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HOPX homeobox methylation in differentiated thyroid cancer and its clinical relevance

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Abstract

Background: The inactivation of the tumor-suppressor homeodomain-only protein X (HOPX) usually involves promoter methylation in several cancer types. This study aimed to investigate the *HOPX-β* mRNA expression and promoter methylation and their clinical relevance in differentiated thyroid cancer (DTC).
Patients and methods: Clinicopathological data and paraffin-embedded thyroid tumor tissues from 21 patients with DTC and 6 with benign tumors (T) and their non-tumor parenchyma (NT) were investigated. Tumor cell lines (FTC238, FTC236 and WRO) were treated with demethylating agent. *HOPX-β* mRNA expression was assessed by qRT-PCR and methylation status by Q-MSP. Thyroid cancer data from Cancer Genome Atlas (TCGA) was also collected.
Results: *HOPX-β* mRNA re-expression in two cell lines treated with demethylating agent was observed concomitantly with reduced promoter methylation. Reduced mRNA expression in T group compared to their NT was observed, and reduced protein expression in T compared to NT was observed in three cases. Low mRNA expression with high methylation status was detected in 6/14 DTC samples. High methylation status was associated with older age at diagnosis, recurrent or progressive disease and with the presence of new neoplasm event post initial therapy while hyper-methylation correlated with worse overall survival, worse disease-free status and older age.
Conclusion: A moderate coupling of downregulation of *HOPX-β* mRNA expression in DTC followed by high *HOPX-β* promoter methylation was observed however; high *HOPX* promoter methylation status was associated with the worse prognosis of DTC patients.

Key Words
▶ HOPX
▶ methylation
▶ differentiated thyroid cancer
▶ epigenetic

Introduction

Differentiated thyroid cancer (DTC) has continued to increase over the past several decades, likely due to enhanced detection (1, 2). Cancer research, in general, has benefited greatly from many of the new scientific technologies such as DNA microarray, proteomic techniques and epigenetic profile analysis (3) which have ushered in the discovery of novel biomarkers and targets for drug design. More specifically, thyroid carcinogenesis and progression has been shown to be affected by a growing number of DNA alterations in tumor-suppressor genes, especially via gene promoter methylation (4) causing a silencing of tumor-suppressor genes that are linked to apoptosis or DNA repair (5, 6). A better understanding of such modifications may open the way to host innovative targeted therapies in DTC.

The homeodomain-only protein X (HOPX) (Ensembl: ENSG00000171476) inhibits gene transcription through sequestration of serum-responsive factor and recruitment of histone deacetylase (7). HOPX has been implicated in contributing to the epigenetic regulation of differentiation-associated genes, encompassing crucial oncoproteins such as angiogenic growth factor of CYR61, cell-surface proteins such as EMP1 and EPHA2, transcriptional factor such as c-Fos, EGR1 and metabolism-related genes like GLUT3 (8, 9, 10). It is omnipresent in a large cluster of normal tissue, but less in malignant samples including choriocarcinoma, endometrial, lung and gastrointestinal (GI) cancers (8, 10, 11, 12, 13). It has been demonstrated that ectopic HOPX expression constrains tumor growth and that RNA interference knockdown of endogenous HOPX rebuilt it, advocating its strong tumor-suppressor action (14, 15). Its inactivation mechanism during cancer pathogenesis usually involves promoter methylation (8, 13, 16). Two *HOPX* promoters have been reported and recent studies indicates that there are five splice variants *HOPX* (16). However, only *HOPX-β* (NM 139211.2) promoter harbors CpG islands including the first exon and intron (13). Its epigenetic control has been correlated with tumorigenesis and worse prognosis in uterine, breast, esophageal, gastric, pancreatic and colorectal cancers (10, 11, 12, 13, 15, 17, 18). However, the correlation between HOPX-β and DTC remains unknown with only one study, involving six papillary thyroid cancer (PTC) samples, which showed upregulation in four PTC tumors, contrasting with previously described downregulation observed in other types of cancer (19). In the present study, we investigated the *HOPX-β* gene expression and promoter methylation status in DTC tissues, cancer cell lines and in thyroid cancer samples from The Cancer Genome Atlas (TCGA) database. The clinical relevance of *HOPX* expression and methylation was also studied.

Methods

Clinical specimens

From August 2013 till September 2013, paraffin-embedded thyroid tumor tissues (T) and paired non-tumor parenchyma (NT) were collected from consecutive patients diagnosed with stage I to III DTC and thyroid benign lesions that were submitted to surgery at São Rafael Hospital. NT tissue was defined as the adjacent area to the site of the lesion with no histologic signs of abnormal pathology. All samples with evidence of chronic lymphocytic thyroiditis were excluded, in an attempt to minimize differences due autoimmune disease. TNM and risk of recurrence classification was made according to the American Joint Committee on Cancer (AJCC) 7th edition and ATA guidelines (American Thyroid Association) staging system, respectively (20, 21).

A total of 27 patients were included in the current study. Of these, 21 patients were diagnosed with stage I to III DTC and 6 patients with thyroid benign lesions (two follicular adenomas and four hyperplastic nodules). Clinical and pathological data of all DTC patients are described in Table 1. Due to the reduced amount of tissue available, three other T (PTC) and NT samples from a previous study (22) were included to investigate protein expression. This study was approved by the Federal University of Bahia – Ethical Committee for Research Projects.

Quantitative polymerase chain reaction

Total RNA was obtained using the TRIzol method, reverse-transcribed with SuperScript III reverse transcriptase kit (Life Technologies) and quantified with Nanoespectrophotometer (KASVI, Curitiba, PR). Quantitative real-time PCR (qRT-PCR) was carried out and normalized to S8 gene expression using Platinum SYBR Green Master Mix (SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, Passive Reference I, Buffer) and a 7500 Real-Time PCR System (Life Technologies) in conditions previously reported (15).

Western blot analysis

Immunoblotting (10% SDS-PAGE) was performed with primary HOPX antibody (1:1000; Cell Signaling)

Table 1 Clinical-pathological characteristics of 21 patients with differentiated thyroid cancer and *HOPXβ* mRNA expression.

Pt. (M/F)	Age (years)	Histology	MF	EE	Invasion Capsular	Invasion Vascular	Invasion Perineural	LN Metastasis	TNM staging T	TNM staging N	TNM staging M	Risk ATA	Risk AJCC	HOPXβ mRNA (AU) Nontumor	HOPXβ mRNA (AU) Tumor
1 (F)	57	PTC[a]	No	No	No	No	No	No	T1a	N0	M0	Low	I	13.319	4.162
2 (M)	41	PTC[a]	Yes	Yes	Yes	Yes	Yes	Yes	T3	N1b	M1	High	II	2.343	1.644
3 (F)	52	PTC[a]	Yes	No	No	No	No	No	T1a	N0	M0	Low	I	0.744	1.552
4 (M)	49	PTC[a]	Yes	No	No	Yes	No	Yes	T1a	N1a	M1	Inter.	III	1.718	2.591
5 (F)	72	PTC[a]	No	Yes	Yes	Yes	No	No	T3	N0	M1	Inter.	III	0.159	0.857
6 (M)	48	PTC[a]	Yes	No	No	No	No	No	T1a	N0	M0	Low	I	1.182	1.974
7 (F)	32	PTC[b]	Yes	Yes	Yes	Yes	Yes	Yes	T3	N1a	M0	High	I	2.272	4.526
8 (F)	30	PTC[a]	No	No	No	No	No	No	T1b	N0	M0	Low	I	3.909	1.463
9 (M)	45	PTC[a]	No	No	No	No	No	No	T1a	N0	M0	Low	I	1.749	0.286
10 (F)	52	HCC	No	No	Yes	No	No	No	T1a	N0	M0	Low	I	3.397	0.743
11 (F)	42	PTC[a]	Yes	Yes	Yes	No	No	No	T3	N0	M0	Inter.	III	12.693	4.155
12 (F)	57	PTC[a]	No	Yes	No	No	No	No	T3	N0	M1	Inter.	I	5.299	1.413
13 (F)	44	PTC[a]	No	No	No	No	No	No	T1a	N0	M0	Low	I	11.817	3.217
14 (F)	27	PTC[a]	Yes	No	No	No	No	Yes	T1a	N1a	M0	Inter.	I	1.592	1.557
15 (F)	27	PTC[c]	Yes	No	Yes	No	No	No	T1b	N0	M0	Low	I	2.408	2.247
16 (F)	55	PTC[a]	Yes	No	No	No	No	No	T1a	N0	M0	Low	I	0.717	1.735
17 (F)	49	PTC[a]	Yes	No	No	No	No	No	T1a	N0	M0	Low	I	6.777	1.475
18 (M)	54	PTC[a]	Yes	Yes	No	No	No		T1a	N1a	M1	Inter.	III	1.62	1.844
19 (F)	38	PTC[a]	No	No	Yes	No	No	No	T3	N0	M0	Inter.	I	1.838	1.344
20 (M)	33	PTC[a]	No	No	No	No	No	No	T1a	N0	M0	Low	I	104.04	51.864
21 (F)	30	PTC[a]	Yes	Yes	Yes	Yes	Yes	Yes	T3	N1b	M0	High	I	6.235	2.643

[a]Classic variant; [b]solid variant; [c]follicular variant.

AJCC, 7th Edition American Joint Committee Cancer Staging System; ATA, American Thyroid Association; F, female; EE, extrathyroid extension; HCC, Hürthle cell carcinoma; LN, lymph nodes; M, male; MF, multifocality; Pt., patient; PTC, papillary thyroid carcinoma.

and tubulin antibody (1:10,000; Sigma-Aldrich). Band densitometry was performed using ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Cell treatment with 5-Aza-dC demethylating agent

To quantify *HOPX-β* gene expression, tumor cell lines FTC238 (catalog no. 94060902), FTC236 (catalog no. 06030202), WRO (metastatic thyroid FTC cell lines, ECACC; Health Protection Agency Culture Collection; depositor, J Köhrle and Orlo Clark) were treated with 5-aza-2′-deoxycytidine (5-Aza-dC, Sigma-Aldrich). Cells (5.0×10^5) were grown at 37°C in 5% CO_2 for 4 consecutive days in adequate culture medium plus 15 µm 5-Aza diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) or DMSO alone as an experimental control. All assays were performed in triplicate. At the end of the treatment, total RNA and genomic DNA were obtained for subsequent analysis of *HOPX-β* and S8 mRNA expression by real-time PCR.

Quantitative methylation-specific PCR (Q-MSP)

Genomic DNA was obtained with the Gentra Puregene Kit (QIAgen). For quantitative methylation analysis, all reactions were performed in triplicate. Primer sequences for *HOPX-β* and β-actin have been previously described (15). With 18 µL total volume reaction containing 20 ng of DNA previously treated with EpiTect Bisulfite (QIAgen), the PCR was performed with 7500 FAST Real-Time PCR System (Life Technologies) in conditions of 95°C for 3 min, followed by 45 cycles of 95°C for 20 s, 60°C for 30 s and 72°C for 30 s. The same previously reported DNA region was chosen for Q-MSP, which examined HOPX-β methylation in gastric and colorectal cancer and enclosed nine CpG sites (10, 17). The samples were considered to be methylated when amplification was detected in at least two triplicates. EpiTect Control DNA (Qiagen) served as a positive control and generated standard curves from 1.5 dilution series. Percent Methylated Reference (PMR) was calculated using a procedure previously described (23). In brief, the PMR was calculated as the ratio of the median value of the *HOPX-β*:β-actin ratio of each sample divided by the median value of the *HOPX-β*:β-actin of the fully methylated control DNA and multiplied by 100 with the Cts (cycle thresholds) for all samples converted to a relative quantity scale using a standard curve of diluted methylated DNA. The ratio of the 'quantity' of *HOPX-β*-methylated DNA to total β-actin DNA was calculated for both the samples of interest and the fully methylated control DNA.

Methylation and expression analysis from The Cancer Genome Atlas (TCGA) database

A total of 564 samples (508 papillary tumors (T) and 56 non-neoplastic tissues (NT)) of the 580 TCGA thyroid samples (THCA) (515 T, 65 NT) were selected in this study (24) (http://cancergenome.nih.gov). Among them, 55 T samples paired with NT adjacent samples. Samples without methylations data, classified as poorly differentiated thyroid cancer, from patients that received neoadjuvant therapy prior to resection or without complete clinical data were not selected.

The *HOPX* methylation beta values from TCGA data (level 2) (24) were collected from Xena Public Data Hubs (https://xena.ucsc.edu/). From the 25 *HOPX* CpG sites with methylation data, six CpG sites within the CpG island of the *HOPX-β* promoter were considered relevant and the methylation value of the island was calculated as the sum of their beta values (25). To categorize T samples as hyper-methylated threshold methylation value of 1.89 was determined considering the distribution of the beta value for normal and T samples as previously described (25).

HOPX expression data were also collected from Xena Public Data Hubs as the RSEM value (normalized expectation maximization value) for gene expression (polyA + Illumina Hiseq) (level 3). The clinical data of these patients was download from Cbioportal for cancer genomics (level 3) (http://www.cbioportal.org/study?id=thca_tcga#clinical) (26). Clinical and pathologic characteristics are presented in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Statistical analysis

All data were analyzed with SSP version 22 or GraphPrim 6. Results were expressed as mean±s.d. or median (maximum–minimum). The χ^2 test or Fisher exact test was used for categorical variables and Student's *t* test, Wilcoxon matched-pairs signed-rank test for non-parametric data, ANOVA and Pearson correlation test were used for continuous variables. Fisher exact tests, χ^2 test and multivariate logistic regression analysis were performed for correlation analysis of *HOPX-β* promoter methylation status with clinic–pathologic parameters. Kaplan–Meier method using log-rank test was used for survival analysis and Cox proportional hazard model for multivariate prognosis analysis. The value of $P < 0.05$ was considered significant.

Results

HOPX-β expression and methylation status in thyroid cancer cells cultures

Cell cultures of FTC236, FTC238 and WRO were treated with 5-Aza-dC to investigate *HOPX-β* expression and methylation. The *HOPX-β* mRNA mean normalized expression was significantly higher in WRO cells treated with 5-Aza compared to untreated cells (2.85 ± 0.44 AU; 8.76 ± 1.05 AU, $P=0.03$). Similar result was observed in FTC236 (0.085 ± 0.04 AU; 1.176 ± 0.055 AU) but not significant (Fig. 1A). The PMR (percent of methylated reference) values of HOPXβ promoter methylation were reduced in FTC238 (1.47 ± 0.38 AU; 0.38 ± 0.11 AU) and in WRO 5-AZA-treated cells (5.08 ± 1.37 AU; 2.74 ± 0.025 AU) (not significant) compared with untreated cells (Fig. 1B). The HOPX protein expression was higher in FTC238 5-AZA-treated cells compared with untreated cells (Fig. 1C). In FTC236 cells no change in *HOPX-β* mRNA and protein expression, and methylation values treatment were observed after 5-Aza treatment.

HOPX-β expression and methylation status in DTC

We next compared mRNA levels of *HOPX-β* in 27 patients with malignant thyroid tumors ($n=21$) or benign thyroid tumors ($n=6$) with a matched adjacent NT tissue from each of these patients. As seen in Fig. 2A, the malignant tumor samples showed a reduced expression of *HOPX-β* mRNA (1.73 (0.28–51.86) AU) compared with their NT tissue (2.34 (0.16–104.0) AU) ($P=0.02$). This pattern was sustained when the outlier (sample #20) was excluded (T: 1.69 (0.28–4.53) AU; NT: 2.37 (0.16–13.32) AU) ($P=0.04$). At all 14/21 (67%) pairs had lower *HOPX-β* mRNA expression in the malignant tumor sample compared to their NT parenchyma tissue (Table 1). As for the six patients with benign lesions (Fig. 2B), there was no meaningful difference between *HOPX-β* mRNA expression in benign tumor tissues (1.07 (0.13–2.68) AU) when compared with their NT tissue (1.45 (1.26–49.22) AU); however, four of the six pairs of samples showed T<NT expression pattern. We found no relationship between *HOPX-β* mRNA downregulation and risk of recurrence classification (21). We then performed methylation analysis in 14 available DTC tumors and matched NT samples. Figure 3 shows a tendency of increased overall PMR in tumor samples (0.31 (0.02–1.88) AU) compared with NT paired sample (0.13 (0.003–1.02) AU) $P=0.055$. Among them, in 10 (71.4%) samples the tumor methylation status was increased compared to paired NT. Figure 4 details
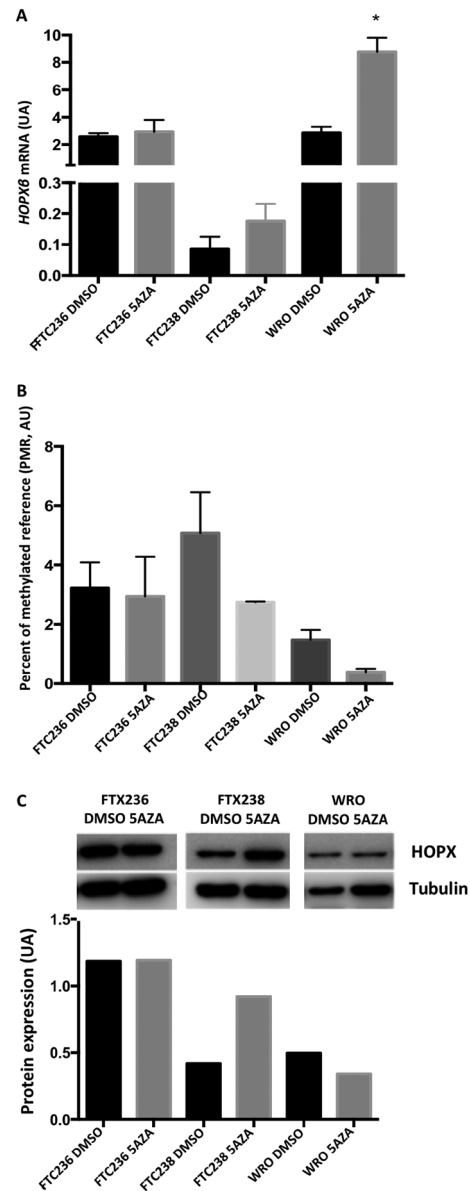

Figure 1

HOPX-β mRNA (A) and protein expression (C), and PMR (percent of methylated reference) values of HOPXβ promoter methylation (B) before (DMSO) and after (5AZA) treatment with the demethylating agent 5-AzadC in cell cultures of FTC236, FTC 238 and WRO. (*$P=0.03$ between control and treated cells.) AU, arbitrary units.

the results by patient in which 6 of the 14 (42.8%) did present downregulation of *HOPX-β* mRNA coupled with promoter methylation. In evaluating this particular subset of patients in comparison to the six patients who did not show a pattern of promoter methylation and gene silencing, we found no evident correlation with any clinical variable. Due to the reduced quantities of tissue available of these samples for HOPX protein expression analysis was investigated in three other thyroid samples
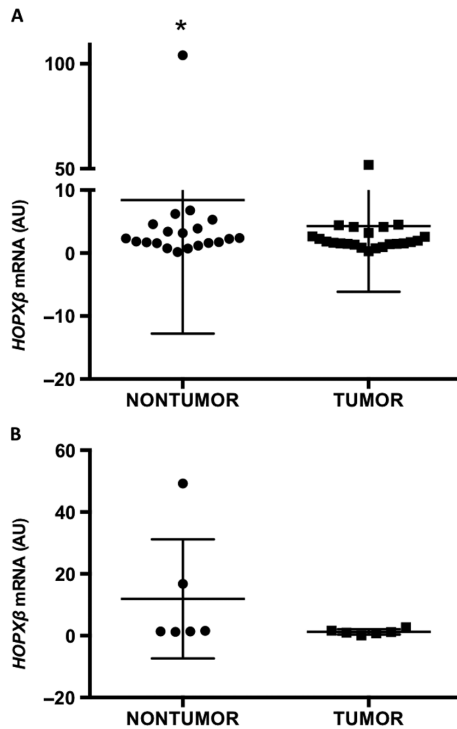
Figure 2

Expression level of *HOPX-β* mRNA in different primary thyroid tumors (A) malignant group, *n* = 21; (B) benign group, *n* = 6; and their matched non-tumor tissues determined by qRT-PCR. Reduced expression in tumor compared with non-tumor samples was observed in malignant groups (*P = 0.03). AU, arbitrary units.

Figure 3

PMR (percent of methylated reference) values of *HOPX-β* promoter methylation in differentiated thyroid cancer (tumor) (*n* = 14) and their matched non-tumor tissues determined by Q-MSP (quantitative methylated PCR). A tendency of high degree of methylation was observed in the malignant tumor samples compared to the matched surrounding non-tumor samples (*P* = 0.055). AU, arbitrary units.

(T/NT) (patients 22, 23 and 24) (22). Reduced *HOPX-β* mRNA expression concomitant with discrete high PMR in T compared with NT was observed in the three samples pairs (mRNA 22-T: 2.497 AU, 22-NT: 122.23 AU; 23 T: 4.436 AU, 23 NT: 4.595 AU; 24 T: 0.959 AU, 24 NT: 3.183 AU) (PRM 22 T: 1.240%, 22 NT: 1.195%; 23 T: 1.185%, 23 NT: 1.180%; 24 T: 0.965 UA, 24 NT: 0.925%). Accordingly, the western blot analysis showed reduced protein expression in the T samples compared with NT samples in the three cases (Fig. 5).

HOPX expression and methylation analysis using TCGA data

The analysis of *HOPX* gene expression from TCGA data showed no differences in T (12.15 ± 8.1 AU) and NT samples (12.14 ± 4.7 AU), as well as for the paired samples (T: 12.02 ± 1.1; NT: 12.14 ± 4.7). Differences in *HOPX* expression were observed between the subtype of PTC, tall cells showed higher expression (12.49 ± 0.7) than follicular variant (11.98 ± 0.8) (*P* = 0.006). The presence of BRAF mutation was associated neither with the expression
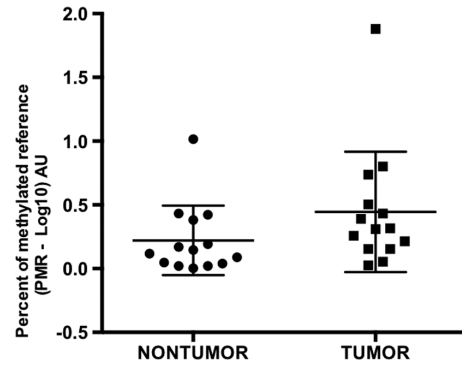
values nor with methylation status. No negative correlation was observed between *HOPX* expression and the methylation value of the CpG island or of each of the six selected CpG site, in tumors, non-tumors, all samples (T and NT) and in the paired samples. Considering the 49 paired samples for which methylation and expression data were available, in 22 cases (44.9%) lower methylation value was observed in T samples compared with NT while 13 T samples (26.5%) showed concomitant reduction of *HOPX* expression.

Regarding *HOPX* methylation from TCGA data, significant high methylation level in T (1.63 ± 0.8) compared with NT samples (1.24 ± 0.3, *P* = 0.0005) was observed. This result was also detected when classical or follicular variants were analyzed individually and between the paired samples (*P* < 0.0001, respectively) (Fig. 6). Considering the histologic types of PTC, the follicular variants showed higher methylation value (1.93 ± 0.07) compared to tall cell variants (1.54 ± 0.1) and classical PTC (1.55 ± 0.04) (*P* < 0.005). We then investigated the correlation between *HOPX* methylation and clinical features. Higher *HOPX* methylation values were associated with older age at diagnosis (≥45 years) (*P* = 0.044), with recurrent or progressive disease (*P* = 0.036), with reduced survival (*P* = 0.013) and with the presence of new neoplasm event post initial therapy (*P* = 0.017). Interestingly when the analysis was reduced to the tumors of the paired samples only reduced survival correlated with high *HOPX* methylation (*P* = 0.05). When the samples were categorized as hyper-methylated (methylation threshold >1.89), 137 of 508 tumor samples (26.9%) remained in this category and hyper-methylation correlated with older age (≥45 years)
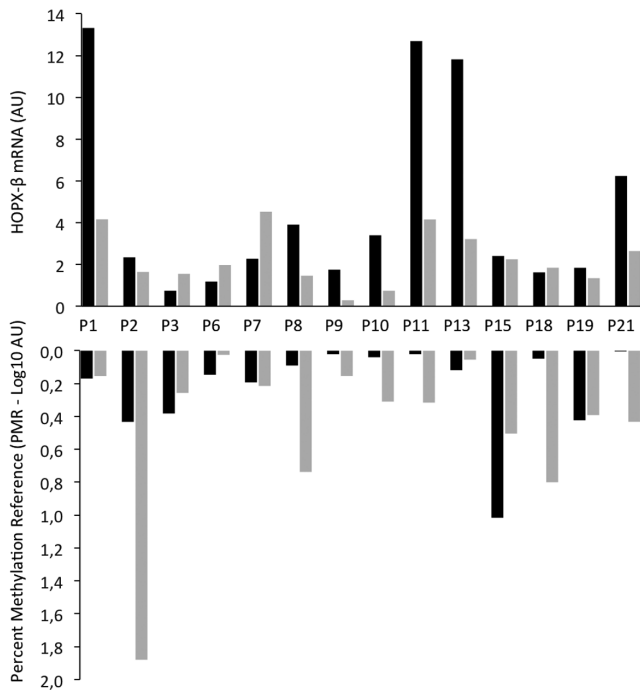
Figure 4

HOPX-β mRNA (top panel) and PMR (percent of methylated reference) values of HOPXβ promoter methylation (bottom panel) in 14/19 pairs of paraffin-embedded DTC samples. AU, arbitrary units; P, patient. Black bar: non-tumor, gray bar: tumor.

($P < 0.001$) and with follicular variant ($P = 0.002$). Kaplan–Meier survival analysis showed significant association of hyper-methylation with worse overall survival ($P = 0.003$), worse disease free status ($P = 0.008$) and older age ($P = 0.006$) (Fig. 7). Multivariate Cox analysis with BRAF status and variables that correlates with worse prognosis and univariate analysis showed that hyper-methylation was an independent prognostic factor for worse prognosis ($P = 0.012$, Hazard ratio = 4.758, 95% CI = 1.4–16.1). No clinical features correlated with hyper-methylation status when the paired group was categorized.

Discussion

HOPX has been suggested as a tumor-suppressor gene candidate gene involved in tumorigenesis, downregulating genes linked to angiogenesis and tumor aggressiveness (10). Indeed, the nature of HOPX action can be dependent upon cellular and extracellular signals, as well as cross-talk between important thyroid cancer signaling pathways, such as PI3K and MAPK (27, 28, 29). A consistent pattern of downregulation of *HOPX-β* mRNA expression coupled with *HOPX-β* promoter methylation levels in cancer cell lines and in various malign tissue
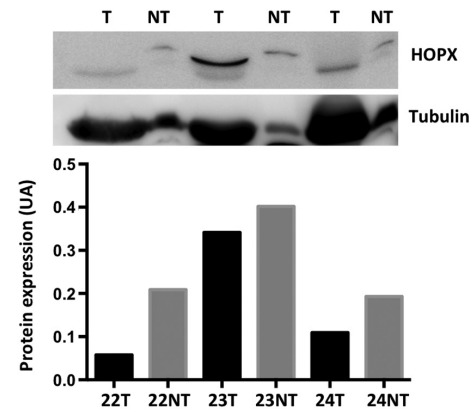

Figure 5

Western blot for HOPX protein expression in three pairs tissue samples (T/NT), 22, 23 and 24 with primary HOPX antibody (1:1000; Cell Signaling) showed reduced expression in the T samples compared with NT samples. Tubulin expression was used as control (1:10,000; Sigma).

types such us esophageal, colorectal, pancreatic in uterine and breast cancers have been previously described (8, 10, 11, 12, 13, 15, 18). On the other hand, in acute myeloid leukemia high *HOPX* expression was an unfavorable prognostic factor associated with low complete remission rate and short survival (30) and in sarcoma cells it was associated to metastases and its knockdown decreased metastatic activity (31). In this study, we interrogated the expression and
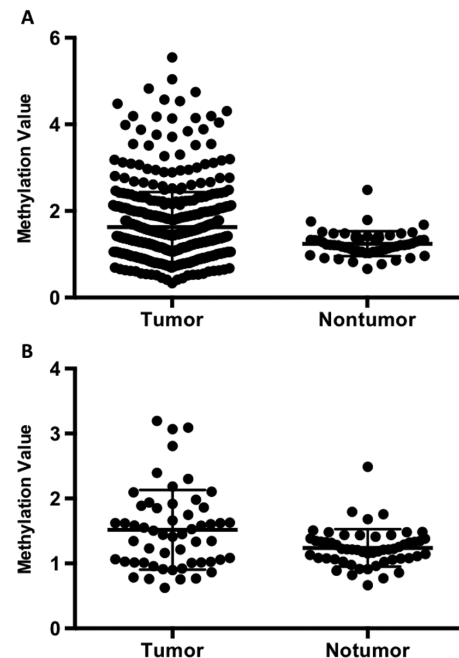

Figure 6

HOPX-β methylation values in tumor (T) and non-tumor (NT) groups in all thyroid cancer TCGA samples (A) and in paired samples (B), *$P < 0.0005$.
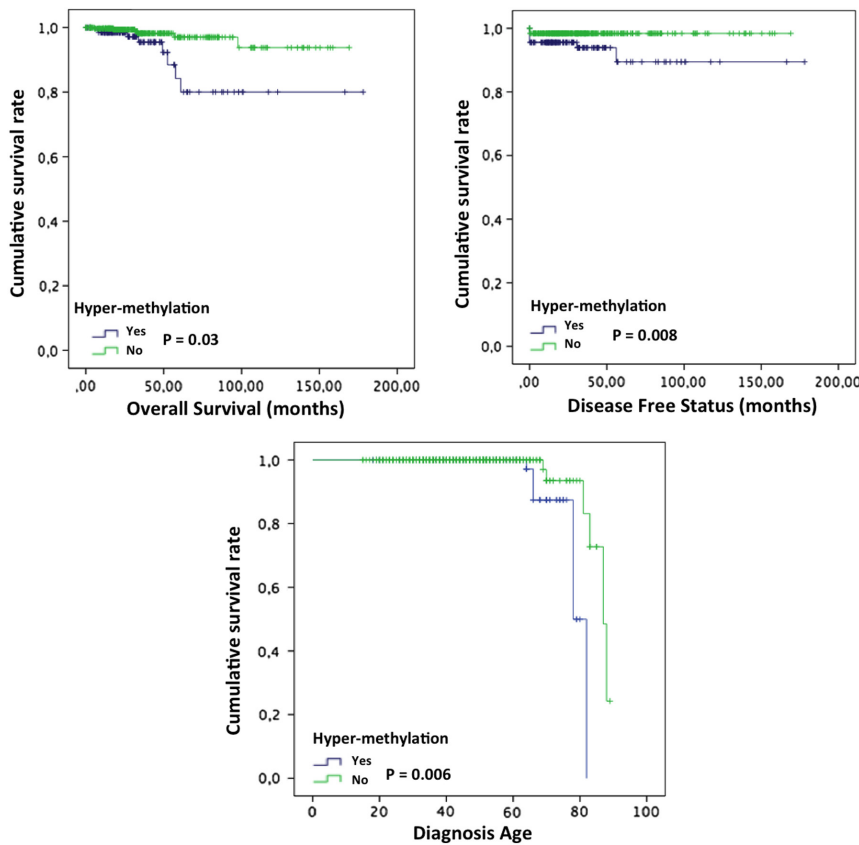
Figure 7

Kaplan–Meier survival analysis using hyper-methylation threshold value of 1.89 showed significant association of *HOPX* promoter methylation with diagnosis age, disease free status and overall survival (*P*=0.006, *P*=0.008, *P*=0.03, respectively).

methylation status and their clinical relevance in DTC. In investigating *HOPX* methylation silencing in DTC cancer cells and re-expression by demethylating agent 5-Aza-dC of *HOPX-β* transcripts evaluated by qRT-PCR, we found that re-expression was recognized in two of the three cancer cell lines analyzed (FTC238 and WRO), concomitantly with reduction in *HOPX-β* promoter methylation. However, protein expression was only increased in FTC238 cells, suggesting that HOPX protein expression depends not only on increased transcription. Our data did also show a considerable downregulation of *HOPX-β* mRNA expression in DTC, along with a trend of high *HOPX-β* promoter methylation. Approximately half of the patient samples studied did find a similar pattern to that reported for other cancers (8, 10, 11, 12, 13). The fact that in this study and others (10, 19, 25) several samples did not show a clear correlation between methylation values and expression suggests that reduction of *HOPX-β* mRNA could be partially explained by epigenetic alterations, though *HOPX-β* promoter methylation is one of mechanism for inactivation. In evaluating *HOPX-β* mRNA expression in benign tumors and their adjacent non-tumor samples, four of six paired samples showed reduced tumor expression, an extended

study using a larger sample is necessary to confirm *HOPX-β* mRNA expression levels in benign tumors.

In order to understand the relationship between methylation and *HOPX-β* expression in thyroid cancer we performed a complete analysis of PTC (THYCA) from TCGA dataset (24). No differences in *HOPX* expression were observed between tumor and non-tumor samples. Nevertheless significant high methylation levels were observed in the tumor samples compared with non-tumor, even more the follicular variants had the highest methylation values. No negative correlation was observed between *HOPX* expression and the methylation status, in contrast, a weak positive correlation between *HOPX* expression and methylation was observed (data not shown). Similarly, in the BRCA dataset, over-expressed *HOPX* showed differentially hyper-methylated promoter in tumors (32) and in head and neck cancer (HNSCC dataset) 17 of 20 paired samples showed reduced *HOPX* tumor expression but only three of them had promoter hyper-methylation (25). As previously mentioned, these data reinforce the concept that *HOPX* methylation may be in part responsible for the loss of HOPX protein and that other mechanisms may be involved in HOPX expression, such as genetic mutation, histone

deacetylation, methylation of other transcription factor genes or inactivation of an upstream tumor-suppressive pathways (18, 32). The direct regulation of *HOPX* through transcription factors has to be also considered.

In the TCGA dataset the presence of BRAF mutation was not associated with the methylation state or gene expression in the tumors. Previously, it was shown that the presence of BRAF V600E mutation promotes aberrant methylation of a large cohort of genes in thyroid cancer cells (33).

Our results showed that high *HOPX* methylation correlated with worse prognosis in thyroid cancer and was associated with older age at diagnosis, recurrent or progressive disease, reduced survival and with the presence of new neoplasm event post initial therapy. Furthermore *HOPX* hyper-methylation could be an independent predictor factor for worse prognosis. These data are in line with previous studies in colorectal cancer (10), esophageal squamous carcinoma (13), gastric cancer (17) and breast cancer (18).

Without a doubt, the HOPX biological activity is more complex than expected, studies to explain the relevance of the isoforms, its tissue specificity, its gene expression regulation, the opposite methylation pattern: hyper-methylated in solid tumor while hypo-methylated in acute myeloid leukemia are needed.

In conclusion, the *HOPX* promoter was methylated in DTC; however, a moderate coupling of downregulation of *HOPX-β* mRNA and protein expression in DTC, followed by high HOPX-β promoter methylation was observed, indicating that other mechanisms may be involved in gene expression regulation. Furthermore, high *HOPX* promoter methylation status was associated with the worse prognosis of DTC patients.

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