Pattern of Melanotransferrin Expression in Human Colorectal Tissues: An Immunohistochemical Study on Potential Clinical Application

KAMILA DUŚ-SZACHNIEWICZ[1], PAWEŁ OSTASIEWICZ[1], MARTA WOŹNIAK[1], PAWEŁ KOŁODZIEJ[1], JACEK R. WIŚNIEWSKI[2] and PIOTR ZIÓŁKOWSKI[1]

[1]*Department of Pathology, Wrocław Medical University, Wrocław, Poland;*
[2]*Department of Proteomics and Signal Transduction, Biochemical Proteomics Group, Max Planck Institute of Biochemistry, Martinsried, Germany*

Abstract. *Background: Our previous liquid chromatography-tandem mass spectrometry (LC-MS/MS) study on colorectal cancer proteome resulted in identification of 10,000 differentially expressed proteins. We observed a significantly changed expression of 25% of all identified proteins between patient and matched adjacent normal mucosa, carcinoma and colorectal adenoma, including melanotransferrin. Herein, we consider this protein as a potential biomarker of colorectal cancer. Materials and Methods: Immunohistochemical detection of melanotransferrin was carried-out to localize its expression pattern within the colorectal tissues by tissue microarray. The diagnostic utility of melanotransferrin was evaluated in patient serum by enzyme-linked immunosorbent assay (ELISA). Results: Strong melanotransferrin expression was found to be related to clinicopathological characteristics, lymph node involvement (p=0.008), tumor localization in colon (p=0.001), presence of mucin (p<0.013) and increasing tumor grade (p<0.001). Melanotransferrin level in serum from patients with colorectal cancer was significantly higher than that in healthy controls (p<0.001). Conclusion: We provide novel evidence that melanotransferrin may be involved in transformation from benign tumor to malignancy and is a marker of an invasive tumor phenotype.*

In 2012 in Europe, over 446,800 new colorectal cancer (CRC) cases were reported and 214,700 deaths occurred (1). Most CRC develops from colonic adenoma, which is a benign tumor of glandular epithelial tissue. Approximately 5% of all adenomas progress into cancer and this process takes 10-20 years (2). Very recently, randomized controlled trials on over 7,000 patients with adenoma and colorectal cancer demonstrated significant reductions in colorectal cancer incidence and mortality associated with polypectomy (3). A number of studies have also reported the strong relationship between the stage at which CRC is detected and the clinical outcome; the diagnosis of CRC at an early stage increases the chance for recovery and has improves the survival rate (4-7). As the lifetime risk of developing CRC reaches up to 5% in the general population, investigations leading to detection of new biomarkers for early-stage CRC are still justified (8). From the molecular point of view, the progression from colorectal adenoma to carcinoma is strongly associated with an accumulation of genomic changes, including chromosomal alterations (gains of 8q, 13q, and 20q and losses of 8p, 15q, 17p and 18q) (9,10). These genetic alterations have an enormous impact on the proteome composition of cancer cells. Nowadays, the clinical proteomic platform can be used for quantitative comparisons between samples representing different stages of diseases with a high reliability and repeatability. Our previous studies reported the liquid chromatography-mass spectrometry (LC-MS/MS)-based proteomic analysis of microdissected tissue from formalin-fixed and paraffin-embedded CRC specimens (11-13). We identified more than 10,000 proteins differentially expressed in patient matched adjacent normal mucosa, primary carcinoma and colonic adenoma (13). Moreover, we recorded significantly changed expression levels of approximately 20% of all identified proteins between normal and pathologically changed tissues, including melanotransferrin (MFI2), although this protein has not been considered as a potential biomarker of CRC.

Human MFI2 is a cell-surface glycoprotein that belongs to the transferrin superfamily and is related to transferrin in tertiary structure and iron-binding properties (14). MFI2 has been also described as one of the first melanoma cell-surface markers, and therefore it is also named melanoma-associated

Correspondence to: Kamila Duś-Szachniewicz, Department of Pathology, Wrocław Medical University, ul K. Marcinkowskiego 1, 50-368 Wrocław, Poland. Tel: +48 717841225, e-mail: kamila.dus@gmail.com

Key Words: Melanotransferrin, colorectal carcinoma, poorly differentiated adenocarcinoma, pathogenesis, adenoma–carcinoma sequence, tumorigenesis.

antigen p97 (15). Previous studies considered the essential function of MFI2 in iron uptake and transport in cancer cells, however, this hypothesis was not confirmed (16, 17). Additionally, the role of MFI2 in cell differentiation, proliferation, migration and angiogenesis was described (18-22). Although the MFI2 was discovered to be significantly overexpressed in melanoma tumors over 30 years ago (23), the exact role of this protein in the pathogenesis of several types of cancer remains unclear. Therefore, it is important to intensify the investigation on the role of MFI2 protein in proliferation, migration and tumorigenesis.

To study whether this candidate biomarker of CRC observed in the LC-MS/MS analysis is overexpressed in a larger cohort of samples, we analyzed the MFI2 immunoreactivity in CRC, corresponding to adjacent normal tissue and adenoma samples using immunohistochemical (IHC) staining of tissue microarrays (TMAs). Additionally, we performed western blot and by enzyme-linked immunosorbent assay (ELISA) in considering the potential role of MFI2 in clinical applications. As far as we are aware of, this study is the first to evaluate MFI2 protein expression in colorectal tissues.

Materials and Methods

Patient samples. The study group included 140 patients with CRC who had undergone surgery at the Department of Surgery, Wrocław Medical University, Poland, between 2012-2013 (71 men and 69 women; mean age=69.05±10.46 years; age range=34-88 years). All samples were collected prior to chemotherapy or radiation. In our study, the grade, stage, tumor size and localization were evaluated for all cases. Patients were classified as having right CRC if the primary tumor was located in the cecum, ascending colon, hepatic flexure or transverse colon, and left CRC if the tumor site was within the splenic flexure, descending colon or sigmoidal colon. Adenoma tissues were obtained from 39 controls during colonoscopy (25 men and 14 women; mean age=57.79±10.26 years; age range=38-75 years). All specimens were fixed in 10% formalin, paraffin embedded and stained by eosin and hematoxylin for routine pathological evaluation. The cancer specimens were classified according to the Union for International Cancer Control Tumor-Node-Metastasis (TNM) Classification of Malignant Tumors (24). The investigation was conducted in accordance with ethical standards, according to the Declaration of Helsinki and according to national and international guidelines and was approved by the Authors' Institutional Review Board. The specimens were processed upon approval by the Bioethics Committee of the Wroclaw Medical University (Decision no. KB-598/2011). Clinicopathological data are presented in Tables I and II.

TMA construction. Representative areas of colonic adenocarcinoma, adjacent normal mucosa and colorectal adenoma were marked on hematoxylin and eosin-stained slides. Cores of 2 mm in diameter were punched from corresponding areas of paraffin blocks and inserted into the recipient block. The complete TMA block was placed in an oven at 40˚C overnight, and then on the cold plate of a tissue-embedding station with subsequent two to three 1-hour cycles of heat/cold to temper the array. Next the TMA block was cut into 5 μm-thick sections for the IHC study.

Plasma sample collection. For the ELISA study, serum from 15 patients with CRC who had not undergone any form of preoperative chemotherapy or radiation therapy were collected. This group comprised of seven males and eight females, with a mean age of 60.62±9.55-years (range=41-72 years). Sera obtained from 15 healthy individuals were used as controls. All control samples were age and sex matched. The healthy volunteers had no obvious evidence of malignancy, based on abdominal ultrasound examinations, routine blood tests, biochemistry tests and questionnaires. Serum samples were collected in heparin-containing tubes and centrifuged for 10 min at 2,000 ×g. Then samples were separated and immediately stored in new tubes at −80˚C until use. The storage time was less than 6 months.

Immunohistochemistry. IHC was performed on automated Dako EnVision™ FLEX detection system (Dako Co., Tokyo, Japan) using rabbit polyclonal antibody to MFI2 (Sigma-Aldrich, Seelze, Germany). Sections (5-μm) of CRC TMA were submitted to the analysis. The Dako EnVision™ FLEX detection system included pretreatment with Dako PT LINK (pre-treatment module) and high pH Target Retrieval solution at 97˚C for 20 min. In a preliminary study to determine appropriate antibody dilution, TMA samples were incubated with MFI2 antibody diluted by 25, 50, 100 and 200 with antibody diluent at room temperature for 60 min. Antibody incubation was followed by standard signal amplification including rabbit LINKER at room temperature for 15 min, horseradish peroxidase (HRP)-conjugated EnVision™ FLEX at room temperature for 20 min, 3,3'-diaminobenzidine (DAB) reaction for 10 min and counterstaining with hematoxylin for 3 min. Antibody dilution of 50 was selected as appropriate and slides were scored semiquantitatively for staining intensity and distribution.

Sample validation. Immunostaining of tumor sections for MFI2 was reported according to the following standards (Table III). For each specimen, the immunoreactivity score (IRS) was calculated by multiplying the staining intensity value (score 0-3) by percentage of stained cells (score 0-4) as described in Table III. The resulting IRS then ranged from 0-12. The percentage of stained cells was calculated from five neighboring fields (×100) in the center of lesions, divided by the total number of cells in evaluated areas, and then divided by the number of fields. For MFI2 immunoreactivity, the results were considered as a negative (score 0), weakly positive (score 1-4), positive (score 5-8) and strongly positive (score 9-12). The above criteria were blindly determined by the co-author with clinical data using a light microscope (Eclipse Ci, Nikon, Tokyo, Japan) at ×200 magnification. The scores were then compared between two pathologists and their validation was in agreement in 82.7% of cases. A final 100% consensus was assigned after second evaluation of discordant cases by pathologists.

Verification of MFI2 in human plasma by ELISA. A human MFI2 ELISA detection kit (Cloud Clone Corp., Houston, TX, USA) was used to measure MFI2 in human serum. The tests were performed according to the manufacturer's recommendations. In brief, 100 μl of each dilution of standards, blank and 1:50 diluted samples were added into the appropriate microtiter plate wells with a biotin-conjugated antibody specific to MFI2 and incubated at 37˚C for 2 h. Avidin conjugated to HRP was used as a secondary antibody. Detection was accomplished using tetramethylbenzidine substrate and the enzyme-substrate reaction was terminated by adding

Table I. *Clinicopathological characteristics of patients with colorectal cancer.*

Feature		No. of cases	%
Gender	Male	71	50.7
	Female	69	49.3
Age (years)	<65	45	3.1
	>65	95	67.9
pT status	T1	2	1.4
	T2	36	25.7
	T3	85	60.7
	T4	17	12.2
pN status	N0	75	53.6
	N1-3	65	46.4
pM status	Mx	130	92.9
	M1	10	7.1
Localization	Left colon	75	53.6
	Right colon	28	20.0
	Rectum	37	26.4
Tumor size	<5 cm	87	62.1
	>5 cm	42	30.0
	No data	11	7.9
Mucinous phenotype	Yes	12	8.6
	No	128	91.4
Grading	G1	31	22.1
	G2	86	61.4
	G3	23	16.5

Table II. *Clinicopathological characteristics of patients with adenoma.*

Features		No. of cases	%
Gender	Male	25	64.1
	Female	14	35.9
Age (years)	<65	28	71.8
	>65	11	28.2
Dysplasia	Absent	15	38.5
	Low	17	43.6
	High	7	17.9
Adenoma type	Tubular	27	69.2
	Tubulovillous	12	30.8

sulphuric acid solution. The absorbance was determined at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Final quantities of corresponding antigen in plasma samples were calculated from an eight-point standard curve (0-2000 pg/ml). All sera were measured from triplicates and are indicated as the mean value. The limit of detection was 12.9 pg/ml. The inter- and intra-assay coefficients of variation were below 15% for all tests.

Western blotting assay. To examine the level of MFI2 protein in tissue lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting analysis were applied. For the western blot, formalin-fixed and paraffin-embedded tumor blocks were used. Firstly, the tissue blocks were cut into 10 sections of 20 μm and put on microscope slides. Next a pathologist marked the cancerous parts of the section in accordance with the image visible in the light microscope and the appropriate parts of each section were macrodissected. Adjacent normal tissues (control) were macrodissected from the tumor resection margins (surgical and histological tumor-free margins). Collected tissues were de-paraffinized in three changes of xylene in a heating block with agitation, 10 min each, cleared in three changes of absolute alcohol and dried in concentrator (Eppendorf, Hamburg, Germany). Samples were then homogenized in a lysis buffer [0.1 M Tris-HCl (pH 8.0), 0.1 M DL- dithiothreitol, 4% SDS] at 99˚C in a heating block with agitation (600 rpm) for 1 h. Next, the crude extracts were clarified by centrifugation at 16,000 × *g* at 18˚C for 10 min. The protein concentration in the supernatant was measured at 280 nm using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Hybond, GE Healthcare Bio-sciences AB, Uppsala, Sweden) by NuPage System as recommended by Invitrogen (Life Technologies). Following washing in PBS, incubation with 2.5% glutaraldehyde (Sigma-Aldrich, Seelze, Germany) and then with 5% goat serum (Sigma-Aldrich), the membrane was probed with primary polyclonal rabbit antibody against MFI2 (dilution 1:250) at 4˚C. Anti-β-actin polyclonal antibody was purchased from Abcam (Abcam, Cambridge, UK). The following day, after washing with PBS-Tween 0.1%, blots were incubated with the appropriate HRP-conjugated secondary antibody (Abcam) and the bound antibodies were visualized using DAB Enhanced Liquid Substrate System for Immunohistochemistry (Sigma-Aldrich). Documentation of bands was performed using Molecular Imager Gel Doc TMXR+ (Bio-Rad).

Table III. *Immunoreactivity score calculation.*

Staining intensity	% Staining
0: None	1: <25%
1: Weak	2: 25-50%
2: Moderate	3: 51-75%
3: Strong	4: >75%

Statistical analysis. The association between MFI2 immunoreactivity and clinicopathological parameters were analyzed by Pearson's Chi-square test or Fischer's exact test. A value of $p<0.05$ was considered as statistically significant in all analyses. Statistical analysis was performed by STATISTICA v.10.0 (StatSoft, Kraków, Poland).

Results

MFI2 protein is overexpressed in human CRC. To investigate the oncogenic properties of MFI2, IHC was performed on 319 formalin-fixed and paraffin-embedded colorectal tissues (Figures 1 and 2). The MFI2-positive cytoplasmic staining was observed in 88.6% (124/140) of CRC samples, while adjacent normal epithelium was positive in 34.4% (48/140) only, suggesting that CRC cells may be responsible for MFI2

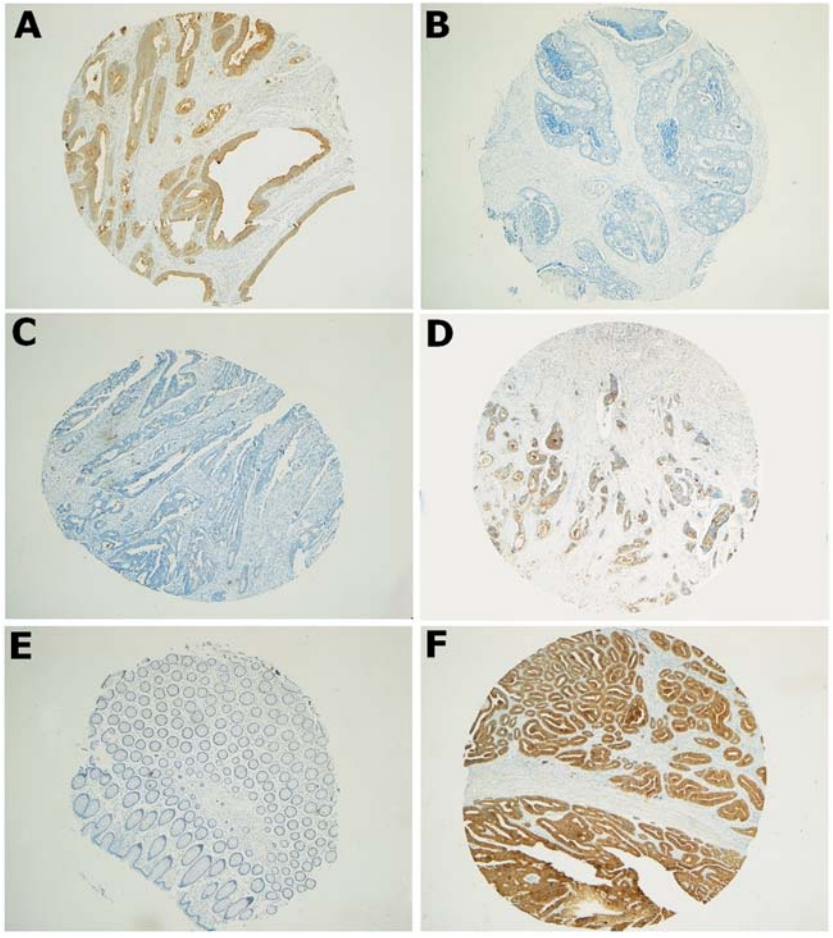

Figure 1. *Immunostaining for melanotransferrin in tissue microarrays constructed from colorectal tissue showing strongly positive immunoreactivity of well differentiated colorectal cancer (CRC; A), negative staining of CRC (B), negative control (C), strong positive staining of poorly differentiated CRC (D), negative staining of adjacent normal epithelium (E), strong immunoreactivity in colorectal adenoma (F). Magnification, ×40.*

Table IV. *Melanotransferrin immunoreactivity in colorectal tissue (CRC), their corresponding adjacent normal epithelium (Norm) and adenoma tissues (Ad) by immunoreactive score (IRS).*

		IRS, n (%)					*p*-Value	
Tissue	Number	0	1-4	5-8	9-12	All positive	*vs*. Norm	*vs*. Ad
Norm	140 (100%)	92 (65.7%)	37 (26.4%)	11 (7.9%)	0 (0%)	48 (34.3%)		
Ad	39 (100%)	10 (25.6%)	14 (35.9%)	14 (35.9%)	1 (2.6%)	29 (74.3%)	<0.001	
CRC	140 (100%)	16 (11.4%)	2 (1.4%)	56 (40%)	66 (47.2%)	124 (88.6%)	<0.001	<0.001

overexpression (Table IV). The average MFI2 IRS for cancerous tissues was 6.19±3.59 and was almost four-times higher than that for matched adjacent normal tissue (M_{IRS}=1.6±2.57). The average MFI2 IRS for adenoma tissues was 3.87±2.74 (Figure 3).

Correlation with clinicopathological characteristics. Statistical analysis of the association between MFI2 immnunoreactivity and clinicopathological features is shown in Table V. Strong MFI2 expression (IRS 9-12) was found to be significantly related to certain clinicopathological

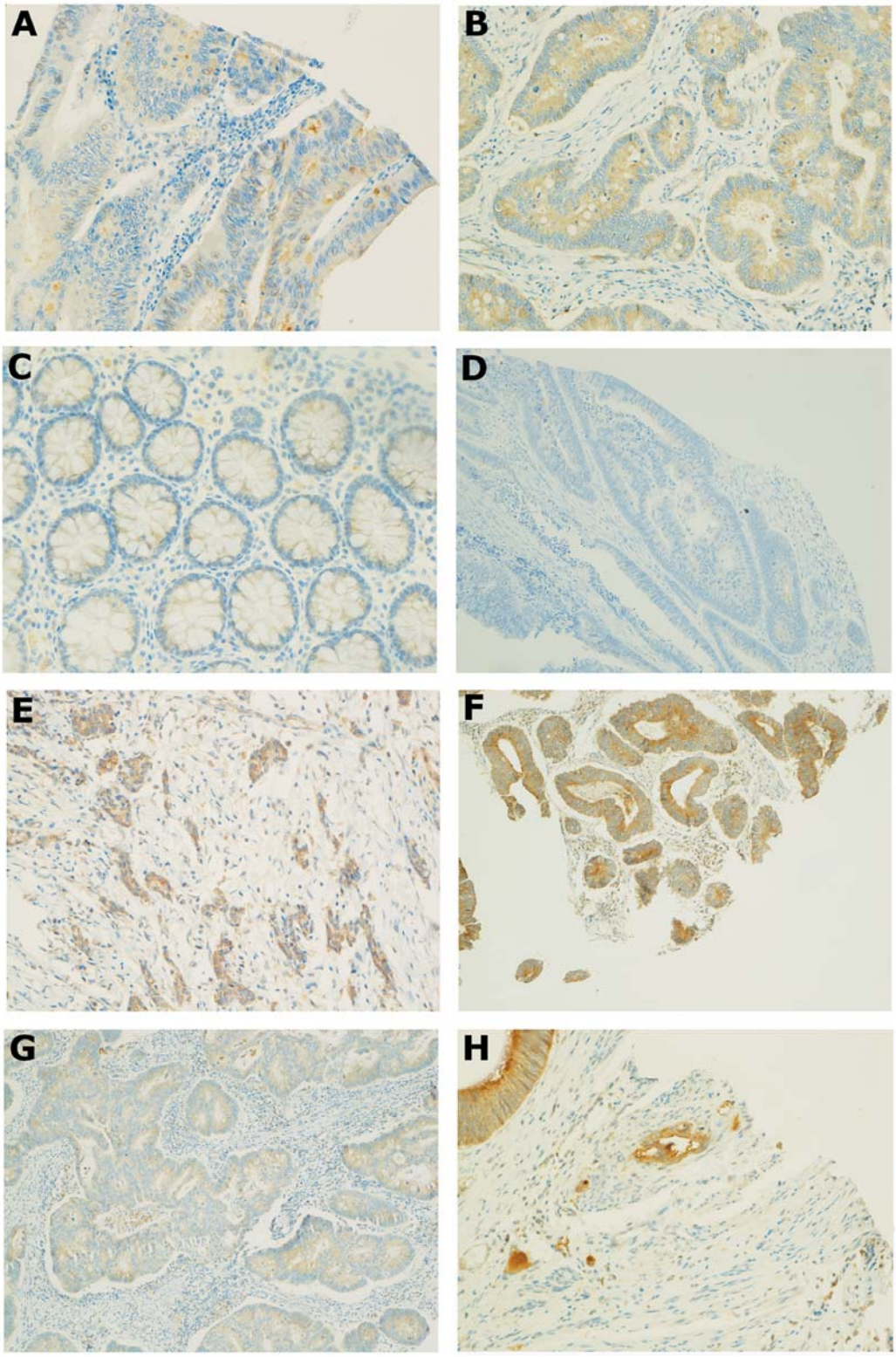

Figure 2. *Representative immunohistochemical staining for melanotransferrin expression. A: Weakly positive staining [immunoreactivity score (IRS)=2] of adenoma; B: weakly positive staining (IRS=4) of colorectal carcinoma (CRC, C: negative staining (IRS=0) of adjacent normal epithelium, D: negative control, E: strong positive staining (IRS=9) of poorly differentiated CRC, F: strong positive staining (IRS=12) of well-differentiated CRC, G: weakly positive staining (IRS=2) of CRC, H: immunodetection of small cancer nest. Magnification, ×100.*

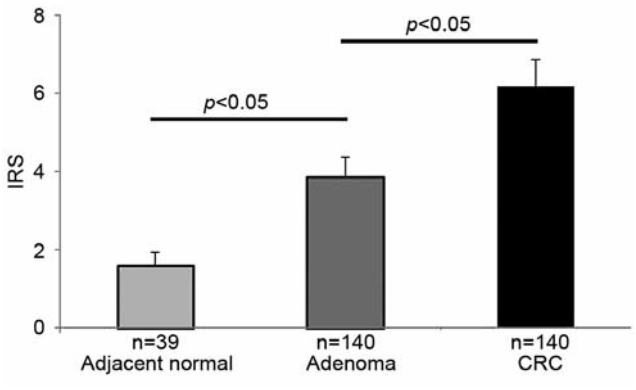

Figure 3. *The immunoreactivity score (IRS) for melanotransferrin expression in tumor (CRC), matched adjacent normal tissue and colorectal adenoma. Data represent the mean±SEM.*

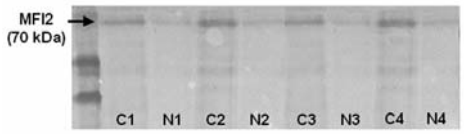

Figure 4. *Western blot analysis of melanotransferrin (MFI2) expression in colorectal cancer tissue with paired adjacent normal epithelium. C_1, C_2: Well-differentiated tumor; C_3, C_4: poorly differentiated tumor; N_{1-4}: matched adjacent normal mucosa.*

Table V. *The association of melanotransferrin (MFI2) expression with patient clinicopathological characteristics.*

Characteristic			High MFI2 expression (IRS 9-12)		
		No. of cases (n=140)	No.	%	*p*-Value
Gender	Male	71	35	49.3	0.728
	Female	69	31	44.9	
Age (years)	<65	45	22	48.9	0.919
	>65	95	44	46.3	
pT status	T1	2	0	0.00	0.610
	T2	36	17	47.2	
	T3	85	41	48.2	
	T4	17	8	47.1	
pN status	N0	75	27	36.0	0.008
	N1-3	65	39	60.0	
pM status	Mx	130	60	46.1	0.516*
	M1	10	6	60.0	
Localization	Left colon	75	42	56.0	0.001
	Right colon	28	16	57.1	
	Rectum	37	8	21.6	
Tumor size	<5 cm	87	38	43.7	0.191
	>5 cm	42	20	47.6	
	No data	11	8	72.7	
Mucinous phenotype	Yes	12	10	83.3	0.013*
	No	128	56	43.7	
Grading	G1	31	2	6.4	<0.001
	G2	86	43	50.0	
	G3	23	21	91.3	

*Fischer's exact test, other parameters: Chi-square test.

characteristics, including lymph node involvement (pN), tumor localization, overexpression of mucin in tumor cells and tumor grade (G). No significant correlations were observed among patients age and gender. Additionally parameters like depth of tumor invasion (pT status), presence of distant metastases and tumor size had no relationship with MFI2 expression.

The relation of MFI2 protein immunoreactivity to different tumor location was investigated in detail (Table V). The difference in the frequency of strong MFI2 expression in tumors located in colon *vs.* rectum was significant (p=0.001). Out of the 16 MFI2-negative cancer samples, 14 cases were located in the rectum. The average MFI2 IRS for cancer located in the colon was 7.01±3.04 and was 1.8-times higher than that for rectal tumors (M_{IRS}=3.91±4.03).

Regarding tumor histological grade, there was a statistically significant difference in MFI2 expression depending on increasing tumor grade (p<0.001). MFI2 was strongly overexpressed in 91.3% of high-grade tumors (G3). The differences in IRS between G1, G2 and G3 cancer are presented in Table VI.

MFI2 immunoreactivity was found to be related to presence of lymph node metastases (pN) (p=0.008). Percentage of specimens with high MFI2 expression was 36% (27/75) for cancer without lymph node involvement and 60% (39/65) for tumors where metastasis to lymph nodes was observed. Additionally, MFI2 was negative in 13.3% (10/75) and 9.2% (6/65) of N_0 and N_{1-3} cancer samples, respectively (data not shown).

Finally, this study showed that MFI2 expression is related to a mucin-producing phenotype in cancer cells. Twelve patients with mucin-producing cancer (including mucinous cancer and signet-ring cell cancer) were enrolled in the study and tumor in 10 of them showed strong MFI2 immunoreactivity. The average IRS for mucin-producing cancer was also clearly higher compared to non-mucin-producing adenocarcinoma (9.17±2.79 *vs.* 5.91±3.53) and the average IRS for all adenocarcinoma samples (6.19±3.59).

ELISA. To examine whether MFI2 is overexpressed in human serum, its expression levels were measured by using a commercial ELISA kit. Samples from 15 patients with CRC and 15 healthy controls were analyzed. MFI2 was detected in all cancer samples and in 12 controls. MFI2 levels in sera from patients with CRC (median=523.14 pg/ml, range=65.76-

Table VI. *Melanotransferrin expression pattern by immunoreactive score (IRS) according to tumor grade.*

		IRS, n (%)				
Grade	N (=140)	0	1-4	5-8	9-12	Mean±SD
G1	31 (100%)	8 (25.8%)	21 (67.7%)	0 (0.0%)	2 (6.5%)	3.19±2.32
G2	86 (100%)	9 (10.5%)	34 (39.5%)	0 (0.0%)	43 (50.0%)	6.29±3.27
G3	23 (100%)	0 (0.00%)	2 (8.7%)	0 (0.0%)	21 (91.3%)	9.87±2.36

1075.08 pg/ml) were significantly higher than those in healthy controls (median 181.34 pg/ml, range=0-423.60 pg/ml; $p<0.001$).

Western blot. MFI2 protein expression in 10 formalin-fixed and paraffin-embedded samples was confirmed by the western blot technique. MFI2-corresponding bands were found with different intensity in both, adjacent normal and cancerous samples. As showed in the Figure 4, there was no difference in MFI2 levels detected between well- (G1) and poorly-differentiated (G3) cancer.

Discussion

Recently, much effort has been put into proteomics studies on CRC (25-30). As a result, numerous proteins have been found to be overexpressed in cancer tissues. We investigated the quantitative changes between formalin-fixed paraffin-embedded cancer tissues, adenomas and adjacent normal epithelium by LC-MS/MS (11-13). Expression levels of 1,808 proteins were significantly different between normal and cancer tissues (11). The aim of the present study was, from the long list of up-regulated proteins, to find those with real impact on CRC tumorigenesis.

Colorectal cancer treatment is effective in 95% of patients surviving over 5 years if diagnosed and removed at an early stage (31). Consequently, the aim is to develop a convenient diagnostic tool that is sensitive and specific for both adenomas with low- or high-grade dysplasia and the earliest stages of CRC. Particular attention should be paid to the expression pattern of colorectal adenoma as there is a gap in the literature on adenoma biomarkers.

Melanotransferrin is a glycoprotein expressed at the cell membrane; it has been identified in several normal adult tissues as well as in fetal tissues (32, 33). MFI2 shares sequence and iron-binding function with the serum iron transport protein, transferrin (34). The investigations on the relationship between MFI2 protein expression and neoplasia development have mainly focused on melanoma: it has been found that MFI2 is significantly overexpressed in malignant melanoma, whilst its expression in normal tissue is usually low (14, 17, 23). In the melanoma cell line model, Rahmanto and co-workers reported that MFI2 overexpression was associated with increased cellular proliferation, whereas MFI2 down-regulation resulted in the opposite effect (20). Considering the iron-binding capacity of MFI2, it was hypothesized that overexpression of this protein in melanoma cells is related to their increased iron requirements for proliferation, however, several studies demonstrated that MFI2 did not play an essential role in iron uptake by cancer cells (15-17).

To further decipher the role of MFI2 in tumorigenesis, investigation of the effect of purified recombinant MFI2 for its capability to induce angiogenesis in the chick chorioallantoic membrane (CAM) was performed (18). Sala and co-workers during macroscopic and microscopic evaluation of the vascular density of the CAM assay found that MFI2 exerts an angiogenic response quantitatively similar to that elicited by fibroblast growth factor-2. Additionally, they observed an overexpression of vascular endothelial growth factor-receptor-2 (VEGFR2) in newly-formed vessels, suggesting that angiogenic activity of MFI2 may depend on activation of endogenous VEGF.

MFI2 is attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor and this fact may have importance for its functional characteristics. The GPI anchor is a complex post-translational modification that is implicated in the regulation of a host of physiological processes by conveying properties to the protein to which it is attached (35). The overall abundance of GPI-anchored proteins is estimated to comprise 1-2% of all translated proteins in the human proteome (36) and the levels of GPI-anchored proteins in normal cells are under tight control, as evidenced by the low levels of glycosylphosphatidylinositol transamidase (GPIT) complex mRNA (37, 38). Studies by Nagpal *et al*. indicate that the GPIT levels are amplified in 19 human cancer types (including breast, ovarian, stomach and uterus and cancer) due to chromosomal amplifications acquired during malignant transformation (39), however, the impact of how the amplification of GPIT subunits influence cancer progression is just beginning to be assessed. It is worth considering whether MFI2 as a GDP-anchored protein may play a role as an activation antigen in the immune system and may be involved in signal transduction.

Finally, the role of MFI2 in inhibition of cancer metastasis has been confirmed (40). Rolland and co-workers showed that administration of a single dose of a monoclonal antibody directed against human MFI2 significantly reduced the development of human melanoma brain metastases in nude mice. They suggested membrane-bound MFI2 as an attractive target for cancer therapy. All the above-cited research defined a role for MFI2 in tumorigenesis but further study to examine the exact molecular processes and novel signaling pathways is needed. To our knowledge, this work is the first report of MFI2 expression pattern in colorectal tissues. We confirmed that MFI2 is differentially expressed in both adenoma and cancer samples compared to adjacent normal tissues. The IRS for MFI2 gradually changes from control normal epithelium, through adenoma to CRC, suggesting that MFI2 protein overexpression is associated with neoplastic progression. These results are in concordance with our previous investigations on colorectal tissues by LC-MS/MS (11-13). Additionally, the presence of aberrant MFI2 expression in human adenoma tissues compared to adjacent normal mucosa indicates that overexpression of this protein is an early event in CRC tumorigenesis.

Herein, we observed significant correlations between MFI2 expression and clinicopathological parameters of patients with CRC. The feature strongly associated with poor prognosis in CRC is presence of lymph node metastasis and the pN status is a strong independent prognostic factor for survival of patients with CRC. Our investigations confirmed a statistically important MFI2 overexpression in cancer where lymph node involvement was observed compared to specimens without lymph node involvement. Interestingly, MFI2-negative specimens were observed with similar frequency in both types of samples.

A statistically important association between tumor grade and MFI2 expression level was also found. We showed that the IRS for G3 was over three-times higher than that for well-differentiated cancer. Taken together, the strong MFI2 immunoreactivity in poorly-differentiated and pN1-3 cancer indicates the role of this protein in tumor progression and its potential utility as a marker of a more aggressive and invasive tumor phenotype.

In 1990, Bulfil drew attention to the developmental and biological differences in the proximal and distal colon that may reflect differing susceptibilities to neoplastic transformation (41). Subsequently, a number of articles focused on differences observed in the molecular biology, pathology, epidemiology and clinical data between right-sided and left-sided colon cancer. The relationship of right-sided cancer with older age, larger tumor size, mucinous subtype and microsatellite instability has been proven (42-46). Our study showed no dissimilarity in MFI2 expression between tumors located in the left and right colon, however, we did notice another feature regarding tumor location: 37.8% of the studied rectal carcinomas did not exhibit MFI2 immunoreactivity, while only 1.9% of colonic tumors were MFI2-negative. This shows a large disparity in MFI2 immunoexpression pattern between cancer located in the colon and that in the rectum. The Cancer Genome Atlas Network published in 2012 a comprehensive characterization of human colon and rectal carcinomas (47). In-depth analyses showed that adenocarcinomas of the colon and rectum are not distinguishable at the genomic level, however, tumors from the right/ascending colon were more likely to be hypermethylated and to have higher mutation rates than were other CRCs. Despite the similarity at the genome level, several studies confirmed that rectal cancer is different from colonic cancer regarding their clinical behavior (48). Compared to colonic cancer, the lymph node involvement status in rectal cancer has a higher impact on the choice of neoadjuvant therapy (49). Furthermore, a molecular profiling study of 115 pairs of primary and metastatic tissues of patients with CRC indicated high discordance rates in the mutational profile of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) and F-box/WD repeat-containing protein 7 (*FBXW7*) between the primary tumor location and corresponding metastases (50). Due to this evidence, Tamas and co-workers suggested that rectal and colonic cancer have different potential drug targets (48). The differences in MFI2 expression between colonic and rectal cancer observed in our study also confirm the hypothesis that CRC is not one single disease and further studies on the molecular composition of CRC are needed.

In our study, we found very interesting relationship between prominent mucin (MUC) production by cancer cells and MFI2 overexpression. Mucinous colorectal carcinoma represents a relatively rare sub-type of CRC, with distinct clinical and pathological features. Mucinous CRC is associated with a higher expression of MUC2 and MUC5AC, but a lower expression of MUC1 (51). Recently, it was shown that mucinous differentiation is associated with particular genetic and molecular features such as increased B-Raf proto-oncogene serine/threonine kinase (*BRAF*) mutation rate (52) and microsatellite instability (53) but the mechanisms responsible for this altered pattern of expression remain unclear. Regarding survival of patients with mucinous CRC, some authors suggests that a mucinous component may be associated with increased mortality (54, 55). Herein, we established that the average IRS for mucin-producing cancer was statistically significantly higher than in cases where mucin overproduction was not observed. Taking into consideration the fact that 9 out of 12 mucinous carcinomas enrolled in this study were accompanied by lymph node metastases, overexpression of MFI2 may determine an invasive tumor phenotype. However, the main limitation of this study is the small number of mucinous carcinomas involved, therefore investigations should be extended to a larger cohort of samples.

A candidate cancer-specific biomarker should be a secreted protein produced by the tumor with capability of entering the circulation. MFI2 is mainly expressed at the cell membrane, so it can be easily secreted into the extracellular matrix and then may penetrate into the blood vessels. Therefore, MFI2 indeed has the characteristics required to be a blood marker of cancer. Brown *et al*. reported a very low level of soluble melanotransferrin in normal blood (23). Soluble melanotransferrin was also immunodetected in urine and saliva (17): the amount was higher in urine (25-fold) and in saliva (10-fold) than in serum. The only study concerning MFI2 in relation to CRC tumorigenesis was a report by Shin *et al*., where they demonstrated overexpression of MFI2 in plasma of 228 patients with CRC by LC-MS/MS and ELISA (56). In our study, the plasma level of MFI2 was 1.9-times higher in patients with CRC compared to healthy controls. In our investigation, MFI2 was also detected in serum obtained from patients with cancer and in the control group and, as in the cited study, the concentrations of MFI2 in patients with CRC patients were significantly increased in comparison with healthy controls.

Our results indicate that MFI2 is aberrantly expressed in colorectal adenomas and CRCs and may have value as a marker for early detection of CRC. Regarding the relationship between MFI2 overexpression and certain clinicopathological characteristics, including lymph node involvement, presence of mucin and tumor grade, we propose this marker be considered as an indicator of aggressive and invasive tumor phenotype. However, further studies should be performed to investigate the molecular mechanism underlying the involvement of MFI2 in CRC development and progression.

Acknowledgements

The Authors would like to thank Małgorzata Kubicka for technical assistance. This work was supported by the Polish National Center of Science (DEC-2011/01/N/NZ5/04253) and Max-Planck Society for the Advancement of Science.

References

1 Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D and Bray F: Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. Eur J Cancer *49*: 1374-1403, 2013.

2 Leslie A, Carey FA, Pratt NR and Steele RJC: The colorectal adenoma-carcinoma sequence. British Journal of Surgery *89*: 845-860, 2002.

3 Coleman HG, Loughrey M, Murray LJ, Johnston BT, Gavin A, Shrubsole MJ, Bhat S, Allen PB, McConnell V and Cantwell MM. Colorectal cancer risk following adenoma removal: a large prospective population-based cohort study. Cancer Epidemiol Biomarkers Prev *24(9)*: 1373-1380, 2015.

4 Zauber AG, Winawer SJ, O'Brien MJ, Lansdorp-Vogelaar I, van Ballegooijen M, Hankey BF, Shi W, Bond JH, Schapiro M, Panish JF, Stewart ET and Waye JD: Colonoscopic polypectomy and long-term prevention of colorectal cancer deaths. Engl J Med *366(8)*: 687-696, 2012.

5 Simunic M, Perkovic N, Rosic-Despalatovic B, Tonkic A, Ardalic Z, Titlic M and Maras-Simunic M: Colonoscopic Polypectomies and Recommendations on the Colonoscopy Follow-up Intervals Depending on Endoscopic and Histopathological Findings. Acta Inform Med *21(3)*: 166-169, 2013.

6 Patel SG and Ahnen DJ: Prevention of interval colorectal cancers: what every clinician needs to know. Clin Gastroenterol Hepatol *12(1)*: 7-15, 2014.

7 Gatta G, Ciccolallo L, Capocaccia R, Coleman MP, Hakulinen T, Møller H and Berrino F: Differences in colorectal cancer survival between European and US populations: the importance of sub-site and morphology. Eur. J Cancer *39(15)*: 2214-2222, 2003.

8 Siegel R, Naishadham D and Jemal A: Cancer statistics, 2012. CA Cancer J Clin *62*: 10-29, 2012.

9 Hermsen M, Postma C, Baak J, Weiss M, Rapallo A, Sciutto A, Roemen G, Arends JW, Williams R, Giaretti W, De Goeij A and Meijer G: Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. Gastroenterology *123*: 1109-1119, 2002.

10 Nakao K, Mehta KR, Fridlyand J, Moore DH, Jain AN, Lafuente A, Wiencke JW, Terdiman JP and Waldman FM: High-resolution analysis of DNA copy number alterations in colorectal cancer by array-based comparative genomic hybridization. Carcinogenesis *25*: 1345-1357, 2004.

11 Wiśniewski JR, Ostasiewicz P, Duś K, Zielińska DF, Gnad F and Mann M: Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma. Mol Syst Biol *8*: 611, 2012.

12 Wiśniewski JR, Duś K and Mann M: Proteomic workflow for analysis of archival formalin-fixed and paraffin-embedded clinical samples to a depth of 10,000 proteins. Proteomics Clin Appl *7(3-4)*: 225-233, 2013.

13 Wiśniewki JR, Duś-Szachniewicz K, Ostasiewicz P, Rakus D, Ziółkowski P and Mann M. Absolute proteome analysis of colorectal mucosa, adenoma and cancer reveals drastic changes in fatty acid metabolism and plasma membrane transporters. J Proteome Res *14(9)*: 4005-4018, 2015.

14 Rose TM, Plowman GD, Teplow DB, Dreyer WJ, Hellstrom KE and Brown JP: Primary structure of the human melanoma-associated antigen p97 (melanotransferrin) deduced from the mRNA sequence. Proc Natl Acad Sci USA *83*: 1261-1265, 1986.

15 Rahmanto SY, Bal S, Loh KH, Yu Y and Richardson DR: Melanotransferrin: search for a function. Biochim Biophys Acta *1820(3)*: 237-243, 2012.

16 Richardson DR and Baker E: The uptake of iron and transferrin by the human malignant melanoma cell. Biochim Biophys Acta, *1053*: 1-12, 1990.

17 Richardson DR: The role of the membrane-bound tumour antigen, melanotransferrin (p97), in iron uptake by the human malignant melanoma cell. Eur. J Biochem *267*: 1290-1298, 2000.

18 Sala R, Jefferies WA, Walker B, Yang J, Tiong J, Law SK, Carlevaro MF, Di Marco E, Vacca A, Cancedda R, Cancedda FD and Ribatti D: The human melanoma associated protein melanotransferrin promotes endothelial cell migration and angiogenesis *in vivo*. EUR J Cell Biol *81*: 599-607, 2002.

19 Dunn LL, Sekyere EO, Rahmanto YS and Richardson DR: The function of melanotransferrin: a role in melanoma cell proliferation and tumorigenesis. Carcinogenesis *27*: 2157-2169, 2006.

20 Rahmanto SY, Dunn LL and Richardson DR: Identification of distinct changes in gene expression after modulation of melanoma tumor antigen p97 (melanotransferrin) in multiple models *in vitro* and *in vivo*. Carcinogenesis *28*: 2172-2183, 2007.

21 McNagny KM, Rossi F, Smith G and Graf T: The eosinophil-specific cell surface antigen, EOS47, is a chicken homologue of the oncofetal antigen melanotransferrin. Blood *87*: 1343-1352, 1996.

22 Suardita K, Fujimoto K, Oda R, Shimazu A, Miyazaki K, Kawamoto T and Kato Y: Effects of overexpression of membrane-bound transferrin-like protein (MTf) on chondrogenic differentiation *in vitro*. J Biol Chem *277*: 48579-48586, 2002.

23 Brown JP, Woodbury RG, Hart CE, Hellstrom I and Hellstrom KE: Quantitative analysis of melanoma-associated antigen p97 in normal and neoplastic tissues. Proc Natl Acad Sci USA *78*: 539-543, 1981.

24 Sobin L, Gospodarowicz M and Wittekind C: TNM Classification of Malignant Tumors. 7th edition. UICC International Union Against Cancer. New Jersey, Wiley-Blackwell, 2009.

25 Uzozie A, Nanni,P, Staiano T, Grossmann J, Barkow-Oesterreicher S, Shay JW, Tiwari A, Buffoli F, Laczko E and Marra G: Sorbitol dehydrogenase overexpression and other aspects of dysregulated protein expression in human precancerous colorectal neoplasms: a quantitative proteomics study. Mol Cell Proteomics *13*: 1198-1218, 2014.

26 Wiśniewski JR, Ostasiewicz P and Mann M: High recovery FASP applied to the proteomic analysis of microdissected formalin-fixed paraffin-embedded cancer tissues retrieves known colon cancer markers. J Proteome Res 10: 3040-3049, 2011.

27 Lee JG, McKinney KQ, Pavlopoulos AJ, Park JH and Hwang S: Identification of anti-metastatic drug and natural compound targets in isogenic colorectal cancer cells. J Proteomics 113: 326-336, 2015.

28 Karagiannis GS, Pavlou MP, Saraon P, Musrap N, Xie A, Batruch I, Prassas I, Dimitromanolakis A, Petraki C and Diamandis EP: In-depth proteomic delineation of the colorectal cancer exoproteome: Mechanistic insight and identification of potential biomarkers. J Proteomics *103*: 121-136, 2014.

29 Jimenez CR, Knol, JC, Meijer GA and Fijneman RJ: Proteomics of colorectal cancer: overview of discovery studies and identification of commonly identified cancer-associated proteins and candidate CRC serum markers. J Proteomics *73*: 1873-1895, 2010.

30 Van Houdt WJ, Emmink BL, Pham TV, Piersma SR, Verheem A, Vries RG, Fratantoni SA, Pronk A, Clevers H, Borel Rinkes IH, Jimenez CR and Kranenburg O: Comparative proteomics of colon cancer stem cells and differentiated tumor cells identifies birc6 as a potential therapeutic target. Mol Cell Proteomics *10(12)*: M111.011353, 2011.

31 Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B and Starling N: Colorectal cancer. Lancet *375(9719)*: 1030-1047, 2010.

32 Woodbury RG, Brown JP, Yeh MY, Hellstrom I and Hellstrom KE: Identification of a cell surface protein, p97, in human melanomas and certain other neoplasms. Proc Natl Acad Sci USA *77*: 2183-2187, 1980.

33 Sekyere EO, Dunn L and Richardson DR: Expression profile of the transferrin homologue, melanotransferrin (tumour antigen p97), in human and mouse tissues. Biochim Biophys Acta *1722*: 131-142, 2005.

34 Brown JP, Hewick RM, Hellström I, Hellström KE, Doolittle RF and Dreyer WJ: Human melanoma-associated antigen p97 is structurally and functionally related to transferrin. Nature *296(5853)*: 171-173, 1982.

35 Paladino S, Lebreton S and Zurzolo C: Trafficking and membrane organization of GPI-anchored proteins in health and diseases. Curr Top Membr *75*: 269-303, 2015.

36 Eisenhaber B, Bork P and Eisenhaber F: Post-translational GPI lipid anchor modification of proteins in kingdoms of life: analysis of protein sequence data from COMPLETE genomes. Protein Eng *14*: 17-25, 2001.

37 Zhao P, Nairn AV, Hester S, Moremen KW, O'Regan RM, Oprea G, Wells L, Pierce M and Abbott KL: Proteomic identification of glycosylphosphatidylinositol anchor-dependent membrane proteins elevated in breast carcinoma. J Biol Chem *287(30)*: 25230-25240, 2012.

38 Dolezal S, Hester S, Kirby PS, Nairn A, Pierce M and Abbott KL: Elevated levels of glycosylphosphatidylinositol (GPI) anchored proteins in plasma from human cancers detected by *C. septicum* alpha toxin. Cancer Biomark *14(1)*: 55-62, 2014.

39 Nagpal JK, Dasgupta S, Jadallah S, Chae YK, Ratovitski EA, Toubaji A, Netto GJ, Eagle T, Nissan A, Sidransky D and Trink B: Profiling the expression pattern of GPI transamidase complex subunits in human cancer. Mod Pathol *21(8)*: 979-991, 2008.

40 Rolland Y, Demeule M, Fenart L and Béliveau R: Inhibition of melanoma brain metastasis by targeting melanotransferrin at the cell surface. Pigment Cell Melanoma Res *22(1)*: 86-98, 2009.

41 Bufill JA: Colorectal cancer: evidence for distinct genetic categories based on proximal or distal tumor location. Ann Intern Med *113(10)*: 779-788, 1990.

42 Shen H, Yang J, Huang Q, Jiang MJ, Tan YN, Fu JF, Zhu LZ, Fang XF and Yuan Y: Different treatment strategies and molecular features between right-sided and left-sided colon cancers. World J Gastroenterol *21(21)*: 6470-6478, 2015.

43 Fuccio L, Spada C, Frazzoni L, Paggi S, Vitale G, Laterza L, Mussetto A, Rojas F, Radaelli F, Hassan C, Petruzziello L and Frazzoni M: Higher adenoma recurrence rate after left- *versus* right-sided colectomy for colon cancer. Gastrointest Endosc *82(2)*: 337-343, 2015.

44 Amri R, Bordeianou LG, Sylla P and Berger DL: Variations in metastasis site by primary location in colon cancer. J Gastrointest Surg *19(8)*: 1522-1527, 2015.

45 Bara J, Nardelli J, Gadenne C, Prade M and Burtin P: Differences in the expression of mucus-associated antigens between proximal and distal human colon adenocarcinomas. Br J Cancer *49*: 495-501, 1984.

46 Arai T and Kino I: Morphometrical and cell kinetic studies of normal human colorectal mucosa. Comparison between the proximal and the distal large intestine. Acta Pathol Jpn *39*: 725-730, 1989.

47 Cancer Genome Atlas Network: Comprehensive molecular characterization of human colon and rectal cancer. Nature *487*: 330-337, 2012.

48 Tamas K, Walenkamp AM, de Vries EG, van Vugt MA, Beets-Tan RG, van Etten B, de Groot DJ and Hospers GA: Rectal and colon cancer: Not just a different anatomic site. Cancer Treat Rev *41(8)*: 671-679, 2015.

49 MERCURY Study Group: Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: prospective observational study. BMJ *333*: 779, 2006.
50 Kopetz S, Overman MJ, Chen K, Lucio-Eterovic AK, Kee BK, Fogelman DR, Dasari A, Raghav KPS, Sanchez EV, Phillips J, Shureiqi I, Garrett CR, Wolff RA, Patel K, Aldape KD, Luthra R, Routbort M, Maru DM, Meric-Bernstam F and Eng C: Mutation and copy number discordance in primary *versus* metastatic colorectal cancer (mCRC). J Clin Oncol *32 (suppl 5)*: 3509, 2014.
51 Perez RO, Bresciani BH and Bresciani C: Mucinous colorectal adenocarcinoma: influence of mucin expression (MUC1, -2 and -5) on clinico-pathological features and prognosis. Int J Colorectal Dis *23(8)*: 757-765, 2008.
52 Chen D, Huang JF and Liu K: *BRAF*V600E mutation and its association with clinicopathological features of colorectal cancer: a systematic review and meta-analysis PLoS One *9(3)*: e90607, 2014.
53 Renaud F, Vincent A and Mariette C: *MUC5AC* hypomethylation is a predictor of microsatellite instability independently of clinical factors associated with colorectal cancer. Int J Cancer *136(12)*: 2811-2821, 2015.
54 Negri FV, Wotherspoon A, Cunningham D, Norman AR, Chong G and Ross PJ: Mucinous histology predicts for reduced fluorouracil responsiveness and survival in advanced colorectal cancer. Ann Oncol *16(8)*: 1305-1310, 2005.
55 Catalano V, Loupakis F, Graziano F, Torresi U, Bisonni R, Mari D, Fornaro L, Baldelli AM, Giordani P, Rossi D, Alessandroni P, Giustini L, Silva RR, Falcone A, D'Emidio S and Fedeli SL: Mucinous histology predicts for poor response rate and overall survival of patients with colorectal cancer and treated with first-line oxaliplatin- and/or irinotecan-based chemotherapy. Br J Cancer *100(6)*: 881-887, 2009.
56 Shin J, Kim HJ, Kim G, Song M, Woo SJ, Lee ST, Kim H and Lee C: Discovery of melanotransferrin as a serological marker of colorectal cancer by secretome analysis and quantitative proteomics. J Proteome Res *13(11)*: 4919-4931, 2014.

Received July 29, 2015
Revised October 22, 2015
Accepted October 23, 2015