 DDR1 regulates thyroid cancer cell differentiation via IGF-2/IR-A autocrine signaling loop

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*(V Vella and M L Nicolosi contributed equally to this work)

**Abstract**

Patients with thyroid cancers refractory to radioiodine (RAI) treatment show a limited response to various therapeutic options and a low survival rate. The recent use of multikinase inhibitors has also met limited success. An alternative approach relies on drugs that induce cell differentiation, as the ensuing increased expression of the cotransporter for sodium and iodine (NIS) may partially restore sensitivity to radioiodine. The inhibition of the ERK1/2 pathway has shown some efficacy in this context. Aggressive thyroid tumors overexpress the isoform-A of the insulin receptor (IR-A) and its ligand IGF-2; this IGF-2/IR-A loop is associated with de-differentiation and stem-like phenotype, resembling RAI-refractory tumors. Importantly, IR-A has been shown to be positively modulated by the non-integrin collagen receptor DDR1 in human breast cancer. Using undifferentiated human thyroid cancer cells, we now evaluated the effects of DDR1 on IGF-2/IR-A loop and on markers of cell differentiation and stemness. DDR1 silencing or downregulation caused significant reduction of IR-A and IGF-2 expression, and concomitant increased levels of differentiation markers (NIS, Tg, TSH, TPO). Conversely, markers of epithelial-to-mesenchymal transition (Vimentin, Snail-2, Zeb1, Zeb2 and N-Cadherin) and stemness (OCT-4, SOX-2, ABCG2 and Nanog) decreased. These effects were collagen independent. In contrast, overexpression of either DDR1 or its kinase-inactive variant K618A DDR1-induced changes suggestive of less differentiated and stem-like phenotype. Collagen stimulation was ineffective. In conclusion, in poorly differentiated thyroid cancer, DDR1 silencing or downregulation blocks the IGF-2/IR-A autocrine loop and induces cellular differentiation. These results may open novel therapeutic approaches for thyroid cancer.

**Key Words**
- thyroid cancer
- Discoidin Domain Receptor 1
- insulin receptor isoform-A
- insulin-like growth factor-2
Introduction

In patients with thyroid cancer from follicular thyroid cells, metastatic spread is the most frequent cause of death (Mazzaferrri & Kloo 2001). Differentiated thyroid cancers (DTCs) with papillary and follicular histotypes account for 85–90% of total cancers originated from thyroid follicular cells. First-line therapy of DTCs includes surgery followed or not by radioiodine administration for residue ablation (Lamartina & Cooper 2015, Haugen 2017). Radioiodine administration is also used to treat distant metastases that occur in 5–10% of patients with DTCs. However, approximately two-thirds of these patients will eventually become refractory to radioactive iodine (RAI) therapy with poor prognosis and a mean life expectancy of less than 5 years (Durante et al. 2013, D’Agostino et al. 2014, Gruber & Colevas 2015).

Moreover, poorly differentiated thyroid cancers (PDTCs), which represent 2–10% of total thyroid cancers, and anaplastic thyroid carcinomas (ATCs) (<1% of total cases) have reduced/absent capacity to concentrate iodine and often develop distant metastases (Kondo et al. 2006, Antonelli et al. 2008, Asioli et al. 2010). Advanced RAI-refractory thyroid cancers are rarely responsive to conventional therapies and are often treated with multikinase inhibitors (MKIs) with anti-angiogenic activity. In particular, sorafenib and lenvatinib, the two MKIs approved for the treatment of these patients, have been shown to improve progression-free survival but not overall survival (Brose et al. 2014, Ferrari et al. 2015, Schlumberger et al. 2015, Nagarajah et al. 2016). Thus, novel and more effective therapies are urgently needed for PDTC/ATC and RAI-refractory DTCs.

In addition to tumor angiogenesis, several mutational and non-mutational events in survival signals (i.e. BRAF/MAPK, PI3K, mTOR) and/or tyrosine kinase receptors (i.e. Ret, EGFR, HGF/SF receptors, insulin and insulin-like receptors (IR and IGF-1R)) have been implicated in thyroid cancer progression and may represent suitable targets (Vella et al. 2001, Espinosa et al. 2007, Knauf & Fagin 2009, Frasca et al. 2013, Malaguarnera et al. 2014).

Recently, the inhibition of the mitogen-activated kinase (MAPK) pathway has been proposed as a differentiation strategy, in order to re-activate iodine uptake and cancer cell sensitivity to RAI (Ho et al. 2013).

We previously demonstrated that human thyroid cancers often overexpress the isoform-A of the insulin receptor (IR-A) (Vella et al. 2001, 2002, Malaguarnera et al. 2011). IR-A, also known as the ‘fetal’ IR isoform, differs from the ‘metabolic’ IR-B isoform for the exclusion of exon 11 (Belfiore et al. 2017). In addition to insulin binding, the IR-A is a high-affinity receptor for insulin-like growth factor 2 (IGF-2), which is often produced locally by cancer cells by mechanisms including loss of imprinting (Hajdu et al. 2010). Moreover, overactivation of the IGF-2/IR-A loop is a hallmark of several cancer histotypes and is associated with cancer progression, metastases and resistance to therapies (Sciaccà et al. 1999, Vella et al. 2002, Law et al. 2008, Malaguarnera et al. 2012, Malaguarnera et al. 2017).

The IGF-2/IR-A loop has also been implicated in epithelial-to-mesenchymal transition (EMT) (Zelenko et al. 2016) and other stem-like features (Malaguarnera et al. 2011), which play a key role in cancer development and recurrence. In human thyroid cancer, IR-A overexpression and autocrine IGF-2 levels were strongly associated with tumor aggressiveness, loss of differentiation (Vella et al. 2002) and with stem-like phenotype and self-renewal when cancer cells were cultured as thyrospheres (Malaguarnera et al. 2011, Giani et al. 2015).

Taken together, these data suggest that targeting the IR-A/IGF-2 loop in poorly differentiated thyroid cancer might result in significant switch to differentiation. Unfortunately, this is not an easy target as specific IR-A inhibitors are not available.

Notably, we recently found that Discoidin Domain Receptor 1 (DDR1) functionally interacts with the IR and is a novel important regulator of IR expression (Vella et al. 2017, Belfiore et al. 2018). DDR1 silencing downregulated IR expression and reduced insulin and IGF-2-induced IR-A-dependent biological responses in human breast cancer cells (Vella et al. 2017).

Herein, we aimed at investigating whether targeting DDR1 in thyroid cancer cells might affect the IGF-2/IR-A loop, thus inhibiting stemness and favoring cell differentiation. We also evaluated the impact of a combination therapy targeting both DDR1 and the IR/IGF-1R pathways.

Indeed, our results confirm the hypothesis that the IGF-2/IR-A/DDR1 pathway is an important regulator of cell stem-like phenotype and differentiation in human thyroid cancer.

Materials and methods

Materials

Bovine serum albumin, fibronectin, collagen IV from Sigma-Aldrich; Metafectene PRO from Biontex Laboratories GmbH (Germany); lipofectamine 2000,
### Table 1  PCR primers used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDR1 total (Vella et al. 2017)</td>
<td>Fw 5′-GGGCTCTGTCGCAGGACTAG-3′</td>
</tr>
<tr>
<td>IR total (Vella et al. 2017)</td>
<td>Rv 5′-AGGCATGAGTAATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>IR isoforms (Malaguarnera et al. 2011)</td>
<td>Fw 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>IGF-1 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-TTGCGAGGACAGGTCATCGAGATGTT-3′</td>
</tr>
<tr>
<td>IGF-2 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GGGCTCTGTCGCAGGACTAG-3′</td>
</tr>
<tr>
<td>HMGA1 (Vella et al. 2017)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>Sp1 (Vella et al. 2017)</td>
<td>Rv 5′-TTGCGAGGACAGGTCATCGAGATGTT-3′</td>
</tr>
<tr>
<td>β-ACTIN (Vella et al. 2017)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>hRP-S9</td>
<td>Rv 5′-TTGCGAGGACAGGTCATCGAGATGTT-3′</td>
</tr>
<tr>
<td>IGF-1R (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>hnRNP-A2B1 (Chettouh, et al. 2013)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>hnRNP-A1 (Chettouh, et al. 2013)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>hnRNP-F (Chettouh, et al. 2013)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>hnRNP-H (Chettouh, et al. 2013)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>SF2/ASF (SRSF1) (Chettouh et al. 2013)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>CUGBP-1 (Masuda, et al. 2012)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>MBNL1 (Cheng, et al. 2014)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>VIMENTIN</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>N-CADHERIN (Cheng et al. 2012)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>ZEB1 (Hugo et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>ZEB2 (Kang et al. 2014)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>SNAIL2</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>NANOG (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>OCT4 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>ABCG2 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>SOX2 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>CD133 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>TG (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>NIS (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>TPO (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>TTF1 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>TSHR (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>PAX8 (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
<tr>
<td>GAPDH (Malaguarnera et al. 2011)</td>
<td>Rv 5′-GCTGAGAGAAGATATACATCGAGATGTT-3′</td>
</tr>
</tbody>
</table>

Fw, Forward; Rv, Reverse.
lipofectamine RNAiMax, Opti-MEM, fetal calf serum (FCS), TRIzol Reagent, ThermoScript RT kit, SYBR Green MasterMix from Life Technologies, Inc. Laboratories; methyl thiazolyl tetrazolium (MTT), nitrocellulose membranes, HRP-conjugated secondary antibodies from Amersham Biosciences. Constructs encoding either an empty vector (pCMV6-Entry with C-terminal Myc-DDK Tag), turbo Green Fluorescent Protein (pCMV6-AC-tGFP) and the human WT DDR1 isoform α (DDR1 WT) cDNAs were purchased from OriGene (Rockville, MD, USA). The specific silencer Select Pre-designed pool of four siRNA oligos for DDR1 (Human DDR1 siGENOME SMARTpool Cat M-003111-04) and the negative control, consisting of a pool of four scramble siRNAs were from Thermo Fisher Scientific Dharmacia (NYSE: TMO). DDR1 tyrosine kinase inhibitors DDR1-IN-1 dihydrochloride and 7RH were obtained respectively by Tocris Bioscience (Bristol, UK) and by Sigma. The BRAF inhibitor vemurafenib and the IR tyrosine kinase inhibitor NVP-AEW541 were purchased from Selleck (Houston, TX, USA). The DDR1 mutant K618A (DDR1/KD) in the pCMV6-Entry vector was generated with the QuickChange II XL Site-directed Mutagenesis Kit (Agilent Technologies).

Thyroid cell cultures and human tissue specimens

Anaplastic thyroid cancer cells SW1736, Hth74 and C643, were provided by Dr N E Heldin (Uppsala, Sweden); the FF1 cell line was established in our laboratory. Papillary 8505C thyroid cancer cell line was purchased from EACC (Salisbury, UK). Papillary thyroid cancer cell lines TPC1, and BcPap were provided by Drs A Fusco and M Santoro (Naples, Italy). 4A1 cell line was obtained from normal thyrocytes immortalized with SV-40 virus, as previously described (Belfiore et al. 1991). These cell lines were grown in complete RPMI 1640 (Sigma), with the exception of SW1736, C643, Hth74 and BcPap cells, which were grown in complete MEM (Sigma). 4A1 cells were maintained in complete DMEM (Sigma). Key molecular abnormalities found in cancer cell lines are shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). TPC1, BcPap, 8505C, SW1736, Hth74 and C643 cell lines have been authenticated for correct origin by STR profile analysis.

Thyrospheroids were generated as previously described (Fierabracci et al. 2008). Briefly, single cells from tissue digestion were cultured at a density of 10,000–30,000 cells/mL in low-attachment flasks in DMEM/F12 (Sigma), B27 supplement (Life Technologies) and human recombinant epidermal and basic fibroblast growth factors (20 ng/mL each).

Normal thyroid primary cultures from tissue specimens were established from normal thyroid tissue, as previously described (Milazzo et al. 1992). Thyroid tissue specimens were collected at surgery, immediately frozen and stored in liquid nitrogen until processing. Thyroid cancer specimens (two differentiated and four less differentiated) and six normal thyroid specimens were homogenized using gentle MACS dissociation for protein and RNA analysis. The study was approved by the Local Ethics Committee of the University Magna Graecia of Catanzaro (Reg. prot. n. 229 of 21th September 2017) and all patients provided written informed consent.

Western blot analysis

Cell lysates were subjected to western blot analysis as previously described (Malaguarnera et al. 2008). The following antibodies were used: anti-DDR1 (C-20, sc-532), anti-IRβ (C-19, sc-711), anti-tubulin (H-235, sc-9104) (Santa Cruz Biotechnology); anti-p-DDR1 (Tyr792); anti-p-Akt (Ser473), anti-AKT, anti-p-ERK1/2 (T202/Y204), anti-ERK1/2 (Cell Signaling Technology).

IR isoform mRNA expression

IR isoform mRNA expression was measured by RT-PCR analysis (Bioline PCR Kit) using primers for the flanking exons 10 and 12 and resolved on 2.5% agarose gel. The 167-bp and 131-bp DNA fragments, representing Ex11+ (IR-B isoform) and Ex11– (IR-A isoform), respectively, were quantified by densitometry analysis. The proportion of IR-A was calculated as densitometric value of band IR-A/densitometric values of bands IR-A+IR-B.

Real-time PCR

Total cellular RNA was extracted using TRIZol Reagent according to the manufacturer’s protocol, as previously described (Malaguarnera et al. 2015). qRT-PCR was used to confirm the expression levels of mRNAs. Total RNA (2 µg) was reversely transcribed using the ThermoScript RT kit and oligo (dT) primers. Synthesized cDNA was combined in a qRT-PCR reaction using primers for the gene of interest (Table 1). Real-time PCR was performed with the ABI 7500 Real-Time PCR System (Applied Biosystems) using probe, primer sets and TaqMan Universal Master Mix (Taqman gene Expression Assay, Applied Biosystems) or SYBR Green chemistry. Human GAPDH and β-ACTIN were used for normalization in both TaqMan and SYBR
Green chemistry. mRNA quantification was performed using the comparative CT method (ΔΔCT).

Gene silencing by siRNA and gene overexpression

For siRNA experiments, cells were transiently transfected with a mixture containing Opti-MEM, Lipofectamine RNAiMax and either a pool of four scramble siRNA oligos (10 nM) or a pool of four specific siRNA oligos for DDR1 (10 nM).

For overexpression experiments, cells were transiently transfected with a mixture containing Opti-MEM, Metafectene PRO (Biontex) and the DNA of interest or the corresponding control empty vectors. Most experiments of DDR1 overexpression were performed by transiently transfecting cells with plasmids encoding either the human constitutive pCMV6-DDR1 or the corresponding empty vector (pCMV6-EV). Cells were then processed after 48 h, according to the aim of the experiment.
Generation of human IR promoter-Luc+ plasmid

The human IR promoter (hIRp) region was previously described (Seino et al. 1989). We used pG3-Luc+ basic plasmid (from Promega) to generate pGL3-Luc+-hIRp containing −877 to −2 promoter fragments. Genomic DNA extracted from HeLa cells by TRIZol Reagent was subjected to PCR reaction by Pfx DNA polymerase (all from Invitrogen) using the following oligonucleotides:
Fw (−877) 5′-CGACGCGTCTCGAGTCAACAAAATAAACAATT-3′
Rv (−2) 5′-CATGTCAGCTGCGGAGGAGCGCGG-3′

The PCR fragment obtained, which is the minimal transactivation region having the 99.7% of the maximal promoter activity (Seino et al. 1989), was purified and cloned in pGL3-Luc+ reporter plasmid using MluI-NheI restriction sites.

Generation of pLEX FLAG-tagged Sp1v1 vector

Total RNA was extracted from HeLa cells by TRIZol Reagent and then reverse-transcribed using Superscript III (all from Invitrogen). The resulting complementary DNA was subjected to PCR reaction by Pfx DNA polymerase (Invitrogen) using Sp1-specific oligonucleotides with FLAG sequence in the forward primer:
Fw: 5′-ATA AGA ATG CGG CCG CGC CAC CAT GGA TTA CAA GGA TGA CGA CGA TAA GAT GAG CGA CCA AGA TCA CTC C-3′
Rv: 5′-CGA CGC GTT CAG AAG CCA TTG CCA CTG ATA-3′

The PCR fragment of ~2300 bp was purified and then cloned in pLEX vector by NotI-MluI (New England Bio labs) restriction sites. To select pLEX FLAG-tagged Sp1 variant 1 vector (pLEX FLAG-tagged Sp1v1), DNA obtained from ten bacterial colonies was subjected to PCR amplification using the Fw primer mentioned above and the following reverse primer: 5′-ACTGCTGCCACTCTGTTCCT-3′. DNA PCR fragment corresponding to 381 bp was selected since identified the Sp1v1.

IR promoter activity

Cells were transiently transfected, as described earlier, with either scramble or specific siRNA oligos for DDR1 (10 nM). Twenty-four hours after transfection, cells were incubated with fresh medium and transfected with 1 µg of the firefly luciferase reporter plasmid containing the hIR promoter (pGL3-Luc+–hIRp) together with the empty vector or Sp1, using Metafectene PRO (Biontex). As an internal control of transfection efficiency, pCMV6-AC-tGFP plasmid vector was used. Luciferase assay was performed 48 h post transfection and assayed using the Dual Luminescence Assay Kit (Promega) as described by the manufacturer.

Cell viability

Cell viability was measured by MTT test. Thyroid cell lines were plated in 48-multiwell plates under standard culture conditions. The number of viable cells was determined with the MTT assay 48 h post transfection.
Endocrine-Related Cancer

Table 2  IR expression and IGF-2 secretion in thyroid cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total IR (ELISA) (ng/100 µg membrane protein)</th>
<th>%IR-A (RT-PCR)</th>
<th>IR-A (ng/100 µg membrane protein)</th>
<th>IGF-2 expression (RT-PCR)</th>
<th>IGF-2 in conditioned medium (ng/mL/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW1736</td>
<td>15.95 ± 1.16</td>
<td>58.8 ± 3.84</td>
<td>9.37 ± 0.61</td>
<td>Positive</td>
<td>1.65 ± 0.17</td>
</tr>
<tr>
<td>C643</td>
<td>1.13 ± 0.11</td>
<td>50.9 ± 4.78</td>
<td>0.57 ± 0.05</td>
<td>Positive</td>
<td>16.66 ± 0.23</td>
</tr>
<tr>
<td>Hth74</td>
<td>28.71 ± 1.68</td>
<td>32.1 ± 3.94</td>
<td>9.21 ± 1.13</td>
<td>Positive</td>
<td>21.19 ± 0.31</td>
</tr>
<tr>
<td>FF1</td>
<td>7.56 ± 0.21</td>
<td>70.9 ± 0.33</td>
<td>5.36 ± 0.02</td>
<td>Positive</td>
<td>10.69 ± 0.12</td>
</tr>
<tr>
<td>8505C</td>
<td>21.51 ± 1.46</td>
<td>65.4 ± 0.72</td>
<td>14.06 ± 0.15</td>
<td>Positive</td>
<td>1.03 ± 0.21</td>
</tr>
<tr>
<td>BcPap</td>
<td>19.99 ± 0.23</td>
<td>59.3 ± 2.42</td>
<td>11.85 ± 0.48</td>
<td>Positive</td>
<td>1.40 ± 0.10</td>
</tr>
<tr>
<td>TPC1</td>
<td>9.21 ± 0.62</td>
<td>42.8 ± 8.92</td>
<td>3.94 ± 0.82</td>
<td>Positive</td>
<td>4.95 ± 0.65</td>
</tr>
<tr>
<td>4A1</td>
<td>15.67 ± 0.15</td>
<td>38.4 ± 0.85</td>
<td>6.01 ± 0.13</td>
<td>Positive</td>
<td>0.98 ± 0.11</td>
</tr>
<tr>
<td>NPC</td>
<td>1.7 ± 0.3</td>
<td>34.3 ± 3.05</td>
<td>0.583 ± 0.05</td>
<td>Negative</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Densitometric and statistical analysis

Densitometry results were obtained using NIH ImageJ. IC₅₀ values were calculated from linear transformation of dose–response curves. To define drug–drug interactions (in terms of synergism, additivity or antagonism), the combination index (CI) was calculated with the isobologram equation (Chou et al. 1994). The effect of drug–drug interactions was defined synergistic when the CI was <0.9; additive when it was 0.90 < CI < 1.10 and antagonistic when CI was >1.10 (Manara et al. 2010).

Differences between means were evaluated by one-way ANOVA followed by post hoc analysis of significance (Bonferroni test) for the comparison between more than two groups, whereas the Student’s t test for unpaired samples was used for comparisons between two groups. The level of significance was set at P<0.05. Statistical analysis was performed with GraphPadPrism6 (GraphPad Software). Data were expressed as mean ± s.e.m.

Results

DDR1, IR-A and IGF-2 expression in human thyroid cancer cells and cancer specimens

We first evaluated the expression of DDR1 and IR in several human thyroid cancer cell lines including papillary cancer cells (TPC1, BcPap) and anaplastic cancer cells (SW1736, C643, Hth74, 8505C, FF1). Immortalized, non-malignantly transformed human thyroid cells (4A1) and primary cell cultures obtained from normal thyroid tissues (NPCs) were also studied. In addition, DDR1 and IR expression was evaluated in human thyroid cancer specimens and in contralateral normal thyroid tissues obtained at surgery.

In thyroid cells, DDR1 was expressed at various levels. The highest expression was detectable in one cell line from anaplastic cancer (SW1736) and in two papillary cancer

Invasion assay

The ability of cells to invade the extracellular matrix was measured in Boyden’s chambers as described (Malaguarnera et al. 2015). Cells were placed on polycarbonate filters (8 µm pore size, Corning Costar) coated on the upper side with 25 µg/mL fibronectin or 250 µg/mL collagen IV. After incubation for 6 h, cells on the upper surface of filters were removed with a cotton swab, and the filters were stained for 30 min with crystal violet (0.05% crystal violet in PBS plus 20% ethanol). After three washes with water, crystal violet was solubilized in 10% acetic acid for 30 min at room temperature, and its concentration was evaluated by absorbance at 595 nm.

IGF-2 secretion in conditioned medium

Cell culture supernatants from the different cell lines were harvested after 72 h of culture and stored at −20°C until use. The concentrations of hIGF-2 were quantified using sandwich ELISA kits (Mediagnostic, Germany), according to the manufacturer’s instructions.

IR measurement

IR content was measured from membrane fractions, prepared as previously described (Vella et al. 2002). Lysates from membrane fraction of thyroid cell lines were subsequently used for IR content measurement by ELISA (Invitrogen).

conditions. After 24 h, cells were transfected with a pool of four DDR1 siRNA oligos for DDR1-expressing vectors and the relative negative controls. After 72 h, transfected cells were then incubated with medium containing 5 mg/mL MTT and processed as previously described (Vella et al. 2009).

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confirmed that DDR1 was clearly overexpressed at the level of follicular thyroid cells while scarcely expressed at the stromal level (not shown).

Levels of IR and DDR1 mRNA were also evaluated by qRT-PCR and mirrored protein levels both in thyroid cancer cells, primary cultures (Fig. 1C) and tissue specimens (Fig. 1D).

Thyroid cancer cell lines, with the exception of Hth74 cells, predominately expressed IR as the ‘fetal’ IR-A isoform rather than the ‘metabolic’ IR-B isoform (Fig. 2A). The median IR-A relative abundance in cancer cells was 58.8% (range 32.1–70.9%), while the immortalized human thyroid 4A1 expressed approximately 40% of IR-A. In cancer specimens (C), IR-A relative abundance ranged from 50 to 60% as compared to 38–40% of normal thyroid specimens (N) (Fig. 2B).

Measurement of total IR in cell lysates was performed by ELISA and confirmed the high expression of IR protein in thyroid cancer cell lines. As specific antibodies for the IR-A are not available, the absolute amount of IR-A protein could be crudely estimated by combining ELISA data with the proportion of IR-A isoform as measured by RT-PCR (Table 2).

IGF-2 transcript was found in all thyroid cells, as evaluated by RT-PCR (Fig. 3A), while IGF-1 transcript was barely detectable only in Hth74 cells (Fig. 3B). IGF-2 protein was additionally measured by ELISA in media conditioned by the various thyroid cancer cell lines, as shown in (Fig. 3C and Table 2). IGF-2 mRNA levels were also analyzed in tissue specimens where IGF-2 mRNA levels were upregulated in thyroid cancer specimens compared to normal controls (Fig. 3D).

Taken together, these data indicate that DDR1 and IR-A are expressed at high levels in human thyroid cancer cells and tissue specimens and support previous data indicating that an autocrine/paracrine IGF-2/IR-A loop is active in human thyroid cancer (Vella et al. 2002).

Three ATCC cell lines (SW1736, 8505C and Hth74) and one PTC cell line (TPC1) that co-express IR and DDR1 at various levels were chosen for all subsequent experiments aiming at evaluating whether DDR1 silencing or overexpression might affect the IGF-2/IR-A autocrine loop and the cell biology of differentiation. Hth74 cells were also chosen for their high levels of IGF-2 expression (Fig. 3C).

**DDR1 regulates the IGF-2/IR-A loop in thyroid cells**

In all four thyroid cancer cell lines (SW1736, 8505C, Hth74 and TPC1), DDR1 silencing by siRNA approaches determined a marked reduction of IR protein (median reduction 61.5%,
Figure 4
DDR1 affects IR expression levels. (A) Western blot after DDR1 silencing. SW1736, 8505C, Hth74 and TPC1 thyroid cancer cell lines were transiently transfected with either a pool of four scramble siRNAs or a pool of four siRNA oligos against DDR1. After 48 h, cells were lysed and analyzed by SDS-PAGE and immunoblotted with the indicated primary antibodies. Tubulin was used as control for protein loading. Blot is representative of three independent experiments. Histograms represent the mean ± S.E.M. of DDR1 and IR densitometric analysis after normalization against tubulin. (B) qRT-PCR after DDR1 silencing. In DDR1 silenced cells (siDDR1), IR mRNA levels were evaluated by qRT-PCR analysis and values were normalized using human β-ACTIN as housekeeping control gene. In parallel, DDR1 mRNA was evaluated by qRT-PCR to confirm DDR1 silencing. (C) Western blot after collagen stimulation. SW1736 and 8505C cells were transiently transfected with either a constitutive empty (pCMV6-EV) or a human DDR1 (pCMV6-DDR1) expressing vectors. After 24 h cells were stimulated with 10 µg/mL collagen IV for 48 h. Cells were then lysed and analyzed by SDS-PAGE and immunoblotted with the indicated primary antibodies. Tubulin was used as control for protein loading. Blot is representative of three independent experiments. Histograms represent the mean ± S.E.M. of densitometric values after normalization against tubulin. (D) Western blot after DDR1 overexpression. Thyroid cancer cell lines were transiently transfected with either a constitutive empty (pCMV6-EV) or a human DDR1 (pCMV6-DDR1) expressing vectors. After 48 h, cells were lysed and analyzed by SDS-PAGE and immunoblotted with the indicated primary antibodies. Tubulin was used as control for protein loading. Blot is representative of three independent experiments. Histograms represent the mean ± S.E.M. of densitometric analysis after normalization against tubulin. (E) qRT-PCR after DDR1 overexpression. In DDR1-transfected cell lines, IR mRNA levels were evaluated by qRT-PCR analysis and values were normalized using human β-ACTIN as housekeeping control gene. DDR1 overexpression was confirmed measuring DDR1 mRNA by qRT-PCR. (F) Thyroid cancer cells were transiently transfected with either a constitutive empty (pCMV6-EV) or the human DDR1 (pCMV6-DDR1) or the mutant K618A DDR1 expressing vectors. After 48 h, cells were lysed and analyzed by SDS-PAGE and immunoblotted with the indicated primary antibodies. Tubulin was used as control for protein loading. Blot is representative of three independent experiments. Histograms represent the mean ± S.E.M. of densitometric values after normalization against tubulin. (G) IR isoforms transcripts after DDR1 silencing. IR isoform (IR-A and IR-B) transcripts were evaluated in all four thyroid cancer cell lines after DDR1 silencing, as described in (A). (H) qRT-PCR expression of IGF-2 after DDR1 silencing. IGF-2 transcript was evaluated in six cell lines. Normalization was done using human β-ACTIN as housekeeping control gene. Data are presented as the mean ± S.E.M. (error bars) from three independent experiments. (A, B, C, D, E and F) *0.01 < P < 0.05; **0.001 < P < 0.01; ***P < 0.001; statistical significance was calculated using one-way ANOVA followed by Bonferroni test.
a decrease of IR-A (ranging from 28 to 42%) with a corresponding increase of IR-B levels (Fig. 4G) and was associated with a significant inhibition of autocrine IGF-2 expression in several thyroid cancer cell lines, which included two additional cell lines, C643 and FF1, which together with Hth74 cells, express the highest levels of autocrine IGF-2 production (Fig. 4H). IGF-2 expression was not significantly affected by collagen stimulation (48 h) in untransfected cells (Supplementary Fig. 2A) whereas was upregulated by transfection with DDR1, both WT and K618A mutant (Supplementary Fig. 2B).

The inhibition of IGF-2/IR-A loop associated with DDR1 silencing determined a significant inhibition of downstream signaling, as demonstrated by a significant reduction of AKT and ERK1/2 phosphorylation (Fig. 5A). On the contrary, DDR1 overexpression induced strong increase of AKT and ERK1/2 phosphorylation (Fig. 5B). However, modulating DDR1 levels by either siRNA approaches or overexpression strategies did not affect total AKT and ERK1/2 expression levels (Fig. 5A and B).

To further investigate the mechanisms regulating IR-A levels, we demonstrated that DDR1 silencing reduced the activity of the IR promoter (Fig. 6A) and significantly diminished the expression of Sp1 and HMGA1, two key positive regulators of IR transcription (Fig. 6B).

Additionally, several splicing factors, including CUGBP1, hnRNP-H, hnRNP-A2B1, SF2/ASF, which are involved in exon 11 skipping of the IR gene and in the preferential generation of the IR-A isoform were significantly downregulated by DDR1 silencing in thyroid cancer cells (Fig. 7). Conversely, MBNL1, hnRNP-F and SRp20, factors inhibiting exon 11 skipping were upregulated (Fig. 7).

Collectively, these data indicate that, in human thyroid cancer cells, DDR1 by acting at multiple levels is a potent modulator of the IGF-2/IR-A autocrine loop.
DDR1 affects the biology and phenotype of thyroid cancer cells

Having shown that reducing DDR1 levels downregulates the IGF-2/IR-A loop, we next investigated whether it might also affect some relevant characteristics of thyroid cancer cell biology. As shown in Fig. 8A and B, DDR1 silencing significantly reduced the proliferation rate and the invasive potential of thyroid cancer cells, whereas DDR1 overexpression enhanced both features. In cell lines SW1736 and 8505C, proliferation and invasion experiments were performed both in the presence or absence of collagen. However, collagen did not affect the results (Fig. 8C).

Significantly, DDR1 silencing was associated with a more differentiated cell phenotype, as shown by a significant increase of essential markers of thyroid cell differentiation, such as thyroglobulin (Tg), thyroperoxidase (TPO) and sodium-iodine symporter (NIS), TTF1 and PAX8, two key transcription factors involved in thyroid cell differentiation were also significantly increased (Fig. 8D). Finally, DDR1 silencing caused a general and significant decrease of several mesenchymal markers (Vimentin, Snail-2, Zeb1, Zeb2 and N-Cadherin) associated with EMT (Fig. 8D), and markers associated with cell stemness, including OCT-4, SOX-2, ABCG2 and Nanog (Fig. 8D). The presence of collagen did not affect these results (Supplementary Fig. 3A and B). Transfection with either DDR1 wild type or DDR1 K618A mutant was associated with changes in markers expression suggestive of a less differentiated and more stem-like phenotype (Supplementary Fig. 4A and B), whereas collagen stimulation of untransfected cells was ineffective (Supplementary Fig. 5A, B and C).
Combination treatment with inhibitors of IR/IGF-1R and DDR1 has additive effects on thyroid cancer proliferation and invasion

Given the positive feedback between DDR1 and the IGF-2/IR-A loop, we next evaluated whether the combined pharmacological inhibition of DDR1 and IR could be a useful therapeutic tool to target the most aggressive TCs. To this aim, we first explored the ability of a DDR1 inhibitor (DDR1-IN-1) to affect the IGF-2/IR-A loop. Exposure of SW1736 and 8505C cells to DDR1-IN-1 was associated with a significant reduction of DDR1, IR and IGF-2 (Fig. 9A and B). Similar results were obtained with a second DDR1 inhibitor, 7RH (Supplementary Fig. 6A and B), supporting the specificity of these effects. Exposure to DDR1-IN-1 was also associated with reduction of the relative abundance of IR-A (Fig. 9C). SW1736 and 8505C cancer cells were transiently transfected with a pool of four scramble siRNAs or of four siRNA oligos against DDR1. After 24 h they were stimulated with collagen IV for 48 h. Cell viability was evaluated by MTT assay (left). For invasion assay, 48 h after transfection cells were removed from plates with 0.01% trypsin and seeded on polycarbonate filters coated with 10 μg/mL collagen IV. Cells were allowed to migrate for 6 h (right). (D) Thyroid-specific differentiation, EMT, and stemness markers in cells silenced for DDR1 expression. After DDR1 silencing cells showed a significant increase of thyroid cell differentiation markers and two key transcription factors (left). DDR1 silencing caused also a significant decrease of several markers associated with EMT (middle) and of cell stemness markers (right). (A, B, C and D) ns, P > 0.05; *0.01 < P < 0.05; **0.001 < P < 0.01; ***P < 0.001; statistical significance was calculated using one-way ANOVA followed by Bonferroni test.

Figure 8
DDR1 silencing affects the biological effects of thyroid cancer cells. (A) Cell proliferation after either DDR1 silencing or DDR1 overexpression. Thyroid cancer cells were transiently transfected with a pool of four scramble siRNAs or of four siRNA oligos against DDR1 (left). Alternatively cells were transiently transfected with plasmids encoding either the empty vector (pCMV6-EV) or the human DDR1 cDNA (pCMV6-DDR1) (right). Cell viability was evaluated by MTT assay. Values are expressed as percentages of untreated scramble-transfected cells (basal) and represent the mean ± S.E.M. of three independent experiments in triplicate. (B) Cell invasion after either DDR1 silencing or DDR1 overexpression. For invasion assay, 48 h after transfection cells were removed from plates with 0.01% trypsin and seeded on polycarbonate filters coated with 25 μg/mL fibronectin. Cells were allowed to migrate for 6 h. Values are expressed as percentages of empty vector-transfected cells (basal) and represent the mean ± S.E.M. of three independent experiments in triplicate. (C) Cell proliferation and invasion after collagen stimulation. SW1736 and 8505C cancer cells were transiently transfected with a pool of four scramble siRNAs or of four siRNA oligos against DDR1. After 24 h they were stimulated with collagen IV for 48 h. Cell viability was evaluated by MTT assay (left). For invasion assay, 48 h after transfection cells were removed from plates with 0.01% trypsin and seeded on polycarbonate filters coated with 10 μg/mL collagen IV. Cells were allowed to migrate for 6 h (right). (D) Thyroid-specific differentiation, EMT, and stemness markers in cells silenced for DDR1 expression. After DDR1 silencing cells showed a significant increase of thyroid cell differentiation markers and two key transcription factors (left). DDR1 silencing caused also a significant decrease of several markers associated with EMT (middle) and of cell stemness markers (right). (A, B, C and D) ns, P > 0.05; *0.01 < P < 0.05; **0.001 < P < 0.01; ***P < 0.001; statistical significance was calculated using one-way ANOVA followed by Bonferroni test.

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IGF-2/IR-A/DDR1 signaling is enhanced in cancer thyrospheres and associated with dedifferentiation: effects of inhibitors of IR/IGF-1R and DDR1

Thyroid cancer cells cultured as thyrospheres become enriched in stem-like features (Malaguarnera et al. 2011, Giani et al. 2015, Nagayama et al. 2016), thus representing an excellent model for further investigating the role of IGF-2/IR-A and DDR1. In fact, thyrospheres from 8505C cells showed increased expression of IR and IGF-2 (Fig. 10A), and increased IR-A relative abundance, as compared to cell cultured in monolayers (Fig. 10B).

DDR1 expression also increased (Fig. 10A), suggesting that IGF-2/IR-A/DDR1 signaling is amplified when thyroid cancer cells acquire stem-like features. Noteworthy, IGF-2 is able to upregulate DDR1 protein and mRNA expression dose-dependently (Vella et al. 2017) and Supplementary Fig. 8). Exposure of 8505C thyrospheres to DDR1-IN-1 (10 µM) and NVP-AEW541 (1 µM) either as single agents or in combination was associated with reduced DDR1, IR and IGF-2 (Fig. 10C), increased expression of differentiation markers (Fig. 10D) and reduced expression of stemness markers (Fig. 10E). The two inhibitors showed additivity and were similarly or more effective than vemurafenib.
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Figure 10

The crosstalk between the IGF-2/IR-A loop and DDR1 is more relevant in thyrospheres from cancer cells. (A) Relative quantification of DDR1, IR, and IGF-2 mRNAs in cell monolayer compared to thyrospheres. RNA levels were evaluated by qRT-PCR analysis and values were normalized using human β-ACTIN as housekeeping control gene. The experiment shown is representative of three independent experiments. Values are mean ± S.E.M. of three separate experiments. (B) IR isoforms transcripts in cell monolayer (M) compared to thyrospheres (S). IR isoform (IR-A and IR-B) transcripts were evaluated in thyrospheres derived from thyroid cancer cells, as described in Methods. (C, D and E) Effect of DDR-IN-1, NVP-AEW541 and vemurafenib on DDR1, IR and IGF-2 expression in thyrospheres. 8505C cells were grown as thyrospheres in the presence or absence of collagen IV (10 µg/mL), and exposed to DDR1-IN-1 (10 µM), NVP-AEW541 (AEW541) (1 µM), and vemurafenib (1 µM) either as single agents or in combination. Cells were analyzed for DDR1, IR and IGF-2 (C), differentiation markers (D), and stemness markers (E) expression. All the effects described in (C), (D), and (E) were not influenced by the presence of collagen. (A, B, C, D and E) *0.01 < P < 0.05; **0.001 < P < 0.01; ***P < 0.001; statistical significance was calculated using one-way ANOVA followed by Bonferroni test.

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Discussion

Herein we demonstrated that DDR1 expression is upregulated in human thyroid cancer cell lines compared to primary cultures from normal thyroid. DDR1 was also overexpressed in human cancer specimens from thyroid cancer as compared to specimens from normal thyroid tissue. DDR1 is upregulated in a variety of malignancies, but the mechanisms regulating its expression levels are not well understood (Vialiathan et al. 2012). We now provide evidence that IGF-2 upregulates DDR1 levels in a dose-dependent manner in thyroid cancer cells. These data are in agreement with our previous findings indicating that IGF-1, IGF-2 and insulin upregulate DDR1 in breast cancer cells and in murine fibroblasts through the activation of the Akt/miR199a-5p pathway (Mata et al. 2016, Vella et al. 2017).

Therefore, our data suggest that autocrine IGF-2 production might partially account for DDR1 overexpression in human thyroid cancer. Consistently, thyroid cancer cells cultured as thyrospheres and acquiring stem-like features showed upregulation of the IGF-2/IR-A loop and DDR1.

Conversely, DDR1 regulated IR-A levels in human thyroid cancer cells. DDR1 silencing markedly reduced the expression levels of both IR protein and mRNA while DDR1 overexpression had opposite effects. These findings are reminiscent of data observed in human breast cancer cells (Vella et al. 2017) further corroborating the concept that DDR1 is a novel and important regulator of IR expression in various cell models. Interestingly, DDR1 silencing altered the relative abundance of IR isoforms favoring the less ‘mitogenic’ and more ‘metabolic’ IR-B isoform. In fact, DDR1 stimulation of IR-A expression appears to involve multiple mechanisms including IR gene transcription, mRNA stability and splicing as well

(1 µM), an inhibitor of BRAF advocated as a differentiating agent for BRAF-mutated thyroid cancer (Ali et al. 2014, Cheng et al. 2016). These effects were not influenced by the presence of collagen (Fig. 10C, D and E).
as regulating IR protein stability (Vella et al. 2017). Autocrine expression of IGF-2 was also reduced by targeting DDR1 expression.

We previously reported that the IGF-2/IR-A loop is strongly associated with dedifferentiation and stem-like features in thyroid cancer cells (Vella et al. 2002, Malaguarnera et al. 2011). Therefore, we evaluated whether DDR1 silencing could lead to thyroid cancer cell differentiation. Indeed, DDR1 silencing was associated with increased expression of TTF1 and PAX8, tissue-specific transcription factors expressed in the thyroid follicular cells, which contribute to the maintenance of the differentiated phenotype by activating transcription of the thyroglobulin (Tg), thyroperoxidase (TPO) and sodium-iodine symporter (NIS) genes. Accordingly, gene expression for Tg, TPO and NIS was also increased following DDR1 silencing in all cell lines studied. These findings indicate that DDR1 silencing favors cell differentiation, at least in part by downregulating the IGF-2/IR-A loop and inhibits cell proliferation and invasion. In contrast, DDR1 overexpression was associated with dedifferentiation and increased proliferation and invasion. Notably, all these effects of DDR1 are independent from its collagen-binding activity, as they were not mimicked by collagen exposure whereas can be reproduced by the kinase inactive K618A DDR1 variant. These data are in agreement with our previous findings showing that, in human breast cancer cells, the functional crosstalk between DDR1 and IGF-1 receptor is largely independent of the DDR1 kinase (Malaguarnera et al. 2015). Moreover, independent studies have also shown that cell invasion induced by DDR1 is also collagen independent (Juin et al. 2014).

Recently, given the increasingly recognized relevance of DDR1 in cancer, several small molecules with specific DDR1 kinase-inhibiting activity have been characterized (Kim et al. 2013). At least some of these inhibitors have been shown to downregulate DDR1 expression (Gao et al. 2013, Lu et al. 2016), although the mechanism is unclear and may involve ERK1/2 activation (Ruiz & Jarai 2011). We reasoned, therefore, that these inhibitors, might mimic the effects of DDR1 silencing to some extent. In fact, both the DDR1 inhibitors tested (DDR-IN-1 and 7RH) shared the ability to downregulate DDR1 expression. We also found that in cells cultured either as monolayers or as thyrospheres inhibition of DDR1 kinase partially recapitulates the effects of DDR1 silencing causing downregulation of the IGF-2/IR-A loop, cell differentiation and inhibition of cell proliferation and invasion. We also investigated whether a combined treatment with DDR-IN-1 and an inhibitor of the IR/IGF-IR signaling pathway had additive or synergic activity toward the inhibition of insulin and IGF-2 biological effects and found an additive effect on cell proliferation in two different cell lines while the effect on cell invasion was more than additive. When tested in thyrospheres from BRAF-mutated 8505C ATC cells, this treatment was equally or more efficacious than vemurafenib in stimulation re-differentiation. It is worth mentioning that the use of vemurafenib has been previously advocated as re-differentiating agent in BRAF mutated cells (Ali et al. 2014, Cheng et al. 2016).

Recurrent/metastatic disease from poorly differentiated or undifferentiated histotypes as well as RAI-refractory DTCs is resistant to conventional therapies including radioactive iodine and chemotherapy, and it is often treated with multikinase inhibitors with angiogenic activity (Brose et al. 2014, Ferrari et al. 2015, Schlumberger et al. 2015, Nagarajah et al. 2016). However, these drugs have major shortcomings including severe side effect and clonal selection associated with tumor recurrence. Moreover, these drugs are cytostatic, and do not improve patients’ overall survival (Brose et al. 2014, Ferrari et al. 2015, Schlumberger et al. 2015, Nagarajah et al. 2016).

A recent clinical trial showed the efficacy of a selective MAPK inhibitor (Selumetinib) in reversing resistance to radioiodine in patients with metastatic thyroid cancer, especially in patients with RAS mutant-disease, supporting the rationale for therapies inducing re-differentiation (Ho et al. 2013).

In conclusion, our present study identifies the IGF-2/IR-A/DDR1 functional interaction as a novel molecular network upregulated in stem-like thyroid cancer cells, which could represent a suitable target inducing a re-differentiation program in advanced thyroid cancer. Whether these changes are sufficient for reverting RAI resistance in patients with advanced and metastatic disease is still unknown. Our present work provides the rationale for such studies, which may have profound social impact in improving the management and quality of life for patients with RAI-refractory advanced thyroid cancer.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-18-0310.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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