CLINICAL STUDY

Evaluation of the sensitivity to chemotherapeutics or thiazolidinediones of primary anaplastic thyroid cancer cells obtained by fine-needle aspiration

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Abstract

Objective: Anaplastic thyroid cancer (ATC) is often unoperable and chemotherapy and radiotherapy are the main treatments. Until now 'primary ATC cell cultures' (ANA) have been developed from surgical biopsies. The possibility to obtain ANA from fine-needle aspiration (FNA-ANA) and to test their sensitivity to different drugs could increase the effectiveness of treatments and avoid unnecessary surgical procedures.

Design: To obtain FNA-ANA from six ATC patients before undergoing surgery and to evaluate the chemosensitivity of FNA-ANA to chemotherapeutic agents and thiazolidinediones (TZD).

Methods and results: FNA-ANA from the six ATC patients were cultured in RPMI 1640 and propagated in DMEM. Chemosensitivity was evaluated by inhibiting the proliferation with increasing concentrations of five different chemotherapeutic agents (bleomycin, cisplatin, gemcitabine, etoposide, and carboplatin) or TZD (rosiglitazone). Chemotherapeutic agents significantly inhibited ($P < 0.0001$) FNA-ANA proliferation, such as TZD ($P < 0.001$); etoposide was the most effective in reducing cell growth. Another ANA culture for each patient was obtained from a biopsy specimen; the results for the chemosensitivity tests were similar to those obtained with FNA-ANA. The ^{V600E}BRAF mutation was observed in two ATC patients; the inhibition of proliferation by drugs was similar in tumors with or without ^{V600E}BRAF mutation.

Conclusions: Our study demonstrates 1) the possibility to obtain FNA-ANA, and opens the way to the use of FNA-ANA to test the chemosensitivity to different drugs (chemotherapeutic agents or TZD; and possibly the radiosensitivity) in each patient, avoiding unnecessary surgical procedures and the administration of inactive chemotherapeutics; and 2) that etoposide is highly effective in reducing ATC cell growth *in vitro*.

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Introduction

The medical treatment of patients with anaplastic thyroid cancer (ATC) has still not been well defined. Some experts advocate aggressive multimodal treatment (surgery, chemotherapy (CT), radiotherapy) for ATC. In some studies, patients treated with total thyroidectomy, followed by radiotherapy and CT (1–3), survived longer than the other patients; but these results have not been confirmed by other authors (4–6).

Disease orientated *in vitro* drug screening using human tumor cell lines has some predictive value for the activity of clinical responses (7, 8). A positive predictive value has been reported for *in vitro* drug screening of ~60% of patients and a negative predictive value of 90% (9). Thus, *in vitro* drug screening might help to prevent the administration of inactive chemotherapeutics to patients. Based on this model, the

potential activity of gemcitabine (10), vinorelbine, and paclitaxel (11, 12) has been recently reported in ATC. These studies underline the potential value of preclinical drug screening.

The most recent development in the treatment of ATC seems to be represented by the possibility offered by peroxisome proliferator-activated receptor- γ (PPAR γ) agonists, by adenovirus-mediated p53 tumor suppressor gene therapy or by the use of vascular and growth factor-targeted agents (13). All these new therapies need further confirmation coming from ongoing *in vitro* studies and clinical trials.

In particular, newly developed synthetic PPAR γ agonists include anti-diabetic drugs, thiazolidinediones (TZD) (troglitazone, rosiglitazone, pioglitazone, etc.) (14), that elicit both anti-neoplastic (14) and anti-inflammatory effects (15) in several types of mammalian cells. Recently, it has been shown that TZD exert

anti-proliferative effects on human papillary carcinoma cells (16), and it has been proposed that PPAR γ agonists may be effective for the treatment of patients with thyroid cancer that fails to respond to traditional treatments (17–20). Moreover, it has been shown that TZD inhibited cell proliferation and down-regulated the invasive potential in five continuous human anaplastic cancer cell lines (21). These results have been recently confirmed by other studies (19).

The possibility to test the sensitivity of 'primary ATC cell' (ANA) cultures from each subject to different drugs could permit an increase in the effectiveness of the treatment. Until now, primary ANA cultures have been obtained from surgical biopsies (biopsy-ANA) performed for therapeutic or diagnostic procedures. The possibility to develop primary ANA cultures from fine-needle aspiration (FNA) could avoid unnecessary surgical procedures and allow the sensitivity to different chemotherapeutics to be evaluated in each patient.

Here, we report the establishment of ANA cultures from cells obtained by FNA cytology, and the results of chemosensitivity tests with chemotherapeutic agents or PPAR γ agonists TZD.

Subjects and methods

Patients source for thyroid tissue

FNA samples were obtained from six patients with ATC by FNA cytology (23 gauge needle). Surgical thyroid tissue was obtained from the same six patients with ATC (the tissue was obtained from a thyroid biopsy during first surgical operation). The diagnosis was established on commonly accepted clinical, laboratory, and histological criteria (22). Immunohistochemistry showed the absence of expression of thyrotropin (TSH) receptor, thyroperoxidase (TPO), thyroglobulin (Tg) and sodium/iodide symporter (NIS).

The study subjects gave their informed consent to the study, which was approved by the Ethical Committee of the University of Pisa.

Microdissection and DNA extraction

Serial 5 μ m sections were taken from paraffin blocks for histological examination on glass slides and for DNA extraction on membrane slides (Nikon, Firenze, Italy). The presence of the tumor tissue was confirmed in the first and the last section for each section series. Unstained sections were deparaffinized with Bio-Clear (Bio-optica, Milano, Italy), rehydrated in graded ethanol, and stained with H&E. Microdissections were performed using the laser-assisted SL microcut Microtest (MMI GmbH distributed by Nikon). For each sample, 3–5 microareas of 5 μ m were obtained. Each area contained 200–500 cells. Particular care was taken in microdissecting areas of the tumor. Samples from the

non-neoplastic thyroid parenchyma, generally from the contralateral lobe, were dissected as control reference. The microdissected cells were placed in the SL microcut transfer film (Nikon) and the DNA was extracted overnight in a humidified chamber at 56 °C in 200 μ l tissue lysis buffer (ATL DNeasy Tissue kit; Qiagen GmbH) containing 20 μ l proteinase K. DNA was isolated by Qiagen spin column; carrier tRNA was added to improve DNA recovery. Finally, DNA was eluted in 40 μ l TE buffer and immediately processed for PCR amplification. A mock control, in which no tissue was added, was processed in parallel with each sample (23).

Detection of BRAF mutation by PCR-SSCP and direct DNA sequencing

PCR-SSCP screening of *BRAF* mutations was performed by amplifying exon 15 according to a standard procedure (24). DNA was used as a template in a 20 μ l PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂ (pH 8.3), 0.2 mM dNTPs, 8 pmol amplimers, and 1.25 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). PCR primers for the *BRAF* exon 15 were as follows: 5'(F)-TCCTTTACTTACTACACCTCAGAT-3' and 5'(R)-AGTG-GAAAAATAGCCTCAAT-3'.

The amplicon size was 167 bp. Cycling conditions were as follows: initial denaturation (94 °C, 5 min), then 35 cycles (denaturation, 94 °C for 40 s; annealing, 55 °C for 40 s; synthesis, 72 °C for 40 s), followed by a final extension of 5 min at 72 °C. All PCR products were visualized by electrophoresis in 2% w/v agarose gel and purified using PCR purification kit (Qiagen). Purified products were then diluted 1:1 with denaturing solution (1% v/v xylene cyanol, 1% v/v bromophenol blue, 0.1 mM EDTA, and 99% v/v formamide), boiled for 5 min and immediately placed in ice to prevent the annealing of single-strand products. SSCP screening was carried out on the GenePhor Electrophoresis Unit using GeneGel Excel 12.5/24 (12.5% T, 2% C), according to the manufacturer's instructions (GE Healthcare, Milano, Italy). Electrophoresis (600 V, 25 mA, 15 W) was performed at 18 °C for 100 min. Gels were stained with PlusOne Silver Staining Kit (GE Healthcare), according to the manufacturer's instructions. Altered migration patterns in two or three independent PCR-SSCP runs were indicative of DNA mutations. Purified PCR products were then sequenced by an ALF II automated sequencer (GE Healthcare) using the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (GE Healthcare). DNA sequences were compared with those of the normal *BRAF* gene exon 15 using the Basic Alignment Search Tool software (BLAST) available at www.ncbi.nlm.nih.gov/BLAST/. As a control, two human thyroid cancer cell lines, ARO and TPC, heterozygous and negative for the *BRAF* mutation respectively, were used (24).

ATC cell culture

FNA samples were obtained from six patients with ATC by FNA cytology (23 gauge needle). FNA materials were spread out in a sterile environment (about 10 000 cells were seeded) in six-well plates and cultured in RPMI 1640 (Sigma–Aldrich) containing gentamicin (20 µg/ml; Sigma–Aldrich), penicillin G (100 IU/ml; Sigma–Aldrich), glutamine (1% w/v; Sigma–Aldrich), and FCS (20% v/v; Seromed, Biochrom). After 2 weeks, cells were trypsinized and propagated in 75 cm² flasks in DMEM (Sigma–Aldrich) containing penicillin/streptomycin (50 mcg/ml; Sigma–Aldrich), glutamine (1% w/v; Sigma–Aldrich), and FCS (20% v/v; Seromed, Biochrom), and then incubated at 37 °C in 5% CO₂. Cells were used for chemosensitivity tests at the fourth passage, after 4–5 weeks of controlled *in vitro* growth in order to obtain an adequate number of cells.

Neoplastic tissue was finely minced into 1 to 3 mm pieces with a scalpel or scissors. Fragments were washed three to five times in M-199 media (Sigma–Aldrich) supplemented with penicillin (500 IU/ml; Sigma–Aldrich), streptomycin (500 IU/ml; Sigma–Aldrich), and nystatin (1000 IU/ml; Sigma–Aldrich). Tumor tissues were suspended in DMEM containing penicillin/streptomycin (50 mcg/ml; Sigma–Aldrich), glutamine (1% w/v; Sigma–Aldrich), and FCS (20% v/v; Seromed, Biochrom), and incubated at 37 °C in 5% CO₂.

Primary cultures reached confluence. Thereafter, the cells were detached with a trypsin solution and transferred to primary tissue-culture flasks (Becton Dickinson Labware, Bedford, MA, USA). At passage 3, cells were coated in methocel (25) to evaluate the colony-forming efficiencies. The biggest colonies were picked up and expanded in tissue-culture flasks. Cells were used for chemosensitivity tests at the fourth passage.

Immunohistochemistry showed the absence of expression of TSH receptor, TPO, Tg, and NIS. The presence of cytokeratin was investigated by immunocytochemistry on destained smears in FNA-ANA cells and a partial and focal positivity was obtained.

Cell viability test

A viability and proliferation assay was used by analyzing the number of viable cells by the cleavage of tetrazolium salts added to the culture medium (Cell Proliferation Reagent WST-1; Roche) (26). Proliferation assays have become available to analyze the number of viable cells by the cleavage of tetrazolium salts added to the culture medium. The tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture (26). The formazan dye produced by metabolically

active cells was quantified by a scanning multiwell spectrophotometer (ELISA reader) by measuring the absorbance of the dye solution at 450 nm. Cells were seeded in a 96-well microtiter plate at a concentration of 35 000 cells/ml in a final volume of 100 µl per well, followed by a 48-h incubation period with chemotherapeutic agents or PPARγ activator, as mentioned above, in a humidified atmosphere (37 °C, 5% CO₂). After the incubation period, 10 µl of the Cell Proliferation Reagent WST-1 was added to 100 µl culture medium in each well and the absorbance of the samples was measured at 450 nm against the control (the same cells without any treatment) using a microtiter plate (ELISA) reader. The same volume of culture medium and Cell Proliferation Reagent WST-1 (10 µl Cell Proliferation Reagent WST-1/100 µl culture medium) was added to one well, in order to use this background control (absorbance of culture medium plus WST-1 in the absence of cells) as a blank position for the ELISA reader.

The absorbance was measured again after 1 and 2 h, from the start of tetrazolium reaction.

The measured absorbance of blank was subtracted from the control and treatments; the control was normalized to 100% for each assay and treatments were expressed as a percentage of the control.

The experiments were conducted in triplicate for each FNA. The mean percentage of inhibition of proliferation was calculated with respect to the control for each FNA. The results are the mean percentage with respect to the control in the six FNA.

Proliferation assay: cell counting

Since 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (used in the MTT assay) measures mitochondrial cell activity and it has already been demonstrated that there is not always a direct relationship with cell number, the proliferation was evaluated using cell number counting (27, 28), as well.

Cells were seeded at a density of 13 000 cells per well in 24-well tissue-culture plates in medium supplemented with 10% FBS v/v with or without the indicated factors (12). The medium was changed every other day. After 72 h in an atmosphere of 5% CO₂–95% air at 37 °C, the cells were detached from plates by incubation with 500 ml PBS containing 100 mg trypsin and 1 mmol/l EDTA. The cells were counted using a hemocytometer.

Treatment of human cells with chemotherapeutic agents

Human cells were treated in standard culture media as above for 48 h with different drugs: bleomycin (3.5 µM; Crinos IF, Villa Guardia, Como, Italy) (29), carboplatin (100 µM; Sigma–Aldrich) (30), cisplatin (10 µM; Sigma–Aldrich) (31), gemcitabine (50 µM; Lilly) (32),

and etoposide (10 μ M; Bristol-Myers Squibb spol. sro, Olivova, Praha, Czech Republic) (33), at concentrations equivalent to peak plasma levels achieved *in vivo* (29, 31–33).

Treatment of human cells with PPAR γ activator

Human cells were treated in standard culture media as above for 48 h with PPAR γ activator, BRL49653 (rosiglitazone; gift from SmithKline Beecham) (0, 0.1, 1, 5, 10, 20, 30, 40 μ M) (15).

Results

FNA-ANA and biopsy-ANA grew to reach the confluence and we were able to obtain a sufficient quantity of material to perform the chemosensitivity tests; the morphology of cells was quite similar in FNA-ANA and biopsy-ANA (Fig. 1).

FNA-ANA were sensitive to all chemotherapeutic agents (Fig. 2). The anti-proliferative effect of chemotherapeutic agents became apparent after a minimum of 24 h of incubation and increased with longer incubation times. Shorter exposure times (10 and 18 h) did not show any anti-proliferative effect on all tested cells;

Figure 1 Anaplastic thyroid cancer cells cultured from (A) fine-needle aspiration cytology or (B) biopsy (Papanicolaou staining; 630 $\times$).

Figure 2 The results of WST-1 assay (2 h from the start of tetrazolium reaction) (mean $\pm$ s.e.m. of all samples) in ANA cells from FNA showed a significant reduction ($P < 0.001$, by ANOVA) of proliferation with respect to the control with all the tested chemotherapeutic agents (after 48 h of incubation).

therefore, results are not reported. The anti-proliferative effects were calculated after 2 days (48 h) of incubation with chemotherapeutic agents. Gemcitabine and etoposide were significantly more effective in inhibiting the proliferation than carboplatin, cisplatin, and bleomycin ($P < 0.05$). The results of chemosensitivity tests in individual FNA-ANA are presented in Fig. 3. A similar trend for the sensitivity to each antineoplastic was observed in individual FNA-ANA (the inhibition was higher with etoposide and gemcitabine), even if differences were present among the preparations (with a higher sensitivity in preparations 3B and 3F). No significant differences were observed between the sensitivity to antineoplastics in FNA-ANA and biopsy-ANA from the same patient.

Cell counting confirmed the above-mentioned results in the whole group of FNA-ANA. In control FNA-ANA, the cell number was $39\,200 \pm 3200/100$ mcl, per well; after the treatments, the cell number was: $13\,700 \pm 4000$ (35%, with respect to the control) with bleomycin; $26\,300 \pm 2350$ (67%) with carboplatin; $17\,270 \pm 3800$ (44%) with cisplatin; 9000 ± 2450 (23%) with etoposide; and $12\,100 \pm 1855$ (31%) with gemcitabine ($P < 0.001$, with respect to control, for all the tested drugs; ANOVA) (Fig. 4). Similar results were observed in biopsy-ANA cells.

The results for ANA cells cultured from biopsy were quite similar to those obtained from FNA-ANA (Fig. 5). Also in this case, gemcitabine and etoposide were significantly more effective in inhibiting the proliferation than cisplatin, bleomycin, and carboplatin ($P < 0.05$). There were no significant differences in sensitivity to chemotherapeutic agents between the tested cells from FNA cytology or biopsy.

The results of WST-1 assay in FNA-ANA after 48 h of incubation with rosiglitazone showed a reduction of

Figure 3 (A–F) The results of WST-1 assay (2 h from the start of tetrazolium reaction) in ANA cells from each FNA showed a significant reduction ($P < 0.001$, by ANOVA) of proliferation with respect to the control with all the tested chemotherapeutic agents (after 48 h of incubation). A similar trend for the sensitivity to each antitumoral agent was observed in individual FNA-ANA (the inhibition was higher with etoposide and gemcitabine), even if differences were present among the preparations (with a higher sensitivity in preparations 3B and 3F). Preparations 3C and 3D were obtained from V_{600E} BRAF mutation positive cells.

proliferation with respect to the control (2 h (Fig. 6) (from the start of tetrazolium reaction)) of 29, 48, and 49% with rosiglitazone 20, 30, or 40 μ M ($P = 0.0001$, for all); rosiglitazone 0.1, 1, 5, and 10 μ M did not show a significant reduction of cell activity.

The results of WST-1 assay in ANA cultured from biopsy after 48 h of incubation with rosiglitazone were quite similar to those obtained from FNA-ANA (data not shown).

The V_{600E} BRAF mutation was observed in two ATC patients. The results regarding the inhibition of proliferation by chemotherapeutic agents obtained in

biopsy-ANA cells from tumors with V_{600E} BRAF mutation were similar to those from tumors without BRAF mutations (ANOVA).

Discussion

Here, we report the establishment of primary cell cultures from FNA in patients with ATC that could be used to test the chemosensitivity of ANA cells to different chemotherapeutic agents and TZD.

The role of surgery in ATC remains controversial.

Figure 4 The results of cell counting (2 h from the start of tetrazolium reaction) (mean $\pm$ S.E.M. of all samples) in ANA cells from FNA showed a significant reduction ($P < 0.001$, by ANOVA) of proliferation with respect to the control with all the tested chemotherapeutic agents (after 48 h of incubation).

The recently published consensus on the surgical treatment of ATC recommends complete surgical resection; that is however, justified only if postoperative morbidity rate remains low (34).

Even when surgical resection is possible, surgical treatment *per se* is not able to control the disease and much research is now ongoing to find new medical therapies in patients with ATC (13).

However, following an extensive review regarding the results of CT by itself or as part of a multimodal approach, until now, neither the results for doxorubicin (the most commonly used) nor for other drugs, such as cisplatin, bleomycin, etc., have been able to obtain a definitive judgement of any real effectiveness in controlling the disease (13).

Figure 5 The results of WST-1 assay (2 h from the start of tetrazolium reaction) (mean $\pm$ S.E.M. of all samples) in ANA cells from surgical biopsy showed a significant reduction ($P < 0.001$, by ANOVA) of proliferation with respect to the control with all the tested chemotherapeutic agents (after 48 h of incubation).

Figure 6 The results of WST-1 assay (2 h from the start of tetrazolium reaction) (mean $\pm$ S.E.M. of all samples) in ANA cells from FNA showed a reduction of proliferation with respect to the control of 29, 48 and 49% with rosiglitazone (after 48 h of incubation) 20, 30, or 40 μ M (respectively; $P < 0.0001$, for all).

The effect of chemotherapeutic agents has been reported for *in vitro* CT in ANA (9, 10), and recently the potential activity of paclitaxel, vinorelbine and gemcitabine in ANA has been shown. Disease orientated *in vitro* drug screening using human tumor cell lines has some predictive value for the activity of clinical responses (7), with a negative predictive value of 90% (9), and might help to prevent the administration of inactive chemotherapeutics to patients.

The possibility of testing the sensitivity of ANA from each subject to different chemotherapeutic agents or other drugs could increase the effectiveness of the treatment; in fact, *in vitro* chemosensitivity tests are able to predict *in vivo* effectiveness in 60% of cases (9). There are several reasons for the discrepancy between *in vitro* and *in vivo* results: 1) the drug may be metabolized and/or inactivated in the body by different organs (liver, kidney, etc.) or in the tumor; 2) the cells may become resistant to the drug; and 3) the growth curve of certain tumors is very important in determining a response to chemotherapeutics. However, it is well known that a negative chemosensitivity test *in vitro* is associated with 90% of ineffectiveness of the CT *in vivo* (9), allowing the administration of inactive chemotherapeutics to these patients to be avoided. Moreover, this new technique will permit the evaluation of the efficacy of other drugs, different from traditional antineoplastics (e.g., tyrosine kinases inhibitors), in patients with ATC in the future.

Until now, primary ANA cultures have been obtained from surgical biopsies performed for therapeutic or diagnostic procedures. Here, we report a technique to obtain cell cultures from FNA cytology in ATC. Another paper by Bravo *et al.* (35) reported the establishment of primary cultures by needle aspiration biopsy in only one patient. However, the reports of cutaneous needle track seeding after fine needle aspiration biopsy (FNB) in patients with thyroid cancer have been published (36, 37). FNA cytology overcomes this problem; in fact, in our

patients we did not find any sign of needle track seeding after FNA.

The cells were submitted to CT which revealed an *in vitro* sensitivity to the chemotherapeutic agents used at IE50 with a higher sensitivity for etoposide and gemcitabine. The material obtained by FNA is obviously the expression of a cell population present in a limited area of the tumor, and theoretically this could determine the selection of a cellular population not representative of the entire tumor. To exclude this possibility, CT was repeated under the same conditions with primary cell cultures obtained from bioptical material. The results were quite similar to those observed in FNA-ANA, excluding that FNA sampling might have brought a cell population selection.

The results of the MTT assay and of cell counting were quite similar for most of the CT used; a discrepancy was observed with carboplatin, in fact MTT assay was much more affected than cell counting. This discrepancy may be explained by the differences between the two methods, because it has already been demonstrated that there is not always a direct relationship with the cell number (27, 28).

Regarding the effectiveness of CT, a difference was observed among them. In fact, carboplatin reduced cell growth with respect to the control, but the cell number increased significantly with respect to the initial value (from 13 000 cells per well to about 26 000 cells per well), suggesting that the drug *per se* was not effective in inhibiting tumor growth, accordingly with clinical results. Regarding bleomycin, cisplatin, and gemcitabine, the number of cells (13 700, 17 250, and 12 200 cells/well respectively) after the treatments were similar to the initial value of seeded cells, suggesting a cell cycle arrest (38–41). It could be hypothesized that if the CT had been washed out the cells would have grown again. After the treatment with etoposide about 9000 cells/well were present, less than the number seeded, suggesting a partial apoptotic effect (42); the future studies will be needed to verify this point. This last result opens the possibility to study new combinations of drugs with etoposide in this *in vitro* cultures and looks in the future for effective treatment for ATC.

Furthermore, the results of our study have demonstrated the anti-proliferative effect of PPAR γ agonist rosiglitazone in FNA-ANA.

It was demonstrated that ATC cell lines express the PPAR γ gene and protein (21, 43). Moreover, it was found that TZD-inhibited cell growth and invasion of ATC cell lines in a dose-dependent manner. The inhibitory effect on proliferation or invasion was particularly significant in cell lines which showed higher level of the PPAR γ gene or protein expression (21, 43, 44). TZD mediate growth inhibition through induction of apoptosis. However, there is controversy regarding the induction of apoptosis by TZD in relation to proto-oncogene, *c-myc*, or cell-cycle inhibitor protein p27 (16, 43) in thyroid carcinoma. Other findings

suggested that PPAR γ agonists should induce apoptosis in ATC cell lines through the up regulation of Bax protein (21).

One of the established modes of action of the TZD is the activation of PPAR γ (45). However, Ohta *et al.* (16) demonstrated no correlation between effects mediated by PPAR γ and expression of PPAR γ in carcinoma cells. In breast cancer cells, it has also been shown that troglitazone can repress cyclin D1 independently from PPAR γ activation (46).

More extensive studies are needed to clarify the exact molecular mechanisms that specifically down regulate the proliferation and invasion by PPAR γ agonists.

The results of our study have demonstrated the inhibitory effect on cell activity of TZD in FNA-ANA cells. The results were quite similar to those observed in primary cell cultures obtained from bioptical material, excluding a cell population selection, according to what was observed with chemotherapeutic agents.

Recently, it has been shown that troglitazone treatment overcomes the resistance to doxorubicin in the doxorubicin-resistant K562 human leukemia cells (47). Furthermore, it has been shown that RS5444 (a new PPAR γ agonist) has an additive anti-proliferative activity with paclitaxel in ATC continuous cell line cultures (12). Our results showing a sensitivity of FNA-ANA cell cultures to TZD as well as to chemotherapeutic agents stimulate verifying if these drugs may have a synergic anti-proliferative effect in this type of cancer.

BRAF provides crucial signals for the proliferation of thyroid carcinoma cells spontaneously harboring the ^{V600E}BRAF mutation and, therefore, BRAF suppression might have therapeutic potential in ^{V600E}BRAF-positive thyroid cancer (48, 49).

Our results show that chemotherapeutic agents and TZD act both in ATC with or without ^{V600E}BRAF mutation, suggesting that their effect is mediated by other mechanisms. However, owing to the small number of patients (two with and four without BRAF mutation), further studies in larger series will be needed.

In conclusion, 1) a technique to obtain primary cell cultures from ATC from FNA-ANA cytology has been found, which allows unnecessary surgical procedures to be avoided; 2) primary cells obtained by FNA-ANA have a chemosensitivity to chemotherapeutic agents and TZD quite similar to that observed in primary cells from biopsy and 3) etoposide is highly effective in reducing cell growth, probably due to a partial apoptotic effect; this result opens the possibility to study new combinations of drugs with etoposide in these *in vitro* cultures and looks in the future for effective treatment for ATC. The possibility to test the chemosensitivity to different drugs in each patient is able to increase the efficacy of treatments, avoiding the administration of ineffective drugs.

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