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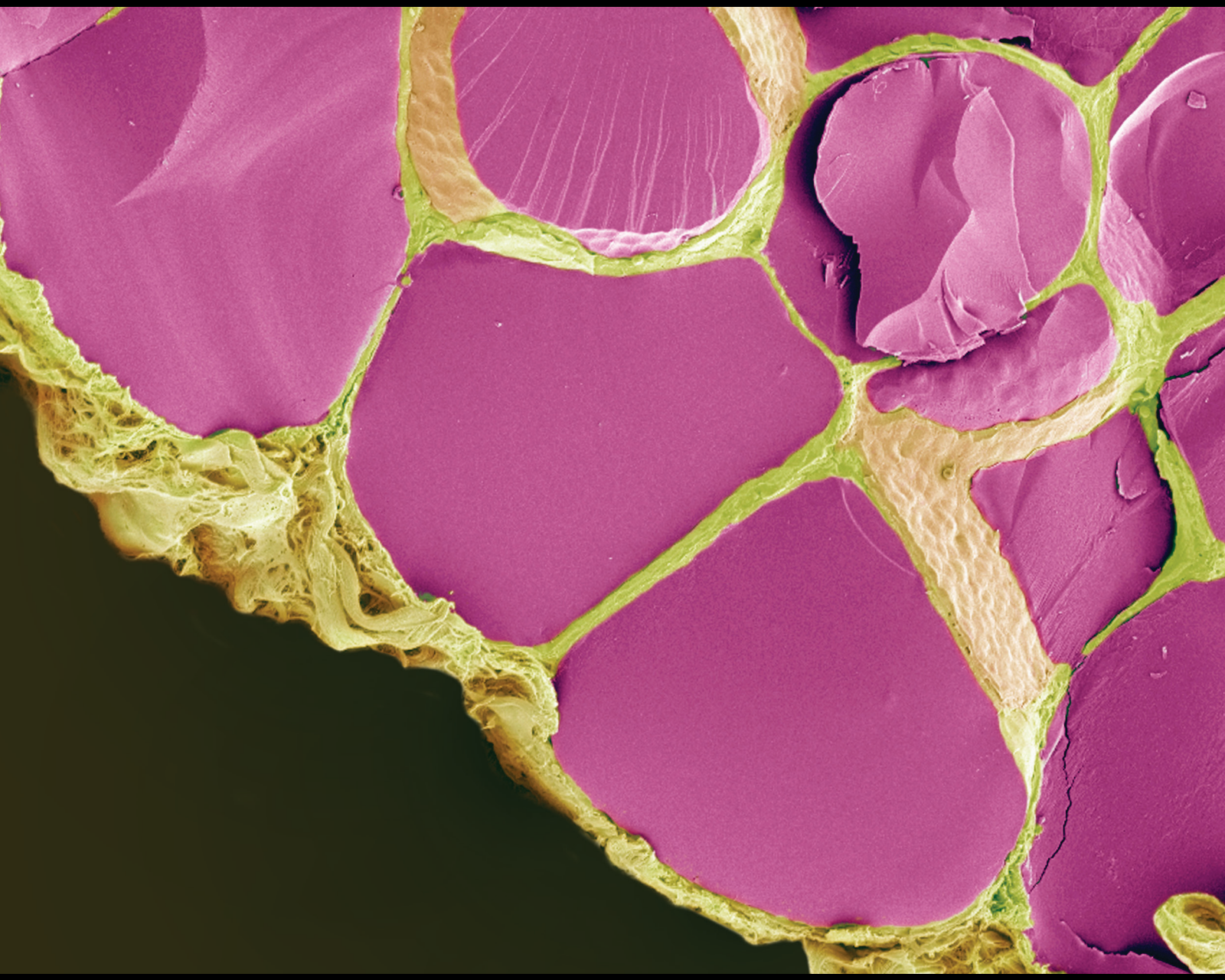
Anaplastic Thyroid Carcinoma: Molecular Tools for Diagnosis and Therapy

Guest Editors: Ginesa Garcia-Rostan, Giovanni Tallini, and Giuliana Salvatore



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Editorial Board

Contents

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Editorial

Anaplastic Thyroid Carcinoma: Molecular Tools for Diagnosis and Therapy

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Anaplastic thyroid carcinoma (ATC) is one of the most lethal human malignancies, with a median overall survival of less than six months. While most of the genetic mutations occurring in papillary thyroid carcinoma (PTC) were recently discovered through an integrated genomic approach, molecular genetics of ATC is still in part unknown. ATC is refractory to conventional therapies, including surgery, chemotherapy, radiotherapy, and radioiodine therapy. New target agents are currently being evaluated in clinical trials. Under this frame, there is an urgent need to investigate the molecular biology and the therapeutic opportunities of this understudied, rare cancer.

The aim of this special issue is to gather a collection of papers focusing on molecular tools for diagnosis and therapy of anaplastic thyroid carcinoma. The issue contains eight papers including four review articles and four research papers.

The review article of C. S. Fuziwara and E. T. Kimura analyzes the role of miRNA in anaplastic thyroid carcinoma. miRNAs are a class of small noncoding RNAs that regulate posttranscriptional gene expression. Increasing evidences show that they may drive oncogenesis. The article underscores the families of miRNA that are deregulated in ATC, namely, the miR-200 family, miR-30 family, let-7 family, miR 17–92 cluster, miR-146a/b, and miR 221/222. Modulation of miRNA levels, using miRNA mimics or anti-miRs, may open new therapeutic options for ATC.

In the review article of M. Ragazzi et al. the focus is on the histopathology of ATC. The authors discussed the typical molecular features of ATC cells, with an elegant description of the main histological subtypes and the differential diagnostic criteria. The review included also an update of the molecular genetics of ATC.

The other two review articles included in the special issue focus on new proteins overexpressed in ATC, which may represent novel therapeutic targets, that is, Aurora kinases and transferrin receptor.

In detail, the work of E. Baldini et al. focuses on Aurora kinases in ATC. The review is a comprehensive update of the structure, expression, localization, and functions of Aurora proteins and their role in human cancers. Further, the review summarizes the findings, mainly made by the authors group, of Aurora kinases in thyroid cancers. Importantly, preclinical data indicate that Aurora kinase inhibitors may have a therapeutic potential for ATC treatment.

The review of R. Parenti et al. focuses on type 1 transferrin receptor (TfR1/CD71) in ATC. The TfR1/CD71 is a cell membrane glycoprotein involved in iron homeostasis and cell growth. The authors showed immunohistochemical data demonstrating the overexpression of TfR1/CD71 in

ATC. They discuss the opportunities to target TfR1/CD71 by monoclonal or recombinant antibodies or transferrin-gallium-TfR1/CD71 molecular complexes or small interfering RNAs (siRNAs).

Four research articles are included in the special issue. Two of these articles are from the group of Sipetic. In the first research paper, V. Zivaljevic et al. analyze the risk factors for ATC. The study, done on 126 ATC patients, showed that independent risk factors for ATC are low education level, type B blood group, and goitre. A similar study, included in the special issue, from the same group of authors, addresses the importance of age in patient survival. The study demonstrates that the best prognosis for ATC is in patients younger than 50 years old.

In the research article by F. Baldan et al. the synergy between HDAC inhibitors and PARP inhibitors has been investigated in an ATC-derived cell line SW1736. The authors showed that both compounds synergize in activation of apoptosis and induction of thyroid-specific gene expression. Thus, this work suggests that the combined use of HDAC and PARP inhibitors may be a useful strategy for treatment of ATC.

Finally, W. Arancio and et al. conducted a competing endogenous RNA analysis to investigate the role of the stem cell factor SOX2 in ATC cell lines. The authors identified a functional network of SOX2 interactors including genes involved in the biogenesis of microRNAs (DICER1, RNASEN, and EIF2C2), in the control cell cycle (TP53 and CCND1), and in mitochondrial activity (COX8A).

In conclusion, within this special issue, we gather articles underlining several aspects of molecular biology and therapeutic options for ATC. We hope that this special issue could contribute to the advancement of the knowledge of this rare and aggressive cancer.

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Giovanni Tallini
Giuliana Salvatore

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Research Article

Synergy between HDAC and PARP Inhibitors on Proliferation of a Human Anaplastic Thyroid Cancer-Derived Cell Line

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Anaplastic thyroid carcinoma (ATC) is a very aggressive human malignancy, having a marked degree of invasiveness and no features of thyroid differentiation. It is known that either HDAC inhibitors or PARP inhibitors have antiproliferative effects on thyroid cancer cells. Therefore, in this study the possible synergy between the two types of compounds has been investigated. The ATC-derived cell line SW1736 has been treated with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the PARP inhibitor PJ34, alone or in combination. In terms of cell viability, the combination index value was always lower than 1 at various tested dosages, indicating, therefore, synergy in a wide range of doses for both compounds. Synergy was also observed in induction of apoptosis. In terms of thyroid-specific gene expression, synergy was observed for TSHR mRNA levels but not for NIS, TTF1, TTF2, and PAX8 mRNA levels. Altogether, these data suggest that the combined use of HDAC and PARP inhibitors may be a useful strategy for treatment of ATC.

1. Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence has continuously increased in the last three decades all over the world [1]. Thyroid cancers are typically classified as papillary (PTC), follicular (FTC), medullary (MTC), or anaplastic (ATC) carcinomas.

ATC is one of the most aggressive human malignancies. These tumors have a marked degree of invasiveness and extensive necrosis and there are no features of thyroid differentiation [2]. The mechanisms underlying the development of ATCs are incompletely understood. Currently, available therapy for ATCs includes chemotherapy, radiotherapy, and surgery [3]. Nonetheless, patients with ATC still have a median survival of 5 months and less than 20% survive 1 year. Furthermore early tumor dissemination results in 20–50% percent of patients having distant metastases and 90% having adjacent tissue invasion on presentation [2].

HDAC inhibitors (HDACIs) are a group of small molecules that promote gene transcription by chromatin remodeling and have been extensively studied as potential drugs for treating cancer. Luong et al. have established that the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), already FDA-approved for the treatment of several neoplastic diseases [4, 5], has antitumor activities against thyroid cancer [6].

Inhibitors of the poly(ADP-ribose) polymerases (PARPs) family are currently being evaluated as potential anticancer drugs. PARPs have a key role in a large number of cell viability processes as DNA repair, genome integrity, regulation of transcription, proliferation, and apoptosis [7].

Different independent studies have demonstrated that the combination of both HDAC inhibitors and PARP inhibitors with other drugs could result in synergistic effects on their antitumor activities if compared to those observed using single agents [8, 9].

Current cancer therapy should satisfy requirements for targeted elimination of cancer cells simultaneously with life-compatible adverse effects [10]. One of the main tenets of cancer therapeutics is that combinations of anticancer agents with different targets or different mechanisms of action and varied normal tissue toxicities will produce better therapeutic outcomes [11] by decreasing single drugs doses and minimizing or slowing drug resistance development. In this study, we investigated the possible use of SAHA, an HDAC inhibitor, and PJ34, a PARP inhibitor, in combination, in a cellular model of anaplastic thyroid cancer.

2. Material and Methods

2.1. Cell Line and Treatments. SW1736, human cell line derived from anaplastic thyroid cancer, was grown in RPMI 1640 medium (EuroClone, Milan, Italy) supplemented with 10% fetal bovine serum (Gibco Invitrogen, Milan, Italy) and 50 mg/mL gentamicin (Gibco Invitrogen, Milan, Italy) in a humidified incubator (5% CO_2 in air at 37°C). The identity of SW1736 cells was demonstrated by evaluating the following STRs: D16S539, THO1, vWA, D3S1358, D21S11, and D18S51; the obtained genotype was identical to those reported by the CLS Cell Lines Service GmbH (http://www.cell-lines-service.de/). Cultured cells were treated with the following agents, either alone or in combination, as described in the text: SAHA (1–4 μM in DMSO) (Cayman Chemical, Michigan, USA) and PJ34 (5–30 μM in nuclease-free water) (Merck Chemicals Ltd). These concentrations are consistent with those utilized for *in vivo* studies [12, 13]. All treatments were done for 72 hours.

2.2. Cell Viability. To test cell viability, CellTiter-Blue Cell Viability assay (Promega, Milano, Italy) was used according to the manufacturer's instructions. Cells were seeded onto 96-well plates in 200 μL medium. The next day, the growth medium was replaced with fresh medium containing DMSO as vehicle (untreated cultures) or SAHA and PJ34 alone or in combination. For each treatment quadruplicate wells were used.

2.3. Combination Index (CI Value). Effects of drugs combination used in this study were evaluated using the combination index equation based on the multiple drug-effect equation of Chou-Talalay [14, 15]. In all cases where CI value could be determined the following diagnostic rule was applied: CI < 1 indicates synergism, CI = 1 indicates additive effect, and CI > 1 indicates antagonism. The analysis was obtained on CompuSyn software (ComboSyn Inc., Paramus, USA).

2.4. Annexin V Staining. Cells were treated with appropriate drugs as described and then they were washed with cold PBS, transferred to a polystyrene round-bottomed flow tube (Falcon, Becton Dickinson, Franklin Lakes, NY, USA), and resuspended in 195 μL of 1× binding buffer (BB-10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). To the suspension, 5 μL of fluorescein-conjugated Annexin V (Annexin V-FITC; Bender Med Systems, Wien, Austria) was added and samples were incubated for 10 min at room temperature. After washing, cells were resuspended in 190 μL of BB in which 10 μL of propidium iodide stock solution (final concentration 1 μg/mL) was added. Flow cytometry analysis was done on CyAN, Dako Cytomation using the Summit software.

2.5. Quantitative RT-PCR. Total RNA from cell line, treated for 72 h with SAHA 1 μM and PJ34 15 μM alone or in combination, was extracted with RNeasy mini kit according to manufacturer's instructions (Qiagen, Hilden, Germany). 500 ng of total RNA was reversely transcripted to cDNA using random exaprimers and MMLV reverse transcriptase (Invitrogen). Real-time PCRs were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) with the ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). The ΔΔCT method, by means of the SDS software (Applied Biosystems), was used to calc ulate the mRNA levels. Oligonucleotide primers were purch ased from Life Technologies and were as follows: β-actin 5′ primer CGAGCGCGGCTACAGCTT, β-actin probe ACCACCACGGCCGAGCGG, and β-actin 3′ primer TCCTTAATGTCACGCACGATTT; PAX8 5′ primer CAACAGCACCCTGGACGAC, PAX8 3′ primer AGGGTGAGTGAGGATCTGCC, and PAX8 probe CTGACCCCTTCCAACACGCCACTG; NIS Hs00166567_m1; TTF1 Hs00968940_m1; TTF2 Hs00916085_s1; and TSHR Hs01053846_m1.

2.6. Statistical Analysis. Cell viability, apoptosis, and mRNA levels were expressed as means ± SD, and significances were analyzed with the *t* test performed with GraphPAD Software for Science (San Diego, CA, USA).

3. Results

In a first set of experiments, single effects of the HDAC inhibitor SAHA and the PARP inhibitor PJ34 on cell viability of the human anaplastic thyroid cancer-derived cell line SW1736 were investigated. Cell viability was assessed after treatment with different doses of SAHA and PJ34 for 72 hours (Figure 1). Both SAHA and PJ34 alone inhibited cell proliferation in a dose-dependent manner; however, at the utilized doses, SAHA seemed to have a greater effect, causing a more significant decrease in cell viability compared to cells treated by PJ34. Thus, both compounds alone were able to inhibit proliferation of SW1736 cells. We then tested synergy of the two compounds by measuring CI values of different drug combinations according to the Chou-Talalay equation [14, 15]. As indicated in Table 1, all combinations used showed a very high decrease in cell growth compared to untreated cells (always the CI values were lower than 1). Our results indicated that SAHA and PJ34 have a synergic effect in decreasing cell proliferation in a quite high range of utilized doses.

We focused on the SAHA 1 μM and PJ34 15 μM combination which generated the lowest CI value. As represented in Figure 2, the treatment with SAHA 1 μM had only a light effect on SW1736 viability, while PJ34 15 μM reduced cell proliferation more effectively. Using these doses in combination

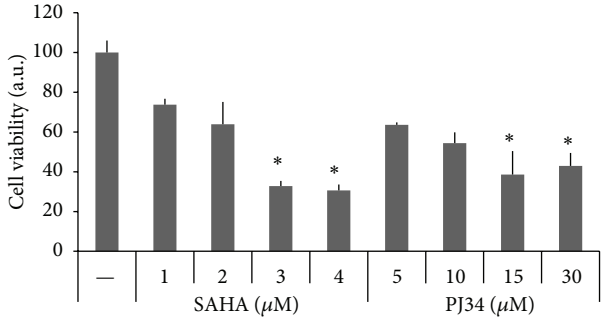

Figure 1: Effects of HDAC and PARP inhibitors on SW1736 cell viability. Cells were treated for 72 h with SAHA (1 μM–4 μM) or PJ34 (5 μM–30 μM), and CellTiter-Blue Cell Viability assay was performed as described in Section 2. Bars indicate the percentage of viable cells versus controls (untreated cells) and represent means ± SD of three experiments. ∗ indicates values significantly different compared to control.

Table 1: Combination index data for SAHA and PJ34 combination.

Dose SAHA (μM)	Dose PJ34 (μM)	Combination effect (% cell viability)*	CI value
1.0	5.0	26	0.26979
1.0	10.0	37	0.60235
1.0	15.0	12	0.13878
1.0	30.0	13	0.18473
2.0	5.0	18	0.33123
2.0	10.0	17	0.33414
2.0	15.0	19	0.40111
2.0	30.0	11	0.25341
3.0	5.0	13	0.36504
3.0	10.0	26	0.75320
3.0	15.0	16	0.47175
3.0	30.0	14	0.45413
4.0	5.0	17	0.60763
4.0	10.0	17	0.62785
4.0	15.0	10	0.40318
4.0	30.0	13	0.53865

*Mean value of four replicates. In each condition standard deviation is less than 10%.

we obtained a strong lowering of cell viability, almost 90% compared to untreated cells.

Subsequently, we evaluated apoptosis of SW1736 by measurement of Annexin V by fluorescence-activated cell sorting after treatments with SAHA 1 μM and PJ34 15 μM, alone or in combination (Figure 3(a)). The percentage of apoptotic cells (Annexin V-positive/PI-negative) was 1.98% with SAHA 1 μM and 1.99% with PJ34 15 μM while after the combination treatment, it reached 8.16% (Figure 3(b)).

We next tested if synergy between the two compounds was present on expression of several thyroid-specific genes. Effects on mRNA levels were evaluated by real-time PCR.

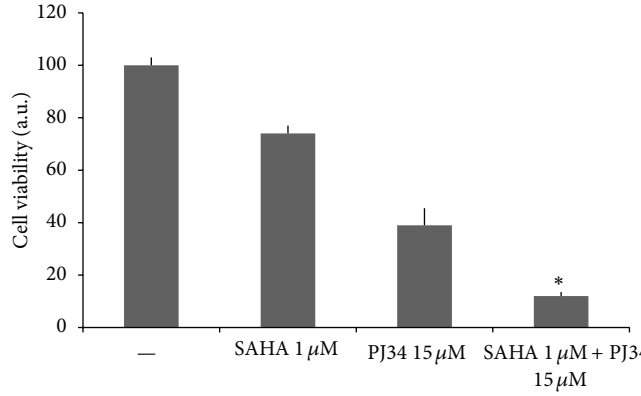

Figure 2: Effect of HDAC and PARP inhibitors combination on SW1736 cell viability. Cells were treated for 72 h with SAHA 1 μM and PJ34 15 μM, alone or in combination. CellTiter-Blue Cell Viability assay was performed as described in Section 2. Bars indicate the percentage of viable cells versus controls (untreated cells) and represent means ± SD of four experiments. ∗ indicates values significantly different compared to all other conditions.

Among all genes analyzed synergy between the two compounds was detectable only for TSHR gene (Figure 4). The 72-hour treatment with SAHA 1 μM induced a marked effect on TSHR mRNA expression, while PJ34 15 μM did not have any remarkable effect compared to the control cells. However, by using the two drugs in combination we obtained a strong effect on TSHR mRNA levels, significantly higher than all other conditions, with an increment of 36-fold of induction compared to the control.

4. Discussion

Developing a pharmacological treatment against cancer, the central issue consists in increasing therapeutic index and, at the same time, limiting development of resistance. One solution is to combine multiple drugs that act synergistically, and, in fact, a large number of ongoing clinical trials are investigating the effects of combined therapy against different types of cancer [16, 17]. In developing new strategies for treatment of anaplastic thyroid cancer, combinations of HDAC inhibitors and other drugs have been attempted [18–24]. However, neither in preclinical nor in clinical settings of thyroid cancer treatment, the combination between HDAC and PARP inhibitors has been investigated. It is increasingly clear that cancer is not only caused by genetic factors but can also be considered a epigenetics disease [25] and epigenetic enzymes can, therefore, be considered as novel therapeutic targets. Accordingly, both HDAC and PARP inhibitors can be considered as epigenetic drugs. Combinations of HDAC and PARP inhibitors have been tested in different kinds of neoplastic diseases. By using hepatocellular carcinoma cell lines, a synergistic inhibition of cell growth by SAHA and the PARP inhibitor olaparib has been demonstrated [26]. Inhibition of cell proliferation was associated with increase of apoptosis levels, accumulation of DNA damage, and modification of the cAMP signaling pathway. Combined

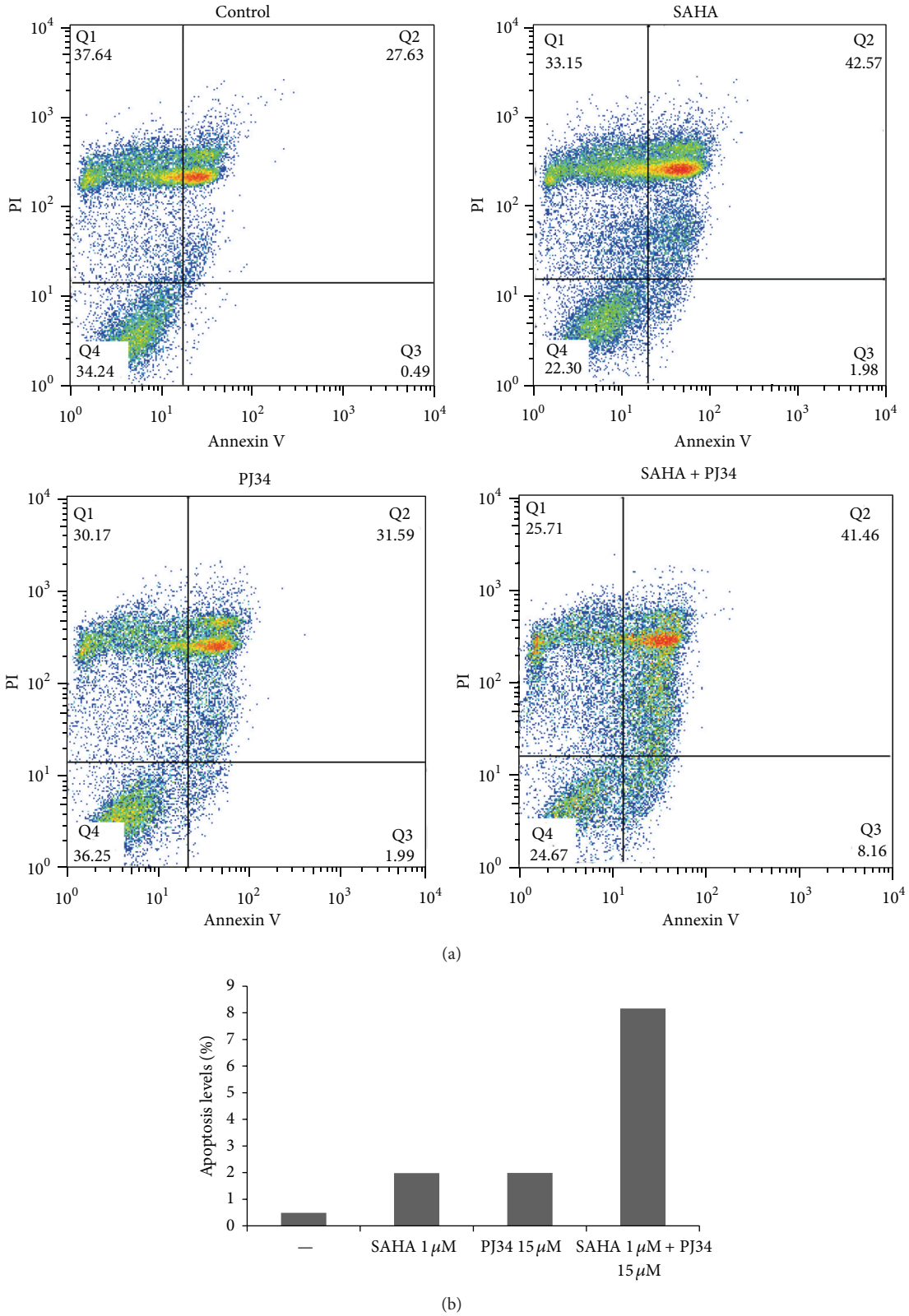

Figure 3: Combination of SAHA and PJ34 induces apoptosis in SW1736 cell line. In (a) Annexin V (FITC) and propidium iodide (PI) staining of anaplastic thyroid cancer cell line after 72 h treatment with SAHA 1 μM, PJ34 15 μM alone or in combination. In (b) representation of Annexin V-positive/PI-negative cells.

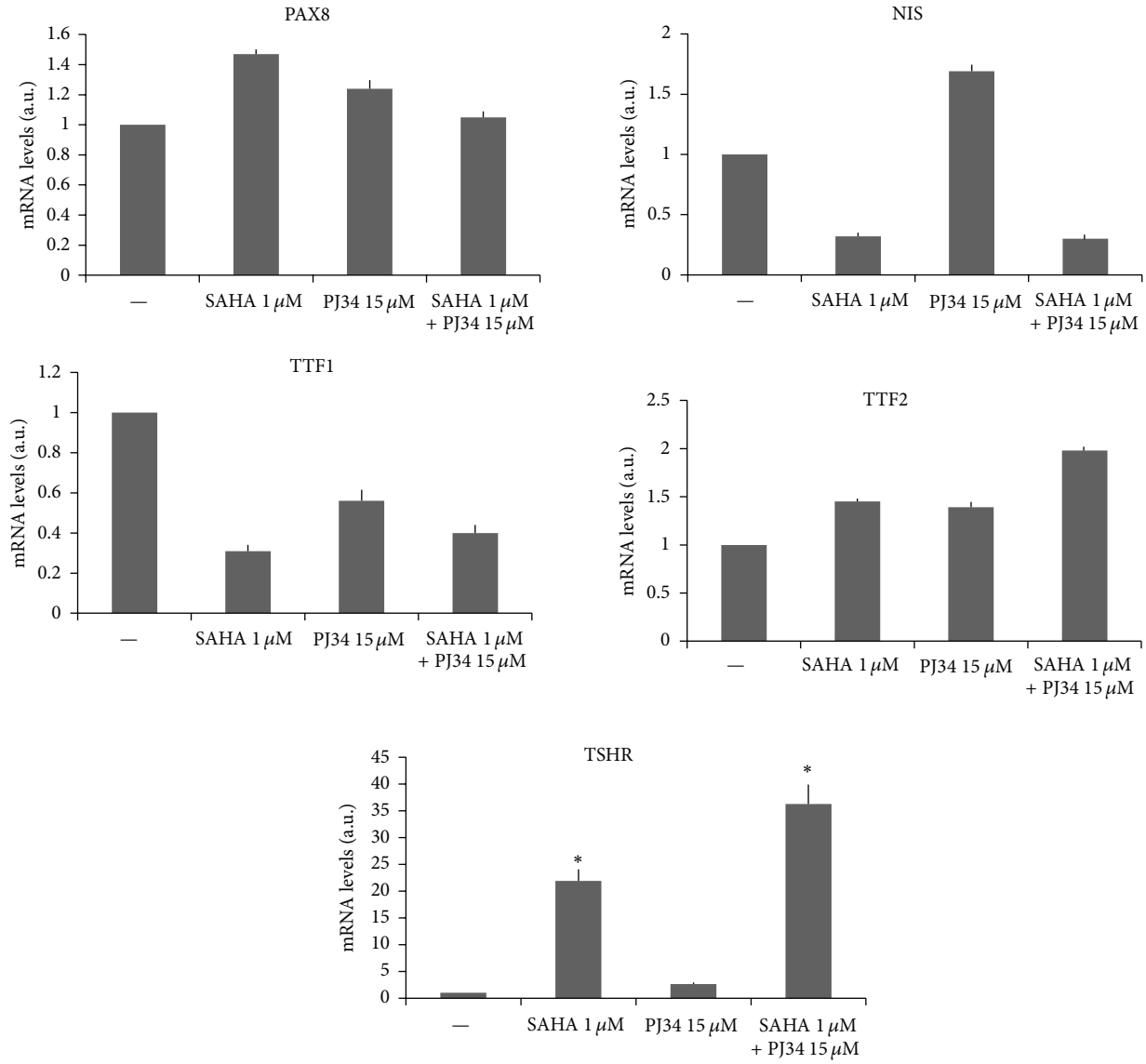

Figure 4: Expression levels of PAX8, NIS, TTF1, TTF2, and TSHR genes in SW1736 cell line. RNA extraction and real-time PCR are described in Section 2. For each gene the results were normalized against β-actin and expressed in arbitrary unit ($2^{-\Delta Ct}$). Each bar represents the mean value of three different determinations. For each bar standard deviation is not above 10% of each value. $*$ indicates values significantly different compared to all other conditions.

effects of SAHA and PJ34 on leukemia cell lines have been investigated [27]. Also in that study, synergistic effects on proliferation inhibition and apoptosis increase have been observed. Recently, synergy between SAHA and olaparib has been observed even in ovarian cancer cell lines [28]. We have recently obtained similar effects on breast cancer cell lines (unpublished data).

Effects of HDAC and PARP inhibitors alone have been previously investigated by our group. We have shown that HDAC inhibitors affect cell proliferation and expression of various genes in several thyroid cancer cell lines [29, 30]. Moreover Lavarone et al. have recently shown that PJ34 inhibits cell growth and increases NIS expression in various thyroid cancer cell lines [31]. In this research we demonstrate that HDAC and PARP inhibitors have a synergistic effect on proliferation of a human anaplastic thyroid-derived cell line. Thus, synergy between these two classes of compounds appears to be a common phenomenon in cancer cell lines of various origins, underlying the potential role of these combinations as an interesting strategy for cancer therapy. Our obtained CI values indicate that synergy between SAHA and PJ34 occurs in a wide range of doses, suggesting that the combined effect could probably be observed also *in vivo*.

In addition to the impact on cell proliferation, we have investigated effects of the SAHA-PJ34 combination on thyroid-specific genes expression. Regarding thyroid-specific transcription factors, the SAHA-PJ34 combination induces a TTF1 slight decrease, a TTF2 slight increase, and no change in PAX8. Such behavior indicates that the control of these genes expression occurs through distinct mechanisms. This

view agrees with previous studies in which control of thyroid-specific transcription factors expression has been investigated [32].

Synergy between SAHA and PJ34 in increasing mRNA levels of TSHR was observed. The TSHR is localized in the plasma membrane, and, thus, it has been proposed as a target to direct therapeutic compounds into thyroid cancer cells [33–36]. Our data would suggest that the combined use of HDAC and PARP inhibitors may facilitate such approach.

Differently from TSHR, NIS gene expression is reduced by SAHA alone and in combination with PJ34. Such a different effect between these genes expression is not unexpected. TSHR and NIS have a different regulation in terms of gene expression. It is well known, for example, that during tumorigenesis NIS is one of the earliest downregulated genes, while TSHR is among the latest ones [37, 38].

In conclusion, considering our data on cell proliferation and gene expression altogether, the combined use of HDAC and PARP inhibitors can be a useful strategy for ATC treatment. Preclinical *in vivo* studies are required to validate such a possibility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Anaplastic Thyroid Carcinoma: A ceRNA Analysis Pointed to a Crosstalk between *SOX2*, *TP53*, and microRNA Biogenesis

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It has been suggested that cancer stem cells (CSC) may play a central role in oncogenesis, especially in undifferentiated tumours. Anaplastic thyroid carcinoma (ATC) has characteristics suggestive of a tumour enriched in CSC. Previous studies suggested that the stem cell factor *SOX2* has a preeminent hierarchical role in determining the characteristics of stem cells in SW1736 ATC cell line. In detail, silencing SOX2 in SW1736 is able to suppress the expression of the stem markers analysed, strongly sensitizing the line to treatment with chemotherapeutic agents. Therefore, in order to further investigate the role of SOX2 in ATC, a competing endogenous RNA (ceRNA) analysis was conducted in order to isolate new functional partners of SOX2. Among the interactors, of particular interest are genes involved in the biogenesis of miRNAs (*DICER1, RNASEN,* and *EIF2C2*), in the control cell cycle (*TP53, CCND1*), and in mitochondrial activity (*COX8A*). The data suggest that stemness, microRNA biogenesis and functions, p53 regulatory network, cyclin D1, and cell cycle control, together with mitochondrial activity, might be coregulated.

1. Introduction

Anaplastic thyroid carcinoma (ATC) is a rare endocrine tumour. Its morphological features resemble undifferentiated neoplasm. Due to severe metastasis development and to the rapid fatal course, surgery is rarely performed. Radiotherapy and chemotherapy are also not very effective. It has been suggested that those standard therapies are ineffective because they are not able to efficiently target a subpopulation of ATC cells, called the cancer-initiating cells or cancer stem cells (CSCs). It has been proposed that CSCs possess stem-cell-like features, are at the core of the development of many tumours, especially undifferentiated ones like ATC, are responsible for the recurrence of the tumour and metastasis formation, and usually are very resistant to classical therapies.

Despite many controversies regarding the cancer stem cell model, it has the potential to drive the discovery of innovative treatments that may eradicate the very chemoresistant core of cancer [1]. In this connection, the CSC model is the sum of many hypotheses that have arisen to explain the most vexing aspects of cancer: metastasis, relapse, and therapeutic resistance [2]. In this perspective, CSC research holds out promise for improved treatment outcomes, in particular, as regards overcoming resistance to chemotherapy on solid tumours [1].

The most accepted CSC model makes use of a new paradigm of cellular differentiation, in which cancer cells can dedifferentiate toward more primitive, stem-like phenotypes [2]. The dedifferentiation seems to be highly heterogeneous, giving an explanation to the observed discontinuous behaviour of many cancers [2]. Alternatively, CSCs might arise from transformed stem cells in the stem niche [1, 2].

Similar to normal stem cells, CSCs have the ability both to self-renew and to give rise to differentiated tumour cells, are responsible for the organization of a tumour mass [3], and are tumorigenic when transplanted into an animal host [4]. CSCs

Table 1: microRNAs that have been reported in the literature to regulate the main transcript from the SOX2 locus.

hsa-let-7a	hsa-miR-125b-2*	hsa-miR-1914*	hsa-miR-30a*
hsa-let-7a*	hsa-miR-126	hsa-miR-1915	hsa-miR-30b
hsa-let-7b	hsa-miR-126*	hsa-miR-1915*	hsa-miR-30b*
hsa-let-7b*	hsa-miR-134	hsa-miR-200c	hsa-miR-30c
hsa-let-7c	hsa-miR-137	hsa-miR-200c*	hsa-miR-30c-1*
hsa-let-7c*	hsa-miR-142-3p	hsa-miR-203	hsa-miR-30c-2*
hsa-let-7d	hsa-miR-143	hsa-miR-204	hsa-miR-30d
hsa-let-7d*	hsa-miR-143*	hsa-miR-205	hsa-miR-30d*
hsa-let-7e	hsa-miR-145	hsa-miR-206	hsa-miR-30e
hsa-let-7e*	hsa-miR-145*	hsa-miR-21	hsa-miR-30e*
hsa-let-7f	hsa-miR-155	hsa-miR-21*	hsa-miR-452
hsa-let-7f-1*	hsa-miR-155*	hsa-miR-223	hsa-miR-452*
hsa-let-7f-2*	hsa-miR-17	hsa-miR-223*	hsa-miR-9
hsa-let-7g	hsa-miR-17*	hsa-miR-296-3p	hsa-miR-9*
hsa-let-7g*	hsa-miR-183	hsa-miR-296-5p	hsa-miR-92a
hsa-let-7i	hsa-miR-183*	hsa-miR-302a	hsa-miR-93
hsa-let-7i*	hsa-miR-1908	hsa-miR-302a*	hsa-miR-93*
hsa-miR-100	hsa-miR-1909	hsa-miR-302b	
hsa-miR-100*	hsa-miR-1909*	hsa-miR-302b*	
hsa-miR-106b	hsa-miR-1910	hsa-miR-302c	
hsa-miR-106b*	hsa-miR-1911	hsa-miR-302c*	
hsa-miR-125a-3p	hsa-miR-1911*	hsa-miR-302d	
hsa-miR-125a-5p	hsa-miR-1912	hsa-miR-302d*	
hsa-miR-125b	hsa-miR-1913	hsa-miR-302f	
hsa-miR-125b-1*	hsa-miR-1914	hsa-miR-30a	

have been identified in a wide range of human tumours [3]. At the molecular level, CSCs are usually enriched in cell surface markers such as CD44, CD24, and CD133, while Wnt/β-catenin, Notch, and Hedgehog signalling pathways seem to have key roles in CSC properties [4]. Specific microRNA signatures have been identified in many CSCs [4] that seem to play a role in the epithelial-mesenchymal transition [4].

Regarding ATC, it has been hypothesized that the tumour initiates from transformed thyroid stem cells, rather than from differentiated thyrocytes undergoing a conventional multistep carcinogenesis model [5–7].

The rarity and rapid fatal nature of ATC has led to limited *ex vivo* studies. Here we describe an *in vitro* study on a well-validated ATC cell line: SW1736. The SW1736 cell line is characterized by a high percentage of population with stem cell-like properties and high expression of several stem markers (SOX2, OCT4, NANOG, C-MYC, SSEA4, and the ABCG2 transporter) [8]. Interestingly, *SOX2* silencing downregulates *in trans* the expression of other stem cell markers and sensitizes ATC cells to treatment with classical chemotherapeutics such as cisplatin and doxorubicin [8]. This suggests that the stem cell factor *SOX2* could have a pre-eminent hierarchical role in determining the characteristics of stem cells in SW1736 ATC cell line.

Therefore, in order to further investigate the role of *SOX2* in ATC, a bioinformatic analysis of the functional network of *SOX2* was performed. In detail, a competing endogenous RNA (ceRNA) analysis was conducted. This kind of analysis is able to predict genes functionally correlated with the *bait* gene rather than physically associated with it [9, 10]. The ceRNA hypothesis is based on the rationale that RNA molecules can regulate one another via microRNAs [9, 10]. ceRNAs are RNAs that share miRNA recognition elements, thereby regulating each other by influencing the available level of miRNA [9, 10]. In the past, ceRNA analysis made it possible to isolate several genes and functional networks related to cancer development, ageing, and homeostasis [11–19].

2. Materials and Methods

2.1. MirWALK Analysis. miRWalk is a comprehensive database that provides information on miRNA from humans, mice, and rats on their predicted as well as validated binding sites on their target genes. The validated targets module [20] hosts experimentally verified miRNA interaction with associated genes.

Using the miRWalk [20] data and embedded tools, we collected the microRNAs that have been reported in the literature to regulate the main transcript from the SOX2 locus (Table 1).

This set of miRNAs was inserted into the miRWalk analysis tool [20] to collect any human mRNA that has been reported to be regulated by them. Then the genes collected were organized in a hierarchical order for the number of validated microRNA hits (Table 2). The more microRNAs are

Table 2: ceRNA organized in hierarchical order for the number of validated microRNA hits.

Gene	Hits	Gene	Hits
DICER1	35	SLC27A4	10
TP53	26	RUNX1	10
RNASEN	22	RRBP1	10
EIF2C2	22	PAK3	10
COX8A	22	NFKB1	10
CCND1	22	LIN28	10
MYC	20	KLF4	10
CDKN1A	20	FRAP1	10
BCL2	20	EIF2C1	10
AKT1	19	CREB1	10
PTEN	18	CDK6	10
CDKN2A	18	APC	10
VEGFA	16	TWIST1	9
EGFR	16	SYNE1	9
TGFB1	14	SIRT1	9
KRAS	14	PRDM1	9
JUN	14	MCL1	9
HMGA2	14	HMOX1	9
ERBB2	14	DNMT1	9
ZEB1	13	DDX20	9
TLR4	13	CKAP4	9
SSSCA1	13	CDKN1C	9
MET	13	BRCA1	9
TNF	12	ZNF828	8
SOCS1	12	TP63	8
PIK3CA	12	TIMM8A	8
ESR1	12	STMN1	8
DGCR8	12	SCPEP1	8
CEBPB	12	ROS1	8
CD4	12	PSAT1	8
TGFBR2	11	PDCD4	8
STAT3	11	MYCN	8
PROM1	11	MAPK3	8
NPC1	11	JAK2	8
IL6	11	IL1B	8
EPHB2	11	IFNG	8
E2F3	11	IFNA1	8
E2F1	11	HMGA1	8
CDKN1B	11	GEMIN4	8
		CTNNB1	8
		CD19	8
		BCL2L11	8
		BAX	8

shared between the bait *SOX2* gene and the candidate genes, the higher the possibility that the candidate gene transcripts can act as *SOX2* ceRNAs. All analyses were updated to December 15, 2013.

2.2. GeneMANIA Analysis. Arbitrarily, the top 6 genes together with *SOX2* were analysed using the GeneMANIA [21] tool that helps to predict the functions of a set of genes and to predict in which gene ontology (GO) functions the set of genes might be involved (Figure 1) (Table 3). The GO functions reported are the ones with a false discovery rate (FDR) < 0.1. All analyses were updated to December 15, 2013.

2.3. Cell Cultures. The SW1736, 8505C, C643, FRO, BCPAP, TPC-1, and WRO cell lines were cultured in Dulbecco's modified Eagle's medium high glucose medium supplemented with 10% fetal bovine serum and 5% glutamine. Cultures were maintained in 5% carbon dioxide at 37°C in a humidified incubator.

2.4. Small Interfering RNA (siRNA) Transfection. siRNA transfection in SW1736 cells was performed using INTERFERin transfection agent (Polyplus-Transfection, Illkirch, France), according to the manufacturer's instructions. Briefly, the transfection agent and the siRNA complex were added to the cells and incubated for 72 hours for RNA extraction and analysis. The final concentration of *SOX2* siRNA was 100 nM. Each assay was performed in triplicate in at least three independent experiments. *SOX2* was silenced using Stealth SiRNA SOX2 HSS144045 (Invitrogen, Milan, Italy). siCONTROL Stealth siRNA Negative Control was used as a control (Invitrogen, Milan).

2.5. SOX2 Coding Sequence Vector and Transient Transfection. The vector used was taken from Addgen (http://www.addgene.org/) (Plasmid 26817): pcDNA3.3_SOX2; and transformation into SW1736 cells was performed using Xfect transfection agent (Clontech Laboratories, Inc. A Takara Bio Company) according to the manufacturer's instructions. The transfection agent and plasmid were added to the cells and incubated for 72 hours for RNA extraction and analysis.

2.6. SOX2 3′ Untranslated Region (3′UTR) Vector and Transfection. The vector was synthesized in service by Eurofins genomics (https://www.eurofinsgenomics.eu) using a pcDNA 3.1 backbone and a chemically synthesized 3′UTR (as reported in http://mybioinfo.info/exon_display.php?tax_id=9606&gene_id=GeneID: 6657) (Table 4).

2.7. Reverse-Transcription PCR and Real-Time Quantitative PCR. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Milan, Italy), including a digestion step with DNase I. RNA quantity and quality were assessed using the Nanodrop 2000 (Thermo Scientific, Wilmington, USA). The RNA extracted was reverse-transcribed with Random Hexamers (Applied Biosystems, Darmstadt, Germany) and Improm II Reverse Transcriptase (Promega Italia, Milan, Italy), according to the manufacturer's protocol. Primer pair sequences are reported in Table 5.

The reactions were performed as follows: 5′ at 94°C, 30 cycles (30″ at 94°C, 30″ at 55°C, 30″ at 72°C), 5′ at 72°C, and stocked at 4°C. The only exception was the amplification of the SOX2 3′UTR, for which the following was done: 5′ at 94°C, 30 cycles (30″ at 94°C, 30″ at 55°C, 90″ at 72°C), 5′ at 72°C, and stocked at 4°C. Expression was analyzed by real-time quantitative PCR (qRT-PCR) using Quantitect SYBR

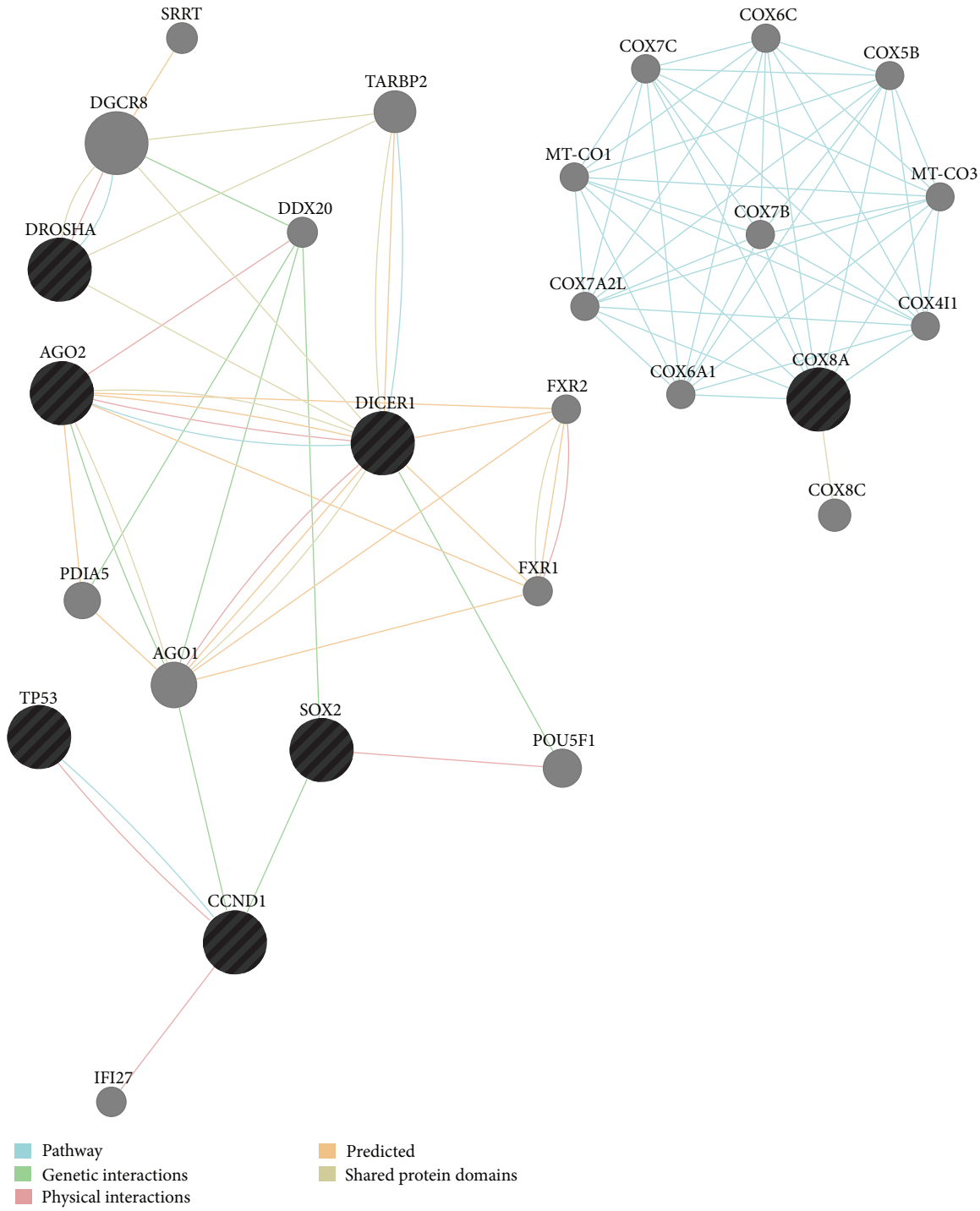

Figure 1: SOX2 ceRNA network by GeneMANIA.

Green PCR kit (Qiagen, Milan, Italy). All reactions were performed using a Rotor-gene Q Instrument (Qiagen, Milan, Italy). The data were analysed using the REST software [22].

2.8. Pool of Normal Thyroid Tissue. A pool of RNA from normal thyroid tissue specimens was used, as described in [23].

2.9. Limbal Stem Cell. A pool of RNA from limbal stem cells specimens was used, as described in [24].

2.10. Lymphocytes. Peripheral blood samples of a healthy volunteer were collected in tubes containing ethylenediaminetetraacetic acid (EDTA, 1 mg/mL) after 8 hours' fasting. Lymphocytes were isolated by lympholyte (CEDARLANE,

Table 3: Gene ontology of SOX2 ceRNA network by GeneMANIA.

Function	False discovery rate	Coverage
Query genes	n/a	7/7
Gene silencing by RNA	$3.14e-15$	9/33
Gene silencing	$5.01e-13$	9/59
Gene silencing by miRNA	$1.3e-11$	7/25
Posttranscriptional gene silencing by RNA	$1.92e-11$	7/28
Posttranscriptional gene silencing	$1.92e-11$	7/28
Regulation of gene expression, epigenetic	$5.32e-9$	8/110
Production of miRNAs involved in gene silencing by miRNA	$1.24e-8$	5/13
dsRNA fragmentation	$1.49e-8$	5/14
Production of small RNA involved in gene silencing by RNA	$1.49e-8$	5/14
Cellular response to dsRNA	$7.77e-8$	5/19
Response to dsRNA	$1.16e-7$	5/21
Respiratory electron transport chain	$1.16e-7$	7/102
Electron transport chain	$1.16e-7$	7/103
Cellular respiration	$7.68e-7$	7/136
Mitochondrial membrane	$3.2e-6$	8/274
ncRNA metabolic process	$5.44e-6$	7/185
Mitochondrial envelope	$5.44e-6$	8/297
Mitochondrial inner membrane	$7.4e-6$	7/195
Organelle inner membrane	$1.1e-5$	7/208
Posttranscriptional regulation of gene expression	$4.71e-5$	7/259
Energy derivation by oxidation of organic compounds	$7.46e-5$	7/279
Cellular response to organic cyclic compound	$2.23e-4$	5/101
ncRNA processing	$2.59e-4$	5/105
Endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5′-phosphomonoesters	$6.97e-4$	3/14
Endoribonuclease activity	$1.25e-3$	3/17
Response to organic cyclic compound	$3.56e-3$	5/183
Stem cell maintenance	$1.09e-2$	3/35
Stem cell development	$1.25e-2$	3/37
Ribonuclease activity	$1.97e-2$	3/44
Endonuclease activity	$1.97e-2$	3/44
Stem cell differentiation	$4.17e-2$	3/57
Somatic stem cell maintenance	$5.63e-2$	2/11
Germplasm	$5.63e-2$	2/11
P granule	$5.63e-2$	2/11
Pole plasm	$5.63e-2$	2/11
Ribonucleoprotein granule	$6.92e-2$	3/71
Endodermal cell fate commitment	$7.54e-2$	2/13

Burlington, Ontario, Canada), according to the manufacturer's instructions.

2.11. Statistical Analysis. We used the SPSS 13 software, Windows edition, for all our statistical analyses. Correlations were determined using Spearman's test (nonparametric equivalent for Pearson's test). $P < 0.05$ was considered statistically significant.

3. Results

Previous data [8] suggested that the stem cell factor *SOX2* possesses a preeminent hierarchical role in determining stemness characteristics in the SW1736 ATC cell line. With the final aim of investigating the role of *SOX2* in ATC, a bioinformatic ceRNA analysis [9, 10] of the functional network of *SOX2* was performed.

Using the miRWalk [20] data and embedded tools, we collected the microRNAs that have been reported in the literature to regulate the main transcript from the *SOX2* locus (Table 1).

This set of miRNAs was inserted into the miRWalk analysis tool [20] to collect any human mRNA that has been reported to be regulated by them. Then the collected genes were organized in a hierarchical order for the number of validated microRNA hits (Table 2). The more microRNAs are shared between SOX2 and the candidate genes, the stronger the putative competitive effect that is at the core of the ceRNA hypothesis. The first six top level interactors were arbitrary selected for further analyses. The top level *SOX2* interactors in this ceRNA analysis are *DICER1*, *EIF2C2,* and *RNASEN*, involved in miRNA biogenesis and functions [25]; the most studied antioncogene *TP53*, worthy of note because of its suggested role in stemness [26]; the nuclear-coded mitochondrial Cytochrome C Oxidase Subunit VIIIA *COX8A* [27]; and *CCND1*, the cyclin D coding gene [28].

Amongst the lesser interactors reported in Table 2, other genes might be worth studying in the future, especially for their involvement in oncogenesis (such as *MYC*, *BCL2*, *PTEN*, *KRAS*, *JUN*, and many others).

This study aimed to analyse whether a relationship might exist between the 6 top level interactors (*DICER1*, *EIF2C2*, *RNASEN*, *TP53*, *COX8A,* and *CCND1*) and *SOX2* in the ATC cell line SW1736.

With this purpose in mind, the six interactors together with *SOX2* were analysed by GeneMANIA software [21] to verify whether their putative network (Figure 1) might be enriched in some GO annotations. Unsurprisingly, the analysis revealed a statistically significant enrichment of miRNA-mediated, posttranscriptional gene silencing activities (Table 3).

Then we tried to establish in the SW1736 ATC cell line whether perturbations in the transcriptional state of *SOX2* might alter *in trans* the transcriptional state of the ceRNAs identified. When we knocked down *SOX2* transcripts via specific siRNA, all the ceRNAs were coherently downregulated *in trans* in RT-PCR analyses, as expected. The effect of the downregulation varied from one ceRNA to another but was always statistically significant [22] (Figure 2(a)) (Table 6). To further investigate whether the effect could be mediated by the impaired transcriptional factor activity of the protein coded by *SOX2*, we evaluated whether the overexpression of

Table 4: *SOX2* 3′ untranslated region (3′UTR).

5′GGGCCGGACAGCGAACTGGAGGGGGGAGAAATTTTCAAAGAAAAACGAGGGAAATGGGAGGGGTGCAAAA
GAGGAGAGTAAGAAACAGCATGGAGAAAACCCGGTACGCTCAAAAAGAAAAAGGAAAAAAAAAAATCCCATC
ACCCACAGCAAATGACAGCTGCAAAAGAGAACACCAATCCCATCCACACTCACGCAAAAACCGCGATGCCGAC
AAGAAAACTTTTATGAGAGAGATCCTGGACTTCTTTTTGGGGGACTATTTTTGTACAGAGAAAACCTGGGGA
GGGTGGGGAGGGCGGGGGAATGGACCTTGTATAGATCTGGAGGAAAGAAAGCTACGAAAAACTTTTTAAAAG
TTCTAGTGGTACGGTAGGAGCTTTGCAGGAAGTTTGCAAAAGTCTTTACCAATAATATTTAGAGCTAGTCTCC
AAGCGACGAAAAAAATGTTTTAATATTTGCAAGCAACTTTTGTACAGTATTTATCGAGATAAACATGGCAAT
CAAAATGTCCATTGTTTATAAGCTGAGAATTTGCCAATATTTTTCAAGGAGAGGCTTCTTGCTGAATTTTGA
TTCTGCAGCTGAAATTTAGGACAGTTGCAAACGTGAAAAGAAGAAAATTATTCAAATTTGGACATTTTAATT
GTTTAAAAATTGTACAAAAGGAAAAAATTAGAATAAGTACTGGCGAACCATCTCTGTGGTCTTGTTTAAAAA
GGGCAAAAGTTTTAGACTGTACTAAATTTTATAACTTACTGTTAAAAGCAAAAATGGCCATGCAGGTTGACA
CCGTTGGTAATTTATAATAGCTTTTGTTCGATCCCAACTTTCCATTTTGTTCAGATAAAAAAAACCATGAAAT
TACTGTGTTTGAAATATTTTCTTATGGTTTGTAATATTTCTGTAAATTTATTGTGATATTTTAAGGTTTTCCC
CCCTTTATTTTCCGTAGTTGTATTTTAAAAGATTCGGCTCTGTATTATTTGAATCAGTCTGCCGAGAATCCAT
GTATATATTTGAACTAATATCATCCTTATAACAGGTACATTTTCAACTTAAGTTTTTACTCCATTATGCACAG
TTTGAGATAAATAAATTTTTGAAATATGGACACTGAAA3′

Table 5: RT-PCR primer pairs.

Gene	Forward primer 5′ > 3′	Reverse primer 5′ > 3′
SOX2 CDS	GGAGACGGAGCTGAAGCCGC	GACGCGGTCCGGGCTGTTTT
DICER1	CTTTGCAACCCCTCAGCAT	TCATGAATTGCTTCTTGTTGC
TP53	ATCTACTGGGACGGAACAGC	GTGAGGCTCCCCTTTCTTG
RNASEN	CACCGAGATCACAGTCATGG	TGTCTTCTCCTGTCGGGACT
EIF2C2	TCCACCTAGACCCGACTTTG	AACTCTCCTCGGGCACTTCT
COX8A	TTACCTCCTGCTTCGTGACC	CACTCTGGCCTCCTGTAGGT
CCND1	ATGCCAACCTCCTCAACG	GGACCTCCTTCTGCACACAT
SOX2 3′UTR	CACCGGGCCGGACAGCGAACTGGAGGGGGG	TTTCAGTGTCCATATTTCAAAAATTTATTTATC
β-ACTIN	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG

the coding sequence of *SOX2* could have some *in trans* effects on ceRNAs. The coding sequence lacks the 3′ untranslated region (3′UTR) that mainly bears the regulation mediated by miRNAs [29]. Our data indicate that no such effect occurs (Figure 2(b)) (Table 7), so the trans effect highlighted in (Figure 2(a)) is likely to be due to the endogenous miRNA competition, as in our hypothesis, rather than a classical interaction mediated by the proteic transcriptional factor *SOX2*. Finally, we investigated whether the overexpression of *SOX2* 3′UTR might have any effects *in trans* in the SW1736 ATC cell line. The effects were very modest, if present at all, but in line with the modulation that occurs during the competing events [9, 10]. The most notable effect was the positive correlation, as expected, with the expression of *EIF2C2* and *SOX2* itself (Figure 2(c)) (Table 8).

The experiments previously described looked into the effects of perturbation of the expression of SOX2 on the expression of ceRNA genes in an ATC cell line. We then endeavoured to see whether any correlation might exist between the basal expression of *SOX2* and the ceRNA genes in different specimens. In detail, we analysed by RT-PCR the relative expression of *SOX2* and *SOX2* ceRNAs compared to *β-ACTIN* expression in SW1736 ATC cell line, in 8505C ATC cell line, C643 ATC cell line, FRO ATC cell line, BCPAP papillary thyroid carcinoma (PTC) cell line, TPC-1 PTC cell line, WRO follicular thyroid carcinoma, and a pool of normal thyroid tissues present in the laboratory from previous experiments [23], a pool of limbal stem cells [24], and isolated lymphocytes from a male donor of 36 years old (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/439370). Interestingly, this analysis suggested a correlation in the basal expression of *DICER1*, *RNASEN,* and *EIF2C2* (Table 9), as can be expected of genes whose functions are strictly coregulated in the biogenesis and function of microRNA, but surprisingly their basal expression seemed also to be somehow related to the basal expression of *TP53* (Table 9), suggesting interesting scenarios that will be discussed shortly.

4. Discussion

The ceRNA bioinformatics analysis pointed to a list of genes that could be functionally coregulated with the stem transcriptional factor *SOX2* by a crosstalk mediated by several miRNAs. In our analysis, we used interactions reported in the literature instead of bioinformatically predicted ones as done in the past [17–19]. This approach makes it possible to harvest

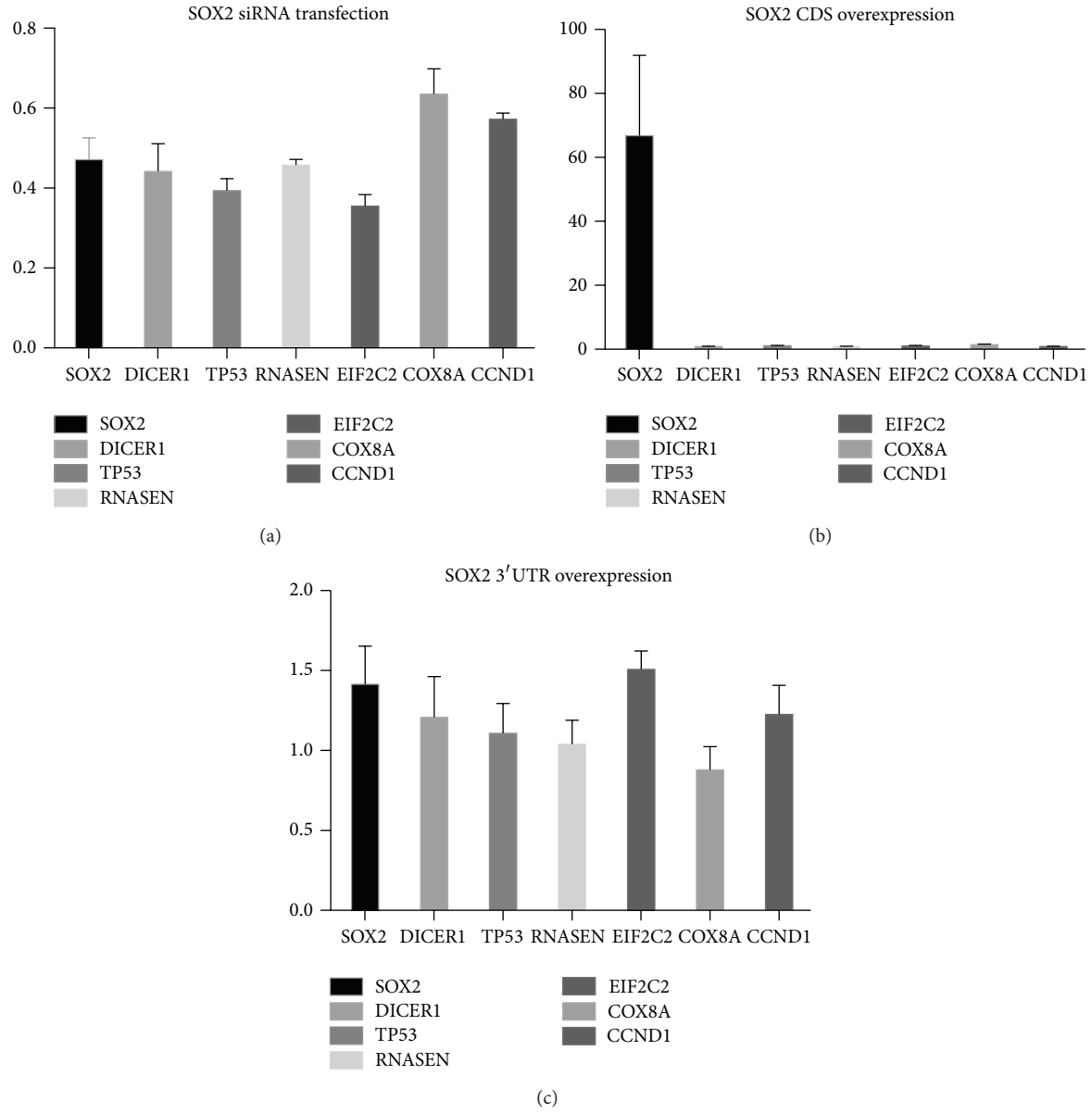

Figure 2: Example of SOX2 and SOX2 ceRNAs levels of transcription quantified by RT-PCR compared to controls and normalized against β-actin expression in SW1736 ATC cell line. Whiskers represent the standard errors. (a) Analysis of SOX2 silencing. (b) Analysis of SOX2 coding sequence overexpression. (c) Analysis of SOX2 3′UTR overexpression.

Table 6: Example of REST analysis on SOX2 and SOX2 ceRNAs levels of transcription quantified by RT-PCR compared to controls and normalized against β-actin expression in SW1736 ATC cell line after SOX2 silencing. P(H1) is the probability of the alternative hypothesis that the difference between sample and control groups is due only to chance.

Gene	Reaction efficiency	Expression	Std. error	95% C.I.	P(H1)	Result
SOX2	0.6375	**0.471**	0.416–0.538	0.384–0.580	0.000	DOWN
DICER1	0.6125	**0.442**	0.373–0.523	0.366–0.533	0.000	DOWN
TP53	0.7025	**0.394**	0.364–0.427	0.348–0.445	0.170	DOWN
RNASEN	0.73	**0.458**	0.444–0.472	0.444–0.472	0.000	DOWN
EIF2C2	0.7475	**0.355**	0.326–0.386	0.320–0.394	0.000	DOWN
COX8A	0.69	**0.635**	0.572–0.705	0.560–0.720	0.000	DOWN
CCND1	0.6875	**0.572**	0.556–0.588	0.556–0.588	0.000	DOWN

Table 7: Example of REST analysis on SOX2 and SOX2 ceRNAs levels of transcription quantified by RT-PCR compared to controls and normalized against β-actin expression in SW1736 ATC cell line after SOX2 coding sequence (CDS) overexpression. P(H1) is the probability of the alternative hypothesis that the difference between sample and control groups is due only to chance.

Gene	Reaction efficiency	Expression	Std. error	95% C.I.	P(H1)	Result
SOX2	0.63	**66.783**	41.627–98.592	37.461–120.196	0.000	UP
DICER1	0.67	**0.847**	0.750–0.981	0.677–1.034	0.131	
TP53	0.8175	**1.099**	1.000–1.198	0.953–1.261	0.136	
RNASEN	0.7475	**0.840**	0.707–1.001	0.672–1.059	0.131	
EIF2C2	0.7725	**1.015**	0.829–1.246	0.788–1.320	0.854	
COX8A	0.765	**1.430**	1.197–1.699	1.136–1.788	0.000	UP
CCND1	0.755	**0.861**	0.763–0.947	0.721–1.056	0.080	

Table 8: Example of REST analysis on SOX2 and SOX2 ceRNAs levels of transcription quantified by RT-PCR compared to controls and normalized against β-actin expression in SW1736 ATC cell line after SOX2 3′UTR overexpression. P(H1) is the probability of the alternative hypothesis that the difference between sample and control groups is due only to chance.

Gene	Reaction efficiency	Expression	Std. error	95% C.I.	P(H1)	Result
SOX2	0.655	**1.414**	1.176–1.710	1.102–1.818	0.000	UP
DICER1	0.7025	**1.207**	0.952–1.536	0.912–1.600	0.680	
TP53	0.77	**1.105**	0.918–1.332	0.881–1.386	0.661	
RNASEN	0.75	**1.039**	0.889–1.222	0.833–1.299	0.830	
EIF2C2	0.765	**1.508**	1.393–1.641	1.314–1.733	0.169	UP
COX8A	0.785	**0.879**	0.733–1.068	0.669–1.160	0.680	
CCND1	0.7425	**1.226**	1.045–1.462	0.937–1.611	0.341	
SOX2 3′UTR	0.64	**3.092**	2.577–3.762	2.330–4.119	0.000	UP

Table 9: Spearman two-tailed test correlations between basal gene expressions (as reported in Supplementary Table 1) among SW1736, 8505C, C643, FRO, BCPAP, TPC-1, WRO, normal thyroid pool, limbal stem cells, and lymphocytes.

	SOX2	DICER1	TP53	RNASEN	EIF2C2	COX8A	CCND1
SOX2							
Rho	1	0.152	0.2	−0.176	0.042	0.321	−0.467
P		0.676	0.580	0.627	0.907	0.365	0.174
DICER1							
Rho	0.152	1	0.939	0.709	0.952	0.030	−0.067
P	0.676	—	<0.001	0.022	<0.001	0.934	0.855
TP53							
Rho	0.200	0.939	1	0.770	0.939	−0.115	−0.042
P	0.580	<0.001	—	0.009	<0.001	0.751	0.907
RNASEN							
Rho	−0.176	0.709	0.770	1	0.842	−0.382	0.261
P	0.627	0.022	0.009	—	0.002	0.276	0.467
EIF2C2							
Rho	0.042	0.952	0.939	0.842	1	−0.127	0.067
P	0.907	<0.001	<0.001	0.002	—	0.726	0.855
COX8A							
Rho	0.321	0.030	−0.115	−0.382	−0.127	1	−0.127
P	0.365	0.934	0.751	0.276	0.726	—	0.726
CCND1							
Rho	−0.467	−0.067	−0.042	0.261	0.067	−0.127	1
P	0.174	0.855	0.907	0.467	0.855	0.726	—

more solid and reliable data, though it is easier to collect genes that have been previously analysed.

Our experiments were pursued in an ATC cell line that has previously been demonstrated to constitutively express *SOX2* that functionally possesses a preeminent hierarchical role on many other stem cell factors [8], suggesting a leading role in the maintenance of the stemness feature in this cell line. ATC represents a very good candidate for a cancer highly enriched in CSCs, which probably are at the core of its unfavourable outcome [1–7]. For these reasons, it is both important to understand the regulatory network that underlies the functions of *SOX2* in ATC, and at the same time an ATC cell line is a very good candidate for studying the *SOX2* network.

Looking at the cross-regulation between *SOX2* and the most probable ceRNAs that we isolated, many if not all the ceRNAs analysed seem to be responsive to alterations in the transcriptional state of *SOX2* transcripts, independently of the coded protein, suggesting a regulatory network strictly based on noncoding-RNAs (ncRNAs). The most striking evidence is the effect of siRNA-mediated silencing on *SOX2*, where all the ceRNAs are accordingly downregulated (Figure 1(a)) (Table 6). By contrast, the overexpression of the *SOX2* CDS alone seems to have almost no effect at all (Figure 1(b)) (Table 7). In contrast, the overexpression of the 3′UTR of the *SOX2* transcripts seems to have an upregulation effect *in trans*, even if not to a great degree (Figure 1(c)) (Table 8). The 3′UTR of transcripts is the portion of messengers that is likely to bear the vast majority of regulation mediated by microRNAs [30]. The data reported here are consistent with our hypothesis, so it is reasonable to point to the ceRNAs isolated as potential functional interactors with *SOX2*, at least in the SW1736 ATC cell line.

The interactors isolated pointed to a central role of microRNA biogenesis and functions in *SOX2* activities (Table 3) and hence in stemness, as other authors have recently suggested [29]. Here we report that probably the transcription of *SOX2* stem factors and of Dicer (*DICER1*), Ago2 (*EIF2C2*), and Drosha (*RNASEN*) is coregulated by a microRNA network. In detail, Drosha is a RNA-specific endoribonuclease that is involved in the initial nuclear step of microRNA biogenesis. Dicer is a cytoplasmic endoribonuclease that plays a central role in the production of short interfering RNAs (siRNA) and mature microRNAs. SiRNAs and microRNAs serve as a guide to directing the RNA-induced silencing complex (RISC) to complementary RNAs to degrade them or prevent their translation. Ago2 is the essential proteic core of the RISC complex. Overall, the miRNA pathway is a means to specifically regulate the expression of target genes that seem to directly and indirectly affect tumorigenesis [31].

The *SOX2* ceRNA *TP53* gene codes for p53, one of the most studied genes in relation to cancer development [32]. It is also often mutated in ATC towards a nonfunctional form [33]. Nevertheless, even a mutated form, if transcribed, can still exert its regulatory functions via its transcript (e.g., its 3′UTR). In this perspective, homozygous deletion of the locus or full silencing of the gene perturbs the network differently from a null mutation, which still permits transcription from the locus. In the authors' opinion, this distinction is often not taken into account. It is interesting to note that some authors have suggested a role of p53 in homoeostasis of the stem niche [26] and in microRNA biogenesis [34], setting it at a crossroads between cancer, stemness, and microRNA biogenesis and functions. Our data are in support of this interpretation, all the more so because the basal transcription of *TP53* seems to be correlated with the basal transcription of *DICER1*, *EIF2C2,* and *RNASEN* in the specimens that we analysed, many of them from ATC and other thyroid cell lines (Table 9).

The *SOX2* ceRNA *CCND1* codes for cyclin D1, the regulatory subunit that promotes G1/S cell-cycle progression and is involved in oncogenesis. It has been reported that cyclin D1 induces Dicer expression *in vitro* and *in vivo* and vice versa and their expression significantly correlates each other (at least in some subtypes of human breast cancer). It has been suggested that cyclin D1 induction of Dicer coordinates microRNA biogenesis [28, 35]. Our data are in line with the previous results and add a new level of possible crosstalk between *DICER1* and *CCND1*, suggesting novel actors in the network previously isolated, such as *SOX2* or *TP53*. It is likely that cross-regulation between cyclin D1 and Dicer might occur in other cancers, especially in ATC, which are enriched in *SOX2* producing cells [8], which we suggest is part of the network.

The role of *COX8A* is more difficult to appraise. The protein encoded by this gene is the terminal enzyme of the respiratory chain that leads to the production of the electrochemical gradient across the inner mitochondrial membrane. Recent discoveries suggest central roles of mitochondria in the maintenance of pluripotency, differentiation, reprogramming, and ageing [36]. Our data suggest possible crosstalk between a crucial nuclear coded mitochondrial factor and cell fate determinants such as *SOX2* and *TP53*.

5. Conclusions

The SW1736 ATC cell line was used to investigate functional *SOX2* interactors isolated by a novel bioinformatics analysis. Because *SOX2* seems to have a central role in the maintenance of stem features in the SW1736 ATC cell line, the interactors are likely to play a role in stemness regulation.

The analysis pointed to *DICER1, EIF2C2, RNASEN, TP53, COX8A,* and *CCND1* genes, suggesting that stemness, microRNA biogenesis and functions, p53 regulatory network, cyclin D1, and cell cycle control, together with mitochondrial activity, might be coregulated as a whole in their functions. Our data and previous data from the literature indicate that those functions are strictly interlinked and that deregulation of them might lead to cancer transformation, especially in cancers such as ATC that possess an undifferentiated nature suggestive of cancer stem cell enrichment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Update on Anaplastic Thyroid Carcinoma: Morphological, Molecular, and Genetic Features of the Most Aggressive Thyroid Cancer

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Anaplastic thyroid carcinoma (ATC) is the most aggressive form of thyroid cancer. It shows a wide spectrum of morphological presentations and the diagnosis could be challenging due to its high degree of dedifferentiation. Molecular and genetic features of ATC are widely heterogeneous as well and many efforts have been made to find a common profile in order to clarify its cancerogenetic process. A comprehensive review of the current literature is here performed, focusing on histopathological and genetic features.

1. Introduction

Anaplastic thyroid carcinoma (ATC) represents the most aggressive extreme of the clinical spectrum of thyroid epithelial neoplasms, being one of the most lethal human tumors.

It constitutes less than 5% of clinically recognized thyroid malignancies but it accounts for more than half of the deaths for thyroid cancer, with a mortality rate that is over 90% and a mean survival of six months after the diagnosis.

It is defined by the WHO as a highly malignant tumor wholly or partially composed of undifferentiated cells that retain features indicative of an epithelial origin, on immunohistochemical or ultrastructural ground [1]. It usually affects elderly people, with a mean age in the mid-60s, and shows a female predominance [1].

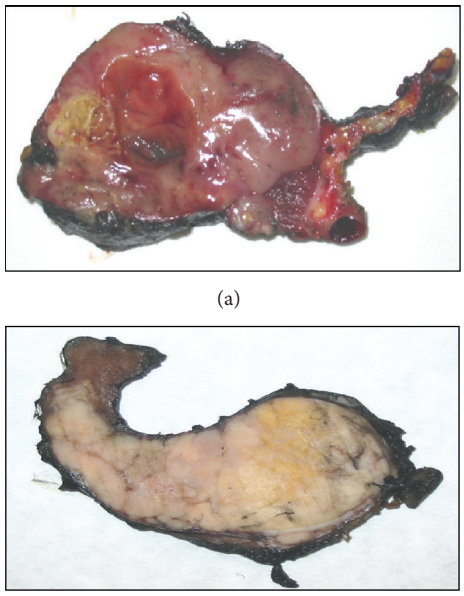
In this review we tried to summarize the current knowledge on ATC from both morphological and biological points of view.

2. Morphological Features

Grossly, ATC is well recognized as a large, necrotic, and hemorrhagic mass that is typically widely invasive, often replacing most of the thyroid gland parenchyma with infiltration of the surrounding soft tissue and adjacent structures of the neck (Figures 1(a) and 1(b)).

The morphological spectrum depends on the admixture of three main histological patterns: spindle cell, giant cell, and squamoid [2–4]. These patterns often coexist and are not predictive of patients' outcome but are historically used to group ATC in major histological categories and to define their main differential diagnoses. The histological categories are sarcomatoid and epithelioid-squamoid.

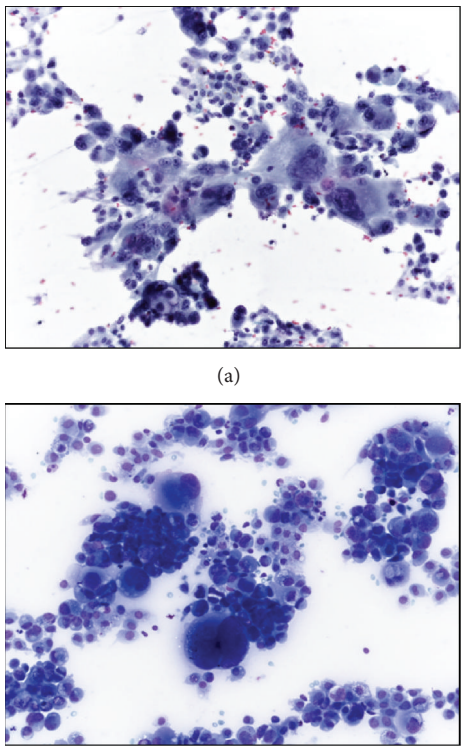
The small cell category, that was included in older classification of ATC, is no longer considered, as it comprised cases of bona fide lymphomas, medullary carcinomas, and insular carcinomas [2, 3, 5].



(a)

(b)

Figure 1: Grossly, ATC shows a diffusely infiltrative pattern of growth. The cut surface can be brownish (a) or whitish (b); in both specimens discrete yellowish areas of necrosis are evident.



(a)

(b)

Figure 2: FNAB smears are usually made up of polymorphic neoplastic cells in a dirty necrotic background ((a) Papanicolaou stain, (b) May-Grunwald Giemsa stain).

Common features to all patterns of ATC are hypercellularity, large foci of necrosis, marked invasiveness, and angiotropism with a tendency to infiltrate medium-sized veins and arteries, replacing their muscular wall [2, 3].

For diagnostic purposes, fine needle aspiration biopsy (FNAB) is an important tool and can provide a correct diagnosis of ATC in up to 84% of cases [6].

FNAB smears are usually composed of a pleomorphic cellular population in a necrotic background (Figures 2(a) and 2(b)). The tumor cells are bizarre, oval to spindle-shaped, dyscohesive elements showing anisocytosis, and irregular sometimes multiple nuclei, perfectly reflecting the sarcomatoid or epithelioid histological morphology.

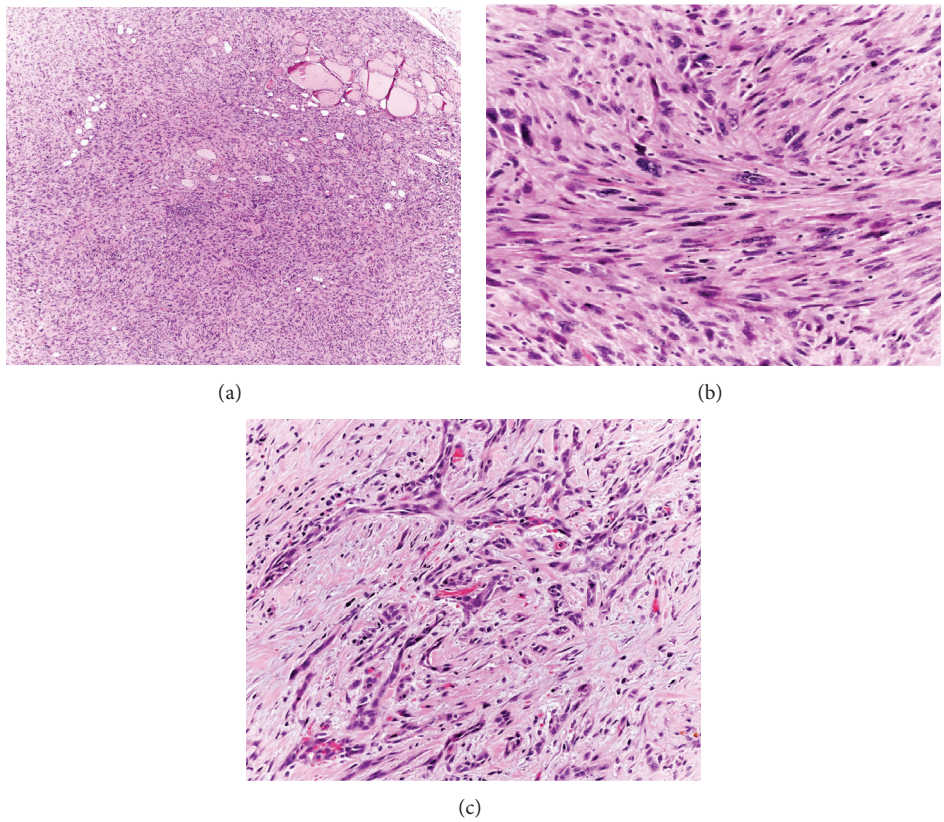
2.1. Sarcomatoid Category

2.1.1. Histology. Anaplastic thyroid carcinomas with sarcomatoid appearance are characterized by spindle cells and giant cells, the most frequent patterns seen in ATC. In fact, spindle and giant cells have been found, alone or in combination, in at least 50% of cases reported by Carcangiu and colleagues [2].

Spindle cells show a fascicular or storiform pattern of growth, indistinguishable from a true sarcoma (Figures 3(a) and 3(b)). These neoplasms are generally well vascularized often resulting in a hemangiopericytoma-like pattern or forming anastomosing channels lined by tumor cells, resembling an angiosarcoma (Figure 3(c)). An odd variation on the theme of the spindle cell form is the paucicellular variant [7, 8]. This infrequent entity was first described by Wan et al. in 1995 as a peculiar subtype of ATC with gross and histological features closely mimicking Riedel's thyroiditis [7]. It is characterized by low cellularity with striking degree of fibrosis and hyalinization, presence of spindle cells resembling fibroblasts or myofibroblasts, absence of obvious nuclear atypia, and sprinkling of lymphocytes. Features allowing a diagnosis of ATC are (1) presence of coagulative necrosis with ghost shadows of preexisting blood vessels, (2) recognition of scattered atypia and mitosis in more cellular areas at the periphery of the fibrosis, (3) detection of blood vessels obliterated by neoplastic spindle cells, and (4) positivity for epithelial markers [7].

Giant cells are characterized by deep pleomorphism, having bizarre sometimes multiple hyperchromatic nuclei, abundant eosinophilic cytoplasm, and a plump, oval, or round shape (Figure 4(a)). They are typically interspersed among smaller mononuclear tumor cells with similar cytoplasmic features showing a solid architecture. The formation of alveolar, pseudoglandular, or pseudovascular structures can also be seen, probably due to an artefactual separation of the cells. The cytoplasm of the tumor cells can sometimes assume a clear or granular appearance simulating a clear cell or an oncocytic carcinoma, respectively; the presence of striking pleomorphism, high mitotic activity, and necrosis is strongly suggestive for ATC [2].

Osteoclast-like multinucleated giant cells are occasionally present and could be prominent, resembling similar tumors described in breast and pancreas. Osteoclast-like multinucleated giant cells are known to be reactive elements of



(a) (b) (c)

Figure 3: (a) In sarcomatoid ATCs, neoplastic cells are morphologically indistinguishable from a primary sarcoma. At higher power view (b), spindle cells are pleomorphic and show a storiform pattern of growth. (c) Anastomosing cords of neoplastic cells resembling neoplastic vessels are the dominant features in this case.

monocytic/histiocytic lineage, immunohistochemically positive for CD68-KP1 (Figure 4(b)) and apparently derived from histiocytoid mononuclear cells via cellular fusion [9]. They give the tumors an appearance reminiscent of giant cell tumor of bone and soft tissue.

Huge inflammatory infiltrate is often present, sometimes predominantly neutrophilic in type, giving the tumor an appearance resembling inflammatory variant of malignant fibrous histiocytoma.

Heterologous elements, such as bone, cartilage, and skeletal muscle, can also be found. Matrix formation with chondro- and osteosarcomatous differentiation has been reported in up to 5% of anaplastic carcinoma [10]. Rhabdomyosarcomatous appearance has also been described [2, 11]. Carda et al. reported two cases, in which the skeletal muscular differentiation was demonstrated by electron microscopy and immunohistochemistry with positivity for muscle-specific actin, desmin, myogenin, and MyoD1 [11].

2.1.2. Differential Diagnosis. Sarcomatoid ATC closely simulates a large variety of soft tissue sarcomas. When a well-differentiated component is lacking and immunohistochemistry fails to demonstrate an epithelial differentiation, this distinction could be really difficult. Two characteristic histological features are helpful to differentiate sarcomatoid anaplastic carcinoma from a true sarcoma: the presence of angulated necrotic foci with neoplastic cells palisading around them as seen in glioblastoma of the central nervous system and the tendency of the spindle neoplastic cells to infiltrate the wall of large-sized veins and arteries [2].

It should be kept in mind however that primary sarcomas of the thyroid are indeed very rare so that it has been suggested that all sarcomatoid tumors of the thyroid gland should be regarded as ATC [12].

Primary sarcomas simulating a sarcomatoid ATC have been reported as case reports: fibrosarcoma [13], leiomyosarcoma [14], chondrosarcoma [15], osteosarcoma [16], and angiosarcoma (including epithelioid variant) [17, 18]. Metastases are possible as well and should be clinically ruled-out [19–21].

In addition, various spindle cell neoplastic and nonneoplastic thyroid lesions could simulate a sarcomatoid pattern and they should be taken into consideration by pathologist during diagnostic process. Differential diagnoses are described in Table 1.

2.2. Epithelioid-Squamoid

2.2.1. Histology. Anaplastic thyroid carcinomas with epithelioid-squamoid appearance are histologically less heterogeneous than sarcomatoid tumors. They are characterized by polygonal cells with a clearly epithelial appearance, growing

Table 1: Differential diagnoses of sarcomatoid category.

Thyroid lesions simulating a sarcomatoid pattern		Differential features
Other malignancies	Primary or metastatic sarcoma	It is an exclusion diagnosis: (i) lack of a well-differentiated component; (ii) no epithelial markers; (iii) absence of palisading necrosis and neoplastic spindle cells infiltrating the wall of large-sized vessels; (iv) presence of extrathyroidal sarcoma clinically detected (in metastatic disease).
	SETTLE (spindle epithelial tumor with thymus-like elements)	(i) Adolescent or young adults (mean age 15 years); (ii) biphasic pattern of growth with a predominant spindle cell component merging with mucin-secreting glandular elements; both components have an epithelial phenotype; (iii) generally indolent behavior.
	Spindle cell variant of papillary thyroid carcinoma	Metaplastic variant of PTC: (i) spindle cells retain even if focally nuclear features of PTC; (ii) consistent immunoreactivity for thyroglobulin.
	Spindle cell variant of medullary carcinoma	(i) Presence of amyloid deposits; (ii) immunoreactivity for calcitonin and/or calcitonin gene-related peptide.
Benign processes	Solitary fibrous tumor	(i) Low mitotic rate (4 mitoses or fewer per 10 high-power fields); (ii) no necrosis or vascular invasion; (iii) positivity for bcl-2, CD34, CD99, and vimentin and negativity for all epithelial markers.
	Riedel thyroiditis	(i) Absence of necrosis; (ii) evidence of occlusive phlebitis (no angioinvasion); (iii) absence of neoplasm; (iv) negativity for epithelial markers; (v) generally benign self-limiting disease.
	Post-fine-needle aspiration Spindle cell nodules of the thyroid	(i) History of FNA biopsy; (ii) size ranging from 3 to 10 mm; (iii) not encapsulated but relatively circumscribed and located mostly in the center of preexisting thyroid nodules; (iv) low mitotic rate; (v) immunoreactivity for smooth muscle actin (myofibroblastic origin).

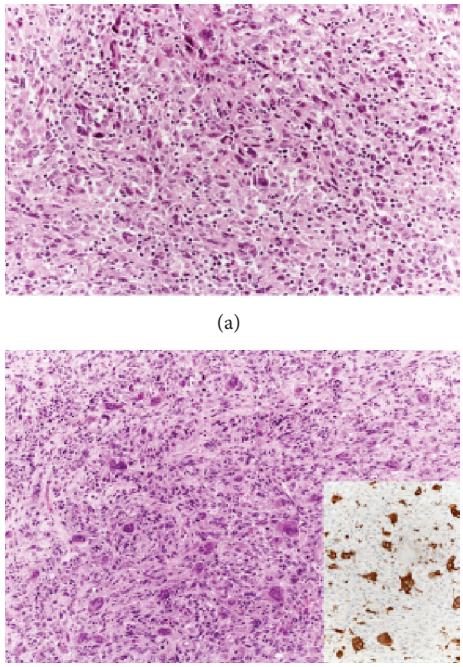
in solid nests, intermingled by desmoplastic stroma (Figures 5(a), 5(b), and 5(c)). Keratinization could be seen even if rarely. Squamoid pattern was present in about 20% of ATCs described in the largest series reported in literature [2, 4] and it is most frequently seen in combination with spindle and/or giant cells patterns.

Two peculiar variants of ATC belong to epithelioid-squamoid category and are described below.

Anaplastic spindle cell squamous carcinoma is a variant of ATC with both spindle cell elements and squamous islands with focal keratinization, similar to its counterpart described in the breast [22] and oropharynx [23, 24]. It was originally described by Bronner and LiVolsi as a unique subtype of ATC associated with the tall cell variant of papillary thyroid carcinoma (TCV PTC) [25]. In a recent series it was pointed out that this variant of ATC could clinically and histologically mimic a laryngeal squamous cell carcinoma. Therefore, caution is warranted in evaluating laryngeal squamous lesions in patients with known history of TCV PTC and without known risks factors for head and neck carcinogenesis [26].

Lymphoepithelioma-like ATC is a subtype of epithelioid-squamoid ATC characterized by histologic features similar to those of lymphoepithelioma of the nasopharynx and lymphoepithelioma-like carcinoma (LELC) of other sites [27]. It is composed of sheaths of epithelial cells in a rich inflammatory background including lymphocytes and some plasma cells. Tumor cells are immunoreactive for epithelial membrane antigen and keratin but are negative for thyroglobulin. Notably there is not association with EBV infection, as in LELC of organs that are not embryologically derived from primitive pharynx or foregut such as skin [28], urinary bladder [29], and uterine cervix [30].

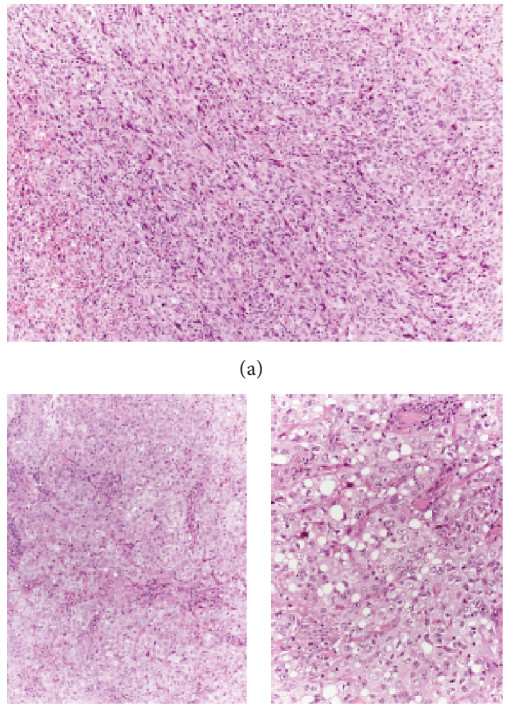
2.2.2. Differential Diagnosis. Pure squamous cells carcinoma of the thyroid is exceedingly rare and is listed as a separate entity in the WHO [1]. Its clinical presentation and behavior are the same of ATC. It is by definition not associated with other types of thyroid carcinoma.



(a)

(b)

Figure 4: Neoplastic giant cells are characterized by deep pleomorphism, with bizarre multiple hyperchromatic nuclei (a). They differ from reactive osteoclast-like giant cells (b) that show bland cytological features and are typically immunoreactive for CD68-KP1 (inset).



(a)

(b) (c)

Figure 5: In the epithelioid-squamoid category, neoplastic cells show a solid (a) or nested (b) architecture. They are plump and have abundant cytoplasm with a variable degree of squamous differentiation (c).

When a thyroid tumor is almost composed of squamoid elements it could be also necessary to rule out a direct invasion from an upper airway primary or a metastatic process firstly from the lung. Careful clinical examination is the most important clue, particularly to exclude metastases. The search of a well-differentiated component, by extensive sampling of the surgical specimen, is also a helpful feature in identifying the tumor origin (Figure 6) and it is mandatory in these cases. Notably Toner et al. reported some cases of ATC with endotracheal presentation, showing metaplasia or atypical, probably regenerative, epithelial changes in the adjacent airway epithelium that could be easily misinterpreted as an in situ component [31].

In thyroid, squamous differentiation may be seen in other neoplastic settings, without the meaning of anaplastic transformation. Squamous differentiation can be present as a result of a metaplastic process in papillary carcinoma, most commonly in the diffuse sclerosing variant [32], in medullary carcinoma, in mucoepidermoid carcinoma, and in sclerosing mucoepidermoid carcinoma with eosinophilia [1]. Squamous differentiation is also present in most cases of "carcinoma showing thymus-like differentiation" (CASTLE), which is thought to arise either from ectopic thymus or remnants of branchial pouches [33, 34].

On the other hand, nonneoplastic squamous cells can be present as embryonic remnants in the thyroglossal duct or structures derived from the branchial pouch (e.g., thymic epithelium) and as squamous metaplasia in thyroiditis or as a reparative phenomenon following FNAB [35] and postradiation therapy.

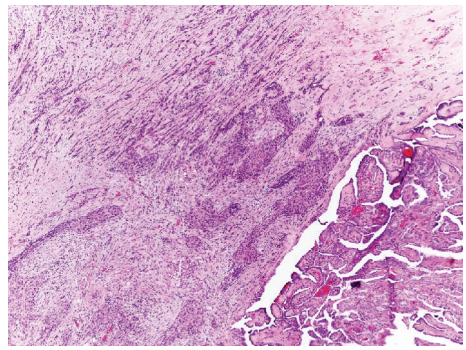


Figure 6: A significant rate of ATC is associated with WDTC. In this case, residual foci of papillary carcinoma are seen in the lower right corner but the main bulk of the tumor is composed of strands of squamoid atypical cells and spindle neoplastic elements.

Differential diagnoses are summarized in Table 2.

2.3. Immunohistochemical Features. ATCs show a variable immunophenotype. Immunoreactivity for cytokeratin is present in 40% to 100% of cases according to the different series [2, 36–38]. Vimentin is consistently present in the spindle cell component, whereas EMA and CEA are particularly expressed in the squamoid cells [2].

Table 2: Differential diagnoses of squamoid category.

Thyroid lesions simulating a squamoid pattern	Differential features
Pure squamous cells carcinoma	(i) Entirely composed of squamous cells; (ii) no evidence of another type of thyroid carcinoma in close proximity.
Primary head and neck squamous cell carcinoma	(i) Presence of in situ component in head and neck structures; (ii) features of "ab extrinseco" involvement of thyroid parenchyma; (iii) PAX8 is consistently negative.
Metastatic squamous cell carcinoma of the lung	(i) Presence of a lung nodule clinically detected; (ii) PAX8 is consistently negative.
Diffuse sclerosing variant of papillary thyroid carcinoma	(i) Abundant psammoma bodies; (ii) neoplastic cells retain nuclear features of PTC; (iii) immunoreactivity for thyroglobulin.
Mucoepidermoid carcinoma	(i) Combination of squamoid and mucinous components; (ii) thought to represent papillary carcinoma with extreme degree of squamous and mucinous metaplasia; (iii) low grade thyroid neoplasm.
Sclerosing mucoepidermoid carcinoma with eosinophilia	(i) Fibrohyaline stroma; (ii) striking infiltration of eosinophils; (iii) mucin secretion is often present; (iv) typically arising in Hashimoto thyroiditis (thought to derive from metaplastic squamous nests associated with inflammatory infiltrate).
CASTLE (carcinoma with thymus-like elements)	(i) Lymphoepithelioma-like carcinoma with foci of squamous differentiation; (ii) pushing margins; (iii) immunoreactivity for CD5, bcl-2, high molecular weight keratin, mcl-1 (thought to be ectopic thymic carcinoma arising from remnants of the branchial pouch); (iv) usually indolent behavior with tendency to late recurrences.

Table 3: Immunohistochemical features of ATCs.

Immunostain	Percentage of positive cases
Cytokeratin	40–100%
Vimentin	100% (in spindle cells)
EMA	30–50% (in squamoid cells)
CEA	Rarely (in squamoid cells)
Thyroglobulin	0% (false positivity due to nonneoplastic thyroid follicular cells entrapped in the tumor or diffusion from destroyed normal follicles)
TTF-1	0%
RET/PTC oncoprotein	0%
Calcitonin	0%
PAX8	0–79% of ATCs (probably depending on antibody used); 92% of ATCs with squamoid features

Typically, ATC cells are not immunoreactive for thyroglobulin, calcitonin, TTF-1, or RET/PTC oncoprotein [36, 37, 39]. False positive reaction to thyroglobulin may result from nonneoplastic thyroid follicular cells entrapped in the tumor or diffusion from destroyed normal follicles (Figures 7(a), 7(b), 7(c), and 7(d)). PAX8 (also known as paired box gene 8) is a transcription factor expressed in nuclei of normal and neoplastic tissue of the thyroid, kidney, and female genital tract [40–42], being retained also in most sarcomatoid renal cell carcinomas. The few studies evaluating PAX8 staining of ATCs have had widely disparate results [40, 43, 44]. Nevertheless PAX8 seems to have a useful diagnostic role in specific settings, having been found in 79% of ATCs and in up to 92% of ATCs showing squamoid features, whereas it is negative in head and neck squamous carcinoma and lung carcinoma [40, 45].

Immunohistochemical features are summarized in Table 3.

3. Genetic Features

Even though ATC is a rare disease, a consistent amount of information is currently available on the genetic alterations that are most frequently associated with this tumor [46, 47] (Figure 8).

3.1. Somatic Gene Mutations. Mutations in the components of the principal oncogenic pathways (MAPK, PI3K, Wnt, etc.) have been described to occur with high frequency in ATC. It is known that more than 90% of thyroid cancer harbor mutations in the MAPK pathway [48]. RAS mutations that occur both in benign and malignant thyroid cancers are detected also in ATCs, with variable frequency ranging from 6 to 50% of cases depending on series [49–53]. By contrast, RET/PTC rearrangements, which account for about 15–20% of PTCs, are rarely found in ATCs [47, 52]. Mutations in the BRAF gene, which occur in more than 50% of well-differentiated PTCs [54–58], are only detected in 25% of ATC cases [59, 60]. This lower frequency is in apparent contrast

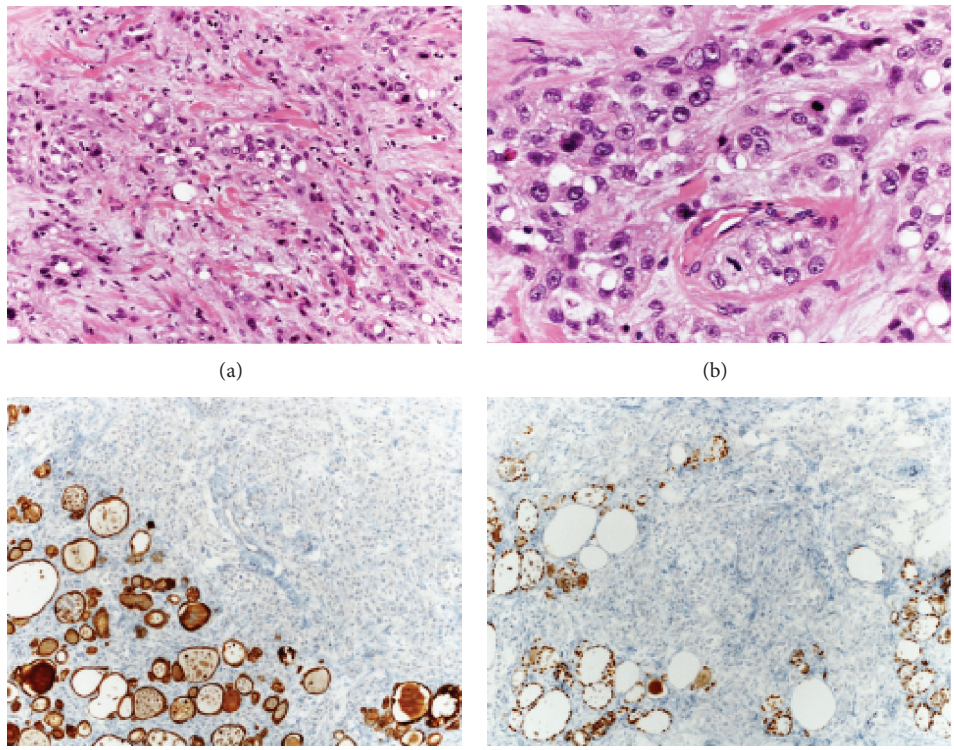


Figure 7: An ATC made up of pleomorphic epithelioid cells arranged in loosely cohesive nests; focally, intracytoplasmic vacuoles are present ((a), (b)). Immunohistochemical stains with keratin 7 (c) and TTF1 (d) highlight entrapped thyroid follicles.

with the role of this mutation in driving aggressiveness of thyroid tumors, which has been proposed and largely debated in the past decade [61–63].

3.1.1. PIK3CA. Gain of function mutations in the PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) gene is found in 25–40% of cancers, with alterations mainly clustering in two hotspots within the helical (exon 9) and catalytic (exon 20) domains [64]. In thyroid cancer, PIK3CA mutations are rare in PTCs (0–5% depending on series) but more frequent in poorly differentiated and anaplastic thyroid cancer (from 11 to 23%). As well, amplification of the PIK3CA genomic locus in 3q26.3 is found in about 40% of ATC suggesting that alteration of the PI3K depending pathway plays a pivotal role in the pathogenesis of ATCs [50, 51, 53, 65].

3.1.2. TERT. Somatic mutations in the promoter of the TERT (Telomerase Reverse Transcriptase) gene have been described as highly recurrent in different types of cancer including thyroid cancer [66–69]. Up to 50% of ATCs (33–50%) have been shown to carry these mutations. Intriguingly, TERT promoter mutations seem to occur prevalently in those tumors harboring mutated BRAF or RAS, suggesting that TERT alteration is acquired later during tumor development and may provide a functional advance to BRAF or RAS-driven tumors by enabling acquisition of additional genetic defects leading to disease progression.

3.1.3. CTNNB1. Wnt pathway appears also to play a relevant function in ATC development. Mutations in *CTNNB1 (β-Catenin)* gene leading to a constitutively active Wnt-signaling have been reported in 25–60% of ATCs [70]. CTNNB1 is a major component of the E-cadherin cell-cell adhesion complexes and a role of this protein in the epithelial-mesenchymal transition process has been demonstrated [71]. Intriguingly, the transdifferentiation of well-differentiated thyroid tumor cells toward a nondifferentiated status has been proposed as one of the major processes in the pathogenesis of ATCs.

3.1.4. p53 and PTEN. Besides gain of function alterations in key oncogenes, tumor development and progression rely significantly on the inactivation of tumor suppressor genes. p53 and PTEN genes are involved in the negative regulation of cell proliferation and in promoting apoptosis and are frequently impaired during tumor progression. More than 50% of ATCs have been reported to carry loss of function mutations in the p53 gene. As well, the overexpression of p53, which may reflect altered function of the protein in the absence of mutation, has been frequently observed in ATC. Loss of function alterations in the PTEN gene, which inhibit the activation of the PI3K pathway, has been reported to occur in 4 to 16% of ATC [50, 51, 53].

3.2. Somatic Chromosomal Aberration. It is well established that the accumulation of genetic alterations is a driving mechanism of tumor growth and spread to distant sites.

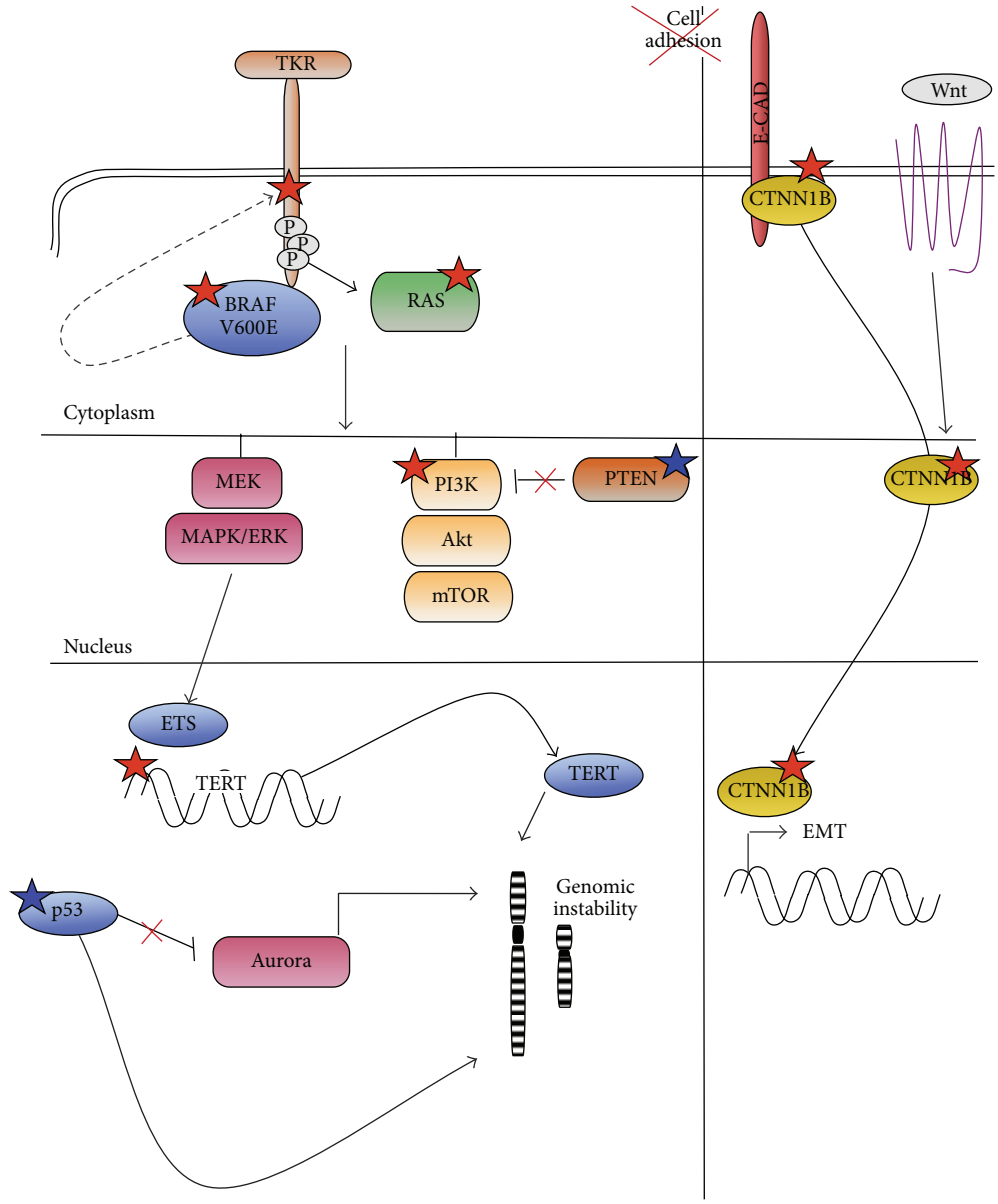
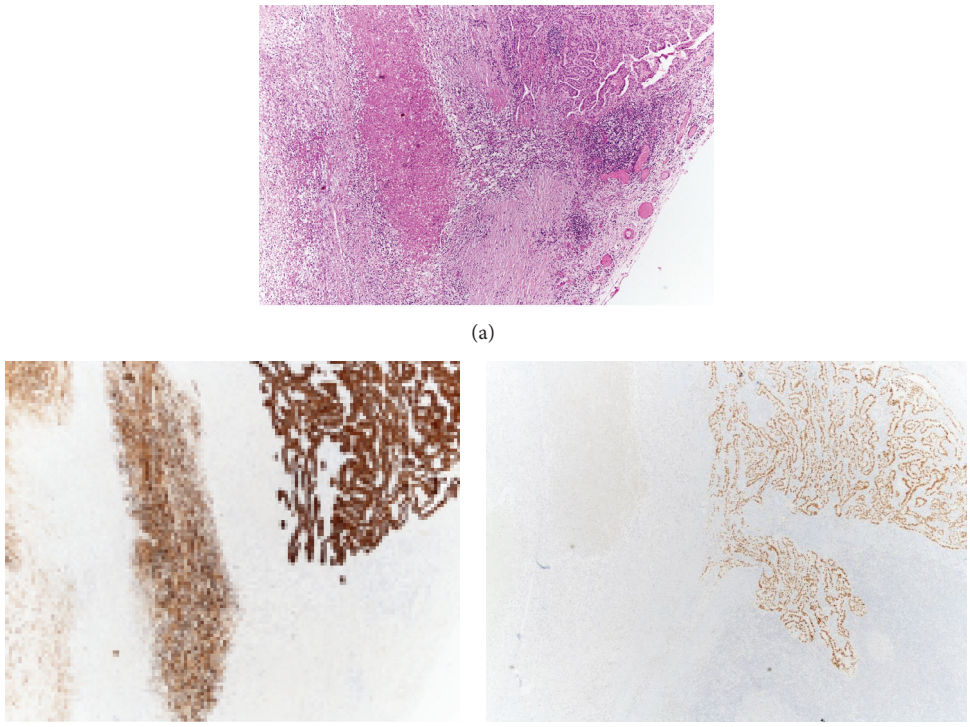


Figure 8: Schematic representation of molecular pathways altered in ATCs.

Several studies have investigated genomic instability and DNA copy number variations in ATC with the intent to understand the impact of genomic damage on the genesis and progression of this tumor. Liu and colleagues used real-time PCR analysis to investigate the copy number of a panel of genes involved in MAPK and PI3K pathway in thyroid cancer including a series of 51 ATCs. They observed that genes coding for tyrosine kinase receptors (RTK) like EGFR, PDGFR, VEGFR, KIT, and MET are frequently amplified in thyroid cancer and in particular in the ATC histotype [51]. Wreesman and colleagues used CGH technique to investigate the molecular-cytogenic profile of different histotypes of thyroid cancer to define chromosomic regions that could be specifically associated with the development of ATC [72]. These authors observed several chromosomal abnormalities that were common to both well-differentiated and nondifferentiated thyroid cancer (like gain of 5p15, 5q11–13, 19p, and 19q and loss of 8p) and that could represent early event in the genesis of these tumors. Furthermore, they found alterations like gain in 3p13-14, loss of 5q11–31, and gain in 11q13 that were exclusive of the genome of the 15 ATCs analyzed and that may represent late genetic events driving the transformation of a preexisting thyroid cancer into the aggressive ATC histotype. Using the same approach, Rodrigues et al. investigated the chromosomal profiles of 7 ATCs, showing that chromosomal imbalances affect the genome of all cases analyzed [73]. Intriguingly, the chromosomal regions affected by the alterations were extremely heterogeneous, suggesting the existence of a high-grade genetic interneoplastic diversity in ATCs. Besides



(a) (b) (c)

Figure 9: Lymph node metastasis of WDTC with anaplastic areas. Residual foci of papillary carcinoma are present in the right upper corner but the metastatic deposits are constituted mainly by spindle cells with wide necrotic areas (a). While both WDTC and ATC are immunoreactive with pankeratin (b), TTF1 is positive only in the WDTC (c).

chromosomal imbalances, Miura and colleagues reported that 6 out of 10 ATCs showed aneuploidy [74].

Summarizing the data currently available, two major considerations emerge: (1) the type of chromosomal alterations that characterize ATCs is widely heterogeneous and up to now it is not possible to define a "common" profile of alterations that is specific of this tumor type. This observation implies that different kinds of genetic damage may contribute to the genesis of this tumor. (2) ATCs are characterized by a higher number of chromosomal alterations with respect to well-differentiated and poorly differentiated thyroid cancer. However, several studies reported that the amount of genetic damage does not directly correlate with the grade of aggressiveness or the outcome of ATCs. Based on these considerations, we may hypothesize that the high-grade genomic instability observed in ATCs is a side effect of the loss of restraining mechanisms of cell proliferation rather than being the cause of tumor progression. Indeed, a number of mitotic proteins involved in cell cycle check points or engaged in chromosome assembly and segregation have been shown to be deranged in ATCs [75, 76]. These include the three members of the Aurora kinase family. Aurora kinases are implicated in several aspects of chromosome segregation and cytokinesis. Expression of all Aurora kinases and in particular of Aurora A is strongly induced in ATC cells [75, 76] and overexpression of Aurora A has been shown to induce centrosome amplification and to potentiate the oncogenic function of Ras [77, 78].

Evidences exist of a negative cross-talk between Aurora A kinase and p53 [79, 80]. Considering the fact that p53 is mutated or aberrantly expressed in a wide proportion of ATCs, it is likely to suppose that these alterations may affect the balance between p53 and Aurora A with relevant consequences on chromosome stability. The possibility to counteract the misfunctioning mitotic proteins has been considered a potential therapeutic strategy for cancer with high grade genetic damage. Indeed, inhibitors of Aurora kinases alone or in combination with other drugs, including microtubule inhibitors, showed an important anticancer effect in preclinical models of ATCs indicating this approach as a possible therapeutic strategy for ATCs treatment [75, 81].

4. Histogenesis

In literature there are indirect, although convincing, evidences that ATC represents a terminal dedifferentiation of preexisting well-differentiated thyroid carcinoma (WDTC) in most, if not all, cases. A large portion of ATC develops in longstanding goiters or in the context of preexisting, incompletely treated papillary or follicular thyroid cancers. Likewise, careful examination of primary ATC tumors reveals coexisting areas of WDTC in 80% to 90% of cases [82, 83]. This better differentiated tumor is usually a papillary carcinoma or one of its variants (particularly Warthin-like and tall cell variant), but it may also be a follicular carcinoma, as well

as an oncocytic carcinoma, or an insular carcinoma [4, 26, 84, 85]. It has been suggested that if an extensive sampling is performed, foci of WDTC are eventually found in every specimen of ATC [83]. Furthermore, it has been postulated that the sharply outlined sclerohyaline nodules sometimes present within undifferentiated carcinoma represent the burn-out residue of such well-differentiated components [86].

Anaplastic transformation may also take place in a metastatic focus, (Figures 9(a), 9(b), and 9(c)) thus supporting the idea that these lesions originate through the dedifferentiation of preexisting well-differentiated cancer [87–89].

Nevertheless, according to the current genetic data it is conceivable that not all the ATCs arise as temporal aggressive evolution of a preexisting WDTC. If the ATC phenotype was always the temporal aggressive evolution of a preexisting WDTC then common founding alterations between the ATCs and the WDTC subgroups should be identified. Whole genome studies showed that the chromosomal asset of ATCs and WDPTC is widely different [51, 72–74] supporting the hypothesis that not all thyroid cancers start as indolent lesions but some of them may originate as already aggressive nondifferentiated cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

MicroRNA Deregulation in Anaplastic Thyroid Cancer Biology

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Anaplastic thyroid cancer (ATC) is among the most lethal types of cancers, characterized as a fast-growing and highly invasive thyroid tumor that is unresponsive to surgery and radioiodine, blunting therapeutic efficacy. Classically, genetic alterations in tumor suppressor *TP53* are frequent, and cumulative alterations in different signaling pathways, such as MAPK and PI3K, are detected in ATC. Recently, deregulation in microRNAs (miRNAs), a class of small endogenous RNAs that regulate protein expression, has been implicated in tumorigenesis and cancer progression. Deregulation of miRNA expression is detected in thyroid cancer. Upregulation of miRNAs, such as *miR-146b*, *miR-221*, and *miR-222*, is observed in ATC and also in differentiated thyroid cancer (papillary and follicular), indicating that these miRNAs' overexpression is essential in maintaining tumorigenesis. However, specific miRNAs are downregulated in ATC, such as those of the *miR-200* and *miR-30* families, which are important negative regulators of cell migration, invasion, and epithelial-to-mesenchymal transition (EMT), processes that are overactivated in ATC. Therefore, molecular interference to restore the expression of tumor suppressor miRNAs, or to blunt overexpressed oncogenic miRNAs, is a promising therapeutic approach to ameliorate the treatment of ATC. In this review, we will explore the importance of miRNA deregulation for ATC cell biology.

1. Introduction

Anaplastic thyroid cancer (ATC) is the most lethal histotype of thyroid cancer, responsible for more than one-third of thyroid cancer-related deaths [1]. The fast-growing nature of this type of cancer and its refractoriness to radioiodine treatment due to tumors not concentrating iodine limit the efficacy of therapeutic interventions [2]. Thus, ATC patients display a rapidly progressive disease that may cause death within six months [3].

ATC's clinical pathology is characterized by the aggressive behavior of cancer cells, which results in a rapid enlargement of the neck tumor to invade adjacent tissue and migrate as distant metastases, usually with secondary sites in the lungs, bone, and brain [4]. During this process, activation of epithelial-to-mesenchymal transition (EMT) is a key feature of anaplastic transformation. Moreover, loss of expression of differentiation markers (iodine metabolizing genes) and, consequently, loss of iodine uptake are markers of this process that negatively impact on ATC radioiodine responsiveness.

Unlike the differentiated histotypes of thyroid cancer, papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) that show a single driver mutation pattern, the tumorigenesis of ATC is not completely understood: it may arise *de novo* or from a preexisting well-differentiated cancer (PTC/FTC). The most prevalent genetic alterations in ATC are mutations in the *TP53* gene at codon 273 as observed in more than 70% of ATC samples [5, 6], leading to p53 loss of function. Moreover, mutations in the telomerase gene (*TERT*) are frequently seen in aggressive thyroid cancers, including in poorly differentiated and anaplastic thyroid cancers [7, 8], and lead to increased transcriptional activity of TERT. However, additional mutations in MAPK signaling (RAS and BRAF genes), in PI3K signaling (PIKCA and PTEN), and in Wnt signaling (β-catenin and APC) have also been detected in ATC [9–12].

Animal models have contributed to an understanding of the molecular transformation of ATC. The thyroid cancer progression hypothesis is corroborated by a transgenic mouse model for the early activation of the $BRAF^{T1799A}$ oncogene

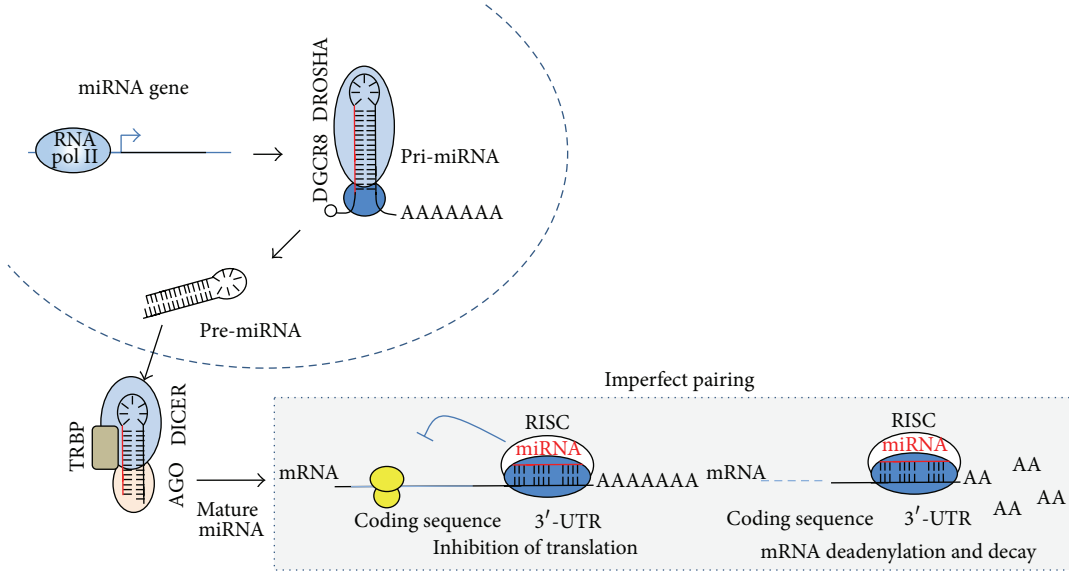

Figure 1: Biogenesis of miRNA. Transcription of miRNA by RNA polymerase II yields a long primary transcript (pri-miRNA) that contains a cap 5′ and poly-A tail. The complex DROSHA/DGCR8 cleaves pri-miRNA and gives rise to an miRNA precursor (pre-miRNA) that is exported to the cytoplasm and further processed by DICER endonuclease. An miRNA duplex associates with the RISC complex and retains the mature strand of miRNA. This complex directs imperfect binding to 3′-UTR region of target mRNA, leading to a reduction in protein levels via translation blockage and mRNA deadenylation and decay.

restricted to the thyroid gland (Tg-BRAF) that is affected by high penetrance PTC that undergoes temporal dedifferentiation [13]. Molecular analysis of Tg-BRAF mice-derived tumors reveals the deregulation of TGFβ signaling upon prolonged stimulation of the BRAF oncogene, with enhanced TGFβ signaling transduction and a shift to EMT by ZEB1 and ZEB2 transcription factor activation [14]. Another transgenic model with BRAF activation in adult mice also gives rise to differentiated thyroid cancer; however, progression to ATC requires *TP53* gene deletion [15], corroborating the multistep cancer progression hypothesis for ATC. A mouse model harboring defective PI3K signaling gives rise to aggressive thyroid cancer. Transgenic mice carrying deletions of *Pten* and *TP53* $[Pten, p53]^{(\text{thyr}-/-)}$ develop ATC with loss of expression of the thyroid transcription factors, *Nkx2-1*, *Pax-8*, and *Foxe1*, and thyroid differentiation markers, *Nis*, *Tpo*, *Tg*, *Tshr*, and *Duox* [16]. $[Pten, p53]^{(\text{thyr}-/-)}$ mice thyroid tumors undergo TGFβ signaling induced EMT and demonstrate increased pSMAD2 and vimentin levels, but loss of E-cadherin expression.

Recently, a pivotal role for microRNAs (miRNAs) in cancer has emerged with increasing evidence showing that they may drive and potentiate oncogenesis. The miRNAs are a class of small noncoding RNAs (~20 nt) that regulates post-transcriptional gene expression. These miRNAs are transcribed as long primary RNAs that undergo sequential cleavages by Drosha and Dicer ribonucleases in the nucleus and cytoplasm, respectively, to yield mature miRNA. In turn, mature miRNA associates with the protein complex RISC (RNA-induced silencing complex), which directs pairing with the 3′-UTR region of target mRNAs. Imperfect pairing leads to translational blockage of target mRNA and, also, mRNA deadenylation and decay (Figure 1).

Essentially, miRNA deregulation in cancer is oncogenic (oncomiR) under two different conditions: (a) when overexpressed, miRNAs may block tumor suppressor gene translation or (b) when underexpressed, miRNAs may derepress protooncogene mRNA translation (Figure 2).

Deregulation of miRNA in thyroid cancer was initially described by He et al. in a group of PTCs [17]. The same group of upregulated miRNAs, such as *miR-146b*, *miR-221*, and *miR-222*, is commonly detected in ATC and in differentiated thyroid cancers (papillary and follicular histotypes) [18] and also in a fraction of benign nodules [17, 19], indicating that persistent expression of these miRNAs may be necessary to maintain tumorigenesis. However, ATC also shows exclusive reduction of certain miRNAs with tumor suppressor properties, indicating a role for these miRNAs in tumor aggressiveness (Figure 3). Investigating tumour samples from a group of ten ATC patients by microarray analysis, the seminal study of Visone et al. [20] revealed the repression of *miR-30d*, *miR-125b*, *miR-26a*, and *miR-30a-5p*. In addition, another study showed a reduction of several members of the *let-7* and *miR-200* family of miRNAs [21] and overexpression of *miR-221*, *miR-222*, and *miR-125a-3p*.

In this review, we will explore some aspects of the deregulated miRNA found in anaplastic thyroid cancer to address the molecular biology and signaling pathways implicated in the clinical-pathological characteristics of this type of cancer (Table 1).

Table 1: Validated targets for deregulated miRNAs in ATC.

miRNAs		Validated targets			Cellular processes	References
Downregulated miRs	*miR-200* family	ZEB1	ZEB2	β-Catenin	EMT and proliferation	[21, 67]
	miR-30 family	Beclin1	EZH2	VIM	Autophagy, chromatin condensation, and EMT	[30, 33, 67]
	let-7 family	RAS	HMGA2	LIN28	Proliferation, histone modification, and stemness	[67, 68]
	miR-25	EZH2	BIM	KLF4	Chromatin condensation and apoptosis	[30, 67]
	miR-125	MMP1	HMGA2	LIN28A	Invasion, histone modification, and stemness	[20, 67, 69]
Upregulated miRs	*miR-221/miR-222*	p27	RECK	PTEN	Cell cycle, growth, and invasion	[20, 67]
	miR-17-92 cluster	p21	TIMP3	PTEN	Cell growth and invasion	[45, 67]
	miR-146a/miR-146b	NFκB	THRB	SMAD4	Cell differentiation, proliferation, and invasion	[52, 67, 70, 71]

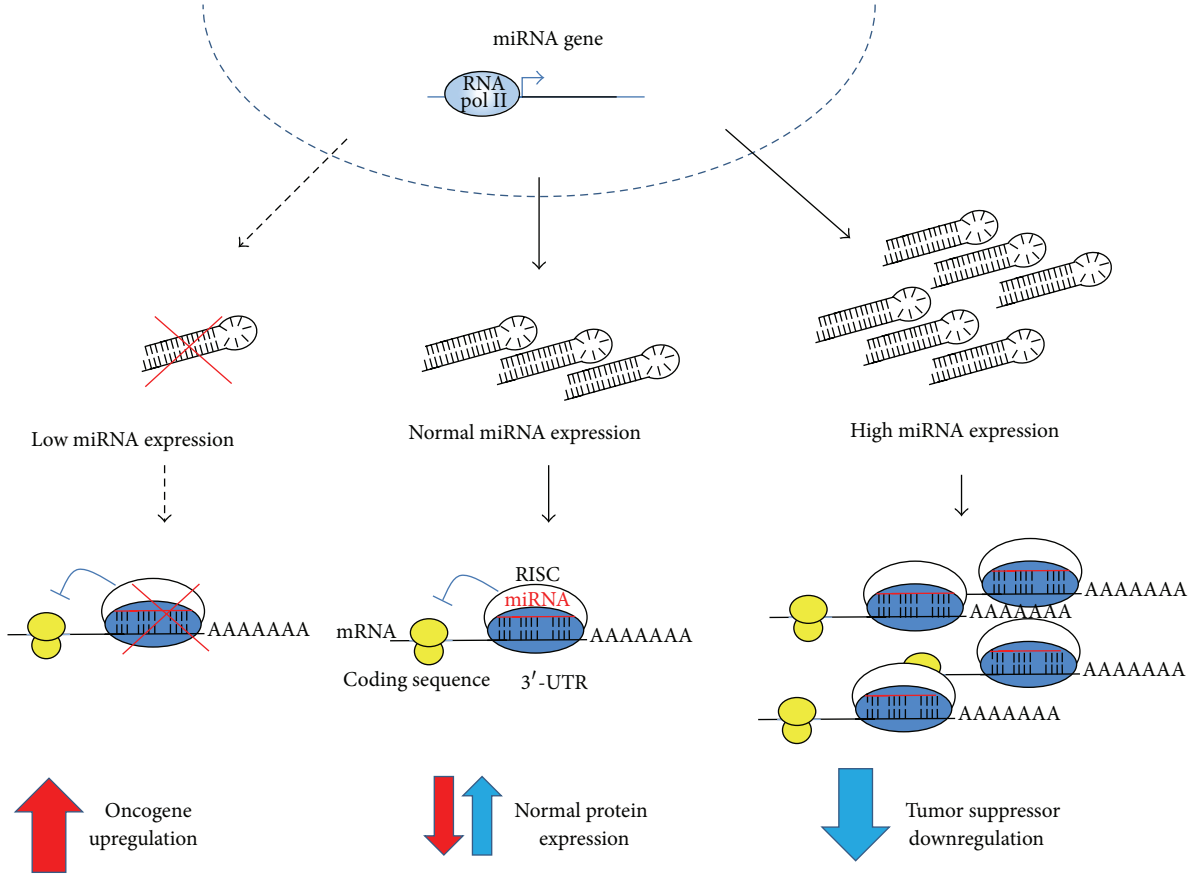

Figure 2: Some miRNAs act as oncomiRs. Deregulation of miRNA changes physiological protein level balances and enhances the oncogenic process where (1) low expression of an miRNA may enhance protein translation of an oncogenic protein or (2) high expression of an miRNA may repress the translation of a tumor suppressor gene. Both situations may occur concomitantly in cancer as observed in anaplastic thyroid cancer.

2. Downregulated miRNAs in ATC

Specific miRNAs are reduced in thyroid cancer, such as the *let-7* family, but other miRNAs, such as the *miR-200* and *miR-30* families, are exclusively downregulated in ATC, indicating that the latter miRNAs may play a role in the acquisition of more aggressive tumor characteristics (i.e., enhanced cell invasion and migration).

2.1. miR-200 Family. The *miR-200* family is composed of the *miR-200a*, *miR-200b*, and *miR-200c* genes, which are usually downregulated in ATC [21]. The *miR-200a* and *miR-200b*

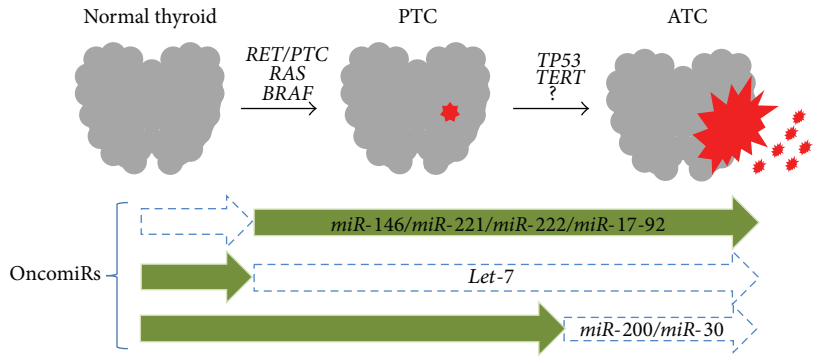

Figure 3: Thyroid oncogenesis and miRNAs. Activation of MAPK oncogenes by mutations or rearrangements leads to PTC. Progression to ATC may be associated with the acquisition of additional genetic alterations such as *TP53* mutations. Deregulation of miRNA occurs during thyroid oncogenesis, with specific upregulation of miRNAs such as *miR-146*, *miR-221*, *miR-222*, and *miR-17-92* cluster, and loss of *let-7* expression, in both PTC and ATC. Exclusive downregulation of miRNAs, such as *miR-200* and *miR-30*, is observed in ATC.

genes make up a cluster located on chromosome 1, while the *miR-200c* gene is located on chromosome 12. An important transcriptional activator of *miR-200* is p53, which binds to the promoter region of *miR-200c* at multiple sites. Interestingly, a *TP53* mutation at a DNA binding domain as present in ATC impairs downstream transcriptional activation of *miR-200* [22]. Moreover, p53 is an important controller of tumor suppressor miRNAs, such as those of the *miR-34* family, and also influences miRNA processing [23], besides having a classical role in DNA repair and genomic stability. Another signaling pathway that influences *miR-200* expression is the EGF pathway. Overexpression of the EGF receptor, EGFR, is observed in ATC [24], while EGFR knockdown in ATC cells restores *miR-200* expression and represses the expression of mesenchymal markers [25]. Classically, TGFβ signaling induces epithelial-to-mesenchymal transition (EMT), via the transcriptional activation of ZEB1 and ZEB2 [21], inducing a mesenchymal phenotype, with the expression of vimentin and repression of E-cadherin as observed in ATC. Interestingly, the *miR-200* family is an important regulator of the EMT process by regulating ZEB1 and ZEB2 protein levels. Downregulation of *miR-200* in ATC would potentiate the TGFβ-mediated EMT switch and enhance aggressiveness.

2.2. miR-30 Family. The *miR-30* family of tumor suppressor miRNAs is composed of five members: *miR-30a*, *miR-30b*, *miR-30c*, *miR-30d*, and *miR-30e*. Downregulation of the *miR-30* family is observed in several types of cancer such as breast, bladder, and colon [26, 27]. Moreover, decreased expression of *miR-30* is observed in metastasis compared to the primary tumor [28], suggesting a role in aggressive disease. In ATC, *miR-30* expression is also reduced in tumor samples [20, 29]. Indeed, modulation of *miR-30* levels in ATC cells has a great impact in cancer cell biology. Importantly, *miR-30d* ectopic expression in an ATC cell line reduced monolayer cell growth and impaired anchorage-independent cell growth [30].

Downregulation of *miR-30* derepresses the expression of EZH2, an important component of the polycomb repressive complex 2 (PRC2) that regulates chromatin condensation and gene expression. EZH2 is the enzymatic subunit of histone methyltransferase that trimethylates histone H3 lysine 27. EZH2 is overexpressed in ATC and enhances cell proliferation, migration, and invasion, while repressing the expression of thyroid transcription factor PAX8 [31]. Another important cellular process regulated by the *miR-30* family is autophagy through targeting the key autophagy-promoting protein, Beclin1 (gene *BECN1*). In ATC, *miR-30d* restoration sensitizes cancer cells to cisplatin treatment by repressing Beclin1, which participates in the early stages of autophagosome formation [32]. An *miR-30d* mimic enhanced the apoptotic effects of cisplatin as shown by increased cleaved caspase-3 and PARP levels and annexin V staining [33]. Moreover, cisplatin treatment in a xenograft model also showed shrinkage of ATC tumor derived from *miR-30* overexpressing cells. The blockage of autophagy using specific inhibitors exerts similar effects to the reintroduction of *miR-30* into ATC cells, indicating that the autophagy process is important for ATC cells' resistance to chemotherapy.

2.3. let-7 Family. Family genes of *let-7* are located on different chromosomes and are abundantly expressed in a normal thyroid gland (*let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, and *let-7g*) [34]. Deregulation of *let-7* is observed in several types of cancer, and its tumor suppressor effects are usually abolished by its downregulation [35]. *let-7* was the first miRNA identified as having a role in cancer through validation of RAS protooncogene mRNA targeting and the association of low levels of *let-7* with a poor prognosis in lung cancer [36]. Downregulation of several members of the *let-7* family is observed in well-differentiated thyroid cancer (PTC and FTC) [37–39], but a marked decrease in the expression of *let-7a*, *let-7c*, *let-7d*, *let-7f*, *let-7g*, and *let-7i* is also observed in ATC [20, 21, 29].

Modulation of *let-7* levels alters thyroid cancer cell biology. Ectopic expression of *let-7f* in a PTC cell line inhibits cell proliferation and viability, while it reduces the activation of MAPK signaling, an important marker of thyroid cancer [40]. Moreover, *let-7* enhances the expression of thyroid transcription factor-1 (TTF1/NKX2-1), a key factor in maintaining the expression of iodine metabolizing genes and thyroid

differentiation, usually lost in ATC. The recovery of *let-7a* expression in an FTC cell line changes cell morphology to an epithelial-like phenotype (flat and adherent), while it reduces cell migration by targeting *FXYD5*, an important regulator of cell adhesion [39]. Moreover, in aggressive lung cancer, loss of *let-7* is associated with a poorer prognosis [36], and the reduction of *let-7c* is associated with refractoriness to chemo- and radiotherapy treatments. Indeed, the ectopic expression of *let-7c* in a lung cancer cell line restores the cells' response to chemo- and radiotherapy and represses the EMT process [41], indicating an important role for *let-7* in tumor aggressiveness.

3. Upregulated miRNAs in ATC

Common miRNAs such as *miR-146*, *miR-221*, *miR-222*, and *miR-17-92* are upregulated in aggressive ATC and in well-differentiated thyroid cancer, indicating that reinforced expression of these miRNAs is important in maintaining the oncogenic process.

3.1. Cluster miR-17-92. The *miR-17-92* cluster is located on chromosome 13 and transcribes a polycistron that yields seven different mature miRNAs: *miR-17-5p*, *miR-17-3p*, *miR-18a*, *miR-19a*, *miR-19b*, *miR-20a*, and *miR-92a*. In normal thyroid follicular cells, early $BRAF^{V600E}$ oncogene activation induces the expression of an *miR-17-92* cluster [42]. The BRAF oncogene is the most frequent genetic alteration in thyroid cancer (i.e., PTC) and is also detected in ATC, associated with poor clinical-pathological features of cancer such as extrathyroidal invasion, short time recurrence, and distant metastases [43, 44]. High levels of *miR-17-92* components are expressed in ATC [45], similar to that observed in other types of cancer, such as lung, colon, pancreatic, and lymphoma [46], especially in the aggressive forms of disease [47].

The molecular modulation of endogenous levels of these miRNAs using *LNA* (locked nucleic acid) modification resulted in important effects in ATC cell biology. Specific blockage of *miR-17-5p*, *miR-17-3p*, and *miR-19a* resulted in a pronounced growth inhibition of ATC cells and apoptosis through activation of caspase-3 and caspase-9 [45]. Inhibition of the *miR-17-92* cluster in ATC leads to the recovery of PTEN protein levels [45], an important negative regulator of PI3K growth signaling, which is repressed in ATC. Moreover, among several validated targets for the *miR-17-92* cluster are proteins associated with tumor aggressiveness. A key target that influences tumor invasion is TIMP-3, an important inhibitor of metalloproteinase activation, targeted by *miR-17-5p* and *miR-17-3p*.

3.2. miR-146a and miR-146b. The *miR-146* family, *miR-146a* and *miR-146b*, is overexpressed in ATC [18, 29, 48]. Despite sharing the same seed region, and therefore targets, *miR-146a* and *miR-146b* are transcribed by two independent genes located on chromosomes 5 and 10, respectively, and regulated by the transcription factor NFκB. The promoter region of both miRNAs contains binding sites for the NFκB complex, part of a key oncogenic signaling pathway, usually overactivated in ATC, which shows increased nuclear staining for RelA (p65), the subunit of the NFκB dimer [49]. Ectopic expression of the inhibitory protein of this signaling, IκB, decreases *miR-146a* and *miR-146b* levels in an ATC cell line [48]. Moreover, inhibition of *miR-146a* leads to an abrogation of anchorage-independent growth and invasion by ATC cells [48]. Interestingly, NFκB activation is observed at the invasive front of aggressive PTC showing local invasion compared to the central region of the tumor [50] and also in response to a $BRAF^{V600E}$ oncogene, leading to cell migration and invasion [51], and *miR-146b* upregulation [52]. Moreover, the introduction of *miR-146b* into PTC mutated cells ($BRAF^{V600E}$ or RET/PTC1) enhances cell invasiveness and migration [53]. Therefore, the NFκB signaling pathway and its transcriptionally activated miRNAs, *miR-146a* and *miR-146b*, play an important role in thyroid cancer aggressiveness and progression. Indeed, increased plasma circulating levels of *miR-146b* can be detected in papillary thyroid cancer before surgery, which also correlates with tumor aggressiveness and poor prognosis [54].

3.3. miR-221 and miR-222. *miR-221/miR-222* is a cluster of miRNAs, located on chromosome X, which is deregulated in thyroid cancer. Although *miR-221* and *miR-222* overexpression is detected in differentiated (PTC and FTC) [17, 37] and anaplastic thyroid cancer cells [18, 29, 55], the expression of these miRNAs is associated with poor clinical-pathological features of cancer. In PTC and FTC, levels of *miR-221* and *miR-222* positively correlate with tumor aggressiveness, increased extrathyroidal invasion, tumor size, higher tumor node metastasis stage, and papillary thyroid cancer recurrence [18, 56–58]. Indeed, higher expression of *miR-221* and *miR-222* is present in metastatic, in comparison to nonmetastatic, cancers [59]. Moreover, *miR-222* increased circulating plasma level is associated with the presence of the BRAF mutation and recurrent papillary thyroid cancer [54].

Ectopic expression of *miR-221* in cancer cells results in a robust increase in anchorage-independent growth in soft-agar medium [37], pointing to a role for this cluster of miRNAs in the process of invasion and cell migration. Indeed, one target of the cluster is RECK, an inhibitor of metalloproteinase. Inhibition of *miR-221* impairs cell migration and invasion via upregulation of RECK while it reduces metastases in a colon cancer mouse model [60]. Both *miR-221* and *miR-222* also influence cell proliferation, once overexpression deregulates the cell cycle, by targeting the $p27^{kip1}$ (CDKN1B) protein, a key regulator of cell cycle progression [61].

4. Concluding Remarks

Currently, there is no effective therapy to blunt the lethal course of ATC, therefore prompting trials of additional and innovative therapies for ATC. Molecular targeted therapy for ATC seems to be a promising approach to retard cancer growth and increase patient survival. The molecular modulation of miRNA levels using miRNA mimics or antimiRs, allied to a novel class of highly specific inhibitors of MAPK and PI3K signaling, for instance, may enhance the ATC response to conventional treatment [62].

Systemic miRNA injection has showed promising results using lipid and other carrier molecules for treating lung and prostate cancer in animal models. Intratumoral injection or tail vein injection of a lipid-based *miR-34a* inhibited orthotopic prostate cancer tumor growth and metastases in immune-deficient mice [63], and lentivirus mediated *miR-34a* delivery to prostate cancer cells completely inhibited tumor growth. Moreover, the growth of prostate cancer bone metastases was significantly inhibited by systemically injecting *miR-16* complexed with atelocollagen [64]. In lung cancer, systemic injection of a neutral lipid emulsion of *miR-34a* and *let-7* significantly decreased in tumor burden in a mouse model of non-small cell lung cancer (NSCLC) [65]. Interestingly, a promising drug called miravirsen (SPC3649) is under phase II clinical trial for treating hepatic cancer. Miravirsen is a 15-nucleotide locked nucleic acid-modified antisense oligonucleotide with high affinity and specificity to *miR-122*, an miRNA used by HCV for hepatic cells infection [66].

Further studies *in vitro* and in animal models concerning the functional role of miRNAs and the impact of modulating oncomiR endogenous levels in ATC are one key to their future application as therapeutic adjuvant treatments. In particular, ATC patients would benefit from the concomitant reintroduction of tumor suppressor miRNAs *miR-200* and *miR-30* and the inhibition of the oncogenic *miR-146*, *miR-221*/*miR-222*, and *miR-17-92* clusters, as these miRNAs target several deregulated processes such as cell growth, invasion, migration, and EMT.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Anaplastic Thyroid Carcinoma: Current Treatments and Potential New Therapeutic Options with Emphasis on TfR1/CD71

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Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human cancers. Actually, ATC is refractory to conventional therapies, including surgery, chemotherapy, radiotherapy, and radioiodine (^{131}I) therapy. Accordingly, genetic and molecular characterizations of ATC have been frequently and periodically reviewed in order to identify potential biological markers exploitable for target therapy. This review briefly focuses on main molecular events that characterize ATC and provides an update about preclinical studies. In addition, the overexpression of transferrin receptor 1 (TfR1/CD71) by neoplastic cells of ATC is emphasized in that it could represent a potential therapeutic target. In this regard, new therapeutic approaches based on the use of monoclonal or recombinant antibodies, or transferrin-gallium-TfR1/CD71 molecular complexes, or lastly small interfering RNAs (siRNAs) are proposed.

1. Introduction

Thyroid cancer represents the most frequent malignancy among all endocrine tumors [1]. Well-differentiated thyroid carcinomas, including papillary (PTC) and follicular (FTC) carcinomas, are characterized by a favorable prognosis, while undifferentiated/anaplastic carcinoma (ATC) is an uncommon and highly aggressive form, which usually results in the death of the patient [2–4]. The 5-year survival ranges from 0 to 14%, with a median survival of 2–6 months [5–9]. ATC arises more commonly in female patients, with a mean age of 70 years, usually affected by nodular goiters or with a history of well-differentiated thyroid carcinoma or with nodal or distant metastases [3]. The patients usually complain of hoarseness due to a large-sized and rapidly expanding neck mass, which, at the time of presentation, is often surgically unresectable due to the invasion of surrounding thyroid structures, such as the laryngeal nerve, esophagus and trachea, and/or documentation of distant metastases [3]. The most important prognostic factor is the degree of the extent of disease at diagnosis. Small-sized ATCs or foci of ATC arising in the context of well-differentiated thyroid carcinomas have a better prognosis [9–11]. Obviously the prognosis also depends on the ability to eradicate the disease by surgery [7, 12]. In fact, if the eradication surgery is associated with radiotherapy and adjuvant or neoadjuvant chemotherapy with doxorubicin, survival may slightly increase [7, 9, 13–15]. Unfortunately wide surgical resection usually fails to provide benefits due to the local spread of tumor, while tracheostomy is often performed to ensure the patent of upper airway, invaded and/or obstructed by massive tumor [3]. Grossly, thyroid parenchyma is widely or completely replaced by a fleshy mass, whitish in color, with multiple areas of necrosis and hemorrhage, which diffusely infiltrates adjacent tissues [3, 5, 6]. Histologically, the tumor is composed of a variable mixture of spindled, epithelioid, and large pleomorphic/bizarre giant cells exhibiting different growth patterns such as solid, trabecular, and fascicular patterns [2, 3, 5, 6, 10]. The overall appearance of ATC is usually closely reminiscent of a high-grade pleomorphic

sarcoma. Mitotic figures are frequently observed, including atypical mitoses. Hemorrhage and necrosis, sometimes with palisading configuration, are often seen [10]. There may be an inflammatory infiltrate, predominantly of granulocytes, which occasionally can invade the cytoplasm of tumor cells. Although the above mentioned features represent the common basic morphological aspects of ATC, several morphological variants have been described over time, some of which appear to be rather uncommon [16]: (i) squamous cell carcinoma variant (tumor consisting of dominant/pure squamous differentiation); (ii) adenosquamous carcinoma variant (in addition to squamous differentiation, tumor contains foci of glandular differentiation with mucin production); (iii) lymphoepithelioma-like carcinoma variant (tumor sharing morphological features with the nasopharyngeal undifferentiated carcinoma); (iv) rhabdoid variant (tumor exhibits cells with clear-cut rhabdoid morphology); (v) osteoclastic variant (tumor contains reactive CD68+ osteoclast-like multinucleated giant cells intermixed to cancer cells); (vi) carcinosarcoma variant (tumor with a mixture of carcinomatous and heterologous mesenchymal components, such as cartilage, bone, or skeletal differentiation); (vii) paucicellular variant (hypocellular tumor with diffuse sclerosis, mimicking Riedel thyroiditis); (viii) angiomatoid variant (tumor mimicking angiosarcoma). Despite the poor morphological differentiation, the epithelial nature of ATC is demonstrable in 45–80% of cases by staining for cytokeratins, especially using cytokeratin AE1/AE3. Approximately half of the cases express epithelial membrane antigen (EMA). Only rarely there is TTF-1 expression, while thyroglobulin is almost invariably negative. Notably, a significant expression of TP53 is commonly observed [16].

As ATC is refractory to conventional chemotherapy, radiotherapy, and radioiodine (^{131}I) therapy [17], new therapeutic approaches are urgently needed in the future. In this regard, some original or review articles about genetic mutations, chromosomal instability, and identification of potential biomarkers exploitable against ATC are emerging in the literature [17–24]. However, while for PTC several potential gene and protein therapeutic targets have been identified [25–29], only a few options seem to be available for ATC in the literature [30]. Waiting for the advent of new genomewide approaches, such as next-generation sequencing (NGS), the analysis of the molecular mechanisms involved in the pathogenesis of ATC still remains the only available tool for planning any target therapy. There is increasing evidence that follicular cell-derived thyroid carcinomas represent a biological continuum of the same disease that progresses from the curable well-differentiated thyroid carcinomas (PTC and FTC) to fatal ATC. In fact, although ATC may derive *de novo*, many cases seem to arise from preexisting PTC or FTC [31–33]. This is supported by morphological evidence showing the gradual loss of papillary and follicular growth patterns associated with a concurrent increase in the presence of solid growth pattern, mitoses, necrosis, and nuclear pleomorphism that is typically observed in ATC. Moreover, most of ATCs exhibit residual foci of differentiated thyroid carcinoma, including both PTC and FTC [16]. Notably, ATC may also develop as a recurrence months or years after the removal of a well-differentiated neoplasm [5, 34]. Apart from this morphological evidence, it has been previously demonstrated that the development of chromosomal instability underlies the progression to more aggressive phenotypes of thyroid cancer [35]. Recurrent gains at 3p13-14 and 11q13 and loss of 5q11–31 were identified exclusively in ATC, suggesting they may be markers for anaplastic transformation [35].

For ATC with minor PTC or FTC components, it is likely that the mutations typically occurring in the latter tumors (e.g., RAS and BRAF mutations) may represent only early events in the tumorigenesis of ATC, while others, including TP53, catenin beta 1, and PIK3CA, may contribute later to the acquisition of a phenotype responsible for the extremely aggressive behavior of ATC [32, 36–38].

Generally, the genes coding proteins differently involved in the transduction pathway, such as RET, RAS, BRAF, PI3K, PTEN, and AKT, are mutated or aberrantly expressed in ATC, providing conditions for uncontrolled cellular proliferation and carcinogenesis via the MAP kinase pathway. RAS point mutations involving specific regions (codons 12, 13, and 61) of the three RAS oncogenes, H-RAS, K-RAS, and N-RAS, by activating both the MAP kinase pathway and the PI3K/AKT pathway, are associated with aggressive thyroid tumor phenotypes including ATC [39–42]. *BRAF*, which belongs to the RAF family of serine/threonine kinases, by regulating the MAP kinase/ERKs signaling pathway, affects cell division, differentiation, and secretion. The most frequent BRAF mutation involves nucleotide 1799 and results in substitution of valine for glutamate at residue 600 (V600E). This point mutation leads to constitutive activation of BRAF kinase and chronic stimulation of the MAPK pathway, playing tumorigenic activity for thyroid cells [30, 32, 43]. Inhibition of BRAF V600E by using vemurafenib has shown promising clinical responses in metastatic PTC [44]. Although BRAF mutation (V600E) is reported in approximately only 25% of ATC, suggesting its involvement in tumor progression together with other genetic markers, it could be exploitable as potential therapeutic target. In this regard a dramatic response to vemurafenib has been obtained in a 51-year-old man with BRAF-mutated anaplastic thyroid cancer [45]. This single case report provides evidence for testing ATCs for BRAF mutation (V600E), treating the positive cases by using vemurafenib. This approach could be suggested, at least, as empirical treatment in rapidly progressive cases. Anyway, the results need to be confirmed in larger series of ACTs.

Different alterations of PTEN/PI3K/AKT pathway that regulates several cellular processes, including cell cycle progression, adhesion, and motility, are also commonly observed in ATC, and then they could be exploitable as potential therapeutic targets. In this regard the missense mutations of *PIK3CA*, which encodes the p110α catalytic subunit of phosphatidylinositol 30-kinase (PI3K), have been frequently detected [32, 38, 46]. Aberrant activation of PI3K/Akt pathway has been suggested to promote progression of a thyroid adenoma to FTC and/or ATC [47], while activation of Akt has been observed in most of the ATCs with PIK3CA mutation [32, 38, 46].

Molecular mechanisms involved in tumor cell dedifferentiation are thought to be mediated by loss/inactivation or

mutation of tumor suppressor gene, *p53* [30, 32, 36, 38, 48–51]. It has been suggested that, unlike RAS and BRAF gene alterations, p53 mutations are crucial in accelerating genomic instability, triggering tumor dedifferentiation toward ATC [36, 51]. Redifferentiation of tissues from ATC upon the reintroduction of wild type p53 and the restoration of cellular responsiveness to physiologic stimuli, such as thyroid stimulating hormone and reexpression of thyroid peroxidase [38, 52], strongly support this hypothesis.

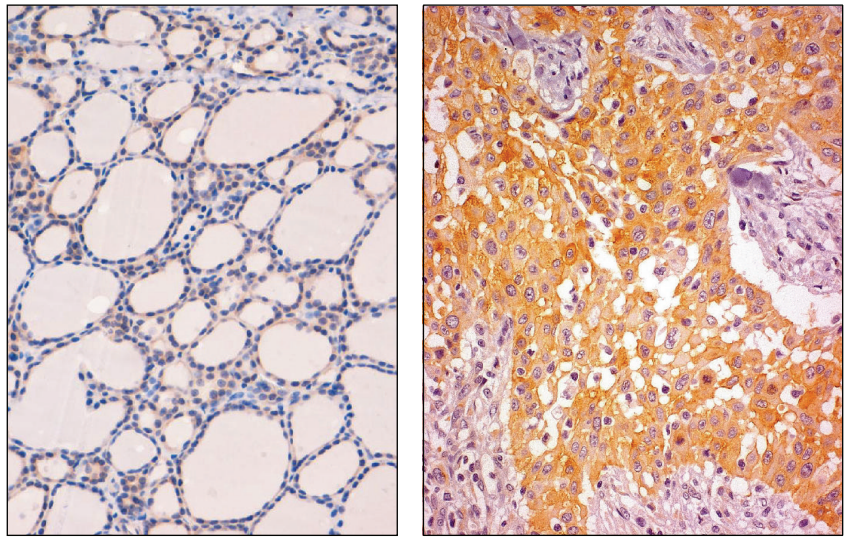
The biological process of dedifferentiation from well-differentiated thyroid carcinomas toward ATC is also underlined by *β-catenin* expression. β-Catenin acts as cell-cell adhesion molecule that complexes with E-cadherin proteins in normal epithelium. Derangement of the E-cadherin/catenin complex, as well as low membrane β-catenin expression or its nuclear localization, is associated with transformation of differentiated carcinomas into ATC [38, 53–57].

New diagnostic and therapeutic opportunities are emerging by the analysis of *microRNAs* (miRNAs). miRNAs are a heterogeneous class of small noncoding but functional RNAs involved in posttranscriptional regulation of target genes, playing a control role in development, proliferation, apoptosis, and stress response [58, 59]. As miRNA expression is frequently altered in several tumors, they are recently emerging as promising prognostic biomarkers and therapeutic agents for many tumors [60, 61]. Specific miRNA profiles have been associated with thyroid tumors [62–66]. Visone et al. [63] by performing miRNA-chip-microarray analysis demonstrated aberrant miRNA expression profile, especially decrease of some of them (miRNAs-30d, -125b, -26a, and 30a-5p), which clearly differentiates ATC from normal thyroid tissues and PTC. Subsequently, Mitomo et al. [64] confirmed downregulation of some miRNAs, such as -26a and -138, but they also noticed upregulation of others, including miRNAs-21, -146b, -221, and -222. The association of specific miRNAs deregulation with ATC transformation is the rational approach for further challenging investigations. In fact, miRNA-125b has a different expression in the human tumors, being upregulated in pancreas and stomach carcinomas, whereas it is downregulated in breast cancer and ATC, suggesting that it can act in different ways depending on the cellular context [67]. Moreover, miRNA-125b and others, which significantly decreased in ATC [63], have, among the predicted regulated target genes, also HMGA1 and HMGA2, which are proteins expressed at very high levels in several malignant tumors, including thyroid carcinomas [68, 69]. Again, miRNA-21, described to be upregulated [64], targets E2F (involved in cell cycle and apoptosis) and inhibits PTEN; miRNA-138, found to be downregulated [63, 64], targets the human telomerase reverse transcriptase (hTERT) gene which is also found to be totally downregulated in both ATC and PTC cell lines in comparison with normal thyroid tissues [64]. Nevertheless, miRNAs are emerging as promising new strategy with therapeutic potential for many aggressive cancers, such as ATC.

Preclinical studies, through *in vitro* and *in vivo* analyses, are providing helpful information in the therapeutic approach of ATC, especially the analysis of the mouse model closely recapitulating the clinic-pathological features of human ATC. While most genetically engineered mouse models gave significant advancements about differentiated thyroid carcinomas, such as PTC [70–72] and FTC [73–76], only recently ATC mouse models have been developed.

Antico Arciuch et al. [77] firstly obtained a mouse model of ATC by combining, in the mouse thyroid follicular cells, two molecular hallmarks of human ATC, namely, activation of PI3K (via Pten deletion) and inactivation of p53. By the age of 9 months, over 75% of the compound mutant mice developed aggressive, undifferentiated thyroid tumors, displaying all the features of their human counterpart, including pleomorphism, epithelial-mesenchymal transition, aneuploidy, local invasion, and distant metastases. It was shown that the tumors developing in this animal model undergo the glycolytic shift known as Warburg effect and are highly sensitive to the therapeutic use of glycolytic inhibitors, which synergize with standard chemotherapy [77]. Later, Nehs et al. [78] elegantly demonstrated the remarkable efficacy of PLX4720 compound, which is an ATP analog that selectively inhibits B-Raf^{V600E} by stabilizing it in an inactive conformation [79], to induce significant regression in an orthotopic mouse model of ATC even when administered at a very late therapeutic intervention stage. This result seems to be particularly promising. It is well known, in fact, that ATC tends to be resistant to traditional approaches such as standard chemotherapy, radiation, and radioiodine (^{131}I) due to loss of the sodium iodide symporter through malignant dedifferentiation [80]. If downregulation of BRaf with anti-BRaf^{V600E} therapy also causes sodium iodide symporter upregulation, as suggested by *in vitro* data, it could be expected that patients treated with anti-B-Raf^{V600E} therapy may undergo both reduction of tumor size/invasiveness and possible redifferentiation, thus making radioactive iodine administration possible to control an additional metastatic burden [78, 81–83]. A detailed description of an approach establishing an orthotopic mouse model of ATC has been reported by Sewell et al. [84], which mainly emphasized thyroid tumor metastasis and disease related cachexia and respiratory distress. Just recently, McFadden et al. [85] have genetically engineered a mouse model of BRAF-mutant ATC and demonstrated that combination treatment with MEK and BRAF inhibitors results in enhanced antitumor activity as compared to treatment with a BRAF inhibitor alone, suggesting that this combination could be useful as a component of treatment regimens also in human.

Given these results, it must be stressed that the animal model is a tool of unquestionable benefit for the development of appropriate therapeutic approach against complex diseases. Orthotopic mouse models seem to be ideal and commonly used for preclinical and translational studies of compounds and therapies, not only because of the fact that they may mimic key aspects of human diseases (e.g., metastasis), but also because of their reproducibility and the possibility to evaluate systemic effects of treatments [86]. Thus, even if aggressive thyroid tumors, such as ATC or poorly differentiated thyroid carcinoma, carry several complex genetic alterations, likely explaining disease progression



(a) (b)

Figure 1: (a) Cells of a nodular goiter showing a weak and cytoplasmic staining for TfR1/CD71. (b) In ATC TfR1/CD71 is diffusely expressed both in the cytoplasm and in the cell surface of neoplastic cells.

and resistance to single-compound approaches, orthotopic models of human thyroid cancer also hold the potential to be good models for testing novel combinatorial therapies [86].

2. TfR1/CD71: A Potential Therapeutic Target

Among the biomarkers which have been identified in ATC, by using different approaches, and that can be exploitable as potential therapeutic targets, we focus on type I receptor for transferrin (TfR1/CD71) [82]. TfR1, also known as CD71, is a type II cell membrane-associated glycoprotein involved in iron homeostasis and cell growth [87–89]. Although ubiquitously expressed on the cell surface, TfR1/CD71 is commonly upregulated in cells with high proliferative index, including cancer cells that need iron as cofactor of many enzymatic reactions, such as DNA synthesis [87–90]. TfR1/CD71 overexpression has been reported in several human malignant tumors, including lymphomas, carcinomas, neuroendocrine, and brain tumors [91]. Among carcinomas, TfR1/CD71 overexpression has been documented in colon, stomach, pancreas, breast, lung, liver, bladder, oral cavity, and uterus [91]. Notably a close correlation of TfR1/CD71 expression level and tumor proliferation index, histological grading, stage, and prognosis has also been largely demonstrated [87–91].

Based on our previous studies which showed an increased transferrin expression by PTC cells in comparison with thyroid cells from benign tissues [25], we performed PCR, western blotting, and immunohistochemical studies on fresh, paraffin-embedded thyroid tissues, as well as in thyroid cell lines, to assess whether TfR1/CD71, the receptor for transferrin, is upregulated in malignant thyroid tumors [91]. Conventional RT-PCR revealed the presence of TfR1/CD71 mRNA in all thyroid samples examined, suggesting that this receptor is transcribed in both benign and malignant tissues but differently expressed in malignant versus

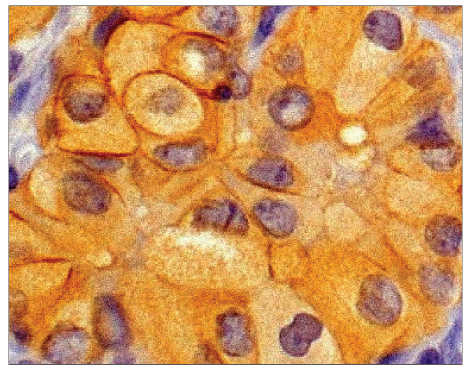
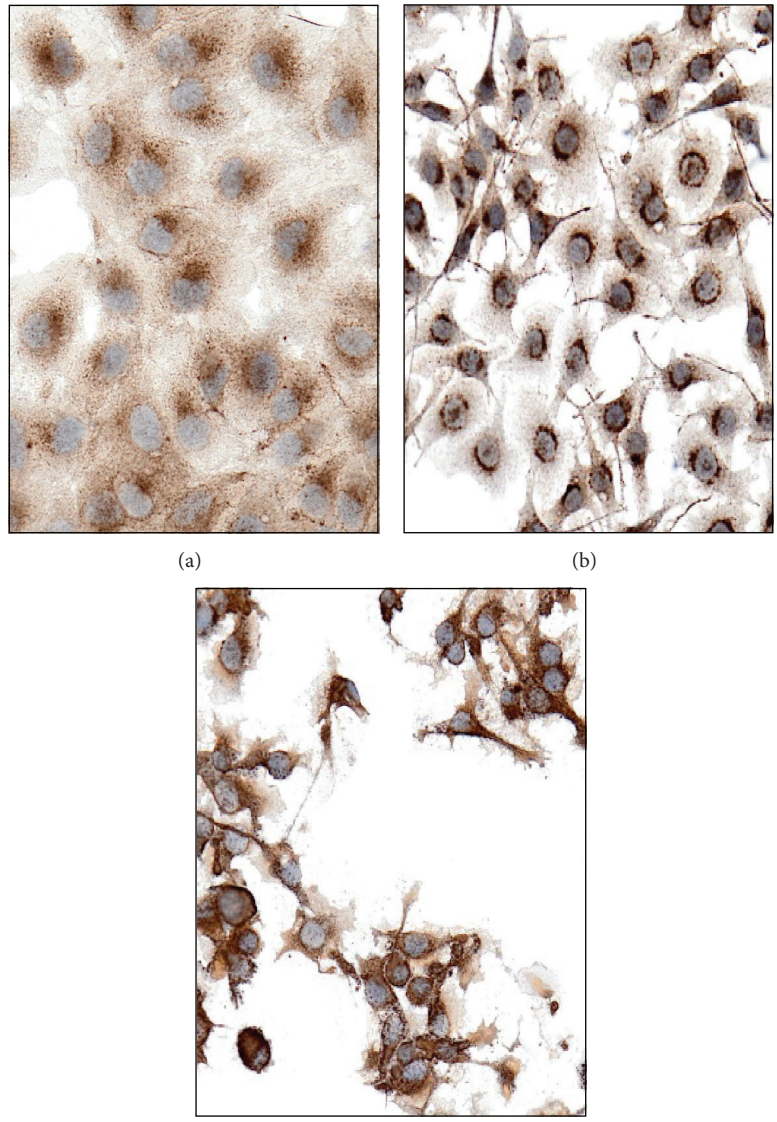


Figure 2: Higher magnification showing concurrent cytoplasmic and cell membrane immunostaining for TfR1/CD71 in ATC.

benign tissues. In fact, western blot analyses showed that although TfR1/CD71 protein was detected in all examined samples, its relative abundance appeared substantially higher in malignant tissues, especially PTC and ATC, when compared to their benign counterparts. Immunohistochemical results paralleled the findings of western blot, revealing an overall overexpression of the receptor in malignant tissues as compared to benign tissues which, by contrast, did not or only weakly and focally showed low levels of expression [91]. All the above mentioned findings suggest that synthesis and membrane incorporation of TfR1/CD71 occur at low levels in normal thyroid tissues, whereas it becomes part of an aberrant gene/protein expression pattern upon neoplastic transformation and malignant progression [91]. In particular, TfR1/CD71 overexpression was observed in all cases of ATCs tested (10 out 10 cases), and similarly to most cases of PTC, a combined strong and diffuse cytoplasmic, as well as, cell membrane immunostaining was observed



(a) (b) (c)

Figure 3: TfR1/CD71 is differentially expressed in thyroid tumor cell lines: increasing cytoplasmic expression is seen in neoplastic cells from PTC (a), FTC (b), and ATC (c).

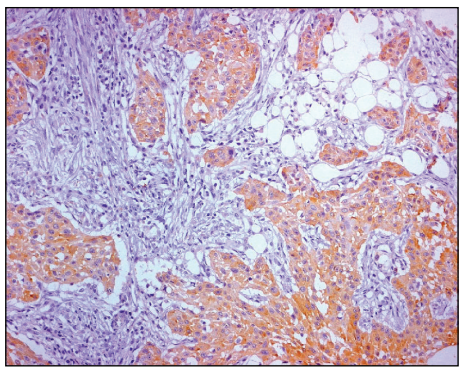


Figure 4: An example of ATC: transferrin is diffusely and strongly expressed in the cytoplasm of neoplastic cells.

(Figures 1 and 2) [91]. Similar results were also obtained in ATC cell lines (Figure 3). Our unpublished immunohistochemical data, showing that most neoplastic cells of ATC exhibit strong and diffuse cytoplasmic staining for transferrin (Figure 4), suggest that the overexpression of this protein concurs with the increased expression of its cognate receptor. Thus, it could be speculated that, as already seen in PTC [25], an autocrine and/or paracrine regulatory loop of transferrin-TfR1/CD71 does exist also in ATC. However we admit that this hypothesis needs to be confirmed by mRNA analyses to exclude the possibility that immunohistochemically detected transferrin can derive from internalized protein present in blood or outside the cell through interaction with its receptor. Interestingly, more than 30 years ago, it was largely known that gallium-67 citrate scintigraphy was helpful in identifying

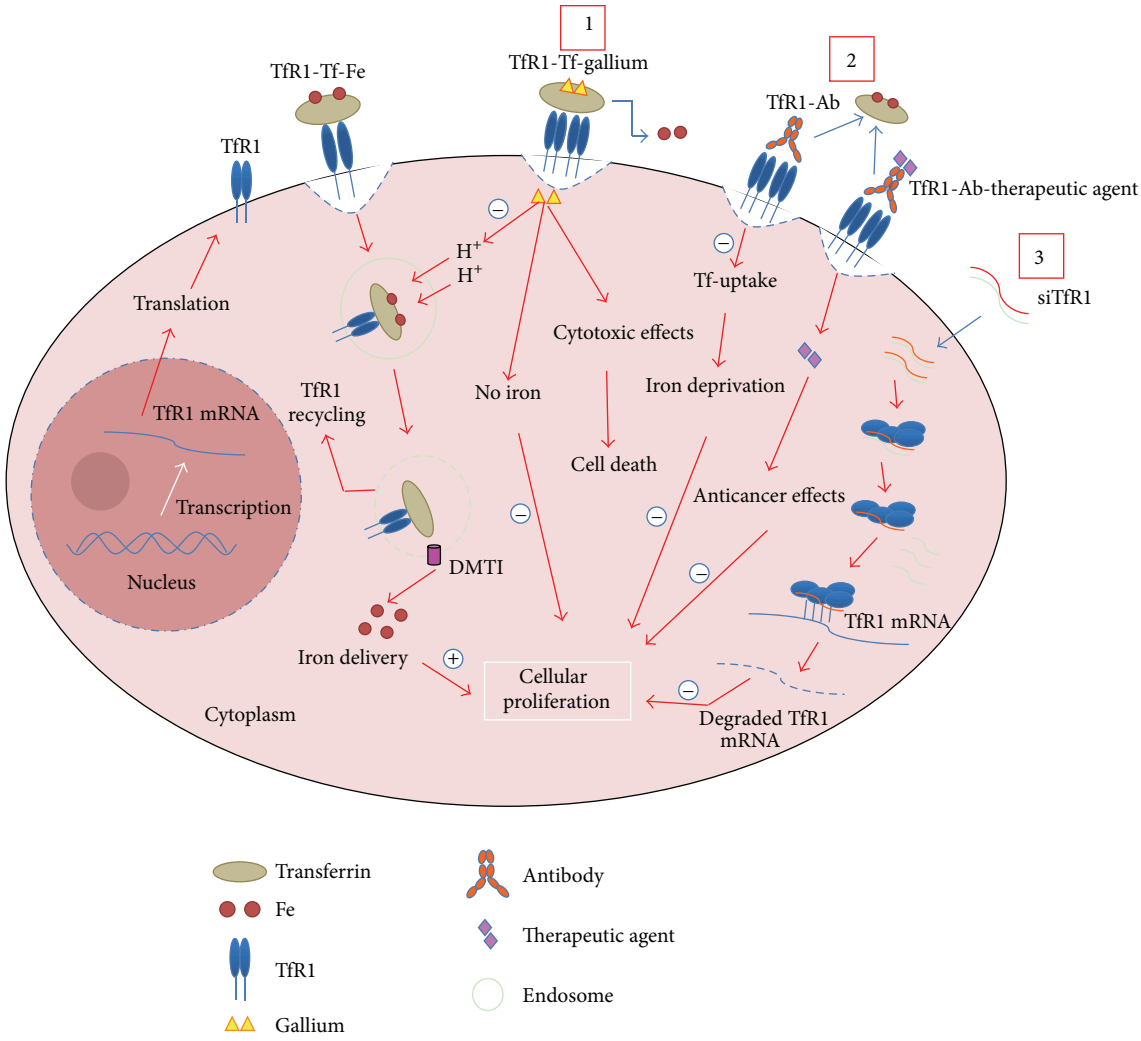


FIGURE 5: The diagram shows cellular uptake of iron by internalizing the transferrin-iron complex through TfR1-mediated endocytosis and summarizes the main therapeutic strategies through TfR1. 1: gallium binds avidly to transferrin, competing with iron (Fe), to form transferrin-gallium complexes, which in turn binds TfR1/CD71 on the surface of neoplastic cells containing high density of TfR1. Gallium, delivered in the cytoplasm, may be capable of interfering with the intracellular release of iron from the endosome to cellular compartments. Gallium also has direct cytotoxic effects resulting in cell death. 2: monoclonal antibody (Ab) against TfR1 binds TfR1 on the cell surface overexpressing the receptor, like in tumor cells, by competing or not with Fe-transferrin complexes depending on the location of the epitope on the receptor to which the antibody binds. This activity, in turn, results in iron deprivation and cell proliferation inhibition. Other types of antibodies have been engineered to deliver therapeutic agents with anticancer effects. 3: small interfering RNA (siRNA) approach. siRNA, alone or complexed, by transit across cellular membrane, are delivered to cytoplasm, where by classical Dicer-RISC-pathway-unwinding-mRNA recognition-cleavage-mRNA degradation could drive TfR1 downregulation. Both three proposed mechanisms potentially conduct to the reduction of iron in the cytoplasm of neoplastic cell and thus to the inhibition of cellular proliferation.

malignant thyroid tumors, especially primary and metastatic ATCs [92, 93]. At that time, it was hypothesized that there was a close correlation between gallium-67 uptake and degree of malignancy of thyroid tumor cells, even if the mechanism of tumor localization of gallium-67 was still unclear [92, 93]. Nowadays it is commonly accepted that gallium-67 citrate is preferentially uptaken by high-grade malignant tumors, through its ability to bind, in place of transferrin, TfR1/CD71 [94–96]. This is supported by the evidence that only malignant tumors that overexpressed TfR1/CD71 at a tissue level were also marked *in vivo* by gallium [97–99]. Based on our findings, it is likely that the high uptake of gallium-67 citrate by ATC cells might be explained by the high expression of TfR1/CD71 in this aggressive neoplasm.

Because of its upregulation in malignant tissues, extracellular accessibility, and constitutive ability to internalize into cells, TfR1/CD71 is currently attracting wide interest as potential direct or indirect therapeutic target [87–89, 91]. Firstly, TfR1/CD71 can be targeted by direct interaction with conjugates of its ligand transferrin (Tf), the iron transporting protein. In this regard, gallium nitrate binds avidly to transferrin to form transferrin-gallium complexes, which

in turn bind TfR1/CD71 on the surface of neoplastic cells [94, 100] (Figure 5). Gallium nitrate or alternatively gallium compounds, a group IIIa metal salt, have been described to inhibit the proliferation of tumor cells *in vitro* and *in vivo* [101]. Antitumoural activity by novel organogallium (III) through induction of apoptosis has also been described in 8505C anaplastic thyroid cancer cell line [102].

Gallium nitrate cytotoxicity may be due to its capability to interfere with the release of iron from endocytic vesicles, depending on not only TfR1/CD71 receptor density on cellular surface but also TfR1/CD71 cycling [95]. In addition, cytotoxicity of gallium may be due, at least in part, to the inhibition of iron uptake. Gallium nitrate binds transferrin and thus, iron cannot bind and is not taken up by the cell [103, 104]. Thus, the transferrin-gallium-TfR1/CD71 molecular complex may represent a promising therapeutic approach against ATC.

TfR1/CD71 can be also specifically targeted by monoclonal or recombinant antibodies (Figure 5). In this regard, there are two different types of antibodies: (i) directly cytotoxic antibodies and (ii) therapeutic agents delivery antibodies. The former, binding TfR1, inhibit the function of the receptor by inducing its sequestration and subsequently degradation in sensitive cells. It has been shown that TfR1 level reduction on the cell surface results in decreased transferrin uptake and induction of lethal iron deprivation (LID) in hematopoietic malignancies [105, 106].

Other monoclonal or recombinant antibodies have been developed to target TfR1/CD71 for delivering chemotherapeutic drugs, protein toxins, radionuclides, liposomes, modified viral particles, and nanoparticles to kill malignant cells [87–89, 107, 108] (Figure 5). The combinations of such antibodies against TfR on human tumor cells have been demonstrated to have antiproliferative effects both *in vitro* and *in vivo* [106, 109–114].

Lastly, TfR1/CD71 may be exploitable as specific target for small interfering RNA (siRNA) approach (Figure 5). This technology represents a powerful genetic tool for sequence-specific inhibition of target proteins capable of modulating cell growth, apoptosis, chemoresistance, and chemosensitivity. In this regard the use of transferrin should ensure specific targeting of siRNA-containing complexes to ATC cells in situ and the consequent uptake by TfR1/CD7-mediated endocytosis of the delivered particles. Recently a siRNA clinical trial has successfully targeted nanoparticles containing transferrin, which engage TfR on the surface of cutaneous melanoma cells [115]. However, the targeting specificity reported in this study has been questioned by other authors who failed to demonstrate the overexpression of TfR1/CD71 in a large series of cutaneous melanomas [116]. Accordingly it was contemplated the possibility that neoplastic cells might internalize nanoparticles conjugated with transferrin through a mechanism independent of the activity of the cognate receptor [116].

In conclusion, although these potential TfR1/CD71-based therapeutic strategies appear to be intriguing, the question of whether this receptor will remain accessible *in vivo* in ATC is still to be elucidated. For these reasons we advise that future *in vitro* and preclinical studies will be performed to confirm the idea of using TfR1/CD71 as a meaningful molecule for target therapy against ATC, which continues to be one the most aggressive human tumors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

A New Aurora in Anaplastic Thyroid Cancer Therapy

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Anaplastic thyroid cancers (ATC) are among the most aggressive human neoplasms with a dire prognosis and a median survival time of few months from the diagnosis. The complete absence of effective therapies for ATC renders the identification of novel therapeutic approaches sorely needed. Chromosomal instability, a feature of all human cancers, is thought to represent a major driving force in thyroid cancer progression and a number of mitotic kinases showing a deregulated expression in malignant thyroid tissues are now held responsible for thyroid tumor aneuploidy. These include the three members of the Aurora family (Aurora-A, Aurora-B, and Aurora-C), serine/threonine kinases that regulate multiple aspects of chromosome segregation and cytokinesis. Over the last few years, several small molecule inhibitors targeting Aurora kinases were developed, which showed promising antitumor effects against a variety of human cancers, including ATC, in preclinical studies. Several of these molecules are now being evaluated in phase I/II clinical trials against advanced solid and hematological malignancies. In the present review we will describe the structure, expression, and mitotic functions of the Aurora kinases, their implications in human cancer progression, with particular regard to ATC, and the effects of their functional inhibition on malignant cell proliferation.

1. Aurora Kinases: From Genes to Proteins

The Aurora kinases belong to a family of serine/threonine kinases having in the Ipl1p (increase in ploidy 1) gene, subsequently named Aurora gene, the founding member discovered in the budding yeast *Saccharomyces cerevisiae* during a genetic screening for mutations causing defective chromosomal segregation [1]. In yeast, the Ipl1 remains the only Aurora kinase so far identified, while two Aurora kinases have been found in *Drosophila melanogaster* and in *Caenorhabditis elegans* [2–4]. In mammals, three Aurora kinases have been identified and characterized: Aurora-A, Aurora-B, and Aurora-C [5]. The catalytic domains of these three proteins are highly related in sequence, showing 67–76% identity, but their N-terminal domains have little similarity, which is held responsible for their distinct intracellular localizations, substrate specificity, and functions (Figure 1). In addition, the amino acid sequence of the catalytic domains of Aurora-A, Aurora-B, and Aurora-C is highly conserved across different organisms suggesting its relevance for protein functions and regulation mechanisms across species [5]. The expression of all three human Aurora kinases is cell cycle regulated being low in the G1/S phase and maximal in the G2/M phase. In the next three paragraphs, we will briefly summarize our knowledge regarding the characteristics of the Auroras' encoding genes, their promoter regulation, and protein structure.

1.1. Aurora-A. The Aurora-A is encoded by the AURKA gene (also known as AIK, Aurora/IPL1-like kinase; ARK1, Aurora related kinase 1; AURA, AURORA2; BTAK, breast tumor-amplified kinase; PPP1R47, protein phosphatase 1 regulatory subunit 47; STK15, serine/threonine-protein kinase 15; STK6, serine/threonine kinase 6), located at 20q13.2 and consisting of 11 exons (Gene ID: 6790).

The AURKA promoter contains a putative TATA-box at −37 to −14 and two CCAAT-boxes at −101 to −88 and at −69 to −56 (Eukaryotic Promoter Database, Swiss Institute of Bioinformatics). Tanaka and colleagues analyzed the 1.8 kb 5′-flanking region of the Aurora-A gene and found two distinct *cis*-regulatory elements: one positively regulates transcription of the Aurora-A gene, while the other is a cell cycle dependent transcriptional repressor [6]. The former is the 7 bp sequence CTTCCGG, located at −85 to −79 and essential for the transcription of the Aurora-A gene, which is bound by the E4TF1, a ubiquitously expressed ETS family protein. The cell cycle

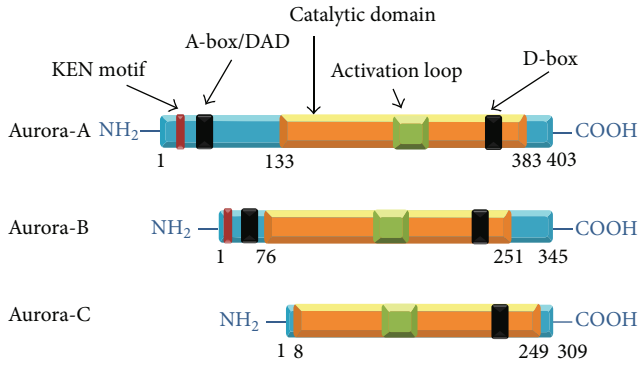

Figure 1: Schematic representation of Aurora kinase proteins. D-box, destruction box; DAD, D-box activating domain; KEN motif, amino acidic K-E-N which serves as targeting signal for the Cdh1-anaphase promoting complex.

dependent transcriptional repressor is formed by tandem repression elements, consisting of a cell cycle dependent element (CDE) located at −44 to −40 (CGCCC) and a cell cycle gene homology region (CHR) located at −39 to −35 (TTAAA) [6]. It is worth mentioning that, in addition to Aurora-A, the G2/M specific transcription of different genes such as cyclin A, cdc25C, cdc2, polo-like kinase, and others is regulated by similar tandem repression elements [6–9]. Over the last few years, a number of transcription factors capable of modulating the expression of AURKA gene have been identified. These include the p53, the HIF-1, and the INI1/hSSNF5, all reported to negatively regulate the activity of the AURKA promoter [10–12]. Conversely, the transcriptional activity of the AURKA promoter has been shown to increase following the interaction with the ΔEGFR/STAT5 complex in glioblastoma cells [13]. Moreover, the oncogene MYCN, a member of the MYC family of basic helix-loop-helix transcription factors, has been described to bind the AURKA promoter either alone or in complex with the DNA methyl binding protein MeCP2 in the neuroblastoma derived cell line Kelly [14]. EWS-Fli1, a fusion gene resulting from the chromosomal translocation (11; 22, q24; q12), encodes a transcriptional activator capable of promoting cellular transformation in Ewing sarcoma cells. Experimental evidence showed that EWS-Fli1 protein upregulates Aurora-A and Aurora-B expression by binding to regulatory ETS-binding sites located at −84 and −71 bp upstream of the transcription initiation sites in both Aurora-A and Aurora-B promoters [15]. Similarly, it has been suggested that activation of the MAPK in pancreatic cancer cells leads to the transcriptional activation of the AURKA and AURKB promoters via ETS2 transcription factors [16].

The 2.4 kb transcript from AURKA gene encodes a protein of 403 amino acids with a predicted molecular mass of 45.8 kDa (Figure 1). Like all Aurora proteins, it is characterized by a C-terminal catalytic domain containing the activation loop. In the latter, an Aurora kinase signature (xRxTxCGTx) is present in which the autophosphorylation of the Thr288 is required for kinase activation [17]. In addition, the Thr288 is positioned within a protein kinase A (PKA) consensus sequence, and *in vitro* experiments indicated a potential role of PKA in Aurora-A phosphorylation [18, 19]. The phosphatase PP1 has been shown to dephosphorylate and inactivate Aurora-A [19]. The C-terminal located destruction box (D-box), containing the motif RxxLxxG, and the N-terminal A-box/D-box activating domain (DAD), containing the motif RxLxPS, play an essential role in Aurora-A degradation by the anaphase promoting complex/cyclosome- (APC/C-) ubiquitin-proteasome pathway. Aurora-A degradation occurs in late mitosis/early G1 phase, when the D-box is targeted by Fizzy related proteins that transiently interact with the APC, and is hCdh1 dependent [18–21]. In the N-terminal region the amino acidic sequence K-E-N, known as KEN motif, is also present, which serves as targeting signal for Cdh1-APC required also for the degradation of other mitotic proteins such as Nek2 and B99 [22]. However, this does not seem to be crucial for Aurora-A degradation [22].

1.2. Aurora-B. The Aurora-B is encoded by the AURKB gene (also known as AIK2; AIM1; ARK2; AurB; IPL1; STK5; AIM-1; STK12), mapped to chromosome 17p13.1, and consisting of 9 exons (Gene ID: 9212).

The AURKB promoter contains three putative CAAT-boxes at −99 to −86, at −66 to −53, and at −30 to −17 (Eukaryotic Promoter Database, Swiss Institute of Bioinformatics). By primer extension two major transcription initiation sites were identified [23]. As for the Aurora-A promoter, also the Aurora-B promoter possesses the CDE and CHR elements, though responsible for the cell cycle regulation of its expression, and several CDE-binding proteins have been identified by means of electrophoretic mobility shift assay and biotin-streptavidin pull-down assay, including the E2F-1, E2F-4, and DP-2 [23]. As above described, the AURKB promoter may be positively regulated by transcription factors such as the ETS2 via ETS-binding sites present in the promoter sequence [15, 16].

The 1.4 kb transcript encodes a protein of 345 amino acids with a predicted molecular mass of 39 kDa (Figure 1) [5]. As described for Aurora-A, also the Aurora-B protein is characterized by a catalytic domain, a C-terminal D-box, and an N-terminal A-box/DAD [18–21]. However, although Aurora-B possesses the same D-box as Aurora-A, it is not degraded by the same ubiquitin ligase but undergoes degradation following its binding to the human proteasome α-subunit C8 in a proteasome dependent manner [24].

1.3. Aurora-C. The Aurora-C is encoded by the AURKC gene (also known as AIE2; AIK3; ARK3; AurC; SPGF5; STK13), which localizes at chromosome 19q13.43 and consists of 7 exons (Gene ID: 6795).

The AURKC promoter contains a CCAAT-box at −36 to −23 (Eukaryotic Promoter Database, Swiss Institute of Bioinformatics). Expression of AURKC is downregulated by PLZF, a transcriptional repressor, through recruitment to its promoter region, and it is of interest to note that the expression levels of PLZF and AURKC mRNAs display opposite patterns in human cervical and colorectal cancers [25].

The 1.3 kb transcript encodes a protein of 309 amino acids with a predicted molecular mass of 35.6 kDa (Figure 1)

[5]. As the other members of the family, also Aurora-C is characterized by a catalytic domain present in the C-terminal region of the molecule (Figure 1). However, differently from Aurora-A and Aurora-B, Aurora-C does not contain the KEN and the A-box/DAD motifs in its N-terminal region, while the C-terminal D-box is present. The mechanism(s) underlying its degradation, however, still remains to be elucidated and represents an interesting area of investigation.

2. Expression, Subcellular Localization, and Functions of the Aurora Kinases

The three Aurora kinases play relevant functions during the mitotic phase of the cell cycle [18, 19]. As mentioned, these proteins display distinct intracellular localizations, substrate specificity, and functions during mitosis and their expression and activity are tightly regulated at the transcriptional or posttranscriptional level, through phosphorylation/dephosphorylation and protein degradation [26]. In the next paragraphs, we will briefly discuss the main mitotic functions of the Aurora kinases and we will mention recent reports suggesting their extramitotic functions.

2.1. Aurora-A. The expression of Aurora-A is cell cycle regulated, being very low during the G1 phase and starting to accumulate at the centrosome in the late S phase to be maximal at the G2-M transition. During mitosis it localizes at the spindle poles to be inactivated and degraded before cytokinesis, as above mentioned [19, 22]. Aurora-A regulates centrosome separation and maturation, cell mitotic entry, and bipolar spindle construction. Centrosome recruitment of Aurora-A is controlled by the Cep192/Spd-2 (centrosome protein of 192 kDa/spindle defective 2) which is also involved in kinase activation during mitosis [27]. Once on the centrosome, Aurora-A promotes the recruitment to the pericentriolar mass (PCM) of a number of proteins required for proper centrosome maturation and function. These include centrosomin, γ-tubulin, LATS2 (large tumor suppressor, homolog 2), TACC3 (transforming acidic coiled coil 3), and NDEL1 (nuclear distribution element-like 1) [19, 22, 28].

While promoting centrosome maturation, Aurora-A activates the CDK1/cyclin B complex allowing the transition of the cell from the G2 to the M phase [29–31]. In particular, Aurora-A in association with the G2 induced Bora protein phosphorylates the PLK1 (polo-like kinase 1). Both Aurora-A and PLK1 phosphorylate CDC25B (cell division cycle 25 B), a member of the CDC25 family of phosphatases which activates cyclin dependent kinases by removing two phosphate groups, leading to CDK1/Cyclin B complex activation and finally promoting mitotic entry [19, 29–31]. PLK1 facilitates this process also by inactivating the CDK1 inhibitor WEE1 (Figure 2). Inactivation of Aurora-A or Plk1 individually shows no significant effect on Cdk1 activation and entry to mitosis, while their simultaneous inactivation produces a marked delay in both Cdk1 activation and mitotic entry, suggesting that the two kinases have redundant functions [32].

A central role of Aurora-A during mitosis is to support the microtubule-organizer center (MTOC) responsible for the formation of the bipolar spindle. In this context Aurora-A has been shown to form complexes with TACC1 and TACC3, which in turn, by binding to ch-TOG/XMAP215 proteins, stabilize microtubules at the centrosome [33–35]. In addition, Aurora-A interacts with and phosphorylates TPX2, which is capable of promoting spindle microtubule polymerization [22].

2.2. Aurora-B. Aurora-B mRNA and protein levels peak at G2/M phase, and the maximum kinase activity is reached from metaphase to the end of mitosis [18, 19]. Aurora-B exerts its action mainly in concert with three other proteins, that is, INCENP (inner centromere protein), survivin, and borealin/Dasra B, with which it associates in the chromosomal passenger complex (CPC). In early prophase, the CPC is located on chromosomal condensing arms where it seems to displace the heterochromatin protein-1 from DNA and to recruit condensin proteins (Figure 3) [36, 37]. From early G2 phase to prophase, Aurora-B phosphorylates histone H3, but its physiological meaning remains unclear. From late prophase to metaphase CPC localizes to the inner centromere, playing a role in formation and stability of the bipolar mitotic spindle, kinetochore assembly, correction of nonbipolar chromosome-spindle attachments, and control of the spindle checkpoint (Figure 3). At the beginning of anaphase CPC relocates to the midzone of the mitotic spindle and to the cell cortex, remaining evident in the midbody of telophasic cells where it modulates the activity of several proteins involved in spindle dynamics, cleavage furrow formation, and completion of cytokinesis (Figure 3) [18, 19, 36, 37].

Aurora-B activation requires its autophosphorylation and binding to INCENP, while all CPC components are necessary for its proper localization during mitosis. Several kinases, such as BubR1 and Bub1 (checkpoint kinases), Mps1 (monopolar spindle 1), Chk1 (checkpoint kinase 1), tousled-like kinase-1, Plk1, and TD-60/RCC2 (regulator of chromosome condensation 2), have been recently shown to be involved in Aurora-B activation. TD-60, a protein with a chromosomal passenger-like localization pattern, seems to be also important for centromeric localization of Aurora-B [38]. The phosphorylation status and activity of Aurora-B are modulated by PP1 and PP2A phosphatases; depending on the regulatory subunit they are bound to, these enzymes can directly modulate Aurora-B activity through dephosphorylation or they may dephosphorylate Aurora-B substrates [38].

2.3. Aurora-C. Expression of Aurora-C is maximal during the G2/M phase. The observation that Aurora-C is expressed at relative high levels in germ cells during spermatogenesis and oogenesis and at very low levels in somatic cells is of interest. Aurora-C is highly similar to Aurora-B in sequence (61% identity), which may explain why the two kinases display similar localization patterns and share interacting proteins and substrates such as INCENP, survivin, and borealin [18, 39]. Interestingly, when ectopically expressed in cells depleted of Aurora-B, Aurora-C is capable of rescuing the Aurora-B-dependent mitotic functions [40]. It is also worth noting that Aurora-C has been shown to interact with and phosphorylate

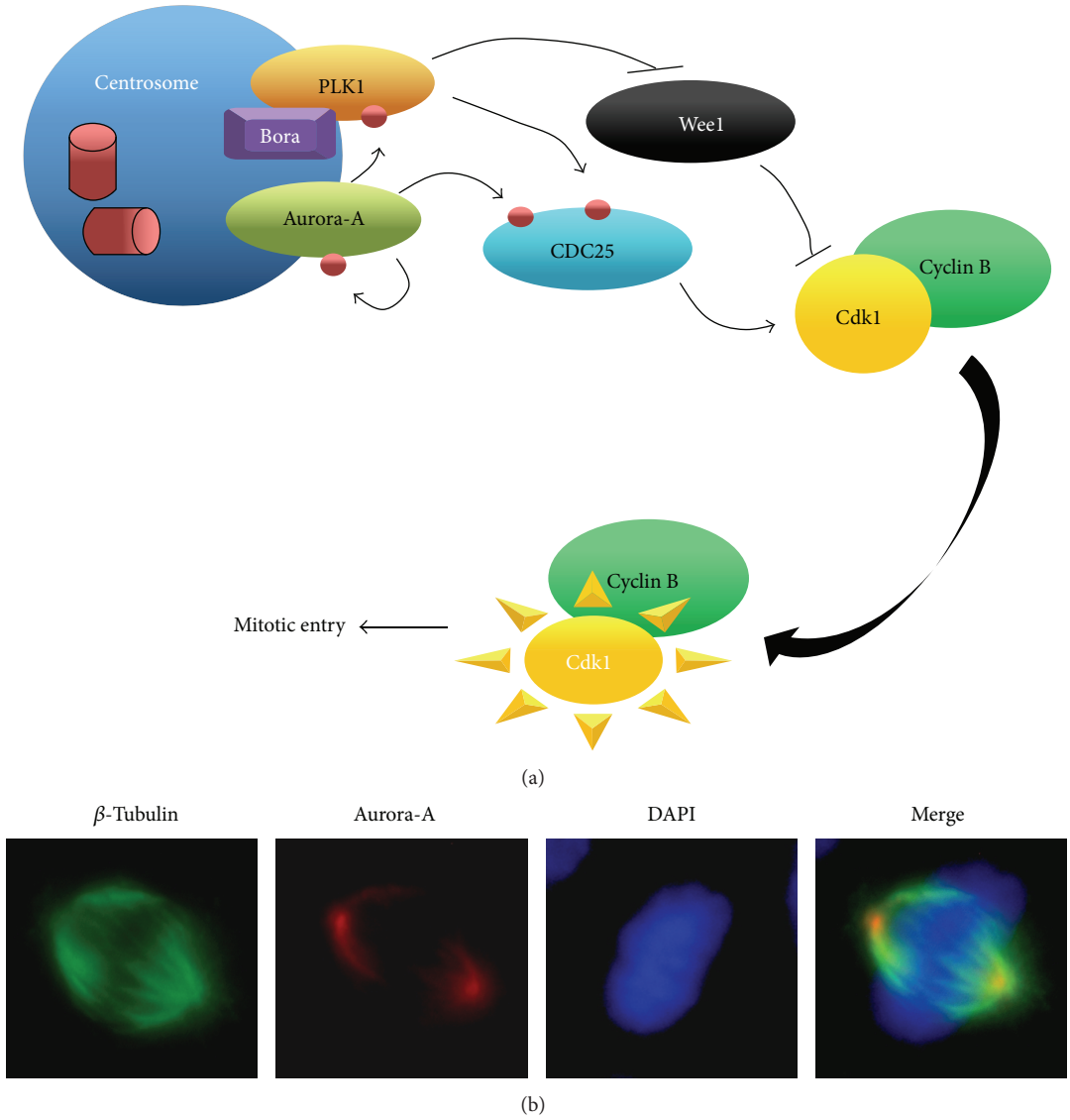

Figure 2: (a) Schematic representation of the pathway induced by Aurora-A to activate the CDK1/cyclin B complex allowing the transition of the cell from the G2 to the M phase. Aurora-A in association with Bora phosphorylates the PLK1. Both Aurora-A and PLK1 phosphorylate CDC25B (cell division cycle 25 B) allowing cyclin dependent kinase 1(CDK1)/cyclin B complex activation and thus promoting mitotic entry. PLK1 facilitates this process also by inhibiting the CDK1 inhibitor WEE1. Inactivation of Aurora-A or Plk1 individually shows no significant effect on Cdk1 activation and entry to mitosis, while their simultaneous inactivation produces a marked delay in both Cdk1 activation and mitotic entry, suggesting that the two kinases have redundant functions. (b) Immunofluorescence showing Aurora-A localization at the spindle pole of an anaplastic thyroid cancer cell in metaphase.

TACC1 in thyroid cells [40]. In the latter, TACC1 and Aurora-C have been shown to colocalize in the cytokinetic bridge [41].

2.4. Extramitotic Functions of Aurora Kinases. Over the last few years, different experimental findings suggest extramitotic functions for Aurora-A and Aurora-C. In particular, Aurora-A has been proposed to affect microtubule dynamics, cell migration and polarity, cilia disassembly, and regulation of intracellular calcium signaling in interphasic cells [22]. Regarding Aurora-C, it has been shown that, along with its interacting protein TACC1, it may be involved in telomere stability. In particular, TACC1 has been shown to bind the LSM7 and SmG proteins involved in telomere formation, while the TFR2 protein, a component of the telomeric complex shelterin, has been shown to form a complex with Aurora-C [42, 43]. Thus, it may be speculated that TACC1 and/or Aurora-C may contribute to telomere homeostasis.

3. Aurora Kinases and Cancer

Genetic instability, a hallmark of solid tumors, is thought to represent the mean by which premalignant cells acquire novel functional capabilities responsible for cancer cell growth and

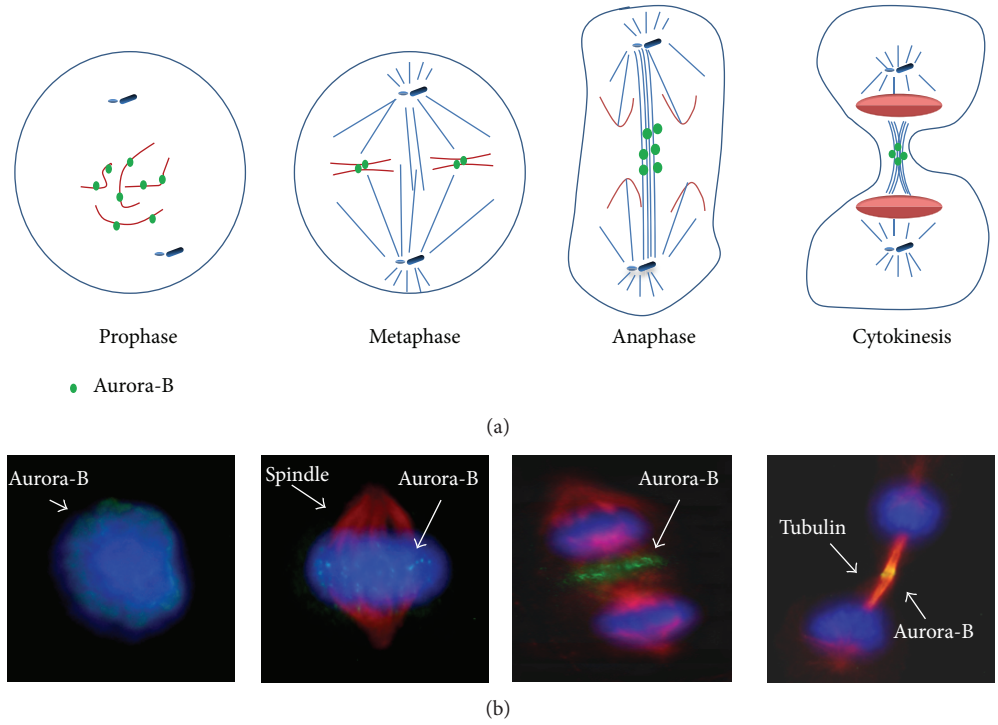

FIGURE 3: Schematic representation (a) and immunofluorescence images (b) of Aurora-B localization during mitosis in an anaplastic thyroid cancer cell. In (b) Aurora-B is in green, microtubules are in red, and DNA, stained by DAPI, is in blue.

tumour progression [44]. In fact, aberrations in chromosome number and structure characterize the majority of human cancers and follow alterations of cellular functions required for appropriate chromosome segregation and integrity of cellular checkpoints [45]. Given the crucial tasks of Aurora kinases in all mitotic stages, their dysfunction and/or dysregulation may well lead to abnormal cell divisions and aneuploidy. However, whether Aurora kinases have a role in cancer initiation is still a matter of debate. It has been reported that the overexpression of either Aurora-A, Aurora-B, or Aurora-C induces cell malignant transformation [46–48]. In different studies, however, although the ability of Aurora-A or Aurora-B to potentiate Ras-induced transformation was demonstrated, the transforming ability of either Aurora-A or Aurora-B overexpression alone was not observed [49, 50].

Aurora-A kinase has been often implicated in cancer progression and its hyperactivation has been demonstrated to induce resistance to microtubule-targeted chemotherapy [51–53]. The AURKA gene is often amplified in many malignancies, and its overexpression has been reported to be significantly associated with a higher tumor grade and a poor prognosis in a number of cancers, such as chondrosarcoma, nasopharyngeal carcinoma, ER-positive breast cancer, glioblastoma, colorectal cancer, gastric cancer, and endometrioid ovarian carcinoma [54–60]. In addition, somatic mutations located within the catalytic domain of Aurora-A, altering kinase activity and subcellular localization, have been described in human cancer cells [61]. The oncogenic potential of Aurora-A derives from a sum of several spatially and temporally distinct actions. Unlike normal cells, in many cancer cells the expression of Aurora-A becomes constitutive throughout the cytoplasm, regardless of the cell cycle phase; this can trigger a plethora of phosphorylated proteins, improper interactions, and mislocalization. Aurora-A may also represent the downstream target of mitogenic pathways, such as MAPK/ERK (mitogen-activated protein kinases), and hence be overexpressed because of their constitutive activation in tumors [52]. The Aurora-A excess interferes with different cell cycle checkpoints, that is, the late G2 checkpoint, which restrains genetically aberrant cells to enter mitosis, the spindle assembly checkpoint, which blocks the metaphase-anaphase transition in cells with defective spindles, and the postmitotic G1 checkpoint, which arrests cell cycle in aneuploid cells [51, 53]. Centrosome amplification and unrestrained multinucleation, leading to abnormal mitotic spindle, are also observed in Aurora-A overexpressing cells [62]. Moreover, Aurora-A may significantly contribute to tumor progression by interacting with and inhibiting several tumor suppressor proteins such as p53, BRCA1 (breast cancer 1), and Chfr (checkpoint with forkhead and ring finger domains).

Aurora-B plays a less clear role in tumorigenesis. An increased level of Aurora-B in normal cells has been shown to induce premature chromosome separation and segregation errors, to promote generation of tetraploid and aneuploid cells, which develop a transformed phenotype *in vitro* and *in vivo*, and, as above mentioned, to potentiate Ras oncogenic activity [47, 50, 63–65]. Neither amplification nor specific mutations of its gene have been shown in tumors; nevertheless, Aurora-B overexpression has been demonstrated in

Table 1: Aurora kinase inhibitors in clinical trials.

	Inhibitor (company) commercial name	Clinical trial
Pan-Aurora inhibitors	VX-680/MK-0457 (Vertex/Merck) Tozasertib	Phase II (terminated due to severe toxicity)
	PHA-739358 (Pfizer/Nerviano) Danusertib	Phase II
	PHA-680632 (Pfizer/Nerviano)	Phase II
	CYC-116 (Cyclacel)	Phase I
	SNS-314 (Sunesis)	Phase I
	R763 (Rigel)	Phase I
	AMG-900 (Amgen)	Phase I
	AT-9283 (Astex)	Phase II
	PF-03814375 (Pfizer)	Phase I
	GSK1070916 (GlaxoSmithKline)	Phase I
Aurora-A inhibitors	MLN8237 (Millennium)	Phase II
	EMD-2076 (EntreMed)	Phase II
	MK-5108 (Vertex)	Phase I
Aurora-B inhibitors	AZD1152 (AstraZeneca)	Phase II

several cancer types, like breast, colorectal, kidney, lung, and prostate cancer, and it has been reported to correlate with the level of genomic instability and with poor prognosis in advanced colorectal cancer, astrocytoma, head and neck squamous cell cancer, and endometrial and hepatocellular carcinomas [51, 63–65]. At present, very little information is available about the role of Aurora-C in cancers. Despite Aurora-C is hardly detected in normal somatic cells, it is highly expressed in various tumor cell lines [66–69]. One study has described the transforming potential of overexpressed Aurora-C in NIH-3T3 cells and a correlation between the level of active kinase and tumor aggressiveness of the cells injected in nude mice [48].

The overexpression of Aurora kinases in human cancers and their relevance in controlling the mitotic process have led to the development of small-molecule inhibitors as putative anticancer drugs. Aurora inhibition results in cytokinesis failure and generation of tetraploid cells, which, depending on the postmitotic checkpoint activation, may be unable to proceed in cell cycle or rather proliferate and become polyploid. The exit from cell cycle is likely to generate viable quiescent cells, whereas endoreplicating cells have greater tendency to undergo apoptosis. Nowadays the Aurora kinase inhibitors are considered a promising therapeutic option, especially against those cancers that do not respond to currently available anticancer therapies [70–77]. About 30 small molecule inhibitors of Aurora kinases are under preclinical and clinical evaluation with some of them, reported in Table 1, undergoing phase I-II clinical trials for solid and hematological cancers [70–77]. The responses obtained in these clinical trials showed either disease stabilization or less frequently partial responses in patients with solid cancers, while more promising activity has been observed in patients with hematological malignancies [70–77]. However, a number of side effects, most of which reversible upon drug withdrawal, were observed [74]. On-target toxicity encountered in the different clinical trials included grade 3/4 neutropenia, leukopenia, and myelosuppression, while off-target effects included hypertension, somnolence, mucositis, stomatitis, proctalgia, grade 3 increase in aspartate aminotransferase, and ventricular dysfunction [70–77]. Cardiotoxicity, associated with death of one patient, has been recorded in a phase II clinical trial with tozasertib (VX-680) [74].

4. Thyroid Cancers

Epithelial thyroid cancer (TC) accounts for about 1% of all human tumors and represents the most common endocrine malignancy, the fifth most common cancer in women in the United States [78, 79]. The majority of TC (90–95%) is differentiated carcinomas (DTC), occurring as papillary (PTC) or follicular (FTC) histotypes, the incidence of which has been increasing over recent years [80]. Following dedifferentiation DTC are assumed to generate the poorly DTC (PDTC) and the highly aggressive and invariably fatal anaplastic thyroid carcinomas (ATC) [81, 82]. Relevant molecular alterations encountered in DTC progression comprise gene rearrangements of tyrosine kinase receptors, such as the RET/PTC and NTRK1 (neurotrophic receptor-tyrosine kinase 1), or activating point mutations of proteins mediating cellular responses to growth and differentiation signals, including RAS, BRAF, phosphatidylinositol 3-kinase (PI3K), or the oncogenic fusion protein PAX8-PPARγ, as well as inactivating mutations in the tumor suppressor phosphatase and tensin homolog (PTEN) and TP53 [83–85]. The conversion of early-stage thyroid tumors to more aggressive and invasive malignancies occurs through an epithelial-to-mesenchymal transition (EMT), which implies the loss of cell-cell contacts, remodeling of cytoskeleton, and the acquisition of a migratory

phenotype [86, 87]. In fact, abnormal expression of integrins, Notch, MET, TGFβ, NF-κB, PI3K, TWIST1, matrix metalloproteinases (MMP), components of the urokinase plasminogen activating system (uPAS), and p21-activated kinase (Pak), involved in the EMT, has been identified in PTC progression [86–91]. Genomic instability, a hallmark of solid tumors including TC, is thought to represent the driving force responsible for the generation and accumulation in the malignant cells of the above-mentioned molecular alterations [44, 92–94]. According to this, hypothesis is the evidence that the number and the frequency of chromosomal abnormalities, observed during thyroid cancer progression, increase from the DTC to the PDTC and ATC [92–94].

While the prognosis for DTC patients is favorable, with 10-year survival rate of about 90%, that for patients affected by PDTC and ATC is very poor, with a median survival of only few months from diagnosis [94–96]. It has to be noted, in fact, that the surgical resection of the tumor mass is not effective in ATC patients and treatment of recurrent or persistent PDTC and ATC with conventional radiotherapy and/or chemotherapy provides little or no benefit. Therefore, novel therapeutic approaches are sorely needed for these neoplasms [85, 97].

5. Aurora Kinases and Thyroid Cancers

Normal human thyrocytes express all three Aurora kinases in a cell cycle dependent manner [67]. The expression of Aurora-A and Aurora-B in this cell type is mainly regulated at the transcriptional level, while that of Aurora-C appears to be modulated at the posttranscriptional level [67]. An increased expression of all three Aurora kinases has been shown in various cell lines originating from different epithelial thyroid tumor histotypes, compared to normal thyrocytes, as well as in DTC and ATC tissues, compared to normal matched tissues [33, 67, 98]. In addition, a study aimed at evaluating the gene expression profile in ATC, by means of tissue microarray and immunohistochemistry, identified AURKA as one of the most frequently and most strongly overexpressed genes in these tumors [99]. This is consistent with the observation that gain of chromosome 20q, where AURKA is located (20q13.2), is frequently encountered in ATC [100]. Based on these findings, the potential therapeutic value of Aurora kinase inhibition on the proliferation and growth of ATC cells has been evaluated in preclinical studies [101–105]. In particular, the *in vitro* effects of different pan-Aurora kinase inhibitors, including the MK-0457 (VX-680), the SNS-314 mesylate, and the ZM447439, have been investigated on proliferation, apoptosis, cell cycle, ploidy, and anchorage-independent growth of a panel of ATC-derived cell lines [102–104]. These molecules were found to inhibit proliferation of ATC cells in a time- and dose-dependent manner and to impair cancer cells colony formation in soft agar. Cytofluorimetric analysis of cell cultures exposed to the pan-Aurora kinase inhibitors revealed an accumulation of tetra- and polyploid cells because of endoreplication events followed by the activation of caspase-3 and accumulation of a sub-G0/G1 cell population, both indicative of apoptosis [102–104]. Treated cells showed mitotic alterations consistent with the inhibition of Aurora kinases, including major impairment of centrosome functions, with abnormal spindle formation characterized by the presence of short microtubules, inhibition of histone H3 phosphorylation, and inability to complete the cytokinesis. In addition, the selective inhibition of either Aurora-A or Aurora-B has been investigated [101, 105, 106]. The selective inhibition of Aurora-B expression by means of RNA interference, or of Aurora-B function by AZD1152, has been reported to significantly reduce growth and tumorigenicity of ATC-derived cells, both *in vivo* and *in vitro* [101]. Similarly, functional inhibition of Aurora-A by MLN8054 in a panel of ATC-derived cell lines has been shown to inhibit cell proliferation and to induce cell cycle arrest and cell apoptosis [105]. In xenograft experiments, the Aurora-A inhibitor was found to reduce tumor volume by 86% [105]. Interestingly, the combined treatment with MLN8054 and bortezomib, targeting the ubiquitin-proteasome system, showed additive effects on ATC-derived cell proliferation and apoptosis, compared to monotherapy [106]. Pazopanib, an inhibitor of kinases including the VEGFR (vascular endothelial growth factor receptor) shown to have impressive therapeutic activity in patients affected by radioactive iodine-refractory DTC, was tested in a phase II clinical trial on ATC patients [107, 108]. Despite several pazopanib treated ATC patients showed a transient disease regression, no RECIST (response evaluation criteria in solid tumors) response was obtained [106]. More recently, in a preclinical study on a panel of ATC derived cell lines, pazopanib was found to potentiate the cytotoxic effects of paclitaxel *in vitro* and in xenograft experiments [109]. The effects of this pazopanib were attributed to an unexpected off-target inhibition of Aurora-A in ATC derived cell lines. In fact, the same results were obtained when combining paclitaxel and MLN8237, a selective Aurora-A inhibitor. In the same study, the authors also showed that the combined administration of pazopanib and paclitaxel attained a marked and durable regression of lung metastasis, in a single ATC patient [109].

In conclusion, the preclinical and clinical data so far available indicate that Aurora kinase inhibitors may have a therapeutic potential for ATC treatment either in monotherapy or, more likely, in combination therapy with anti-microtubule drug.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Enke Baldini, Massimino D'Armiento, and Salvatore Ulisse contributed to writing the first draft and all of them contributed to the revisions and approved the final version of the paper.

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Research Article

Age as a Prognostic Factor in Anaplastic Thyroid Cancer

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Background. Anaplastic thyroid cancer (ATC) is one of the tumors with the shortest survival in human medicine. *Aim.* The aim was to determine the importance of age in survival of patients with ATC. *Material and Methods*. We analyzed the data on 150 patients diagnosed with ATC in the period from 1995 to 2006. The Kaplan-Meier method and log-rank test were used to determine overall survival. Prognostic factors were identified by univariate and multivariate Cox regression analysis. *Results.* The youngest patient was 35 years old and the oldest was 89 years old. According to univariate regression analysis, age was significantly associated with longer survival in patients with ATC. In multivariate regression analysis, patients age, presence of longstanding goiter, whether surgical treatment is carried out or not, type of surgery, tumor multicentricity, presence of distant metastases, histologically proven preexistent papillary carcinoma, radioiodine therapy, and postoperative radiotherapy were included. According to multivariate analysis, besides surgery ($P = 0.000$, OR = 0.43, 95% CI = 0.29–0.63), only patients age ($P = 0.023$, OR = 0.68, 95% CI = 0.49–0.95) was independent prognostic factor of favorable survival in patients with ATC. *Conclusion*. Age is a factor that was independently associated with survival time in ATC. Anaplastic thyroid cancer has the best prognosis in patients younger than 50 years.

1. Introduction

Anaplastic thyroid cancer (ATC) is one of the most aggressive tumors in human medicine. This aggressiveness is reflected in the local infiltrative growth and early metastatic spread. Therefore, the prognosis of ATC patients is one of the worst among the malignant tumors in general.

Despite multimodal treatment, which includes surgery, radiotherapy, and chemotherapy, survival time is discouraging.

Fortunately, ATC is a rare tumor. Its annual incidence is about 2 per million residents and its share in the structure of patients with thyroid cancer is decreasing and now stands at 1-2% [1, 2]. ATC occurs most frequently in the elderly but has been described in all age groups [3, 4].

As one of the most aggressive tumors, ATC draws a lot of attention, but it has not been sufficiently researched. Due to its low incidence, there are not many institutions that have single series with more than 100 patients. Analysis of prognostic factors is possible only in this larger series because most patients unfortunately die in the first few weeks or months after diagnosis, so one might get wrong impression that there is no significant survival in patients with ATC. In larger series, significant survival was recorded, which enables analysis of prognostic factors associated with longer survival in patients with ATC.

The aim of this study was to determine the role and importance of age in survival of patients with ATC.

2. Material and Methods

We analyzed retrospectively the data on 150 patients with ATC (96 women and 54 men) diagnosed in the period from 1995 to 2006. In 85 operated-on patients, the diagnosis was obtained on the basis of definitive histopathological finding,

Table 1: Demographic and clinical characteristics of patients with anaplastic thyroid cancer.

Variables	Number	Percent (%)
Gender		
Male	54	36.0
Female	96	64.0
Residence in endemic goiter area	11	11.3
Goiter	53	35.5
Duration of goiter		
0	97	64.7
<10	18	12.0
10–20	18	12.0
>20	17	11.3
Surgery		
Yes	85	56.7
No	65	43.3
Type of surgery		
Open biopsy	12	14.1
Tracheotomy	2	2.4
Tumor reduction	27	31.8
Lobectomy	4	4.7
Thyroidectomy and dissection	40	47.1
Multicentricity of tumor	5	6.0
Presence in one or both thyroid lobes		
One	58	68.2
Both	27	31.8
Tumor size		
T1–T3	3	3.6
T4	82	96.4
Metastases in lymph nodes	54	63.5
Distant metastases	28	32.9
Preexisting well-differentiated tumor (papillary)	49	57.6
Lymphocyte infiltration	7	8.2
Preoperative radiotherapy	2	2.4
Postoperative radiotherapy	67	78.7
Chemotherapy	2	78.7
Radioactive iodine therapy	8	9.4
Other malignant tumors	6	7.1
Diabetes	13	15.3
Arterial hypertension	26	30.6

and in 65 nonoperated-on patients diagnosis was based on cytological findings after fine needle aspiration. Patients with histologically proven preexisting papillary thyroid carcinoma (49) were also included in the study.

Reviewing medical records, we collected data on demographic characteristics of patients (age, sex, place of residence, and life on endemic goiter area), duration of disease before diagnosis, whether the patients knew of goiter before and if so for how long back, and whether the operation was done or just fine needle aspiration biopsy. In the operated-on patients, the following has been analyzed: the type of surgery that was done, then the size of the tumor, its multicentricity, the involvement of one or both lobes of the thyroid gland, the presence of regional lymph node metastases, or distant metastases proven.

Special attention was focused on the histopathological findings in the operated-on patients and the presence of preexisting well-differentiated thyroid cancer, as well as the degree of lymphocytic infiltration of the tumor.

Data on whether in the operated-on patients radiotherapy (preoperative or postoperative), chemotherapy, or radioactive iodine therapy was implemented has been gathered.

Some other diseases in patients with anaplastic thyroid cancer which could have an impact on survival were taken into consideration, other malignant tumors, diabetes, and hypertension. Basic demographic and clinical characteristics of ATC patients are shown in Table 1.

Table 2: Distribution of patients with anaplastic thyroid cancer according to age.

Age groups (years)	Number	Percent (%)
≤40	2	1.3
41–50	9	6.0
51–60	29	19.3
61–70	81	54.0
>70	29	19.3
Total	150	100.0

Data on whether patients were still alive or when they died were gathered through interviews of patients or their families.

The Kaplan-Meier method and log-rank test were used to determine the overall survival. Potential prognostic factors were identified by univariate and multivariate Cox regression analysis. SPSS (version 17.0) was used for statistical analysis.

3. Results

Distribution of patients with anaplastic thyroid carcinoma is presented in Table 2.

The largest number of patients with anaplastic thyroid carcinoma was in the seventh decade of life (more than half of the patients) and then in the sixth and eighth decade of life (almost 20%). Under 40 years of age, anaplastic carcinoma was detected in just two affected (1.3%) and in younger than fifty in 11 patients (7.3%). The youngest patient was 35 years old and the oldest was 89 years old. The average age of patients was 64 years (standard error ± 8.5 years).

Table 3 and Figure 1 show the survival rates of patients with anaplastic carcinoma of the thyroid gland in relation to the age. The patients were divided into three age groups: the first consisted of patients younger than 51 years, the second consisted of patients aged 51 to 70 years, and the third consisted of patients older than 70 years. The three age groups <50 ys, 51–70 ys, and >70 were arbitrarily chosen.

The longest survival in all periods was found in the youngest age group and the lowest survival in the oldest group of patients in which no one had a survival of more than 3 years. In the youngest age group, one-year survival was observed in more than half of the patients, whereas in both the older age groups one-year survival was 3-4 times less. During the first month from diagnosis of anaplastic thyroid carcinoma, almost 30% of patients older than 70 years died and less than 10% in the two younger age groups. Five-year survival rates were three times more often in the youngest age group than in the middle one. In the oldest group, there were no patients with the five-year survival rate. The middle and the oldest age group were compared for other characteristics described in Material and Methods section, but the observed differences did not affect the survival according to multivariate analysis.

Mean survival and median survival in patients with anaplastic carcinoma of the thyroid gland in relation to age are presented in Table 4. In the youngest age group the median survival was 62 weeks and in the middle age group 16 weeks, which was the median for all patients with anaplastic thyroid cancer overall, while the shortest median of 12 weeks was found in the oldest age group of patients. This difference was statistically highly significant according to the log-rank test ($P = 0.008$).

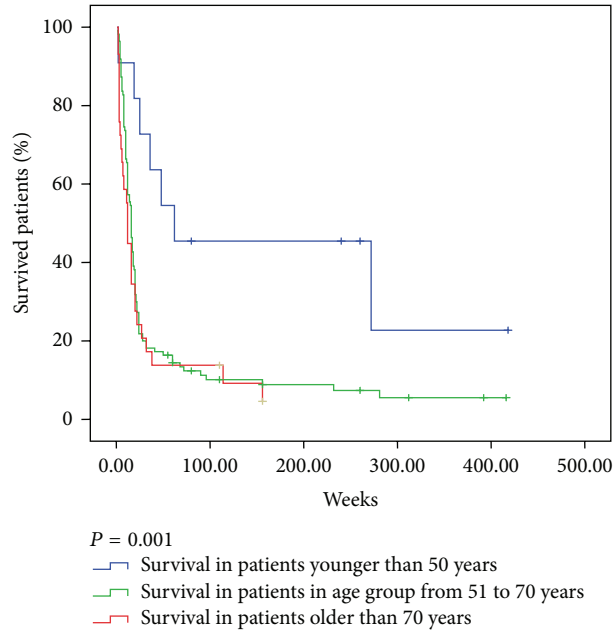

Figure 1: Kaplan-Meier survival curve in patients with anaplastic thyroid cancer in relation to age.

According to univariate Cox regression analysis, among the factors that influence the survival of patients with anaplastic thyroid cancer, patients age was significantly statistically associated with longer survival ($P = 0.000$, OR = 0.96, 95% CI = 0.94–0.99). All the factors associated with longer survival in patients with ATC at the level of statistical significance ($P < 0.1$) according to univariate model were then included in the model of multivariate analysis: patients age, presence of longstanding goiter before the appearance of ATC, surgery, type of surgery, tumor multicentricity, presence of distant metastases at the time of diagnosis, histologically proven preexistent papillary carcinoma, radioiodine therapy, and postoperative radiotherapy. According to multivariate analysis, after eliminating the confound effect, besides surgery ($P = 0.000$, OR = 0.43, 95% CI = 0.29–0.63), only patients age ($P = 0.023$, OR = 0.68, 95% CI = 0.49–0.95) was independent prognostic factor of favorable survival in patients with ATC.

4. Discussion

The average age of patients with ATC in our study was 64 years, and more than half of the patients were in their seventh decade of life. Only two affected were younger than 40 years of age, and the youngest patient was a 35-year-old woman. Our oldest patient with anaplastic thyroid cancer was an 89-year-old man. According to the literature, the largest number of patients with anaplastic carcinoma of the thyroid gland

Table 3: Survival rates in patients with anaplastic thyroid cancer according to age.

Follow-up period (weeks)	The survival rate for patients under the age of 50 years (%)	The survival rate in patients with 51–70 years (%)	The survival rate in patients older than 70 years (%)
4 weeks	90.0	91.8	72.4
16 weeks	81.8	46.4	34.5
52 weeks	54.5	16.4	13.8
260 weeks	22.7	7.4	0

Table 4: Mean and median survival in patients with anaplastic thyroid cancer (in weeks) in relation to age.

Age (years)	Mean survival (weeks)	Standard error	95% confidence interval	Median survival (weeks)	Standard error	95% confidence interval
<50	174.3	52.7	71–277.5	62.0	86.6	0–231.8
51–70	50.2	10.1	30.5–70.1	16.0	1.1	13.8–18.2
>70	30.0	8.8	12.8–47.3	12.0	0.9	10.2–13.7

Log-rank $P = 0{,}008$.

is in the seventh and eighth decade of life [5]. The average age of patients ranges from 65 to 72 years, and there is no significant difference between men and women in relation to the age at which anaplastic thyroid cancer usually occurs [6, 7]. More than 90% of patients in all the major series are older than 50 years [5, 8]. The oldest person with anaplastic thyroid cancer was 104 years old [6]. Anaplastic thyroid cancer is rare under 40 years of age [9]. In childhood, its appearance is quite rare and has been described only in few cases in the world. Unfortunately, in the childhood, it has the same fatal outcome, as well as in adult patients [10–12]. Age of patients with anaplastic thyroid cancer at the time of diagnosis, according to our results, is an independent factor that was significantly associated with longer survival ($P < 0.05$, RR = 0.65, 95% CI = 0.49 to 0.95). In other words, survival is significantly longer in younger than in older patients. However, it should be noted that in the youngest age category of patients with ATC (younger or at the age of 50) in whom the best survival was found, there is only 11 patients. Almost half of the patients died during the first year (5/11); a five-year survival was observed only in two patients.

According to the literature, data on the impact of age on survival in patients with ATC are controversial, although longer survival can be expected in younger patients, and it is known that anaplastic thyroid cancer usually occurs in older patients [13]. Besic et al. [14] point out that in patients with anaplastic thyroid cancer, who are older than 70 years, the risk of shorter survival is 1.5 times higher than in patients younger than 70 years, but the age is not an independent predictor according to the multivariate regression analysis. Yau et al. [6] found that survival was significantly longer in patients younger than 65 years, as a reason for cited comorbidity that is present in the elderly. Giuffrida and Gharib [15] and Kebebew et al. [16] found longer survival in patients with anaplastic thyroid cancer who are younger than 60 years. Gilliland et al. [17] found that one-year survival in patients with anaplastic thyroid cancer declines in older patients. Li and colleagues [18] by analyzing 12 patients with ATC under the age of 55 found that the prognosis is better than in older patients but only if the remaining of histologically proven preexistent papillary carcinoma exists. Sugitani et al. [19] in the largest of all studies of prognostic factors in ATC, which included 677 ATC patients from 38 institutions, in multivariate regression analysis among other factors independently associated with a worse prognosis state also the age over 70 years. Unlike the most, according to the results by Haigh et al. [20], years of age had no influence on survival in patients with anaplastic thyroid carcinoma.

According to our study, besides patient age, only surgery was independent prognostic factor of favorable survival in patients with ATC. Among the nonoperated-on patients, no one survived longer than one year, and among the operated-on ones the mean survival rate was seven times higher. However, the independent predictor significantly associated with survival is the type of operation performed, that is, how radical the operation was. As many as one-half of the patients that underwent radical surgery lived longer than one year and one-fourth lived longer than five years. Yau et al. [6] found a significant link between surgical treatment and the length of survival. In their series, 68% of the patients underwent surgical treatment, and half of them had complete removal of the tumor. The results of Haigh et al. [20] prove that radical surgical treatment is an independent survival predictor, and it was possible to carry it out in 31%. According to literature, radical surgical treatment is an independent survival predictor [6, 20]. The best results are obtained by multimodal treatment [5, 8, 13]. However, some of the authors did not find that multimodal treatment had an impact on the survival, where the only positive prognostic factor was the complete resection of the tumor [7, 21]. Their study, like ours, showed that none of the nonoperated-on patients reached the point of one-year survival. Multiannual survival in patients with complete resection of the tumour occurs irrespective of the extent of operation on the thyroid, that is, irrespective of the fact whether a total thyroidectomy or only hemithyroidectomy was performed, the only condition

being that the tumour was totally removed [11]. According to Venkatesh et al. [13], surgery is vital but not sufficient enough for longer survival, as one-year survival is noted in operated-on patients but almost never in nonoperated-on ones, and radical operation without multimodal treatment does not significantly prolong the survival. The mean survival of patients, who undergo multimodal treatment, is 11 months [5].

5. Conclusion

Age is one of the most important factors in the survival of patients with ATC. The best prognosis has patients younger than 50 years, but the smallest number of patients is in this age category. The limitation of our study is relatively small number of patients although it is one of the single institution series with the greatest number of patients in the world. Nevertheless, the results of this study should be verified as many cases as possible by conducting multicenter study out of different centers in the world.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Risk Factors for Anaplastic Thyroid Cancer

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Background. Anaplastic thyroid cancer (ATC) is a form of thyroid cancer with very poor prognosis, but is fortunately quite rare. Its aetiology is unknown and not well researched. *Aim.* The aim of this study was to identify potential risk factors for ATC. *Material and Method.* Case-control study of 126 ATC patients (77 females and 49 males) and 252 controls individually matched by gender, age, and place of abode. In statistical analysis we used a Cox regression model. *Results.* Univariate logistic regression showed that the risk factors for ATC are low education level, type B blood group, goitre, other nonthyroid malignancies, diabetes, late menarche, and an early first pregnancy. Multivariate logistic regression analysis showed that independent risk factors for ATC are low education level (OR = 1.42, 95% CI = 1.09–1.86), type B blood group (OR = 2.41, 95% CI = 1.03–5.66), and goitre (OR = 25–33, 95% CI = 5.66–126.65). *Conclusion.* Independent risk factors for ATC are: low education level, type B blood group, and goitre.

1. Introduction

Anaplastic thyroid cancer (ATC) is one of the most aggressive tumours known in medicine. Even though a multimodal approach is used in treating these patients, average survival rate is expressed in months, and one-year survival is 15% [1, 2]. Fortunately, ATC is a very rare malignancy with an incidence of roughly 2 per 1,000,000 residents. Although ATC represents only 2% of all thyroid cancers, it is the cause of death in half of patients whose definitive cause of death is thyroid malignancy [3]. Given that ATC is a very rare malignancy, it has not been extensively researched. Risk factors for ATC have not been sufficiently studied, and its aetiology essentially remains unknown. There have been numerous case-control studies for all histopathological types of thyroid cancer, even medullary thyroid cancer [4, 5]. Apart from our previous study, we did not encounter in the literature other case-control studies of ATC; even though being such an aggressive tumour, it attracts great attention in oncology and endocrinology. Probably, one of the possible reasons for this is that a long period of observation is required to gather a significant number of cases, given that ATC is quite rare. We published the first part of our research, with a smaller number of cases and one control group, ten years ago [6]. Following that study, this research was continued and we published our results with a greater number of cases and two control groups. The first control group consisted of patients with papillary thyroid cancer, while the second control group was formed from patients with goitre [7, 8]. In this paper we will briefly recapitulate the results of our previous studies and present our new results where the control group was formed from examinees from the general population. This paper gives the final results of our long-time research that aimed to identify potential risk factors for the development of ATC.

2. Material and Method

A case-control study was used to identify potential risk factors for ATC. The study group consisted of 126 consecutive

newly discovered patients with ATC. The diagnosis of ATC was based on the definite histopathological findings for operated patients (55) and on the basis of the results of fine needle aspiration biopsy for patients that were not operated on (71). All patients were diagnosed at the clinic where the study was carried out, from 1993 to 2005. The control group was formed from the general population with the same place of residence as the patients from the studied group. There were twice as many examinees in the control group, which amounts to 252. Examinees that were included in the control group were chosen from neighbours of patients from the studied group in the exact same manner. They were individually matched by place of abode, gender, and age (±2 years). All examinees (cases and controls) were interviewed by the same doctor. Before the interview, all examinees were made familiar with the aim of the study and voluntarily decided whether they would take part in the research. Nobody refused to participate in the study. A specialized epidemiological questionnaire was used. It consisted of questions regarding sociodemographic characteristics, habits, life in endemic goitre areas, exposure to radiation, and professional exposure to chemicals. The next batch of questions consisted of questions about personal history in regard to previous thyroid diseases and thyroid function, other endocrine disorders, other malignancies, various health disorders, diagnostic and therapeutic procedures, previous operations, and use of medicaments. Another group of questions examined family history in regard to thyroid diseases, other diseases of endocrine glands, malignant tumours, and hereditary diseases. Also, detailed data was gathered for menstrual, hormonal, and reproductive characteristics of female examinees. If a significant health disorder was reported, it was double checked in available medical documentation; or, if not available, it was double checked with the closest siblings. In statistical analysis we first used conditional univariate logistic regression analysis (ULR). All variables that were statistically related to ATC, at a level of significance of $P \leq 0.10$, were included in a multivariate conditional logistic regression analysis model (MLR), to identify independent risk factors for ATC.

3. Results

In Table 1 basic sociodemographic characteristics, blood type groups, and habits of examinees are shown. Most of the cases were female with a ratio of 1.5 : 1. In the cases group, age ranged from 37 to 88 years, with an average age of 67 years. Only 10% of cases were younger than 50 years, and only one case was younger than 40 years. More than half of the cases were in their seventh decade (51.2%). More patients resided in an urban area (69.8%) than in a rural area (30.2%). Most cases and even controls had completed only primary school (52.4% and 44.0%, resp.) or had not completed primary school (30.2% and 24.6%). In general, cases had a lower education level (only partial or full primary school) compared to the controls, and this was statistically significant according to ULR. A statistical significant difference was observed in the number of examinees with blood group type B. This blood group was present in 10.3% of cases and 4.4% of controls.

Table 1: Sociodemographic characteristics, blood group type, and habits of examinees.

	Cases		Controls		P^*
	N	%	*N*	%	
Total	**126**	**100.0**	**252**	**100.0**	
Gender					
Males	49	38.9	98	38.9	Matched
Females	77	61.1	154	61.1	
Age					
<40	1	0.8	2	0.8	
41–50	10	8.0	16	6.4	
51–60	30	24.0	40	16.0	Matched
61–70	65	51.2	150	60.0	
71–80	19	15.2	42	16.0	
80+	1	0.8	2	0.8	
Place of abode					
Rural	38	30.2	76	30.2	Matched
Urban	88	69.8	176	69.8	
Education					
No education	38	30.2	62	24.6	
Primary school	66	52.4	111	44.0	0.0124
Secondary school	17	13.5	59	23.4	
High education	5	4.0	20	7.9	
Occupation					
Housewife	38	30.2	80	31.7	
Farmer	18	14.3	19	7.5	
Blue collar worker	12	9.5	17	6.7	0.3948
White collar worker	8	6.3	31	12.3	
Pensioner	48	38.1	93	36.9	
Professional	2	1.6	12	4.8	
Marital status					
Unmarried	4	3.2	2	0.8	
Married	99	78.6	213	84.5	0.2222
Divorced	0	0	7	2.8	
Widow	23	18.3	30	12.0	
Blood group type					
O+	52	41.3	114	45.2	
A+	54	42.9	120	47.6	0.0066
B+	14	10.3	11	4.4	
AB+	7	5.6	7	2.8	
Smoking status					
Smoker	43	34.1	98	38.9	0.3671
Nonsmoker	83	65.9	154	61.1	
Former smoker					
Yes	5	7.1	15	9.7	0.5111
No	78	92.9	139	90.3	
Alcohol					
Yes	3	2.4	2	0.8	0.2254
No	123	97.6	250	99.2	
Coffee					
Yes	110	87.3	222	88.1	0.8240
No	16	12.7	30	11.9	

*Univariate logistic regression analysis.

Of the 13 cases with type B blood group, twelve had B+, while one had B− blood group. We did not find any statistically

significant associations within the habits of the examinees (cigarette smoking, alcohol, and coffee consumption).

Personal and family history of examinees is presented in Table 2. Life in an endemic goitre area did not prove to be statistically significant. Among the cases, 30.2% had a previously diagnosed goitre or thyroid nodules, on average for 18 years (ranging from 1 to 50 years). Six cases had a previous thyroid operation (three for well-differentiated thyroid cancer and three for benign thyroid diseases). On average, the time between the operation and ATC presentation was a bit longer than five years. There was not a single examinee with goitre in the control group, except for one with hyperthyroidism and one with Hashimoto thyroiditis. One of the cases, who had been previously operated for well-differentiated thyroid cancer, received I131 therapy, before the anaplastic transformation occurred. Five patients in the cases group were on substitution or suppressive therapy with L-thyroxine. We found that examinees from the cases group had twice as many nonthyroid malignancies (8.7% versus 4.4%) than the examinees from the control group. This proved to be statistically significant, according to ULR. The most common malignancy was cervical cancer (three cases) and skin cancer (basocellular cancer) in three cases. This was followed by breast cancer (two cases), lung cancer (one case), colorectal cancer (one case), and prostatic cancer (one case). Professional exposure to chemicals was not statistically significant. In comparison to the control group, there were twice as many examinees in the cases group with diabetes, and according to ULR this difference was statistically significant. Even though cases were exposed to radiotherapy more often than controls (1.6% versus 0.4% as it is in Table 2), according to ULR this difference was not statistically significant. A family history of thyroid diseases or other malignancies did not show statistical significance. Hormonal and reproductive characteristics of female examinees are presented in Table 3. Menarche was more common in both of the groups (cases and controls), before the age of 15 years. The number of female examinees who had menarche at the age of 15 years or later was much greater in the cases group (20.8% versus 9.1%). A statistical difference was not observed, according to ULR, in relation to normal menstruations, pregnancy, number of children, number of miscarriages, and deliberate terminations of pregnancy. The same applied to the use of oral contraceptives and menopause estrogens. In the cases group, 19.2% of examinees were pregnant before the age of 19, while in the control group there were 9.2%, and this proved to be a statistically significant difference. Variables that were, according to ULR, statistically significantly related to ATC were included in a MLR model. These are education level, type B blood group, goitre, personal history of nonthyroid malignancies, diabetes, late menarche, and an early first pregnancy. The results of MLR are shown in Table 4. According to MLR three variables are independently related to ATC and represent independent risk factors for ATC: low education level, type B blood group, and goitre.

Table 2: Personal and family history of examinees.

	Cases		Controls		P^*
	N	%	N	%	
Total	**126**	**100.0**	**252**	**100.0**	
Life in endemic goitre area					
Yes	29	23.0	47	18.7	0.3190
No	97	77.0	205	81.3	
Goitre					
Yes	38	30.2	0	0	0.0000
No	88	69.8	252	100.0	
Hyperthyroidism					
Yes	1	0.8	0	0	0.6623
No	125	99.2	252	100.0	
Thyroiditis					
Yes	1	0.8	0	0	0.6623
No	125	99.2	252	100.0	
I131 therapy					
Yes	1	0.8	0	0	0.6623
No	125	99.2	252	100.0	
L-Thyroxin therapy					
Yes	5	4.0	0	0	0.1858
No	121	96.0	252	100.0	
Nonthyroid malignancies					
Yes	11	8.7	11	4.4	0.0936
No	115	91.3	241	95.6	
Exposure to chemicals					
Yes	3	2.4	12	4.8	0.2731
No	123	97.6	240	95.2	
Diabetes					
Yes	15	11.9	15	6.0	0.0476
No	111	88.1	237	94.0	
Radio therapy					
Yes	2	1.6	1	0.4	0.2555
No	124	98.4	251	99.6	
Thyroid disease in family					
Yes	5	4.0	12	4.8	0.7260
No	121	96.0	240	95.2	
Malignancies in family					
Yes	16	12.7	25	9.9	0.4141
No	110	87.3	227	90.1	

*Univariate logistic regression analysis.

4. Discussion

According to the results of this study, when the control group was formed from the general population, independent risk factors for ATC were low education level (OR = 1.42, 95% CI = 1.09–1.86), type B blood group (OR = 2.41, 95% CI = 1.03–5.66), and goitre or thyroid nodules (OR = 25.33, 95% CI = 5.66–126.65). A low education level is probably a risk factor for ATC for two reasons. People with a lower level of education have generally lower health awareness, and they rarely and/or much later seek medical attention. Also, adequate health care is less frequently available. Bakiri et al. and Gaitan et al. point out that a lower education and a lower socioeconomic status are associated with a higher incidence of ATC and that other thyroid cancers are usually discovered

Table 3: Hormonal and reproductive characteristics of female examinees.

	Cases		Controls		P^*
	N	%	N	%	
Menarche before 15					
Yes	61	79.2	140	90.9	0.0151
No	16	20.8	14	9.1	
Normal menstruations					
Yes	73	94.8	149	96.8	0.4747
No	4	5.2	5	3.2	
Pregnancy					
Yes	73	94.8	152	98.7	0.1041
No	4	5.2	2	1.3	
First pregnancy before 19					
Yes	14	19.2	14	9.2	0.0376
No	59	80.8	138	90.8	
Number of children					
<2 children	37	48.1	84	54.4	0.2751
≥2 children	40	51.9	70	45.6	
Miscarriages					
Yes	4	5.5	4	2.6	0.2905
No	69	94.5	148	97.4	
Abortions					
Yes	58	75.4	109	70.6	0.4158
No	19	24.6	45	29.4	
Oral contraceptives					
Yes	20	26.0	34	22.1	0.5099
No	57	74.0	120	77.9	
Menopause estrogens					
Yes	5	7.0	8	5.6	0.6678
No	66	93.0	136	94.4	

*Univariate logistic regression analysis.

Table 4: Results of multivariate conditional logistic regression analysis.

Variable	P	OR	95% CI
Education	0.0105	1.42	1.09–1.86
Blood group type B	0.0431	2.41	1.03–5.66
Goitre	0.0000	25.33	5.66–126.65

in a more advanced stage [9, 10]. The importance of education is also observed in the paper of Memon et al., where they find that twelve or more years of education significantly decreases the risk of thyroid cancer (OR = 0.6, 95% CI = 0.3–0.9) [11].

Searching the available literature, we did not encounter any papers that associate type of blood group with ATC or any other histopathological type of thyroid cancer. On the other hand, a link between type of blood group and other malignancies has been described. Su et al. find that type B blood group increases the risk of oesophageal cancer, especially in men [12]. Type A blood group is associated with a risk for cancer for various localisations, such as gastric cancer (especially in patients with a positive family history), cancer of the larynx and hypopharynx, pancreatic cancer, breast cancer, and rectal cancer [13–18]. According to the study of Marinaccio et al., type A blood group, apart from increasing the risk for cervical and ovarian cancer, is a bad prognostic factor for these types of cancer [19].

We will now compare the results of this study with the results of our previous studies that also researched risk factors for ATC, but with different control groups. Our first case-control study of risk factors for ATC had a slightly smaller number of cases (110) and one hospital control group [6]. According to that study, independent risk factors for ATC were goitre (OR = 37.55, 95% CI = 4.86–290.11), life in an endemic goitre area (OR = 2.56, 95% CI = 1.05–6.22), a personal history of other nonthyroid malignancies (OR = 5.51, 95% CI = 1.04–29.25), diabetes (OR = 4.06, 95% CI = 1.29–12.81), and a lower level of education (OR = 2.44, 95% CI = 1.17–5.06). Our present study identified three independent risk factors for ATC, compared to our previous study where we identified five. Two of these risk factors are the same in both studies, and those are a lower level of education and goitre. Our present study could not identify life in an endemic area as a risk factor, since the control group was matched by place of abode. Practically, the main differences between these two studies are that one found diabetes and a personal history of other nonthyroid malignancies as independent risk factors, while the other identified type B blood group as an independent risk factor for ATC. Similar results were obtained when we conducted a case-control study with the same 126 cases of ATC that were included in the present study, but the control group was formed from 252 patients with goitre [8]. In fact, the development of ATC is preceded by long-term goitre in nearly half of patients [20]. This is one of the main reasons that we decided to form the control group from patients operated for goitre whose definite pathohistological findings confirmed a benign disease. This allowed us to establish potential risk factors for the development of ATC in patients with goitre. These risk factors could further help us, as additional criteria, when deciding which patients with benign goitre should undergo operative treatment. When patients operated for goitre were used as the control group, there were five variables independently related to the development of ATC. Among these were a lower level of education (OR = 1.85, 95% CI = 1.21–2.82) and type B blood group (OR = 3.69, 95% CI = 1.10–12.49), which were identified in our present study as well, using a different control group. Apart from these two factors, the other three factors were a personal history of other nonthyroid malignancies (OR = 4.37, 95% CI = 1.11–17.31), late menarche, at the age of 15 or later (OR = 2.63. 95% CI = 1.15–5.88), and an early pregnancy, before the age of 19 (OR = 2.96, 95% CI = 1.26–6.96). These two reproductive factors, as found in our present study, were also statistically significantly associated with ATC, according to ULR, but were not found to be independent risk factors. A late menarche and an early pregnancy have been identified as a risk factor for thyroid cancer in women, where its incidence is several times higher than in men [21–23].

Significantly different results were found when the control group consisted of the same number of cases with ATC

(126) but where the controls were formed from examinees with papillary thyroid cancer [7]. ATC can develop from preexistent well-differentiated thyroid cancer, which is found in 18–71% of cases with ATC [24–26]. Since, in a significant number of cases with ATC, well-differentiated thyroid cancer has been proven to coexist, on definitive histopathological findings, we decided to form the control group from patients with papillary thyroid cancer, to try and identify which factors could be responsible for the dedifferentiation, that is, anaplastic transformation of well-differentiated thyroid cancer. Based on the results of MLR, when the control group consisted of patients with papillary thyroid cancer, age proved to be the only independent risk factor for the development of ATC (OR = 1.11, 95% CI = 1.05–1.15). Therefore, in comparison to other observed variables, there were no statistically significant differences between patients with ATC and patients with papillary thyroid cancer.

At the end, we would like to point out that in our case-control research of risk factors for ATC we tried to surpass one of the greatest problems in the design of case-control studies and that is the choice of the control group. We overcame this problem by forming several control groups with different criteria and with a bigger number of controls than cases. However, one of the greatest problems of these studies is the fact that they consist of a relatively small number of cases with ATC; but, on the other hand, there are just a few centres in the world that published papers with more than 100 cases of ATC, and unfortunately they have not conducted any case-control studies for risk factors for ATC. Therefore, the results of our study should be tested in a study with a higher number of cases, which could only be achieved by a multicentric study with several high volume centres in the world.

Conflict of Interests

There was no conflict of interests for this study.

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