Targeting RAS-MAPK-ERK and PI3K-AKT-mTOR signal transduction pathways to chemosensitize anaplastic thyroid carcinoma

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Anaplastic thyroid carcinoma (ATC) is a rare, but aggressive and chemo-resistant tumor with dismal prognosis. Most ATCs harbor mutations that activate RAS/MAPK/ERK and PI3K/AKT/mTOR pathways. Therefore, we investigated and correlated the expression of phosphatase and tensin homolog, pERK, and pAKT proteins as well as mutations of \textit{BRAF}, \textit{RAS}, and \textit{p53} genes in samples of patients with ATC. Furthermore, we evaluated the potential of inhibition of these pathways on chemosensitization of ATC using 2 thyroid carcinoma cell lines (FRO and SW1736). Our results revealed a negative correlation between the activity of RAS-MAPK-ERK and PI3K-AKT-mTOR pathways in samples of patients. To be specific, the PI3K-AKT-mTOR pathway was suppressed in patients with activated \textit{NRAS} or high pERK expression. \textit{In vitro} results suggest that the inhibition of either RAS-MAPK-ERK or PI3K-AKT-mTOR components may confer sensitivity of thyroid cancer cells to classic chemotherapeutics. This may form a basis for the development of novel genetic-based therapeutic approach for this cancer type. (Translational Research 2014;164:411–423)

Abbreviations: ATC = anaplastic thyroid carcinoma; ATP = adenosine triphosphate; C\textit{Pt} = cisplatin; DOX = doxorubicin; DTC = differentiated thyroid carcinoma; FBS = fetal bovine serum; F\textit{T}I = farnesyltransferase inhibitor; GAP = GTPase-Activating Protein; GTP = Guanosine-5’-triphosphate; IHC = Immunohistochemical; MTT = (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); PCR-SSCP = polymerase chain reaction–single-strand conformational polymorphism; PTX = paclitaxel

INTRODUCTION

Thyroid carcinoma is the most common malignancy of the endocrine system\textsuperscript{1,2} and represents about 1\% of all human cancers. Most thyroid malignancies, papillary thyroid carcinoma and follicular thyroid carcinoma, are well differentiated and have favorable prognosis. On the other hand, anaplastic thyroid carcinoma (ATC) is one of the most aggressive human cancers, with an intrinsic resistance and dismal prognosis despite various therapeutic modalities.\textsuperscript{3,5} Multimodality treatment consisting of surgery when feasible combined with radiation and chemotherapy is
Anaplastic thyroid carcinoma (ATC) is a chemoresistant tumor. Most ATCs harbor mutations that activate RAS-MAPK-ERK and PI3K-AKT-mTOR pathways. The role of these pathways was analyzed in the pathogenesis and chemoresistance of ATC.

Translational Significance

Most patients with ATC analyzed showed mutation of NRAS. It was followed by mutation of p53 and negatively correlated with pAKT. Positive correlation between phosphatase and tensin homolog and pERK suggested that either RAS-MAPK-ERK or PI3K-AKT-mTOR pathway governs carcinogenesis of ATC. The effects of signal transduction inhibitors on chemosensitization to doxorubicin and paclitaxel were examined in vitro. Dual mTOR inhibitor could be a potential therapeutic strategy for ATC patients.

generally recommended. Multimodality treatments appear to afford long-term survival. Usually administered chemotherapy in these treatments encompasses doxorubicin (DOX), paclitaxel (PTX), and cisplatin (CPl). Patients who had R0/R1 resection and underwent protocol chemoradiation had a local complete response rate of 89%. As more data become available regarding the molecular pathogenesis of ATC, more targeted therapies are appearing in the clinic. Promising classes of agents are the small-molecule tyrosine-kinase inhibitors, angiogenesis inhibitors, and vascular disrupting agents. Although ATC accounts only for 2% of thyroid cancer incidence, it results in 14%–39% of thyroid cancer deaths. To improve overall clinical outcome, it is of great importance to explore more thoroughly the genomic alterations associated with ATC and to obtain molecular markers that could serve as diagnostic tools or treatment targets.

Most ATCs harbor mutations that activate one or both pathways: RAS-MAPK-ERK and PI3K-AKT-mTOR cascade. These signaling pathways are cell’s chief mechanisms for controlling cell survival, differentiation, proliferation, and metabolism. Besides, changes in the activity of these signaling pathways can lead to drug resistance, which is a common feature of ATC. The mutations in RAS-MAPK-ERK and PI3K-AKT-mTOR pathways are usually already present in the well-differentiated tumor component from which most ATCs develop. ATC shows few additional specific gene mutations in contrast to differentiated thyroid carcinoma (DTC) indicating that many ATCs derive from pre-existing DTCs by a process of dedifferentiation, acquiring new mutations in the tumor suppressor genes: p53 and phosphatase and tensin homolog (PTEN). Genetic alterations in p53 gene are the most frequent in ATCs (55%). p53 is a tumor suppressor gene, which plays a key role in regulation of the cell cycle, DNA repair, and apoptosis. PTEN is a tumor suppressor gene that dephosphorylates the phosphatidylinositol-3,4,5-triphosphate, which reduces the downstream activity of protein kinase B-AKT kinase, thereby inducing cell-cycle arrest and apoptosis.

PTEN has been found to be mutated or deleted in different types of thyroid tumors, but the real incidence of PTEN alterations in ATC has not been well established. The mutations in other genes occur with the following frequencies in ATC: CTNNB1 (38%), BRAF (26%), RAS (22%), and PIK3CA (17%). The mutant BRAFV600E protein results from a transversion (T1799A) in exon 15 of the BRAF gene, which causes a valine for glutamate substitution. This mutation induces a conformational change in the protein that constitutively activates the MAPK pathway (ie, ERK1/2). This oncprotein is implicated in the pathogenesis and progression of ATC. RAS family oncogenes are important regulators of cell growth and have a role in thyroid tumor differentiation. Mutations in all 3 cellular RAS genes (KRAS, HRAS, and NRAS) have been identified in benign and malignant thyroid tumors. They seem to be common in follicular thyroid carcinoma and ATC, but occur less frequently in papillary thyroid carcinoma.

The aim of this study was to investigate the role of the key components of RAS-MAPK-ERK and PI3K-AKT-mTOR pathways in the pathogenesis of ATC using set of 12 patient samples. The following alterations were investigated and correlated: expression of PTEN, pERK, and pAKT proteins, mutations of BRAF and RAS oncogenes (HRAS, KRAS, and NRAS) and p53 tumor suppressor gene. Further study was performed on poorly DTC cell line (FRO cell line) and ATC cell line (SW1736) to elucidate the role of inhibition of RAS-MAPK-ERK and PI3K-AKT-mTOR pathways in chemosensitization. Five inhibitors of key components of RAS-MAPK-ERK and PI3K-AKT-mTOR pathways were used in combination with classic chemotherapeutics: tipifarnib—RAS inhibitor, GSK690693—adenosine triphosphate-competitive pan-Akt kinase inhibitor, AZD2014—a dual mTORC1 and mTORC2 inhibitor, dabrafenib—a mutant BRAFV600E specific inhibitor, and wortmannin—specific inhibitor of phosphoinositide 3-kinases (PI3Ks).
METHODS

Tissue samples. Cancer tissue samples from 12 patients with ATC who underwent surgical resection at the Institute for Oncology, Clinical Center of Serbia, were analyzed. The samples were collected and used after obtaining informed consents and approval from the Ethics Committee, in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Surgically removed tissue was fixed in 10% buffered formalin and embedded in paraffin. Diagnosis of ATC was established by histologic examinations of the surgical specimens. Twelve patients included 8 women and 4 men, with a median age of 67.2 years (range, 46–82 years). The surgery had mainly palliative character with the median patient survival of 68 days (35–441 days). Patients received neither radiotherapy nor chemotherapy before surgery.

Cells and cell culture. FRO, poorly DTC cell line, was generous gift from Prof. Alfredo Fusco from Institute of Endocrinology and Experimental Oncology, National Centre of Research from Naples, Italy. SW1736, ATC cell line, was purchased from the American Type Culture Collection (Rockville, MD). SW1736 cells were maintained in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 10,000 U/mL penicillin, 10 mg/mL streptomycin, 25 mg/mL amphotericin B solution at 37°C in a humidified atmosphere at 5% CO2, whereas FRO cell line was cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 4.5 g/L glucose, 10,000 U/mL penicillin, and 10 mg/mL streptomycin at 37°C in a humidified atmosphere at 5% CO2. All cell lines were subcultured at 72-hour intervals using 0.25% trypsin-EDTA and seeded into a fresh medium at the following densities: 16,000 cells/cm2 for SW1736 and 8000 cells/cm2 for the FRO cell line.

Drugs. PTX was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). DOX solution was obtained from EBEWE Arzneimittel GmbH, (Vienna, Austria). Cpt was obtained from Pfizer (Perth) Pty Ltd (Bentley, Australia). GS6K90693, tipifarnib, AZD2014, dabrafenib, and wortmannin were kindly provided by SelleckChem. PTX was diluted in absolute ethanol and 1 mM aliquots were stored at −80°C. DOX was diluted in deionized water and 1 mM aliquots were stored at −20°C. Cpt was diluted in deionized water and 1 mM aliquots were stored at room temperature. GS6K90693, tipifarnib, AZD2014, dabrafenib, and wortmannin were diluted in dimethyl sulfoxide and 10 mM aliquots were stored at −20°C. Before treatment, all drugs were freshly diluted in sterile water.

DNA and RNA extraction. DNA from paraffin-embedded tumor material was extracted using Kappa Express Extract DNA extraction kit (KapaBiosystems) according to the manufacturer’s instructions. RNA from tissue samples was extracted using RecoverAll Total Nucleic Acid Isolation kit (Ambion) according to the manufacturer’s protocol. RNA isolation from cell lines was carried out using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The quality of DNA and RNA was verified by electrophoresis on 0.8% and 1.3% agarose gels, respectively. The concentrations of isolated nucleic acids were assessed spectrophotometrically.

Polymerase chain reaction–single-strand conformational polymorphism analysis of p53 gene and DNA sequencing of p53, BRAF, and RAS genes. Frequently mutated exons (5–9) of the p53 gene were amplified and screened for mutations by polymerase chain reaction–single-strand conformational polymorphism (PCR-SSCP) analysis according to Orita et al.26 Primers and PCR conditions were as described previously.27 All samples were analyzed for the presence of mutations from at least 3 independent PCR amplifications and tested under at least 2 different experimental SSCP conditions to avoid false positives and negatives. DNA isolated from paraffin-embedded material of normal thyroid tissue was used as a negative control. A final analysis for the presence of mutated single-strand conformers was performed by polyacrylamide gel electrophoresis followed by silver staining. To confirm the results of PCR-SSCP, samples with detected mutations were subjected to sequencing. Sequencing was also performed to detect mutations in BRAF and RAS genes. Primers and PCR conditions for BRAF and RAS genes (HRAS, KRAS, and NRAS) are shown in Table I. All sequences were determined with Applied Biosystems Incorporated dye terminator sequencing kit according to the manufacturer’s specifications on an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City). Sequencing was carried out in both directions. The obtained sequences were analyzed and compared with wild-type p53, BRAF, and RAS (HRAS, KRAS, and NRAS) sequences using BLAST software in the NCBI GenBank database.

Immunohistochemistry. Tumor samples were fixed in buffered 10% formalin, embedded in paraffin blocks, and cut in 3-μm sections for routine analysis. Cells (FRO and SW1736) were grown on chamber slides and fixed with 4% paraformaldehyde before hematoxylin-eosin staining and immunohistochemistry. The following antibodies were used according to the manufacturer’s instructions: PTEN (1:50, clone: PN37; Invitrogen), pERK (1:100, clone: P44/42-MAPL-ERK1/2(137 FS); Cell Signaling), and pAKT (1:40, clone: HCL-1 AKT-Phos; Novocastra, Leica Biosystems). Immunostaining was performed by incubating tissue...
sections with appropriate serum for 30 minutes at room temperature in humidity chamber, using the streptavidin-biotin technique (LSAB + Kit, Peroxidase Labeling, K0690; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized with diaminobenzidinehydrochloride (No. K3468; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized with diaminobenzidinehydrochloride (No. K3468; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized with diaminobenzidinehydrochloride (No. K3468; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized with diaminobenzidinehydrochloride (No. K3468; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized with diaminobenzidinehydrochloride (No. K3468; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized with diaminobenzidinehydrochloride (No. K3468; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized with diaminobenzidinehydrochloride (No. K3468; DakoCytomation, Glostrup, Denmark).

Immunohistochemical (IHC) results were independently evaluated by 2 pathologists (Z.M. and J.S.) on microscope Leica DM2500 (Leica Microsystems, Wetzlar, Germany). The immunoreactivity of PTEN was assessed using the semiquantitative method based on the score of percentage of stained cells-cytoplasm or nuclei (P) (0, no immunoreactivity; 1, 1%–10%; 2, 11%–50%; and 3, 51%–100%) and intensity of staining (0, no immunoreactivity; 1, reduced staining intensity relative to the corresponding normal cells; 2, same as normal cells staining; and 3, increased staining). Because cutoff levels for reduced PTEN expression by IHC methods have not been defined so far, we used the mean PTEN score as a cutoff point to designate reduced expression. Accordingly, PTEN status was defined as follows: low expression, if score was ≤4 and high expression, if score was >4. The pAKT staining was evaluated by an H-score, which was calculated by multiplying the P by the corresponding intensity of staining (1 = weak, 2 = moderate, and 3 = strong), giving a maximum score of 300 (100% × 3). A positive control for pAKT (human skin) was included in each analysis. H-scores >50 were considered positive. The pERK immunoreactivity levels of each case were assessed semiquantitatively under light microscope by assessing the average signal intensity (on scale of 0-3) and the proportion of cells showing a positive nuclear stain (0, none; 0.1, less than one tenth; 0.5, less than one half; and 1, and greater than one half). The intensity and proportion scores were then multiplied to give the H-score. H-score >1 was considered as elevated pERK immunoreactivity.

**Chemosensitivity by MTT assay.** MTT assay is based on the reduction of 3-(4, 5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide into formazan dye by active mitochondria of living cells. Cells grown in 25 cm² tissue flasks were trypsinized, seeded into flat-bottomed 96-well tissue culture plates (1000 cells/well FRO cell line and 2000 cells/well for SW1736 cell line), and incubated overnight in 100 μL of appropriate medium. After 24 hours, the cells were treated with GSK690693 (1-25 μM), tipifarnib (0.05-1 μM), AZD2014 (0.01-0.25 μM), dabrafenib (0.1-5 μM), and wortmannin (2.5-50 μM) as well as with PTX (0.01-1 μM), DOX (0.01-1 μM), and Cpt (2.5-50 μM) and incubated for 72 hours in complete medium. The combined effects of GSK690693, tipifarnib, AZD2014, dabrafenib, and wortmannin with PTX or DOX after 72 hours were also studied. In simultaneous treatments, 3 concentrations of GSK690693 (1, 2.5, and 5 μM), tipifarnib (0.25, 0.5, and, 1 μM), AZD2014 (0.025, 0.05, and 1 μM), dabrafenib (0.01, 0.05, and 0.1 μM),

### Table I. Oligonucleotide primers and PCR conditions for Ras and BraF genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Primer sequence*</th>
<th>PCR cycling conditions</th>
<th>PCR reaction mixture in final volume of 25 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>K RAS</td>
<td>Exon 2</td>
<td>(a) 5′-GTAAGCCAGTCTGATGAGAA-3′</td>
<td>5′ at 95°C; 35 cycles: 1′ at 95°C, 1′ at 56°C, 1′ at 65°C; 7′ at 65°C</td>
<td>MgCl2 1.5 mM dNTP 2 mM each Primers 0.6 μM Taq1 U/reaction</td>
</tr>
<tr>
<td>K RAS</td>
<td>Exon 3</td>
<td>(a) 5′-AGTGTTTGATGAGGAAGAG-3′</td>
<td>5′ at 95°C; 35 cycles: 1′ at 95°C, 1′ at 56°C, 1′ at 65°C; 7′ at 65°C</td>
<td>MgCl2 2 mM dNTP 2 mM each Primers 0.16 μM Taq1 U/reaction</td>
</tr>
<tr>
<td>H RAS</td>
<td>Exon 1</td>
<td>(a) 5′-GTGGATGATGAGGG-3′</td>
<td>5′ at 95°C; 35 cycles: 1′ at 95°C, 1′ at 56°C, 1′ at 65°C; 7′ at 65°C</td>
<td>MgCl2 1.5 mM dNTP 2 mM each Primers 0.6 μM Taq1 U/reaction</td>
</tr>
<tr>
<td>H RAS</td>
<td>Exon 2</td>
<td>(a) 5′-AGTGTTTGATGAGGAAGAG-3′</td>
<td>5′ at 95°C; 35 cycles: 1′ at 95°C, 1′ at 56°C, 1′ at 65°C; 7′ at 65°C</td>
<td>MgCl2 2 mM dNTP 2 mM each Primers 0.16 μM Taq1 U/reaction</td>
</tr>
<tr>
<td>N RAS</td>
<td>Exon 2</td>
<td>(a) 5′-CTGTGTTGATGAGGAAGAG-3′</td>
<td>5′ at 95°C; 35 cycles: 1′ at 95°C, 1′ at 56°C, 1′ at 65°C; 7′ at 65°C</td>
<td>MgCl2 2 mM dNTP 2 mM each Primers 0.16 μM Taq1 U/reaction</td>
</tr>
<tr>
<td>N RAS</td>
<td>Exon 3</td>
<td>(a) 5′-CTGTGTTGATGAGGAAGAG-3′</td>
<td>5′ at 95°C; 35 cycles: 1′ at 95°C, 1′ at 56°C, 1′ at 65°C; 7′ at 65°C</td>
<td>MgCl2 2 mM dNTP 2 mM each Primers 0.16 μM Taq1 U/reaction</td>
</tr>
<tr>
<td>BRAF</td>
<td>Exon 15</td>
<td>(a) 5′-CTGTGTTGATGAGGAAGAG-3′</td>
<td>5′ at 95°C; 35 cycles: 1′ at 95°C, 1′ at 56°C, 1′ at 65°C; 7′ at 65°C</td>
<td>MgCl2 2 mM dNTP 2 mM each Primers 0.16 μM Taq1 U/reaction</td>
</tr>
</tbody>
</table>

*Primer (a) is the 5′ primer used in the PCR and primer (b) is the 3′ primer used in PCR.

Abbreviation: PCR, polymerase chain reaction.
and wortmannin (2.5, 5, and 10 μM) were combined with PTX (0.01-0.05 μM) or DOX (0.025-0.5 μM). After incubation time, 100 μL of MTT solution (1 mg/mL) was added to each well and plates were incubated at 37°C for 4 hours. Formazan product was dissolved in 200 μL dimethyl sulfoxide. The absorbance of obtained dye was measured at 540 nm using an automatic microplate reader (LKB 5060-006 Micro Plate Reader, LKB, Vienna, Austria). Half maximal inhibitory concentration (IC50) values were defined as the concentration of the drug that inhibited cell growth by 50% and calculated by linear regression analysis using Excel software.

**Statistical analysis.** A statistical analysis was performed using STATISTICA 6.0 software (StatSoft, Inc, Tulsa). To test correlations between different parameters (RAS alterations and levels of immunoperoxidase expression of pAKT, pERK, and PTEN) the Fisher exact test was used. Statistical differences were considered significant when P < 0.05.

**Median effect analysis.** The nature of the interaction between inhibitors (tipifarnib, GSK690693, AZD2014, dabrafenib, and wortmannin) and PTX or DOX was analyzed using CalcuSyn software (Biosoft, Cambridge, United Kingdom) that uses the combination index (CI) method, based on the multiple drug effect equation. This analysis requires that at least 3 or more data points for each single drug were available in each experiment. The nonconstant ratio combination design was chosen to assess the effect of both drugs in combination. We illustrated the obtained results by fraction-affected CI graphs. Values of CI <1 point to a pronounced additive effect or synergism, that is, the smaller the value, the greater the degree of synergy. A value of CI = 1 indicates an additive effect, and values of CI >1 point to an antagonistic effect.

**RESULTS**

**pAKT, pERK, and PTEN protein expression.** According to established IHC scoring, IHC studies showed the high level of pAKT in 5 (41.6%), high level of pERK in 7 (58.3%), and low level of expression of PTEN in 6 of 12 patients with ATC (50%). FRO and SW1736 cell lines had high expression of pERK and PTEN, whereas pAKT expression was low. IHC results are summarized in Table II. Representative examples of staining intensities for pAKT, pERK, and PTEN are demonstrated in Fig 1.

**Mutational status of p53 tumor suppressor gene.** PCR-SSCP analysis for p53 hot spot exons 5–9 was conducted to detect potential mutations in the set of 12 samples of patients with ATC. It revealed aberrations in 9 samples, which were further sequenced to confirm and identify mutations. A DNA sequencing procedure confirmed mutations in 5 of 9 samples. The exon locations, codon positions, and nucleotide changes of all mutations are summarized in Table III. One sample had silent mutation in codon 213 of exon 6, a rare naturally occurring polymorphism, whereas the rest of identified mutations caused changes in the amino acid sequence. Two of them have never been reported in ATC before. One is the change in codon 286 of exon 8 (GAA → AAA), which caused the change of amino acid glycine to lysine and the other is deletion...
of 17 nucleotides in exon 5 (Del 12409–12426), which led to the frameshift mutation in p53 gene.

**Mutational status of BRAF and RAS oncogenes.** Twelve samples of patients with ATC were analyzed for the presence of hot spot mutation V600E in BRAF. Only one of the patients was a carrier of this mutation (Table II). RAS exons 1, 2, and 3 (containing most frequently mutated codons 12, 13, and 61) were amplified and subjected to sequencing. No mutations were detected in codons 12, 13, and 61 of all 3 RAS (KRAS, HRAS, and NRAS) genes in any of 12 ATCs.

Interestingly, we found that NRAS was mutated in codon 50 in 9 patients with ATC (75%). Four of them carried transversion of C to T (ACC → ATC), whereas the other 5 had the changes in 2 nucleotides of codon 50 (ACC → ATG) (Table IV). Identified mutations have never been reported in ATC before.

**Table III.** Detected and confirmed mutations in p53 tumor suppressor gene

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Exon/intron</th>
<th>Codon number</th>
<th>Nucleotide change</th>
<th>Predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Exon 5</td>
<td>141</td>
<td>TG→TC</td>
<td>Cys→Tyr</td>
</tr>
<tr>
<td>3</td>
<td>Exon 5</td>
<td>141</td>
<td>Del 12409–12426</td>
<td>Frameshift</td>
</tr>
<tr>
<td>4</td>
<td>Exon 5</td>
<td>154</td>
<td>GC→GT</td>
<td>Gly→Val</td>
</tr>
<tr>
<td>8</td>
<td>Exon 8</td>
<td>286</td>
<td>AAA→AA</td>
<td>Glu→Lys</td>
</tr>
<tr>
<td>16</td>
<td>Exon 6</td>
<td>213</td>
<td>CGA→CGG</td>
<td>Polymorphism</td>
</tr>
</tbody>
</table>

*Mutations that have not been reported in anaplastic thyroid carcinoma before (http://p53.bii.a-star.edu.sg).

**Table IV.** Mutations in NRAS oncogenes

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Exon/intron</th>
<th>Codon number</th>
<th>Nucleotide change</th>
<th>Predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 3, 8</td>
<td>Exon 2</td>
<td>50</td>
<td>ACC→ATC</td>
<td>Thr→Ile</td>
</tr>
<tr>
<td>5, 6, 7, 9, 10</td>
<td>Exon 2</td>
<td>50</td>
<td>ACC→ATG</td>
<td>Thr→Met</td>
</tr>
</tbody>
</table>

*Mutations that have not been reported in anaplastic thyroid carcinoma before.

**Associations among NRAS, PTEN, pAKT, and pERK alterations.** The Fisher exact test revealed a statistically significant positive correlation between PTEN and pERK expressions \( P = 0.04 \); Table V) meaning that patients with high PTEN expression have also high expression of pERK. Besides, the frequency of NRAS activation was significantly lower in tumors with high expression of pAKT \( P = 0.04 \); Table VI). Analysis of alterations in other genes did not show any statistically significant correlation.

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**Fig 1.** Immunocytochemical analysis of pAKT, PTEN, and pERK expression in anaplastic thyroid carcinoma. Representative examples of (A) antibody controls: pAKT epidermis (20× objective), PTEN breast tissue (10× objective), pERK breast tissue (10× objective); (B) low nuclear pAKT immunoreactivity (10× objective), low cytoplasmatic PTEN immunoreactivity (10× objective), and low cytoplasmatic pERK immunoreactivity (10× objective); (C) high nuclear pAKT immunoreactivity (10× objective), high cytoplasmatic PTEN immunoreactivity (10× objective), and high cytoplasmatic pERK immunoreactivity (10× objective). PTEN: phosphatase and tensin homolog.
Enhancement of PTX and DOX sensitivity in combined treatments with GSK690693, tipifarnib, AZD2014, wortmannin, and dabrafenib. We investigated the potential of inhibitors (pan-AKT inhibitor [GSK690693], RAS inhibitor [tipifarnib], dual mTOR inhibitor [AZD2014], PI3K inhibitor [wortmannin], and mutant BRAFV600 inhibitor [dabrafenib]) to sensitize ATC cell line SW1736 to DOX and PTX in simultaneous combined treatments (Fig 3). The effects of tested combinations on cell growth were assessed by MTT assay. We subjected these results to computerized synergism or antagonism CalcuSyn software analysis. All combinations that produced decrease in IC₅₀ value for DOX/PTX by 50% or more demonstrated additive (CI = ~1) and synergistic (CI <1) effects (Fig 3).

Table V. Correlation between pERK and PTEN expression

<table>
<thead>
<tr>
<th>Variables</th>
<th>High NP (%)</th>
<th>Low NP (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>5 (83.3)</td>
<td>1 (16.7)</td>
<td>0.004*</td>
</tr>
<tr>
<td>Low</td>
<td>1 (16.7)</td>
<td>5 (83.3)</td>
<td></td>
</tr>
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</table>

Abbreviations: NP, number of patients per group.
*Bold indicates statistically significant values, P < 0.05.

Table VI. Correlation between mutated NRAS and pAKT expression

<table>
<thead>
<tr>
<th>Variables</th>
<th>Yes NP (%)</th>
<th>No NP (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAKT expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2 (40.0)</td>
<td>3 (60.0)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Low</td>
<td>7 (100)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: NP, number of patients per group.
*Bold indicates statistically significant values, P < 0.05.

Cell growth inhibition by chemotherapeutics and signal transduction inhibitors. The effects of chemotherapeutics (DOX, PTX, and CPT) and inhibitors of signaling molecules (pan-AKT inhibitor [GSK690693], RAS inhibitor [tipifarnib], dual mTOR inhibitor [AZD2014], mutant BRAFV600 inhibitor [dabrafenib], and PI3K inhibitor [wortmannin]) on cancer cell growth were evaluated by the MTT assay. The results are summarized in Fig 2. The IC₅₀ values obtained in FRO and SW1736 cells for DOX were 0.04 and 0.347 μM, respectively (Fig 2, A). PTX exerted the highest potential for cell growth inhibition with similar inhibitory profile in both cell lines (Fig 2, B). The IC₅₀ values obtained in FRO and SW1736 cells for PTX were 0.044 and 0.093 μM, respectively. The IC₅₀ values obtained for CPT were 15 μM in FRO and 38 μM in SW1736 (Fig 2, C). Dabrafenib induced similar effect regarding cell growth inhibition in both ATC cell lines (Fig 2, D). Interestingly, dabrafenib reached the maximum inhibitory potential at 0.5 μM showing 40% inhibition with concentrations between 0.5 and 5.0 μM. The IC₅₀ values for GSK690693 were 7.5 μM in FRO and 25 μM in SW1736 (Fig 2, E), whereas the IC₅₀ values obtained in FRO and SW1736 cells for tipifarnib were 2 and 8 μM, respectively (Fig 2, F). Regarding signal transduction inhibitors, AZD2014 showed the highest potential for cell growth inhibition in tested cell lines with the IC₅₀ values of 0.2125 μM in FRO and 0.15 μM in SW1736 (Fig 2, G). Although wortmannin induced better effect in FRO with lower concentrations, the IC₅₀ value for both cell lines was 30 μM (Fig 2, H).

DISCUSSION

The aim of this study was to evaluate the impact of molecular alterations in key components of the RAS-MAPK-ERK and PI3K-AKT-mTOR pathways as well as their mutual effect on pathogenesis of ATC. We analyzed and correlated following alterations: immunoeexpression of PTEN, pERK and pAKT proteins, mutations of BRAF and RAS (HRAS, KRAS, and NRAS) oncogenes, and p53 tumor suppressor gene in patients with ATC.

Predominantly altered gene in our set of ATC samples was NRAS, whereas BRAF mutation was determined in only 1 patient. This is considerably lower in comparison with reported data, but it might be attributed to the population differences. However, confirmation is...
Fig 2. Cell growth inhibition of ATC cell lines induced by different anticancer agents. Cell growth inhibition of FRO and SW1736 cell lines induced by DOX (A), PTX (B), CPt (C), dabrafenib (D), GSK690693 (E), tipifarnib (F), AZD2014 (G), and wortmannin (H). The results were assessed by MTT assay. All values represent average ± standard deviation obtained from 3 independent experiments, n = 3. DOX, doxorubicin; PTX, paclitaxel; CPt, cisplatin.
needed on a larger set of samples. NRAS was mutated in 75% of our patients suggesting its key role in the development of this tumor. According to the literature, NRAS mutation was restricted to the group of thyroid tumors with low or absent thyroglobulin expression, suggesting that this genetic change is prevalent in less differentiated thyroid tumors. Aberrant NRAS function is associated with a single mutation typically at codon 12, 13, or 61. Mutation at these conserved sites favors GTP binding and produces constitutive activation of RAS gene. We found that NRAS was mutated in codon 50 in our set of ATC samples. These mutations have never been reported in genesis of thyroid cancer, but they are described in patients with Noonan syndrome. The change of codon 50 does not substantially affect intrinsic GTPase activity, GAP association, or GAP-stimulated hydrolysis, but it activates MAPK signaling, indicating a different mechanism of functional deregulation of this gene. In addition, mutant NRAS suppresses apoptosis and further contributes to the development of different tumors.

The status of p53 in ATC has been subjected to intensive investigation showing that this tumor type harbors p53 mutations at high frequency. However, only 33% of patients in our set of samples carry p53 mutations in hot spot exons, and 2 of them have never been reported in ATC before. Notably, mutations in p53 tumor suppressor were found only in patients with NRAS mutations, whereas NRAS mutations were also found independently. It was already reported that in anaplastic carcinomas, mutations of both RAS and p53 genes may be observed, whereas in well-differentiated thyroid cancer, RAS mutations, but not p53 mutations, are frequent. The further suggestion was that p53 mutation may occur as a secondary event, concomitant with the loss of cell differentiation. Our results comply with the previous reports confirming the possibility that NRAS mutations arise earlier than p53 alterations in ATC development.

We have found high pAKT expression in 41.6%, high pERK expression in 50%, and low PTEN expression in 50% of patients indicating that both pathways RAS-MAPK-ERK and PI3K-AKT-mTOR cooperate in the development of ATC. Moreover, our results showed a significant positive correlation between PTEN and pERK, which is in accordance with previous studies reporting that the activation of the PI3K-AKT-mTOR signaling pathway inhibits ERK1/2 activation, which may also stand for our results. In addition, we have found a significant negative correlation between activated NRAS gene and the expression of pAKT. “Oncogene addiction” was observed in some cases in which only one signaling pathway appears to drive cancer progression. As an example, combinations of hot spot mutations in PIK3CA (phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha) and RAS genes were not found in the same patient with bladder tumor. In addition to their independent signaling programs, RAS-MAPK-ERK and PI3K-AKT-mTOR pathways extensively crosstalk and either positively or negatively regulate each other sometimes providing compensatory mechanisms.

ATC is known to be resistant to anticancer therapy. Usually administered chemotherapy for patients with ATC encompasses DOX, PTX, and Cpt. DOX and PTX are natural products with different mechanism of action. DOX is a classic topoisomerase IIα poison, whereas PTX increases microtubule polymerization and stability and induces mitotic arrest. Cpt, such as DOX, is considered as DNA damaging agent. These 3 cytostatics are the mainstream therapy of numerous cancer types.

RAS-MAPK-ERK and PI3K-AKT-mTOR pathways are thought to participate in the development of drug resistance. The involvement of the PI3K-AKT-mTOR pathway in promoting resistance against DOX was reported, whereas the RAS-MAPK-ERK pathway was shown to be involved in the development of PTX resistance. Inhibitors of the PI3K-AKT-mTOR and RAS-MAPK-ERK pathways are rapidly evaluated in preclinical models and clinical studies to determine whether they could restore the sensitivity to conventional anticancer therapeutics. Hence, we investigated the effects of Ras (tipifarnib), pan-AKT (GSK690693), dual mTOR (AZD2014), mutant BRAFV600 (dabrafenib), and PI3Ks (wortmannin) inhibitors alone and in combination with classic chemotherapeutics in vitro. Tipifarnib is a farnesyltransferase inhibitor that inhibits the RAS kinase in a post-translational modification step before the kinase pathway becomes hyperactive. GSK690693 binds to and inhibits Akt 1, 2, and 3, preventing downstream protein phosphorylation in the PI3K-Akt signaling pathway. AZD2014 blocks activity of both mTOR complexes and is considered as mTORC1/mTORC2 dual inhibitor. Dabrafenib is a mutant BRAFV600 specific inhibitor with 4- and 6-fold less potency against BRAF (wt) and C-RAF, respectively. Wortmannin is a specific inhibitor of PI3Ks.

Two thyroid cancer cell lines with mutated BRAF (FRO, poorly DTC cell line and SW1736, ATC cell line) were tested immunohistochemically for the expression of pAKT, pERK, and PTEN. Both cell lines demonstrated high pERK and PTEN expressions, whereas the expression of pAKT was low. FRO cell line was generally more sensitive to the applied chemotherapeutics with the exception of AZD2014 and dabrafenib, which could be attributed to its relatively more...
Fig 3. Specific inhibitors (GSK690693, tipifarnib, AZD2014, wortmannin, and dabrafenib) enhance the sensitivity of SW1736 cells to PTX and DOX. GSK690693, tipifarnib, AZD2014, wortmannin, and dabrafenib decrease the IC_{50} values for DOX (A) and PTX (B). IC_{50} values are normalized to IC_{50} value of DOX/PTX. The applied
differentiated phenotype when compared with SW1736. Unlike DOX and PTX, Cpt was effective only at high concentrations. As for the signal transduction inhibitors, dual mTOR inhibitor was the most potent. On the other hand, pan-AKT and PI3K inhibitors showed the weakest effect, which may be because of the low target (pAKT) expression.

Furthermore, we studied the simultaneous combination of signal transduction inhibitors with classic chemotherapeutics DOX and PTX in anaplastic thyroid cell line SW1736 that was more resistant to applied agents. All combinations with PTX and DOX demonstrated additive and synergistic effects. The enhancement of PTX activity by RAS inhibitor tipifarnib was high showing the potential for PTX resistance reversal. Previous work by Marcus et al also showed that farnesyltransferase inhibitors, such as tipifarnib, synergize with taxanes. This was because of the inhibition of the tubulin deacetylase function of histone deacetylase 6, thereby enhancing tubulin acetylation.54 On the other hand, pan-AKT inhibitor strongly sensitized cells to DOX. Accordingly, Wallin et al showed that DOX treatment caused an increase in the amount of nuclear pAKT in cancer cells favoring their survival. Therefore, PI3K-AKT inhibition significantly increased apoptosis and enhanced the antitumor effects of DOX in a set of breast and ovarian cancer models.59 We have further confirmed this by the synergy obtained after combined application of PI3K inhibitor (wortmannin) and DOX. Considering the role of the PI3K-AKT-mTOR pathway in DOX resistance,50,51 our result implies that both pan-AKT and PI3K inhibitors could be a promising tool for overcoming DOX resistance in ATC.

The most promising results in our study were obtained with dual mTOR inhibitor AZD2014, which inhibits all kinase functions of both mTOR complexes thereby completely blocking PI3K-AKT-mTOR signaling.50 To be more specific, all 3 AZD2014 concentrations in a nanomolar range considerably sensitized cells to either PTX or DOX showing no concentration-dependent effect. The synergistic effect of mTOR inhibitor and PTX has already been reported by Shafer et al. The authors suggested 2 mechanisms: one through the enhancement of PTX effect on polymerization of tubulin and the other through abolished phosphorylation of S6 kinase, which is a target of mTOR kinase.51 Because it is known that the PI3K-AKT-mTOR pathway is involved in DOX resistance,50 inhibition of this pathway by dual mTOR inhibitor contributes to DOX sensitization.

The application of dabrafenib significantly sensitized ATC cells with mutated BRAF to both DOX and PTX. Unlike actual treatment options for ATCs with mono- or combined therapy of dabrafenib and kinase inhibitors, we tried to show how intracellular inhibition of signaling molecules might help in overcoming ATCs’ chemoresistance as a multimodal approach suggested previously.17 Therefore, these results may help in planning new therapeutic strategies for this incurable disease.

SPECULATIONS

Although ATC accounts only for 2% of thyroid cancer incidence, it is very aggressive and results in 14%–39% of thyroid cancer deaths. In addition, it is resistant to anticancer therapy. This prompted us to examine RAS-MAPK-ERK and PI3K-AKT-mTOR in samples of patients with ATC, 2 usually altered signaling pathways that are involved in resistance to chemotherapy. Herein, we report for the first time activating mutation of NRAS in codon 50 associated with ATC. This mutation was followed by mutation of p53 and negatively correlated with pAKT expression. Moreover, we observed positive correlation between PTEN and pERK suggesting that either RAS-MAPK-ERK or PI3K-AKT-mTOR pathway governs carcinogenesis of ATC. Then, we transferred our investigation in vitro and pointed to therapeutic modalities useful for sensitization of ATC to classic chemotherapeutics. We have shown that inhibition of either RAS-MAPK-ERK or PI3K-AKT-mTOR pathway components may form a basis for the development of novel genetic-based therapeutic approach for ATC. Our results implicate that inhibition of RAS-MAPK-ERK signaling is more rational in combination with PTX, whereas inhibition of PI3K-AKT-mTOR is more effective in combination with DOX. The application of dual mTOR inhibitor AZD2014 seems to be a logical therapeutic strategy for patients with ATC because it equally synergizes with both DOX and PTX. The usage of mutated BRAF inhibitor dabrafenib in combination with classic cytostatics must be considered for patients with this specific mutation. Further investigation including preclinical and clinical trials is needed to evaluate the clinical relevance of these findings.
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REFERENCES