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PHF21B as a candidate tumor suppressor gene in head and neck squamous cell carcinomas

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ABSTRACT

A significant association between DNA losses on 22q13.31 and head and neck squamous cell carcinomas (HNSCC) was previously reported by our group. Our data indicated that *PHF21B* gene, mapped on 22q13.31 and encoding a protein with function of chromatin-mediated transcriptional regulation, might be a putative tumor suppressor gene. To test this hypothesis, gene copy number was assessed in 75 HNSCC and 49 matched peripheral blood samples. *PHF21B* losses were detected in 43 tumors and were significantly associated with patients with familial history of cancer ($P < 0.0001$); i.e., 36/43 cases showed a positive family history of cancer and 22/36 had first-degree relatives with cancer ($P = 0.049$). In attempt to investigate other mechanisms for *PHF21B* loss of function, DNA sequencing was performed and no mutations were detected. We next evaluated the gene expression levels after inhibition of DNA methylation in nine HNSCC and breast carcinoma cell lines. Additionally, *PHF21B* expression levels were evaluated in colon cancer HCT116 cells as well as in its counterpart DKO (double knockout of DNMT1 and DNMT3B). The higher expression levels of *PHF21B* gene detected in DKO cells were inversely correlated with the DNA

Abbreviations: 5-Aza-dC, 5-AZA-2′-deoxycytidine; ENCODE, ENCyclopedia Of DNA Elements; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; MS-HRM, Methylation Sensitive-High Resolution Melting; qPCR, quantitative real time PCR; RT-qPCR, reverse transcription-quantitative real time PCR; TSA, trichostatin A.

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methylation. Further, DNA methylation in the specific promoter-associated CpG Island was investigated. Interestingly, gene hypermethylation was detected in 13/37 tumors: 5/13 HNSCC cases had family history of cancer in first-degree relatives and 8/13 showed both, DNA methylation and *PHF21B* losses in the tumor sample. One patient had *PHF21B* loss in the peripheral blood cells and *PHF21B* methylation in the tumor sample. Additionally, overexpression of *PHF21B* in cell lines drastically reduces clonogenic and migratory abilities. These data suggest that *PHF21B* is a novel tumor suppressor gene that can be inactivated by genetic and epigenetic mechanisms in the human cancer.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous and multi-causal group of diseases accounting for 3%–6% of all human cancers; being the 6th most frequent malignancy and the 8th most common cause of cancer-related deaths worldwide (Ferlay et al., 2010; Kamangar et al., 2006).

Chronic exposure to tobacco and alcohol and human papillomavirus (HPV) infection are considered the main risk factors for HNSCC (Cardesa and Nadal, 2011; Lambert et al., 2011). Other risk factors include diet, socioeconomic status, and genetic susceptibility determined by low penetrance gene polymorphisms (Hopkins et al., 2008; Olivieri et al., 2009; Sturgis and Wei, 2002).

An inherited component for predisposition of risk for head and neck cancer has been suggested by case reports of families with multiple affected members (Ankathil et al., 1996; Hara et al., 1988; Tashiro et al., 1986), by a significant proportion of familial aggregation of oral carcinoma or other cancers of the head and neck (Copper et al., 1995; Foulkes et al., 1995, 1996; Garavello et al., 2008; Goldstein et al., 1994; Negri et al., 2009; Yu et al., 1999), and by segregation analysis data in first-degree relatives (De Andrade et al., 1998).

The first reports of familial aggregation of head and neck cancer were published over 20 years ago. Most researchers shared the concept that similar lifestyle factors (e.g., diet, tobacco and alcohol use) within the family setting accounted for findings of familial aggregation, but even after adjusting for these factors, individuals with a family history of HNSCC had approximately two-to three-fold increased risk of developing the disease (Brown et al., 2001; Copper et al., 1995; Foulkes et al., 1995; Goldstein et al., 1994; Yu et al., 1999). However, despite of the effort devoted to characterizing the genetic predisposition for head and neck cancer, few studies have shown an association with germline *TP16* mutations and familial HNSCC. In contrast, Jefferies and Foulkes (2001) found no *TP16* mutations in 40 patients with HNSCC and a second primary tumor. Two subsequent reports associated *TP16* mutations in families with HNSCC (Lange et al., 2002; Yu et al., 2002).

Previous studies in oral cancer have suggested the existence of a putative tumor suppressor gene mapped at 22q13.3 (Miyakawa et al., 1998; Reis et al., 2002a). Subsequently, our group reported a significant association between family history of oral cancer and a chromosome 22 deletion flanked by the *DIA1* gene and D22S274 marker (Reis et al., 2002b). This evidence was reinforced in a second study in 64 cases of untreated primary HNSCC. We observed a high frequency of chromosome 22 loss, mainly del(22)(q13), which correlated with survival and family cancer history (Bérgamo et al., 2005). Taken altogether, these data indicate that this region may harbor a tumor suppressor gene that might be involved in familial HNSCC.

Based on the significant association between 22q13.31 losses, preferentially at the D22S274 locus (mapped at 7.7 Kb downstream of *PHF21B*), and family cancer history in HNSCC probands, the *PHF21B* gene was further examined aiming to determine its role as a novel tumor suppressor gene. *PHF21B* encodes the PHD finger protein 21B, composed of 531 amino acids (NP_612424.1) with no previously established function. Our results demonstrate that *PHF21B* is a nuclear protein, whose overexpression reduces cell migration and colony formation, supporting its role as a tumor suppressor gene.

2. Materials and methods

2.1. Patients

This retrospective cohort study consisted of 75 HNSCC selected samples stored at the biobank of A.C. Camargo Hospital, São Paulo, Brazil. None of the patients had received radiotherapy or chemotherapy prior to surgery and sample collection. Eligibility criteria included the absence of previous histological diagnosis of any cancer type, a tumor larger than 1 cm in size and availability of complete clinicopathological data. Lip, nasopharynx, thyroid and salivary gland tumors were all excluded, as well as patients having systemic predisposing diseases, such as Epidermolysis bullosa, Xeroderma pigmentosum, Juvenile papillomatosis and Fanconi's anemia. All patients provided written informed consent. The Institutional Review Board at A.C. Camargo Hospital approved this study (CEP 792/2006B).

The age of HNSCC probands ranged from 33 to 87 years old with a median of 56 years old and a mean of 56.9 ± 12.8 years old. Between the 40 cases with family history, the age ranged from 33 to 87 years old, with a median of 56.5 years old and a mean of 57.9 ± 11.8 years old. Among the 35 cases without family history, the age ranged from 39 to 73 years old, with a median of 56 years old and a mean of 55.7 ± 13.8 years old.

The male-to-female ratio was 4.4:1; 53.3% were tobacco users, 53.3% were alcohol users and 46.7% used both tobacco and alcohol. The length of the follow-up period ranged from 6.1 days to 185 months, with a median of 31.4 months and a mean of 54.9 ± 55.4 months.

The histopathological classification was performed according to the WHO International Classification of Diseases for Oncology (Louis et al., 2007). The clinical staging was determined using the tumor-node-metastasis staging system (Edge et al., 2009). Medical records were examined to obtain detailed clinicopathological data, including information on family cancer history, consumption of alcohol and tobacco and demographic data (Table 1). Family cancer history was ascertained from pedigrees after interviews with patients and family members concerning the number of brothers and sisters, the first-and-second degree relatives with a history of cancer, the anatomic tumor location and data available in medical records. Family members had tumors of the lung, esophagus and HNSCC; such tumors are associated with tobacco consumption. Other tumor sites seen in family members (referred to as HNSCC-associated tumors) included stomach, kidney, urinary bladder, breast, uterine/cervical cancers and skin melanoma (Negri et al., 2009).

2.2. Definition of familial HNSCC risk

In an attempt to establish clinical criteria to identify patients belonging to high-risk cancer families associated with a predisposition to HNSCC development, we adopted the following requirements for patient classification into two groups (positive or negative for familial history of cancer: 1) at least two first-degree relatives in the family affected with head and neck cancer and/or other related cancers as described by Negri et al. (2009); 2) age of cancer onset lower than 45 years old in at least one of the affected family members and/or the proband; 3) any age at onset when the HNSCC patient reported no tobacco and/or alcohol consumption or other related well-known etiological factor. According to these criteria, 40 out of 75 (53.3%) patients with HNSCC had a family history of cancer: 12 showed a family history of HNSCC, of which 7 had at least one first-degree relative with HNSCC (mainly oral cavity or larynx carcinoma); 18 had at least two affected relatives (5 had at least one HNSCC relative, 1 had two first-degree relatives affected by HNSCC, 8 had at least one tobacco-associated tumor in the affected relatives, and 10 had at least one HNSCC-associated tumor in the affected relatives); and 10 showed only one affected relative with other tumor type than HNSCC. Other cancer sites involved on those 18 cases have included colon (5 cases, all in first-degree relatives), thyroid (1 first-degree relative), pancreas (2 first-degree relatives), prostate (1 first-degree relative), lymphoma (1 second-degree relative), breast (3 first-degree relatives), and non-melanoma skin cancer (2 brothers).

Table 1 – Clinical data from all 75 HNSCC patients.

Characteristics		Number of cases	%
Age	≤45 years	16	21.3
	>45 years	59	78.7
Gender	Male	61	81.3
	Female	14	18.7
Tumor site	Oral Cavity	38	50.6
	Oropharynx	16	21.3
	Hipopharynx	8	10.7
	Larynx	13	17.4
Tobacco	Yes	40	53.3
	No	10	13.3
	Na	25	33.4
Alcohol	Yes	40	53.3
	No	9	12.0
	Na	26	34.7
Tobacco + alcohol	Yes	35	46.7
	No	16	21.3
	Na	24	32.0
Stage T	T1 + T2	22	32.5
	T3 + T4	52	65.1
	Na	1	2.4
Stage N	N0	38	50.7
	N+	35	46.7
	Na	2	2.6
Clinical Evolution	Dead (by disease or not)	43	57.3
	Alive	19	25.3
	Loss of follow-up	16	17.4

Na: Information not available.

2.3. DNA extraction

Genomic DNA from tumor tissue ($n = 75$) and matched peripheral blood samples ($n = 49$) was prepared after digestion with proteinase K, extraction with phenol-chloroform and precipitation with 100% ethanol. Figure 1A describes each experimental assay performed to characterize germline or acquired genetic and epigenetic alterations of *PHF21B*.

2.4. HPV detection

The presence or absence of human papillomavirus (HPV) DNA has been determined in 59/75 HNSCC samples (78.7%) using the Linear Array HPV Genotyping Test kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA).

2.5. Analysis of PHF21B copy number by quantitative real time PCR (qPCR)

PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Life Technologies, Foster City, CA, USA). Primer sequences used in each PCR-based approach are listed in Supplementary Table S1. PCR reactions using Power® SYBR® Green (Applied Biosystems, Life Technologies) were performed as described previously (Reis et al., 2002b). Tumor samples and matched peripheral blood samples were analyzed after high-precision automated PCR setup by QIAgility (Qiagen, GmbH, Hilden, Germany). The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as copy number reference. Relative quantification was calculated by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The cell lines KG-1a (derived from chronic myeloid leukemia, having chromosome 22 monosomy) and Hs578T (derived from human breast cancer, with chromosome 22 trisomy) were obtained from the

American Type Culture Collection (ATCC) (Rockville, MD, USA) and used as references to determine the cut-off values for copy number alterations. Cell lines were cultured according to ATCC recommendations to confirm their karyotypes. Five DNA blood samples from volunteers without cancer were also used as normal DNA copy number controls. Initially, the target and control gene mean value ratios were determined for all cell lines and normal controls after eight repetitions. The confidence interval obtained for the normal tissues (disomic), was calculated using the mean and standard deviation of the $2^{-\Delta\Delta CT}$ values, ranging from 0.78 to 1.28. For the cell line KG-1a (chromosome 22 monosomy), the interval ranged from 0.52 to 0.69, and for the cell line Hs578T (chromosome 22 trisomy), the interval determined ranged from 1.46 to 1.73. Thus, DNA copy number analysis defined $2^{-\Delta\Delta CT}$ values <0.69 as losses and $2^{-\Delta\Delta CT}$ values >1.46 as gains in the *PHF21B* target region. Each PCR plate included control reactions with the DNA from the cell lines, the normal control DNAs and a non-template control. All samples were analyzed in duplicate, and experiments were repeated when Cq differences between replicates were higher than 0.5.

2.6. PHF21B *gene sequencing and analysis*

Six exons of the *PHF21B* gene were directly sequenced (exons 3, 6–9, and 11) (Figure 1A). Each exon was amplified individually using 100 ng of genomic DNA obtained from 26 matched tumoral and peripheral blood samples. The polymerase chain reactions contained 1× Pfu Ultra™ II reaction buffer, 1 mM of each dNTP, 200 mM of each primer and 0.5 μL of Pfu Ultra™ II Fusion HS DNA Polymerase (Stratagene, Agilent Technologies, USA) in a final reaction volume of 25 μL. PCR products were purified with ExoSAP-IT (USB Products, Cleveland, OH) and sequenced in both directions. Sequencing reactions of purified products were performed with the Big Dye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Life Technologies) and separated in the ABI3130 DNA Sequencer (Applied Biosystems, Life Technologies). The sequences were analyzed using CLC Main Workbench 5.0.2 software (CLC Bio, Aarhus, Denmark) and compared with the *PHF21B* reference sequence (NCBI GenBank NM_138415.4).

2.7. 5-Aza-2′-deoxycytidine *and trichostatin A treatment of cell lines*

To investigate if alternative epigenetic mechanisms could lead to the down-regulation/inactivation of the *PHF21B*, the human cell lines SCC4, SCC9, SCC15 (derived from squamous cell carcinoma of the tongue), the FaDU cell line (derived from squamous cell carcinoma of the pharynx), and the breast cancer cells lines SK-BR-3, Hs578T, MDA-MB-231, MDA-MB-415 and MDA-MB-453, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA), cultured according recommended instructions, and treated with the DNA demethylating nucleoside analog 5-Aza 2′deoxycytidine (5-Aza-dC), either alone or in combination with the histone deacetylase inhibitor Trichostatin A (TSA), as previously described by Da Costa Prando et al. (2011). We then evaluated the potential for these epigenetic drugs to modulate *PHF21B* expression.

The effect of DNA methylation on the *PHF21B* transcription level was also evaluated in HCT116 colorectal cancer cells and in their counterparts, DKO cells, which are a double knockout of DNMT1 and DNMT3B. DKO cells are considered a biologically demethylated cell line, characterized by loss of 95% of DNA methylation (Rhee et al., 2002). Genomic DNA and total RNA were extracted from the cell cultures and were used for MS-HRM (Methylation Sensitive – High Resolution Melting) analysis and reverse transcription-quantitative real time PCR (RT-qPCR).

2.8. *Total RNA extraction and detection of* PHF21B *gene expression by RT-qPCR*

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) and subsequently treated with DNase I. The reverse transcription was performed from 2 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). The reaction was primed with random primers in a total volume of 20 μL, according to the manufacturer's instructions. After amplification of cDNA obtained from treated and untreated cells with specific primer sets for the *GAPDH*, *HPRT1* and *ACTB* genes, the most stable genes under these experimental conditions was selected as the endogenous control. The relative expression levels of *PHF21B* gene were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to *HPRT1* (hypoxanthine phosphoribosyltransferase 1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) expression levels.

2.9. *Sodium bisulfite treatment and Methylation Sensitive – High Resolution Melting (MS-HRM) analysis*

Genomic DNA (1 μg) was treated with sodium bisulfite using the EpiTect Bisulfite Modification Kit (Qiagen) according to the manufacturer's instructions. After sodium bisulfite treatment, PCR amplification and MS-HRM were performed on the Step One Plus Real Time PCR System (Applied Biosystems, Life Technologies) to assess *PHF21B* promoter-associated CpG island methylation (CpG island 273, nucleotide position chr22: 45403037-45406372, 3336 bp, CG content of 55.4% and ratio of observed to expected CpG equal to 1.07, UCSC Genome Browser on Human, GRCh37/hg19 Assembly). MS-HRM is a screening methodology that enables rapid analysis of locus-specific DNA methylation based on the differential melting behavior of PCR amplification products derived from methylated and unmethylated templates after bisulfite treatment. Primer sequences were designed according to Wojdacz et al. (2008) (Supplementary Table S1). PCR was performed in a 20 μL volume containing 1× MeltDoctor HRM Master Mix (Applied Biosystems, Life Technologies), 200 nM of each primer, and 25 ng of bisulfite-treated DNA template. A standard curve with known methylation ratios (100%, 50%, 25%, 10%, 5%, 2.5%, and 0%) was included in each assay to estimate the methylation ratio of each sample using the EpiTect PCR Control DNA Set, containing both bisulfite-converted methylated and unmethylated DNA and unconverted unmethylated DNA (Qiagen) as references. Methylation standard reactions were performed in duplicates, and cell lines and tissue sample reactions were performed in triplicates. The cycling

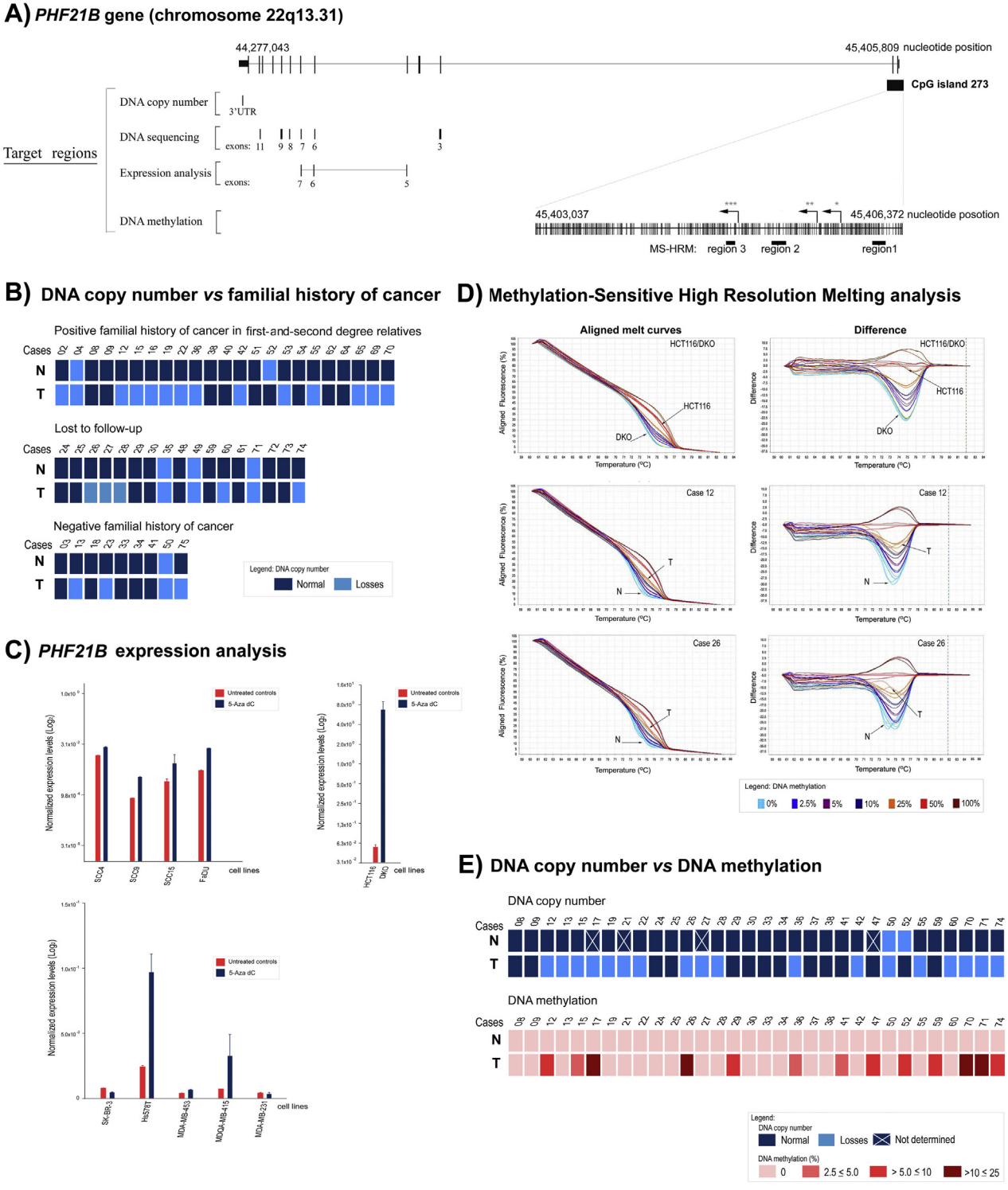

Figure 1 – Experimental assays for characterization of *PHF21B*. (A) Scheme of the *PHF21B* gene and its promoter-associated CpG islands organized according to the physical location on 22q13.31. This gene contains 13 exons spanning approximately 128 Kb of DNA (UCSC Genome Browser on Human, assembly GRCh37/hg19, http://genome.ucsc.edu) and a highly dense promoter-associated CpG island. This island spans 3336 bp, including the 5′ region, with the first two exons of this gene overlapping three alternative transcription start sites: (*) transcript variant 1, NM_138415.4, (**) transcript variant 2, NM_001135862.2, and (***) transcript variant 3, NM_001242450.1. The comprehensive analysis of the *PHF21B* gene in HNSCC patients included several target regions for PCR-based assays for detection of the DNA copy number (3′UTR), gene expression (exons 5–7), and mutations (exons 3, 6, 7, 8, 9, and 11). Changes in DNA methylation of CpG Island 273 were screened for three regions in HNSCC-derived cell lines. (B) DNA copy number changes in the *PHF21B* gene in matched normal (peripheral blood) and tumoral samples obtained from HNSCC patients. Constitutive losses of *PHF21B* were detected in cases showing a positive family cancer history (case 04), in case 50 (negative for family cancer history), and in cases 35, 49, and 71 (unknown information). Tumor-specific acquired losses were detected in

conditions were as follows: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min; followed by an HRM step of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and continuous acquisition from 60 to 95 °C at 0.3%. MS-HRM data were analyzed using High Resolution Melting Software (Applied Biosystems, Life Technologies). Data were visualized as normalized melting curves and difference plots.

2.10. *Statistical analysis*

Associations between *PHF21B* gene copy number alterations and clinic pathological characteristics were determined by Pearson's Chi-Square and Fisher's exact tests, using SPSS v.17.0 software (SPSS, Chicago, IL, USA). Tumors were grouped as T_1 and T_2 *versus* T_3 and T_4, as well as N_0 *versus* N_1, N_2, N_3 and N_4. Patients were followed between surgery and death or the last date of follow-up in December 2012. The null hypothesis was rejected when the two-tailed *P*-value was <0.05. Graphical representations were made using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

2.11. *Protein sequence alignment*

Amino acid sequence alignment between PHF21A (AAH15714.1) and *PHF21B* (NP_612424.1) was performed with BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PAGE=Proteins). PHF21A functional domains were identified using Uniprot (http://www.uniprot.org/).

2.12. PHF21B *overexpression*

The plasmid containing the human ORF for *PHF21B* (NM_138415) GFP-tagged was purchased from Origene. Empty vector was obtained by digesting the plasmid with NotI and HindIII followed by ligation with T4 DNA ligase. Cell lines expressing the lowest *PHF21B* mRNA levels were chosen for overexpression experiments. MDA-MB231 breast cancer cell line and SCC9 head and neck cancer cell line were plated in 100 mm dishes in approximately 60% confluence and transfected with 10uL lipofectamine (Invitrogen, Life Technologies) and 10ug of DNA for 6 h. Cells stably expressing the vectors were isolated by Neomycin (Sigma–Aldrich, St. Louis, MO, USA) resistance (MDA-MB231 1000 μg/mL and SCC9 750 μg/mL) and two rounds of sorting by flow cytometry in a BD FACS Aria.

2.13. *Subcellular localization of* PHF21B

Cell lines with the highest *PHF21B* mRNA expression levels were chosen for subcellular localization studies. Cells were plated in glass coverslips, fixed with 4% paraformaldehyde and permeabilized with PBS 0.5% Triton-X100. Blocking of non-specific antigens was performed with PSB 5%BSA for 1 h at room temperature. Anti-*PHF21B* (Sigma–Aldrich) was used at 1:100 in PBS 1% BSA for 1 h at room temperature. Secondary antibody (anti-rabbit Alexa 488, Molecular Probes) was used at 1:1000 in PBS 1% BSA for 1 h at room temperature. Nuclei were labeled by Draq5 (Molecular Probes, Life Technologies). Alternatively, MDA-MB231 and SCC9 expressing GFP (green florescent protein) or GFP-*PHF21B* were plated in coverslips, fixed and permeabilized. Nuclei were labeled by Draq5 (Molecular Probes, Life Technologies). Images were taken by confocal microscopy in LeicaSP5 equipment.

2.14. *Cellular migration and clonogenic assays*

10^4 cells expressing GFP of GFP-*PHF21B* were plated in Transwell chambers (Corning-Costar, NY, USA) of 0.8um pore in DMEM (Gibco, Life Technologies) without fetal calf serum (Sigma–Aldrich). Chemoattraction was induced by DMEM containing 10% fetal calf serum and cells were allowed to migrate for 6 h. Cells were fixed in 4% paraformaldehyde and stained with DAPI (Molecular Probes, Life Technologies) for 30 min. The upper portion of the transwell chamber was cleaned with the aid of a cotton swab. Ten pictures were taken from each well and all green cells in each field were considered.

For clonogenic assays, 25 cells expressing GFP or GFP-*PHF21B* were counted using fluorescence to reassure the expression of the vectors. Cells were plated in 35 mm plates and the number of green colonies was evaluated after 10 days of culture.

2.15. *Cell viability after treatment with chemotherapeutic agent*

The possible effect of *PHF21B* in drug response was evaluated by preliminary tests of cell viability after treatment of cell lines with cisplatin. Cell viability was determined by the MTT colorimetric assay, as follow: 5×10^3 cells were added in 24-wells plates and after 12 h were treated with cisplatin at concentrations of 1, 10, 50, and 100 μM. After 24 h at 37 °C, the MTT dye [3-(4,5)–dimethylthiahiazo-(-z-y1)-3,5-diphenytetrazolium bromide] was added to the medium (0.5 mg/ml), followed by incubation during 3 h. Cells were lysed with acid isopropanol and absorbance was measured at a wavelength of 595 nm. The values were expressed relative to untreated controls. Experiments were made in triplicates.

21 out of 49 cases. (C) Effect of epigenetic treatment with 5-Aza-dC on *PHF21B* gene expression: two cell lines were positively modulated by treatment with 5-Aza-dC (Hs578T, MDA-MB-415). The results are expressed as normalized levels relative to HPRT and represent the mean of real time PCR analyses. The expression levels in DKO cells (DNA methyltransferases deficient) were compared to those of their counterpart, the HCT166 cell line. (D) MS-HRM (Methylation Sensitive-High Resolution Melting) region 1 was selected for the DNA methylation analysis in matched samples obtained from peripheral blood and tumoral tissues. This comparative analysis showed complementary genetic and epigenetic mechanisms of *PHF21B* gene disruption in HNSCC samples (cases 12, 15, 17, 26, 36, 70, 71, and 74). (E) Aligned melt curves and difference plot obtained from MS-HRM analysis demonstrating the presence and absence of DNA methylation in HCT116 and DKO cell lines, respectively. (N) DNA from matched normal and (T) tumoral tissues from individual patients.

3. Results

3.1. PHF21B copy number in HNSCC patients

To confirm if *PHF21B* loss was associated with HNSCC, gene copy number alterations were evaluated by qPCR in two groups of patients: positive ($n = 40$) and negative ($n = 35$) for familial history of cancer. Losses were detected in 43/75 HNSCC tumor samples (57.3%). Significant higher frequency of *PHF21B* losses was detected in patients with family history of cancer (90%, 36/40 cases) in comparison with patients without familial history of cancer (20%, 7/35) ($P < 0.0001$). Among the 36 cases with loss of *PHF21B* and positive for family history of cancer, 22 had first-degree relatives affected by HNSCC/HNSCC-associated cancers ($P = 0.049$), being four cases with first-degree relatives with HNSCC (Table 2).

DNA from matched peripheral blood samples of a subgroup of 49 HNSCC patients was also analyzed by qPCR for relative *PHF21B* copy number alterations (Figure 1B). Constitutional losses were detected in six patients (cases 04, 35, 49, 50, 52 and 71), of which one had a family history of cancer (case 04) and three (cases 35, 49 and 71) have lost follow-up. Of these, five cases were concordant for *PHF21B* losses in the paired tumor samples (Figure 1B).

No significant association was observed between copy number alterations and gender ($P = 0.538$), age (≤45 and >45 years old; $P = 0.216$), tumor site ($P = 0.361$), tumor size (T1 + T2 *versus* T3 + T4; $P = 0.290$), lymph node involvement (N_0 *versus* N+; $P = 0.366$), tobacco ($P = 0.494$) and alcohol usage ($P = 1.000$) (Table 2). Family history of cancer in tobacco users (25 out of 40 patients; 62.5%) was associated with a high frequency of losses in the *PHF21B* locus (56.7%) in these tumors.

Table 2 – Distribution of tumor and blood samples according to qPCR results for *PHF21B* gene. N°: total sample number; Nd: Data not determined.

Variable		Tumor samples			Blood samples		
		Cases (N° = 75)	Cases with *PHF21B* loss (%)	P-value	Cases (N° = 49)	Cases with *PHF21B* loss (%)	P-value
Age	≤45 years	16	7 (43.8)	0.216[a]	11	0 (0.0)	0.315[b]
	>45 years	59	36 (61.0)		38	6 (15.8)	
Gender	Male	61	36 (59.0)	0.538[b]	40	4 (10.0)	1.000[b]
	Female	14	7 (50.0)		9	1 (11.1)	
Tobacco	Yes	40	25 (62.5)	0.494[a]	20	0 (0.0)	0.259[b]
	No	10	5 (50.0)		7	1 (14.3)	
	Nd	25			22		
Alcohol	Yes	40	24 (60.0)	1.000[a]	20	0 (0.0)	0.231[b]
	No	9	5 (55.6)		6	1 (16.7)	
	Nd	26			23		
Tobacco + alcohol	Yes	39	23 (59.0)	1.000[a]	16	0 (0.0)	0.157[b]
	No	11	7 (63.6)		11	1 (9.1)	
	Nd	25			22		
At least one criteria for familial risk	Yes	46	27 (58.7)	0.764[b]	28	1 (3.6)	0.150[b]
	No	29	16 (55.2)		21	4 (19.0)	
Family history of cancer	1 degree	30	22 (73.3)	0.049[b]	19	0 (0.0)	0.067[b]
	No	29	14 (48.3)		14	3 (21.4)	
	Nd	16			16		
Family history of HNSCC	1 degree	7	4 (57.1)	1.000[a]	1	0 (0.0)	1.000[b]
	No	50	30 (60.0)		31	3 (9.7)	
	Nd	18			17		
N stage	N0	38	24 (63.2)	0.366[b]			
	N+	36	19 (52.8)				
	Nd	1					
Clinical stage	I + II	17	13 (76.5)	0.080[b]			
	III + IV	57	30 (52.6)				
	Nd	1					
HPV16+	Yes	5	3 (60.0)	1.000[a]			
	No	54	29 (53.7)				
	Nd	16					
Recurrence	Yes	18	12 (66.7)	0.551[b]			
	No	31	18 (58.1)				
	Nd	26					
Death	Yes	43	24 (55.8)	0.814[b]			
	No	29	17 (58.6)				
	Nd	3					

a Pearson's Chi-square.
b Fisher's exact test.

Five out of 59 tumor samples (7.8%) evaluated for HPV infection were positive for HPV16, being four oropharyngeal carcinomas (47, 50, 52, and 53) and one oral cavity carcinoma (case 21). Three out of five patients (60%) were negative for family history of cancer (cases 21, 47, and 50) and presented no alcoholic and smoking habits. Only one patient (21) was smoker and alcohol consumer.

3.2. PHF21B *mutation screening*

We screened for mutations in the *PHF21B* gene in a set of 26 matched DNA samples obtained from peripheral blood and tumors. Ten patients had a family history of cancer; nine out of 10 had first-degree relatives affected by cancer. Six exons were screened by direct sequencing, including exons 6, 7, and 8, corresponding to the protein's PHD domain. Sequences were matched to DNA reference sequences from a primary sequence database (NCBI GenBank, using RefSeq NM_138415.4). No germline or somatic acquired mutations were detected.

3.3. *Epigenetic analysis of* PHF21B *promoter-associated CpG islands*

Initially, we performed *PHF21B* transcript expression analysis in a panel of nine human cancer cell lines treated with a demethylating agent. Four of the cell lines were derived from HNSCC tumors (SCC4, SCC9, SCC15, and FaDU), and five cell lines were derived from breast cancers (SK-BR-3, Hs578T, MDA-MB-231, MDA-MB-415, and MDA-MB-453). These cell lines were treated with 5-Aza-dC alone or in combination with TSA. We then assessed the alterations in *PHF21B* expression levels to test our hypothesis of gene silencing by epigenetic mechanisms. Low expression levels were observed in all cells lines prior to the treatment. HNSCC-derived cell lines showed a discrete augmentation of expression after treatment with 5-Aza-dC alone (Figure 1C). In two breast cancer cell lines (Hs578T and MDA-MD-415), we observed significant overexpression after treatment with the demethylating agent, but the opposite effect was observed in the SK-BR-3 and MDA-MB-231 cell lines. The effect of DNA methylation in the regulation of the *PHF21B* gene was clearly observed in the biologically demethylated DKO cells, which are deficient in DNA methyltransferase function due to the double knockout of DNMT1 and DNMT3B. Compared with their counterparts, HCT116 cells, DKO cells showed significant overexpression of *PHF21B* (Figure 1C).

Based on these results, an MS-HRM assay was designed to screen the DNA methylation status in three sites within the *PHF21B* CpG Island (Figure 1A). According to data from ENCODE project, region 1 is associated with an epigenetic status compatible with active regulatory elements, namely the presence of RNA polymerase II (Pol2) and acetylation of lysine 27 of histone H3 (H3K27Ac) (online data available in the UCSC Genome Browser at http://genome.ucsc.edu). As expected from expression analysis, HNSCC-derived cell lines were unmethylated. However, the presence of methylation in this CpG island was confirmed in the MDA-MD-231 (25%) and HCT116 cell lines (50%). Additionally, as expected, DKO cells were unmethylated (Figure 1D).

In parallel, the methylation analysis of CpG dinucleotides in the *PHF21B* promoter (Figure 1D and E) was investigated in matched DNA samples (peripheral blood and tumor) from 32 patients. No methylation was detected in DNA samples from peripheral blood. Tumor-specific methylation was detected in 13 out of 32 samples (ranging from 2.5% to 25% of methylation). Interestingly, in 8/13 cases (cases 12, 15, 17, 26, 36, 70, 71, and 74), promoter methylation was detected concurrently with the loss of *PHF21B* gene (Figure 1D). Among these 13 cases, five had first-degree relatives affected by cancer, three showed no family history of cancer, and five did not have a follow-up. One case (50) showed *PHF21B* loss in both blood and tumor samples without changes in DNA methylation. A second case (52) showed *PHF21B* loss in the blood sample and promoter methylation in the tumor sample. However, the presence of aberrant methylation of the *PHF21B* gene in HNSCC was not associated with clinicopathological parameters.

3.4. *Functional aspects of* PHF21B

Since the functions of *PHF21B* are not known, we performed a sequence alignment and searched for domain functions using another member of the family, PHF21A (Figure 2A). PHF21A is transcriptional repressor in the nervous system during development (Klajn et al., 2009; Lan et al., 2007). *PHF21B* shares with PHF21A the PHD zinc finger domain, the DNA binding domain and the region required for transcriptional repression, suggesting that *PHF21B* may have similar functions. Accordingly, both endogenous and transfected *PHF21B* localize to the nucleus, further implying a role in transcriptional control (Figure 2B–H).

To address the role of *PHF21B* in tumor cells, MDA-MB231 and SCC9 cell lines that express low levels of *PHF21B* were transfected with a fusion protein of *PHF21B* tagged with GFP or GFP alone. Cells were evaluated by their clonogenic and migration abilities. Overexpression of *PHF21B* drastically reduced the migratory ability of both MDA-MB231 and SCC9 cells when compared to the control with GFP (Figure 2I and J). The clonogenic ability of MDA-MB231 cells was also drastically reduced when compared to cells transfected with GFP alone (Figure 2K). These data suggest that *PHF21B* is a novel tumor suppressor gene with functions in migration and colony formation.

The response of GFP transfected in MDA-MB-231 cells exposed to cisplatin was significantly different from GFP-*PHF21B* transfected cells ($P = 0.0013$, two way ANOVA test followed by Bonferroni correction test), indicating that the presence of *PHF21B* decreases cellular resistance to this chemotherapeutic agent (Supplementary Figure S1).

4. Discussion

Although the genetic basis of familial HNSCC is unknown, previous studies have indicated an increased risk of developing cancer in first-degree relatives of patients with HNSCC. Evidence for a hereditary syndrome associated with HNSCC development includes the occurrence of these tumors in family groups having different lifestyle factors, the early onset of HNSCC cancer in non-smokers or individuals lacking any risk factors, and/or aggregation in families by tumor site (Toner

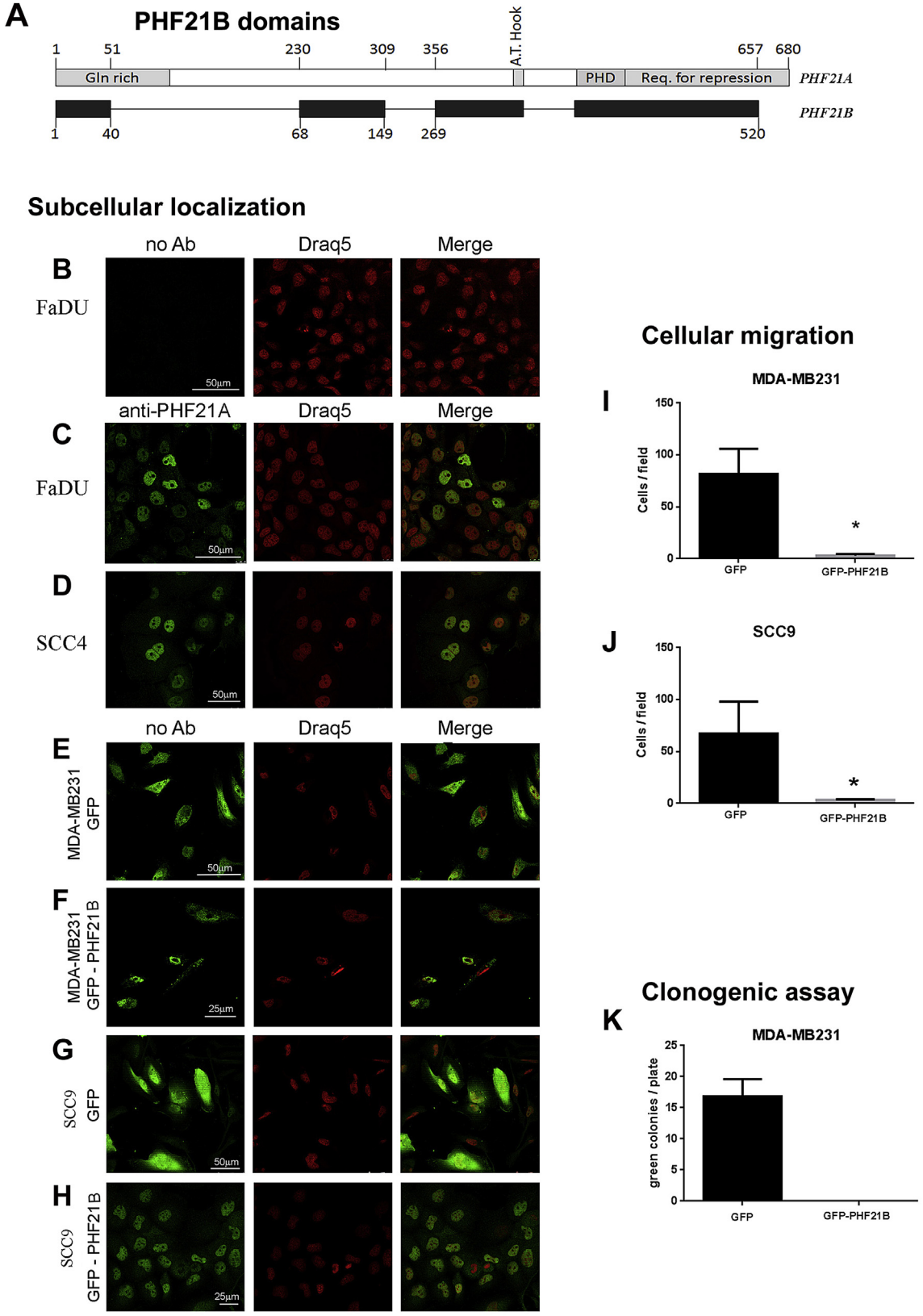

Figure 2 – Clonogenesis and migration assays performed for PHF21B. (A) Amino acid sequence alignment between PHF21A and *PHF21B* reveals conservation of domains important for transcriptional repression. The sequences from the two proteins were aligned using BLASTP. Important functional domains from PHF21A were visualized by UNIPROT: a GLN-rich region is found between residues 4-108; a DNA binding domain A.T Hook is found between 425 and 437; a PHD-type zinc finger domain is found between 488 and 535; a region required for transcriptional repression is found between 486 and 680. (B–H) Subcellular distribution of endogenous and transfected *PHF21B*. FaDU (B and C) and SCC4 (D)

and O'Regan, 2009). Although these reports have indicated that family history of head and neck cancer is a strong determinant of cancer risk, only one study has demonstrated germline mutations in *TP16* in a family with members having melanoma, HNSCC and lung cancer (Yu et al., 2002).

Previous data of our group showed an association between high frequencies of chromosome 22 deletions and family cancer history in patients with HNSCC (Reis et al., 2002a, 2002b; Bérgamo et al., 2005). Thus, we hypothesize that *PHF21B* gene mapped at this region might be a putative tumor suppressor gene involved in HNSCC development.

PHF21B is located 7.6 Kb from the D22S274 marker and is a conserved gene that encodes a potential histone modification reader with a PHD zinc finger domain (Collins et al., 2003; Gerhard et al., 2004). Initially, we detected expression of the *PHF21B* transcript in normal gingival tissue, in several human cell lines (derived from breast carcinomas, hepatoblastoma, colorectal carcinoma, oral carcinoma) and in solid tumor samples (uterine leiomyomas, breast and head and neck carcinomas) (data not shown).

We further characterized the association between chromosome 22 deletions and family cancer history based on the two-hit hypothesis of tumor suppressor inactivation (Knudson, 1971). We first examined the occurrence of *PHF21B* losses in a subset of matched peripheral blood and tumor DNA samples taken from each individual. Constitutional deletion of *PHF21B* was detected in five paired cases, with family cancer history confirmed in one (case 04). Nonrandom losses detected in the *PHF21B* gene in matched peripheral blood and tumor samples and the association with family cancer history suggested that this gene is a novel candidate in the predisposition for HNSCC. However, confirming if *PHF21B* loss predisposes for HNSCC development is a difficult task due to the lack of accurate information on affected family members and unavailable follow-up in several families. In addition, it is very difficult to obtain samples from the relatives of affected family members.

To investigate another potential mechanism of gene inactivation, *PHF21B* mutations were assessed in 26 paired samples (normal and tumoral). This analysis included exons 6, 7, and 8, encoding for the PHD domain. The absence of mutations in the PHD domain of the *PHF21B* gene are in accordance with the literature for other proteins containing the same domain, such as the inhibitor of growth (ING) family. Mutations in genes encoding PHD finger-containing factors have been found to be associated with immune diseases and also with the pathogenesis of several cancers (Baker et al., 2008). In head and neck tumors, previous studies have indicated that *ING1* (inhibitor of growth family, member 1, mapped on 13q34) (Gunduz et al., 2000) and *ING3* (7q31) (Gunduz et al., 2002) are rarely mutated but possessed a high allelic deletion rate. Missense mutations were found with low frequency within the PHD finger domain and in the nuclear localization motif in the ING1 protein; it is likely that these mutations abrogate the protein function in HNSCC (Gunduz et al., 2000). Frequent allelic losses and reduced expression of *ING3* (Gunduz et al., 2002), *ING4* (Gunduz et al., 2005; Li et al., 2011), *ING2* (4p35.1) (Borkosky et al., 2010), and *ING5* (2q37.3) (Cengiz et al., 2010) were also reported in HNSCC. Down-regulation of *ING* family members has been associated with HNSCC progression (Borkosky et al., 2010; Li et al., 2011). Additionally, the decreased expression of the *ING* genes belonging to the PHD finger family suggests that transcriptional mechanisms, such as promoter methylation, could be associated with their down-regulation. These findings described in ING family members are similar to those found in the present study.

The *PHF21B* gene and its flanking regions are highly GC-rich. We thus sought to determine whether aberrant DNA methylation of the *PHF21B* gene promoter was a mechanism in the inactivation of this gene. To test this hypothesis, we initially performed an *in vitro* assay by treating HNSCC and breast cancer cell lines with the demethylating nucleoside 5-Aza-dC, alone or in combination with the histone deacetylase inhibitor trichostatin A (TSA). With the exception of MDA-MB-231, the MS-HRM assay failed to demonstrate the presence of DNA methylation in the promoter CpG islands in HNSCC and other breast cancer cell lines analyzed. As expected from the expression analysis following demethylating treatment, it was observed the lack of promoter methylation in HNSCC cell lines. However, in the Hs578T and MDA-MB-415 breast cancer cells, the treatment with 5-Aza-dC resulted in an increase in the *PHF21B* transcript levels not associated with changes in DNA methylation. Komashko and Farnham (2010) reported that most changes in gene expression caused by the 5-Aza-dC treatment are from genes whose promoter regions are not silenced by DNA methylation or repressive histone marks. Instead, most alterations were found in genes that reside in active chromatin. Alternatively, it is possible that alterations in *PHF21B* gene expression observed in treated cells is the result of a secondary effect in which transcription factors are demethylated, resulting in gene up-regulation. These transcription factors could initiate a cascade of transcriptional alterations resulting in the *PHF21B* up-regulation we observed in Hs578T and MDA-MB-415 breast cancer cells. In addition, 5-Aza-2-dC treatment should induce the expression of genes involved in the response to DNA damage, including p53-activated genes, as a secondary effect of DNA adducts and double-strand breaks (Pulukuri and Rao, 2005).

The hypothesis of epigenetic regulation of the *PHF21B* gene was supported by the combined analysis of the HCT116 cell line and its derivative, the biologically demethylated DKO cell line. *PHF21B* overexpression was associated with loss of DNA methylation in DKO cells and with the inverse pattern

cells were plated and subjected to immunohistochemistry with no primary antibody (B) or anti-*PHF21B* (C and D) in green. Nuclei were labeled by Draq5 in red. Alternatively, MDA-MB-231 (E and F) or SCC9 (G and H) cells transfected with GFP (E and G) of GFP-*PHF21B* (F and H) were fixed and nuclei were labeled with Draq5 in red. Images were taken by confocal microscopy. (I and J) *PHF21B* impairs cellular migration and colony formation. (I) MDA-MB-231 and SCC9 (J) cells transfected with GFP or GFP-*PHF21B* were plated in transwells chambers without fetal calf serum. Chemoattraction was stimulated by the addiction of 10% serum in the lower chamber. The number of green cells that migrated to the lower chamber was quantified after 6 h. (K) Twenty-five MDA-MB-231 cells transfected with GFP or GFP-*PHF21B* were plated in 35 mm dishes. After 10 days, the number of green colonies was quantified.

in HCT116 cells. Furthermore, DNA methylation analysis by MS-HRM was conducted in matched DNA samples from peripheral blood and tumors from the same patients. Tumor-specific DNA methylation was detected in 13/32 HNSCC cases (40%). Among these 13 cases, five had first-degree relatives affected by cancer and three cases had no family history. In 8/13 tumors, methylation status was associated with the loss of expression of *PHF21B*. These results suggested that genetic and epigenetic changes are involved in loss of function of the *PHF21B* gene in HNSCC.

PHF21B encodes for PHD finger protein 21B of 531 amino acids (NP_612424.1) which function is yet unknown. The other member of the family, *PHF21A/BHC80*, encodes for a member of BHC [core-BRCA2-associated factor (BRAF)-histone deacetylase (HDAC)], a repressive complex in neurons (Klajn et al., 2009). PHF21A protein binds unmethylated histone H3 lysine 4 and participates in the lysine-specific demethylase 1 complex, being implicated in histone-methylation dynamics (Lan et al., 2007). Amino acid sequence alignment with PHF21A, confirmed the presence of domains involved in transcriptional repression, which is further strengthened by the presence of *PHF21B* in the nucleus. Impressively, *PHF21B* overexpression reduces cellular ability of clonogenesis and migration, suggesting a role for *PHF21B* in tumorigenesis.

Our results point to an increased appearance of tumors in families with reduced expression of *PHF21B*, suggesting a mechanism of increased cellular transformation. In fact, our results in cells point that in the absence of *PHF21B* there is an increase in transformation ability of cells, evidenced by increased colony formation and migration. In addition, we evaluated chemotherapy sensitivity and observed that the presence of *PHF21B* decreases cellular resistance to cisplatin in MDA-MB-231 cells but not in SCC9 cells (Supplementary Figure S1). Although only two cells lines have been evaluated, the presence of *PHF21B* protein suggested a cell-type specific effect. If the presence of *PHF21B* does not change drug sensitivity, this gene may still have other mechanisms of promoting tumorigenesis, as demonstrated herein. We believe that *PHF21B* can be considered a tumor suppressor gene whose loss is related to the acquisition of tumorigenic abilities such as colony formation and migration. Future studies will elucidate the underlying mechanisms of loss of *PHF21B* function in cancer cells.

5. Conclusion

Our data suggest that *PHF21B* may be a novel tumor suppressor gene. *PHF21B* losses in matched blood and tumor samples, together with the acquired DNA methylation, suggest that this gene is involved in the HNSCC development. Cell assays demonstrate that *PHF21B* overexpression reduces clonogenic ability and migration capability, confirming its potential role as a novel tumor suppressor gene.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.09.009.

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