

Altered Expression of MBNL Family of Alternative Splicing Factors in Colorectal Cancer

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Abstract. *Background/Aim:* Colorectal cancer is currently the third leading cause of cancer-related deaths and recently, alternative splicing has risen as its important regulator and potential treatment target. In the present study, we analyzed gene expression of the MBNL family of regulators of alternative splicing in various stages of colorectal cancer development, together with the MBNL-target splicing events in *FOXP1* and *EPB41L3* genes and tumor-related *CD44* variants. *Materials and Methods:* Samples of tumor tissue and non-malignant mucosa from 108 patients were collected. After RNA isolation and reverse transcription, the relative gene expression of a selected gene panel was tested by quantitative real-time PCR, followed by statistical analysis. *Results:* MBNL expression was decreased in tumor tissue compared to non-tumor mucosa. In addition, lower expression was observed for the variants of *FOXP1* and *EPB41L3*, while higher expression in tumor tissue was detected both for total *CD44* and its cancer-related variants 3 and 6. Transcript levels of the MBNL genes were not found to be related to any of the studied clinicopathological characteristics. Multiple significant associations were identified in the target gene panel, including higher transcript levels of *FOXP1* and *CD44v3* in patients with distant metastases and connections between recurrence-free survival

and altered levels of *FOXP1* and *CD44v3*. *Conclusion:* Our results identified for the first-time deregulation of MBNL genes in colorectal cancer. Down-regulation of their transcripts in tumor tissue compared to matched non-tumor mucosa can lead to transition of alternative splicing patterns towards a less differentiated phenotype, which highlights the importance of alternative splicing regulation for tumor growth and propagation.

Colorectal cancer (CRC) remains the third most commonly diagnosed malignancy in males and the second most commonly diagnosed cancer in females (1, 2). Globally, colorectal cancer is among the leading causes of cancer-related deaths (3, 4). Although the incidence of CRC has been steadily rising worldwide over the last few decades, especially in developing countries (3), the overall survival rate has increased as well with advances in targeted agents and cytotoxic chemotherapy (4, 5). Also, improvements in CRC screening result in disease detection at lower stages when it is easier to remove the tumor by surgery before metastatic spread occurs (6). In general, treatment choice is based on multiple factors, including clinical features (*e.g.* tumor location, stage at detection), demographic variables (*e.g.* sex, age at diagnosis, and familial risk), and characteristics of the tumor (*e.g.* microsatellite instability, somatic mutations) (7, 8).

In addition to common prognostic factors and therapeutic targets, evidence accumulated during the past decades has revealed novel markers. In particular, the importance of alternative splicing dysregulation during colorectal cancer development has been observed, opening new aspects of specific modifications of splicing regulatory proteins and pathologic splicing events in CRC (9, 10).

Alternative splicing (AS) is known as an important post-transcriptional process that enables formation of multiple

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distinct mRNAs from a single gene, which results in increased proteomic diversity with distinct variants having various structural and functional properties (11). AS represents a crucial regulatory mechanism of gene expression, cellular differentiation, and signal transduction (12), mediating different biological processes including erythropoiesis, neuronal differentiation or embryonic stem cell programming (13). Under normal conditions, AS is known as a versatile and powerful mechanism that is tightly regulated by splicing regulatory proteins. Regulation of AS is a complicated process and its misregulation drives production of aberrant protein isoforms, which may lead to cancer pathogenesis, muscular dystrophies or neurological diseases (14).

Splicing regulation is generally directed by regulatory sequences, which are cis-acting, and RNA-binding proteins (RBPs), which are trans-acting. The interactions of regulatory sequences and RBPs determine the resulting relative synthesis rate of protein isoforms. The most common mechanisms of alternative splicing misregulation originate from alterations in the levels and activity of RBPs (14). Multiple families as well as individual RBPs exist, some with ubiquitous expression and some highly restricted to particular tissue types or developmental stages. For instance, the heterogeneous nuclear ribonucleoproteins (hnRNPs), RBFOX2 or MATR3, are known as part of a larger RBP complex and are examples of ubiquitous splicing regulators (15). Examples of more specific RBPs include CELF (CUG-BP and ETR-3-like factors) and muscleblind-like (MBNL) families. The latter is the major focus of the presented study and consists of three genes, namely MBNL1, MBNL2 and MBNL3. Besides the primary MBNL function of AS regulators during mRNA maturation (16), they can also be involved in regulation of mRNA subcellular localization (17), mRNA stability control and alternative polyadenylation (18, 19).

The MBNL proteins are key regulators of precursor mRNA as they influence both alternative splicing (AS) and overall mRNA stability in mammals. During embryonic development, increased concentrations of MBNL1 and 2 lead to differentiation of embryonic stem cells by promoting a shift from embryonic to fetal and then to adult splice patterns of target mRNAs (20). Although all three paralogs of MBNL share homologous structures, including four zinc finger (ZnF) domains essential for recognizing the same consensus sequence in pre-mRNA and mRNA targets, they are significantly different with respect to their functional specializations and distribution of expression patterns during embryonic and postnatal development (20). Both MBNL1 and 2 are expressed in a diverse range of adult tissues. MBNL1 appears to be the predominantly expressed member of the MBNL family, it promotes muscle differentiation and in most tissues it facilitates transitions from embryonic to fetal and from fetal to adult splicing patterns (21, 22). A significant exception to this rule is brain tissue where

MBNL2 is predominantly expressed (20). MBNL3 is mostly present at early stages of development, and later in the placenta, spleen and lung. In addition, MBNL3 appears to function in an opposing manner to MBNL1 and 2, as it has been reported to inhibit muscle differentiation (23). In pathophysiology, MBNLs' functional insufficiency results in mis-regulation of alternative splicing, alters target genes' isoform ratios and can cause cancers, muscular dystrophies, and neurological diseases (24). A majority of studies focus on pathologies associated with aberrant MBNL1 function while the roles of MBNL2 and MBNL3 have been less investigated (16, 21). The best-described disease related to the MBNL protein family dysregulation is myotonic dystrophy (20, 25). Deregulation of MBNL activity can also influence alternative splicing of factors controlling pluripotency and reprogramming, such as FOXP1 and CD44, which have been linked to cancer (26, 27). Recently, MBNL1 splicing deregulation in colorectal cancer was linked to colorectal cancer cells' resistance to oxidative stress, showing importance of MBNL1 regulated alternative splicing for survival of cancer cells (28).

In the present study, we address the question of whether alterations in the MBNL family expression can be linked to pathological and clinical behavior of colorectal cancer and whether they can be used as markers of prognosis and prediction of treatment efficiency. In addition to MBNL genes themselves, we analyzed a number of alternatively spliced isoforms of EPB41L3, FOXP1 and CD44 genes that were previously confirmed to be regulated by MBNL or are known for their abundant alternative splicing (25, 26).

Materials and Methods

Selection of genes studied. In the present study, we analyzed the transcript levels of three MBNL paralogs in colorectal cancer and selected variants of Forkhead Box P1 (FOXP1), Erythrocyte Membrane Protein Band 4.1 Like 3 (EPB41L3) and CD44 transcript variants 3 and 6. To test the effect of MBNL activity on the alternative splicing of the target genes, we selected FOXP1 and EPB41L3 exons reported to be regulated by MBNL according to the study by Hong Han *et al.* (26). CD44 transcript variant 3 and CD44 transcript variant 6 were selected as particular isoforms of CD44 related to cancer diseases (29). In addition to serving as a potential marker for MBNL functional status, analysis of FOXP1, EPB41L3 and CD44 alternative splicing could bear important information given their own oncogenic potential.

Selection of reference genes – hypoxanthine phosphoribosyltransferase1 (HPRT1), Histidyl-TRNA Synthetase (HARS) and actin beta (ACTB) was based on existing literature data (30, 31) and our own experience.

Primer design. Quantitative real-time polymerase chain reaction (PCR) primers for the selected genes were designed using Primer3 software (32, 33) with special emphasis on exon/exon-junction-spanning assays. In case of special splicing variants, primers were designed to align into the exon of interest, common assays were

Table I. List of primers used and their characteristics. Primers designated as var are specific for particular splice variants known to be regulated by MBNL; primers labeled as com are used to amplify other mRNA splice isoforms except the specific MBNL-regulated variant.

Gene name	Primer type	Primer sequence [5'-3']	Amplicon size [nt]	GC content [%]	Predicted melting temperature [°C]
ACTB	Forward	CCAACCGCGAGAAGATGA	97	56	60
	Reverse	CCAGAGGCGTACAGGGATAG		60	59
HPRT1	Forward	TGACCTTGATTTATTTGCATACC	102	33	59
	Reverse	CGAGCAAGACGTTTCAGTCCT		55	60
HARS	Forward	ATTGGGGTGGAGCGGATTTT	78	0.5	60
	Reverse	TGTCTCCGTGGTCCGTATCT		0.55	60
MBNL1	Forward	ATCGCCTGCTTTGATTCATT	76	40	59
	Reverse	TTAAATGTGGGGGTGGATGA		45	60
MBNL2	Forward	GCCCAGCAGATGCAATTTAT	137	45	60
	Reverse	AACCCAACCTCCAGGGGTAC		55	60
MBNL3	Forward	ATTCTGGAACCCACCTCT	89	50	59
	Reverse	TGAAATTCTCGGCAAACTC		45	60
EPB41L3com	Forward	AAAGAGGCCAAAGAGCAGCA	115	50	60
	Reverse	GCAAGCTAAGTTATTCCTCTGGTC		46	59
EPB41L3var	Forward	ACCATGACCAGGAATAACTTAGCT	101	42	60
	Reverse	AGTCAGTTGGGTTAGAAGAGGG		50	59
FOXP1com	Forward	CACGTGGAAGAATGCAGTGC	88	55	60
	Reverse	CACTGTCCATACTGCCCTT		55	59
FOXP1var	Forward	ACGTGGAAGGGTGCCATTC	114	58	60
	Reverse	GCGGCCACGTTTAACTCTT		50	59
CD44	Forward	CCCAGATGGAGAAAGCTCTG	113	55	60
	Reverse	GTTGTTTGCTGCACAGATGG		50	60
CD44v3	Forward	TCCCTGCTACCAATATGGACTC	122	50	60
	Reverse	ACTCTGCTGCGTTGTCATTG		50	60
CD44v6	Forward	AGGAACAGTGGTTTGGCAAC	68	50	60
	Reverse	CGAATGGGAGTCTTCTTTGG		50	60

designed on exons typical for all other variants included in the NCBI RefSeq database at the time of primer design (10/2016). Designed primers were aligned to a non-redundant mRNA database using BLAST (34) to check for putative amplification of off-target sequences and to validate priming of desired variants. Primer sequences used in the study are summarized in Table I.

Selection of patients and collection of tissue samples. In total, 108 patients who underwent surgery for colorectal cancer at the Department of Surgery of University Hospital in Pilsen between years 2008 to 2016 were included in this retrospective study. All enrolled patients agreed to the processing of the samples by signing informed consent. The study was approved by the Ethical committee of the Faculty of Medicine and University Hospital in Pilsen and complied

with the International Ethical Guidelines for Biomedical Research Involving Human Subjects. Clinical data were collected on 01/2020 by clinicians and anonymized before transfer for statistical analysis.

Tissue collection was performed within 20 min after the removal of the tissue from the patient for both tumor and non-tumor mucosa (anatomically the most distant tissue that was macroscopically healthy, in the range of 15-20 cm from the tumor location). Tissue samples with maximal size of 5 mm in the longest axis were gathered, immediately frozen in cryotubes (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80°C (35).

Isolation of RNA and quality validation. A standard protocol for RNA isolation using TriReagent RT (MRC, USA) was used to isolate RNA from frozen samples. The tissue was removed from the cryotube and

placed in liquid nitrogen in a mortar. The tissue was pulverized and transferred into an Eppendorf tube with 1ml TriReagent RT chilled to 4°C and total RNA was isolated according to the manufacturer’s protocol. Total isolated RNA was dissolved in nuclease-free water (Ambion, Carlsbad, CA, USA). RNA concentration was assessed by absorbance measurement using the Infinite M200 (Tecan, Männedorf, Switzerland) in the NanoQuant setting. RNA quality was assessed by agarose gel electrophoresis. Only samples with a 230 nm/260 nm ratio >1.7 and samples with proper bands without degradation on agarose gel electrophoresis were used for further analysis.

Reverse transcription (DNase, PCR control). cDNA was synthesized according to the manufacturer’s protocol, using 500 ng of total RNA in 20 µl reaction by RevertAid First Strand cDNA Synthesis Kit (THERMO SCIENTIFIC, Waltham, MA, USA). For priming of reverse transcription reaction, a combination of oligo(dT)18 and random hexamer primers, each at 2.5 µM final concentration, was used. The quality of cDNA and possible contamination by genomic DNA was assessed by a control PCR reaction (GAPDH amplification, 40 cycles) and agarose gel electrophoresis.

Quantitative real-time PCR. 5x HOT FIREPol® EvaGreen® qPCR SuperMix (Solis BioDyne, Tartu, Estonia) was used for the quantitative PCR. cDNA was diluted 100x and 2 µl of cDNA were used in each reaction, having a final volume of 10 µl. Optimal cycling parameters and annealing temperatures were assessed by the measurement of sensitivity, specificity and efficiency of individual quantitative PCR reactions. CFX 96 quantitative real-time PCR cyclers (Biorad, CA, USA) was used for the analysis.

Cycling parameters were: initial hold at 50°C for 20 s and initial denaturation at 95°C for 12 min followed by 45 cycles consisting of denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s. Results were analyzed and basic statistical analysis was carried out using the Bio-Rad CFX Manager software.

Statistical analysis. Standard frequency tables and descriptive statistics were used to characterize the patient group. Relative transcript levels of measured genes in the tumor tissue (relative to adjacent non-tumor tissue) were expressed by $-\Delta\Delta Ct$ values according to Livak *et al.* (36), with mean Ct of the above-listed reference genes used as internal control. Because of their mostly normal distribution (as reviewed in histograms and using Shapiro-Wilk test), data were analyzed using mainly parametric methods. One sample *t*-test against zero reference was used to test for significant up- or down-regulation.

In order to directly explore the relationship between MBNL expression and the levels of their target gene variant transcripts, $-\Delta Ct$ values (*i.e.* differences between mean reference gene Ct and MBNL Ct) of MBNL 1-3 were tested for correlation (using Kendall’s tau) with the difference between the Ct values of FOXP1com and FOXP1var, and also with the Ct difference between EPB41L3com and EPB41L3var. The differences between the “com” and “var” Ct values represent the ratio of the variant transcript levels and the fact that they do not involve reference genes prevents the occurrence of potential false correlations caused by measurement noise in the reference genes’ Ct values. This analysis was independently performed in tumor tissue and non-tumorous mucosa.

Associations between the transcript levels and clinical characteristics of the patients (T – tumor stage, N – lymph node invasion, M – metastases, G – histological grade, UICC – classification

Table II. *Characteristics of the analyzed colorectal cancer patients.*

Characteristic	Category	Number	%
Gender	F	44	40.7
	M	64	59.3
Age (in years)	30-50	7	6.5
	50-70	57	52.8
	>70	44	40.7
T stage	T1	3	2.8
	T2	23	21.3
	T3	66	61.1
	T4	14	13.0
	Unknown	2	1.9
	N	N0	62
N1		21	19.4
N2		21	19.4
N3		1	0.9
Unknown		3	2.8
M	M0	79	73.1
	M1	26	24.1
	Unknown	3	2.8
Histological grade	G1	13	12.0
	G2	74	68.5
	G3	14	13.0
	Unknown	7	6.5
UICC staging	I	23	21.3
	II	33	30.6
	III	27	25.0
	IV	24	22.2
	Unknown	1	0.9
Tumor location	Right or transverse colon	41	38.0
	Left or sigmoid colon	30	27.8
	Rectum or rectosigmoid junction	36	33.3
	Non-specific	1	0.9

F: Female; M: male; tumor classification based on TNM classification on malignant tumors with following categories: T stage: size of the primary tumor; N: degree of tumor spread in lymph nodes; M: presence of distant metastasis; G: histological tumor grade; UICC staging: tumor classification according to Union for International Cancer Control.

according to Union for International Cancer Control) were analyzed by two-sample t-test (difference between two groups), one-way ANOVA (testing for non-uniform means in multiple groups) and Kendall’s tau (testing for increasing/decreasing trends across multiple ordinal categories). Recurrence-free survival (RFS) was determined from the date of surgery until the date of first documented disease recurrence or death. Overall survival (OS) was determined from the date of surgery until the date of death, regardless of its cause. Patients who had not progressed or died were censored at the date of the last follow-up. Significance of associations between transcripts and survival times was assessed using univariable Cox proportional hazards model (except for EPB41L3var, which was removed from survival analysis due to a small subset of patients with EPB41L3var measurements and survival data available). Prerequisites of the Cox model were checked by testing the scaled Schoenfeld residuals for correlation with observation time (which should not occur under the proportionality assumption), and by visually assessing the Martingale residuals (in relation to the covariate value) and the distribution of

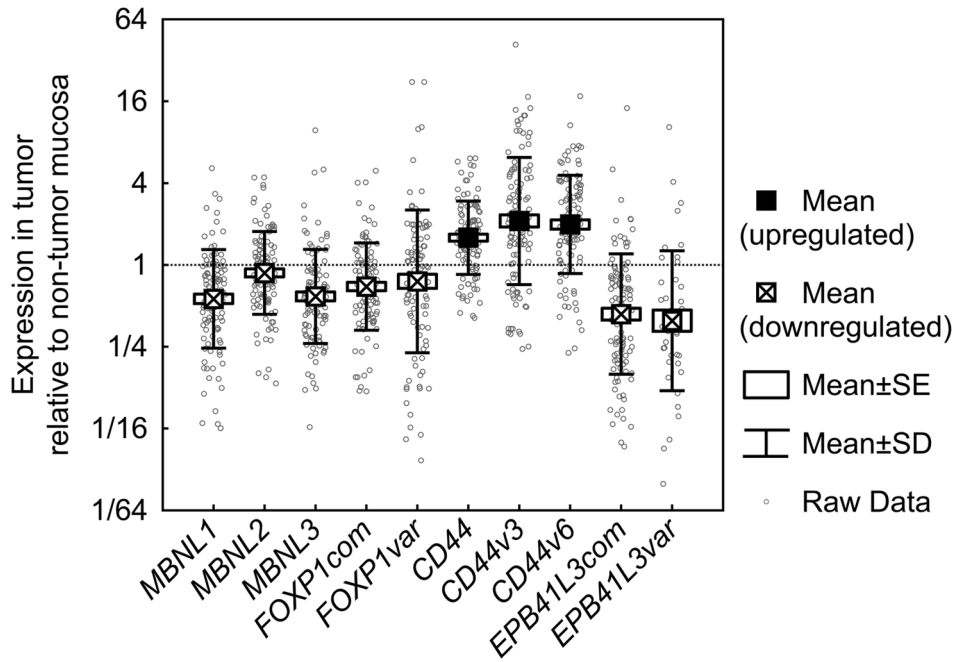


Figure 1. Expression ratio of candidate genes in tumor vs healthy tissue. All genes show significant deregulation in tumor tissue.

deviance residuals in order to confirm linearity and absence of overly influential observations, respectively. Discovered prognostic genes were subsequently tested for independence of other clinical factors using the multivariable Cox proportional hazards model.

In order to visualize the expression-survival associations and to detect possible non-proportional ones, significant or borderline results were reviewed using automated stratification. In this procedure, a threshold (cut-off) expression ($-\Delta\Delta Ct$) value was independently determined for OS and RFS for each gene by an automated optimization process finding the threshold providing the lowest Cox-Mantel p -value in two-sample Kaplan-Meier survival analysis, which was finally verified using the Gehan-Wilcoxon test. This was done using a custom-written script in Matlab enumerating all possible stratifications (with a minimum group size empirically set to 20% of the whole cohort) according to the variable of interest.

All reported p -values are two-tailed, and the level of statistical significance was set at $\alpha = 0.05$. False discovery rate (FDR) was controlled using the Benjamini-Hochberg procedure carried out upon the results of all significance test performed within the study. At the baseline significance level of 0.05, the estimated FDR is 10%, indicating 90% of the presented significant results to be true positives. A conservative overall FDR of 5% would require the individual significance level of 0.022. Statistical processing and testing were performed in STATISTICA data analysis software system (StatSoft, Inc. 2013, Version 12, www.statsoft.com) and Matlab (2019b, MathWorks Inc., Natick, MA, USA).

Results

In the current study, we analyzed the expression of 3 MBNL paralogs and variants of 3 target genes with transcription-

variant-specific primers on a sample set of 108 patients. Descriptive statistics of the enrolled patients are summarized in Table II.

Focusing on the MBNL family, we observed a decrease in the level of all MBNL genes in tumor tissue compared to paired non-tumor samples (MBNL1 $p < 0.001$, MBNL2 $p = 0.047$, MBNL3 $p < 0.001$). It represents the first concise evidence that MBNL genes are deregulated in colorectal cancer. Despite the deregulation on the transcript level, no correlation between MBNL1, 2 and 3 transcripts and clinical parameters was observed in our patient set.

Deregulation of transcripts was detected also in the target gene panel, with CD44 ($p < 0.001$), CD44v3 ($p < 0.001$) and CD44v6 ($p < 0.001$) showing higher levels in tumor tissue, and FOXP1com ($p < 0.001$), FOXP1var ($p = 0.024$), EPB41L3com ($p < 0.001$) and EPB41L3var ($p < 0.001$) being up-regulated in non-tumor samples (Figure 1).

Subsequent correlation analysis between MBNL expression and the target gene variant transcript ratios showed higher expression of MBNL1 and MBNL3 to be significantly associated with increased variant transcript levels of FOXP1var (MBNL3 in tumor tissue $p = 0.046$; MBNL1 in non-tumor mucosa $p = 0.024$; MBNL3 in non-tumorous mucosa $p = 0.011$). Similarly, MBNL3 overexpression was linked to increased variant transcript ratio of EPB41L3var in non-tumorous mucosa ($p = 0.002$). Contrastingly to that, higher expression of MBNL2 in the tumor was linked to lower transcript levels of EPB41L3var ($p = 0.011$).

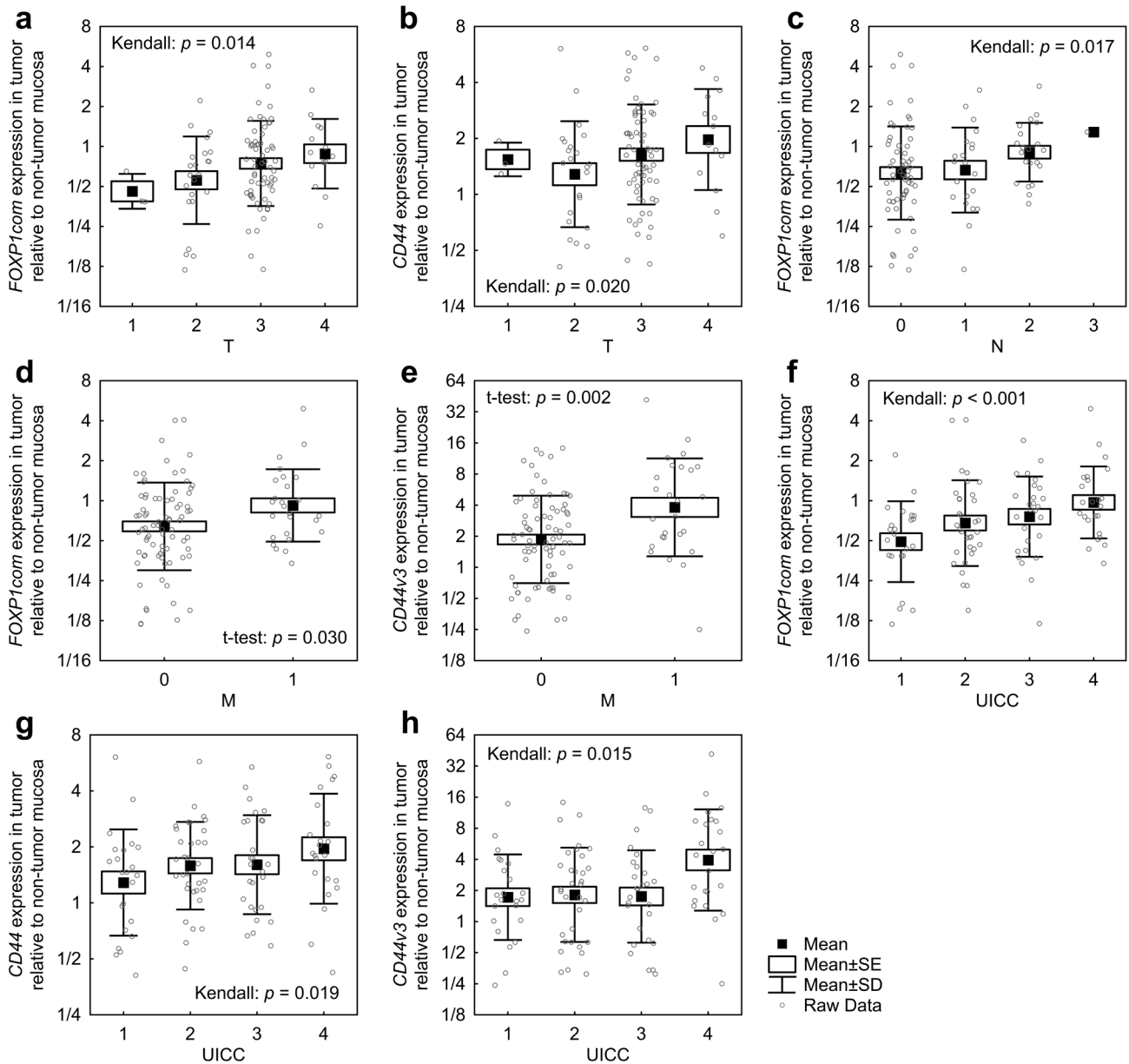


Figure 2. Expression of candidate genes according to T, N, M, G, and UICCC in tumor tissue relative to healthy tissue. (a) distribution of FOXP1com expression change according to T stage; (b) distribution of CD44 expression change according to T stage; (c) distribution of FOXP1com expression change according to N; (d) distribution of FOXP1com expression change according to M; (e) distribution of CD44v3 expression change according to M; (f) distribution of FOXP1com expression change according to UICC score; (g) distribution of CD44 expression change according to UICC score; (h) distribution of CD44v3 expression change according to UICC score.

Next, we analyzed the associations of the expression of target genes with clinical parameters. The analysis detected increasing trends for FOXP1com with both T and N advancement (T staging: Kendall correlation: $\tau=0.16$, $p=0.014$; N: Kendall correlation: $\tau=0.16$, $p=0.017$) and also for CD44 with T-stage advancement (Kendall correlation: $\tau=0.15$, $p=0.020$). Furthermore, the comparison of

expression between groups with and without distant metastasis at the time of surgery revealed significantly higher levels of FOXP1com ($p=0.030$) and CD44v3 ($p=0.020$) expression in the metastasis group. The overall UICC staging score was significantly correlated with expression of FOXP1com (Kendall correlation: $\tau=0.23$, $p<0.001$), CD44 (Kendall correlation: $\tau=0.15$, $p=0.019$) and CD44v3

Table III. Results of the univariable Cox proportional hazard models.

Predictor variable	OS			RFS		
	<i>p</i> -Value	HR ¹	prop. test ²	<i>p</i> -Value	HR ¹	prop. test ²
MBNL1	0.315	1.184	0.155	0.227	1.190	0.398
MBNL2	0.924	1.019	0.040	0.926	0.985	0.073
MBNL3	0.928	0.984	0.377	0.553	0.911	0.731
FOXP1com	0.002	1.732	0.514	0.070	1.309	0.977
FOXP1var	<0.001	1.503	0.298	0.030	1.262	0.902
CD44	0.225	1.320	0.918	0.268	1.236	0.608
CD44v3	0.023	1.358	0.647	0.016	1.333	0.864
CD44v6	0.097	1.356	0.335	0.449	1.114	0.236
EPB41L3com	0.583	1.080	0.778	0.281	1.124	0.440

¹HR: Hazard ratio; relative change of risk per each unit of $-\Delta\Delta\text{Ct}$ value increase; ²proportionality test; *p*-values of the Spearman correlation analysis between the scaled Schoenfeld residuals and observation times. Significant values (in bold) indicate that the proportionality assumption of the Cox model might not have been met.

(Kendall correlation: $\tau=0.16$, $p=0.015$). When considering tumor grading, we observed significantly non-uniform expression of CD44v6 across different grades (G1-G3) (one-way ANOVA: $F=3.90$, $p=0.024$) (Figure 2).

Using a univariable Cox proportional hazards model, proportional associations of the measured expression levels with overall survival were found for FOXPcom ($p=0.002$) with a hazard rate (HR) of 1.73 per unit of $-\Delta\Delta\text{Ct}$ value (*i.e.* each doubling of the expression ratio between the tumor tissue and macroscopically healthy mucosa increases the risk of death 1.73-fold), FOXP1var ($p<0.001$, $\text{HR}=1.50$), and CD44v3 ($p=0.023$, $\text{HR}=1.36$) (Table III and Figure 3). For FOXP1com and FOXP1var, the association with OS was proven to be independent of other clinical factors by the multivariable Cox model (Table IV). Recurrence-free survival was significantly influenced by FOXP1var ($p=0.030$, $\text{HR}=1.26$) and CD44v3 ($p=0.016$, $\text{HR}=1.33$), both of which showed independent prognostic ability in the multivariable setting (Table V). Automated determination of threshold values providing the best separation of groups confirmed these results (Figure 4).

Discussion

In the current study, we report significant deregulation of transcripts of the MBNL gene family in colorectal carcinoma. The MBNL proteins (MBNL1, MBNL2, MBNL3) are important regulators of alternative splicing and their increased activity promotes a switch from embryonic to adult AS pattern, represses pluripotency network and promotes differentiation. It is known that the expression profile of three MBNL paralogs and their correlated effect with the set of transcription factors might alter multiple splicing events (26, 37). Our data show a decrease of MBNL

expression in tumor tissue compared to matched non-tumorous intestinal mucosa, and also simultaneous changes in the expression of splice-variants that are known to be regulated by MBNL (24, 38). Also, our results show a weakly significant, yet consistent relationship between the expression of MBNL1 and 3, and the levels of their target variant transcripts of FOXP1 and EPB41L3. Marginally significant associations were observed between the expression of the studied genes and T, N, M, G and UICC clinical factors, with minute differences in gene expression among individual groups.

The observed down-regulation of transcripts of all three genes of the MBNL family in colorectal cancer tissue may promote a less differentiated phenotype of the malignant cells. Previous studies proposed a role of the MBNL proteins in the genesis of various tumor types. It has been shown that MBNL1 inhibits invasiveness and migratory capacity of cancer cells (39), including metastatic potential of colorectal cells *in vitro* (40). Down-regulation of MBNL3 was observed to be a crucial step in leukaemia stem cell renewal (27). On the other hand, MBNL2 is believed to be a driver of hypoxia adaptation, which is necessary for proliferation and migration of cancer cells in lung and breast cancer, and MBNL2 is thus considered a pro-oncogene in these cancer types (41). The function of the individual MBNL family members may therefore be very different and they can regulate particular alternative splicing patterns depending on the disease type and cellular context. In addition, the function of MBNL can be regulated by their own alternative splicing, as was described for MBNL exon 7 in the case of prostate cancer (42). Although we detected down-regulation of all members of the MBNL family, which is in line with their supposed tumor suppressor role, we detected neither an association of MBNL expression and T, N, M, G and UICC clinical factors in our

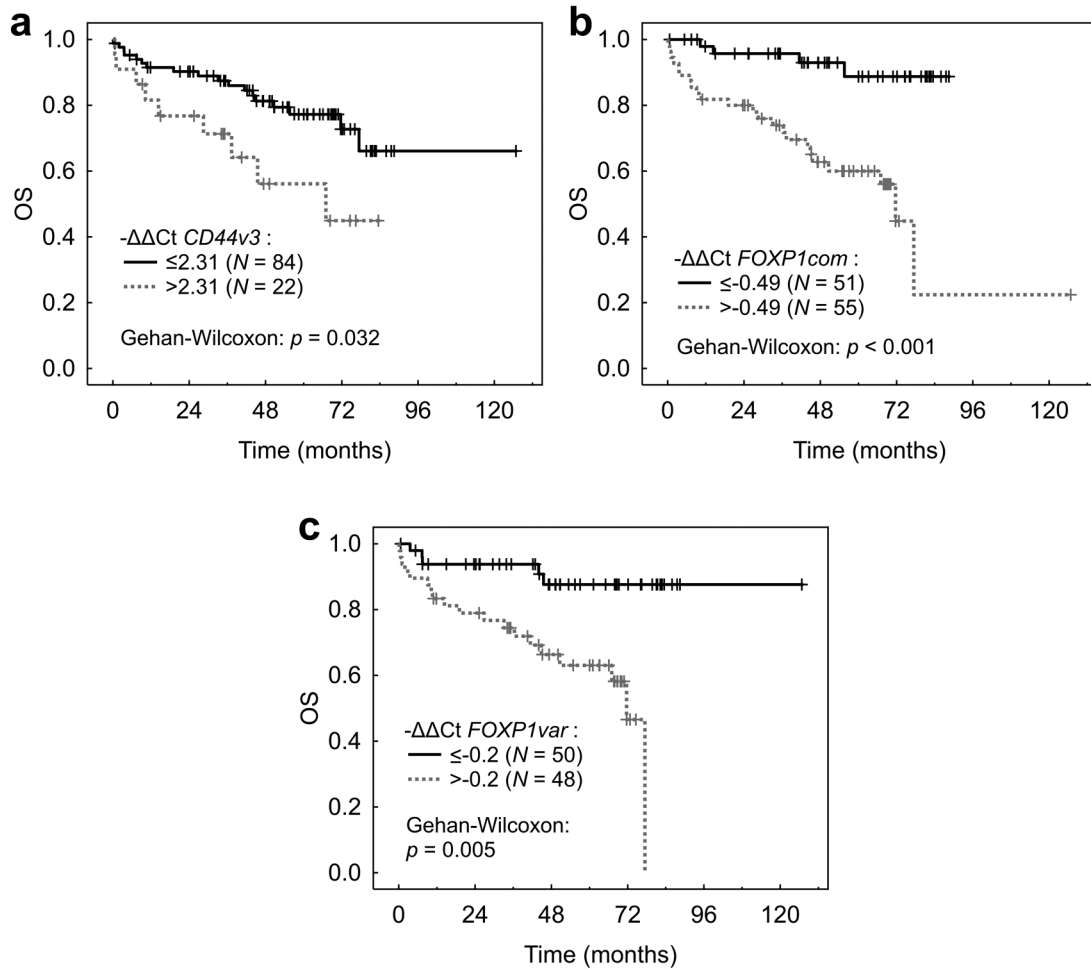


Figure 3. Kaplan-Meier overall survival curves based on optimized gene expression level groups; (a) *CD44v3* (b) *FOXP1com* (c) *FOXP1var*.

data, nor any significant influence on patient survival. This indicates MBNL down-regulation as a significant marker of colorectal malignancy independent of disease progression, without direct manifestation of their down-regulation at the level of clinicopathological parameters.

We further examined expressions of additional genes, whose alternative splicing was shown to be under the MBNL proteins' regulation, and with implied role in cancer pathophysiology. The MBNL activity influences splicing of *FOXP1* by promoting exclusion of exons that activate pluripotency genes and correspond to a stem cell-like phenotype (26, 43). Down-regulation of MBNL might thus contribute to pluripotency network engagement in cancer cells through *FOXP1*. Overall, the results of previous studies on the role of *FOXP1* in cancer are controversial, suggesting both roles – as an oncogene as well as tumor suppressor – depending on the tumor type. In our data, we observed

significantly lower expression of *FOXP1* in tumor cells, supporting previous findings in colorectal cancer (43, 44). Unexpectedly, *FOXP1* expression was positively associated with disease progression in terms of tumor invasion, node involvement and presence of distant metastasis. Moreover, higher expression of *FOXP1* was independently associated with shortened overall survival. These results seem to be in contrast with a previous study, where absence of *FOXP1* was associated with decreased survival (43). Our results support the view of a more complex pattern of the *FOXP1* factor involvement, possibly dependent on disease stage and on expression of particular isoforms. Under the condition of MBNL down-regulation, transcript levels of *FOXP1* might correlate with a less differentiated cell phenotype.

Cancer tissue cells also displayed enhanced expression of *CD44* and its alternative splice variants *CD44v3* and *CD44v6*. *CD44* functions as an adhesion molecule in cell-cell and cell-

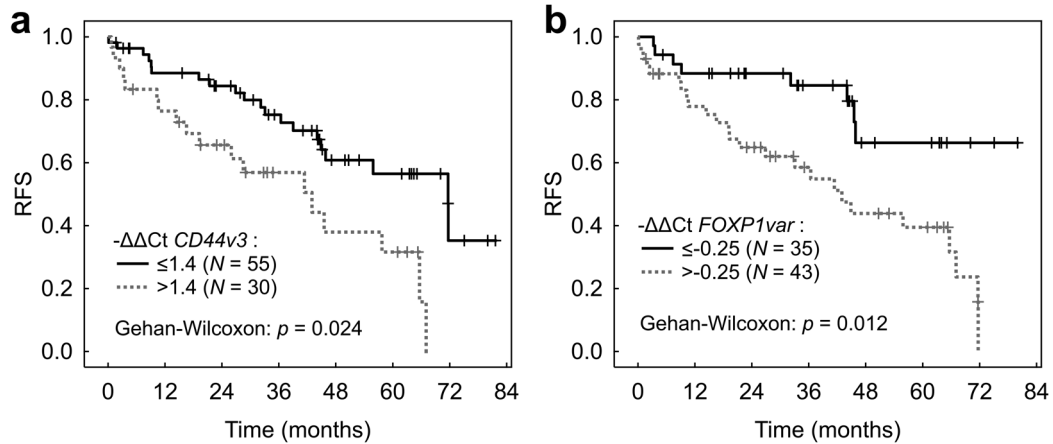


Figure 4. Kaplan-Meier recurrence free survival curves based on optimized gene expression level groups; a) CD44v3 and b) FOXP1var.

Table IV. Results of the multivariable Cox proportional hazard model for OS.

Predictor variable	Category	Relative risk (95% CI)	p-Value
Gender	Female	1	0.100
	Male	2.271 (0.855-6.028)	
Age	<70	1	0.013
	≥70	3.781 (1.329-10.757)	
T	T1 or T2	1	0.151
	T3 or T4	2.707 (0.695-10.541)	
N	N0	1	0.132
	N1 or higher	2.035 (0.807-5.133)	
M	0	1	0.338
	1	28.072 (0.031-25 740)	
Surgical radicality	R0	1	0.711
	P	0.276 (0-250.506)	
Chemo/radiotherapy	Yes	1	0.687
	No	1.236 (0.441-3.465)	
-ΔΔCt CD44v3	≤2.31	1	0.083
	>2.31	2.531 (0.887-7.226)	
-ΔΔCt FOXP1com	≤-0.49	1	0.010
	>-0.49	4.630 (1.438-14.907)	
-ΔΔCt FOXP1var	≤-0.2	1	0.000
	>-0.2	8.383 (2.677-26.248)	

Significant p-Values are shown in bold.

Table V. Results of the multivariable Cox proportional hazard model for RFS.

Predictor variable	Category	Relative risk (95% CI)	p-Value
Gender	Female	1	0.209
	Male	1.669 (0.751-3.713)	
Age	<70	1	0.038
	≥70	2.420 (1.052-5.568)	
T	T1 or T2	1	0.714
	T3 or T4	1.218 (0.426-3.483)	
N	N0	1	0.048
	N1 or higher	2.222 (1.006-4.91)	
M	0	1	0.815
	1	0.761 (0.078-7.436)	
Surgical radicality	R0	1	0.099
	P	6.489 (0.705-59.75)	
Chemo/radiotherapy	Yes	1	0.513
	No	1.337 (0.560-3.194)	
-ΔΔCt CD44v3	≤1.4	1	0.036
	>1.4	2.345 (1.059-5.196)	
-ΔΔCt FOXP1var	≤-0.25	1	0.015
	>-0.25	3.006 (1.234-7.325)	

Significant p-Values are shown in bold.

matrix interactions and as a receptor in signal transduction, proposed to regulate tumor invasiveness and replication. The observation of CD44 up-regulation is consistent with a vast body of previous studies, however its role as a prognostic marker is not fully clear (45). Recent results of a meta-analysis proposed its role as a negative prognostic marker (46). This supports our observation of high CD44 expression being associated with tumor invasion and overall UICC staging.

As the CD44 is expressed in various alternatively spliced variants, we further focused on an analysis of CD44v3 and CD44v6, which have been proposed to play a direct role in cancerogenesis (47, 48). Down-regulation of MBNL3 was shown to activate the pluripotency network and CD44v3 overexpression in chronic myeloid leukemia (27). A study with colon cancer cell lines proposed a role of CD44v3 in tumor invasiveness (49). CD44v6 is present in colorectal

cancer stem cells, required for their ability to form metastasis (47) and enhancing their resistance to chemotherapy (50, 51). Both variants have been suggested as prognostic markers in colorectal cancer, with a probably more established role of CD44v6 (38, 52, 53).

We show associations of alternative CD44 variants with several clinicopathological parameters of disease progression. The CD44v6 correlated with tumor grading progression, while expression of CD44v3 was higher in patients with distant metastasis and correlated with the overall UICC score. The higher expression of CD44v3 was also associated with a worse survival rate, although without conclusively proven prognostic independence. This finding highlights the role of CD44v3 next to CD44v6 as an important marker with potential prognostic value.

The up-regulation of stem cell-like variants CD44v3 and CD44v6 is in line with the observed down-regulation of MBNL and concurrent switch to stem cell phenotype with high potential towards pluripotency and invasiveness.

Another gene whose expression is associated with extensive alternative splicing is the gene coding for cytoskeletal adaptor protein EPB41L3, which also acts as an important proliferation regulator. It has been demonstrated that alternative splicing of EPB41L3 is involved in differentiation of epithelial cells (54). The existing evidence is consistent with the role of EPB41L3 as a tumor suppressor (55). In line with this, we observed down-regulation of EPB41L3 in colorectal cancer tissue.

Conclusion

In conclusion, our results highlight the role of alternative splicing as an important mechanism in colorectal cancer pathogenesis. Alternative splicing, in addition to transcription changes, modulates gene expression to promote oncogenesis. Specifically, our data suggest a potential shift of the observed gene expressions towards stem-cell variants, associated with lower differentiation, pluripotency network engagement and enhanced invasiveness.

Altogether, in this study we have tried to advance our understanding of alternative splicing regulators as important markers of cancer progression and open the door for the further systematic study of alternative splicing regulation in colorectal cancer and other malignancies.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization, P.P.; Methodology, N.N., P.P.; Formal analysis, P.H.; Funding acquisition, P.P., P.K. and V.L.; Investigation, N.N.,

P.K., R.P. and J.R.; Project administration, P.P.; Resources, O.V., J.B., R.P. and J.R.; Supervision, P.P. and V.L.; Visualization, P.H.; Writing – original draft, N.N., F.Z. and A.N.; Writing – review and editing, P.P. and P.H.

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