Histopathology 2009, **55**, 77–90. DOI: 10.1111/j.1365-2559.2009.03330.x

The helicase-like transcription factor is a strong predictor of recurrence in hypopharyngeal but not in laryngeal squamous cell carcinomas

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Date of submission 25 July 2008
Accepted for publication 16 December 2008

Capouillez A, Debauve G, Decaestecker C, Filleul O, Chevalier D, Mortuaire G, Coppée F, Leroy X, Belayew A & Saussez S

(2009) *Histopathology* **55**, 77–90

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Aims: To examine the immunohistochemical expression of helicase-like transcription factor (HLTF) in relation to the prognosis of hypopharyngeal (HSCCs) and laryngeal (LSCCs) squamous cell carcinomas, and to characterize the HLTF protein variants expressed in biopsy specimens of head and neck squamous cell carcinoma (HNSCC) as well as the HeLa cell line.

Methods and results: HLTF expression was determined by immunohistochemistry on a series of 100 hypopharyngeal (stage IV) and 56 laryngeal SCCs (stages I, II and IV). The HLTF variants were defined using reverse transcriptase-polymerase chain reaction and Western blots in 13 fresh HNSCC biopsies and in HeLa cells. High levels of HLTF expression were associated with rapid recurrence rates in a subgroup of 81 stage IV hypopharyngeal SCCs (with complete follow-up). A 95-kDa HLTF variant truncated at the carboxyl-terminal domain was detected in addition to the 115-kDa full-size protein in HNSCC biopsies, while six variants were observed in HeLa cells.

Conclusions: Our results demonstrate, for the first time, that hypopharyngeal SCCs presenting high levels of HLTF have a worse prognosis. The quantitative determination of HLTF in hypopharyngeal SCCs was an independent prognostic marker alongside tumour node metastasis staging. HNSCCs expressed the truncated HLTF variant lacking the domains involved in DNA repair.

Keywords: head and neck carcinoma, HIP 116, HLTF, prognosis, SMARCA 3, SWI/SNF protein, ZBU 1

Abbreviations: ABC, avidin–biotin–peroxidase complex; AU, arbitrary unit; CA, carcinoma; DES, diethylstilbestrol; DMEM, Dulbecco's Modified Essential Medium; dNTP, deoxyribonucleotide triphosphate; FBS, fetal bovine serum; HLTF, helicase-like transcription factor; HNSCC, head and neck squamous cell carcinoma; HSCC, hypopharyngeal squamous cell carcinoma; LI, labelling index; LSCC, laryngeal squamous cell carcinoma; MOD, mean optical density; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcriptase; SDS, sodium dodecyl sulphate; SWI/SNF, mating-type switching/sucrose non-fermenting; TFE, tumour-free epithelium; TNM, tumour node metastasis; TNT, Transcription/Translation; UV, ultraviolet

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C.D. is a Senior Research Associate of the Belgian National Fund for Scientific Research.

Introduction

Head and neck squamous cell carcinoma (HNSCCs) is a significant cause of morbidity worldwide, with approximately 500 000 new cases diagnosed each year.[1–3] HNSCCs constitute a collection of diseases that, despite the common location (head and neck areas) and histology (SCCs), include various types of tumours that differ in their pathogenesis, biology, sub-location (i.e. hypopharynx or larynx), treatment, patient survival rate and impact on patients' quality of life.[2,3] Although significant progress has been made since combined treatments came into practice,[4,5] a number of statements remain valid concerning HNSCCS: (i) almost two-thirds of patients have advanced forms (stages III and IV) of the disease at diagnosis, (ii) 50% of patients die from their disease within 2 years of initial diagnosis, (iii) 5% of patients every year develop additional second primary tumours, and (iv) survival for patients with HNSCC has not improved in the past 30 years.[2,3,6] Therapies currently used (surgery, radiation and/or chemotherapy) have been associated with only modest improvements in patient survival. Novel approaches are thus required to provide head and neck oncologists with a more effective armamentarium against this extremely challenging disease. The application of analytical approaches based on genetics, proteomics and bioinformatics has facilitated the identification of critical genomic and proteomic changes in HNSCCs, several of which have been linked to clinical outcome.[6]

A workshop on research and therapeutic opportunities for HNSCCs came to an overall consensus that HNSCC is a relatively understudied malignancy, and further investigations focusing on the biology of this tumour have the potential to influence significantly the prevention and treatment of this type of tumour.[2]

Helicase-like transcription factor (*HLTF*) is a member of the mating-type switching/sucrose non-fermenting (SWI/SNF) family implicated in chromatin remodelling. HLTF has been shown to be involved in cancer progression in different ways either through epigenetic silencing by DNA methylation or through overexpression.[7] *HLTF* promoter methylation was first reported by Moinova *et al.*[8] in all colonic cancer cell lines and in 43% of primary colonic cancers. Other studies have confirmed *HLTF* promoter hypermethylation in human colorectal cancers,[10–14] and other groups have described the same features in gastric,[15–18] oesophageal[19] and uterine cancers.[20] Based on the finding that *HLTF* methylation was detected in only one oesophageal carcinoma out of 40 samples, one might consider that HLTF is not a common target for methylation in oesophageal SCC.[19] In sharp contrast to this epigenetic silencing, Gong *et al.*[21] have demonstrated that *HLTF* transcript levels were higher by approximately 20-fold in a variety of transformed cell lines, compared with those in non-transformed human fibroblast cells or human heart tissue. Unk *et al.*[22] have demonstrated that human HLTF is an ubiquitin ligase that targets proliferating cell nuclear antigen. HLTF can complement the ultraviolet (UV) sensitivity of *rad5*– yeast cells, thus strongly supporting a role for HLTF in postreplication DNA repair.[22] We have previously investigated HLTF expression in a hamster model of kidney tumours induced by diethylstilbestrol (DES) and demonstrated that HLTF induction is detected very early (after 2 months of DES treatment) during tumour progression in small preneoplastic buds.[23]

Using computer-assisted microscopy, we investigated the prognostic value derived from quantitative determination of the levels of immunohistochemical expression of HLTF in a homogeneous series of 81 stage IV hypopharyngeal SCCs (HSCCs) and 56 stage I, II and IV laryngeal SCCs (LSCCs). Furthermore, because previous data have shown the expression of HLTF protein variants,[24] we investigated the nature of such variants expressed in a series of 16 fresh HNSCC biopsy specimens and the HeLa cell line using reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting.

Materials and methods

PATIENTS' CHARACTERISTICS

A total of 156 patients (including 100 with HSCC and 56 with LSCC) who underwent surgery aimed at curative tumour resection were studied. The patient database was obtained by retrospective compilation (January 1989–December 2001) from the records of the ENT Department at the Hôpital Claude Huriez (Lille, France). Surgical margins were evaluated for all patients. After CO_2 laser resection, the surgeon most frequently performed additional small resection to help the pathologist in analysis of the margin. Twenty percent of stage IV HSCC and LSCC patients presented positive margins as well as 10% of stage I and II LSCC patients. The description of tumour status was based on the histopathological grade of tumour differentiation [criteria defined in[25]] and the tumour node metastasis (TNM) staging classification.[26] Detailed information on the patient's age, gender, tumour histopathology, type of laryngeal or hypopharyngeal surgery, response to treatment at the primary tumour site as well as follow-up data up to last contact with the patient and the status of the disease at that time were available for 81 HSCC and 56 LSCC patients (Table 1). All 100 HSCC

Table 1. Clinical data

Variable	High-stage HSCCs Stage IV 81 cases	Low-stage LSCCs Stages I and II 40 cases	High-stage LSCCs Stage IV 16 cases
Age (years)			
Range	40–78	36–88	43–78
Average	55	57	57
Sex (cases)			
Male	76	40	16
Female	6		
Site (cases)			
Supraglottic area		5	9
Glottic area		30	
Supraglottic and glottic areas		5	4
Subglottic and glottic areas			3
Piriform sinus	61		
Postcricoid area	17		
Posterior wall	3		
Histological grade (cases)			
Well differentiated	42	34	10
Moderately differentiated	28	5	6
Poorly differentiated	11	1	
TNM stage (cases)			
T1N0M0		31	
T2N0M0		11	
T2N2M0	8		
T3N2M0	8		
T4N0M0	12		9
T4N1M0	7		3
T4N2M0	43		4
T4N3M0	3		
Tumour treatment (cases)			
Co_2 laser cordectomy		8	
Frontolateral laryngectomy		2	
Vertical partial laryngectomy		4	
Supracricoid partial laryngectomy		23	3
Supraglottic laryngectomy		3	

Table 1. (*Continued*)

Variable	High-stage HSCCs Stage IV 81 cases	Low-stage LSCCs Stages I and II 40 cases	High-stage LSCCs Stage IV 16 cases
Total laryngectomy			13
Partial pharyngolaryngectomy	9		
Total pharyngolaryngectomy	54		
Circular pharyngolaryngectomy	8		
Oesopharyngolaryngectomy	10		
Treatment of the neck (cases)*			
Functional neck dissection	**69**	19	23
Radical neck dissection	**49**	–	2
Recurrence (cases)			
Local recurrence	17	3	4
Distant recurrence	11	3	7
Follow-up			
Range (months)	2–122	2–130	5–74
Average (months)	37	43	30

*Some patients underwent bilateral neck dissection.

and 56 LSCC cases under study were from patients who had not undergone chemotherapy and/or radiotherapy before surgery. All stage IV HSCC and LSCC patients received additional postoperative radiotherapy. For stage I and II LSCC patients, four patients (10%) received postoperative radiotherapy because they had positive margins. This meant that both series of HSCCs and LSCCs in the study were homogeneous in terms of histopathological as well as clinical criteria. Patients suffering from SCCs localized at other sites of the head and neck area were excluded from the study. Patients were asked to participate (human biopsies) in the study as approved by the local Institutional Review Board. Written informed consent and a complete medical history were obtained from each patient.

ANTI-HLTF SERUM

Two human HLTF variants were expressed from the same open reading frame and differed only in the translation start site (Met1 or Met123).[24] A rabbit antiserum (ART2) specific for the HLTFMet1 variant was raised against a peptide (VIPPDDFLTSDEEVD) in the amino-terminal sequence missing in the shorter Met123 variant (residues 42–56) as previously described by Debauve *et al.*[22].

CELL CULTURE

HeLa cells were grown in Dulbecco's Modified Essential Medium (DMEM; BioWhittaker Europe, Verviers, Belgium) with Phenol Red, 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (DMEM–FBS) (supplements from BioWhittaker or Gibco-Invitrogen, Merelbeke, Belgium).

IMMUNOHISTOCHEMISTRY

All tumour samples were fixed for 24 h in 10% buffered formaldehyde, dehydrated and then embedded in paraffin. Immunohistochemistry was performed on 5-μm-thick sections mounted on silane-coated glass slides as recently detailed.[27] Before starting the immunohistochemistry protocol, dewaxed tissue sections were briefly exposed to microwave pretreatment in a 0.01-M citrate buffer (pH 6.0) for 2 × 5 min at 900 W. The sections were then incubated with 0.4% hydrogen peroxide solution for 5 min to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS; 0.04 M Na_2HPO_4, 0.01 M KH_2PO_4 and 0.12 M NaCl, pH 7.4) and successively exposed for 20 min to

solutions containing avidin (0.1 mg/ml in PBS) and biotin (0.1 mg/ml in PBS) to avoid false-positive staining resulting from endogenous biotin. After a thorough washing step with PBS, the sections were incubated for 20 min with a solution of 0.5% casein in PBS and sequentially exposed at room temperature to solutions of (i) specific primary anti-HLTF antibody; (ii) corresponding biotinylated secondary antibody (polyclonal goat antirabbit IgG); and (iii) avidin-biotin-peroxidase complex (ABC kit). Incubation steps were alternated with thorough washing steps to remove unbound proteins. Antigen-dependent presence of the peroxidase complex in the sections was visualized by incubation with the chromogenic substrates containing diaminobenzidine and H_2O_2. After rinsing, sections were counterstained with luxol fast blue and mounted with a synthetic medium. To exclude antigen-independent reactivity, the incubation step with primary/secondary antibodies was omitted from the protocol in the controls. In all cases these controls were negative. Moreover, competition with the HLTF immunogenic peptide completely suppressed the ART2 labelling (data not shown). The biotinylated secondary antibodies and the ABC kit came from DakoCytomation (Glostrup, Denmark).

COMPUTER-ASSISTED MICROSCOPY

After the immunohistochemical steps, the levels of HLTF expression were quantitatively determined using a computer-assisted KS 400 imaging system (Carl Zeiss Vision, Hallbergmoos, Germany) connected to a Zeiss Axioplan microscope as detailed previously.[27] For each microscopic field, we focused the analyses on the neoplastic cells or tumour-free epithelia (TFE) using computer-assisted morphometry after interactive identification. These tissue areas are delimited precisely with the computer mouse. 15 fields covering a surface area ranging from 60 000 to 120 000 μm^2 were scanned, after which the quantitative analysis of immunoreactivity for a given marker yielded data on two variables: the labelling index (LI), i.e. the percentage of immunopositive tissue areas, and the mean optical density (MOD), i.e. the intensity of reactivity of positive cells.[27]

RT-PCR AND CLONING IN *pCR4-TOPO*

Isolation of HeLa total RNA was performed using the Versagene RNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Total RNAs were retro-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Total RNA (1 μg) was incubated in a 20-μl volume with 2.5 μM anchored oligo$(dT)_{18}$ primers, 40 U Protector RNase Inhibitor, 1 mM deoxyribonucleotide triphosphate (dNTP) mix, Transcriptor RT Reaction Buffer 1× and 10 U Transcriptor Reverse Transcriptase. The mixture was incubated for 45 min at 50°C, 5 min at 85°C and then kept on ice.

The resulting cDNAs were amplified by two successive PCRs using one forward primer specific to the Met1 translation start codon (no. 1: 5′-ggg gta cca tgt cct gga tgt tca ag-3′) and either one of two reverse primers specific to the intron 21 sequence (no. 2: 5′-ttt ccc ctc aaa ttc acc ac-3′, outer; and no. 3: 5′-gcc agt ggt caa caa cag aa-3′, inner). Two microlitres of RT products were mixed with 10 μl PrimeSTAR Buffer 5× (Takara, Seraing, Belgium), 200 μM dNTPs, 15 pmol of primers 1 and 2, 1.25 U PrimeSTART HS DNA Polymerase, and water to a final volume of 50 μl. The cDNA was amplified using three-step thermocycling (98°C, 10 s; 55°C, 5 s; and 72°C, 4 min) following Takara's instructions. One microlitre of the PCR product was then amplified with primers 1 and 3 following the same procedure.

The PCR products were analysed by electrophoresis on a 1% agarose gel and ethidium bromide staining, and the DNA band of interest was then extracted and purified (QIAquick extraction kit; Qiagen, Valencia, CA, USA). In order to generate 3′-A overhangs, 2.5 U *Taq* Polymerase (Fermentas, Saint Leon-Rot, Germany), 5 μl 10× *Taq* Buffer and 20 μM dNTPs were added to 45 μl purified PCR product and incubated for 10 min at 72°C. This fragment was cloned into the *pCR4-TOPO* plasmid (Invitrogen, Carlsbad, CA, USA) and used for transformation into Top10 *Escherichia coli* following the manufacturer's instructions (TOPO TA cloning kit; Invitrogen). Twelve clones were selected, amplified in 5 ml liquid culture in LB/ampicillin (100 μg/ml) and the plasmid DNA was extracted with the Wizard Miniprep Kit (Promega, Madison, WI, USA). The sequences were determined by the dideoxy chain termination method with the Dye Terminator Cycle Sequencing quick start kit (Beckman Coulter, Fullerton, CA, USA) and with primers either in the vector or in HLTF cDNA. The reaction products were analysed on the Beckman CEQ2000 sequencer and aligned with Bioinformatics software available at the Belgian EMBnet node (Wemboss package, http://www.be.embnet.org/services/WEMBOSS).

IN VITRO TRANSCRIPTION/TRANSLATION

Two of the 12 clones described in the previous section, *pCR4-HLTFMet1ΔA* and *pCR4-HLTFMet1ΔB* (6569

and 6798bp, respectively), were used as templates to express the encoded protein variants [Transcription/Translation (TNT) Quick Coupled Transcription/Translation Systems; Promega]. The *pGEM-4Z-HLTF* positive control has been described previously[24] and encodes the HLTF Met1 and Met123 variants. First, 1 μg of plasmid DNA, 2 μl of[35] S-Cys, 40 μl TNT Quick Master mix and nuclease-free water up to a final volume of 50 μl were mixed and incubated at 30°C for 1.5 h. Then, 3 μl of the transcription/translation products were added to 17 μl of 1× sodium dodecyl sulphate (SDS) buffer (Fermentas)/1× Reducing Agent (Fermentas) mix, heated at 95°C and analysed by SDS–polyacrylamide gel electrophoresis (PAGE) (8%). After electrophoresis (1 h at 120 V), the gel was fixed for 30 min in an isopropanol:water:acetic acid (25:65:10) solution. The gel was then incubated for 30 min in Amplify solution (Amersham Pharmacia Biotech, Freiburg, Germany), dried (Air Dry; Bio-Rad Laboratories, Hercules, CA, USA) and finally exposed to an MR Kodak Biomax film at −80°C for 2 days.

IMMUNOPRECIPITATION/WESTERN BLOT

Tissues from HNSCC biopsy specimens were homogenized at 4°C in BugBuster Protein Extraction Reagent (Novagen, Darmstadt, Germany) with protease inhibitors added. Tissue homogenates were then centrifuged at 12 000 ***g*** for 20 min at 4°C, and the protein concentrations were measured using Bio-Rad protein assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Then, 800 μg of protein was resuspended in 990 μl lysis buffer and incubated overnight at 4°C in the presence of 100 μl of protein A-Sepharose suspension (64 mg per 500 μl lysis buffer; Amersham Pharmacia Biotech) for a preclearing step. After centrifugation, the supernatant was harvested and 10 μl ART2 antiserum was added and mixed by gentle rotation at 4°C for 1 h. The mixture was centrifuged and the pellet washed three times with 1 ml lysis buffer. Proteins were eluted in 19 μl SDS sample buffer and 1 μl 20× Reducing Agent (Fermentas), heated to 95°C for 5 min and separated by SDS–8% PAGE. After separation, the proteins were electrotransferred from the gel onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech) using an electrophoretic transfer cell (Trans-Blot SD Semi-Dry; Bio-Rad) at 24 V for 30 min. Non-specific protein-binding sites on the membranes were blocked for 1 h at room temperature using PBS, 5% ECL Advance Blocking Reagent (Amersham Pharmacia Biotech) and 0.2% Tween 20. Membranes were then incubated overnight at 4°C in ART2 primary antibody diluted 1:1000 in the blocking buffer described in the immunohistochemistry section above. After incubation, the membranes were washed three times in washing buffer (1× PBS and 0.2% Tween 20) and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat antirabbit antibodies (Amersham Pharmacia Biotech) diluted 1:15 000 in blocking buffer. Finally, after 30 s of incubation in the presence of the ECL kit (Amersham Pharmacia Biotech), immunoreactive bands were visualized by exposure of the membrane to a sensitive film (Hyperfilm ECL; Amersham Pharmacia Biotech). Biotinylated molecular weight markers were analysed in parallel for internal calibration (Bio-Rad Laboratories).

DATA ANALYSIS

Independent groups of quantitative data were compared using the non-parametric Kruskall–Wallis test (more than two groups) [described in 28: describing HLTF expression in TFE, dysplasia and carcinoma (CA)]. In the case of significant Kruskall–Wallis tests, post hoc tests (Dunn's procedure) were used to compare pairs of groups (to avoid multiple comparison effects). As previously described,[29] we applied a decision tree-based technique to determine threshold values on quantitative variables that could be used to discriminate between two groups of patients with different clinical courses such as patients without recurrence versus those with recurrence. Briefly, for each variable of interest (MOD or LI variable) this technique exhaustively investigates all the possible univariate splits between two observed values to identify the one that produces the greatest improvement in the process of distinguishing between the two groups of patients defined above. The selection of the best split from the set of possible candidate splits uses the Gini index, which is a measure of group mixture reaching a value of zero when discrimination is perfect (i.e. the two groups of interest are perfectly separated on the basis of the selected split). We then used standard Kaplan–Meier analysis and the Gehan-generalized Wilcoxon test to validate these thresholds.

All statistical analyses were carried out with Statistica software (Statsoft, Tulsa, OK, USA).

Results

EXPRESSION OF HLTF IN NORMAL EPITHELIUM AND CARCINOMAS

In a recent study, we investigated the immunohistochemical expression of HLTF in relation to the

progression of HSCCs and LSCCs (i.e. comparing TFE with dysplasia and CA) and reported that the levels of HLTF expression were very different during laryngeal tumour progression compared with hypopharyngeal tumour progression.[28])

Morphological examination of different tissue samples (including peritumoral TFE and CA) revealed that the immunoreactivity of HLTF was clearly different in carcinomas (HSCCs and LSCCs) with respect to TFE (Figure 1). In TFE, HLTF was found mostly in the cytoplasm of cells in the basal and suprabasal layers (65% of cases of HSCC TFE and 97% of cases of LSCC TFE).

Table 2 describes the MOD and LI data in hypopharyngeal and laryngeal TFE and CA. For the comparison between the HLTF expression in TFE and CA, we studied 100 cases of stage IV hypopharyngeal CA. However, we obtained the complete clinical data (recurrence and follow-up) for only 81 cases, used in the prognostic paragraph described below. As regards hypopharyngeal lesions, both immunohistochemical variables [intensity of immunoreactivity (MOD, *post hoc* comparison: $P = 0.0005$) and percentage of immunopositive areas (LI, *post hoc* comparison: $P = 0.01$)] that described HLTF expression levels were higher in CA than those in TFE. However, the intensity of reactivity characterizing HLTF expression decreased with laryngeal malignancies (MOD, *post hoc* comparison: $P = 0.03$) (Figure 1).

CONTRIBUTION OF HLTF TO THE PROGNOSIS OF HSCC RECURRENCE

We analysed the data distribution with the aim of discriminating between patients with and without recurrences (Figure 2A–D, Table 1). The use of a decision-tree technique made it possible to define

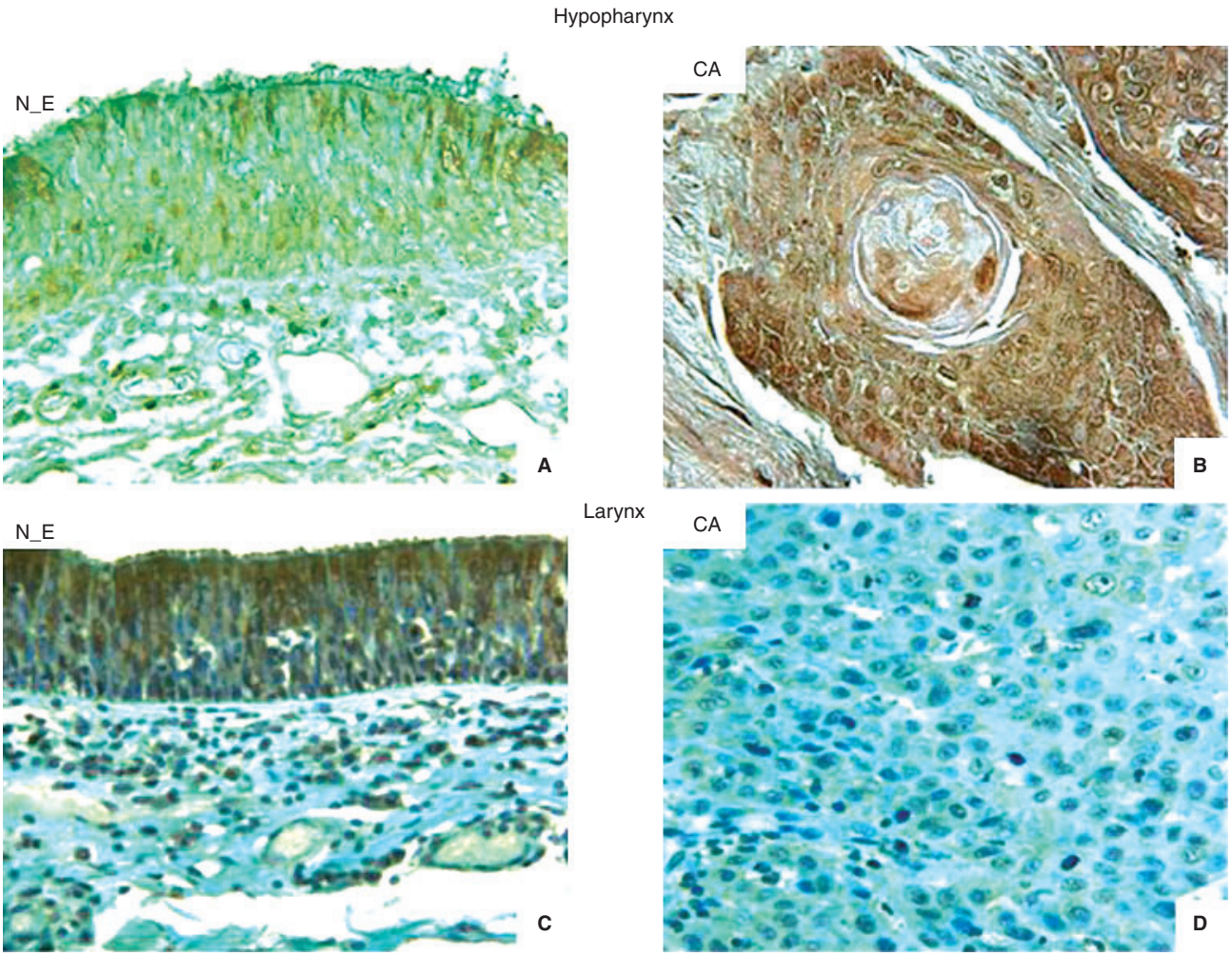

Figure 1. **A–D**, Immunohistochemical reactivity profile for helicase-like transcription factor in the hypopharynx (**A**,**B**) and larynx (**C**,**D**). The antigen was localized in normal epithelium (**A**,**C**) and in carcinomas (**B**,**D**).

Table 2. MOD and LI data in hypopharyngeal and laryngeal TFE and carcinomas

	Variables	Tumour-free epithelium	Carcinoma	*P*-values
Hypopharyx	MOD	72.7 ± 14.1 AU (27 cases)	89.2 ± 14.4 AU (100 cases)	0.0005
	LI	58.8 ± 26.5% (27 cases)	80.1 ± 11.4% (100 cases)	0.01
Larynx	MOD	118.9 ± 40.7 AU (33 cases)	95.7 ± 17.7 AU (56 cases)	0.03
	LI	18.9 ± 17.4% (33 cases)	19.2 ± 16% (56 cases)	NS

MOD, mean optical density; LI, labelling index; AU, arbitrary unit.

efficient thresholds on the basis of the two quantitative variables (LI and MOD) that characterize HLTF expression in each HSCC (see Materials and methods section[28]) (Figure 2A,B). These thresholds corresponded to 80 arbitrary units (AU) of immunoreactivity intensity (MOD variable) and 70% of the HLTF immunoreactive tissue (LI variable), and led to the identification of two groups of patients associated with significantly different risks of disease recurrence as shown by Kaplan–Meier analysis (Figure 2A,B). In fact, patients suffering from stage IV HSCC, who are characterized by a low HLTF levels (i.e. <70% of the LI variable and <80 AU of MOD variable), were associated with a significantly lower risk of recurrence than others (Figure 2C,D). The quantitative determination of HLTF in HSCCs was a prognostic marker alongside TNM staging because the study was a homogeneous series examining only stage IV tumours. Our analysis indicated no significant association with the clinical features (i.e. presence/absence of nodal metastases, margin status or tumour grading).

In LSCC patients, no statistical correlation was observed between HLTF expression (MOD and LI variables) and TNM status, the margin status or the tumour grading. The same quantitative variables (MOD and LI) did not disclose a significant prognostic value in terms of recurrence for patients suffering from LSCC (Figure 3). However, in a small series of 16 cases suffering from stage IV LSCC, we showed that low HLTF levels (i.e. <22% of the LI variable) tended to be associated with a higher risk of recurrence ($P = 0.06$) (Figure 3F). The same threshold (i.e. 22% of LI) did not disclose any significant correlation with the proportion of disease-free patients in our stage I and II LSSC patients (Figure 3E).

EXPRESSION OF HLTF VARIANTS IN HELA CELLS

The need to take into account the existence of protein variants, several of which could be detected with the antiserum we used, added an additional level of complexity in analysing HLTF expression. The two variants initially characterized (HLTFMet1 and Met123) resulted from an alternative translation initiation site in the same reading frame and differed in their amino-terminus.[24] Only the shorter one (Met123) showed transcriptional activity, suggesting that the amino-terminal domain could act as a transcriptional repressor.[24]

We amplified the HLTF mRNAs from HeLa cells by RT-PCR using a forward primer encompassing the Met1 translation start codon and reverse primers specific to intron 21, which was retained in RUSH1-β cDNA. The PCR products were cloned into the *pCR4* plasmid and used for transformation into *E. coli* cells. Twelve individual clones were sequenced and found to contain intron 21. However, four of them had spliced out exon 20, which resulted in a frame shift that introduced a stop codon just 5′ from the sequence coding the RING domain. The conceptually translated protein was named HLTFMet1ΔA (735 amino acids, 83 kDa, Figure 4A,B). The cDNAs containing the exon 20 sequence could encode the HLTFMet1ΔB protein variant (842 amino acids, 95 kDa) that was truncated in its carboxyl-terminus just after the same RING domain as described for RUSH1-β.

Different proteins were produced by transcription/translation of the different cDNAs in a reticulocyte lysate in the presence of ^{35}S-Cys and analysed by both SDS–PAGE and fluorography (see Materials and methods; Figure 4). Two bands with the expected molecular weight for HLTFMet1ΔA and Met1ΔB were detected (83 kDa and 95 kDa, respectively). Furthermore, two bands probably corresponding to the specific Met123 variants were also produced by use of the alternative translation initiation site (69 kDa and 81 kDa for Met123ΔA and Met123ΔB, respectively) (Figure 4B).

EXPRESSION OF HLTF VARIANTS IN FRESH BIOPSIES OF HNSCC PATIENTS

In order to evaluate the presence of these variants, we performed Western blots with the ART2 antibody on

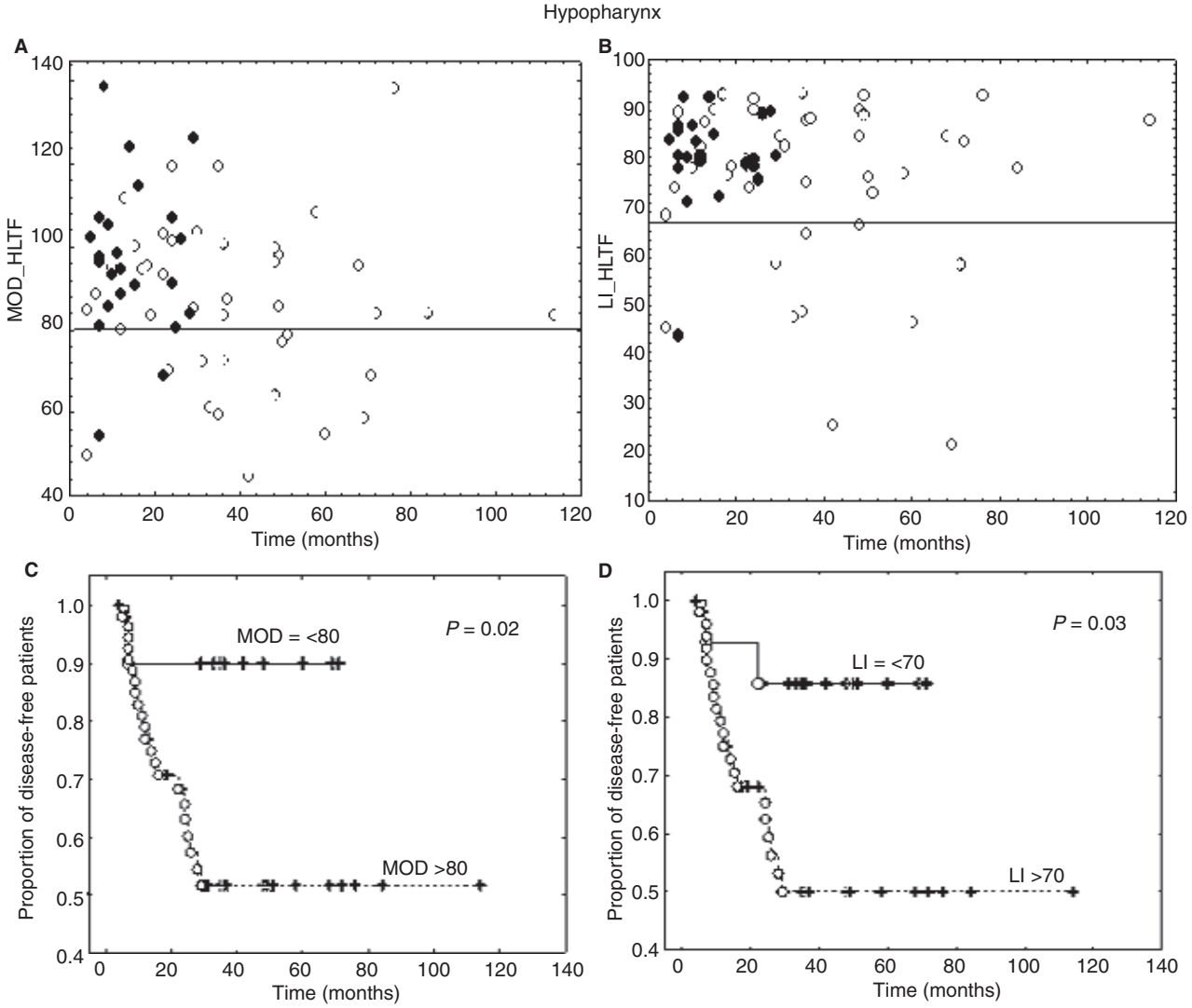

Figure 2. **A**, The patients' recurrence status (open dots, patients without recurrence; filled dots, patients with recurrence) (follow-up on the abscissa) compared with immunohistochemical HLTF expression in terms of intensity of immunoreactivity (MOD_HLTF, *Y*-axis) that was quantified in hypopharyngeal carcinomas (CAs). The horizontal line indicates a threshold value on the MOD_HLTF feature [determined at 80 arbitrary units (AU)] by means of a decision-tree method, which at best distinguished patients with recurrence from those without recurrence. **B**, The results of a similar analysis carried out on the percentage of HLTF-immunopositive tissue area (LI_HLTF) where the vertical line (corresponding to 70%) at best differentiated between patients with recurring and non-recurring malignancies. The group of patients with recurrent pathology generally exhibited a high level of HLTF-specific immunoreactivity in their tumours (i.e. above a mean optical density of 80 AU and/or >70% of immunopositive tissue area). **C**,**D**, The remission curves associated with the two groups of stage IV hypopharyngeal CA patients identified as indicated in **A** and **B**, respectively. In **C** and **D**, patients with tumour recurrences are defined by means of open dots and those without by crosses. *P*-values were derived by means of the Gehan's generalized Wilcoxon test. MOD, mean optical density; HTLF, helicase-like transcription factor.

protein extracts from different specimens with benign and malignant head and neck disease (Figure 4C). Of these 13 biopsy specimens, one patient presented with SCC of the oral cavity (case 5, pT4N0M0), three with SCC of the oropharynx (case 1, T1N1M0; case 2, T3N2bM0; case 12, T3N2bM0), four with HSCC (case 7, T2N1M0; case 8, recurrence after radiotherapy; case 11, pT4N2cM0; case 13, pT4N0M0) and three patients presented with LSCC (case 4, T1N0M0; case 9, pT4aN0M0; case 10, T3N0M0). Cases 3 and 6 corresponded to tumour-free hypopharyngeal epithelium cut off in the vicinity of the CA. All of these specimens expressed a 95-kDa HLTF variant truncated in the carboxyl-terminal domain, whereas only three

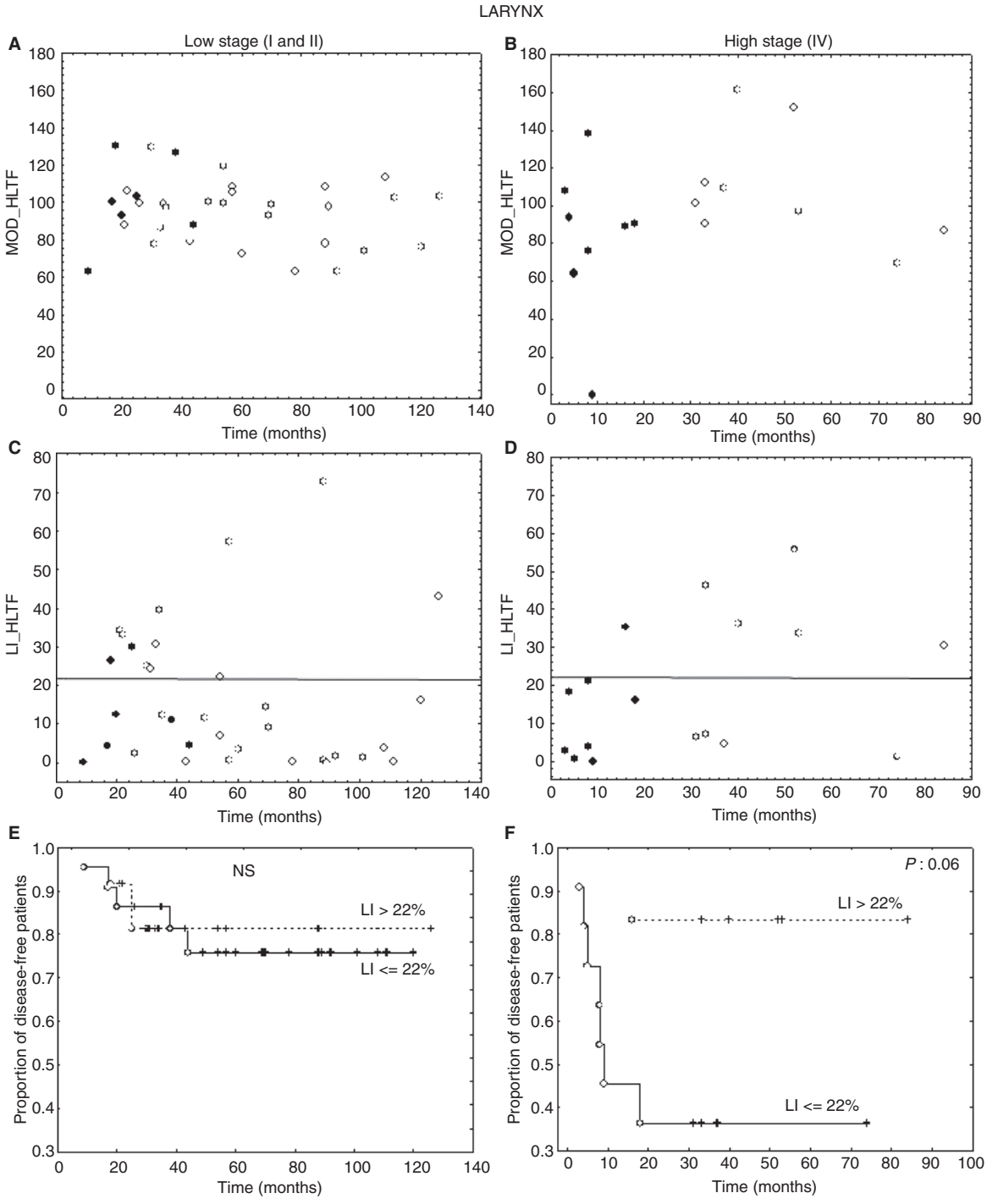

Figure 3. Study of helicase-like transcription factor (HLTF) expression levels and tumour recurrence in laryngeal carcinoma. **A**,**C**,**E**, Low-stage (I and II); **B**,**D**,**F**, high-stage (IV) laryngeal carcinomas. **A–D**, Patients' recurrence status on the ordinate (open dots, no recurrence; filled dots, recurrence) compared with HLTF expression on the abscissa (**A**,**C**, intensity of immunoreactivity, MOD_HLTF; **B**,**D**, percentage of immunopositive cells, LI_HLTF) that was quantified in laryngeal carcinomas. The horizontal line indicates the best threshold value on the LI_HLTF feature (22% of LI_HLTF) that was defined by a decision-tree method, which best distinguished patients with recurrence from those without. **E**,**F**, Remission curves associated with low or high (using the threshold value of 22% of LI_HLTF) HLTF expression. In these figures, patients with tumour recurrences were defined by means of open dots and those without by crosses. *P*-values were derived by the Gehan's generalized Wilcoxon test. MOD, mean optical density; LI, labelling index.

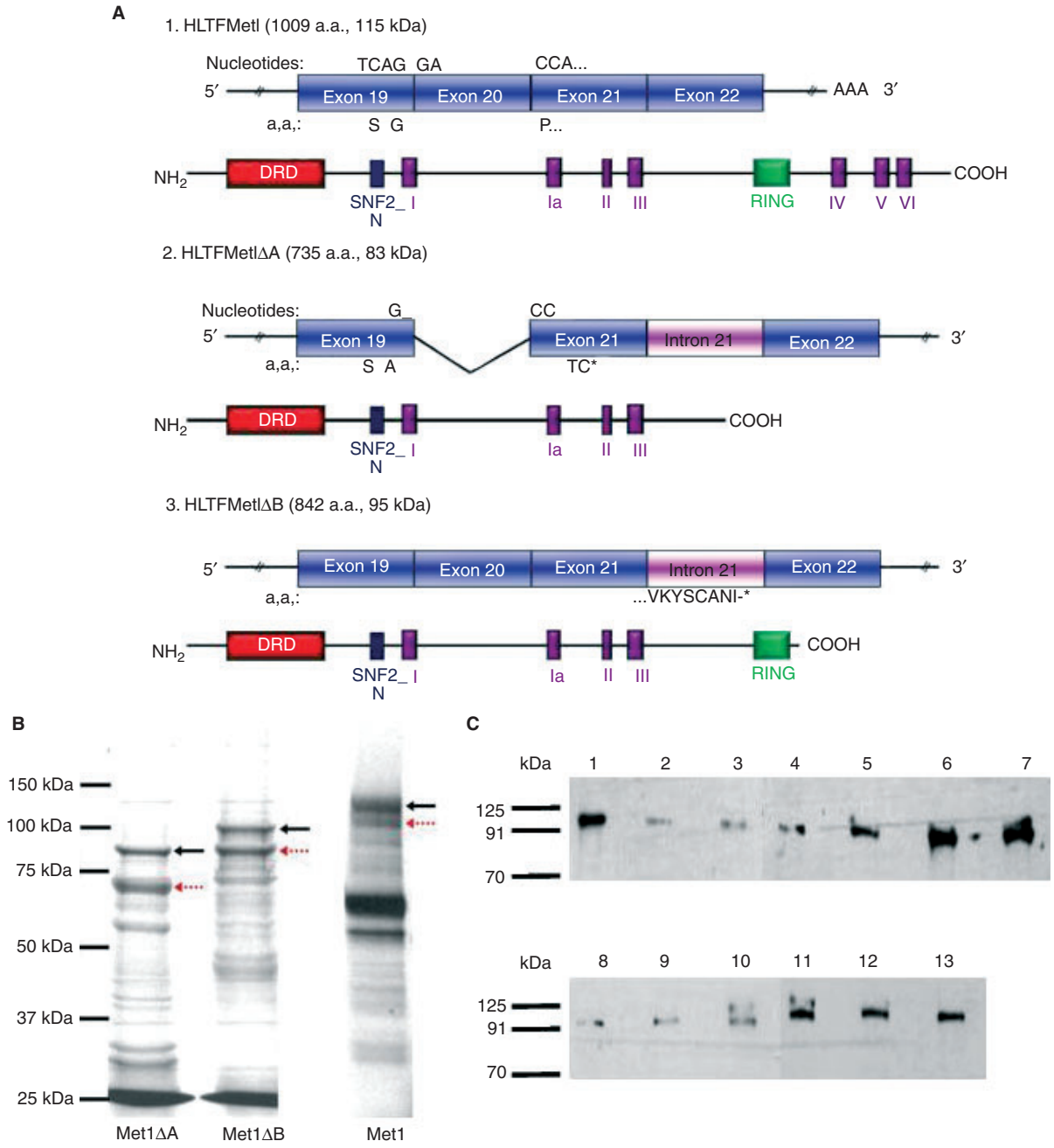

Figure 4. **A**, Schematic representation of the helicase-like transcription factor (HLTF) mRNA variants and their encoded proteins. 1. Upper line: 3′ part of the full-length HLTF mRNA. Lower line: encoded protein (HLTFMet1). The DNA Binding Domain (DBD) is represented by a red rectangle. The SNF2 family N-terminal domain is represented by a blue rectangle. Helicase domains are represented by seven pink rectangles. The RING domain is represented by a green square. 2. Upper line: 3′ part of the spliced mRNA variant lacking exon 20. Lower line: encoded protein (HLTFMet1ΔA). 3. Upper line: 3′ part of the spliced mRNA variant containing intron 21. Lower line: encoded protein (HLTFMet1ΔB). Theoretical average mass (MW) calculated on the expasy website using peptide mass software (http://us.expasy.org/tools/peptide-mass.html). *A stop codon. **B**, Expression of the HLTF variants *in vitro*. Transcription/translation of the cDNAs cloned into *pCR4* (for ΔA and ΔB variants) or *pGEM-4Z* (for HLTF wild type) was performed in a reticulocyte lysate in the presence of polymerase T7 and ^{35}S-Cys. The radioactive products were analysed by electrophoresis on a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (8%) and fluorography: HLTFMet1 (black arrows) and putative HLTFMet123 (red arrows). **C**, Western blotting of head and neck tumours [squamous cell carcinoma (SCC) of oral cavity (case 5), SCCs of oro- (cases 1, 2, 12) and hypopharynx (cases 7, 8, 11, 13), SCCs of larynx (case 4, 9, 10), tumour-free epithelium of the hypopharynx (cases 3, 6)] and visualization of HLTF presence. Note: a 95-kDa HLTF variant truncated in the carboxyl-terminal domain was detected in addition to the 115-kDa full-size protein in fresh head and neck SCC biopsy specimens.

specimens (cases 11, 12 and 13) expressed the 115-kDa full-size protein (Figure 4C).

Discussion

The present study has shown that immunohistochemical HLTF expression levels in a homogeneous series (in terms of histopathological as well as clinical criteria) of stage IV HSCCs were highly correlated with tumour recurrence rates and could be used as a strong prognostic factor alongside TNM staging. At first glance, our observations appear to contradict the notion that HLTF is probably a tumour suppressor. Indeed, the *HLTF* gene was inactivated by hypermethylation in a large percentage of various tumour types (reviewed by Debauve *et al.*[7]), and the HLTF protein is required for error-free postreplication repair of damaged DNA in a mode of action similar to Rad5 in *Saccharomyces cerevisiae*.[23] Inactivation of HLTF results in increased sensitivity to DNA damage by UV irradiation in human cells.[23] One could thus hypothesize that the loss of HLTF expression in cancer cells, from hypermethylation of its gene, would favour mutations in replicated DNA and contribute to the genome plasticity that is associated with cancer. The increased levels of HLTF expression that we observed in HSCCs in fact corresponded to the expression of a truncated protein, as shown by Western blots (Figure 4). This 95-kDa HLTF variant lacked several helicase domains and had probably lost its DNA repair activity. Analogous to what has been described for the TP53 tumour suppressor protein, we hypothesized that the mutated, inactive HLTF protein escaped degradation and accumulated in the tumour cells.[31,32] Further investigations would be needed to characterize the degradation/stabilization mechanisms at stake for the wild-type or truncated HLTF variants. One could also imagine that, similar to TP53, the 95-kDa HLTF variant may behave as a dominant negative mutant interfering with wild-type HLTF activity. The 'choice' for a cancer cell either to fully inactivate the HLTF gene or to overexpress a protein variant may be related to the cell type, as suggested by the different proportions of tumours that exhibit hypermethylated HLTF gene or immunohistochemical reactivity for HLTF (e.g. colon versus oesophagus).[8–20]

Moreover, our previous study described significant up-regulation of HLTF expression throughout the neoplastic progression of HSCCs. This increased expression of HLTF in CAs seems to be accompanied by a shift from the nuclear compartment (TFE and dysplasias) to the cytoplasm.[28] This shuttle could be indicative of a switch in biological activity, such as the loss of chromatin/transcription regulatory functions or, more importantly for cancer progression, the loss of postreplication DNA repair that would contribute to the mutagenic phenotype of tumour cells.

An additional layer of complexity was added in this study by the fact that the *HLTF* gene produced several protein variants, two of which (Met1 and Met123) have been previously characterized and shown to differ in their transcriptional activity.[24] We have now identified four additional HLTF variants and have shown that HLTFMet1ΔB was expressed in all the HNSCC biopsy specimens analysed. The Met1ΔB variant, characterized by the absence of the carboxyl-terminal helicase domains, had lost its ability to repair DNA. Our data, which revealed the prognostic value of HLTF expression in stage IV HSCC cases and the expression of HLTFMet1ΔB in all HNSCC biopsies, allows us to propose this variant as an emerging marker of poorer prognosis in HSCCs.

We have previously reported that the levels of HLTF expression are very different during laryngeal tumour progression compared with hypopharyngeal tumour progression.[28] We have described here that no statistical correlation was observed between HLTF expression (MOD or LI variables) and clinicopathological findings (staging or recurrence) for LSCCs. This finding relates directly to the fact that cancers of the upper digestive tract are heterogeneous in their neoplastic processes and require a unique set of epidemiological, anatomical, pathological and therapeutic considerations. Laryngeal and hypopharyngeal CAs present several significant clinical and biological differences that could partly explain the observed divergence in HLTF prognostic value: (i) most hypopharyngeal CA patients (70–80% of cases) present with advanced stage disease (III and IV) at the time of diagnosis, whereas laryngeal CA patients are most frequently diagnosed at early stages (70–90% of cases presented at stages I and II);[33] (ii) at a similar stage, hypopharyngeal CAs are associated with a worse prognosis than laryngeal CAs;[33] (iii) well-differentiated CAs are common in larynx, whereas poorly differentiated tumours are more frequently located in the hypopharynx;[34] (iv) in laryngeal CAs, there is a significant relationship between the presence of intratumoral lymphatics and nodal metastases, which was not the case for hypopharyngeal CAs;[35] (v) finally, we have shown that levels of HLTF expression are very different during laryngeal tumour progression compared with hypopharyngeal tumour progression (comparison of HLTF expression in TFE, dysplasias and CA). We have demonstrated that the MOD variable describing the intensity of HLTF immunolabelling significantly

decreases with laryngeal malignancy, whereas the same variable increases significantly in hypopharyngeal CAs.[28] Previous studies have reported *HLTF* promoter hypermethylation in a significant number of colorectal and gastric CA cases, but only one case of oesophageal CA has been reported with the same gene silencing.[8–19] HLTF thus appears to play different roles in tumour development depending on the histological type and the anatomical location.

Competing interests

None to declare.

Acknowledgements

S.S. and A.B. are the recipients of a grant from the Fondation pour la recherché médicale dans le Hainaut (Belgium) and A.B. from the Fonds de la Recherche Scientifique-FNRS, (Belgium) and G.D. from Télévie (Belgium). A.C. held pre-doctoral fellowships from the Fonds de Recherche pour l'Industrie et l'Agriculture (FRIA, Belgium). The expert technical assistance of G. Ninfa is also gratefully acknowledged. Finally, we thank Drs Cécile Gerbaux and Fabrice Ribaucour for their help in the development of immunoprecipitation conditions.

References

1. Ferris RL. Progress in head and neck cancer immunotherapy: can tolerance and immune suppression be reversed? *ORL J. Otorhinolaryngol. Relat. Spec.* 2004; **66;** 332–340.
2. Grandis JR, Pietenpol JA, Greenberger JS, Pelroy RA, Mohia S. Head and neck cancer: meeting summary and research opportunities. *Cancer Res.* 2004; **64;** 8126–8129.
3. Shah JP, Patel SG. *Head and neck surgery and oncology*. New York: Mosby, 2003.
4. Posner MR, Hershock DM, Blajman CR *et al.* TAX 324 Study Group. Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer. *N. Engl. J. Med.* 2007; **357;** 1705–1715.
5. Vermorken JB, Remenar E, van Herpen C *et al.* TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene* 2007; **26;** 2157–2165.
6. Saussez S, Camby I, Toubeau G, Kiss R. Galectins as modulators of tumor progression in head and neck squamous cell carcinomas (HNSCCs). *Head Neck* 2007; **29;** 874–884.
7. Debauve G, Capouillez A, Belayew A, Saussez S. HLTF and its implication in cancer progression. *Cell. Mol. Life Sci.* 2008; **65;** 591–604.
8. Moinova HR, Chen WD, Shen L. HLTF gene silencing in human colon cancer. *Proc. Natl Acad. Sci. USA* 2002; **99;** 4562–4567.
9. Versteege I, Sevenet N, Lange J *et al.* Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* 1998; **394;** 203–206.
10. Hibi K, Kodera Y, Ito K, Akiyama S, Nakao A. Aberrant methylation of HLTF, SOCS-1, and CDH13 genes is shown in colorectal cancers without lymph node metastasis. *Dis. Colon Rectum* 2005; **48;** 1282–1286.
11. Hibi K, Nakao A. Highly-methylated colorectal cancers show poorly-differentiated phenotype. *Anticancer Res.* 2006; **26;** 4263–4266.
12. Hibi K, Nakao A. Lymph node metastasis is infrequent in patients with highly-methylated colorectal cancer. *Anticancer Res.* 2006; **26;** 55–58.
13. Hibi K, Nakayama H, Kanyama Y *et al.* Methylation pattern of HLTF gene in digestive tract cancers. *Int. J. Cancer* 2003; **104;** 433–436.
14. Kim YH, Petko Z, Dzieciatkowski S *et al.* CpG island methylation of genes accumulates during the adenoma progression step of the multistep pathogenesis of colorectal cancer. *Genes Chromosomes Cancer* 2006; **45;** 781–789.
15. Hamai Y, Oue N, Mitani Y *et al.* DNA hypermethylation and histone hypoacetylation of the HLTF gene are associated with reduced expression in gastric carcinoma. *Cancer Sci.* 2003; **94;** 692–698.
16. Kim JJ, Chung SW, Kim JH *et al.* Promoter methylation of helicase-like transcription factor is associated with the early stages of gastric cancer with family history. *Ann. Oncol.* 2006; **17;** 657–662.
17. Leung WK, Yu J, Bai AH. Inactivation of helicase-like transcription factor by promoter hypermethylation in human gastric cancer. *Mol. Carcinog.* 2003; **37;** 91–97.
18. Oue N, Mitani Y, Motoshita J *et al.* Accumulation of DNA methylation is associated with tumor stage in gastric cancer. *Cancer* 2006; **106;** 1250–1259.
19. Fukuoka T, Hibi K, Nakao A. Aberrant methylation is frequently observed in advanced esophageal squamous cell carcinoma. *Anticancer Res.* 2006; **26;** 3333–3335.
20. Kang S, Kim JW, Kang GH *et al.* Comparison of DNA hypermethylation patterns in different types of uterine cancer: cervical squamous cell carcinoma, cervical adenocarcinoma and endometrial adenocarcinoma. *Int. J. Cancer* 2006; **118;** 2168–2171.
21. Gong X, Kaushal S, Ceccarelli E *et al.* Developmental regulation of Zbu1, a DNA-binding member of the SWI2/SNF2 family. *Dev. Biol.* 1997; **183;** 166–182.
22. Unk I, Hajdú I, Fátyol K *et al.* HLTF functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination. *Proc. Natl Acad. Sci. USA* 2008; **105;** 3768–3773.
23. Debauve G, Nonclercq D, Ribaucour F *et al.* Early expression of the Helicase-Like Transcription Factor (HLTF/SMARCA3) in an experimental model of estrogen-induced renal carcinogenesis. *Mol. Cancer* 2006; **5;** 23.
24. Ding H, Descheemaeker K, Marynen P *et al.* Characterization of a helicase-like transcription factor involved in the expression of the human plasminogen activator inhibitor-1 gene. *DNA Cell Biol.* 1996; **15;** 429–442.
25. Hyams VJ, Batsakis JG, Michaels L (eds) Tumors of the upper respiratory tract and ear. In *Atlas of tumor pathology*. Washington, DC: Armed Forces Institute of Pathology, 1998; 132–137.
26. Wittekind C, Greene FL, Hutter RRP, Kimpfinger M, Sobin LH. *TNM atlas*, 5th edn. Berlin: UICC Springer, 2004.
27. Saussez S, Decaestecker C, Lorfevre F *et al.* Increased expression and altered intracellular distribution of adhesion/growth-regulatory lectins galectins-1 and -7 during tumour progression in hypopharyngeal and laryngeal squamous cell carcinomas. *Histopathology* 2008; **52;** 483–493.

28. Capouillez A, Decaestecker C, Filleul O *et al.* Helicase-like transcription factor exhibits increased expression and altered intracellular distribution during tumour progression in hypopharyngeal and laryngeal squamous cell carcinomas. *Virchows Arch.* 2008; **453;** 491–499.
29. Saussez S, Cucu DR, Decaestecker C *et al.* Galectin-7 (p53-induced gene-1): a new prognostic predictor of recurrence and survival in stage IV hypopharyngeal cancer. *Ann. Surg. Oncol.* 2006; **13;** 999–1009.
30. Hayward-Lester A, Hewetson A, Beale EG, Oefner PJ, Doris PA, Chilton BS. Cloning, characterization, and steroid-dependent posttranscriptional processing of RUSH-1 alpha and beta, two uteroglobin promoter-binding proteins. *Mol. Endocrinol.* 1996; **10;** 1335–1349.
31. Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene* 2007; **26;** 2157–2165.
32. Courtois S, de Fromentel CC, Hainaut P. p53 protein variants: structural and functional similarities with p63 and p73 isoforms. *Oncogene* 2004; **23;** 631–638.
33. Shah J. *Head and neck surgery and oncology*. London: Mosby, 2003.
34. Roland NJ, Caslin AW, Nash J *et al.* Value of grading squamous cell carcinoma of the head and neck. *Head Neck* 1992; **14;** 224–229.
35. Audet N, Beasly NJ, MacMillan C *et al.* Lymphatic vessel density, nodal metastases and prognosis in patients with head and neck cancer. *Arch. Otolaryngol. Head Neck Surg.* 2005; **131;** 1065–1070.