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ORIGINAL PAPER

Frequent CpG methylation of ubiquitin carboxyl-terminal hydrolase 1 (UCHL1) in sporadic and hereditary Tunisian breast cancer patients: clinical significance

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Abstract Aberrant methylation of the CpG islands in promoter regions is one of the mechanisms for inactivation of tumor suppressor genes in many human cancers including breast carcinoma. In this study, we aimed to assess, by methylation-specific PCR, the CpG methylation pattern of the UCHL1 promoter in 94 sporadic and 44 hereditary breast cancers from Tunisian patients. The percentage of UCHL1 methylation was 67 % in sporadic and 82 % in hereditary breast cancer cases. In sporadic cases, UCHL1 methylation correlated with poor response to treatment ($P = 0.042$) and progesterone receptor status ($P = 0.036$), whereas in patients with hereditary predisposition, the only significant association was found with Her2 expression ($P = 0.024$). Moreover, in patients with sporadic breast cancer, the UCHL1 unmethylated pattern conferred a prolonged overall survival time in particular in the group of patients with advanced TNM stage of the disease (P log rank $= 0.04$). Aberrant CpG methylation of the UCHL1 promoter was significantly associated with transcriptional silencing of this tumor suppressor gene in sporadic breast cancer tissues ($P = 0.001$). On the other hand, the UCHL1 unmethylated pattern correlated with P53 positivity in primary sporadic tumors ($P = 0.032$), supporting the functional link between the two tumor suppressors in breast tumorigenesis.

Keywords UCHL1 · Methylation · Breast cancer · Transcriptional silencing · P53

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Introduction

Breast cancer is the most common malignancy and the major cause of cancer mortality of women worldwide [1]. Although in recent years major improvement in the diagnosis and treatment of breast cancer has been done, early detection methods are still limited. Inactivation of tumor suppressor genes is the major mechanism for tumor development [2], through either genetic or epigenetic alterations or a combination of both [3]. Epigenetic silencing of tumor suppressor genes, including promoter CpG island methylation and histone modifications, leads to the disruption of tumor suppressor gene functions and involves in tumor development [4]. Hypermethylation of gene promoters has been explored as both a mechanism and marker of tumorigenesis including breast cancer [5–7]. Thus, identification of more epigenetically disrupted TSGs in breast cancer is needed.

Ubiquitination has emerged as one of the most versatile of post-translational modifications, with roles in the regulation of various cellular processes such as cell cycle control, DNA damage repair, and membrane trafficking [8]. However, deregulation of protein ubiquitination contributes to tumor initiation and progression [9, 10]. Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1 or PARK5/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 conditions, including Parkinson's

disease, and a wide range of malignancies [11]. The role of UCHL1 in tumorigenesis has been complex, from tumor suppressive to oncogenic, depending on the tumor type [12–16]. Aberrant methylation of the UCHL1 gene promoter has been reported in multiple tumors, including esophageal [17], gastric [18], renal [19], prostate [20], head and neck squamous cell carcinoma [21], hepatocellular [14], ovarian [22], and colorectal cancers [14, 23], supporting a critical role of UCHL1 in tumor suppression. In fact, it was reported that UCHL1 exerts tumor suppressor activities by targeting the p53 deubiquitination and further activating p53 signaling, thus inhibiting cell proliferation and inducing apoptosis of NPC, HCC, and other carcinoma cells [14, 24]. In breast cancer cell lines and primary tumors, Xiang et al. [25] have recently shown that UCHL1 is epigenetically silenced and acts as a functional tumor suppressor by inhibiting cell proliferation and inducing apoptosis. Conversely, it has been reported that high UCHL1 mRNA level is significantly associated with aggressive phenotype, such as high histological grade and negative ER/PR, and has a poorer prognosis than those with a low UCHL1 mRNA level [26]. Therefore, the role of UCHL1 in breast tumorigenesis and its potential as tumor marker remains unclear.

Our study aimed to assess the methylation status of the UCHL1 promoter as well as its epigenetic silencing in sporadic and hereditary breast cancer in Tunisian patients. Further, CpG methylation of the UCHL1 was correlated with the clinicopathological parameters and the overall survival of patients. Finally, we have examined the relationship between UCHL1 methylation and the expression of P53.

Materials and methods

Tumor specimens

A total of 94 women with sporadic breast cancer and 44 unrelated hereditary cases from the south area of Tunisia were included in our study. The group of hereditary cases presented a mean age of 49.3 years, with an age range of 24–80 years, and included women with the following features, based on the Breast Cancer Linkage Consortium criteria [27]: early onset ($\leq$40 years) and/or bilaterality; or more than three cases of breast cancer in the family; or more than one case of ovarian cancer in the family; or more than two first-degree relatives involved; or male breast cancer in the family. The group of sporadic cases presented a mean age of 51.6 years, with an age range of 25–83 years. Frozen tissues from sporadic and hereditary cases were collected at the Departments of Radiotherapy and Anatomo-Pathology from Habib Bourguiba Hospital of Sfax (Tunisia). All patients were graded according to the modified Scarff-Bloom-Richardson system, and the clinical stage was determined according to the TNM classification. All patients gave informed consent prior to specimen collection according to institutional guidelines.

DNA extraction and MSP analysis

Genomic DNA was isolated from fresh frozen tissues using TRIzol according to the manufacturer's protocol [28]. The quantity of DNA was checked by spectrophotometer and stored at −20 °C for further use.

For methylation-specific PCR (MSP) assays, DNA samples (1–2 μg) were treated by sodium bisulfite which converts the unmethylated cytosine to uracil using the EZ Methylation Kit according to the manufacturer's recommendations (Zymo Research). The bisulfite-treated DNA was amplified using specific primers for methylated and unmethylated alleles. The sequence of the primers, annealing temperature, and product size are listed in Table 1.

The amplification of UCHL1 gene was carried out in a thermal cycler (Applied Biosystem) with initial denaturation at 95 °C for 10 min followed by 35 cycles (30 s at 95 °C, 30 s at 55.7 °C for the methylated fragment and 58 °C for the unmethylated fragment, and 30 s at 72 °C) and a final extension at 72 °C for 10 min. The reactions were performed in a total volume of 25 μL, containing 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.

Sequence analysis

PCR products were purified by the SpinKlean^{TM} Gel Extraction Kit (Biomatick) and then sequenced with the ABI Prism^{R} 3100 Genetic Analyzer (Applied Biosystems, USA) using the Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems). Sequence analyses were performed by BioEdit software.

RNA extraction and RT-PCR (reverse transcription-PCR)

Total RNA was isolated from fresh frozen tissues using TRIzol [28]. First-strand cDNA synthesis was performed on total RNA, previously treated with DNase (Amersham Biosciences), using 2 μg of RNA, 0.5 μg oligo dT, 2 mM dNTP, 0.5 unit/μL RNase inhibitor (Amersham Biosciences), 4 μL of 5 × RT buffer, and 200 units of MMLV reverse transcriptase (Fermentas). The reaction mixture

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

Gene		Sequence (5′–3′)	Annealing temperature (°C)	PCR product (bp)	PCR cycles
MSP					
UCHL1 (M)	F	TTTATTTGGTCGCGATCGTTC	55.7	175	35
	R	AAACTACATCTTCGCGAAAACG			
UCHL1 (U)	F	GGGTTTGTATTTATTTGGTTGT	58	184	35
	R	CTTAAACTACATCTTCACAAAACA			
RT-PCR					
UCHL1	F	AGCTCAAGCCGATGGAGATC	55	211	40
	R	CCCTTCAGCTCTTCAATCTG			
GAPDH	F	GCTCTCTGCTCCTCCTGTTC	60	123	40
	R	CGCCCAATACGACCAAATCC			

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

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

Immunohistochemistry

Immunostaining of ER, PR, Her2/neu, and P53 was performed as described previously [29]. Briefly, 4 μm sections attached on silanized slides were dewaxed in xylene, rehydrated in ethanol, covered with 10 mM citrate buffer (pH 6), and heated in a microwave oven for two consecutive 10 min periods, at 500 W. Tissues sections were incubated for 30 min with primary monoclonal antibodies against ER (Dako, clone 1D5, 1:25), PR (Dako, clone PgR636, 1:50), Her2/neu (Dako, clone 124, 1:100), and p53 (clone DO-7, Dako Cytomation 1:50) followed by incubation with biotin-labeled secondary antibody. 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, tissues sections were counterstained by Mayer's hematoxylin, dehydrated, and mounted (Dako). Stained tissues were scored under the light microscope, and the intensity of staining was scored on the basis of the approximate percentage of positive tumor cells and the relative immunostaining intensity as described previously [29].

Statistical analyses

Statistical analyses were performed using the SPSS 17 statistical software for Windows. The two-sided $\chi 2$ test was used to determine associations between the methylation status of gene promoter and various clinicopathological features. The correlation with overall survival was performed using Kaplan–Meier survival plots, and the significance was tested using the log rank test. $P < 0.05$ was considered statistically significant.

Results

Methylation profile of UCHL1 gene promoter

To investigate the CpG methylation of the UCHL1 promoter, we analyzed by MSP 94 sporadic and 44 hereditary breast cancer specimens. The prevalence of the promoter methylation status of UCHL1 was 67 % and 82 % for patients with sporadic and hereditary breast cancer, respectively. The difference between the percentage of UCHL1 methylation in sporadic and hereditary cases was near to the statistically significance value ($P = 0.08$). A representative example of the MSP products of bisulfite-treated samples, using primers for specific methylated and unmethylated sequences of UCHL1 promoter, was shown in Fig. 1a. This was confirmed by sequencing of the UCHL1 promoter region of tumor DNAs with the methylated and unmethylated profiles (Fig. 1b).

Association of UCHL1 promoter methylation with clinicopathological parameters

Table 2 summarizes the relationship between gene promoter methylation of UCHL1 and clinicopathological features. For sporadic breast cancer patients, there was a significant correlation of UCHL1 promoter hypermethylation with a poor response to treatment ($P = 0.042$) and progesterone receptor status ($P = 0.036$). On the other

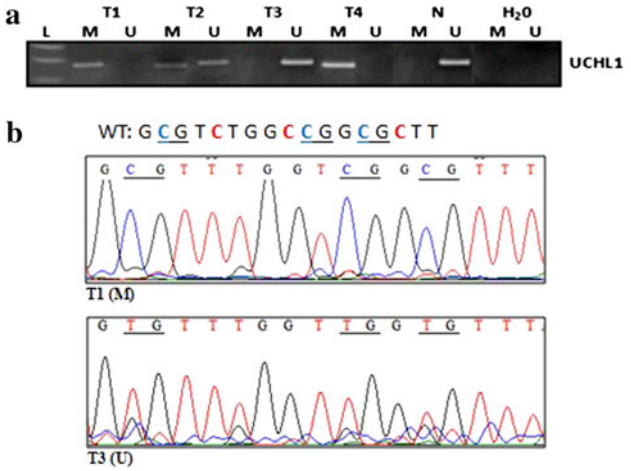

Fig. 1 **a** Representative example of MSP for UCHL1 gene promoter in breast cancer patients. The PCR product in lanes M shows the presence of methylated templates of UCHL1 gene, whereas the product in lanes U indicates the presence of unmethylated templates. L: 100 bp DNA ladder (Fermentas); T1-4: tumor samples; N: nontumoral sample; H_2O: negative control; T1, T4: methylated, T2: hemimethylated, T3, N: unmethylated. **b** Sequence analysis of the UCHL1 promoter upon bisulfite modification. WT: wild-type sequence; T1 (M): methylation of CpG-bit point of cytosine unchanged; T3 (U): the nonmethylated CpG sites of cytosine into thymine

hand, for hereditary breast cancer cases, the only significant association was observed between CpG methylation of UCHL1 and loss of Her2 expression ($P = 0.024$).

Association of UCHL1 promoter methylation with patient survival

For sporadic cases, the survival time was available for only 46 patients and the follow-up time ranged from 3 to 119 months. The Kaplan–Meier plot illustrated a reduction in overall survival for the patients with methylated UCHL1 status (P log rank $= 0.078$, Fig. 2a). In addition, if we consider the group of patients with advanced TNM stage (III + IV), the methylated UCHL1 status becomes significantly related to a shortened patients' survival rate (P log rank $= 0.04$, Fig. 2b). The COX regression analysis revealed that tumor size ($P = 0.038$, 95 % CI $= 1.1$–494), TNM stage ($P = 0.046$, 95 % CI $= 1.06$–701.3), and progesterone receptor status ($P = 0.03$, 95 % CI $= 0.001$–0.669) were dependant factors for prognosis (Table 3). The association of UCHL1 methylation profile with prognosis was not maintained in the multivariate analysis. No relationship between UCHL1 methylation profile and survival was found in patients with hereditary breast cancer.

Table 2 Associations between methylation of UCHL1 gene promoter and clinicopathological features of sporadic and hereditary breast cancer patients

Sporadic cases				Hereditary cases		
	Total	M (%)	U (%)	Total	M (%)	U (%)
Cases	94	63 (67)	31 (33)	44	36 (82)	8 (18)
Age at diagnosis						
≤50	49	32 (65.3)	17 (34.7)	23	20 (87)	3 (13)
>50	42	29 (69)	13 (31)	21	16 (76.2)	5 (23.8)
P value		0.705			0.355	
Tumor size						
≤3 cm	45	31 (68.9)	14 (31.1)	21	18 (85.7)	3 (14.3)
>3 cm	46	30 (65.2)	16 (34.8)	14	10 (71.4)	4 (28.6)
P value		0.710			0.301	
Grade						
I + II	67	45 (67.2)	22 (32.8)	23	19 (82.6)	4 (17.4)
III	24	16 (66.7)	8 (33.3)	8	6 (75)	2 (25)
P value		0.965			0.639	
TNM Stage						
I + II	32	19 (59.4)	13 (40.6)	14	12 (85.7)	2 (14.3)
III + IV	44	31 (70.5)	13 (29.5)	14	11 (78.6)	3 (21.4)
P value		0.315			0.622	
Lymph node						
N0	38	25 (50)	13 (50)	18	16 (88.9)	2 (11.1)
N+	53	36 (52.8)	17 (47.2)	16	12 (75)	4 (25)
P value		0.831			0.289	
Metastasis						
M0	36	28 (70)	12 (30)	21	18 (85.7)	3 (14.3)
M+	19	12 (60)	8 (40)	13	10 (76.9)	3 (23.1)
P value		0.439			0.513	
Survival						
≤3 years	15	10 (66.7)	5 (33.3)	18	14 (77.8)	4 (22.2)
>3 years	31	22 (71)	9 (29)	10	9 (90)	1 (10)
P value		0.766			0.418	
Response to treatment						
Positive	38	9 (23.7)	29 (76.3)	22	18 (81.8)	4 (18.2)
Negative	20	10 (50)	10 (50)	6	5 (83.3)	1 (16.7)
P value		**0.042**			0.932	
ER status						
Positive	55	36 (65.5)	19 (34.5)	15	12 (80)	3 (20)
Negative	36	25 (69.4)	11 (30.6)	12	10 (83.3)	2 (16.7)
P value		0.692			0.825	
PR status						
Positive	52	39 (56.9)	22 (43.1)	19	16 (84.2)	3 (15.8)
Negative	36	29 (78.4)	8 (21.6)	8	6 (75)	2 (25)
P value		**0.036**			0.574	
Her2 status						
Positive	19	14 (73.7)	5 (26.3)	6	3 (50)	3 (50)
Negative	54	32 (59.3)	22 (40.7)	21	19 (90.5)	2 (9.5)
P value		0.263			**0.024**	

M methylated DNA, *U* unmethylated DNA, *ER* estrogen receptor, *PR* progesterone receptor

Bold values indicate statistically significant *P* value

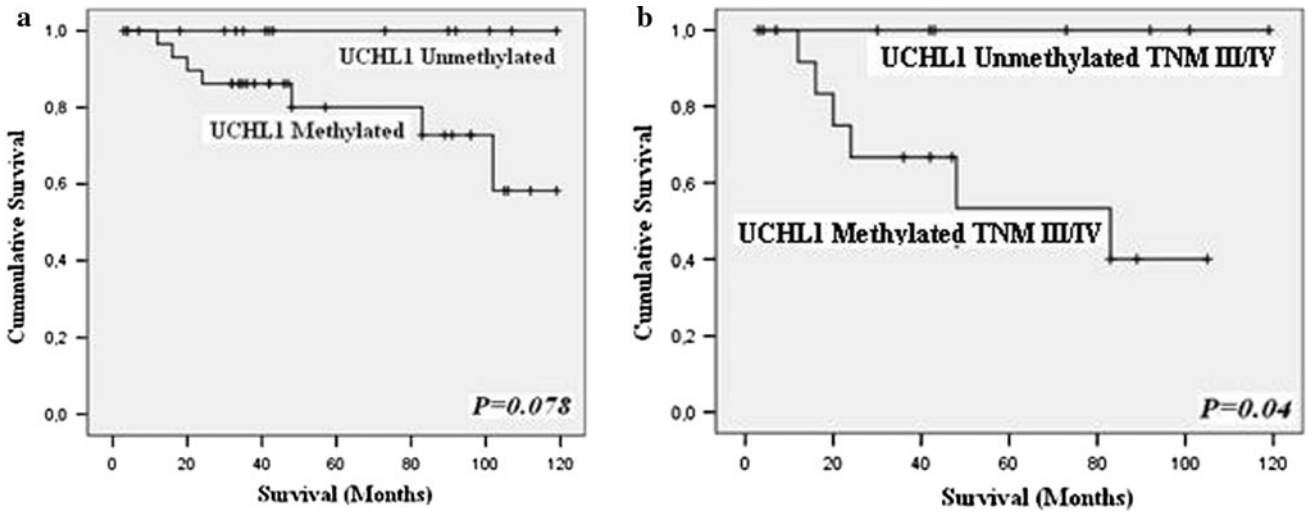

Fig. 2 Kaplan–Meier survival curves correlating overall survival with UCHL1 methylated and unmethylated profiles in all patients **a** and in a group of patients with TNM III + IV **b**

Table 3 Multivariate survival analysis in sporadic breast cancer patients: Cox proportional hazards model

Clinical features	*P*	HR	95 % CI	
			Lower	Upper
Age (≤ 50 vs > 50)	0.817	1.273	0.165	9.81
Tumor size (≤ 3 vs > 3)	**0.038**	24.336	1.1	494
TNM stage (I + II vs III +IV)	**0.046**	27.7	1.06	701.3
RE (positive vs negative)	0.165	0.158	0.012	2.136
RP (positive vs negative)	**0.030**	0.025	0.001	0.669
Her2 (positive vs negative)	0.883	1.385	0.018	104.588
P53 (positive vs negative)	0.527	0.522	0.07	3.91

Bold values indicate statistically significant *P* value

Analysis of mRNA expression of UCHL1 in sporadic breast cancer tumors

In attempt to validate the effect of aberrant methylation on the expression of UCHL1, we performed RT-PCR analysis on 50 available sporadic breast cancer tumors using the GAPDH as internal control (Fig. 3). Analysis of mRNA expression of UCHL1 resulted in a strong correlation between the MSP and RT-PCR ($P = 0.001$). In fact, among 30 cases with the methylated pattern, only eight (26.7 %) expressed the UCHL1 mRNA (Fig. 4). Therefore, we suggest that the CpG methylation is a major event responsible for the silencing of UCHL1 in breast cancer.

Relationship between UCHL1 methylation and P53 expression in breast cancer

As it has been shown previously that the UCHL1 stabilizes the P53 through its deubiquitination, we thought to

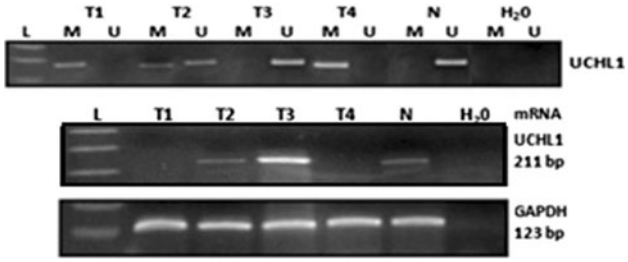

Fig. 3 Representative results of MSP and RT-PCR for UCHL1 in primary breast cancer. GAPDH was used as an endogenous control. T1, T4: tumor samples with methylated pattern and absence of UCHL1 mRNA. T2: tumor sample with hemimethylated pattern and weak level of UCHL1 mRNA. T3: tumor sample with unmethylated pattern and presence of UCHL1 mRNA. N: nontumoral sample. Lanes U and M correspond to unmethylated and methylated DNAs, respectively. H_2O: negative control for MSP and RT-PCR. L: 100 bp DNA ladder (Fermentas)

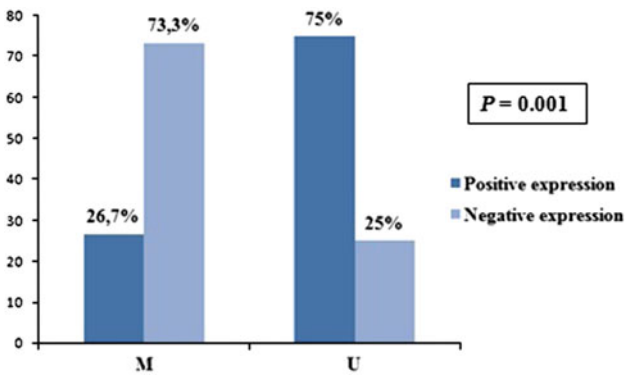

Fig. 4 Histogram representing the methylation profile and the mRNA expression of UCHL1 in tumor samples

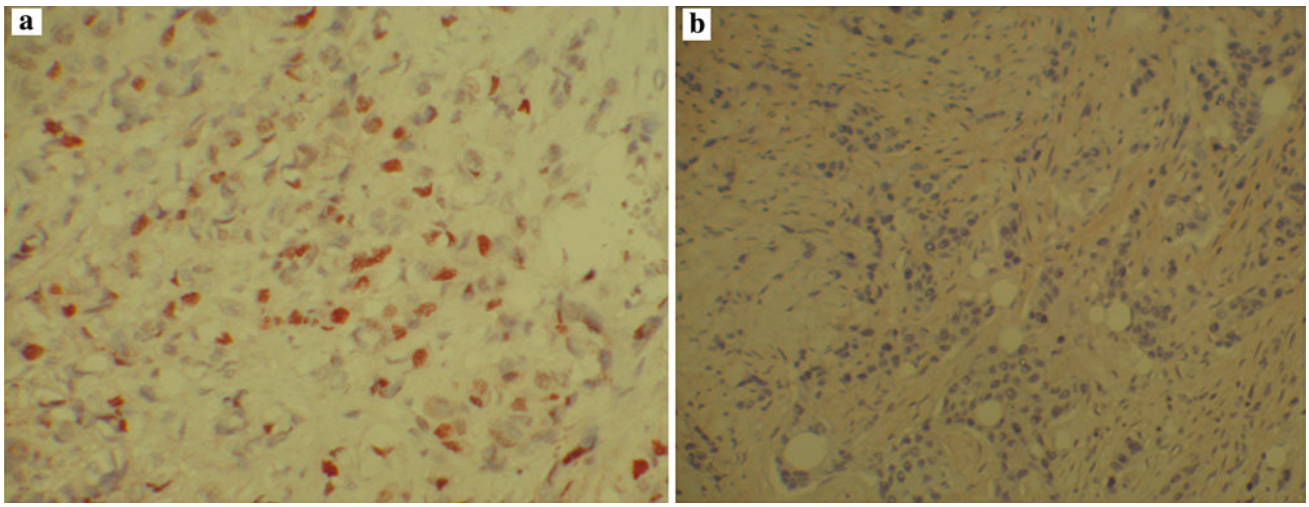

Fig. 5 Immunohistochemical staining for positive (**a**) and negative (**b**) P53 expression in breast cancer

Table 4 Association between P53 expression and UCHL1 DNA methylation status in sporadic and hereditary cases

Methylation status of UCHL1 promoter	P53 expression	Sporadic cases		Hereditary cases	
		Positive	Negative	Positive	Negative
Methylated		18 (41.9 %)	25 (58.1 %)	7 (36.8 %)	12 (63.2 %)
Unmethylated		16 (69.6 %)	7 (30.4 %)	1 (100 %)	0 (0 %)
P value		**0.032**		0.209	

Bold values indicate statistically significant *P* value

examine the possible correlation between the UCHL1 methylation pattern and P53 expression. Immunohistochemical analysis of P53 expression showed that 51.5 and 40 % of samples are positive for sporadic and hereditary breast cancer, respectively (Fig. 5). Interestingly, in sporadic breast cancer, we observed that positive P53 immunostaining correlated with the unmethylated UCHL1 pattern. Indeed, 69.6 % of unmethylated pattern were positive for P53 expression; however, only 30.4 % were negative for P53 expression ($P = 0.032$). This was confirmed by the association between UCHL1 mRNA expression and P53-positive immunostaining in tumor biopsies ($P = 0.0001$). In hereditary breast cancer, this association is also observed without reaching the statistical significance due to the small number of specimens ($P = 0.209$; Table 4).

Discussion

Promoter DNA methylation has been associated with the regulation of the expression pattern of many tumor suppressor genes in breast cancer [30, 31]. In this study, we analyzed the CpG methylation pattern of the UCHL1 promoter in sporadic and hereditary primary breast tumors. We found that UCHL1 is frequently methylated in primary sporadic breast tumors and statistically correlated with poor response to treatment and progesterone receptor (PR) status, which is concordant with the report of Xiang et al. In fact, they have found that promoter methylation of UCHL1 was detected in 9 of 10 breast cancer cell lines (90 %) and 53 of 66 (80 %) primary tumors, but rarely in normal breast tissues, which was statistically correlated with advanced clinical stage and progesterone receptor status [25]. In hereditary cases, the percentage of methylated UCHL1 promoter was higher than sporadic cases (82 % against 67 %, $P = 0.08$) and correlated with loss of Her2 expression. Regarding the prognostic relevance of the UCHL1 methylation pattern, we found that the overall survival is reduced for patients with methylated UCHL1 status in particular in a group of patients with advanced TNM stage of sporadic breast cancer.

Several lines of evidence showed that the ubiquitin–proteasome system (UPS) is altered in cancer and particularly the deubiquitinating enzymes (DUB). Indeed, in vitro studies demonstrated that UCHL1 stimulates

oncogenic transformation and invasion in nonsmall cell lung carcinoma [32] and colorectal cancer cells [33], suggesting that UCHL1 may function as an oncogene. Furthermore, it has been reported that overexpression of UCHL1 in HCT8 colorectal cancer cells enhances cell migration resulting in distant metastasis [34]. Moreover, Kim et al. [32] demonstrated that siRNA-mediated knockdown of UCHL1 reduces H157 lung carcinoma cancer cells migration in vitro and decreases lung metastasis in a murine xenograft model. Conversely, in primary tumors, silencing of UCHL1 through aberrant CpG methylation has been reported in multiple tumors, such as esophageal [17], gastric [18], renal [15, 19], prostate [20], nasopharyngeal [24], and colorectal cancers [22, 23], suggesting a potential tumor suppressor role for UCHL1. In breast cancer, the role of UCHL1 is still ambiguous. Although high expression of UCHL1 was reported to predict early recurrence in patients with invasive breast cancer [26], other studies supported that UCHL1 functions as tumor suppressor in breast cancer [25, 35]. Indeed, Xiang et al. [25] have found that UCHL1 was frequently down regulated in breast cancer cell lines and tumor tissues and have demonstrated that ectopic UCHL1 expression in breast tumor cells suppresses cell growth and induces G0/G1 arrest and apoptosis. Moreover, it has been found that overexpression of UCHL1 induces apoptosis in MCF-7 cells probably through the PIK3/Akt pathway [35]. Our results are in line with this finding since UCHL1 promoter was frequently methylated in both sporadic and hereditary breast cancer of Tunisian patients, and that this aberrant methylation was strongly correlated with the loss of expression of UCHL1 ($P = 0.001$), supporting its potential tumor suppressive role; however, other studies are needed to better define the role of UCHL1 in breast tumorigenesis.

Previous works demonstrated that UCHL1 could activate the p14ARF-p53 signaling pathway by deubiquitinating p53 and p14ARF as well as ubiquitinating MDM2, further resulting in its tumor suppressive role in NPC tumorigenesis [14, 24]. Recently, Xiang et al. [25] have found that UCHL1 induces P53 accumulation and reduces MDM2 protein level, and subsequently upregulates the expression of p21, as well as cleavage of caspase 3 and PARP, supporting its tumor suppressive function in breast tumorigenesis. In our study, we examined the expression of P53 by immunohistochemistry in 66 and 20 available sporadic and hereditary samples, respectively. Among the 66 specimens, P53-positive staining was observed in 51.5 % of cases (34 out of 66). Interestingly, a positive association was observed between P53 positivity and the unmethylated pattern of the UCHL1 promoter as well as with the presence of UCHL1 mRNA in sporadic cases.

In summary, we found that UCHL1 is frequently methylated in both sporadic and hereditary breast cancer in Tunisian patients. Aberrant methylation of the UCHL1 promoter correlated with poor response to treatment, progesterone receptor status, and shortened patients' survival rate for sporadic patients and with loss of Her2 expression for hereditary breast cancer patients. We also identified a positive association of P53 expression with the unmethylated pattern of the UCHL1 promoter and with the positive expression of UCHL1, supporting the evidence that UCHL1 exerts its tumor suppressive functions through P53 deubiquitination in breast tumorigenesis.

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Competing interests The authors declare that they have no competing interests.

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