ORIGINAL PAPER

Nuclear shape in papillary thyroid carcinoma: a role for lamin B receptor?

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Abstract

Irregularity in the nuclear shape, with extensive folds and invaginations of the nuclear membrane (NM), remain the basic diagnostic feature of papillary thyroid carcinoma (PTC). The biological reasons for these irregularities are obscure, but evidence has been presented that they might be linked to RET/PTC gene translocation. In the present study, we have investigated the hypothesis that the NM irregularities in PTC might be linked to alterations in the expression of lamin B receptor (LBR), a component of the inner NM responsible for the distribution of Lamin B and associated chromatin. Fisher AH *et al.* already reported on the lack of LBR in PTC, a finding in contrast with the observation that a reduced expression of LBR because of gene mutation is responsible for the lack of nuclear segmentation of granulocytes in Pelger–Huët anomaly. In the present study, we confirmed the lack of immunohistochemical staining for LBR in PTC nuclei, in contrast to a positive staining in intestinal epithelium and stromal cells. However, Western blot and RT–PCR analysis demonstrated a strongly positive reaction in PTC extracts, thus proving an expression of LBR higher in PTC cases and cells than in follicular carcinoma cells. In conclusion, our data suggest that LBR is heavily expressed in PTC cells, but an abnormal folding of the protein might explain its lack of immunohistochemical reactivity and be associated with the anomalous folding of the NM.

Keywords: nuclear membrane, papillary thyroid carcinoma, lamin B receptor.

Introduction

Papillary thyroid carcinoma is the most frequent carcinoma of the thyroid and of endocrine organs. Its behavior is relatively indolent, but aggressive varieties are known and its frequency alone gives to this entity a prominent clinical and diagnostic interest [1].

Shape of the nucleus, including the presence of nuclear grooves and inclusions, coupled with changes in chromatin distribution, remain the basic microscopic criteria for the cytological diagnosis of papillary thyroid carcinoma (PTC) [2, 3], but these patterns cannot be considered neither specific nor selective. In fact, thyroid carcinomas with dark, round nuclei but displaying papillary fronds (hence, *bona fide* PTC) are occasionally encountered, either as primary tumors or metastases [1]. On the other hand, clear nuclei with intra-nuclear "inclusions" are a feature of several other extra-thyroidal tumors (e.g. hepatomas, nevi). Moreover, clear, irregularly-shaped nuclei are a recognized characteristic of oxyphilic cells in neoplastic and non-neoplastic thyroid lesions, such as Hashimoto's thyroiditis [1].

Still, despite the lack of specificity and of definition of their biological origin and significance, the nuclear alterations of PTC maintain a diagnostic interest, so that their peculiarity and frequency justify a more thorough analytical approach [1, 2, 4, 5].

The light microscopical appreciation of the nuclear shape is indirect, being based on the staining of nucleic acids with basic dyes, such as Hematoxylin. In other words, since part of the heterochromatin is strictly bound to the inner nuclear membrane, by using routine nuclear stains we obtain indirect information on the nuclear envelope itself.

Ultrastructural, immmunohistochemical and molecular studies carried out on the nuclear envelope have revealed the presence of several components: nuclear lamina, inner nuclear membrane, outer nuclear membrane, nuclear pore complexes and statin, a non-proliferation specific protein [6–9]. Theoretically, proteins associated with these components should constitute useful markers of the nuclear membrane (NM). In particular, one might expect tracing of NM-associated proteins to yield a more objective and direct appreciation of the nuclear shape.

In a previous study, we have shown that immunofluorescent demonstration of NM with anti-lamin B antibodies followed by three-dimensional (3D) reconstruction of confocal microscopical images revealed fine alterations of the nuclear shape in PTC [10]. We have expanded this approach and, in addition to lamin B, we have tested emerin, a NM marker that can be traced by immunofluorescence. These two proteins label different structural components, since lamin B is located in the proteinaceous layer at the interface between the chromatin and the inner nuclear membrane, while emerin is a transmembrane protein crossing both the inner and the outer nuclear membrane [8, 11, 12]. Previously, Fisher AH *et al.* [13] already demonstrated by immunofluorescence and immunoblotting procedures the uniform distribution of these two proteins along the NM of PTC and follicular-type epithelium.

Taken together, all these data suggest that the cytological (nuclear) features so typical of PTC are somehow linked to the molecular alterations leading to the development of this tumour, but we do not know how these alterations produce the structural changes. A more profitable approach might be linked to an analytical investigation on the proteins proper of the nuclear envelope, since structural or quantitative modifications of these proteins might explain the morphogenesis of PTC nuclei.

As a working hypothesis, it can be suggested that one or more components of the lamin complex might be involved. Lamins seem to be important in the attachment of chromatin to the nuclear envelope and studies have already been dedicated to the presence and distribution of lamins (A, B and C) and of other nuclear membrane components as emerin in PTC nuclei, but failed to reveal peculiarities of distribution or quantity of these proteins suggesting a role in the genesis of PTC nuclei [10, 13, 14]. However, some data suggest that lamin B receptor (LBR) might be a candidate. In fact, it is the best example of a chromatin- and lamin-binding membrane protein [15] and is an integral membrane protein, playing an active role in positioning the heterochromatin at the nuclear periphery and in the shaping of the nucleus. Mutations in LBR gene are the underlying cause of the Pelger–Huët anomaly, characterized by neutrophil nuclear hypolobulation and modified chromatin distribution [16]. On the other hand, an immunohistochemical investigation on PTC cases by Fischer AH *et al.* [13] concludes on the lack of expression of LBR.

All these data induced us to focus on the search for the distribution and expression of LBR in PTC cases and cells, as a possible candidate responsible for the shaping out of PTC nuclei.

Material and Methods

PTC cases and cells

Following a procedure first published by our group [17] and now used as a standard in our hospital [18], specimens were under-vacuum sealed in plastic bags inside the surgical theatre immediately after surgery, and kept at 4^0C until transfer to the pathology laboratory. Once in the pathology lab the surgical specimens were processed without delays for fixation in 4% Formaldehyde in 0.1 M phosphate buffer pH 7.2 (Histo-Line Laboratories, Milan, Italy), then embedded in paraffin, according to the guidelines approved by the local research ethics committees. From each specimen, one sample was frozen in liquid nitrogen immediately after dissection and stored at -80^0C. For the present study, we investigated four PTC cases and four cases of follicular thyroid carcinoma.

TPC, NPA (papillary thyroid carcinoma), WRO (follicular carcinoma) and HT29 (colon carcinoma) cell lines were cultured at 37^0C, 5% CO_2 in RPMI–1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/fungizone.

Immunofluorescence (IF) and immunohistochemistry (IHC)

Indirect immunofluorescence (IF) analysis was performed on cell lines. Briefly, cells were gently fixed in ice-cold methanol, acetone (1–10 minutes) or in 4% paraformaldehyde for 20 minutes at 37^0C. Cells were then rinsed twice with ice cold PBS 1X and stained for either emerin (NCL-emerin, Novocastra, Newcastle, UK) or lamin B receptor (ab32535, AbCam, Cambridge, MA, USA). Detection was performed with FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI. Images were acquired with motorized Metafer Scanning System (Carl Zeiss MetaSystems GmbH).

Immunohistochemistry (IHC) was performed on tissue sections using mouse monoclonal anti-emerin antibody 1:50, 30 minutes or rabbit monoclonal to lamin B receptor antibody 1:50, 30–60 minutes. IHC conditions are reported in Table 1.

Table 1 – ***Different trials and conditions for immunohistochemistry performed on tissue sections and cells with lamin B receptor antibody. All the different experimental procedures were ineffective in retrieving the antigenic reactivity for LBR in PTC cells.***

Method	Antigen retrieval	Additional treatment 1	Additional treatment 2
1.	EDTA pH 8, 40 minutes, 98^0C	/	/
2.		SDS 1%, 5–15 minutes, RT or 30 minutes, 4^0C	/
3.		DNase, 30 minutes, RT	SDS 1%, 5 minutes, RT
4.		Pronase, 37^0C, 10–30 minutes	/
5.		SDS 1%, 5 minutes, RT	TRITON 0.1% 4^0C o.n.
6.		SDS 1%, 5 minutes, RT	mAb o.n.
7.		SDS 1%, 10 minutes, 98^0C	/
8.		Phosphatase, 30 minutes, 37^0C	/
9.		Neuraminidases, 30 minutes, 37^0C	/

To minimize nonspecific background, a pre-treatment was always performed with goat serum (1:50, 30 minutes). Positive and negative controls (omission of the primary antibody and IgG-matched serum) were included for each immunohistochemical run.

RNA extraction

Total RNA was extracted from cell lines and fresh frozen tissue samples with Trizol® reagent, according to manufacturers' instructions. RNA pellets were resuspended in DEPC-treated water, and RNA concentrations were assessed with a spectrophotometer (BioPhotomer Eppendorf AG, Hamburg, Germany). RNA samples were stored at -80^0C until use.

Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR) and PCR Analysis

RT–PCR was performed after a DNAse treatment step with the TURBO DNA-free™ Kit (Ambion, Foster City, CA, USA). For each sample up to 4 μg of RNA were reverse transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems,

Foster City, CA, USA). RNA samples without reverse transcriptase were reverse transcribed as negative controls of DNA contamination for PCR analyses. To assess LBR (NM_002296.2 and NM_194442.1) expression, oligonucleotide primers were designed with Primer-BLAST (*www.ncbi.nlm.nih.gov/tools/primer-blast*): FW 5'-ACTGCCAATTGGAAAGGTTG-3', REV 5'-ACGCTTCCTCATTCCAGAGA-3' (500 bp, annealing temperature 56^0C). Each PCR reaction was carried out with a mix containing PCR Buffer 10X (1X final), $MgCl_2$ (2.5 mM final), dNTPs mix (0.2 mM final), primers (0.5 μM final), POLYTAQ Taq DNA Polymerase (1.5 U final) (Polymed, Florence, Italy), cDNA (200 ng). The reactions were performed on PTC–100 Peltier Thermal Cycler (MJ Research, Inc., MA, USA). PCR products were separated by electrophoresis on an agarose gel stained with ethidium bromide. To reduce the risk of contamination from previously amplified products, separate bench areas were used for RNA isolation, amplification and electrophoresis.

Western blot analysis

Cell lines were washed twice with cold PBS, suspended in 400 μL of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% SDS) added with protease inhibitors cocktail (complete, EDTA-free, Roche) and sonicated in order to properly solubilize nuclear protein. Subsequently, they were incubated at 4^0C for 30 minutes.

Five-μm section from each frozen sample was examined after Hematoxylin–Eosin staining to verify the tumor/stroma ratio. For protein extraction, we used five sections 20 μm thick from about 1×1 cm of tumor tissue. In those samples in which the Hematoxylin–Eosin staining showed a smaller tumor area, we cut additional sections.

Sections were collected into tubes and washed once adding 1 mL of cold PBS and centrifuged at 14 000 rpm at 4^0C for one minute in order to remove the OCT and blood residues. After removing of supernatant, the tissue pellet was suspended in 100 μL of the same lysis buffer used for cell lines and incubated at 4^0C for one hour.

Cell and tissue samples were centrifuged at 14 000 rpm at 4^0C for 15 minutes to eliminate insoluble residues. Supernatants were collected in new tubes and quantified with BCA protein assay. Then, total extracts were separated into aliquots of 25 μg each, frozen and stored at -80^0C until use.

After adding 5X loading buffer, total extracts were heated at 99^0C for 5 minutes and loaded into a 10% polyacrylamide gel. Electrophoresis run was performed in TGS buffer with 100 V tension. After separation, proteins were transferred on a nitrocellulose membrane for one hour at 300 mA. To verify the correct transfer, the membrane was stained with Ponceau red. Afterwards, saturation was performed with TBS 5% dry milk for one hour at room temperature, under gentle agitation. Afterwards the membrane was immunoblotted with anti-LBR (ab32535, AbCam, Cambridge, MA, USA) 1:500 diluted in TBS 5% BSA and incubated over night at 4^0C.

After the incubation, membrane was washed twice with TBS 1% Triton for 15 minutes and incubated with proper peroxidase-labeled secondary antibody, 1:15 000 in TBS 1% Triton for one hour at room temperature. Membrane was then washed three times with TBS 1% Triton for 10 minutes and developed with ECL luminol (Bio-Rad, Hercules, CA, USA).

SiRNA

SiRNA vector against Lamin B receptor was provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

In a six well tissue culture plate, 2×10^5 per well TPC cells were seeded in 2 mL antibiotic-free normal growth medium supplemented with FBS and incubated at 37^0C in a CO_2 incubator until they reach 60–80% of confluence.

Following manufacturer instructions, siRNA transfection mix was prepared adding 1 μg of siRNA duplex into a mixture composed by 200 μL of the transfection medium and 8 μL of transfection reagent, both provided with the kit. This was incubated for 30 minutes at room temperature. Afterwards 0.8 mL of transfection medium was added and overlaid onto the previously washed cells.

Cells were incubated for six hours in a CO_2 incubator. Following 1 mL of normal growth medium containing two times the normal serum and antibiotics concentration was added without removing the transfection mixture and cells were incubated for additional 24 hours.

Results

Immunohistochemical and immunofluorescence data

Immunofluorescence and immunohistochemistry staining with anti-LBR antibodies gave negative results, both on cultured cells and on tissue sections, in PTC epithelial cells and on thyreocytes of the surrounding normal parenchyma, while positive in the stromal and reactive (inflammatory) cells in the same areas. This surprising result in thyroid epithelial cells was unaffected by changes in the antigen retrieval procedures (Table 1) or fixation processes or enzymatic treatments with peptidases or DNAse, and heavily contrasted with the neat decoration of the NM in epithelial intestinal cells (in control sections) as well as in non-epithelial cells (Figure 1). On parallel sections of PTC cases and on cultured cells preparations, staining with anti-emerin antibody resulted in a strong and selective decoration of NM, thus giving evidence of the marked irregularities and foldings, typical of PTC nuclei (Figure 2).

Western blot analysis

Homogenates of PTC cases and of normal thyroid as well as of cultured cells of PTC (lines TPC and NPA) and of follicular carcinoma (lines WRO) were run in parallel and tested with anti-LBR serum. A single, specific band migrating at approximately 67 kD was observed in all tissues and cells, but a heavier signal, suggesting a higher expression of the antigen, was detected in PTC cases and cells (Figure 3). The signal was approximately of the size, and migrated in parallel, to that given by HT29 colon carcinoma cells.

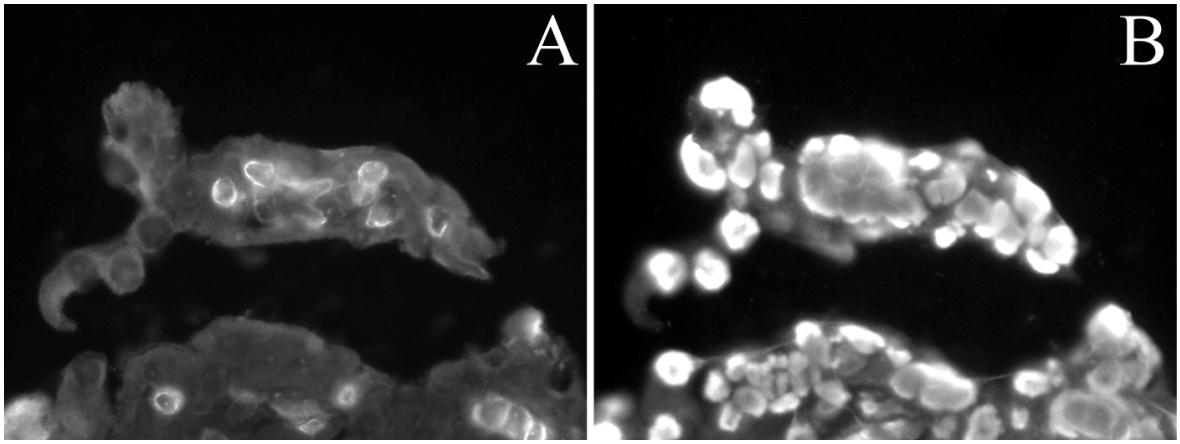

Figure 1 – ***LBR immunofluorescence (IF) on papillary thyroid carcinoma: (A) Section of a case of PTC, stained with anti-LBR antibody. The epithelial cells bordering the papillae did not stain with LBR antibody while stromal cells in the axis of the papillae showed a strong decoration of the nuclear membrane. (B) DAPI nuclear staining, revealing the distribution of all cells (400×).***

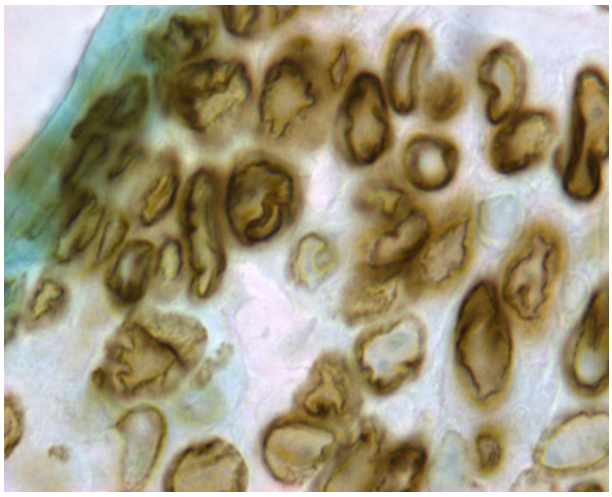

Figure 2 – ***Emerin immunohistochemistry in PTC epithelial cells. Emerin staining highlights nuclear irregularities and foldings typical of PTC (1000×).***

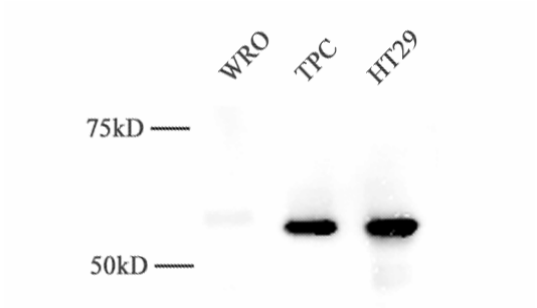

Figure 3 – ***Western blot for LBR on cell lines. Western blot analysis on follicular (WRO) and papillary (TPC) thyroid carcinomas cells compared with colon carcinoma cells (HT29). The procedure reveals that LBR is heavily expressed in TPC cells, to an extent similar to that detectable in HT29 cells. On the contrary, LBR is poorly expressed in WRO cells.***

Gene expression of LBR

RNA extracted from four PTC cases and cells was analyzed by RT–PCR procedures using probes specific for LBR. A specific signal was detected at 500 bp, both in tissue and cell extracts (Figure 4).

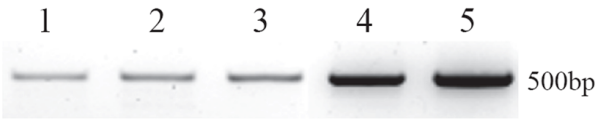

Figure 4 – ***PCR for LBR on cell lines and tissue samples. A specific signal for LBR was detected at 500 bp both in tissue and cells. Lanes 1–3: PTC samples, Lane 4: TPC cell line, Lane 5: HT29 cell line. This data confirms the heavy expression of LBR in papillary thyroid carcinoma cells and tissues.***

siRNA experiments

PTC cells TPC were cultured *in vitro* in presence of siRNA of LBR. Cells (and control untreated cells in parallel) were collected and stained in immunofluorescence with anti-emerin antibodies, in order to check if the block of LBR expression, because of Si–LBR treatment, had any effect on the shape of the nucleus. The percentage of nuclei with infolded NM, as appreciated in these experiments, was not significantly different among related, treated and untreated cells. The correct activation of the siRNA machinery was assessed by western blot and RT–PCR analyses. Treated cells showed a clear reduction of LBR expression compared with controls (Figure 5).

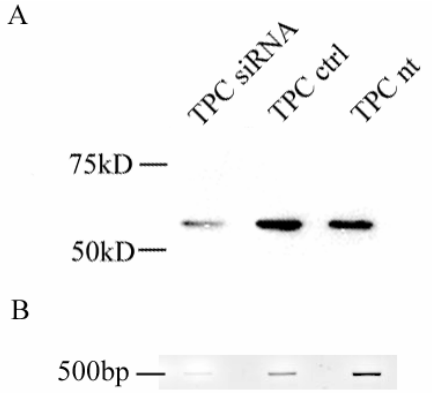

Figure 5 – ***siRNA experiment on TPC cell line. Treated cells showed a reduction on LBR expression either of protein (Western blot analysis, A) and of mRNA (RT–PCR analysis, B).***

Discussion

Shape of the nucleus, as appreciated in histological and cytological preparations, reflects the distribution of chromatin and the related arrangement of the nuclear membrane. Factors influencing the spatial distribution of chromatin in the mammalian nucleus are unknown, but evidence has been presented that such distribution implies functional differences and can influence gene expression [19]. In fact, the nuclear periphery is known to be the site of transcriptional inactivity and gene repression [19], so that chromosomes with the highest gene concentration are sequestrated in the centre of the nucleus while more inactive chromosomes and chromatin are at the periphery [19]. Although the factors that mediate the nuclear positioning of the human chromosomes are unknown, interaction among chromatin and components of the nuclear membrane and lamina is recognized to be important in establishing or maintaining nuclear architecture [19]. Proteins of the nuclear membrane or lamina are candidates for molecules that may anchor regions of the genome at the nuclear periphery.

Data of the literature suggest that peripheral distribution of chromatin, hence gene silencing, involves interaction with proteins of the nuclear envelope, a complex structure composed of the inner and outer nuclear membrane, nuclear pore complexes and lamina, an underlying mesh-like supportive structure. Three proteins proper of the inner nuclear membrane: lamin B receptor, lamina-associated polypeptide 2-beta and emerin, were shown to bind chromatin modifiers and/or transcriptional repressors [20].

The morphogenesis of the nuclear shape in PTC and the biological mechanism underlying these nuclear abnormalities have been studied with *in vitro* models, with the demonstration that induced gene mutations are associated to tumour-specific nuclear changes and appear to mediate the structural changes of the NM and the chromatin organization which are typical of papillary carcinoma [21, 22]. In fact, micro-injection into thyroid cells of RET/PTC oncogene, which leads to activation of a thyrosine-kinase, was shown to induce NM irregularities within hours, without the requirement for a post-mitotic NM reassembly [23]. We have in conclusion to consider that the nuclear features of PTC are a combination of altered distribution of chromatin and of irregularities of the shape of the nuclear membrane. It is tempting to assume that both phenomena are somehow linked to the molecular alterations responsible for PTC. Fusco A *et al.* [24], investigating the spatial distribution of RET/PTC oncogenic activetion in PTC cases, observed that RET activation closely parallels the morphological changes in thyroid nodules being restricted to areas of the tumors featuring the cytological alterations typical of PTC.

In the present study, we have investigated the hypothesis that the NM irregularities in PTC might be linked to alterations in the expression of lamin B receptor (LBR), a component of the inner NM responsible for the distribution of lamin B and associated chromatin. Data of the literature indicate LBR is involved in the shaping of the nuclear membrane, since experiments of nature indicate that a reduced expression of LBR, because of gene mutation, is responsible of the lack of nuclear segmentation of granulocytes in Pelger–Huët anomaly [16, 25].

Fisher AH *et al.* [13] reported negative immunohistochemical results for LBR in PTC. We have been able to confirm the data, but the result of western blot and gene expression analysis indicate that LBR is present and heavily expressed, both in PTC tissue extracts and cells. The molecular weight of LBR from PTC cells is apparently of the same weight as that expressed in intestinal cells, as can be appreciated from the results of western blot analysis (Figure 3), so as to rule out the hypothesis of alternative splicing. Still, in thyroid epithelial cell types the antigen is hidden or inaccessible in its native state when bound to the NM. Several immunohistochemical trials (Table 1), undertaken in order to retrieve the hidden antigen were unsuccessful. The reason for the discrepancy of the accessibility of LBR when present in thyroid epithelial cell (either normal or neoplastic) and stromal or intestinal epithelial cells (where LBR bound to the NM is neatly recognized) are presently unknown. In order to explain the observed paradox it can be suggested that in PTC cells, the protein is heavily expressed but, at least in the native state, it is inaccessible to antibodies being strictly bound to other proteins, such as lamin B.

Conclusions

Although the reasons for the nuclear irregularities typical of PTC remain hidden, the present study indicates that over-expression of LBR, possibly associated with rearrangement of lamin B and related chromatin, is definitely involved.

Acknowledgements

This work was supported by grants from Regione Piemonte "Ricerca Sanitaria Finalizzata".

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Received: October 22nd, 2010

Accepted: November 25th, 2010