Pathology & Oncology Research (2020) 26:2337–2350
https://doi.org/10.1007/s12253-020-00830-2

ORIGINAL ARTICLE

Expression, Epigenetic, and Genetic Changes of HNF1B in Colorectal Lesions: an Analysis of 145 Cases

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Received: 10 January 2020 / Accepted: 21 May 2020 / Published online: 1 June 2020

Abstract

Hepatocyte nuclear factor 1 beta (HNF1B) is transcription factor which plays a crucial role in the regulation of the development of several organs, but also seems to be implicated in the development of certain tumours, especially the subset of clear cell carcinomas of the ovary and kidney. Depending on the type of the tumour, HNF1B may act as either a tumour suppressor or an oncogene, although the exact mechanism by which HNF1B participates in the process of cancerogenesis is unknown. Using immunohistochemical approach and methylation and mutation analysis, we have investigated the expression, epigenetic, and genetic changes of HNF1B on 40 cases of colorectal adenomas and 105 cases of colorectal carcinomas. The expression of HNF1B was correlated with the benign or malignant behaviour of the lesion, given that carcinomas showed significantly lower levels of expression compared to adenomas. In carcinomas, lower levels of HNF1B expression were associated with recurrence and shortened disease-free survival. The mutation analysis revealed three somatic mutations (two frameshift and one nonsense) in the carcinoma sample set. Promoter methylation was detected in three carcinomas. These results suggest that in colorectal cancer, HNF1B may play a part in the pathogenesis and act in a tumour suppressive fashion.

Keywords Colon · Adenoma · HNF-1-beta · Immunohistochemistry · Mutation analysis · Methylation

Introduction

Hepatocyte nuclear factor 1 beta (HNF1B), also known as Transcription Factor-2 (TCF2), is a member of a family of transcription factors which, primarily, plays a crucial role in the regulation of the development of various tissues and organs. Most importantly, it is implicated in the ontogenesis and differentiation of the liver (hence its name), but also the lung, gonads, biliary system, kidney and pancreas [1]. In adults, HNF1B expression is found especially in tubule-forming epithelial tissues such as kidney or pancreatic exocrine duct tubules [2], and also in other tissues such as colon, small intestine, stomach, testis, lung, liver, prostate etc. as described according to the complex RNA-Seq data [3]. As well as cell differentiation, HNF1B is also involved in the regulation of the expression of multiple genes implicated in cell cycle modulation, susceptibility to apoptosis, response to oxidative stress and glucose metabolism [4–6].

As HNF1B expression is found during the embryonic development of the kidneys, pancreas, liver, biliary tract, and reproductive tract, mutations of this gene are associated with various developmental disorders of these systems such as renal cysts and diabetes syndrome (RCAD), or maturity-onset diabetes of the young, type 5 (MODY5) [7, 8]. Furthermore, there is growing evidence that HNF1B may be involved in the tumorigenesis of several types of solid tumours, especially in the subset of clear cell carcinomas of the ovary and renal cell carcinomas of the kidney, but its role in the development of tumours of the gastrointestinal tract, liver, pancreas and prostate is also being discussed [6, 9–15].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-020-00830-2) contains supplementary material, which is available to authorized users.

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Despite the increasing interest and the number of studies dedicated to this topic, little is known about the possible HNF1B mutations which may be found in various tumours, although some single nucleotide polymorphisms (SNPs) have been reported in the non-coding intronic sequences of the *HNF1B* loci associated with risk of kidney cancer [16] and endometrial cancer [17, 18]. Some ancestry-specific *HNF1B* variants associated with prostate cancer have also been reported [19, 20].

Interestingly, it seems that HNF1B may act as either a tumour suppressor or an oncogene depending on the type of tissue and tumour. How this influence is exerted specifically is not yet understood and there are studies which define HNF1B as a pro-differentiation factor with potent tumour-suppressive activity in healthy tissues [21], while other studies point to its role as an oncogene in tissues which have undergone malignant transformation, inducing a cancerous phenotype and activating the formation of invasive phenotypes through epithelial-mesenchymal transition [22]. The exact mechanism by which HNF1B participates in the process of cancerogenesis is unknown and probably differs in various types of tumours.

Given these diverse and often ambiguous results, our aim was to perform a comprehensive analysis of the expression and the epigenetic and genetic changes of HNF1B in benign and malignant lesions of the large intestine, as well as to further investigate the potential differential diagnostic and prognostic value of HNF1B.

Material and Methods

Samples

For the purposes of the study we used FFPE tissue blocks and, where available, the corresponding fresh-frozen tissue (FT) stored in the RNAlater stabilization solution (Qiagen) at −80 °C according to the manufacturer's protocol (Stabilization of RNA in Harvested Animal Tissues; Qiagen). The FFPE tissue blocks were obtained from the archives of our department and the corresponding FT samples were provided by the Bank of Biological Material (BBM) of the First Faculty of Medicine, Charles University in Prague.

A total of 145 FFPE tissue samples were used for the immunohistochemical analysis, including 105 cases of colorectal carcinoma and 40 cases of colonic adenoma. For the 105 cases in the colorectal carcinoma cohort, there were 78 cases with available corresponding FT sample pairs (tumour and non-tumour), which were subjected to DNA isolation for mutation and promoter methylation analysis. In the adenoma cohort, molecular analysis was performed for 34 samples with the use of DNA isolated from FFPE tissue blocks (no FT material was available for this cohort). The clinicopathological characteristics of the analysed samples are summarized in Tables 1 and 2.

Concerning the adenoma cohort, the samples consisted of a variety of adenoma types: tubular adenoma ($n = 24$), tubulovillous adenoma ($n = 9$), sessile serrated adenoma ($n = 3$) and traditional serrated adenoma ($n = 4$). For the carcinoma cohort, the characteristic of 'location' of the tumour was classified as either colon (including tumours found in the appendix, caecum and ascending colon to sigmoid colon) or rectum. The characteristic of 'grade' was divided into four groups (G1, G2, G3 and mucinous) with mucinous carcinomas being assigned a special category, given that the grade of these tumours depends on their microsatellite status, which was not available for all investigated cases (according to the 4th Edition of WHO Classification of Tumors of Digestive System) [23]. The characteristic of 'sidedness' was divided into right-sided (cecum, appendix, ascending colon, hepatic flexure and proximal two thirds of the transverse colon) and left-sided (distal third of the transverse colon, splenic flexure, descending colon, sigmoid colon and rectum) tumors.

The colorectal carcinoma samples were divided according to the TNM classification into four groups (pT1-pT4), while

Table 1 Association of HNF1B expression and clinicopathological characteristics, based on 40 cases of colorectal adenoma

Characteristic	Group	N	H-score mean	H-score median	*p-value*[a]	Expression of HNF1B (N)		
						group 1	group 2	*p-value*[b]
Gender					0.712			0.927
	Male	28	248.3	260		5	23	
	Female	12	246.9	250		2	10	
Age (mean = 70, median = 73)					0.140			0.694
	<70	14	233.2	243		2	12	
	≥70	26	255.8	267		5	21	
Grade of dysplasia					0.129			0.212
	High-grade	20	235.8	250		5	15	
	Low-grade	20	260.1	257		2	18	

[a] – *p*-values are based on the Mann-Whitney U-test

[b] – *p*-values are based on the Pearson chi-square test (categorized expression)

Table 2 Association of HNF1B expression and clinico-pathological characteristics, based on 105 cases of colorectal carcinoma

Characteristic	Group	N	H-score mean	H-score median	*p-value*[a]	Expression of HNF1B (N)		
						group 1	group 2	*p-value*[b]
Gender					0.267			0.162
	Male	61	191.7	210		29	32	
	Female	44	174.5	170		27	17	
Age					0.857			0.896
(mean = 71, median = 71)	<71	50	184.5	190		27	23	
	≥71	55	184.5	190		29	26	
Location					0.167			0.325
	Colon	93	188.6	195		48	45	
	Rectum	12	152.9	155		8	4	
Sidedness					0.451			0.937
	left-sided	54	190.6	190		29	25	
	right-sided	51	178	180		27	24	
Histological subtype					0.781			0.469
	non-mucinous	94	185.7	192.5		49	45	
	mucinous	11	175.0	174.1		7	4	
T stage					0.221			0.152
	T1	10	216.5	240		3	7	
	T2	30	165.8	165		19	11	
	T3	45	194.9	220		21	24	
	T4	20	173.3	180		13	7	
N stage					0.791			0.612
	N0	76	188.4	195		39	37	
	N1	17	177.9	180		9	8	
	N2	12	169.2	180		8	4	
M stage					0.844			0.772
	M0	89	183.8	190		48	41	
	M1	16	188.8	200		8	8	
Dukes staging					0.584			0.436
	A	10	216.5	240		3	7	
	B	50	183.3	178		28	22	
	C	29	173.3	180		17	12	
	D	16	188.8	200		8	8	
Stage TNM grouping					0.469			0.767
	I	34	175.3	170		20	14	
	II	34	201.8	220		16	18	
	III	21	168.3	180		12	9	
	IV	16	188.8	200		8	8	
Grade					0.097			0.161
	G1	26	204.4	225		11	15	
	G2	59	186.9	190		31	28	
	G3	8	113.1	110		7	1	
	mucinous	12	177.1	183		7	5	
LN positivity					0.498			0.502
	Positive	29	174.3	180		17	12	

Table 2 (continued)

Characteristic	Group	N	H-score mean	H-score median	*p-value*[a]	Expression of HNF1B (N) group 1	group 2	*p-value*[b]
	Negative	76	190	200		39	37	
Lymphovascular invasion					0.742			0.392
	Yes	41	179.3	180		24	17	
	No	64	187.9	203		32	32	
Perineural invasion					0.363			0.576
	Yes	9	201.7	220		4	5	
	No	96	182.9	185		52	44	
MMR status*					0.318			
	deficient	11	210.0	260		4	7	0.341
	proficient	42	190.2	195		22	20	
Recurrence*					**0.048**			**0.019**
	Yes	16	147.8	153		13	3	
	No	87	189.5	205		43	44	
MSI*					0.274			0.734
	MSS	83	180.3	190		45	38	
	MSI-H	20	200.3	205		10	10	

*– data are not available for all cases

[a] – *p*-values are based on the Mann-Whitney U-test or Kruskal-Wallis H-test

[b] – *p*-values are based on the Pearson chi-square test (categorized expression)

the adenoma samples were divided according to the grade of the epithelial dysplasia (low grade vs. high grade). For the purposes of the study a total of 145 lesions of the large intestine were selected, including 105 cases of carcinoma of the large intestine (pT1 = 10 cases, pT2 = 30 cases, pT3 = 45 cases, pT4 = 20 cases) and 40 cases of adenoma (LG dysplasia = 20 cases, HG dysplasia = 20 cases).

All the included cases underwent a histologic review of the hematoxylin and eosin-stained slides. During this review the eligible and appropriate areas of the tumour were identified and marked in order to provide tissue cores for the construction of the TMAs. Two tissue cores (each 2.0 mm in diameter) were drilled from the donor block from each case using the tissue microarray instrument TMA Master (3DHISTECH Ltd., Budapest, Hungary).

Ethical Approval

The study has been approved by the Ethics Committee of General University Hospital in Prague in compliance with the Helsinki Declaration (ethical approval number 41/16 Grant VES 2017 AZV VFN). The Ethics Committee waived the requirement for informed consent, because according to the Czech Law (Act. no. 373/11, and its amendment Act no. 202/17) it is not necessary to obtain informed consent in fully anonymized studies.

DNA Isolation and Quality Control

Prior to the isolation of the FT samples, the tissues were thawed, and 10–30 mg were homogenized using MagNA Lyser Green Beads tubes in a MagNA Lyser Instrument (Roche) in the presence of 600 μl of RLT Plus buffer (Qiagen) with 6 μl of 14.3 M 2-mercaptoethanol (Sigma-Aldrich). The total DNAs and RNAs were isolated according to the Simultaneous Purification of Genomic DNA and Total RNA from Animal Tissues protocol by using an AllPrep DNA/RNA Mini kit (Qiagen). The isolated DNA samples were quantified by NanoDrop 2000 (Thermo Fisher).

DNA from the tissue sections from the archived FFPE tissue blocks was isolated using an automatic isolator MagCore® nucleic Acid Extractor, utilizing the MagCore Genomic DNA FFPE One-step kit, Ref MGF-03 (RBC Bioscience). The isolated DNA was quantified by Qubit fluorimeter (Thermo Fisher) and underwent a quality control test of amplification efficacy by qPCR (5 ng DNA of sample was amplified using 5x HOT FIREPol® EvaGreen® HRM Mix NO ROX; Solis Biodyne). Only the samples which passed the

quality criteria (Cp < 35 for a 180 bp product amplification) were used for subsequent analysis.

Immunohistochemical Analysis

The immunohistochemical (IHC) analysis was performed on all samples using the standard 4 μm thick sections of FFPE tissue and the automated staining instrument Ventana BenchMark ULTRA (Roche, Basel, Switzerland) with a rabbit antibody against the HNF1B protein (polyclonal, dilution 1:500, product no. HPA002083, Sigma-Aldrich, Prestige Antibodies, St. Louis, United States). The heat induced epitope retrieval with a citrate buffer (pH 6.0) was used for pretreatment. The detection of the primary antibody was visualized using the OptiView DAB IHC Detection Kit (Ventana, Roche). Only nuclear staining was regarded as positive and the nuclear expression of HNF1B was double-blindly evaluated by two independent pathologists.

The immunohistochemical results were assessed according to the overall percentage of positive cells (0–100%) and then also semi-quantitatively, using the H-score method previously described by others [24]. This method is based on the assessment of the percentage of positive cells based on the level of staining intensity (1+ for weak intensity, 2+ for moderate and 3+ for strong intensity). The final H-score for each case is then calculated by adding the multiplication of the different staining intensities according to the following formula: [1 x (% of cells 1+) + 2x (% of cells 2+) + 3x (% of cells 3+)], resulting in the H-score value of 0–300.

Prior to the construction of TMAs and the use of the staining instruments, we performed a validation of the staining method, for which 10 cases of colorectal carcinoma, 10 cases of normal mucosa of the large intestine, and 10 cases of adenoma were randomly selected. From these we obtained whole-tissue sections which were stained with the antibody against HNF1B in order to assess the quality of the staining and heterogeneity of HNF1B expression. Although the intensity levels of the staining differed among the different lesions, the staining was homogenous throughout the examined tissues and therefore the decision was made to test all the carcinoma samples ($n = 105$) using the constructed TMAs. Even though the expression pattern of adenomas was also homogenous, the amount of tumour tissue available from each case tended to be rather low and that is why only the larger adenomas, which allowed for the extraction of 2 tissue cores, were selected for the construction of TMA ($n = 30$), while the remaining samples ($n = 10$) were processed as whole-tissue sections.

Positive and negative internal controls were evaluated for each of the IHC stained slides. The staining of normal, non-neoplastic intestinal epithelial cells served as a positive control, while the lack of staining of other structures (such as the connective tissue, smooth muscle and adipose tissue) served as a negative control.

Statistical Analyses

Statistical analyses were performed using the software Statistica (StatSoft, Inc., Tulsa, OK). The nonparametric ANOVA approach was used in order to analyse the association between HNF1B expression (H-score as a continuous dependent variable) and clinicopathological characteristics (categorical variables). Depending on the number of categories either the Mann-Whitney U-test (two categories) or the Kruskall-Walis H-test (three and more categories) was used. For the evaluation of the effect of independent clinicopathological characteristics on the categorized H-score, the Pearson chi-square test was used. Survival analyses were plotted using the Kaplan-Meier method and analysed using the log-rank test. The evaluated outcomes included DFS (death from colorectal carcinoma was considered as a failure), LFS (counted as the period from the date of the primary diagnosis to the date of diagnosis of the first local recurrence) and MFS (counted as the period from the date of the primary diagnosis to the date of the diagnosis of the first distant metastasis). The date of primary diagnosis is the date of the confirmation of the biopsy diagnosis. For the purposes of chi-squared tests and survival analyses, the H-score was categorized into two groups (group 1: H-score 0–200; group 2: H-score 201–300). All tests were two-sided and a P value of less than 0.05 was considered as significant.

Genetic and Epigenetic Analysis

Molecular analyses included DNA mutation analysis of the coding parts of the *HNF1B* exons with adjacent intronic sequences (+ − 15 bp) and epigenetic analysis of CpG methylation in the region of the HNF1B promoter.

The *HNF1B* mutation analysis was performed using two different approaches, depending on the origin of the analysed material. High-quality FT DNA samples (78 tumour and 12 paired non-tumour tissues) were analysed by in-house 2-step polymerase chain reaction (PCR) amplicon next-generation sequencing (NGS), while the FFPE DNA samples (20 tumour samples) were analysed by a capture-based panel NGS, which is more suitable for FFPE samples and included all the coding parts of the *HNF1B* gene.

Amplicon NGS Preparation and Sequencing

For the in-house 2-step PCR amplicon approach, 15 primer pairs with universal adaptor sequences were designed (list of primers is provided in Online Resource 1) to fit the specific *HNF1B* gene regions in the first PCR step, including deep intronic regions containing the rs7527210 and rs4430796 variant sites. In the second PCR step, a universal primer pair containing Illumina sequencing adaptor sequences was used (Online Resource 1). The first PCR step covering the HNF1B

target regions was performed in two separate multiplex reactions. Each reaction included a different primer pair set (Online Resource 1) in order to eliminate unwanted primer interactions. Both PCR reactions were amplified using the FastStart High Fidelity PCR System (Roche) according to the recommended standard PCR procedure (FastStart High Fidelity PCR System; Roche) in 20 μl reactions according to the following PCR protocol: 2 min – 95 °C; 10 cycles of 15 s – 95 °C, 20s – 62 °C and 30s – 72 °C (all steps with ramping temperature 2 °C/s) and then 20 cycles of 15 s – 95 °C, 20s – 62 °C and 30s – 72 °C (standard ramping temperature 4 °C/s). After the first PCR step, 10 μl of both first PCR reactions were equimolarly mixed and purified by the AMPure XP system (0,8x; Beckmann Coulter). The purified PCR product was amplified by 10 cycles of the second PCR step using the same protocol, with a standard ramping rate and different primers (the universal primer pair with Illumina adaptor sequences was used).

After the second PCR step, the concentrations of the PCR products were measured using the Qubit fluorimeter (Thermo Fisher) and then equimolarly mixed into one sequencing library. Once the sequencing library was prepared, it was purified using the AMPure XP system (0,8x; Beckmann Coulter), measured for concentration (Qubit) and for fragment length using High Sensitivity NGS Fragment Analysis kit on the Fragment Analyzer (AATI). The amplicon library was then sequenced together with different (capture) libraries in order to increase sequencing heterogeneity. The sequencing was performed either using 50 samples, which were sequenced in one amplicon library by the MiSeq 300 cycles v2 kit or using 90 samples by the NextSeq 300 cycles mid output kit v2.5. The amplicon sequencing approach showed low coverage of 20 bp on the 5′ end of exon 4 in all of the tested samples, and therefore this part was additionally sequenced by the Sanger sequencing method as described elsewhere [25] with the use of a specific primer pair (Online Resource 1).

Capture-Based NGS Preparation and Sequencing

The DNA from the FFPE samples was prepared using the SeqCap custom hybridization probes (257kbp panel of gene targets, NimbleGen, Roche; the list provided in Online Resource 2) and sequenced as described in Ticha et al., 2019 [26]. The libraries were sequenced by the NextSeq 500 instrument using the NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles).

Biostatistical Analysis of NGS Data

Both the amplicon and panel sequencing raw data were demultiplexed and converted into the .fastq format and analysed by the same pipeline using the NextGENe software (Softgenetics) as described elsewhere [27]. For the reads mapping and analysis, the GRCh37 genome and NM_000458.2 reference transcript was used. Only the samples with minimal coverage >200x and variants with variant allele frequency (VAF) > 10% were further evaluated. The identified variants were manually inspected using IGV (Broad Institute) and prioritized according to the mutation impact [27]. Only the mutations of class 3, 4 or 5 were reported.

Microsatellite Instability

Analysis of microsatellite instability (MSI) was performed with the following set of five quasimonomorphic mononucleotide microsatellite markers: BAT-26, BAT-25, NR-21, NR-22, NR-24 [28]. Fragmentation analysis was performed using ABI 3500 (ThermoFisher). The MSI-high or MSI-low phenotypes were defined as the presence of two or more, or a single unstable locus, respectively. MSI stable tumors (MSS) show no instability.

HNF1B Promoter Methylation

Bisulfite conversion of DNA was performed using the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The primers (Online Resource 1) used for PCR amplification of both the methylated and unmethylated alleles were designed using the software Methprimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The amplified promoter region of *HNF1B* covers 15 CpG islands (as illustrated in Online Resource 3, part a) and includes a CpG island (chr17:36105517–36,105,518, GRCh37) whose methylation is associated with a decreased HNF1B expression [29]. In our settings we were able to detect at least 5% of methylated DNA by High Resolution Melting (HRM) Analysis of the amplified PCR products. Each run included the converted DNA samples and a series of 100%, 20%, 10%, 5 and 0% universally methylated DNA controls mixed with non-methylated DNA (Human HCT116 DKO Non-Methylated DNA and Human HCT116 DKO Methylated DNA; Zymo Research). The melting curves of the analysed samples were compared with the melting curves of the control mixes (Online Resource 3) [30].

Results

Immunohistochemical Findings

Overall, virtually all of the colorectal carcinoma and adenoma samples showed some degree of homogenous, constant nuclear expression of HNF1B. The results of HNF1B expression are summarized in Tables 1 and 2. Figure 1 (a, c, d) shows representative examples of HNF1B expression in colorectal

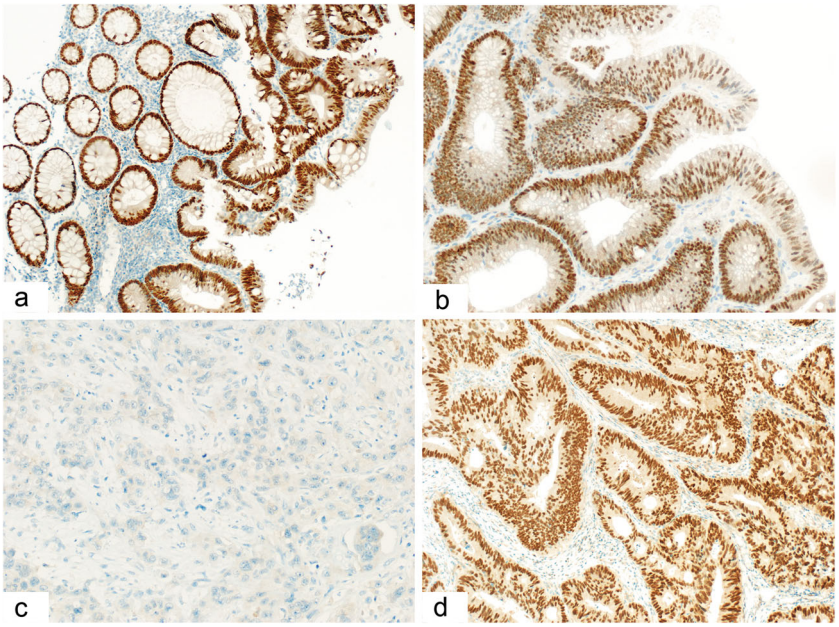

Fig. 1 Immunohistochemical staining of the HNF1B expression in colorectal lesions **a** Strong nuclear expression in the normal epithelial lining of non-neoplastic crypts and equally strong nuclear expression in the adenoma with low grade epithelial dysplasia on the right (100x). **b** Low grade adenoma with superficial areas of high-grade dysplasia, note the decrease of HNF1B expression in the high-grade portions of the epithelium (200x). **c** Complete lack of nuclear expression in colorectal carcinoma (200x). **d** Strong, diffuse nuclear expression in colorectal carcinoma (100x)

carcinomas and adenomas, which was correlated with the benign or malignant behaviour of the lesion. The group of carcinomas showed significantly lower levels of expression compared to adenomas ($p < 0.001$) (Table 3 and Fig. 2). When evaluating the association between HNF1B expression and clinicopathological characteristics (Table 2), a lower HNF1B expression was associated with recurrence ($p = 0.043$) (Fig. 3). None of the other parameters (gender, age, anatomical location, sidedness, T / N / M stage of the tumour, Dukes stage, grade, lymph node positivity, lymphovascular invasion, perineural invasion, and MMR status evaluated by immunohistochemistry where applicable) showed any association on a significant level.

The group of adenomas showed not only significantly higher levels of expression ($p < 0.001$), but also an overall higher intensity of the staining when compared to carcinomas ($p < 0.001$; Table 3). Although there were no statistically significant differences in the H-score based on the grade of epithelial dysplasia [high-grade (HG) dysplasia H-score: 235.8/250 (mean / median) and low-grade (LG) dysplasia H-score: 260.1/257 (mean / median)], in cases of adenomas with predominantly LG dysplasia and only short sections of HG dysplasia there were some interesting changes in the staining pattern. While the epithelial cells with LG dysplasia showed strong, diffuse staining, the superficial portions of the adenoma with HG dysplasia showed a gradual decrease in the intensity of the staining, although the presence of staining positivity was preserved (Fig. 1b). None of the evaluated clinicopathological characteristics in the group of adenomas (gender, age, grade of dysplasia) yielded statistically significant results when assessing the association of these with HNF1B expression (Table 1).

Survival analyses for the three available outcomes [disease-free survival (DFS), local recurrence-free survival (LFS), and distant metastasis-free survival (MFS)] were performed and included 98 carcinoma cases with available follow-up from the sample set of 105 carcinomas. They showed a statistically significant association between HNF1B expression

Table 3 Association of HNF1B expression and the type of diagnosis, based on 145 cases of colorectal lesions

Characteristic	Group	N	H-score mean	H-score median	*p-value*[a]	Expression of HNF1B (N)		
						group 1	group 2	*p-value*[b]
Type of lesion					**< 0.001**			**< 0.001**
	Adenoma	40	247.9	253		7	33	
	Carcinoma	105	184.5	190		56	49	

[a] – *p*-values are based on the Mann-Whitney U-test

[b] – *p*-values are based on the Pearson chi-square test (categorized expression)

(categorized into two groups as described above) and DFS ($p = 0.014$), where lower levels of HNF1B expression were associated with adverse DFS (Fig. 4). The other monitored outcomes did not reach significant values (LFS $p = 0.101$, MFS $p = 0.057$), although there is a trend suggesting an association between lower HNF1B expression and shorter LFS and MFS (Fig. 4).

Genetic and Epigenetic Changes of the HNF1B Gene

Mutation Analysis

Mutation analysis was successfully performed on 84 samples (73 samples of tumour tissue and 11 samples of corresponding healthy tissue) from the total of 90 samples with available fresh-frozen tissue (FT) material (78 tumour and 12 non-tumour) in the sample set of colorectal carcinomas. In the sample set of adenomas, the analysis was successfully performed on 20 samples (from the total of 34 samples, for which only formalin-fixed paraffin-embedded (FFPE) material was available).

Among the 73 colorectal carcinomas we found three somatic *HNF1B* mutations, including two frameshift and one nonsense: c.149delC p.P50LfsTer75 (variant allele frequency (VAF) 38.45%) (hereinafter referred to as Sample A, H-score: 210); c.1006delC, p.H336TfsTer40 (VAF 34.88%) (hereinafter referred to as Sample B, H-score: 240) and c.554C > T, p.Q182X (VAF 26.79%) (hereinafter referred to as Sample C, H-score: 80). Other clinically relevant, either somatic or hereditary mutations were not present. No mutations were revealed among the 11 adenomas.

Given that the effect of HNF1B single nucleotide polymorphisms is also discussed in the literature with often equivocal yet suggestive results, we also evaluated the impact of two of the most commonly reported SNPs (rs4430796 and rs7527210) on colorectal carcinoma survival. Considering rs4430796 SNP, a significantly better DFS was observed in patients with the AA genotype when compared to GA and GG ($p = 0.035$; Fig. 5). No significant effect was detected in the case of rs7527210 ($p = 0.942$, data not shown).

Microsatellite Instability

The MSI analysis was successfully performed on 103/105 carcinoma samples (the remaining 2 samples did not have enough tumour tissue left for molecular analysis). The results showed that 81/103 tumours were MSS, 20/103 were MSI-H and 2/103 were MSI-L. The two MSI-L tumour samples were additionally evaluated using immunohistochemical analysis with antibodies against mismatch-repair proteins (MLH1, PSM2, MSH2, MSH6), which showed preserved expression. Therefore, for the purposes of statistical analyses, those 2 samples were added into the MSS group. Statistical analysis did not show any significant association between HNF1B expression and the two groups (MSI-H versus MSS) of carcinomas (Table 2).

Epigenetic Analysis

Promoter methylation analysis was successfully carried out in 93 (72 carcinomas and 21 adenomas) out of the 99 samples which had undergone the bisulphite DNA conversion. Promoter methylation was detected in 3/72 (4.2%) adenocarcinomas: in two cases a low degree of methylation was detected (5–10%, H-score of the corresponding tumours was 30 and 95), and in one case it was a moderate degree of methylation (approx. 25%, H-score = 295). Non-tumour tissue was available for two of those tumour samples, and no methylation

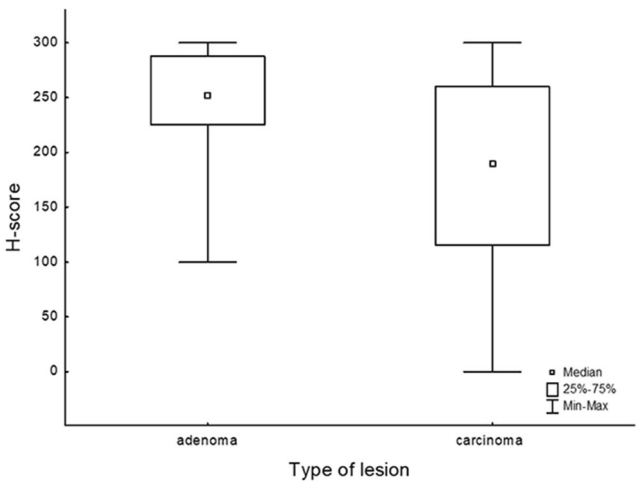

Fig. 2 Association of H-score of HNF1B and type of the lesion. Analysis based on 145 cases (adenoma: $N = 40$, carcinoma: $N = 105$). Mann-Whitney U-test: $Z = 4.069$, $p < 0.001$

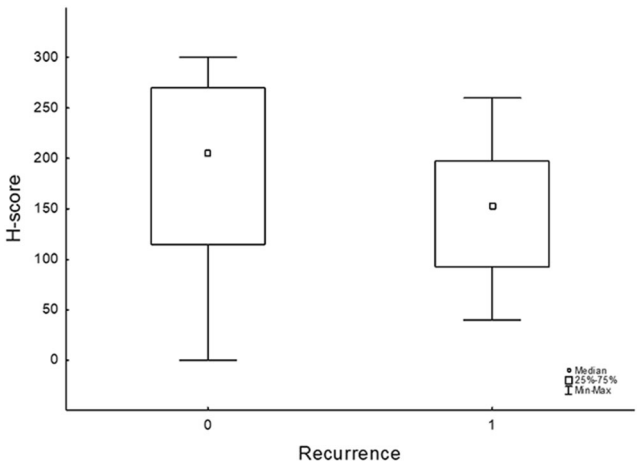

Fig. 3 Association of H-score of HNF1B and disease recurrence. Analyses based on 105 cases of colorectal carcinoma. 0 = no recurrence, 1 = recurrence occurs. Mann-Whitney U-test: $Z = 2.021$, $p = 0.043$

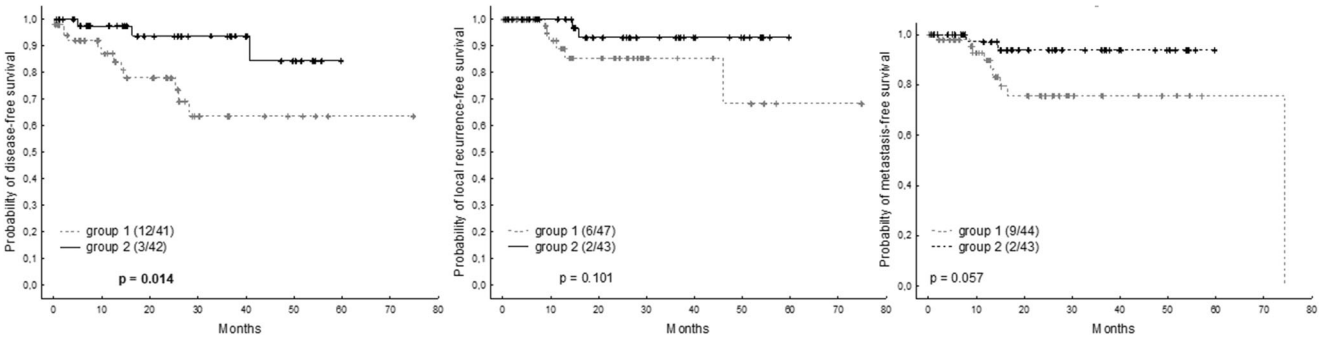

Fig. 4 Correlation of HNF1B protein expression with prognosis. The probability of disease-free survival (a) local recurrence-free survival (b) and metastasis-free survival (c) in relation to category of H-score of HNF1B in 98 cases of colorectal carcinoma. The survival curves are estimated using the Kaplan-Meier method, *p values* are estimated using the log-rank test. The number of complete/censored data are shown in parentheses

was detected in either case. In the cohort of adenomas, methylation was observed in 1/21 adenomas (4.7%). The non-tumour tissue was not available in this case. Of the 87 isolated non-tumour tissue samples the analysis was successfully performed in 61 cases, all of which were non-methylated.

Discussion

HNF1B belongs to one of the four major hepatocyte nuclear factor families, which include HNF1 (HNF1α and HNF1β), HNF3 (or FOXA, made up of FOXA1, FOXA2 and FOXA3), HNF4 (HNF4α and HNF4γ) and HNF6 (or ONECUT, OC, made up of OC1, OC2 and OC3) [31]. Depending on the circumstances, it functions as a bookmarking transcription factor which regulates gene expression by maintaining active transcription or counteracting the epigenetic silencing effect of chromatin condensation [1].

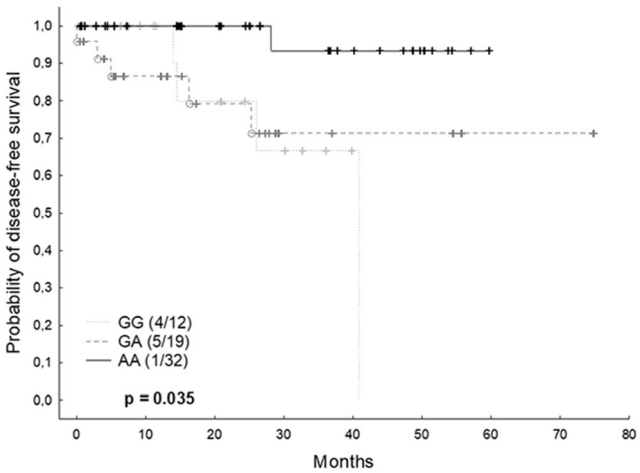

Fig. 5 Correlation of rs4430796 genotype with prognosis. The probability of disease-free survival in relation to rs4430796 SNP genotypes, based on 73 cases of colorectal carcinoma. The survival curve is estimated using the Kaplan-Meier method, the *p value* is estimated using the log-rank test. The number of complete/censored data are shown in parentheses

The key influence of this transcription factor is two-fold. Firstly, HNF1B is involved especially in the development of the kidneys, biliary system, pancreas and gastrointestinal tract [32, 33]. Secondly, its role in the development and progression of cancer (and potentially also chemoresistance in certain tumour types) has been discussed in recent years, with research focusing especially on tumours with clear cell phenotype, given that >90% of these tumours show HNF1B positivity [34].

We have performed a comprehensive, multi-level analysis of the *HNF1B* gene in the setting of benign and malignant lesions of the large intestine. The results of mutation analysis revealed the presence of 3/73 (4.1%) inactivating somatic mutations (VAF around 30% for all three cases) in the colorectal carcinoma cohort, two of which were frameshift (sample A and B) with retained immunohistochemical expression of HNF1B (H-score 210 and 240, respectively). The remaining was a nonsense mutation c.554C > T, p.Q182X (sample C), with a retained but significantly decreased expression of HNF1B (H-score 80). The retained expression could be explained by the incomplete inactivation of one or both alleles or by a possible tumor heterogeneity. There were no mutations detected in the cohort of adenomas. There is very little information in the literature which deals with the presence of either germline or somatic *HNF1B* mutations in malignant tumours, and no information whatsoever regarding specifically colorectal carcinomas. There have been some rare (mostly case report) instances of germline *HNF1B* mutations associated with chromophobe renal cell carcinoma (RCC) [16, 35], confirming that HNF1B acts as a tumour suppressor during carcinogenesis of chromophobe RCC. Similar results were reported for Wilms tumour (WT), which showed decreased HNF1B expression in Wilms´ tumour tissue when compared to the adjacent non-cancerous tissue [1]. Reports of somatic mutations associated with HNF1B-mediated carcinogenesis are equally sparse, and based on an extensive search of the literature it is in fact only our recent study, which described somatic mutations of the *HNF1B* gene in solid tumours of the

female genital tract [36]. In this study, the analysis of genetic variants using HRM (High Resolution Melting) analysis and Sanger direct sequencing of the whole coding sequences of *HNF1B* revealed 1 clinically relevant somatic mutation among 30 endometrial endometrioid carcinomas and one missense variant of unknown significance among 12 ovarian clear cell carcinomas (OCCC) [36].

The prognosis of certain malignant neoplasms may also be influenced by polymorphisms in the non-coding intronic sequences of *HNF1B*. The relationship between *HNF1B* SNPs has already been studied especially for endometrial cancer, ovarian clear cell carcinoma and prostate cancer, but it was yet to be investigated for colorectal cancer [37]. Based on the results of genome-wide association studies, the most commonly implicated SNPs are rs447096 (located in the intron 2 (i2), also known as rs17626333 or rs5876954), rs7527210 (located in i2, also known as rs3786124 or rs60456671) and several other SNPs studied for their association with prostate cancer (rs11649743, rs7501939, rs7405696, rs1016990, rs3094509 and rs4794758) [19, 38, 39]. However, although the number of studies focusing on the association between these intron-situated *HNF1B* SNPs and the risk of certain cancers is steadily increasing, their results are often conflicting and even their clinical role and significance remains unclear.

In the current study, we examined the effect of two selected SNPs (rs4430796 and rs7527210) on colorectal carcinoma survival and found that for SNP rs4430796 there were statistically significant differences in the estimated disease-free survival based on three rs4430796 genotypes. Those patients with the AA genotype showed a significantly better DFS ($p = 0.035$) than patients with GA and GG genotypes, with the GG genotype showing the worst prognosis. This result is in accordance with studies performed on other cohorts, especially endometrial cancer and ovarian cancer. For endometrial cancer, it has been reported that patients with the GG genotype also demonstrated the worst overall survival (OS) [40]. This finding might be associated with the reported effect of HNF1B overexpression on the chemoresistant phenotype of OCCC, given the observed relationship between the GG genotype and decreased OS. The G allele may be implicated in reducing chemosensitivity to cisplatin- or paclitaxel-mediated cytotoxicity, which is particularly interesting in the context of colorectal cancer, given that the backbone of adjuvant therapy for advanced disease lies in fluoropyrimidine- and oxaliplatine-based regimes [41]. In our cohort no significant effect was detected in the case of the SNP rs7527210 ($p = 0.942$, data not shown), which is also mentioned in literature as an ovarian cancer risk- and prostate cancer risk-associated SNP [21].

Epigenetic changes of the *HNF1B* gene, especially epigenetic silencing, have also been reported in several types of human cancers. In general, aberrant methylation of promoter regions of certain tumour suppressor genes is one of the key events in tumour growth initiation and progression. Hypermethylation of the promoter region of *HNF1B* has even been suggested as a possible non-invasive epigenetic marker of colorectal carcinoma [42]. According to this study, *HNF1B* was one of the five genes with the highest average percentages of promoter hypermethylation, along with RUNX3, PCDH10, SFRP5 and IGF2. There are other studies which also mention epigenetic inactivation of *HNF1B* in colorectal cancer cell lines, suggesting its involvement in cancerogenesis [43].

Partial methylation was observed in 3/72 (4.1%) analysed carcinomas and 1/21 (4.7%) adenomas in our study. This single methylated adenoma was a high grade tubulovillous adenoma which despite the methylation showed a strong, diffuse immunohistochemical positivity of HNF1B. Protein expression of HNF1B was also retained in partially methylated carcinomas. Methylation of the promoter did not correlate with HNF1B protein expression, which could be a result of an incomplete inactivation of the *HNF1B* gene. In our sample set of colorectal lesions, the *HNF1B*-promoter methylation was therefore not a frequent phenomenon, which is in accordance with the data gained from The Cancer Genome Atlas (TCGA), which shows very low degrees of methylation (Online Resource 4) in their colorectal carcinoma cohort. On the other hand, these results are in stark contrast with the aforementioned previous findings [42, 43]. However, in the study published by Silva et al. the authors tested only 10 tumours in total and used a different methodical approach from ours (Methyl-Profiler™ DNA Methylation PCR Array System), which may provide an explanation for the significantly different results. The study conducted by Terasawa et al. only examined *HNF1B* methylation in exon 1, which might have produced different results from ours since in our study we tested promoter methylation upstream of exon 1.

To our knowledge, there is very little information on the role HNF1B may play in the development of lesions of the large intestine, specifically colorectal cancer and colonic adenomas. As has already been mentioned, HNF1B is involved in the differentiation of visceral endoderm and therefore in the development of colonic epithelial cells, especially their terminal differentiation and cell fate commitment [44]. In fact HNF1B is reported to be abundant in normal intestinal epithelium, where it cooperates with CDX2 in order to direct key transcriptional programs involved in the dynamic status of intestinal epithelium [45]. Studies on a mouse model showed that while HNF1A deficiency gave rise to mice which were born normally but suffered from functional defects, germline HNF1B embryonic deficiency was found to be lethal, as it leads to a defective differentiation of extraembryonic visceral endoderm [46].

In keeping with these findings, our results did indeed show that there is a strong, diffuse nuclear positivity of HNF1B in the normal colonic mucosa, and this trend is also observed in the staining of colonic adenomas with either low- or high-grade dysplasia. Statistically, there were no differences between high

grade and low-grade adenomas. However, interestingly, in some cases of high-grade adenomas there was an apparent decrease in staining in the high-grade areas when compared to the low-grade areas and normal epithelia. This is particularly interesting given the differences in staining between carcinomas and adenomas, where adenomas showed statistically significantly higher levels of expression than carcinomas ($p < 0.001$). HNF1B positivity was positively correlated with a benign diagnosis, suggesting that in the setting of the large intestine the role of this transcription factor may be in the form of a tumour suppressor and HNF1B may have a protective effect. In our study, on the protein level we observed the highest expression in carcinomas of the T1 stage (tumours invading to the submucosa, mean H-score 216, compared to mean H-score 173 in the T2 stage, 195 in T3 stage and 177 in T4 stage cases). This finding was also observed in TCGA colorectal cohort (Online Resource 4), where the highest expression on the mRNA level was also reported in the T1 stage, although the correlation of those data does not reach statistical significance. Furthermore, lower values of H-score were associated with a recurring disease ($p = 0.043$). It has been suggested that HNF1B could act as a control switch, which in healthy, non-tumour tissue prevents epithelial-mesenchymal transition [21]. This hypothesis is further supported by their finding that the key biological processes related to HNF1B and HNF1B-related gene network (consisting of over 30 genes, especially FLRT3 and SLC14A1) are chemotaxis and cadherin-mediated adhesion to the extracellular matrix. The downregulation of HNF1B in advanced stages of colorectal carcinoma and in recurrent disease could therefore be attributed to a more invasive phenotype of the disease, brought on by a decrease of HNF1B functional effect. This hypothesis is further supported by the results of our survival analyses, which showed that there was a statistically significant association between HNF1B expression and DFS ($p = 0.014$), where lower levels of HNF1B expression were associated with adverse outcome and shorter DFS. However, neither of the other two other monitored outcomes (LFS and MFS) reached significant values.

When focusing on the histological type of studied adenocarcinomas, the analysis of TCGA data revealed that there was a significantly ($p = 0.022$, Online Resource 4) lower expression of HNF1B on mRNA level in the group of mucinous carcinomas when compared to the non-mucinous types. Our data set was limited by the small number of mucinous carcinomas ($n = 11$) and the statistical results did not reach significant levels ($p = 0.469$). Nonetheless, they do suggest a similar trend and also hint at the lower levels of HNF1B expression in mucinous carcinomas.

The detected lower expression of HNF1B in colorectal carcinomas when compared to adenomas could represent a consequence of a defective intestinal cell differentiation, which is reportedly coordinated by a complex network of transcription factors, among which HNF1, CDX2 and GATA4 are the most important [45]. The suggested protective effect of HNF1B in colorectal carcinoma would place CRC, together with chromophobe RCC and WT, amongst the handful of solid cancers, where this tumour suppressive effect has been proposed [1, 35]. In contrast with this group, there is a much larger list of cancers where HNF1B was reported to act as a protooncogene. The most important among these are clearly the tumours characterised by the clear appearance of the cytoplasm, namely OCCC and RCC. It has been suggested that HNF1B may be induced within the stressful environment of endometriotic cysts, leading to alterations in intracellular metabolism and enhanced aerobic glycolysis, which results in a significant survival advantage of cancerous cells, as well as the chemoresistant phenotype of this cancer [47]. Other tumours in this group include prostate carcinoma, yolk sac tumour (YST), hepatocellular carcinoma (HCC) and oncogenic potential is also attributed to HNF1B in malignant glioma [9, 48, 49].

Conclusion

Apart from the role HNF1B plays in the pathogenesis of certain developmental disorders, in recent years it is also being discussed as a tissue-specific tumour suppressor or an oncogene which plays an important role in the development and progression of several tumours. We have performed a comprehensive analysis of the involvement of HNF1B in the setting of benign and malignant lesions of the large intestine, focusing especially on the expression, epigenetic and genetic changes in colorectal carcinoma and its possible use in differential diagnosis or when assessing prognosis. We have revealed infrequent somatic mutations and promoter methylation in our cohort. We have also found that on a protein level, higher HNF1B expression is significantly associated with colonic adenomas when compared to carcinomas. From a prognostic standpoint we observed that there is a significant relationship between the levels of HNF1B expression and recurrence, where a lower expression seems to be associated with a recurring disease. Decreased expression was associated with a decreased DFS. These results suggest that in the context of colorectal cancer, HNF1B may in fact play a part in the pathogenesis and act in a tumour suppressive fashion. However, more studies on larger cohorts are needed to precisely understand the mechanisms at play and the true possible significance of HNF1B in the lesions of large intestine.

Acknowledgements This work was supported by Ministry of Health, Czech Republic (Research project AZV 17-28404A and Conceptual development of research organization 64165, General University Hospital in Prague), by Charles University (Project Progress Q28/LF1 and SVV 260367), and by European Regional Development Fund (CZ.02.1.01/0.0/0.0/18_046/0015959; BBMRI_CZ LM2018125, and OPPK - Research Laboratory of Tumor Diseases, CZ.2.16/3.1.00/24509). Special thanks belong to Zachary H.K. Kendall, B.A. for the English editing.

Author Contributions All authors contributed to the study conception and design. Material preparation, samples and clinical data collection: Michaela Bártů, Vladimír Frýba. Preparation and analysis of samples: Michaela Bártů, Kristýna Němejcová, Nikola Hájková, Jan Hojný, Eva Krkavcová, Karol Simon. Statistical analyses and data interpretation: Romana Michálková, Ivana Stružinská, Michaela Bártů, Kristýna Němejcová, Pavel Dundr. Writing – original draft preparation: Michaela Bártů, Nikola Hájková, Jan Hojný, Ivana Stružinská, Pavel Dundr, Romana Michálková. Writing – review and editing: Ivana Stružinská, Pavel Dundr, Kristýna Němejcová. All authors discussed the results, commented on the manuscript, and approved the final manuscript.

Data Availability The source data are included in this article and its supplementary Online Resource files are available from the corresponding author upon reasonable request.

Compliance with Ethical Standards

Conflict of Interests The authors declare no competing financial and/or non-financial interests.

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