Laboratory Investigation (2014) 94, 788–795

Heterochromatin protein 1 expression is reduced in human thyroid malignancy

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Owing to the loss of heterochromatin integrity that occurs during thyroid tumorigenesis, the expression of Heterochromatin Protein 1 isoforms HP1α and HP1β was assessed by immunohistochemistry in 189 thyroid tumors and non-neoplastic tissues. Expression of HP1β was significantly decreased in all thyroid lesions, except in follicular adenomas, when compared with matched adjacent normal tissue. This loss of HP1β expression may in part be caused by microRNA dysregulation. An example is miR-205, a microRNA that is abundantly upregulated in thyroid carcinomas and shown to reduce the expression of HP1β. In contrast to HP1β, HP1α expression was only reduced in metastatic carcinomas and poorly differentiated lesions. These results suggest the reduction of HP1β followed by a decrease in HP1α contributes to the pathogenesis of thyroid carcinomas, and their loss is a potential marker of thyroid malignancy and metastatic potential, respectively.

Laboratory Investigation (2014) **94,** 788–795; doi:10.1038/labinvest.2014.68; published online 19 May 2014

KEYWORDS: heterochromatin protein 1; thyroid; microRNA

During malignant transformation nuclear architecture becomes more disorganized, with observable changes occurring in heterochromatin aggregation.[1] The human genome encodes three Heterochromatin Protein 1 (HP1) isoforms (α, β, and γ), which are essential chromatin packaging proteins that function to restrict chromatin plasticity.[2] HP1α and HP1β are the isoforms found in heterochromatin and are primarily associated with gene repression. Although generally ubiquitous in normal tissue, differential expression of each HP1 isoform has been observed in both human tumor derived tissues and cells.[3–9] The pattern of expression for HP1 isoforms in solid tumors appears to vary with the tumor context.[3,5,7,9,10] Loss of HP1β expression has been reported in poorly differentiated colon cancers, invasive melanoma lesions, and prostate cancer.[11–13] Loss of HP1α expression has been observed in metastatic lesions,[5] and in medulloblastomas and advanced papillary thyroid carcinomas with poor outcome.[7,9] However, increased HP1α has also been observed in a variety of solid tumors compared with non-tumor tissue.[3]

Thyroid neoplastic lesions range from benign follicular adenomas to malignant but relatively indolent papillary or follicular carcinomas, and to the highly aggressive poorly differentiated or undifferentiated anaplastic carcinomas. Both papillary and follicular well-differentiated carcinomas, comprised of differentiated follicular cells, may metastasize or give rise to the undifferentiated anaplastic carcinomas that have a poor outcome.[14,15] Preoperatively, a common diagnostic problem is distinguishing between benign and malignant thyroid nodules, as this will influence management. Therefore, identifying specific markers that can be useful in improving the accuracy of diagnosis and predicting tumor behavior is of utmost priority.

A myriad of alterations occur at the molecular level across this spectrum of thyroid lesions causing the dysregulation of gene and microRNA (miRNA) expression.[16] It has been proposed that of the miRNAs that are dysregulated only those which are highly overexpressed or strongly downregulated are involved in thyroid tumorigenesis.[17] Thyroid cancers exhibit aberrant miRNA expression that leads to the altered expression of oncogenes as well as tumor suppressor genes.[17,18] Interestingly, several miRNAs that are differentially upregulated in thyroid lesions are also predicted to target the HP1 family.[19]

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Received 22 October 2013; revised 1 April 2014; accepted 13 April 2014

The need for improved accuracy in the diagnosis of thyroid lesions has led us to explore the expression of HP1α and HP1β across benign and malignant thyroid tumors, respectively. Screening of a thyroid tumor tissue microarray (TMA) demonstrated that HP1α expression was reduced in more advanced thyroid carcinomas, but was unchanged in well-differentiated thyroid neoplasms when compared with normal thyroid tissue. However, when compared with normal thyroid tissue, HP1β expression was significantly decreased in all thyroid neoplasms ($P<0.01$), except in follicular adenomas. Results indicate that a significant loss of HP1β may confer a malignant potential. We show that loss of HP1β could in part be due to the upregulation of specific miRNAs that are observed in thyroid tumors. These results suggest that the differential loss of HP1α and HP1β represents potential molecular markers for different stages of thyroid malignancy.

MATERIALS AND METHODS

Tissue Microarrays

TMAs were constructed using 189 formalin-fixed paraffin-embedded tissue blocks from 103 patients with benign and malignant thyroid tumors. Histological classification was based on diagnostic reports and slide re-reviews by two experienced pathologists (T Kroll and M Tretiakova). Questionable cases were not selected for inclusion. Hematoxylin and eosin-stained sections from lobectomy, partial and total thyroidectomy specimens were analyzed to define representative lesional regions and normal adjacent thyroid tissue regions. From these regions, a minimum of two duplicate tissue cylinders with a diameter of 1 mm were obtained and arrayed into recipient blocks using an automated tissue microarrayer (ATA-27, Beecher Instruments, Sun Prairie, WI, USA). The distribution of tissues was as follows: colloid nodules ($N=5$), follicular adenoma ($N=18$), Hurthle cell adenoma ($N=10$), follicular carcinoma primary ($N=14$) and metastases ($N=8$), Hurthle cell carcinoma ($N=8$), papillary carcinoma primary ($N=32$) and metastases ($N=3$), poorly differentiated ($N=4$) and anaplastic carcinoma ($N=7$), and normal matched adjacent thyroid tissue ($N=80$). Recipient blocks were cut into 4-μm-thick sections on Surgipath silane-coated positively charged slides for better adhesion and subjected to immunohistochemistry.

Fresh frozen, matched thyroid normal and papillary carcinoma tissue from four patients was obtained from Christchurch Cancer Society Tissue Bank, Christchurch, New Zealand. This study was approved by the New Zealand Upper South B Regional Ethics Committee and the University of Chicago Institutional Review Board.

Antibodies

The primary antibodies used for this study were the HP1α (C7F11) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA), HP1β (MAC353) rat monoclonal antibody (Abcam, Cambridge, MA, USA), and α-tubulin rat monoclonal antibody (Abcam).

Immunohistochemistry

Immunohistochemical staining was performed using HRP-labeled dextrose-based polymer complex bound to secondary antibody EnVision (Peroxidase/DAB+) from DAKO (Glostrup, Denmark). After deparaffinization and rehydration, slides were blocked for endogenous peroxidase with 3% H_2O_2 for 15 min at room temperature (RT), and then washed in Tris-buffered saline (TBS). Antigen retrieval was carried out by heating sections either in Tris-EDTA buffer (pH 9.0) for 15 min in a microwave oven or in 0.1 M citrate buffer (pH 6.0) for 3 min in a pressure cooker. Non-specific binding sites were blocked using Protein Block (DAKO) for 20 min at RT in a moist chamber. Then slides were incubated, at RT for 1 h or at 4 °C in a moist chamber overnight, with the HP1α (1:200) or HP1β (1:300) antibodies diluted in Antibody Diluent (DAKO) as indicated. After TBS washes, staining was detected with EnVision system (DAKO) developed for 5 min with 3-3′-diaminobenzidine chromogen, counterstained with hematoxylin, and coverslipped. Negative controls were performed by substituting the primary antibody with non-immune mouse immunoglobulins.

Evaluation of Immunostaining

Only nuclear labeling was accepted as specific for HP1. Nuclear expression was quantified manually using 200–500 cells at ×400 magnification, as well as by using Chromavision Automated Cellular Imaging System (ACIS, Clarient Inc, Aliso Viejo, CA, USA). Manual nuclear intensity scoring was graded semiquantitatively as negative (0), weak (1+), moderate (2+), or intense (3+) for each case. Automated ACIS scoring was applied to derive both intensity and percentage staining as previously described.[20] In brief, nuclear staining for HP1 was quantified based on three-color parameters: hue, luminosity, and saturation. Positive brown nuclear staining was recorded as a numerical score between 0 and 225 for each pixel and normalized to an area of 1 μm^2. In addition, the ACIS software was instructed by setting color-specific thresholds to determine brown (positive stain) from blue (negative counterstain) in the nuclei within each TMA core, and calculate the percent of nuclear positivity.

Statistical Analysis

Statistical analysis was performed using STATA (College Station, TX, USA) software package. Nuclear expression levels were calculated as the mean of duplicate measurements ± s.d. *P*-values were calculated using a two-tailed Student's *t* test for continuous variables. Correlations were calculated using the Spearman rank correlation test unless otherwise indicated. $P<0.05$ was considered to be statistically significant. Microarray data from GEO project GSE3467 was analyzed using the websites (http://www.ncbi.nlm.nih.gov/geo/) GEO2R application with the default settings.

Cell Culture

The Nthy-ori 3-1 cell line (ECACC, Wiltshire, UK), derived from the normal thyroid follicular epithelial tissue of an adult that had been immortalized with SV40 large T antigen, was maintained in RPMI 1640 with 10% fetal bovine serum, 1% Penicillin/Streptomycin (Life Technologies, Grand Island, NY, USA) at 37 °C with 5% CO_2 in a humidified atmosphere.

Transfection with Pre-miR miRNA Precursors

Nthy-ori 3-1 cells were transfected with 30 nM Pre-miR miRNA Precursors (Life Technologies) using X-tremeGENE siRNA Reagent (Roche Applied Science, Indianapolis, IN, USA) and Opti-MEM medium (Life Technologies). Media was replaced 24 h post transfection. Cells were plated at a density of 8.5×10^3 cells/cm^2 to permit growth, without reaching confluency after 72 h.

HP1β 3′ UTR Luciferase Reporter Assay

pMIR-REPORT luciferase vectors (Life Technologies) haboring HP1β 3′ UTR sequences with a wild-type (pMIR-REPORT-HP1β WT) or mutated (pMIR-REPORT-HP1β Mut) predicted miR-205 binding site were generated. Annealed oligonucleotides, spanning the region from 240 to 290 bp of the HP1β 3′ UTR, that contained either a WT or Mut miR-205 seed region were cloned into the HindIII and SpeI restriction sites of the pMIR-REPORT vector. The sequences are shown in Figure 4a.

Nthy-ori 3–1 cells were co-transfected, as above, with the 5 nM Pre-miR miRNA Precursors (Life Technologies) and either 150 ng pMIR-REPORT-HP1β WT or Mut vector along with 100 ng pMIR-REPORT β-galactosidase control plasmid (Life Technologies). After 30 h, luciferase measurements were performed using the Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to β-galactosidase activity.

Protein Extraction

Total protein was isolated from frozen matched thyroid normal and papillary carcinoma tissue (31–82 mg) using the mirVana Paris Kit (Life Technologies). The frozen tissue was placed in cold cell disruption buffer supplemented with Complete Protease inhibitor (Roche Applied Science) and homogenized on a Retsch MM301 tissue mill at 1000 rpm for 10 min, then incubated for 10 min at 4 °C. The supernatant was collected after centrifugation at 10 000 *g* at 4 °C.

Total protein from the Nthy-ori 3–1 cell line was obtained by washing cells twice in PBS, incubating the cells in cold lysis buffer (50 mM HEPES pH 8.0, 400 mM NaCl, 1% NP40, Complete Protease Inhibitor) then collecting the supernatant as above. The protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Immunoblot Analysis

Samples were resolved by SDS-PAGE and then transferred to nitrocellulose. The blots were blocked for 1 h at RT with 5% BSA in 25 mM Tris, 150 mM NaCl pH 7.6 and 0.1% Tween-20 (TBS-T), then incubated with antibodies against, either HP1α, HP1β or α-tubulin in 5% BSA/TBS-T for 18 h at 4 °C. Blots were then washed four times in TBS-T, incubated 1 h at RT with the appropriate ECL HRP-linked whole secondary antibody (GE Healthcare, Buckinghamshire, UK) diluted in 5% BSA/TBS-T and washed as before, then developed with ECLplus Western Blotting Detection (GE Healthcare).

RESULTS

HP1α Expression is Reduced in Metastatic Thyroid Neoplasms

To determine the level of HP1α protein expression in thyroid tumors, a TMA containing 189 thyroid lesions and normal adjacent thyroid tissue was screened for HP1α expression by immunohistochemistry. To ensure our results were representative of each tumor, the nuclear expression of HP1 was quantified manually by grading 2–3 cores, from 0 (negative) to 3+ (intense), and then averaging to give the intensity of HP1 staining for each tissue. The results are graphed in Figure 1a, with representative tissue sections shown in Figure 1b. The expression of HP1α in carcinomas was comparable to that observed in normal tissue (Figure 1a), whereas HP1α protein levels were slightly increased in adenomas compared with normal tissue and colloid nodules. A reduction of HP1α expression was observed in metastatic and anaplastic variants, however, this was not significant when compared with normal tissue. When the carcinomas were divided into non-metastatic (primary) and metastatic (either regional or distal) groups, a significant reduction ($P<0.05$) in HP1α expression was observed between papillary carcinomas and their metastatic counterparts (Figure 1c).

Differential Loss of HP1β Expression in Thyroid Lesions

The thyroid TMA was also screened for HP1β protein expression and graded as described above. Normal thyroid tissue that demonstrated strong HP1β nuclear positivity served as a positive control. HP1β expression was significantly decreased ($P<0.01$) in all thyroid lesions, except follicular adenomas and colloid nodules, when compared with normal thyroid tissue by manual scoring for intensity of staining (Figures 2a and b).

Automated HP1β scoring similarly showed a significant decrease in both percent of nuclear positivity and staining intensity for all lesional tissues except follicular adenoma ($P<0.001$, Table 1). Group comparison showed significant differences in staining intensity between normal/benign and malignant lesions with gradual loss of HP1β with malignant progression ($P<0.001$). We found a strong positive correlation between automated and semiquantitative manual grading of nuclear intensities for HP1β ($r=0.62$–0.75).

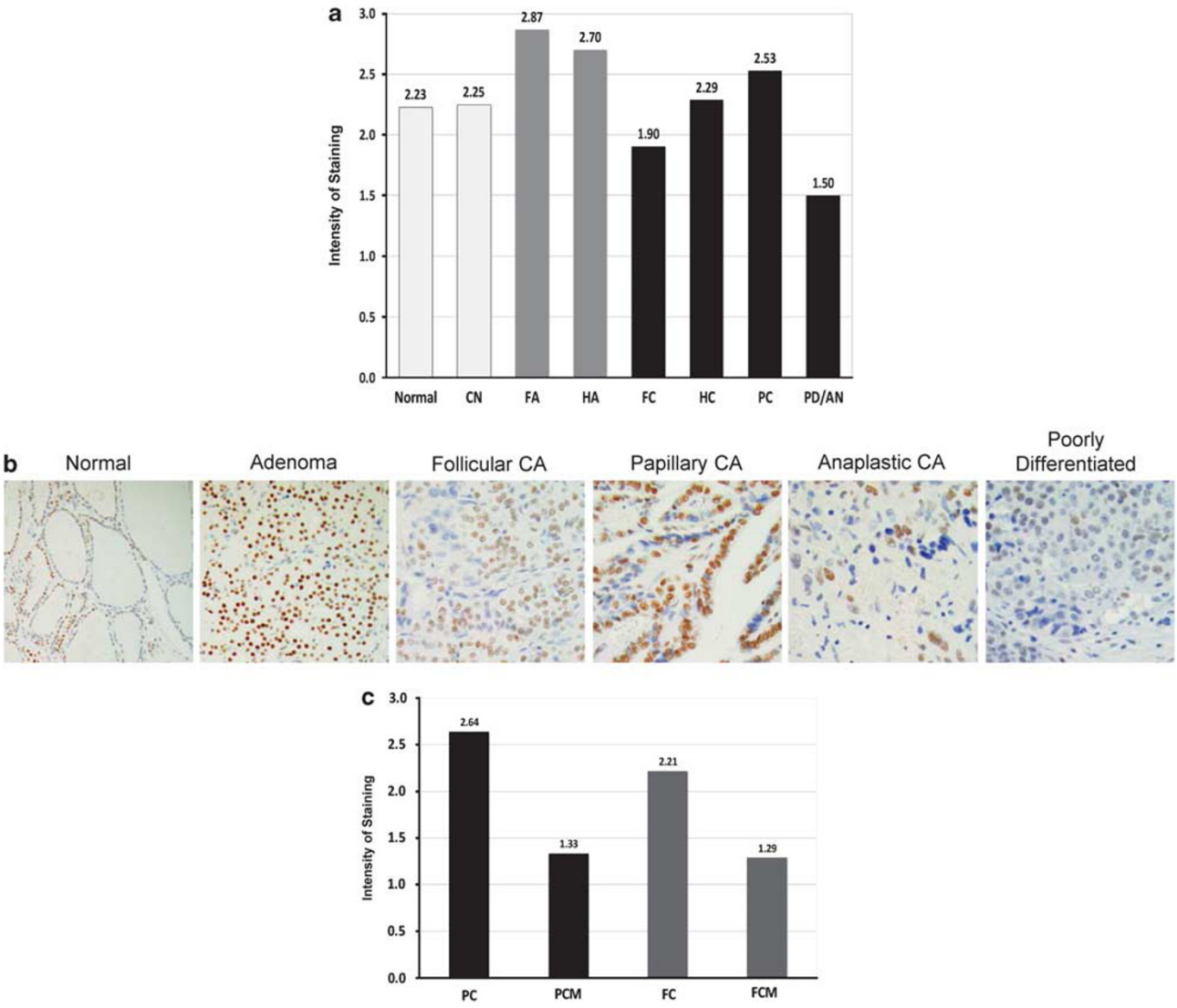

Figure 1 Reduction of HP1α expression in metastatic thyroid carcinomas. (**a**) Graph showing the results from immunohistochemical staining of a 189 sample human thyroid tumor TMA for HP1α. The intensity of HP1 staining was graded as 0, 1+, 2+, and 3+. Colloid nodules (CN), follicular adenomas (FA), Hurthle cell adenomas (HA), follicular carcinomas (FC), Hurthle cell carcinomas (HC), papillary carcinomas (PC), poorly differentiated and anaplastic tumors (PD/AN), and normal adjacent thyroid tissue were stained. (**b**) Representative images from the immunohistochemical staining of the human thyroid tumor TMA are shown for HP1α. Haematoxylin counterstaining (blue) indicates nuclei, HP1α positive nuclei (brown). CA, carcinoma. Original magnification for normal and adenoma × 100, for CA and poorly differentiated × 200. (**c**) Graph showing the intensity of HP1α staining in PC ($N=32$) compared with papillary carcinoma metastatic (PCM, $N=3$) and FC ($N=14$) *versus* follicular carcinoma metastatic (FCM, $N=8$) from immunohistochemical staining of the human thyroid tumor TMA in **a**.

In general, ACIS brown staining intensity scores below 100 were equivalent to negative (0) staining by manual grading, 100–110 was equivalent to weak (1+) staining, 111–120 was equivalent to (2+) staining and >120 was equivalent to (3+) strong nuclear reactivity.

The specificity of the antibodies that recognize HP1β and HP1α was confirmed by immunoblot analysis using total protein extracted from three frozen advanced papillary carcinomas and a Hurthle cell variant, each showed a single band specific for the protein (Figure 2c). This analysis also confirmed the reduction of HP1α and HP1β expression in metastatic papillary carcinomas observed by immunohistochemistry.

HP1β Expression is Altered in Thyroid Cells Overexpressing miR-205

Of the numerous miRNAs differentially upregulated in various histopathological types of thyroid tumors compared with normal tissue, miR-205 is predicted to target HP1β (TargetScanHuman, 2009).[17,19] We observed a strong negative correlation of miR-205 and HP1β expression (Pearson $r=-0.52$) in previously published array data from

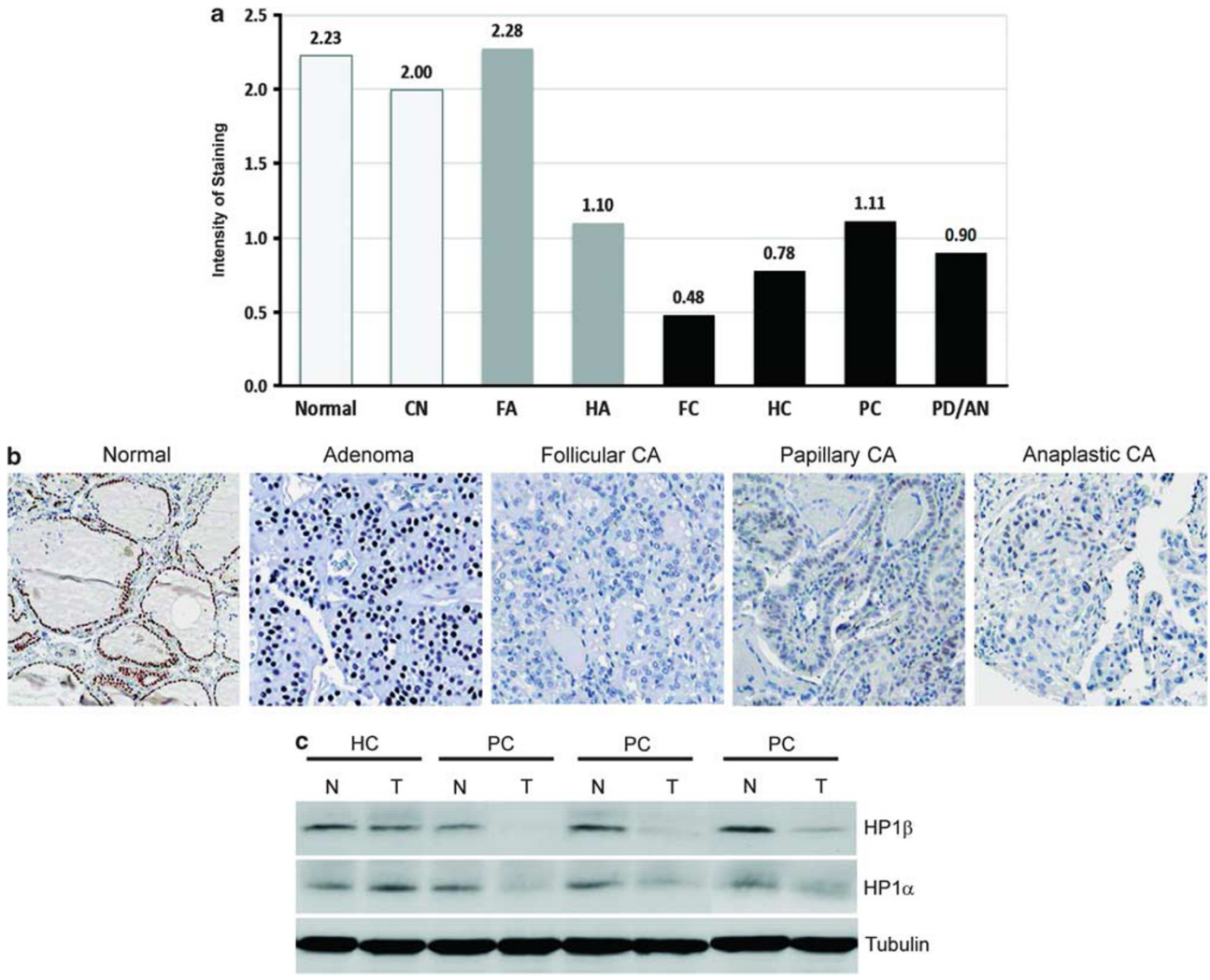

Figure 2 Loss of HP1β expression occurs with increasing grade of thyroid tumor. (**a**) Graph showing the results from immunohistochemical staining of the 189 sample human thyroid tumor TMA for HP1β. The intensity of HP1 staining was graded as 0, 1+, 2+,and 3+. Colloid nodules (CN), follicular adenomas (FA), Hurthle cell adenomas (HA), follicular carcinomas (FC), Hurthle cell carcinomas (HC), papillary carcinomas (PC), poorly differentiated and anaplastic tumors (PD/AN), and normal adjacent thyroid tissue were stained. (**b**) Representative images from the immunohistochemical staining of the human thyroid tumor TMA are shown for HP1β. Haematoxylin counterstaining (blue) indicates nuclei, HP1β-positive nuclei (brown). CA, carcinoma. Original magnification $\times 100$. (**c**) Total protein isolated from three frozen advanced thyroid papillary carcinomas (PC) and a Hurthle cell carcinoma (HC) was analyzed by immunoblotting for expression of HP1β and HP1α and α-tubulin as a loading control. N, normal matched adjacent tissue, T, tumor tissue.

papillary carcinomas (Figure 3a).[21] These data show that miR-205 and HP1β were significantly up and down-regulated ($P<0.05$), respectively in these tumors. Not surprisingly though a negative correlation with HP1β expression was also observed for other upregulated miRNAs such as miR-10a (Pearson $r=-0.73$) even though miR-10a is not predicted to target HP1β.[19,22] Therefore, to determine if increased expression of miR-205 can alter the level of endogenous HP1β protein, immortalized Nthy-ori 3-1 thyroid cells were transiently transfected with a selection of Pre-miR miRNA precursors that are processed by the cell into mature miRNAs. Cells overexpressing miR-205 showed reduced HP1β protein levels compared with mock transfected cells and those transfected with a scrambled pre-miRNA precursor control (NC2) that does not have any predicted endogenous targets (Figure 3b). Overexpression of miR-200a, another miRNA predicted to target HP1β but with reduced complementarity to its seed region in the 3′ UTR of HP1β compared with that of miR-205, only slightly reduced the level of endogenous HP1β. Both miR-10a and miR-223, not predicted to target HP1β, did not alter the level of endogenous HP1β. As a further control, none of the tested miRNAs were predicted to target HP1α and, as shown in Figure 3b, they do not alter the level of endogenous HP1α protein in these cells. Similar results were observed when these Pre-miR miRNAs were

Table 1 Results of HP1β expression analysis by automated cell image analysis (ACIS)

Histology	Group	No. of cases	HP1β %		HP1β intensity	
NT/CN	Normal	85	43.42	43.42	139.36	139.12
FA	Benign	18	63.39	51.18	129.72	120.21
HA		10	29.2		103.1	
FC	Malignant	22	13.62	24.48	79.5	98.62
HC		8	26.49		90	
PC		35	33.5		109	
PD/AC		11	17.94		113.3	
Total		189		$P<0.001$		$P<0.001$

Abbreviations: CN, colloid nodule; FA, follicular adenoma; FC, follicular carcinoma, primary and metastatic; HA, Hurthle cell adenoma; HC, Hurthle cell carcinoma; PC, papillary carcinoma, primary and metastatic; PD/AC, poorly differentiated and anaplastic carcinoma; NT, normal thyroid tissue.

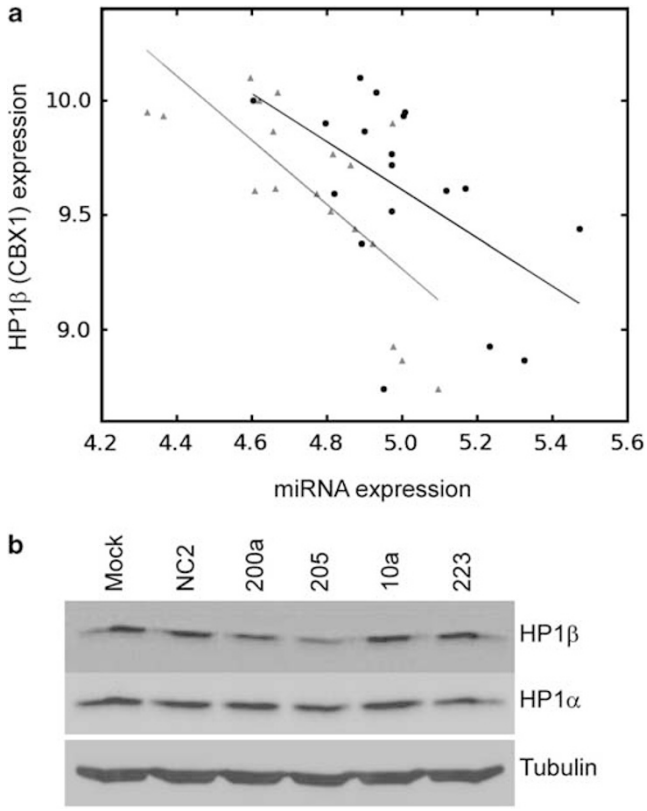

Figure 3 Downregulation of endogenous HP1β expression by miR-205. (**a**) Expression values for miR-205 (closed circles) and miR-10a (gray triangles) are graphed against expression values for HP1β (CBX1 gene) of thyroid papillary carcinomas and normal tissue from published array data. (**b**) Nthy-ori 3-1 cells were transiently transfected with Pre-miR miRNA precursors for either a scrambled control miRNA (NC2), miR-200a, miR-205, miR-10a, miR-223 or without any Pre-miR miRNA precursor (Mock). Total protein was analyzed by immunoblot for expression of HP1β, HP1α and α-tubulin as a loading control.

transiently transfected into HeLa cervical carcinoma cells (data not shown).

To determine if miR-205 post-transcriptionally regulates HP1β expression directly, we used a 3′ UTR luciferase reporter assay. The 3′ UTR region of the CBX1 (HP1β) gene that is a predicted binding site for miR-205 was cloned into the 3′ UTR of the pMIR-REPORT vector (Figure 4a). Nthy-ori 3-1 cells were co-transfected with this reporter vector (WT) or one where the putative HP1β 3′ UTR seed region has been altered to prevent binding of miR-205 (Mut), along with the pre-miRNA precursors. The transfection of pre-miRNA scrambled (NC2) and miR-10a precursors, which do not alter endogenous HP1β levels in Nthy-ori 3-1 cells (Figure 3b), demonstrated no difference in luciferase activity between the pMIR-REPORT-HP1β WT and Mut vectors (Figure 4b).

In the presence of miR-205, there was a 30% reduction ($P<0.05$) in luciferase activity in cells transfected with the pMIR-REPORT-HP1β vector containing the WT HP1β 3′ UTR region compared with the Mut sequence that prevents binding of miR-205 (Figure 4b). This suggests that the observed reduction in HP1β expression in thyroid tumors may in part be due to miR-205 downregulating HP1β post-transcriptionally by binding its 3′ UTR.

DISCUSSION

The altered expression of two HP1 isoforms during thyroid tumorigenesis is not surprising given thyroid cancers generally exhibit a loss of heterochromatin integrity at the microscopic level.[1] Thyroid carcinomas demonstrate a significant reduction in the level of HP1β compared with either normal tissue, proliferative colloid nodules, or benign follicular adenomas (Figure 2). The decrease in HP1β expression between follicular adenoma and carcinoma groups was particularly dramatic (4.7-fold) and could have diagnostic implications. This downregulation of HP1β likely contributes to tumor progression, as knockdown of HP1β in cancer cell lines promotes chromatin disorganization, genomic instability and also increases their invasiveness.[12,23]

In contrast to the changes in HP1β expression, HP1α levels in adenomas and non-metatastic carcinomas were comparable to or increased slightly from those in normal tissue (Figure 1). However, HP1α expression was reduced in metastatic carcinomas from regional or distal sites. This corresponds with observations in breast tissue where non-invasive breast carcinomas were shown to have high levels of HP1α protein,[3] whereas metastatic lesions from distant sites in the body had decreased levels of HP1α.[5] This suggests that HP1α has a role in suppressing metastasis and is supported by the overexpression of HP1α decreasing the invasive potential of a breast cancer cell line.[24] Together these results show a differential loss of HP1 isoform expression

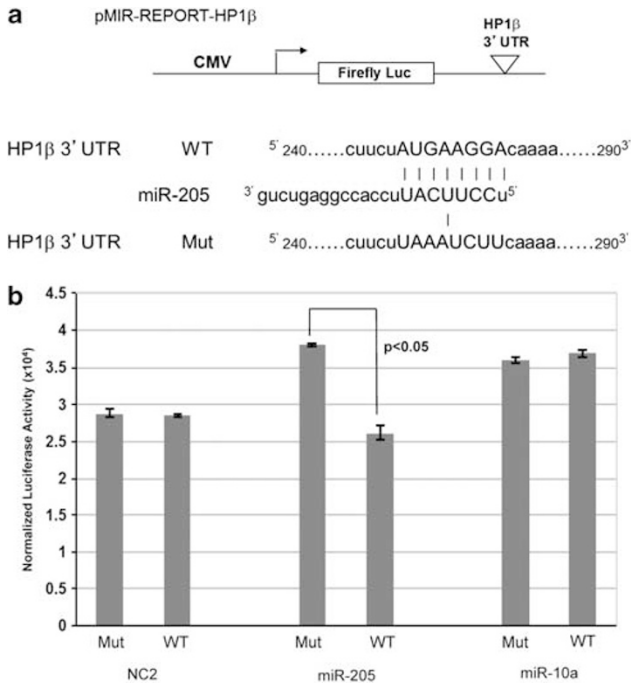

Figure 4 Effects of miR-205 on a luciferase reporter gene bearing a 3′ UTR segment of HP1β. (**a**) Schematic representation of the pMIR-REPORT-HP1β 3′ UTR vector. The CMV promoter drives the constitutive expression of firefly luciferase coding sequence with the region from 240 to 290 bp of the HP1β 3′ UTR sequence containing the predicted binding region of miR-205 cloned downstream of the luciferase gene. Shown below is the predicted seed match between miR-205 and the HP1β (CBX1) 3′ UTR present in the pMIR-REPORT-HP1β WT vector and the mismatches present in pMIR-REPORT-HP1β Mut vector. (**b**) Nthy-ori 3-1 cells were co-transfected with either pMIR-REPORT-HP1β WT or Mut vectors, and the following Pre-miR miRNA Precursors: NC2, miR-205 or miR-10a, and the β-galactosidase control vector. Reporter activity was measured by luciferase assay and normalized to β-galactosidase.

during malignancy in thyroid tissue and preliminary immunohistochemical analyses suggests this pattern is common to other solid tumor types (data not shown).

How the expression of each HP1 isoform is downregulated is poorly understood. In thyroid tumors, reduced expression is unlikely to be due to a decrease in the copy number of HP1β CBX1) or HP1α (CBX5) gene, as loss of 17q21.32 or 12q13.13, respectively, has not yet been observed.[25–27] MiR-205 has been shown to be significantly upregulated in papillary and anaplastic carcinomas compared with hyperplastic nodules and normal thyroid tissue.[19] As miR-205 can directly target the 3′ UTR of HP1β (Figure 4b), loss of HP1β in these carcinomas could be due to miR-205 post-transcriptionally downregulating HP1β expression. This demonstrates that the expression of the HP1 isoforms could be regulated post-transcriptionally. Given that the reduction of HP1β is observed across a range of malignant thyroid lesions, it will be of interest to uncover other transcriptional and post-transcriptional regulators.

In summary, these results suggest that the differential expression of HP1α and HP1β across benign and malignant tumors can be further explored as potential markers, when loss of HP1β and HP1α can be interpreted in favor of malignancy or carcinoma with metastatic potential, respectively. It will be of interest to explore the contribution of the decreased expression of these HP1 isoforms to the pathogenesis and progression of thyroid carcinomas.

ACKNOWLEDGMENTS

This study was supported by grants from the Cancer Society of New Zealand and the Palmerston North Medical Research Foundation. We thank Bruce Lockett and Natisha Magan for critical reading of the manuscript.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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