SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Investigation of certain histopathological and molecular prognostic factors in intestinal tumors

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1. Introduction and review of the literature

1. 1. Epidemiology of colorectal cancer (CRC)

Colorectal cancer (CRC) is one of the most frequent forms of malignancies both in the Western World as well as in Hungary. In Hungary CRC is the second most common malignant tumor in both sexes and is also the second cause of tumor-related mortality.

The Hungarian National Cancer Registry and the National Bureau of Statistics recorded 8658 cases in 2003 and there were 9062 new CRC cases reported in 2005. In 2003 there were 2787 and 2311 CRC related deaths in males and females, respectively. These figures were 2462 (males) and 2095 (females) in 2005. It is well documented that during the last 25 years the absolute figures for CRC-related mortality, frequency of lethal outcome and standardized mortality have all increased both in males and females, although the growth rate has in recent years somewhat slowed.

1. 2. The evolution and molecular biology of CRC

Colorectal carcinogenesis is a multistep process that comprises a host of variable - mostly already well-characterized - genetic abnormalities. In addition to changes that affect the genome epigenetic alterations are also important factors. Genetically CRC is a rather heterogeneous disease hence several tumor types can be differentiated according to the driving and fundamental genetic abnormalities. The specific genetic errors direct CRC-genesis along various pathways but it is of note that the resulting tumor morphology often is rather similar when comparing CRC molecular subtypes. The genetic and epigenetic alterations which are essential features in various CRC forms can be classified into 4 major groups:

1. Chromosomal instability. 2. Microsatellite instability. 3. CpG-island methylator phenotype. 4. Global DNA hypomethylation.

The current data support several specific genetic errors in CRC-genesis together with closely associated signaling pathways.

The fundamental importance of β-catenin and E-cadherin in cell-cell adhesion is well known. E-cadherin is a transmembrane glycoprotein encoded by a gene at the 16q22 chromosomal locus and its atomic (molecular) mass is 120 kDa. E-cadherin's extracellular domain is connected in a Ca-dependent manner to an adjacent cell's cadherin molecule. The intracellular bond with the cytoskeletal actin molecule is ensured by a complex formed between E-cadherin and α-catenin, β-catenin and γ-catenin. β-catenin is also a major participant in the canonical Wnt signaling pathway. In non-stimulated cells (i. e., physiological Wnt signaling) the APC/GSK3b axin destruction complex binds β-catenin and hence it becomes degradable. If, however, receptor-ligand binding takes place GSK3 inhibition follows that liberates β-catenin which in its free form can enter the nucleus and binds to the TCF/LEF transcription factor. The complex thus formed activates transcription of c-myc and cyclin D1, as well as matrix metalloproteinase7 and CD44. In the absence of functional APC protein uncontrolled intranuclear β-catenin accumulation ensues with simultaneous constitutive expression of the c-myc and cyclin D1 genes. The Wnt pathway is a substantial element in the process of continuous mucosal regeneration within the large bowel. APC mutation occurs in about 70% of sporadic CRC and is an early event as is shown by its presence in the adenoma phase (stage). CTNNB1 mutation of β-catenin is less frequent but is also documented.

Mutation(s) of the tumor-suppressor gene TP53 are documented in more than half of CRCs. Its product, p53 is a protein that prevents cells from entering or continuing the cell cycle in case of DNA damage. Mitosis is blocked until the DNA error is not corrected. If correction is not feasible p53 induces apoptosis. Similarly to APC TP53 plays a crucial role in carcinogenesis, however, despite

of extensive researches neither prognostic nor predictive role of p53 has been established.

EGFR is a member of the human epidermal growth factor family (HER-erB). Activation of EGFR plays a critical role in tumor growth and progression and these include angiogenesis, invasion and metastatic spread. When the EGFR receptor binds its ligand a mitogenic cascade is initiated which may get realized via either the RAS-MAPK or the PI3K-Akt or the phospholipase C pathways. Over expression of EGFR is a common event in numerous tumor types and usually indicates unfavorable prognosis. EGFR is the target molecule of several biological tailored therapeutical protocols. Therapy resistance may be brought about by utilization of escape mechanisms which are made available for tumor cells due to the various interactions between the intercellular signaling pathways that are related to EGFR. Therapy resistance or its development explains why finding reliable biomarkers is so important since these markers could prevent unnecessary medication and proper identification of patients with responsive tumors.

1. 3. CRC as a form of familial cancer

The majority (approximately 70%) of colorectal carcinomata are sporadic tumors. Familial CRC (10-30% of all cases) is defined as a carcinoma that develops in a patient who has a first degree relative already affected by the same disease and heredity can be excluded. Inherited cancer syndromes (including CRC) comprise those cases when inheritance of a single mutant gene greatly increases the risk of developing cancer. This predisposition shows an autosomal dominant inheritance pattern, while a smaller group of autosomal recessive disorders is characterized by chromosomal or DNA instability. In both the dominant and recessive cases inheritance follows the mendelian patterns.

The most frequent inherited colorectal cancer syndrome is **h**ereditary **n**on**p**olyposis **c**olorectal **c**ancer syndrome (HNPCC or Lynch syndrome). HNPCC is associated with germ-line mutations of DNA mismatch repair genes. This tumor category accounts for about 2-4% of CRCs. In general the prognosis of HNPCC is better than for sporadic CRCs. There are additional hereditary colorectal cancer syndromes. Li Fraumeni syndrome is a rather rare autosomal dominant disorder. Those suffering from it inherit a mutant *p53* allele. **F**amilial **a**denomatous **p**olyposis (FAP) is also an autosomal dominant disorder characterized by germ line mutation in the APC gene which has been localized to chromosome 5q21-22. Gardner syndrome and Turcot syndrome share the same genetic defect as FAP but differ with respect to extra-intestinal tumors in the latter two. Juvenile polyposis is a familial cancer syndrome with autosomal dominant trait. Germ line mutations in the SMAD/DPC4 tumor suppressor gene account for some of the cases. Cowden syndrome is an autosomal dominant disorder, characterized by mutation of PTEN gene. Peutz-Jeghers syndrome is an autosomal dominant disorder characterized by mutation in STK11(LKB1 gene on chromosome 19p13.

1.4. Prognostic and predictive factors

The classical prognostic factors in CRC are as follows: 1. Tumor stage. 2. In N0 cases the number of lymph nodes analyzed. 3. Age - prognosis is worse in juvenile and elderly patients. 4. Sex: female patients have a significantly better outlook. 5. Localization (anatomic site): tumors affecting the left colon or the rectum carry a worse prognosis. Additional prognostic factors include tumor-free surgical resection, tumor deposits, tumor regression grade, perineural and lymphatic-blood vessel invasion, presence of inflammatory reaction, histological type and grade. Perforation and obstruction are ominous characteristics.

Histological type of the tumor has definitive prognostic significance. The histological classes include mucinous, medullary, signet ring cell and small cell patterns which all carry a worse prognosis than the conventional (NOS) adenocarcinoma. Particularly unfavorable is the prognosis of rhabdoid tumors.

Molecular prognostic factors include microsatellite instability (favorable), chromosomal instability (unfavorable), 18q deletion (unfavorable), BRAF V600E mutation (unfavorable) KRAS mutation and PIK3CA mutation (both unfavorable).

MSI has already been established as a negative predictor for the standard treatment of CRC with 5-FU. For 5-FU there are further potential predictive markers. Included are as the most significant ones thymidilate-synthase (TS), thymidilate phosphorylase, dihydropyrimidine-dehydrogenase and methylen-tetrahydro-pholate reductase (MTHFR). In stage II tumors increased TS expression is associated with unfavorable prognosis. High TS expression is associated with decreased efficacy of 5-FU treatment. The most precise results stem from RT-PCR analysis of TS mRNA.

Oxaliplatin is a standard platinum compound that is used for treatment of metastatic CRC. As part of the nucleotid excision repair (NER) mechanisms excision repair cross complementation group 1 (ERCC1) is responsible for the restoration of 2-30 nucleotid long segments. The magnitude of ERCC1 correlates with oxaliplatin response intensity. High ERCC1 expression predicts high likelihood of oxaliplatin resistance.

1. 5. Feasibility of TMA (multiblock tissue microarray) methodology

Large and thorough literary reports from recent past have provided ample proof for the appropriateness of the TMA method in oncological proteomics.

The cardinal feature of the TMA method is the collection of tissue cylinders from multiple paraffin-embedded tissue blocks and using these, a combined recipient multiblock structure, is created. These multiblocks are then

used for carrying out traditional histological-, immunohistochemical- and FISH reactions. One TMA block may contain several hundreds of tissue cylinders (cores) hence the method is highly cost effective. Since all cylinders are subjected to standardized reaction-milieu, antigen-retrieval, reagent concentration, incubation time and temperature are all uniform for each and every one tissue core TMA can maintain and achieve a heretofore unrealized precision.

2. Aims of the work

Our main goal has been further study of prognostic factors for early, Dukes B2– II. stage CRC. This we planned to realize with the use of TMA methodology, immunohistochemistry and FISH analysis.

The following specific aims had been planned:

1. Validation and control of reliability of the locally introduced TMA methodology using a HNPCC CRC patient cohort. This we planned to achieve by immunohistochemical detection of MSH2, MSH6, MLH1 mismatch repair gene product, i. e., proteins.

2. We intended to gather information on the applicability of immunohistochemistry for β-catenin and E-cadherin expression detection in Dukes B2- II. stage CRC to clarify whether these measures could serve as prognostic indicators and/or predictive factors with regard to the tumors' metastatic potential.

3. Via thorough analysis of a larger Dukes B2- II. stage CRC patient cohort we undertook the evaluation of canonical factors like age, tumor location, sex, histological grade, number of lymph nodes worked up during pathological processing a surgical samples and effects of adjuvant therapy. All these considerations have been complemented with analysis of β-catenin and E-cadherin expression (vide supra) plus further molecular markers like p53, p21, p16, MSH2, MLH1, EGFR, TS in order to clarify their prognostic potential.

4. We also wished to extend this analysis to a patient cohort suffering from an advanced stage CRC. The above mentioned study of β-catenin, E-cadherin, p53, p21, MSH2, MSH6, MLH1, EGFR and TS was furthered with ERCC1 protein detection in order to determine whether immunohistochemical detection of ERCC1 is usable as a predictive marker for the efficacy of oxaliplatin therapy.

5. Another question raised was whether our TMA technique would also allow parallel FISH studies. For this purpose simultaneous analysis of EGFR gene amplification and EGFR expression as measured by immunohistochemistry seemed like an appropriate approach.

6. Since during histopathological routine work we had happened to encounter a very rare rhabdoid tumor detailed histological and immunohistochemical (IHC) findings in this unique entity were compared to the scarce available literary data.

3. Patients, materials and methods

3.1 TMA validation

Validation of our own TMA method was realized through analyzing a large cohort of HNPCC patients collected from the archives of the Medical and Health Science Center of the University of Debrecen (MHSC-UD). Those patients who fulfilled the Amsterdam and Bethesda criteria and had undergone surgery at the 2nd Department of Surgery between 1996 and 2008 were included in this part of the study. Formalin fixed paraffin embedded (FFPE) samples of the surgical specimens were subjected to detection of mismatch repair genes' products (proteins). A total of 75 patients were included with a 34:41 female/male ratio. Conventional IHC was applied to detect MSH2, MSH6 and MLH1 proteins. This step was followed by building TMA multiblocks from the same material. Three cylinders (diameter = 3 mm) were removed from each paraffin block and the cores were used to create 5x10 matrix arrays, i. e., recipient blocks as described by the manufacturer (TMA Master, 3DHistech, Hungary). Finally all IHC reactions were also carried out on these TMA blocks. Results obtained from the above described two sources (FFPE full blocks and TMA cores) were compared to validate the TMA approach.

3. 2. Analysis of the rhabdoid tumor phenotype

Case history:

Anamnestic data on the 81-y-old patient comprised signs and symptoms of subileus accompanied by bloody stools which necessitated hospitalization. Abdominal ultrasound indicated malignancy of the GI tract and corroborative CT findings were obtained. Ileus mandated laparotomy that disclosed an obstructive tumor located in the ileum and adherent to the abdominal wall. This segment was resected with portions of infiltrated adjacent tissues. Surgery was repeated because timorous growths in two segments of the ileum were treated

with surgically radical resections. The patient refused further radical surgery and even refused palliative oncological treatment. His general condition rapidly deteriorated and he died within seven months after the diagnosis.

Review of previously published cases: Thorough search of the computerized data banks available to us disclosed a total of 22 cases of previously reported rhabdoid cancer within the GI tract.

3.3. E-Cadherin- and β-catenin analysis

79 colorectal cancer cases were collected from tissue archives of the Department of Pathology UD-MHSC, covering the time period of 5 years (1996-2001). The fundamental selection criterion required that each tumor had to belong to Dukes B2 stage (pT3N0 TNM stage). The patients' mean age was 65.8 years (range: 35-85 years). The group comprised of 39 women and 40 men. Mean follow-up time was 52 months. All patients who received adjuvant chemotherapy during this period were treated with 5FU+leucovorin (LV). Patients with rectal tumor received additional radiotherapy as well. If metastatic disease was diagnosed the patients received further chemotherapy.

From all tumors 3-3 representative cores were obtained. A total of 237 cores were built in TMA blocks from the 79 tumors.

3.4. Prognostic studies

We retrospectively evaluated the clinical data of 100 patients with stage T3N0 CRC operated at our institute (UD-MHSC) between 1997 and 2003. We analyzed the formalin-fixed paraffin embedded surgical materials using the tissue microarray technique by means of immunohistochemistry for p53, p21 waf, p16, β-catenin, E-cadherin, EGFR, MLH1, MSH2 and TS protein expressions.

The obtained results were compared with patients' survival data (5-year OS, 3-year DFS). We analyzed the prognostic effects for age, tumor localization and for the removed and histologically analyzed lymph nodes. All patients had

undergone a curative surgery and presented tumor free surgical resection margins. All patients who received adjuvant chemotherapy during this period were treated with 5FU+leucovorin (LV). Tumor tissue samples were formalin fixed and paraffin embedded. From the representative areas 1 mm thick core biopsies were retrieved using a TMA master device (3DHistech Budapest, Hungary) and positioned in a 50- core recipient paraffin array block.

3.5. Immunohistochemical detection of ERCC1 expression and analysis of prognostic factors in metastatic CRC

Twenty-eight CRC patients treated at Department of Oncology, UD-MHSC were collected. The fundamental selection criterion required that each patient had metastatic disease and received oxaliplatin chemotherapy. All patients underwent tumor resection, than received adjuvant chemotherapy were treated with 5FU+leucovorin When metastatic disease was diagnosed the patients received oxaliplatin therapy. The average of administered therapeutical cycles was 7 (2-20). From all tumors 3-3 representative cores were obtained and TMA blocks were created as described above. These were used to detect the expression of 10 proteins: p53, p21, MLH-1, MSH-2, MSH-6, EGFR, ß-catenin, E-cadherin, thymidilate-synthase and ERCC1.

3.6. Immunohistochemical (IHC) reactions

5 µm sections from TMA blocks and from whole blocks were used for immunohistochemistry (IHC). The slides were deparaffinated and rehydrated with xylol and graded alcohol. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 for 30 minutes. Antigen retrieval accomplished in 10mM citrate buffer (pH 6.0) in a microwave at 600 W for 5 min. Nonspecific binding was blocked with 1% BSA for 10 min. Subsequently, the slides were incubated with the primary antibody for 1 hour, at 37 ^{0}C. The slides were incubated with a biotinylated rabbit antimouse immunoglobulin as second antibody and subsequently treated with streptavidin peroxidase conjugate for 30 min at 37C

temperature and DAB was used as chromogen substrate. After counterstaining with haematoxylin, the slides were dehydrated and mounted.

3.7.IHC scoring

Normal colonic mucosa exhibited strong membranous staining with both β-catenin and E-cadherin. For both membranous and nuclear positivity of β-catenin those cases in which <10% stained cells were observed the result was classified as negative. If the decorated cells' number fell between 11-50% staining was scored as: 1+. In those instances when positive staining occurred in cells between 51-100% score 2+ was given. For E- cadherin if the number of decorated cells was <75% the reaction was classified as "negative". When the reactive cells' number fell between 76-90% the score was 1+; staining of 91-100% of the cells scored 2+. Only those cases with score 1+ and 2+ were considered as positive for the purpose of statistical data analysis.

Enterocytes of the colonic mucosa and the lymphocytes of lamina propria were positive for MLH1, MSH2 and MSH6. For MLH1 and hMSH2, if nuclear staining was present in tumor cells, the lesion was defined as positive.

Assessment of p53 expression was semi-quantified in terms of percentages: 1+ represented staining in 0-25% of cells; 2+ represented decoration of 26-50% of cells; 51-75% positivity scored 3+ and higher positivity (76-100%) corresponded to 4+.

IHC positivity for p21 required specific strong nuclear decoration. Scoring of EGFR immunostaining by using the DAKO pharmDx kit was performed according to literature. Those cases with score 1+, 2+ and 3+ were considered as positive for the purpose of statistical data analysis.

Semi-quantitative assessment of TS-positive cells was performed regarding the ratio of decorated-nondecorated cells. Staining intensity was also taken into consideration. Mild decoration was scored with the lowest point value (1 point), moderate intensity equaled 2 points, while intense reaction got the highest score

(3 points). A similar semi quantitative scoring scale was applied to the percentage of decorated cells: 0-33% = 1point; 34-66% = 2 points and 64-100% = 3 points. We obtained point values between 0-9 for TS-positivity (intensity × percentage.)

When assessing ERCC1 protein expression point scores were given as follows: when 0-33% of cells was decorated we assigned point 1, 34-66% positivity got point 2 and 67-100% decoration was valued as point 3. In case of zero specific reaction the reaction was considered negative.

3.7. EGFR amplification detected by FISH

Those TMA blocks which had been subjected to E-cadherin and β-catenin analysis were used for EGFR FISH studies. PathVysion EGFR Dual color FISH test was applied with CEP7 as reference gene. Five µm thick sections were cut from all TMA blocks. Deparaffinated sections were digested with Proteinase K for 5 minutes and having added the probe DNA denaturation at 90 degrees followed. DNA hybridization was carried out at 37 Celsius overnight on a Thermobrite manual FISH platform. Following posthybridisation stringency wash background staining with 4,6-diamidino-2-phenylindole (DAPI, Vysis) applied.

Analysis was carried out on Olympus BX51 fluorescent microscope using DAPI/orange/green triple filter. In each case 20 well delineated tumor cells were chosen and the EGFR signal and reference gene signal were counted. The average EGFR signal number per cell value divided by the average signal gene value allows determination whether or not target gene amplification is present. When the averaged CEP7 signal number exceeded 2.5/cell then polysomy of chromosome 7 was present.

Parallel to the FISH reactions the same blocks were also analyzed by EGFR IHC as described above.

3.8. Statistical analysis

We analyzed the expression of the different antigens and other known prognostic factors (age, tumor localization and number of evaluated lymph nodes) in comparison with the 5-year OS and the 3-year DFS. For data analysis we used independent samples t-test and Chi-square test, and for data comparison Pearson's and Spearman's correlations. The Kaplan-Meier method was used to estimate OS at five years and DFS at three years. Some variables were entered in the multivariate analysis and Cox-regression test was used. Statistical analysis was performed using the SPSS 15.0 statistical package (Chicago, IL). $P<0.05$ was considered as significant.

The values obtained by the two different analytical methods were compared and subjected to SPSS-PC 11 statistical software in order to validate the out TMA approach. The Kappa-test allows concordance analysis of data obtained by two or more methods or values generated by two or more diagnostic procedures which are carried out multiple observers (MDs). Kappa is calculated from observed and accidental concordance values. The null-hypothesis (i. e., there is no difference between the two methods or observers) can also be tested by z-probe.

4. Results

4.1. Validation of the TMA method

Evaluating MLH1 the two methods was concordant in 66 cases, differences were observed in 5 cases and in 4 cases one of the methods failed. Kappa =0,758.

Analysis of MSH2 was possible in 72 cases and concordance was found in 64 of them, 8 cases showed differences. The TMA method failed in identifying positivity that had been observed with the traditional approach in 3 cases. On 5 occasions TMA results were negative when the traditional method produced positivity, hence kappa was 0,368.

MSH6 detection could not be evaluated in 18 cases. In 54 cases concordance was found, differences occurred in 3 cases. In 49 cases both methods provided positive results while in the remaining 5 cases both turned out to be negative. In further 3 cases while the traditional method indicated negativity the TMA method failed to provide such result. This run provided kappa = 0,741.

Kappa values obtained in the MLH1 and MSH6 analyses were good, while the same value was weak in the MSH2 analysis. Z-test run on the values obtained indicated p <0,001 for MLH1, for MSH6 p was <0,001 and this corroborates strong agreement. For MSH2 the value of p= 0,002, which only shows weak agreement.

4.2. Studies on the rhabdoid phenotype

In all three surgical sampling provided identically structured tumor. Solid cellular areas with sheeting were present without gland-formation, but conspicuous multifocal necroses and hemorrhages. The tumorous cells had abundant homogeneously eosinophilic cytoplasm with frequent hyaline inclusions. Anisonucleosis was noticeable; the nuclei were often laterally displaced and frequently displayed prominent nucleolus. High mitotic activity was conspicuous and most mitoses were abnormal. Despite extensive multifocal, variably deep exulceration there was no epithelial dysplasia adjacent to these areas.

Diffuse positive reaction was obtained by pan cytokeratin- and vimentin antibodies, while the EMA reaction was only focally positive No decoration (negative reaction) was observed when using the following antibodies: CK7, CK20, LCA, S100, HMB45, SMA, desmin, caldesmon, CD34, C-kit, CD30, bcl6, CD3, CD20, E-cadherin and p27. The high Ki-67 labeling index (30%) (Mib-1 antibody) was congruent with the conspicuous mitotic activity seen in HE stained sections. The rapid deadly progression of the disease could be prognosticated based on the ominously extensive p53 immunoreactivity plus

the significant Her2 decoration (Score ++). Specific and strong nuclear decoration was observed when applying the INI-1 specific antibody.

Both the light microscopic (histology and cytology) and immune-phenotypic features corroborated the rhabdoid character of the tumor that has been maintained throughout the disease-course.

Thorough search of the computerized data banks available to us disclosed a total of 22 cases of previously reported rhabdoid cancer within the GI tract. Out of these seven (and the present case) originated from the small intestine. The others affected the esophagus (3), the stomach (7), large intestine (4), and the mesentery (1). Rhabdoid cancer of the GI tract typically affects elderly (mean age 63.4 years [41-84 y age-range]) people with prominent male preference (male/female ratio = 3.4:1). The mean age of those affected by small intestinal rhabdoid cancer is reported to be 61.7 years (range: 52-81 y) and among these even more significant is the male preponderance (7:1). Rhabdoid tumors of the GI tract are rather aggressive; their prognosis is very poor and over 75% of the patients die within 6 months following the initial diagnosis.

There are 4 reported cases of large bowel rhabdoid tumors. Rhabdoid cancer of the colon affects elderly (mean age 76.7 years [72-84 y age-range]), gender distribution is roughly equal (male/female ratio = 1:1). The survival is poor, three patients died within 3 months following the initial diagnosis, the fourth patient's overall survival was 1 year.

4.3. E-cadherin and β-catenin study

79 archival cases of Dukes B2 stage (pT3N0 TNM stage) colorectal cancer were analyzed. The 5-year OS rate was 59% and the 3-year DFS rate was 64%. Membranous expression of β- catenin was observed in 52 cases while 27 cases were negative. Nuclear expression of β- catenin was detectable in 36 cases while 43 cases were negative. Membranous staining with E- cadherin was observed in 46 cases, 33 cases were negative.

In the whole study population all those cases with tumor cells showing membranous expression of β-catenin had a better OS than those without it, however, this difference was not statistically significant, p=0.280. In case of DFS there was no difference, p=0.442. The nuclear β-catenin expression or E-cadherin expression did not stratify patients with regard to OS or DFS.

During the follow-up period 23 patients developed metastatic disease. The risk of metastases was higher in the β-catenin membranous negative group than in the positive group (p=0.062). When β-catenin showed nuclear expression, the risk of metastases was higher, p=0.022. No relationship between the expression of E-cadherin and development of metastatic disease could be identified. If neither β-catenin nor E-cadherin showed membranous staining, the metastatic disease was significantly more frequent, compared to cases in which both proteins exhibited membranous expression, p=0.047.

48 patients had tumor in the right- or left side of the colon. The clinical outcome was (statistically) independent from β-catenin and E-cadherin expression patterns.

Twelve patients had metastatic disease in this group. There was no difference in the risk of metastases comparing the groups with different protein expression patterns.

31 patients had rectal tumor. There was a trend towards improved DFS and OS in patients with membranous β-catenin expression, but it did not reach statistical significance, [p=0.087 and 0.085, respectively]. There was no difference in OS and DFS either between nuclear β-catenin positive and –negative cases, or between E-cadherin positive and –negative patients. In this group 11 patients had metastatic disease. The risk of metastases was significantly higher in cases which were β-catenin membranous negative than in those which were membranous positive (p=0.024). When β-catenin showed nuclear expression, the risk of metastases was also significantly higher

(p=0.047). No relational pattern could be identified between the expression of E-cadherin and development of metastatic disease.

Finally, we also established that in those patients with metastatic disease (23 cases) there was a significantly better DFS in those with positive membranous staining for β-catenin than in the negative cases [36.58 months vs. 14.55 months, respectively] (p=0.011). The OS was also better in the group of positive membranous staining for β-catenin (p=0.032). There was a trend towards improved DFS and OS in patients with membranous E-cadherin expression, but it was not significant statistically (29.5 months vs. 22.7 months respectively; p=0.851). The loss of membranous β-catenin staining was associated with faster appearance of metastases (27.16 months vs. 13.18 months, respectively; p=0.05). The loss of membranous staining of E-cadherin also lead to faster appearance of metastases (24 months vs. 16.63 months, respectively; p=0.16).

4.4. Results of prognostic analysis

The group consisted of 52 males and 48 females (mean age 66.9 years; range 35–91 years). Mean follow up time was 50.9 ± 30.3 months. 11% of the patients were below 50 years of age, 53% between 51 and70 years and 36% older than 70 years. A significantly shorter 3-year DFS was observed in cases under 50 years (43%, p=0.01), followed by those above 70 years (46%). The 5-year OS of patients above 70 was the least favorable (38%, p=0.002). Localization significantly influenced the 3-year DFS (P = 0.048): right colon 72%, left colon 67% and rectum 45%, and the 5-year OS (p=0.044): right colon 66%, left colon 60%, and rectum 41%. The numbers of removed and processed lymph nodes were between 0 and 12. We found significantly favorable 5-year OS data in patients of the 5-12 group (50% vs. 75%; p=0.035) and also in the 3-year DFS (45.1% vs. 70%; p=0.022). Gender did not influence the prognosis.

Survival rate between G1-G2 and G3 groups was not statistically significant (54.4% vs. 56.6%; p=0.619).

Overall 65% of colon tumor patients received 5-FU adjuvant therapy. The 5-year OS of 5-FU treated patients was 62% compared with 56% for those not receiving 5-FU (p= 0.461).

P53 positivity was present in 59% of the cases. Interestingly, in these early stage cases we could not demonstrate the negative prognostic value of p53 (59.5% vs. 52.5%, p=0.238). p21 positivity occurred in 85%, and the difference in survival was also not significant (p=0.101). In Spearman's correlations test it was strong positive correlation between p21 expression and tumor recurrence (p=0,005). The combined p53 and p21 positive for patients had significantly worse survival (p=0.041).

The p16 positivity of tumors represented 73% of cases, with no significant survival difference (p=0.422) compared with p16 negative cases.

β-catenin membrane expression occurred in 61% of cases. β-catenin membrane expression disappearance accompanied by a lower 5-year OS rate (37.4% vs. 66.4%; p=0.012) and shorter 3-year DFS (45.5% vs. 70.8%; p=0.025).

This result confirms the observations obtained from the β-catenin- E-cadherin study.

Time to the metastasis emergence was associated with loss of membranous β-catenin staining (11.71 vs. 36.23 months; p=0.002). Metastases occurred during follow-up in 31% of patients. In this group, the 3-year DFS was longer in the β-catenin membrane positive patients (11.8 vs. 33.7 months; p=0.021), and the β-catenin membrane negative cases (46%) had lower 5-year OS compared with β-catenin positive cases (5.9% vs. 33.3 %; p=0.008). In those patients, whom during follow-up period metastasis did not occur, the proportion of β-catenin membrane positive cases was 74.4% with a nearly significant difference in 5-year OS (p=0.062).

Nuclear β-catenin staining was positive in 43% of cases, without differences at 5-year OS (46,7% vs.61,9%; p=0.173) and 3-year DFS (60.7% vs. 64.1%; p=0.173).

The E-cadherin positivity was found in 55% of 90 tumor samples with no difference at 5-year OS difference (58.5% vs. 51.8; p=0.247) or 3-year DFS (69.2% vs. 52.3%; p=0.367). In E-cadherin negative patients metastases developed after 19.6 months, while in E-cadherin positive patients the development was after 27.7 months (p=0.194).

The proportion of EGFR positive patients was 54%, and did not influence 5-year OS (p=0.992).

In our MLH1 negative cases (4%) no metastasis, recurrence or death appeared during follow-up. MLH1 and MSH2 positivity was present in 77% with no difference in survival (p=0.934).

TS positivity was found in 29% of cases with no prognostic value (p=0.476).

We entered the variables in the multivariate analysis, and Cox-regression test was used. The membrane β-catenin expression (p=0.026), the number of examined lymph nodes (p=0.005) and age (p <0.001) proved to be significant and independent prognostic markers.

4. 5. Results of immunohistochemical detection of ERCC1 expression and analysis of prognostic factors in metastatic colorectal carcinoma

There were 19 men and 9 women in the cohort of 29 patients (mean age 61.5 years; range 35–91 years) included in this part of the work. There were 17 cases of colon- and 11 rectum carcinomas. The average survival was 28 months, those with metastases in average survived 21.5 months. The five-year overall survival rate was 8%. The results of the IHC study including a total of 10 markers were analyzed individually for each patient.

Positivity of p53 detection was found in half of the cases (50%). Membranous decoration with EGFR antibody occurred in 28%. Nuclear positivity for MLH1

was found in 42%, that for MSH2 was detected in 57%, and for MSH6 this value was 32%.

Membranous reaction was 50% for E-cadherin. Cytoplasmic positivity for TS was found in 17% of the patients. We found no direct relationship between clinical outcome and the results of detection of the following markers: p53, p21, MLH-1, MSH-2, MSH-6, EGFR, E-cadherin, thymidylate- synthase.

Membrane and cytoplasmic positivity was separately evaluated for ß-catenin: 46% membranous positivity and 39% nuclear positivity was detected. Membranous positivity of β-catenin was associated with more favorable survival in metastatic patients (p=0,132).

Intense or moderate ERCC1 reaction was observed in 17 cases. In our system (antigen retrieval, dilution, etc.) the antibody used did not decorate normal mucosal elements.

As indicated by the relationship between ERCC1 positivity and overall survival (p=0,346), ERCC1 decoration and survival of patients with metastases (p=0,098) and ERCC1 positivity and progression free survival (p=0,24) prognosis turned out to be more favorable for ERCC1 negative cases.

4. 6. FISH analysis of EGFR amplification

FISH reaction was applied in TMA blocks construed form 79 tumorous samples. Acceptable reaction was achieved in 74 cases and amplification was detected in 7 cases (9,4 %). The magnitude of amplification varied between 2-4 times.

The average follow-up time for the amplified cases was 94 months (72-124 months). In 6 cases neither metastasis nor local recurrence occurred, these patients were all alive during the follow-up. One patient died from metastatic disease, DFS was 1 month, and OS was 12 months in this case. Based on these observations the likelihood of metastases was 14,3% in the amplified cases,

while without amplification (67 cases, 20 of them with metastases) the same risk was 29.8%.

IHC positivity for EGFR IHC was observed in 55.7% of the cases with score 2-3+ in 15,1% of them. When EGFR gen amplification was verified 71.4% showed IHC positivity with 57.1% frequency of score 2-3+ values. IHC positivity was observed in 50.7 % of amplified tumors with 9% frequency of score 2-3+ values.

5. Discussion

Validation of the TMA methodology

Validation test of the TMA system showed good agreement with data from the literature. This allowed us the conclusion that our system was appropriate for studying the prognostic factor we included among the specific aims of our work.

Analysis of the rhabdoid phenotypic histology

Beckwith and his co-workers published the first description of this kind of neoplasm as a monophasic, solid, rare variant of Wilms' tumor and considered it an unusual form of rhabdomyoid sarcoma and labeled it as malignant renal rhabdoid tumor (MRRT). Not too long after the recognition of the renal form similar neoplasms were recognized in extrarenal locations: in various soft tissues, the CNS, pancreas, liver, urinary bladder. The latter are usually referred to as malignant extrarenal rhabdoid tumors [MERT]. Rhabdoid components are not that uncommon in various adenomatous cancers, sarcomas and melanomas as a secondary phenotypical feature. The rhabdoid forms occur as nonspecific phenotypical characteristics. The deletion of the chromosome portion that harbors the tumor suppressor gene hSNF5/INI-1 (22q11.2) has been shown to occur as a typical genetic alteration in AT/RTs. Nuclear positivity with INI-1 antibody which we observed is in concordance with the literature.

Through literary search revealed a total of 22 rhabdoid cancers that affected the GI tract. Most cases of rhabdoid tumors affecting the GI tract occurred in the elderly population. The mean age of these patients was 63.4 years (ranging between 41-84 years). Primary rhabdoid tumors of GI tract show a strict male preponderance (male/female = 3.6:1). These tumors are aggressive neoplasms and have poor prognosis, over 75% of patients die within 6 months following the diagnosis. At the same time it seems rather likely that rhabdoid tumors of the GI tract represent a unique subtype among the GI cancers, the latter hypothesis needs to stand molecular testing.

E-cadherin/β-catenin study

According to most reports, in case of membranous staining of E-cadherin and β-catenin, better survival can be expected and the occurrence of metastatic disease is less common]. The nuclear expression of β-catenin seems to have potentially adverse prognostic significance in colorectal cancer.

In the whole population, the development of metastatic disease is more frequent, when the β-catenin membranous staining is lost. In case of nuclear β-catenin staining, metastatic disease is also more frequent, this correlation is significant.

In the group of patients with colonic tumours, there is no association between alterations of the expression of β-catenin and E-cadherin on the one hand and OS or DFS on the other. In these patients no difference in the risk of metastases is detectable when comparing groups with different β-catenin and E-cadherin expression patterns.

Negativity of membranous staining of β-catenin in patients with rectal cancer indicates worse OS. The risk of metastatic disease is significantly higher in the β-catenin membranously negative cases than the β-catenin membranously positive rectal cancers. Our results implicate that when β-catenin shows nuclear translocation, the risk of metastases is also significantly.

In the group of patients with metastatic disease the loss of membranous staining of β-catenin leads to faster appearance of metastases and DFS is also worse. The loss of membranous staining of E-cadherin also seems to be associated with rapidly evolving metastatic disease.

It is important to note that we could demonstrate reliably strong association between expression of β-catenin and E-cadherin and clinical outcome only in patients with rectal cancer. For the remaining cohort certain tendencies seem to exist but statistical proof requires further work. It is now believed that there are two pathogenetically distinct pathways for the development of colorectal cancer. Literary data suggest that there are important genetic differences between tumors of the right and left colonic side vs. the rectum. Our observations indicate that β-catenin and E-cadherin both play a crucial role in metastasis development in rectal cancer.

Relevant results from our prognostic study also confirm the observations gained from the E-cadherin/β-catenin analysis since membranous β-catenin expression was associated with significantly better survival. This indicates that β-catenin membranous expression is an independent prognostic factor.

In conclusion our results repeatedly confirm the critical role of β-catenin and E-cadherin complex in colorectal carcinogenesis. In Dukes B2 stage rectal cancers the loss of membranous expression of β-catenin is a predictor of the development of distant metastasis.

Analysis of disease prognosis

In an analysis of 35787 cases of T3N0 colon cancers from the US National Cancer Database, patients were stratified by the number of lymph nodes undergoing pathological examinations. The 5-year OS rate for patients for whom the total number of analyzed lymph nodes varied between 1-7 lymph nodes was 49.8%. When 8-12 lymph nodes were worked up the OS increased to 56.2%. This value climbed to 63.4 % when more than 13 lymph nodes were

pathologically examined (16). These data were also confirmed by the European study.

Our data also suggest that 5-12 examined lymph nodes offer a better prognosis than work-up of only 1-4 lymph nodes.

This marked difference is most likely due to a false negative pNO stage which stems for thorough analysis of numerous lymph nodes because those neglected are likely to harbor metastases.

Seventy percent of colorectal cancers lose a portion of chromosome 17p or 18q or both. The 17p chromosome contains the p53 gene, which is an important tumor suppressor gene and is reported to be mutated in 40-60% of colorectal cancers. P53 mutation is the most common molecular abnormality in malignant tumors. The significance of p53 mutation and over expression can vary in the colon depending on tumor location. A pooled analysis that included survival data from 4416 patients in 28 studies showed that neither p53 over expression nor p53 mutations emerged as powerful prognostic factors. We could not prove the prognostic value of p53 in the early stage tumors (T3N0). The results of many studies on the prognostic value of p53 are very contradictory, because immunohistology is not properly standardized and different antibodies are used for staining.

The role of p21 in colorectal cancer prognosis is controversial. According to the majority of the results in the literature, the over expression of p21 with or without p53 over expression refers to a poor prognosis. We observed p53 and p21 positivity in 4% of cases, and the survival of this group was significantly lower compared to p53 and p21 negative cases ($p=0.041$). Given the small number of cases this result should be considered with circumspection.

In colorectal cancer, the p16 expression correlated with distal location, differentiation and staging of the tumor. We could not prove the prognostic value of p16 in our study.

EGFR activation is a critical initiating event that induces a cascade of responses involved in cell division, proliferation, differentiation, apoptosis and angiogenesis. Although expression of EGFR is of prognostic value in several solid tumors, its prognostic importance in colorectal tumor is unclear. We identified EGFR expression in 52 % of the tumors, but this finding did not influence the prognosis of our patients.

Studies have demonstrated correlation between MSI and improved prognosis in colorectal cancer. Patients with MSI-H have been shown to have longer OS and less tumor recurrence, than MSS patients. A retrospective study suggested that only patients with MSS benefit from 5-FU chemotherapy. In our MLH1 negative cases no metastasis, recurrence or death occurred, but because of the small number of cases we could not perform statistical analysis on survival. We did not find prognostic values when we analyzed the MLH1 together with MLH2.

Several studies have demonstrated that a high level of TS protein expression predicts for a worse survival and is a possible predictor of tumor recurrence. The quantity of intratumoral TS expression is a predictive factor for resistance to 5-FU therapy in colorectal cancer. High levels induced resistance to 5-FU therapy. Among the examined tumor samples 29% were TS positive. TS showed no prognostic value in our study.

It is feasible to detect thymidylate-synthase expression immunoshistochemically on FFPE material. Most reports regard TS gene expression more reliable. There is, beyond doubt, correlation between gene expression and IHC patterns, however, IHC is more problematic to standardize.

Results from IHC detection of ERCC1 expression and analysis of prognostic factors in metastatic CRC

It is fair to conclude that better survival can be expected if membranous staining of E-cadherin and β-catenin is observed, better metastatic survival can

be expected than in non-staining cases. Thus the existence of correlation between β-catenin expression pattern and prognosis has been possible to corroborate.

Increased ERCC1 IHC decoration predicts worse OS, metastatic survival and PFS.

The main culprit for oxaliplatin resistance is DNA-repair. DNA damage caused by platinum compounds is repaired by the intracellular NER complex. The best indicator of response to oxaliplatin is ERCC1 gene expression and intratumoral mRNS synthesis. The lower the ERCC1 mRNS value the better is the therapeutic response. ERCC1 protein can also be detected with IHC and this reaction does have prognostic value. The main reason why our observations were statistically not significant is found in the low number (28) of cases included in the study. NB. There are inherent technical difficulties with IHC methodology as well. All in all our experiences indicate that TS and ERCC1 IHC provides useful information in retrospective studies provided that the sample is appropriately sizeable. Meanwhile it is noteworthy that this diagnostic approach is not adequate for determination of tailored cancer therapy.

FISH detection of EGFR amplification

Success rate of IHC detection of EGFR over expression with is 50-70%. EGFR over expression occurred in 54% and 55.7% of our cases and these results are in total agreement with the literature. In colorectal cancer one of the mechanisms of EGFR activation is amplification of the gene. In most studies the rate of EGFR gene amplification was between 5-17%. We detected gene amplification in 9.4% of our cases and this result is in total agreement with the literature. Although the intensity of protein expression was associated with the likelihood of gene amplification, in colorectal cancer this correlation is not as strong as the one well established for HER2 in breast cancer. EGFR IHC

observations and gene amplification data in our cohort indicate rather a tendency which means that the 2-3+ score in IHC are more frequent parallel with gene amplification. It is believed that patients with increased EGFR gene copy number have poor prognosis. This correlation is not valid in our patients; moreover, the frequency of metastatic disease was lower with amplification than without amplification (14.3% vs. 29.6%). The small size of the sample prevents statistical evaluation.

6. **Summary**

Carcinoma of the large bowel has become one of the most frequent malignancies both in the Western World and in Hungary. Currently colorectal cancer (CRC) represents the second most common malignancy-related cause of death in the developed countries. Development of CRC is a multistep event comprising well characterized genetic alterations which occur often in already established sequences within the epithelial cells of colorectal mucosa preceding the actual appearance of a *sui generis* malignant cell. Our work sums up the results of studies mostly utilizing TMA methods which aimed at the clarification of molecular carcinogenetic events in CRC, mainly at the proteonomic level. The work has been focused on the β-catenin-E-cadherin axis. We have shown that diminished β-catenin expression and to certain degree decreased E-cadherin membranous expression is followed by shorter OS and DFS. At the same time – particularly loss of β-catenin membranous expression – an increased metastatic potential characterizes these cases and these relations are primarily hold true for rectal tumors. We failed to show any significant correlations between the disease outcome and p16, EGFR and mismatch proteins' expression. In p53 and p21 positive cases the survival was significantly lower compared to p53 and p21 negative cases. It was strong positive correlation between p21 expression and tumor recurrence. Our results also show that within the group canonical histopathological characteristics the number of analyzed regional lymph nodes has profound importance is stage II. CRC. Ever since the introduction of biologically tailored antibody therapies prediction of their efficacy has been in the forefront of oncology. The predictive factors in this regard have mostly been represented by expression of EGFR and also by amplification of the EGFR gene. Our results are in total agreement with the literature, not only with regard to EGFR expression but also concerning gene amplification. We have not been able to establish any

prognostic value and predictive evaluation has not been possible due to lack of specific therapy. However, our results provide ample proof for the adequacy of TMA blocks both for immunohistochemical (IHC) analysis of EGFR expression and EGFR gene amplification. In addition to tailored biological therapies the need for genetically based treatment selection is also prevalent. Hence we attempted to utilize TMA blocks in retrospective studies which using immunohistochemistry aimed at the evaluation of detecting TS (a protein assumed to be predictive for 5-FU therapy) and ERCC1 protein (a predictor of oxaliplatin therapy). Our results show that IHC of the two latter proteins has a rather limited value and does not help in determining therapeutical protocols. Direct translation of findings of basic oncological research into clinical practice holds real promises of identification dependable prognostic factors which will allow screening for candidates of recurrence and/or metastatic disease in early stage patients. By the same token there is hope for identifying further predictive factors which will render personalized (tailored) therapy of CRC achievable.

7. New observations

1. Our work provides proof of the feasibility for the use of the TMA methodology introduced in our laboratory to detect molecular prognostic factors with IHC.

2. We have shown that lack of β-catenin membranous expression in rectal carcinoma indicates more frequent occurrence of metastases. The same holds true for nuclear expression of β-catenin. Metastases occur sooner in cases without membranous expression of β-catenin. There is no such relationship between disease outcome and the pattern of β-catenin/E-cadherin expression in carcinoma of the colon.

These new observations further confirm that CRC is a genetically heterogeneous disease and there are fundamental differences between the mechanisms that lead to the appearance of colon or rectal carcinoma.

3. In a larger patient cohort we further confirmed the prognostic importance of the β-catenin/E-cadherin expression pattern. Analyzing disease outcome we have shown that membranous expression of β-catenin is an independent prognostic factor. These results are in concordance with literary data.

Unfortunately the number of pathologically evaluated lymph nodes in our material is much smaller than the International standard. However, even in case of a lower number of lymph nodes analyzed, in Stage II disease the prognostic value of the worked-up lymph nodes' number is unequivocally relevant prognostically. We have not been able to establish any prognostic value for expression of p16, MSH2, or MLH1. Parallel positivity for p53 and p21 has ominous prognostic value. Local recurrence is more frequent when p21 detection results in positivity.

4. IHC detection of either TS and/or ERCC1 protein does not provide reliable help for personalized, tailored therapeutical decisions, however this may still be of use on population-level evaluations. ERCC1 expression is associated with unfavorable prognosis but this we cannot as yet corroborate statistically.

5. TMA blocks can be used for FISH detection of EGFR amplification. Our observations on EGFR amplification agreed as far as frequency is concerned with literary data. By the same token IHC detection of EGFR protein only showed a loose correlation with gene amplification.

6. Rhabdoid phenotype has a particularly unfavorable prognosis within the broad category of GI tumors. Review of the rather scarce literature indicates that rhabdoid phenotype comprises an unfortunate prognostic subgroup of GI tumors affecting elderly patients.

8. List of publications related to the dissertation and other publications

Candidate: László Tóth

Neptun ID: CCJU53

Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. András, C., **Tóth, L.**, Molnár, C., Tanyi, M., Csiki, Z., Dezső, B., Pósán, J., Shemirani, A., Csiki, E., Szántó, J.: Correlations between Clinicopathological Parameters and Molecular Signatures of Primary Tumors for Patients with Stage T3N0 Colorectal Adenocarcinomas: A Single Center Retrospective Study on 100 Cases.
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