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ORIGINAL ARTICLE

CpG methylation of ubiquitin carboxyl-terminal hydrolase 1 (*UCHL1*) and *P53* mutation pattern in sporadic colorectal cancer

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Abstract The ubiquitin-proteasome system plays an essential regulatory role in various cellular processes. Besides its involvement in normal cellular functions, the alteration of proteasomal activity contributes to the pathological states of several clinical disorders, including cancer. Aberrant methylation of the CpG islands has been reported as an alternative way to inactivate gene expression involved in the ubiquitination process and thus protein degradation in tumor tissues. In this study, we aimed to determine the CpG methylation pattern of the *UCHL1* promoter, as well as the mutation spectrum and the expression pattern of *P53* in sporadic colorectal cancer (CRC) from Tunisian patients. We found that *UCHL1* was methylated in 68.57 % and correlated significantly with lymph node metastasis (P=0.029) and transcriptional silencing in tumor tissues (P=0.013). Mutation screening of exons 5–9 of *P53* showed that 42.85 % of cases harbor somatic mutation and are positively correlated with the methylated pattern of *UCHL1* (P=0.001). Furthermore, cytoplasmic accumulation of P53 was strongly associated with the unmethylated *UCHL1* profile (P=0.006), supporting the relationship between these two proteins in CRC.

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Introduction

Colorectal cancer (CRC) is the second most common cancer with 1.2 million new cases/year worldwide [1]. In Tunisia, the incidence of CRC is 2.5–4.5/100,000 [2]. Over the past decade, the prognosis for CRC has been improved through advances in treatments. However, only 30–40 % of patients are diagnosed at an early stage [3]. In most CRC patients, the progression of normal colonic mucosa to invasive cancer requires several molecular changes [4]. Chromosomal and microsatellite instability pathways constitute the major genetic instability events in CRC [5, 6], and aberrant methylation of cancer-related gene promoters is often responsible for transcriptional silencing of tumor suppressor genes (TSG) in tumor tissues [7–9]. Gene promoter's methylation has been extensively explored, and reliable biomarkers have been identified in CRC [7, 8]. On the other hand, CpG DNA methylation often occurs at an early stage during CRC carcinogenesis; therefore, the identification of specifically methylated genes in patients compared to controls could be used for early diagnosis of cancerous lesions. Furthermore, high levels of circulating methylated DNA released by tumor cells have been detected in the peripheral blood of CRC patients, which could serve as biomarkers for tumor cell detection in a non-invasive manner contrary to colonoscopy [10, 11]. Hence, it is useful to make efforts for the identification of epigenetic biomarkers in CRC.

Protein ubiquitination system is responsible for the selective degradation of most intracellular proteins. It has emerged not only as one of the most multifaceted post-translational modifications, which plays an essential regulatory role in many critical cellular processes, but also contributes to tumor

initiation and progression [12]. Hence, targeting the ubiquitin-proteasome pathway constitutes a novel developing concept in cancer therapy [13]. Ubiquitin C-terminal hydrolase-L1 (*UCHL1* or *PGP9.5*), a member of the UCH class of DUBs, is one of the most well-studied DUBs in view of its association with neurodegenerative pathologies and other malignancies [14]. Normally expressed in neurons, the neuroendocrine system, and gonads, aberrant expression of *UCHL1* has been described in non-small cell lung cancer [10, 15], invasive CRC [11], osteosarcoma [16], and esophageal cancer [17]. Overexpression of *UCHL1* was associated with tumor size and invasiveness of neoplastic cells [11, 18]. The expression level of *UCHL1* is either upregulated or downregulated mainly by methylation of CpG islands, depending on the type of tumor. In fact, hypomethylation of the *UCHL1* promoter leads to increased expression in bladder cancer [19]. In contrast, aberrant methylation conducting to transcriptional silencing of the *UCHL1* gene has been described in esophageal squamous cell carcinoma and gastric cancer [20, 21].

P53 is among the most commonly affected TSG during the pathogenesis of human cancers, hence the name of universal TSG. P53 acts as a transcription factor regulating the expression of several genes involved in the control of cell cycle, DNA damage, and apoptosis [22, 23].

The *P53* gene is mutated in about half of human cancers, and in CRC, somatic mutations are found in 40 to 50 % of samples [24, 25]. Regarding its role as a regulator of gene expression, the P53 levels must be strictly controlled in the cells mainly through its interaction with mdm2 [22]. Recent studies tend to find the link between *P53* and *UCHL1* in carcinogenesis. Li et al. showed that UCHL1 could deubiquitinate P53 and p14ARF and ubiquitinate mdm2 for P53 stabilization to promote P53 signaling in nasopharyngeal cancer cells [26]. In breast cancer, it was reported that the *UCHL1* unmethylated pattern correlated with accumulation of P53 in primary sporadic breast tumors [27].

This study was conducted to elucidate whether the *UCHL1* and *P53* genes are associated in the pathogenesis of CRC in Tunisian patients in order to identify novel biomarkers of colorectal carcinogenesis, facilitating personalized therapy. To this end, we analyzed the epigenetic alteration of the *UCHL1* promoter and the mutational status of the *P53* gene in 35 tumors and their association with major clinical parameters. Correlation between P53 expression and *UCHL1* methylation was also investigated.

Material and methods

Patient's characteristics

A total of 35 primary sporadic adenocarcinomas were collected between January 2003 and December 2007 from patients who underwent radical surgical resection at the Department of Digestive Surgery of Habib Bourguiba University Hospital (Sfax, Tunisia). The study was performed in accordance with the ethical standards of the revised Declaration of Helsinki (October 2013), and all patients gave informed consent prior to specimen collection according to institutional guidelines. None of the patients had preoperative or postoperative chemotherapy. At the time of surgery, the age of patients ranged from 35 to 82 years (mean 66.08 years), and the sex ratio was 1:1.05. The histological subtypes were classified according to the World Health Organization criteria. The carcinomas were staged according to the TNM (tumor, lymph node, and metastasis) classification adopted by the American Joint Committee on Cancer.

DNA extraction and methylation-specific PCR

Genomic DNA was isolated from tissue sections by phenol/chloroform procedure as described previously [28], and the quantity was checked by Nanodrop (Thermo Fisher Scientific). DNA samples were stored at −20 °C for further use. For methylation-specific PCR (MSP), 1 μg of DNA was treated with sodium bisulfite which converts the unmethylated cytosine to uracil using the EZ Methylation Kit according to the manufacturer's recommendations (ZymoResearch). The bisulfite-treated DNA was amplified using specific primers for methylated and unmethylated *UCHL1* alleles. The sequences of the primers, annealing temperature, and product size are listed in Table 1. The MSP condition used was described previously [29]. Briefly, after initial denaturation at 95 °C for 5 min, 35 cycles of 30 s at 94 °C, 30 s at optimal annealing temperature, and 30 s at 72 °C were done, followed by a final extension at 72 °C for 10 min. The reactions were performed in a total volume of 25 μl, containing 2 μl of bisulfite-treated DNA, 0.2 μM of each primer, 200 μM dNTP, 1× PCR buffer, and 1 unit of Dream Taq DNA polymerase (Fermentas). The amplified products were analyzed by electrophoresis on 2 % agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

RNA extraction and RT-PCR

Total RNA was isolated from 35 fresh frozen tumor tissues using TRIzol reagent [30]. First-strand cDNA synthesis was performed on 2 μg of total RNA, treated with DNaseI (Amersham Biosciences), 0.5 μg oligo dT, 2 mM dNTP, 0.5 unit/μl RNase inhibitor (Fermentas), 4 μl of 5× RT buffer, and 200 units of MMLV reverse transcriptase (Fermentas). The reaction mixture was incubated at 37 °C for 1 h, followed by 70 °C for 10 min. The cDNA (2 μl) was used as a template in PCR using specific primers for *UCHL1* and *GAPDH* (Table 1). The PCR products were analyzed on 2 % agarose

Table 1 Summary of primer sequences, annealing temperatures, product size, and number of cycles used in the MSP, RT-PCR, and PCR conditions

Gene		Sequence (5′–3′)	Annealing T (°C)	PCR product (bp)	PCR cycles
MSP					
External PCR					
UCHL-1	F	GAAAGGATGGGTTTCCAGAAACT	58	410	40
	R	AAGGCAAAACCGAACCGATC			
Internal PCR					
UCHL-1 (M)	F	TTTATTTGGTCGCGATCGTTC	60	175	35
	R	AAACTACATCTTCGCGAAACG			
UCHL-1 (U)	F	GGGTTTGTATTTATTTGGTTGT	52.5	184	35
	R	CTTAAACTACATCTTCACAAAACA			
RT-PCR					
UCHL-1	F	AGCTCAAGCCGATGGAGATC	58	211	40
	R	CCCTTCAGCTCTTCAATCTG			
GAPDH	F	ACCCACTCCTCCACCTTTG	58	188	40
	R	GAGACAGAATGGAGGTGCTGC			
P53 PCR					
5–6	F	CTGTTCACTTGTGCCCTGACTTTC	60	477	40
	R	CAACCACCCTTAACCCCTCCTCCC			
7–9	F	CCCCTGCTTGCCACAGGTCTCCCCA	55	865	40
	R	TAGACTGGAAACTTTCCACTTGAT			

M methylated DNA, *U* unmethylated DNA, *F* forward primer, *R* reverse primer

gel, stained with ethidium bromide, and visualized under UV light.

Mutation analysis

Exons 5–6 and 7–9 of *P53* gene were amplified in PCR reactions containing 0.2 μM of each primer (Table 1), 200 μM dNTP, 2 mM $MgCl_2$, 1× PCR buffer, 1 unit of Dream Taq DNA polymerase (Fermentas), and 100 ng of DNA in a total volume of 25 μl for 2 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at annealing temperature, 30 s at 72 °C, and then 7 min at 72 °C. PCR products were purified and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI 3130 Genetic Analyser (Applied Biosystems, CA, USA).

Immunohistochemical staining and scoring

Before immunostaining, two pathologists (LA and AK) reviewed hematoxylin- and eosin-stained slides in each case, and blocks containing adenocarcinoma were selected. Briefly, 4-μm-thick sections were cut from each paraffin block, mounted on poly-L-lysine-coated slides, fixed in acetone for 10 min, and left to dry overnight at 37 °C. Slides were deparaffinized in xylene followed by ethanol and subsequent rehydration in graded ethanol. The sections were then pretreated with 3 % hydrogen peroxide for 10 min to inactivate endogenous peroxides and washed in phosphate-buffered saline (PBS) solution. Heat-induced antigen retrieval was performed using epitope retrieval solution (DAKO) at 95 °C for 40 min. After heating, slides were allowed to cool down to room temperature, briefly washed in PBS, and then incubated in blocking solution (protein block serum: 0.25 % casein in PBS containing carrier protein and NaN_3; DAKO) for 5 min. Immunohistochemical staining was performed using the streptavidin-biotin peroxidase system. Slides were incubated for 30 min at room temperature with anti-P53 (Pab 1801: sc-98; Santa Cruz Biotechnology) antibodies diluted at 1:100, washed with PBS before applying the biotinylated secondary antibody (anti-rabbit, DAKO) for 5 min. Sections were incubated with the streptavidin-biotin complex reagent (Universal Quick Kit, DAKO) for 15 min and developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 30 min. Finally, tissue sections were counterstained by Mayer's hematoxylin, dehydrated, and mounted (DAKO).

The immunostained slides were scored as described previously [31]. Briefly, the initial scoring was graded according to the extent of immunostaining as follows: 0: no staining or less than 5 %; 1: 5–25 % staining; 2: 26–75 % staining; and 3: 76–100 % staining. In addition, the intensity of staining was also evaluated as follows: 0: negative; 1: weak; 2: moderate; and 3: intense. In regard to the variability in the intensity of staining, each component of the tumor was scored independently. The sum of the intensity and staining extent scores was used as the final immunoreactive score (0–12). The final scores were regarded as negative (0–1 score) and positive (2–12 score).

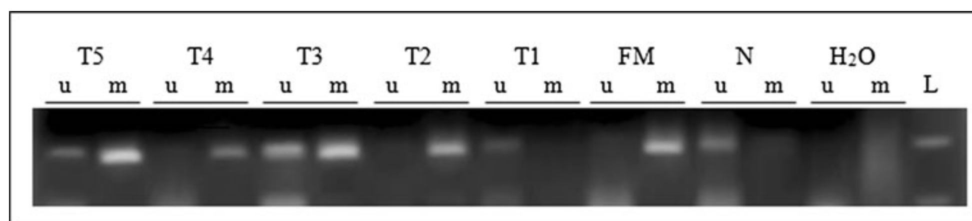

Fig. 1 Representative results of MSP of UCHL1 promoter methylation status in CRC cases. *H2O* negative control for MSP, *T1–T5* tumor DNAs, *FM* fully methylated DNA, *N* non-tumor DNA, *L* 100-bp DNA ladder (Fermentas)

Statistical analysis

The statistical significance of the association between *UCHL1* methylation and *P53* expression and mutational status, as well as clinicopathological parameters, was assessed by a two-sided chi-square test. When needed, Fisher's exact test correction was used in order to validate the chi-square test results. For that statistical analysis, SPSS software version 20 was used.

Results

Methylation profile of the *UCHL1* promoter and correlation with clinicopathological parameters

The *UCHL1* methylated allele was detected in 68.57 % of cases. A representative example of the MSP results is shown in Fig. 1. Using univariate analysis, we analyzed whether clinicopathological characteristics such as age, gender, tumor stage, grading, or localization of the tumor are associated with the methylation status of *UCHL1*. Except for one significant association with lymph node metastasis (P=0.029; t=0.005), no other associations were observed (Table 2).

Correlation of mRNA and methylation status of *UCHL1*

In an attempt to validate the effect of aberrant methylation on the expression of *UCHL1*, we performed semi-quantitative RT-PCR on 35 CRC cases displaying the unmethylated or methylated profiles using the *GAPDH* as internal control (Fig. 2a). We found that among 24 cases with the methylated pattern, the *UCHL1* mRNA was detected in only 5 (20.8 %) cases (P=0.013; t= 0.022; Fig. 2b). Therefore, we suggest that the CpG methylation is a major event responsible for the silencing of *UCHL1* in CRC.

P53 mutation and expression analysis: association with the *UCHL1* methylation pattern

Mutation screening of exons 5–9 of the *P53* gene showed that 15 out of 35 cases (42.85 %) were mutated (Table 3). One mutation was novel: the p.D184EfsX61 (c.552_555del4) in exon 5 (Fig. 3a). Interestingly, the p.R196Q (c.587G>A) mutation in exon 6 was identified in 8 out of 15 patients (Fig. 3b). Besides mutation, several polymorphisms were identified and are listed in Table 3. Furthermore, a positive association was

Table 2 Associations between methylation of the UCHL1 gene promoter and clinicopathological features of CRC patients

Clinicopathological parameters	Total	Methylation status		*P* value
		M (%)	U (%)	
Subjects	35	24 (68.57)	11 (31.43)	
Gender				0.803
Male	18	12 (66.7)	6 (33.3)	
Female	17	12 (70.6)	5 (29.4)	
Age at diagnosis (year)				0.189
≤60	8	7 (87.5)	1 (12.5)	
>60	27	17 (63)	10 (37)	
Tumor size (cm)				0.632
<5	17	11 (64.7)	6 (35.3)	
≥5	18	13 (72.2)	5 (27.8)	
TNM stage				0.127
I+II	12	6 (50)	6 (50)	
III+IV	14	11 (78.6)	3 (21.4)	
Lymph node				0.029
N0	20	11 (55)	9 (45)	
N1	11	10 (90.9)	1 (9.1)	
Vascular invasion				0.155
No	19	14 (73.7)	5 (26.3)	
Yes	5	2 (40)	3 (60)	
Tumor grade				0.148
Well	14	7 (50)	7 (50)	
Poor/moderate	13	10 (76.9)	3 (23.1)	
Tumor site				0.547
Right	7	4 (57.1)	3 (42.9)	
Left	26	18 (69.2)	8 (30.8)	
P53				0.001
Mutant	16	15 (100)	0	
Wild-type	19	9 (45)	11 (55)	
MSI				0.101
MS-low/MS-stable	13	9 (69.2)	4 (30.8)	
MSI-high	7	7 (100)	0	
BRAF				0.918
Mutant	22	14 (63.7)	8 (36.3)	
Wild-type	3	2 (66.7)	1 (33.3)	
KRAS				0.815
Mutant	19	15 (78.9)	4 (21.1)	
Wild-type	6	5 (83.3)	1 (16.7)	

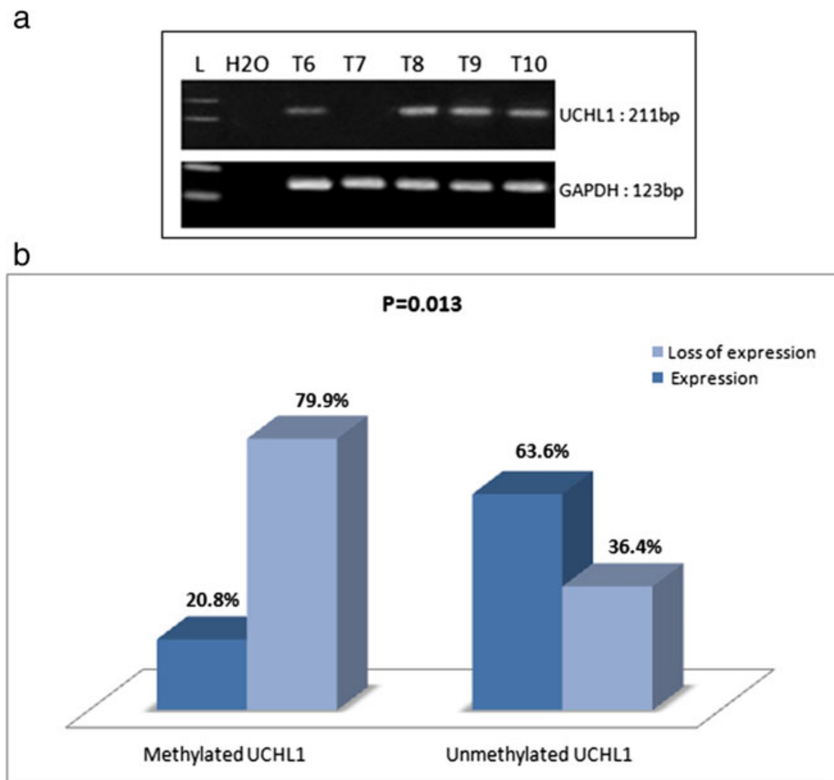

Fig. 2 **a** Representative results of RT-PCR of UCHL1 in CRC cases. GAPDH was used as an endogenous control. *H2O* negative control for RT-PCR, *T1–T5* tumor DNAs, *L* 100-bp DNA ladder (Fermentas), *T7* absence of mRNA, *T6*, *T8–T10* presence of mRNA. **b** Histogram representing the methylation profile and the mRNA expression of UCHL1 in tumor samples

seen between the unmethylated profile of the *UCHL1* promoter and the mutated *P53* gene (P=0.001; t=0.001; Table 1). On the other hand, we studied by IHC the expression of P53 in the 35 CRC specimens analyzed by MSPCR. Aberrant cytoplasmic accumulation of P53 was seen in 57.1 % (20 out of 35) of tumors and significantly correlated with the unmethylated UCHL1 promoter (Fig. 4). In fact, among 15 tumor tissues negative for P53 expression, 14 (58.3 %) were methylated for *UCHL1* (P=0.006; t=0.009), suggesting a relationship between these two proteins in CRC. No association was noted between the P53 immunopositivity and the presence of mutation.

Discussion

Epigenetic modification of the CpG islands in the gene promoter regions has been proposed as an alternative way to inactivate several cancer-related genes in tumor tissues [32]. Recently, many lines of evidence indicated that *UCHL1* is involved in tumor progression. However, the *UCHL1* roles differ depending on the type of cancer. In fact, it has been shown that in some cancers, *UCHL1* acts as an oncogene [18, 33, 34], while in others, it would function as a TSG [26, 35, 36]. In CRC, many lines of evidence indicate that *UCHL1* is related to tumorigenesis, but the mechanism is still ambiguous. Zhong et al. provided evidence that *UCHL1* functions as an oncogene by regulating the β-catenin/TCF pathway [37]. However, other studies have shown that *UCHL1* was more frequently methylated in CRC tissues than in normal colorectal tissues [38], whereas others have indicated that high *UCHL1* expression

Table 3 Mutations in the P53 gene in CRC primary tumors

Case	Validated SNP	Mutation
69	p.D184N (exon 5)	p.L201X (c.601del1) (exon 6)
73	p.D184N (exon 5)	p.R196Q (c.587G>A) (exon 6)
75	p.D184N (exon 5)	p.R196Q (c.587G>A) (exon 6)
77		p.D184EfsX61 (c.552_555del4) (exon 5)[a]
78	p.D184N (exon 5)	p.R196Q (c.587G>A) (exon 6)
81		p.N200KfsX8 c.597_598ins1 (exon 6)
82	p.D184N (exon 5)	p.T231S (c.691A>T) (exon 7)
83	p.D184N (exon 5)	p.T329A (c.985A>G) (exon 9)
87	p.D184N (exon 5)	p.R196Q (c.587G>A) (exon 6)
89	p.D184N (exon 5)	p.R196Q (c.587G>A) (exon 6)
90	p.D184N (exon 5)	p.V157A (c.470 T>C) (exon 5)
		p.R196Q (c.587G>A) (exon 6)
91		p.N288S (c.863A>G) (exon 8)
92	p.D184N (exon 5)	p.R196Q (c.587G>A) (exon 6)
93	p.D184N (exon 5)	p.R196Q (c.587G>A) (exon 6)
94		p.T211FfsX3 (c.630_631del2) (exon 6)

[a] Novel P53 frameshift mutation

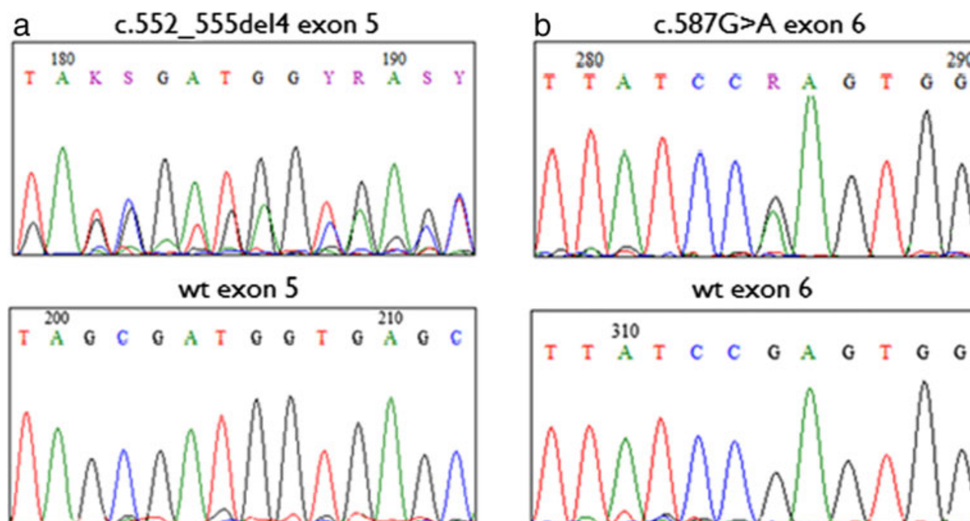

Fig. 3 Chromatograms showing the novel P53 mutation p.D184EfsX61 (c.552_555del4) in exon 5 (**a**) and the recurrent P53 mutation p.R196Q (c.587G>A) in exon 6 (**b**)

was related to colorectal tumor progression, invasion, lymph node metastasis, and poor clinical outcome [39]. In this study, we showed that *UCHL1* is frequently methylated (68.57 %), leading to transcriptional silencing, since a significant correlation was found between the methylated *UCHL1* gene promoter and the loss of its corresponding mRNA (P=0.013; t=0.022). Our results are in agreement with several studies claiming that methylation of *UCHL1* is a common event in colorectal tumors as well as in cell lines of colon cancer [20, 35]. Okochi-Takada et al. showed aberrant methylation of *UCHL1* in 47 % of primary colorectal tumors, and a demethylating treatment with 5-azacitidine resulted in the restoration of its expression in the cell lines [35]. In another study, the percentage of methylation was higher (73 %) in primary colorectal tumors and correlated with lymph node metastasis [20]. In our study, CpG methylation of the *UCHL1* promoter was significantly more frequent in patients with lymph node metastasis than in N0 patients (P=0.029; t=0.05). This result is consistent with the fact that UCHL1 expression is related to lymph node metastases in colorectal patients as previously reported [21, 38]. Regarding its deubiquitinase activity, UCHL1 is involved in different signaling pathways. It was recently shown that in the HCT8 cell line, UCHL1 upregulated the expression of β-catenin by decelerating its degradation [37]. These authors showed that UCHL1 induction increased the accumulation of β-catenin in the cytoplasm and subsequently its translocation into the cell nucleus and activate target genes such as cyclin D1 which is a major regulator of cell proliferation [39]. As a guardian of genome, P53 is strongly regulated, mainly through a feedback loop by MDM2 [40]. The link between UCHL1 and P53 has been reported in breast and nasopharyngeal cancers [26, 27, 41]. It was shown that UCHL1 activates the p14ARF-P53 signaling pathway by deubiquitinating P53 and p14ARF as well as ubiquitinating

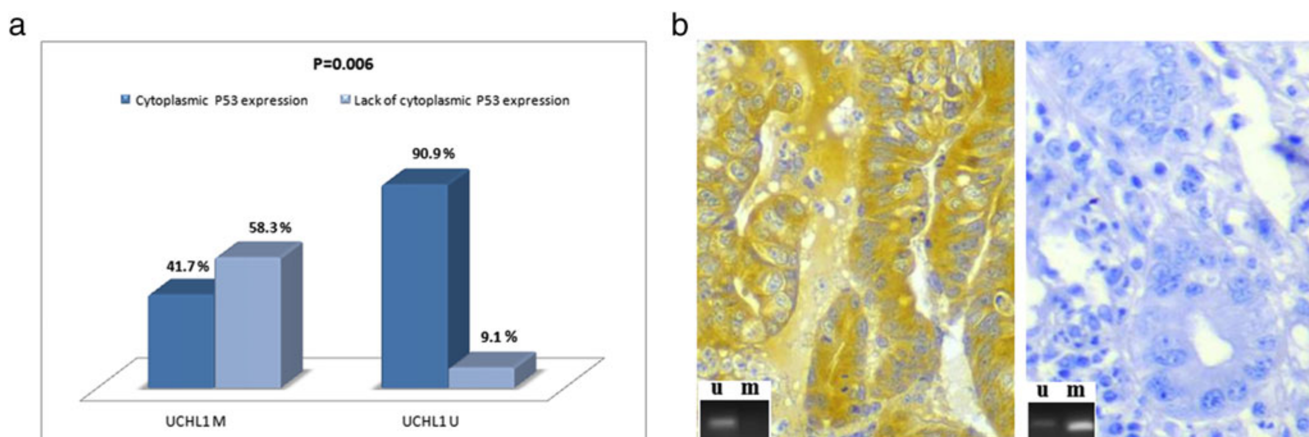

Fig. 4 P53 immunohistochemical staining and methylation status of UCHL1. **a** Histogram representing the cytoplasmic expression of P53 protein depending on the methylation profile of UCHL1 in tumor samples (P=0.006). **b** P53 immunoexpression in tumor tissues showing strong cytoplasmic accumulation and unmethylated UCHL1 (*left panel*) and negative expression and methylated UCHL1 (*right panel*)

MDM2 through its two opposing enzyme activities, hydrolase and ligase [41]. We compared the *UCHL1* methylation status with P53 expression in tumor tissues. Positive association was seen between cytoplasmic accumulation of P53 and the unmethylated *UCHL1* profile. Indeed, among 35 cases, 20 displayed positive immunostaining and 90 % (10 out of 11 cases) of them were unmethylated for the *UCHL1* promoter (P=0.006; t=0.015). Our result supports previous data on breast and nasopharyngeal carcinoma; nevertheless, we will need to confirm it in a larger number of Tunisian CRC patients.

It was reported that the *P53* gene is frequently mutated in CRC cases. The mutations are predominantly of the missense type, located in the DNA binding domain, which leads to the loss of P53 function as a transcription factor [24]. In our series, the *P53* gene is mutated in 42.85 % of cases, which is in line with the rates recorded by the majority of the studies [42–44]. According to previous reports, the mutational spectrum of *P53* in Tunisian patients is largely variable. In fact, while Chaar et al. found that *P53* is mutated in only 17.4 % of patients [45], Aissi et al. showed that *P53* mutations are more frequent, reaching 59.6 % [46]. In our study, among the identified mutations, one was novel: the p.D184EfsX61 (c.552_555del4) located in exon 5. The recurrence of the p.R196Q (c.587G>A) mutation in exon 6 was highly interesting since it has been identified in 8 out of 16 patients. This mutation is likely to be frequent in southern areas of Tunisia as it has not been described in patients from north Tunisia [45, 46].

In summary, we found that *UCHL1* is frequently methylated in Tunisian patients with sporadic CRC and that aberrant methylation correlated with lymph node metastasis. We also observed positive associations of both *P53* expression and mutation in tumor tissues with the unmethylated pattern of the *UCHL1* promoter, supporting the evidence that UCHL1 exerts its tumor-suppressive functions through P53 deubiquitination in colon tumorigenesis. It is well established that the half-life of mutant p53 is increased compared to the wild-type form mainly due to translational modifications of specific residues which prevents its interaction with mdm2, thus escaping p53 degradation. However, the question that remains to be resolved is if UCHL1 can deubiquitinate the mutant p53 in order to increase its stability. This hypothesis can be tested on CRC cell lines expressing either wild-type p53 or the mutant form in the presence of unmethylated UCHL1 in order to confirm our data obtained on primary tumor tissues.

Consequently, our results showed that UCHL1 methylation could be useful as a biomarker for tumor invasion, but we believe that a larger study will allow us to confirm our conclusions.

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Conflicts of interest None

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