tóth

Dr. Csaba Tóth