SPANDIDOS PUBLICATIONS

Underexpression of PPARγ is associated with aneuploidy and lower differentiation of thyroid tumours of follicular origin

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Abstract. Peroxisome proliferator-activated receptor γ (PPARγ) gene is a nuclear receptor that is involved in thyroid tumourigenesis. Recently, our group has shown that follicular carcinomas underexpressing PPARγ protein are more prone to develop distant metastases, to invade locally, to present poorly differentiated areas and to persist after surgery. Aneuploidy is also observed in some thyroid tumours, particularly in the more advanced cases. The aim of the present study was to investigate the association of PPARγ expression with the degree of differentiation and ploidy status of benign and malignant thyroid neoplasias. DNA cytometric studies, ploidy and S-phase fraction (SPF) determination, and quantitative RT-PCR analysis of molecular markers specific for thyroid follicular cells, namely Tg (thyroglobulin), TSHR (TSH receptor) and NIS (Na^+/I^- symporter) were compared between thyroid lesions with positive or negative PPARγ protein expression. We observed that PPARγ-negative tissues expressed lower levels of Tg mRNA [$4.66x10^6$ a.u. (arbitrary units) ± $1.49x10^6$], and were more frequently aneuploid (36%), and presented higher SPF (3.1%±0.4) than PPARγ-positive samples (Tg mRNA = $2.54x10^7$ a.u. ± $9.72x10^6$, P=0.0006; aneuploidy=8%, P=0.0031; SPF=2.2%±0.2, P=0.0430). A similar trend was also observed for TSHR and NIS mRNA, although not reaching statistical significance. This study showed that underexpression of PPARγ is associated with poor tumour differentiation, aneuploidy and higher cell proliferative activity. Therapies designed to modulate expression of PPARγ may have an impact on the growth of thyroid neoplasias.

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; FTC, follicular thyroid carcinoma; FTA, follicular thyroid adenoma; PDTC, poorly-differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; NH, nodular hyperplasia; IHC, immunohistochemistry; SPF, S-phase fraction; Tg, thyroglobulin; TSHR, TSH receptor; NIS, Na^+/I^- symporter; QRT-PCR, quantitative RT-PCR; NT, normal thyroid tissue; ATC, anaplastic thyroid carcinoma; PBS, phosphate-buffered saline; CV, coefficient of variation; SEM, standard error of the mean; a.u., arbitrary units; RB, retinoblastoma

Key words: PPARγ, aneuploidy, thyroid, thyroglobulin, TSH receptor, Na^+/I^- symporter

Introduction

The molecular genetics underlying thyroid carcinogenesis is not fully understood. Recently, rearrangements between PAX8 and PPARγ (peroxisome proliferator-activated receptor γ) genes were associated with follicular thyroid carcinomas (FTCs) and adenomas (FTAs) (1-3). PPARγ is a nuclear receptor that participates in a wide range of cellular processes, such as adipocyte differentiation, lipid storage, insulin sensitization (4-6), cell cycle control, inflammation, atherosclerosis, apoptosis and carcinogenesis (7). PPARγ is activated by natural ligands (e.g., prostaglandin J2 and fatty acids derivatives) or by synthetic ligands (thiazolidinediones). These ligands induce apoptosis and exert anti-proliferative effects on several carcinoma cell lines and inhibit the growth of thyroid carcinoma cells *in vivo* (8-11). In different types of cancer, several studies have shown that, compared to their normal counterparts, tumours can present either over-expression (12,13), underexpression (14) or similar expression of PPARγ (15). Studies in human colon carcinomas, with monoallelic PPARγ mutations, indicated that colon cancer is associated with loss-of-function mutations in PPARγ (16). In addition, PAX8PPARγ fusion protein, resulting from the t(2;3)(q13;p25) translocation between PAX8 and PPARγ genes, is present in a significant proportion of thyroid follicular tumours (1).

Aneuploidy is one of the most frequent genetic abnormalities found in cancer. It results from full or partial aneusomies in which the copy number of entire chromosomes or chromosomal subregions is altered. Aneuploidy *per se* has been proposed to be an early and genetically destabilizing force in cancer development (17), but the

molecular mechanisms underlying aneuploidy are poorly understood. In thyroid lesions, aneuploidy is mostly found in poorly-differentiated thyroid carcinomas (PDTCs), is rare in papillary thyroid carcinomas (PTCs), being observed in a range of 10-57% of follicular tumours (18), and in 10-22% of nodular hyperplasias (NHs) (19-21).

In a previous study (22), we demonstrated that follicular carcinomas that do not express PPARγ protein by immunohistochemistry (IHC) are more prone to develop distant metastases, to invade locally, to present poorly differentiated areas and to persist after surgery. These results suggest an association between underexpression of PPARγ and a less differentiated phenotype. Aiming to investigate this hypothesis, we determined the ploidy status, S-phase fraction (SPF) and the mRNA expression of molecular markers of thyroid follicular cells [Tg (thyroglobulin), TSHR (TSH receptor) and NIS (Na^+/I^- symporter)], by quantitative RT-PCR (QRT-PCR), in benign and malignant thyroid tissues with positive or negative PPARγ staining.

Materials and methods

Materials. In a previous study by our group (22), PPARγ protein was detected by IHC in 77% of normal thyroid tissues (NTs), 71% of NHs (P>0.05, vs. NT), 62% of FTAs (P>0.05, vs. NT), 53% of FTCs (P=0.03, vs. NT) and 49% of PTCs (P=0.01, vs. NT). All PDTCs/ATC were negative (P<0.0001, vs. normal thyroid tissue). Positive staining was usually faint or moderate (in tissues negative for the PAX8/PPARγ rearrangement). From this published series, we selected cases (n=95), from which frozen samples were available, for RNA extraction and quantitative analysis of thyroid specific transcripts. These cases were: 10 NHs, 29 FTAs, 9 FTCs, 37 PTCs (15 classic, 14 follicular, 3 oxyphilic, 3 tall cells and 2 cribiform), 9 PDTCs and 1 anaplastic thyroid carcinoma (ATC). The material was retrieved from the files of the Portuguese Institute of Oncology Francisco Gentil, Lisboa, and institutional ethics guidelines were strictly followed. Samples were immediately frozen at time of surgery to subsequent RNA extractions and DNA flow cytometric analysis, and were also embedded in paraffin for immunohistochemistry. Haematoxylin- and eosin-stained sections were evaluated histologically by pathologists to classify the tumours according to the 2004 World Health Organization Classification of Tumours of Endocrine Organs.

RNA extraction and cDNA synthesis. Total RNA was isolated from frozen tissues by TRIzol reagent (Invitrogen, Paisley, UK), according to the manufacturer's protocol. RNA was quantified by UV spectrophotometry (optical density measured at 260 nm). Complementary DNA (cDNA) was synthesized from 1 or 2 μg [for quantitative real-time PCR (QRT-PCR)] of total RNA at 37˚C for 90 min, using oligo(dT) primers (Invitrogen) and reverse transcriptase (Superscript II, Invitrogen).

Detection of PAX8PPARγ fusion gene. PAX8PPARγ fusion gene was analysed in the entire series by RT-PCR (n=80) or FISH (n=4), and the remaining cases (n=11) by both techniques, as described previously (2,22). All the samples were negative for the PAX8PPARγ fusion gene. This was an important criterion used for sampling selection, since the presence of the PAX8PPARγ fusion protein increases the expression of wild-type PPARγ protein (anti-PPARγ antibody used in this study recognizes PPARγ regions common to both PAX8PPARγ fusion protein and to wild-type PPARγ protein).

QRT-PCR. The mRNA expression levels of Tg, NIS and TSHR were quantified using TaqMan probes for Tg and NIS analysis and SYBR Green dye for TSHR analysis, on an ABI PRISM 7900 HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). To analyze the mRNA expression levels of Tg and NIS by TaqMan probes, specific primers and the probes were selected from the Assay-on-demand products [P/N 4331182; Hs00794359_m1 (Tg), Hs00166567_m1 (NIS); Applied Biosystems]. In order to normalize the differences in the amount of total cDNA used in each reaction, we performed the amplification of GAPDH cDNA as endogenous control (Pre-Developed TaqMan Assay Reagents; P/N 4326317E; Applied Biosystems). Gene expression assays have a FAM (Tg and NIS) or a VIC (GAPDH) reporter dye at 5'-end of the TaqMan MGB probe, and a non-fluorescent quencher at the 3'-end of the probe. To analyse the mRNA expression level of TSHR by SYBR Green dye, we used the following primers: TSHR F (5'-GAA CTGATAGCAAGAAACACCTGG-3') and TSHR R (5'-GTA TCCTGGAACTTGGACTTTT-3') for TSHR gene and PGK F (5'-CAGTTTGGAGCTCCTGGAAG-3') and PGK R (5'-TGC AAATCCAGGGTGCAGTG-3') for PGK-1 gene (23).

cDNA of each sample was synthesized from 2 μg of RNA, as described above. Quantitative RT-PCR was performed in a total reaction volume of 25 μl containing 1X TaqMan Universal PCR Master Mix (P/N 4304437; Applied Biosystems), 1X Assays-on-demand Gene Expression Assay Mix and 2 μl of cDNA diluted in RNase-free water, or 1X SYBR Green PCR Master Mix (P/N 4309155; Applied Biosystems), 10 pmol of each primer and 2 μl of cDNA diluted in RNase-free water. All reactions were performed in a 96-well reaction plate (MicroAmp Optical 96-Well Reaction Plate, Applied Biosystems). Samples were heated for 2 min at 50˚C, followed by 10 min at 95˚C and amplified for 50 cycles of 15 sec at 95˚C and 1 min at 60˚C. SYBR Green assays were followed by a dissociation curve analysis. cDNA from Nthy-ori 3-1 cell line (SV40 large-T-antigen immortalized normal human differentiated thyroid cells - European Collection of Cell Culture, Wiltshire, UK) was serially diluted in dH_2O and amplified in parallel to establish a standard curve for relative quantification. Nthy-ori cell cDNA was also used as calibrator for determining the relative expression of Tg, NIS and TSHR genes. For each sample, the amount of target RNA was normalised to the standard curve, then normalised to the endogenous control RNA expression and then to the calibrator. All quantitative real-time RT-PCR experiments, including a control with no template, were performed in triplicate.

The mRNA expression levels for Tg were analysed in 31 samples (20 FTAs, 7 FTCs and 4 PTCs), for TSHR in 29 samples (20 FTAs, 5 FTCs and 4 PTCs), and for NIS in 20 samples (9 FTAs, 7 FTCs and 4 PTCs).

cytometry. Flow cytometric analysis was performed entative tumour tissue frozen at -80˚C at time of surgery. Tissue samples were disaggregated mechanically using sterile surgery blades in phosphate-buffered saline (PBS) in a Petri plaque. Then, the cell suspension was washed twice and cells were counted in a Bürker's counting chamber. To perform the DNA-specific staining (24), cells were treated with a solution of ribonuclease 1 mg/ml (Sigma-Aldrich, St. Louis, USA) in PBS, for digestion of double-stranded RNA, and a non-ionic detergent Nonidet P40 0.05% (Sigma-Aldrich), for cell lysis. Nuclei were stained with propidium iodide (Sigma), 50 μg/ml in Tris-$MgCl_2$, for 1 h at 4˚C in the dark. After filtration with a 55-μm nylon mesh, the stained cell nuclei were analysed on an Epics Profile II (Flow cytometer, Coulter Electronics, Hialeah, FL, USA) equipped with a 488-nm argon-ion laser. The instrument was aligned daily and considered calibrated when the coefficients of variation (CVs) of standard fluorescent microspheres were below 2% (DNA-Check, Coulter). At least 20000 nuclei were acquired for each case and the results were recorded on a single parameter 256 channel fluorescence histogram.

Cell cycle analysis of DNA histograms was performed with the Multicycle program (Phoenix Flow Systems, San Diego, CA, USA), based on the mathematical method described by Dean and Jett (25) which includes a multiple option cell cycle fitting that automatically determines the DNA index. The DNA ploidy pattern of tumours was expressed by the dichotomy diploid (one G_0/G_1 peak, DNA index = 1.0) vs. aneuploid (one or more abnormal populations, DNA index ≠ 1.0). The internal reference standard was formed by non-malignant diploid cells (fibroblasts, lymphocytes, normal epithelial cells) mixed with the analysed sample. The histograms that presented CVs above 8% were rejected. The S-phase fraction (SPF), a measure of the tumour proliferative activity, was determined according to a polynomial model and corresponds to the percentage of cells in S-phase of the cell cycle. Technical reasons related to excess of background debris, non-specific fluorescence or overlapping of populations impaired the SPF evaluation in 24 of 95 (25.3%) cases.

Cytometric analysis for ploidy was performed in 95 samples (10 NHs, 29 FTAs, 9 FTCs, 37 PTCs, 9 PDTCs and 1 ATC), and S-phase fraction was investigated in 71 samples (8 NHs, 20 FTAs, 4 FTCs, 31 PTCs and 8 PDTCs).

Statistical analysis. The comparison of Tg, TSHR and NIS mRNA expression levels between PPARγ protein negative and positive tissues was performed by the Mann-Whitney test. DNA ploidy and SPF were analysed in the different thyroid histotypes with the χ^2 test, and one-way ANOVA test (with the Dunnett's Multiple Comparison test), respectively. To correlate SPF with ploidy status and PPARγ protein expression we used the unpaired t-test (two-tailed). To correlate the PPARγ protein expression with ploidy, we used the Fisher's exact test (two-tailed). Non-parametric tests were used when the data were not normally distributed. P-values ≤0.05 were considered significant. Statistical analysis was performed using GraphPad Prism statistical software (San Diego, CA, USA). All graphs with error bars indicate means ± standard error of the mean (SEM).

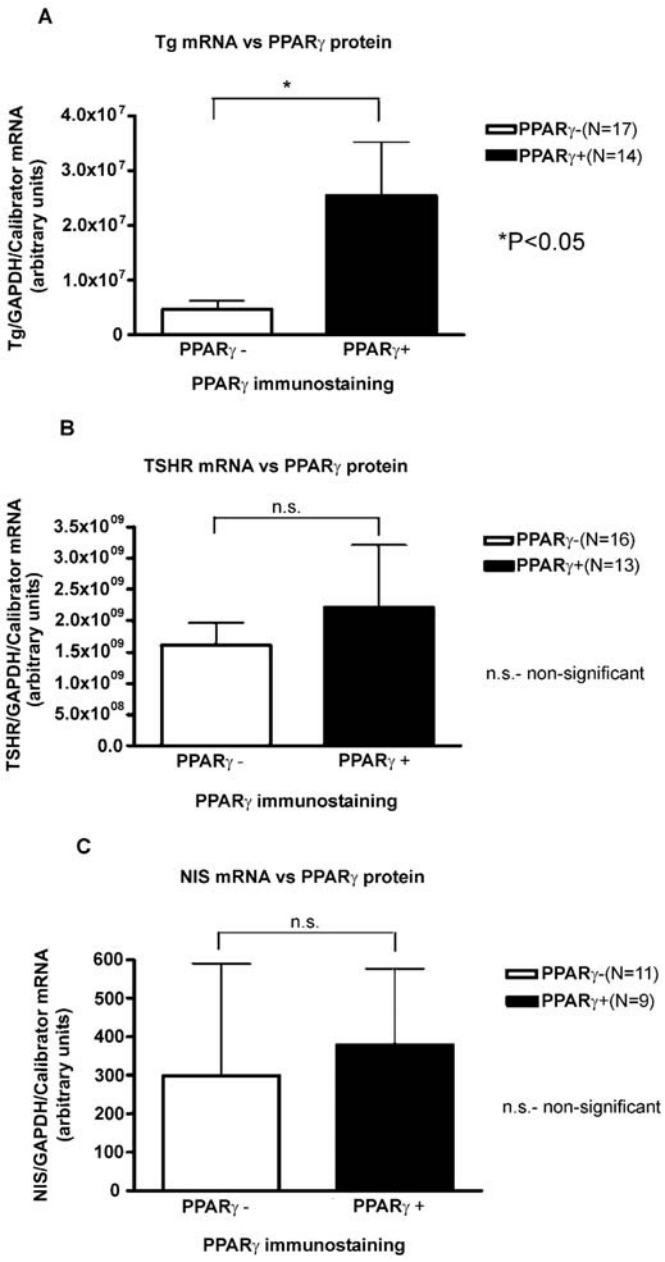

Figure 1. mRNA expression of (A) Tg, (B) TSHR and (C) NIS in PPARγ-negative and PPARγ-positive thyroid tumours (FTAs, FTCs and PTCs), as assessed by real-time RT-PCR, using TaqMan probes (Tg and NIS) and SYBR Green dye (TSHR). Tg, TSHR and NIS mRNA levels were normalized to values of GAPDH (Tg and NIS) or PGK-1 (TSHR) housekeeping genes and to a calibrator (Nthy-ori cells). Graphs with error bars indicate mean value ± SEM. Significant differences between PPARγ-negative and PPARγ-positive cases are indicated.

Results

Correlation between the expression of the PPARγ protein and the expression of Tg, TSHR and NIS mRNAs. The mean Tg mRNA expression level was lower in PPARγ-negative [$4.66x10^6$ a.u. (arbitrary units) ± $1.49x10^6$; n=17] than in PPARγ-positive cases ($2.54x10^7$ a.u. ± $9.72x10^6$; n=14; P=0.0006) (Fig. 1A). This difference in Tg mRNA expression was also statistically significant within the group of FTAs (PPARγ-, $4.06x10^6$ a.u. ± $1.58x10^6$; n=10 vs. PPARγ+, $3.24x10^7$ a.u. ± $1.32x10^7$; n=10; P=0.0021). Although not

Table I. Ploidy analysis in benign and malignant thyroid lesions.

Histotype (n)	Ploidy	
	Diploid (%)	Aneuploid (%)
NH (n=10)	8 (80)	2 (20)
FTA (n=29)	20 (69)	9 (31)
FTC (n=9)	7 (78)	2 (22)
PTC (n=37)	33 (89)	4 (11)
PDTC/ATC (n=10)	3 (30)	7 (70)

χ^2 test, P=0.0039 (frequency of aneuploidy vs histotype); n, number of samples.

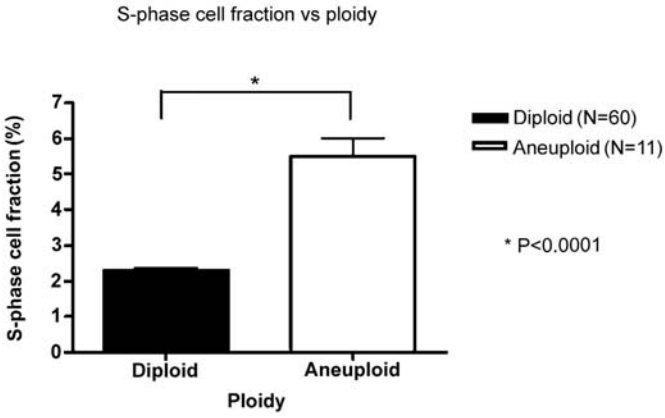

Figure 2. Correlation between ploidy status and the S-phase fraction of thyroid lesions. Aneuploid cases presented higher SPF than diploid cases (P<0.0001). Graph with error bars indicate mean value ± SEM.

Table II. S-phase cell fraction in benign and malignant thyroid lesions.

Histotype (n)	Range (%)	S-phase cell fraction (mean (%) ± SEM)	P-value[a]
NH (n=8)	1.2-5.7	2.3±0.5	<0.01
FTA (n=20)	0.3-4.8	2.3±0.2	<0.01
FTC (n=4)	0.9-7.4	3.7±1.5	n.s.
PTC (n=31)	0.8-4.2	2.3±0.2	<0.01
PDTC (n=8)	1.9-11.7	5.4±1.2	-

One-way ANOVA test, P<0.0001. [a]Dunnett's Multiple Comparison test, each histotype vs. PDTCs; n, number of samples; n.s., non-significant.

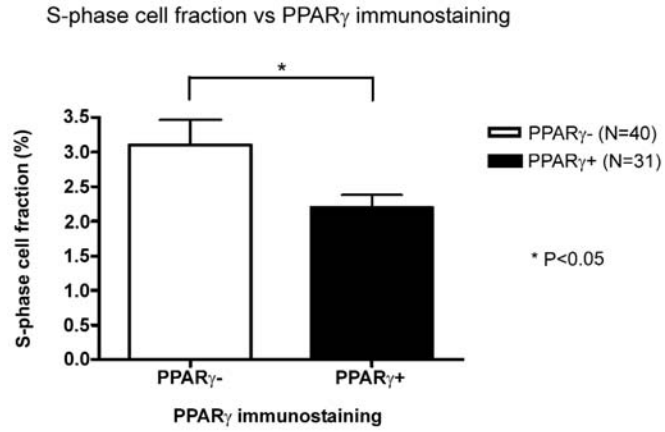

Figure 3. Correlation between S-phase fraction and PPARγ protein expression of thyroid lesions. PPARγ-negative cases presented higher SPF than PPARγ-positive cases (P<0.05). Graph with error bars indicate mean value ± SEM.

statistically significant, we observed that PPARγ-negative tumours expressed lower levels of TSHR and NIS mRNA than PPARγ protein positive cases (TSHR mRNA: PPARγ-, $1.61x10^9$ a.u. ± $7.14x10^8$; n=16 vs. PPARγ+, $2.21x10^9$ a.u. ± $9.99x10^8$; n=13; P=0.2279) (NIS mRNA: PPARγ-, $2.99x10^2$ a.u. ± $2.90x10^2$; n=11 vs. PPARγ+, $3.79x10^2$ a.u. ± $1.98x10^2$; n=9; P=0.1287) (Fig. 1B and C).

Ploidy and SPF analyses. The results obtained in the ploidy and SPF analyses of thyroid lesions are summarised in Tables I and II. Aneuploidy was significantly higher (P=0.0039) in PDTCs (70%) than in the other thyroid lesions. SPF was higher in PDTCs (5.4%±1.2) than in NHs (2.3%±0.5; P<0.01), FTAs (2.3%±0.2; P<0.01), PTCs (2.3%±0.2; P<0.01) or FTCs (3.7%±1.5; n.s.). The differences in SPF values between FTCs and the other histotypes were not statistically significant (P>0.05, Dunnett's Multiple Comparison test). The highest SPF values were observed in 2 PDTCs (11.7 and 9.3%) and in 1 FTC (7.4%). Overall, aneuploid lesions presented higher SPF than diploid lesions (5.7%±0.8; n=11 vs. 2.2%±0.1 n=60; P<0.0001) (Fig. 2).

Correlation between PPARγ protein expression and ploidy. The correlation between PPARγ protein expression and ploidy is summarised in Table III. In the whole series, PPARγ-negative thyroid lesions were more frequently aneuploid (21 of 58; 36%) than PPARγ-positive lesions (3 of 37; 8%) (P=0.0031). After excluding the PDTC/ATC subgroup, which presented aneuploidy in the vast majority of the cases, the difference was still statistically significant: 14 of 48 (29%) in PPARγ-negative vs. 3 of 37 (8%) in PPARγ-positive cases (P=0.0268). If nodular hyperplasias were excluded from the analysis the frequency of aneuploidy was still higher in PPARγ-negative than in PPARγ-positive tumours: 19 of 52 (37%) in PPARγ-negative vs. 3 of 33 (9%) in PPARγ-positive (P=0.0052).

Correlation between PPARγ protein expression and SPF. PPARγ-negative lesions presented higher SPF than PPARγ-positive cases (PPARγ-, 3.1%±0.4; n=40 vs. PPARγ+, 2.2%±0.2; n=31; P=0.0430) (Fig. 3). This finding remained statistically significant considering only the neoplastic tissues (FTAs, FTCs, PTCs and PDTC) (PPARγ-, 3.2%±0.4; n=36 vs. PPARγ+, 2.2%±0.2; n=27; P=0.0449). However, it lost

Correlation between PPARγ protein expression and ploidy.

	PPARγ immunohistochemistry Negative			PPARγ immunohistochemistry Positive		
Histotype (n)	Diploid (%)	Aneuploid (%)	Histotype (n)	Diploid (%)	Aneuploid (%)	P-value[a]
NH (n=6)	4 (67)	2 (33)	NH (n=4)	4 (100)	0 (0)	n.s.
FTA (n=16)	9 (56)	7 (44)	FTA (n=13)	11 (85)	2 (15)	n.s.
FTC (n=7)	5 (71)	2 (29)	FTC (n=2)	2 (100)	0 (0)	n.s.
PTC (n=19)	16 (84)	3 (16)	PTC (n=18)	17 (94)	1 (6)	n.s.
PDTC/ATC (n=10)	3 (30)	7 (70)	PDTC/ATC (n=0)	0 (0)	0 (0)	-
Total (n=58)	37 (64)	21 (36)	Total (n=37)	34 (92)	3 (8)	0.0031

[a]Fisher's exact test (PPARγ immunostaining vs. ploidy); n, number of samples; n.s., non-significant.

significance when the PDTCs, which presented the highest SPF values, were removed (PPARγ-, 2.5%±0.3; n=32 vs. PPARγ+, 2.2%±0.2; n=31; P>0.05).

Discussion

In a previous study (22), we analysed the expression of PPARγ in several types of thyroid lesions and observed that clinically aggressive tumours had lower PPARγ protein expression than less aggressive carcinomas. Based on this finding, we hypothesized that low levels of PPARγ expression might be correlated with lower expression of thyroid specific genes such as Tg, TSHR and NIS. Furthermore, since aneuploidy is more prevalent in poorly-differentiated thyroid carcinomas (26-28), we also investigated the correlation of PPARγ expression with the ploidy status and the proliferative activity of thyroid lesions.

When all thyroid histotypes were grouped together, the levels of Tg mRNA were significantly lower in PPARγ negative lesions than in cases staining for PPARγ. The same was observed in the group of FTAs, which are benign tumours. Although not reaching statistical significance, the levels of TSHR and NIS mRNA were also lower in PPARγ-negative than in PPARγ-positive cases. These results suggest that loss of PPARγ is related to a lower differentiation of thyroid follicular cells. This conforms well with the inhibition of cell proliferation and induction of apoptosis or cell differentiation which is observed in several types of cancers (8-11,15,29-35), following treatment with specific PPARγ agonists. It is well known that the activation of PPARγ suppresses the transcriptional activity of the E2F/DP complex (36). E2F is a transcription factor that regulates the expression of genes involved in entry into S phase and in DNA synthesis (37,38) and DP is an E2F-related protein that stabilizes E2F. According to Altiok *et al* (39) PPARγ activation leads to a loss of binding of E2F/DP to DNA, due to an increase in phosphorylation of this complex. The action of PPARγ on E2F/DP function may also be important at the level of cell differentiation. There are differentiation-promoting agents that cause a decrease in the DNA-binding activity of E2F/DP complex, during the differentiation of several types of cells (40-43).

In general, aneuploid thyroid lesions presented higher SPF than diploid cases (5.7±0.8 vs. 2.2±0.1; P<0.0001) (Fig. 2). Higher proliferative activity has been found in thyroid carcinomas (FTCs and mixed follicular/papillary thyroid carcinomas) as compared to benign lesions (FTAs) and normal thyroid (44). Konarska *et al* (45) observed similar findings in three different thyroid tissues [colloid goiters, diploids (8.6%) vs. aneuploids (13.7%); parenchimatous goiters, diploids (3.7%) vs. aneuploids (6.7%); adenomas, diploids (8.4%) vs. aneuploids (10.2%)]. However, our SPF data should be considered with caution, since there is a lack of correlation with PPARγ expression when the PDTC subgroup, which showed the highest SPF values, was excluded from the SPF analysis.

In our study, the PPARγ-negative cases were more frequently aneuploid (Table III) and presented higher SPF (Fig. 3) than cases expressing PPARγ. The molecular mechanism linking PPARγ to ploidy of thyroid neoplasias remains largely unknown. However, the loss of PPARγ expression may favour chromosomal instability because PPARγ activation induces a block in the G_1-S transition by inhibiting RB (retinoblastoma) phosphorylation and by the attenuation of mitogen-induced p27 degradation (46-48).

An alternative hypothesis is that chromosomal instability could be the driving force that contributes to underexpression of PPARγ and loss of expression of the thyroid-specific genes during the malignant progression of thyroid tumours.

In sum, our results showed that there is an association between underexpression of PPARγ, aneuploidy, and lower cell differentiation of thyroid tumours. Therapies designed to modulate expression of PPARγ may have an impact in tumour growth of thyroid neoplasias.

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