

## Review-II

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# Alternative Strategies in the Management of De-Differentiated Thyroid Cancer

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### SUMMARY

**Over time, up to 30% of differentiated thyroid cancer become de-differentiated and do not respond to traditional therapeutic modalities, posing a therapeutic challenge. Thyroid cancer is characterized by genetic alterations with both, activation of proto oncogenes, and inactivation of tumour suppressors. Over expression and/ or uncontrolled activation of receptor tyrosine kinases, downstream signaling molecules, and inhibition of programmed cell death (apoptosis) are other molecular mechanisms. Currently a number of various therapeutic approaches are being tested in preclinical and clinical studies based on the pathogenesis of the dedifferentiation and target the altered genes or intracellular molecules involved in these processes.**

**It is hoped that some of novel strategies will probably extend the current therapeutic options and provide a hope for otherwise untreatable patients.**

### INTRODUCTION

Differentiated thyroid cancer is the most frequent malignancy of the endocrine system. It accounts for about 1% of all human cancers, with a higher prevalence in women (5 to 9 of 100 000) as compared with men (2 to 4 of 100,000).

Usually, thyroid cancer is a well treatable disease with a good prognosis. The ability to retain features of normal thyroid cells, such as iodine uptake, TSH receptor expression, and thyroglobulin production facilitates monitoring for thyroid cancer recurrence or progression and allow for thyroid-specific treatment. Current therapeutic protocols include surgery to remove the thyroid gland in part or totally; followed, if necessary, by application of  $I^{131}$ . This radioisotope is accumulated in remnant or even disseminated thyrocytes by the thyroidal sodium/iodide symporter (NIS) and destroys them by internal radiation. Another widely used therapeutic measure is the administration of thyroxine (T4) to suppress the production of the thyrotrophic growth factor TSH via hypothalamic and hypophyseal feedback; this aims to reduce the proliferation rate of residual thyroid tissue or metastases. External radiation or chemotherapy do not have much role.<sup>1</sup>

During the natural history of thyroid cancer about 30% of differentiated thyroid cancer de-differentiate and display reduced ability to capture iodine, produce thyroglobulin, and/ or express the TSH receptor; thereby becoming difficult to detect/ monitor and less responsive to traditional therapeutic modalities<sup>2</sup> The presence of de-differentiated cancer, overt or suspected clinically, can be demonstrated/ confirmed by further radiological, (CT, MRI) and/or nuclear medicine investigations (Tl-201 scan,  $^{99m}Tc$ - Tetrofosmin/sestamibi scan or  $^{18}F$ -FDG Positron emission tomography (PET).

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Loss of iodine uptake, makes radioiodine therapy infeasible. Also, dedifferentiated tumours lose the TSH receptor (TSHr) and thus get insensitive to the growth-regulating effects of varying TSH levels; this obliterates any benefit from TSH suppression therapy by T 4. Finally; extensive local tumour growth and/or distant metastases often preclude any further surgical intervention. Following progressive dedifferentiation, the stage of anaplastic thyroid cancer (ATC) is attained, which is associated

with poor prognosis.<sup>3</sup> De-differentiated tumours therefore, pose a therapeutic challenge and there is a need for alternative therapeutic strategies to deal with these patients.

Various therapeutic approaches, currently being tested in preclinical and clinical studies are based on the pathogenesis of the dedifferentiation and target the altered genes or intracellular molecules involved in these processes (Table-1).

**Table-1 Alternative strategies in the management of de-differentiated thyroid cancer, their target,**

Strategy	Target	Mechanism of action	Name of Compound	Current Status of use
<b>1. Gene therapy</b>				
<i>i) NIS gene therapy</i>	NIS gene	NIS gene expression by gene transfer	Viral vectors (Retro/Adeno)	Phase-I Clinical trial
<i>ii) p53 replacement</i>	P53 gene	Introduction of p <sup>53</sup> gene leading to production of p <sup>53</sup> (tumour asuppressor) protein	Viral vectors (etro/Adeno)	Phase-I Clinical trial
<i>iii) Suicide gene therapy</i>	Cell function	Introduction of genes (suicide genes) coding for a sensitizing enzyme which makes tumour cells sensitive against toxin applied as non toxic products	HSVITK/GCN	Used in cell lines and nude mice
<i>iv) Immunotherapy</i>	Host's immune response	Enhancement of host's defense with immuno stimulating agents or genes	Interleukine (IL)-2 or IL-2 genes	Phase-I Clinical trial
<i>v) Genetic immunisation</i>	Immune status	Injection of DNA, coding for tumour antigen, leading to antibody production	Naked plasmid DNA	Animal studies
<i>vi) Anti-sense therapy</i>	Proto oncogenes (c myc)	Blocking of proto oncogene expression	Antisense oligonucleotide	Used in cell lines
<b>2. RA therapy</b>	Genes responsible for differentiation and proliferation	Via receptors acting as transcription factors modulating gene expression	1,3 cis retinoic acid	Phse I-III Clinical trial
<b>3. Targeting of signaling molecules</b>				
<i>i) Ras directed, with</i>				
<i>a) Ras antisense Compound</i>	Ras genes/receptor	Inhibition of cell growth by blocking Ras gene/receptor	ISIS 2503	Phase I-III Clinical trial
<i>b) Phenylacetate</i>	Ras genes/receptor	Inhibition of cell growth by affecting post translational processing of Ras	Phenylacetate	Used in cell lines *Phase I-II Clinical trial
<i>c) Farnesyl transferase inhibition</i>	Ras genes/receptor	Blockade of farnesylation of Ras leading to inhibition of cell growth by stopping translocation of Ras to the cytoplasmic membrane	R 115777, BMS-214662 *L 778,123, *SCH 66336 (Lonafarnib)	*Phase I-II Clinical trial *Phase I-II Clinical trial
<i>ii) Raf inhibition</i>	cRaf	Inhibition of Raf with antisense compounds leading to inhibition of cell growth	ISIS 5132, BAY 43 9006	*Phase I-II Clinical trial

<b>iii) Targeting of receptor (Tyrosine Kinases) with</b>				
a) Antibodies	VEPG, EGF receptor	Receptor blockade with monoclonal antibody	anti EGFR, anti VEGFR (Avastin), anti Her2/nu (Herceptin)	used in cell lines Phase I-III Clinical trial
b) Receptor antagonist	VEGF receptor	Receptor blockade with selective antagonist	SU 5416, SU 5426	Phase I-III Clinical trial
c) Small molecules	VEGF, EGF receptor	Receptor blockade with small molecules	ZD 6474, ZD 1839 (Iressa)	Phase I-II Clinical trial
iv) Therapy with Anti-angiogenesis drugs	Angiogenesis	Angiogenesis inhibition	Thalidomide Combrestatin	Phase I-II Clinical trial *Used in cell lines
v) Targeting Akt/ mammalian target of rapamycin	c Myc, cyclin D1	Cell cycle inhibition	cc1-779	*Phase I-II Clinical trial
vi) Inducing apoptosis with	TRAIL and Bel 2	Induction of apoptosis	G3139 (Oblimerses)	*Phase I-II Clinical trial
a) TRAIL	Caspase pathway	Induction of spoptosis	(TRAIL) APO-2	Used in cell lines
b) BCL-2 antisense	BCL-2	Induction of apoptosis	G3139 (Oblimersen)	*Phase I-III Clinical trial
vii) Other agents				
a) Cyclooxygenase-2 (COX-2) inhibitors	COX-2	Growth arrest by inducing apoptosis and inhibiting agiogenesis	Celecoxib	*Phase I-III Clinical trial
b) Hsp90 inhibitors	Hsp protein	inhibits cellular growth and induces apoptosis through depletion of Akt and Raf and by inducing apoptosis	Geldanamycin	Used in cell lines
c) Demethylation of DNA	promoter region of genes	Re-expression of tumour suppressor and NIS genes	5-Azacytidine	Phase I-II Clinical trial
d) HDAC inhibitors	Histonedeacetylase	Cell cycle arrest	FR 901228 (Depsipeptide)	used in cell lines
e) PPA Rgamma agonist	PPA Regamma gene	Redifferentiation	Rosiglitazone	Phase-I Clinical trial

\* Patients of non-thyroidal cancers NIS : sodium iodide symporter, HSVITK GCN : herpes simplex virus thymidine kinase gancyclovir, RA: retinoic acid, VEGF : vascular endothelial growth factor, EGF: epidermal growth factor, TRAIL : tumour necrosis factor (TNF)-related apoptosis-inducing ligand. HDAC: histone deacetylase

**GENE THERAPY**

Gene therapy has been used a) to induce the expression of genes not normally expressed in particular cells, b) to induce re-expression of silenced genes, or c) to inhibit the expression of abnormal genes.<sup>45</sup> Various strategies used are:

- 1) NIS gene therapy: Re-introduction and expression of the NIS gene, which confers the ability to accumulate radioiodine to non-transporting tissues.
- 2) Tumour suppressor replacement: Reintroduction of a wild-type copy of a lost or mutated gene such as p53 that protects against cancer.
- 3) Suicide gene therapy: Gene transfer is used to introduce into the tumour a vector coding

for a 'sensitising enzyme', which renders tumour cells sensitive against toxins applied as non-toxic prodrugs (also known as pro-drug therapy).

- 4) Immunotherapy: Support of the organism's own defense mechanisms against cancer or triggering of immune responses against tumour-specific antigens or tumour markers by using agents such as IL-2 or introduction of IL-2 gene into the tumour
- 5) Genetic immunization: Generation of an immune response by introduction of 'naked' DNA coding for a cancer associated antigen.
- 6) Antisense therapy: Using sequence-specific oligonucleotides that prevents synthesis of pathogenic proteins.

- 7) **Oncogene silencing:** Shutting off of genes that support uncontrolled growth and formation of metastases.
- 8) **Ribozyme therapy:** Use of sequence-specific 'RNA enzymes', which cleaves mRNAs coding for pathogenic proteins.
- 9) **Chemoprotection:** Introduction of genes coding for proteins that protect normal cells from effects of high-dose chemotherapy.

The cardinal point of gene therapy is the introduction of DNA into target cells. To date, there are two principal strategies to achieve this. Either the cells are manipulated *ex vivo* and afterwards reintroduced into the organism; thereby, only target cells receive the altered genes. Alternatively, the foreign DNA is directly applied, mostly by injecting it into the tumour tissue. As for the methods, various vehicles such as a) viruses (retrovirus & adenovirus), b) liposomes or c) 'naked' DNA can be used. In general, viral vectors are most efficient. This holds true for all retroviruses. Furthermore, they integrate their genome into that of the host whereby long-term gene expression results. However, for the same reason they may disturb the target cell's gene expression in an unforeseen and unwanted way. Another major disadvantage is that they only infect dividing cells. Adenoviruses, in contrast, do not integrate, but this implies that transduced genes are only temporarily active. Also, adenoviruses do not cause serious diseases, but they do trigger an immune response. Finally, they can be used to transduce non-dividing, differentiated cells. DNA transfection by the aid of lipid complexes is less efficient than the virus-based methods but has neither of their disadvantages. This technique may be used for *ex vivo* manipulation as the altered cells can be expanded after transduction, thus compensating for the low efficiency. Finally, even 'naked' DNA in the absence of any vehicle may be used for vaccination against tumour-associated genes.<sup>4,5</sup>

#### GENE THERAPY USING THE SODIUM/IODIDE SYMPORTER (NIS)

A potential clinical application for gene therapy in thyroid carcinomas is restoration of iodine uptake in thyroid cancers by re-expression of

the NIS. A functional NIS protein is fundamental for iodide incorporation in benign and differentiated malignant thyroid cells. Defective/poor iodine uptake may be consequent to (a) NIS gene mutation,<sup>6</sup> (b) reduced/suppressed NIS gene expression<sup>7-8</sup> (c) hypermethylation of the NIS gene promoter,<sup>9</sup> (d) altered subcellular localization of the protein<sup>10</sup> or (e) post-transcriptional modifications of the NIS protein.<sup>11</sup> Reduced expression or functionality of NIS molecule may contribute to the failure of radioiodine therapy due to a lack of iodide accumulation in advanced thyroid carcinomas. Shimura et al. found that re-expression of this transporter molecule after gene transfer leads to a reinduction of iodide transport in non-accumulating thyroid carcinoma cell lines<sup>12</sup>. NIS belongs to a large and widely expressed class of sodium-dependent solute transporters termed after its prototype, a sodium/glucose symporter,

#### REPLACEMENT OF TUMOUR SUPPRESSOR GENE, P53

p53 is intact in the healthy thyroid, in benign thyroid disease or in differentiated carcinomas, but there is a high prevalence of p53 mutations in dedifferentiated carcinomas correlating with the most aggressive histologic tumour types.

The central role of p53 for the pathogenesis of thyroid cancer has made it a potential target for gene therapy. In a couple of studies using thyroid carcinoma cell lines with p53 mutations it has been shown that reintroduction of wild-type p53 causes a more differentiated phenotype of the cells. Expression of the thyroid-specific differentiation markers thyroperoxidase (TPO), thyroglobulin (Tg), and TSHr, of the thyroid-specific transcription factor Pax8, and of MHC class II antigens is stimulated. Furthermore, responsivity to the physiological stimulator TSH is restored and the tumourigenic potential is decreased. Proliferation is inhibited, both in thyrocyte and C-cell derived tumour lines; however, not apoptosis but cell-cycle arrest seems to be the cause.<sup>13-15</sup> Its re-expression in a p53-deficient tumour might enhance the tumour's sensitivity to radiation or chemotherapy.<sup>16</sup> However, this

approach has so far only been tested in vitro, and further studies are needed to show whether it is efficient in experimental animals or, finally, in thyroid cancer patients as it is already done in the case of other epithelial tumours.

### SUICIDE GENE THERAPY

In this approach, gene transfer is used to introduce into the tumour, a vector coding for a 'sensitizing enzyme'. A chemotherapeutic agent is then applied as a non-toxic prodrug, which is activated only in those cells that express the sensitizing enzyme. A positive concomitant is that more cells are killed than have actually been transduced, because the activated drug is secreted and acts on neighbouring cells, too. This is the so-called 'bystander effect'. A number of suicide gene systems have been identified, including the herpes simplex virus type I thymidine kinase gene, the cytosine deaminase gene, the varicella-zoster virus thymidine kinase gene, the nitroreductase gene, the E coli gpt gene, and the E. coli Deo gene.<sup>17-18</sup>

Usually a construct is created in which the gene encoding a certain enzyme such as thymidine kinase (TK) is controlled by a cell type-specific promoter (such as the thyroglobulin promoter), resulting in the expression of TK only in cancer cells.<sup>18</sup> Once expressed, TK is able to activate the antiviral drug, ganciclovir (GCN). GCN is a nucleotide analogue, and HSVI-TK preferentially phosphorylates GCN. Monophosphorylated GCN is then converted to its triphosphate by cellular kinases and interferes with cell propagation by competition with normal nucleotides during replication resulting in DNA strand breaks and subsequent cell death. Because mammalian cells normally do not express TK, only cells that express the enzyme will respond to the drug. Therefore, treatment of patients with GCN after administration of gene therapy could result in cancer-specific cell death. Obviously, such an approach is limited to those cancers that still maintain Tg expression. However, one must bear in mind that dedifferentiation is known to coincide with a decreased expression of thyroid-specific transcription factors. This will then reduce the efficiency of Tg expression and of any transgene controlled by the Tg promoter.

This technique has been tested for brain cancer, because minimal damage of brain function is expected as healthy neurones have stopped proliferation and will not be affected. As the thyroid gland is a very slowly growing tissue, gene therapy using GCN and the TK gene might be a method of choice also in thyroid cancer. Several other prodrug/sensitising enzyme systems have also been tested for thyroid cancer.<sup>19</sup>

### IMMUNOTHERAPY

Although tumours express antigens that may trigger an antitumour immune response, they often evade the surveillance of the immune system. Expression of class I and II MHC antigens may be disturbed, so that neither presentation of processed antigens nor proper T-cell activation occurs. Also, the immune response may be suppressed by products of the tumour or immune tolerance against tumour antigens may develop. Applying immunostimulatory agents such interleukin-2 (IL-2) to enhance defense reactions by the host appears to be a promising strategy against cancer, including de-differentiated thyroid cancer. A candidate in this context is the systemic administration of IL-2 produces antitumoural immunity in animal models. However, the doses required to achieve a therapeutical effect in patients usually causes severe side effects so that this approach is of limited benefit. Local, intratumoural application of the cytokine could provide far higher concentrations and thus circumvent this problem. Alternatively, the IL-2 gene may be introduced into the tumour, an approach that has been tested in animal models for several types of cancers.<sup>20-22</sup>

### GENETIC IMMUNIZATION

Immunization with autologous dendritic cells (DCs) pulsed with tumour antigen results in protective immunity and rejection of established tumours in various human malignancies. Vaccination with calcitonin and/or CEA peptide-pulsed DC resulted in the induction of a cellular, antigen-specific immune response in patients with MTC, leading to

clinical response in some patients.<sup>22</sup> Immune responses can also be triggered by simple injection of naked plasmid DNA coding for an antigen. A DNA vaccine plasmid encoding preprocalcitonin under the control of the cytomegalovirus promoter has been developed.<sup>23</sup>

#### ANTISENSE THERAPY

Antisense compounds are small synthetic DNA sequences of up to 25 oligonucleotides comprised of a complementary sequence to a particular targeted mRNA. When bound to mRNA, these drugs serve as substrates for ribonuclease H that cleaves the mRNA strand, but not the antisense compound. These substances also interfere with ribosomal assembly, blocking gene expression and inhibiting protein synthesis. Antisense compounds designed to inhibit the expression of cell signaling molecules have been synthesized and are being tested as potential drugs for the treatment of cancer.

Two antisense drugs, 1818 2503 and ISIS 5132, target elements from the Ras pathway. Both are phosphorothiotate oligonucleotides in which the presence of oxygen in the phosphodiester bonds is substituted by sulfur, thus conveying nuclease resistance and enhancing bioavailability. The c-myc proto-oncogene coding for a nuclear protein that acts as a transcription factor transducing mitogenic stimuli is amplified and constitutively active in a couple of tumours including thyroid cancer.<sup>24</sup> Blocking c-myc protein expression with antisense oligonucleotides against the translation initiation region of the mRNA significantly reduces the growth rate of the thyroid carcinoma cell lines.

#### II. RETINOIC ACID RE-DIFFERENTIATION THERAPY

Retinoic acids (RA) are biologically active metabolites of vitamin A that play an important role in the morphogenesis, differentiation and proliferation of many cell types. Differentiating effects have been shown in various cell culture models of promyelocytic leukaemia, pheochromocytoma, neuroblastoma and others.<sup>25</sup>

Retinoids have been administered for anti-cancer and tumour-preventive treatment in various clinical trials, and promising results have been achieved in the therapy of acute promyelocytic leukaemia (APL), head and neck cancers and lung and skin tumours.<sup>26</sup> The paradigm of redifferentiation via retinoids is APL, complete remission of disease being achieved in a high percentage of patients.<sup>26</sup>

Redifferentiation therapy of thyroid cancer with RA requires intact receptor pathways in the tumour tissues. The presence of RARs and RXRs and their subgroups has been studied in tumour cell lines and tissues by Northern blot, RT-PCR, electrophoretic mobility supershift and ligand binding assays.<sup>27</sup> RA receptor mRNAs, high-affinity binding sites for RA and functional DNA-binding RA receptors were demonstrated in thyroid tumour cell lines, and mRNAs for most receptor subtypes were seen in thyroid tumour tissues. There were two exceptions. RAR-beta mRNA was strongly reduced in a follicular thyroid tumour cell line and RXRbeta mRNA was undetectable in most of the examined tissues. However, given the known redundancy in the RA receptor signalling pathways,<sup>28</sup> the RA receptor complement of thyroid carcinoma cells should enable them to transduce RA signals required for RA redifferentiation.

According to these considerations, thyroid differentiation markers or specialized thyroid functions, whose expression is reduced or lost in thyroid carcinomas, could be re-induced by RA in thyroid cancer model cell lines. Type I 5'-deiodinase (5'-DI), which deiodinates T<sub>4</sub> to its biologically active form T<sub>3</sub>, is a differentiation marker in thyroid carcinomas. 5'-DI enzyme activity and the corresponding mRNA are stimulated by RA in well differentiated follicular thyroid carcinoma cell lines.<sup>29</sup> Furthermore, the expression of the mRNA coding for NIS was enhanced in these two cell lines. NIS mediates uptake of (radioactive) iodide into the thyrocyte and, therefore, plays a central role not only in iodide metabolism but also in the diagnosis and treatment of thyroid cancer.<sup>30</sup> As NIS expression and function are often lost in thyroid carcinomas, this molecule is a major target for redifferentiation therapy.

E-cadherin is a well-established differentiation marker in various epithelial tumours, and loss of E-cadherin expression correlates with dedifferentiation, increased invasiveness and poor prognosis. This has also been shown in thyroid carcinoma. In thyroid cell culture, RA is able to induce E-cadherin expression. RAs also have antiproliferative effects. Follicular thyroid carcinoma cells (FTC-133) showed a 33% reduction in their number after 3 days of treatment with RA. Induction of expression of fas protein in the thyroid carcinoma cell line FTC-236 might indicate exertion of RA via apoptotic pathway. Finally, tumorigenicity of follicular thyroid carcinoma cells (FTC-133) was found to be reduced after pretreatment with all-trans-RA prior to xenotransplantation onto nude rats.<sup>31</sup>

The theoretical therapeutic implications of RA therapy are: i) Thyroid tumour cells regain former thyroid-specific properties such as iodine uptake, consequently allowing re-application of radioiodine therapy, ii) Pro-apoptotic pathways are restored, reflecting antiproliferative activity, or an improved immune response via cytotoxic activity is obtained and iii) TSH receptors are re-established and TSH responsiveness is re-induced. A no of phase I studies on the administration of 13-cis-retinoic acid in patients with advanced thyroid cancer.<sup>31-32</sup> have been reported. Patients are usually treated with oral doses of 1.0 to 1.5 mg 13-cis-RA per Kg body weight per day over 5 weeks. During RA administration, patients might experience skin and mucosal irritation, nausea, pruritus, nose bleeding, hair loss, arthritis, change in blood counts and increase in liver enzymes. However, these side effects are usually mild and well tolerated and reversible after the end of the therapy.

In one study, 20% of the patients showed an improvement, and another 20% at least did not experience a further progression of their disease.<sup>31</sup> A multicentric clinical study,<sup>32</sup> showed a decreased Tg level in 20% and stable Tg level in 33% patients. Radioiodine uptake increased in 40%, in 11% patients, tumour size regressed, and in 31% no further growth was detectable. In the final results of a pilot study<sup>33</sup> in which fifty patients were evaluated for response, classified

as reduction in tumour size and TG levels, stable disease or disease progression, thirteen patients showed a clear increase in radioiodine uptake, and eight a mild increase. TG levels were unchanged or decreased in 20 patients. Tumour size was assessable in 37 patients. Tumour regression was observed in six, and there was no change in 22. In total, a response was seen in 19 patients (38%). They observed that the response to retinoid therapy did not always correlate with increased radioiodine uptake, so other direct antiproliferative effects also exist.

These encouraging results and a low rate of side-effects, warrant further studies with altered inclusion criteria, employment of other redifferentiating agents or combinations of agents, and other imaging techniques.

### III. TARGETING OF SIGNALING MOLECULES

Ras is a small GTP-binding protein (G protein) regularly expressed in normal thyroid cells,<sup>34</sup> and its protein product is involved in several important functions, including proliferation, differentiation, and cell survival. In normal cells Ras is largely activated by receptor tyrosine kinases, these receptors autophosphorylate at specific residues after the appropriate ligand binds its extracellular domain, leading to activation of several signaling pathways.

In thyroid cancer, overactivation of Ras may occur through activating mutations in the ras gene or by overactivation of receptor tyrosine kinase receptors. Mutations in the gene encoding Ras can result in expression of Ras proteins that are constitutively bound to GTP, i.e. once they are activated they are not able to be turned off. In thyroid carcinoma, activating mutations of ras genes (N-, K-, or H-ras) can be found in as many as 30% of cases. In addition to activating mutations, Ras overactivation can occur secondary to receptor overactivation. Enhanced signaling of receptor tyrosine kinases is a common event in thyroid cancer, particularly papillary thyroid cancer. Thus, when considering Ras as a therapeutic target for thyroid cancers, it appears to be relative thyroid tumour specific, occurs in follicular cancer, and also occurs in papillary cancer, albeit by distinct mechanisms. For these reasons, Ras, Raf and downstream signaling molecules are reasonable

molecular targets to consider for novel forms of thyroid cancer therapy.

### C) FARNESYL TRANSFERASE INHIBITION

Translocation of activated Ras to the cytoplasmic membrane is a key step in its activation. For this step, several posttranslational modifications must occur to allow membrane localization. The initial modification step involves farnesylation (i.e. addition of a farnesyl moiety to a cysteine residue) of Ras by the enzyme farnesyl transferase. Inhibition of farnesyl transferase has been shown to inhibit membrane accumulation of Ras *in vitro* and therefore reduce Ras signal transduction.<sup>35</sup> Currently four farnesyl transferase inhibitors have been developed and are in clinical studies (R1 15777, L-778,123, SCH66336, and BMS-214662). Another inhibitor, manumycin, has been used in laboratory studies, but it has not been evaluated in clinical trials due to toxicity in animal models.

### 2) RAF INHIBITION

The antisense compound designed to inhibit c-Raf such as ISIS 5132 and BAY 43-9006 has been demonstrated to inhibit the growth of malignant cells derived from several different tumours *in vitro* and *in vivo*, inducing apoptosis and cell death. Furthermore, it has been shown in xenograft models that several chemotherapeutic agents enhance their antiproliferative effects.<sup>36</sup> Although this compound may be active against thyroid cancer cells, this has not yet been tested in thyroid cancer cell line.

### 3) INHIBITION OF RECEPTOR (TYROSINE KINASES)

Receptor tyrosine kinases are activated by a wide variety of ligands, are frequently mutated in a manner that results in constitutive activation, and are the most commonly overexpressed receptors in thyroid cancers. In follicular cell-derived thyroid cancers, genetic rearrangements resulting in the expression of chimeric proteins involving the tyrosine kinase domain of RET are common in radiation-related and sporadic papillary thyroid cancer. In

addition, overexpression of other receptor tyrosine kinases, including fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (c-Met), VEGF, insulin, and IGF-I receptors are commonly identified. Several of these are common to many cancers, are expressed at very low levels in non-neoplastic tissues, and may be associated with angiogenesis or progression, making them excellent therapeutic targets.

**VEGF Receptor:** The formation of new blood vessels is a crucial step in determining tumour expansion and is greatly dependent on proangiogenic factors that are produced in a paracrine fashion by tumour cells undergoing hypoxia or mechanical compression. Several growth factors are involved in the process of angiogenesis in malignant tumours; among them, VEGF appears to be the most prominent. Besides the functional activity of stimulating vascular proliferation and permeability and inducing metastasis, VEGF may function as an apoptotic protector for the newly formed vessels via the phosphatidylinositol-3-kinase/Akt signaling pathway.<sup>37</sup> Significantly increased levels of VEGF have recently been demonstrated in the serum of patients with well differentiated metastatic thyroid tumours compared with lower levels found in patients considered to be in a complete remission. Similar results in thyroid tumours have been previously demonstrated by immunohistochemistry,<sup>38</sup> where high levels of VEGF have been associated with the occurrence of metastasis and possibly also with a worse prognosis.<sup>38</sup> In patients with other malignancies, a worse prognosis was observed in those who expressed higher levels of VEGF in their tumours, probably due to increased vessel formation and development of metastasis.<sup>39</sup> Although two VEGF tyrosine kinase receptors (VEGF-1 and VEGF-2) have been identified, VEGF-2 is considered to be the dominant receptor for signal transduction pathway stimulation.

**EGF Receptor (EGFR):** Four structurally related receptors (Her1, ErbB1, or EGFR; Her2/ neu or ErbB2; Her3 or ErbB3; and Her4 or ErbB4) are part of the EGFR superfamily. When bound to ligands, these receptors either homodimerize or



heterodimerize, leading to subsequent signal transduction. The EGFR is commonly expressed in differentiated thyroid tumours, and its overexpression has been associated with a worse prognosis.<sup>40</sup> Her2/neu has been demonstrated to be up-regulated, particularly in papillary thyroid cancer.<sup>41</sup> Several high affinity ligands of the EGFR have been identified, but EGF and TGF are apparently the most prominent. Once bound to a ligand, the EGFR triggers pathways that lead to cell cycle progression. EGFR blocking leads to cell cycle arrest in G1, apoptosis, antiangiogenesis, and down-regulation of matrix metalloproteinase, resulting in a decreased incidence of metastases.

Considering the important role of both EGF and VEGF receptors in the development and progression of malignant tumours, significant attention has been dedicated to new drugs that potentially block pathways related to these receptors. Currently, it is possible to pharmacologically interrupt the activation of these receptors at different levels through neutralization of the ligand with antibodies, blocking the receptor with small molecules, or inhibiting mRNA with antisense compounds.

#### A) ANTIBODIES

- i) Anti-VEGF antibodies (recombinant human monoclonal antibody against VEGF): Monoclonal antibodies directed against VEGF such as bevacizumab (Avastin) have been demonstrated to neutralize VEGF and reduce angiogenesis in several human xenograft models.<sup>42</sup>
- ii) Anti-EGFR antibodies (monoclonal antibodies 528 and 4253): Monoclonal antibodies have been designed to bind exclusively to the extracellular domain of the EGFR, causing internalization of the receptor and thereby reducing its availability. The combination of monoclonal antibodies and chemotherapy or radiotherapy has been shown to act against several human tumour cell lines in xenograft models. In two studies that

evaluated the use of anti-EGF monoclonal antibody in thyroid cancer cell lines, a reduction in tumour growth was noted.<sup>42-43</sup>

- iii) Anti-Her2/neu antibodies: Herceptin (Trastuzumab) is a humanized anti-Her2/neu monoclonal antibody that binds with high affinity to Her2/neu and inhibits the growth of Her2/neu-expressing cancer cells and xenograft models.<sup>44</sup> However, thyroid cancer has not been tested in particular.

#### B) RECEPTOR ANTAGONISTS

VEGF receptor inhibition (VEGFR-2 antagonists): SU5416 (Semaxanib) is a selective VEGF receptor 2 antagonist that has been shown to inhibit tumour growth and metastases in xenograft models for several solid malignancies and is being tested in active trials for patients with several malignancies.<sup>45</sup>

#### C) SMALL MOLECULES

The kinase activity of the EGFR can be blocked by small molecule tyrosine kinase inhibitors, ZD1839, that interfere with ATP binding to the receptor. ZD1839 has been demonstrated to inhibit growth in a wide range of tumour cell lines and human tumour xenografts, although no studies have been conducted to evaluate its effects in thyroid cancer cell lines or in clinical trials. Another agent, ZD6474 was designed to be an orally available alternative small molecule inhibitor of the VEGF receptor family tyrosine kinase domain. Wedge et al. reported that this agent is orally absorbed and exhibited dose-dependent inhibition of tumour growth, VEGF signaling, and angiogenesis in animal xenograft models.<sup>46</sup> This agent, however, is not entirely specific for the VEGF receptor family; It also inhibits the EGFR, TIE-2, and was recently demonstrated to inhibit the family of RET/MEN2B, and RET/PTC3, in vitro and in vivo. These exciting in vitro and animal data suggest potential activity against medullary and papillary thyroid cancer.<sup>47</sup>

#### 4) INHIBITION OF ANGIOGENESIS

- I) **Thalidomide:** Thalidomide has antiangiogenic properties. Although the mechanisms by which thalidomide exerts growth inhibition on new vessels are not yet fully understood, it is currently being tested in several clinical trials for the treatment of different malignancies. A Phase II trial recruiting patients with metastatic follicular, papillary, or medullary thyroid carcinoma is ongoing to evaluate drug toxicity and response in radioiodine-resistant tumours.
- ii) **Combretastatins:** Combretastatins are a family of tubulin-binding proteins derived from the African willow with unique vascular-targeting properties. The agents appear to be most active against endothelial cells, although direct cytotoxic effects have been demonstrated against cancer cells, including thyroid cancer cell lines.<sup>114</sup> Although there are several combretastatins under evaluation, combretastatin A4 phosphate is the compound that has been most carefully tested to date and has been found to have some effect in cases of undifferentiated thyroid cancer.<sup>49</sup>

#### 5) DRUGS THAT ACT ON AKT/MAMMALIAN TARGET OF RAPAMYCIN (MTOR)

Several critical cellular functions are controlled by the mTOR, a serine/threonine member of the phosphatidylinositol 3-kinase-activating cascade. Upon activation, mTOR signals to increase cell cycle progression and cell growth. Several targets of mTOR have been found to be dysregulated in thyroid cancer, such as the cell cycle stimulators c-Myc and cyclin-D1<sup>50</sup> and the cell cycle inhibitor p27kip1 (p27),<sup>51</sup> suggesting a potential role for mTOR in thyroid cancer progression. In particular, c-Myc and cyclin D1 are overexpressed in malignant thyroid tissues, and the level of expression correlates with tumour aggressiveness. mTOR is under control by another important kinase, Akt, which is elevated in several different types of cancer, including anaplastic, papillary, and follicular

thyroid cancer,<sup>52</sup> and may be an important regulator of mTOR in these cancer. These data suggest that mTOR is an appropriate target for thyroid cancer therapy and Rapamycin and its various analogs can be used for this purpose.

#### 6) DRUGS THAT AFFECT APOPTOTIC PATHWAYS (PROGRAMMED CELL DEATH)

Thyroid cancer cells, like many other cancer cells, demonstrate reduced sensitivity to cell death. This ability to resist cell death is thought to lead to the ability of cancer cells to sustain genetic alterations, but continue to grow. Apoptosis is an orderly process leading to cell death through specific signaling pathways. This process can be initiated via intracellular and extracellular stimuli. Several new agents designed to initiate apoptosis in cancer cells are currently being investigated, some of which may have particular utility for thyroid cancer.

- i) **TNF-related apoptosis-inducing ligand (TRAIL; recombinant soluble Apo2 ligand):** TRAIL is a member of the TNF family that is expressed in most benign and malignant tissues and induces apoptosis through activation of the caspase pathway via activation of specific receptors. Compared with Fas ligand, another apoptosis-inducing protein, TRAIL appears to induce apoptosis primarily in cancer cells, suggesting that it might be a potential therapeutic agent for cancer-specific therapy. In benign thyroid cells and differentiated thyroid cancer cell lines, recombinant TRAIL induces apoptosis; however, the presence of the protein inhibitor cycloheximide is required, suggesting the production of an antiapoptotic protein inhibitor.<sup>53-54</sup>
- II) **BCL-2 inhibition:** Phosphorylation of Bcl-2 is associated with resistance to apoptosis, and overexpression of BCL-2 leads to cell proliferation in the absence of growth factors, including in thyroid cancer cells. Overexpression and overactivation of Bcl-2 have been shown to be increased in several cancer cell lines in vitro; treatment with a Bcl-2 antisense compound, G3139, results in cell death and reduced Bcl-2 expression and activity. This agent, although logical for thyroid cancer, has not been tested in vitro.

## 7) OTHER POTENTIAL AGENTS FOR THE TREATMENT OF DE-DIFFERENTIATED THYROID CANCER

- i) Cyclo oxygenase-2 (COX-2) inhibitors: Overexpression and overactivation of COX-2, a key enzyme in the synthesis of prostaglandins, occur frequently in many types of cancers, including differentiated thyroid carcinomas.<sup>55</sup> When activated, COX-2 inhibits apoptosis and enhances angiogenesis. Therefore, COX-2 inhibitors can be considered potential therapeutic agents for the management of different malignant conditions. In vitro, COX-2 inhibitors induce growth arrest and inhibit tumour formation in xenograft models.<sup>127</sup> Several trials are currently open to recruit patients with malignancies, including thyroid cancer, for treatment with celecoxib, a COX-2 inhibitor, alone or in combination with chemotherapeutic agents.
- ii) The 90-kDa Heat shock protein (HSP90) inhibitors: HSP90 protein is a chaperone molecule involved in activation and stabilization of critical proteins in signal transduction pathways. Specifically, the serine/threonine kinases Ran and Akt are proteins that are dependent on Hsp90 for stabilization and localization. Blockade of Hsp90 results in enhanced degradation of these signaling molecules, causing decreased activation. Because the activation of Akt and Raf is implicated in thyroid cell growth and thyroid cancer, inhibition of Hsp90 is a logical consideration for thyroid cancer therapy. Geldanamycin and its related compound, 17-N-allyl amino-17-demethoxy-geldanamycin (17-AAG), inhibit Hsp90 by binding to its ATP-binding domain. In vitro, both have been shown to induce growth arrest in several cancer cell lines. 17-AAG inhibits cellular growth and induces apoptosis in thyroid cancer cell lines cultures through depletion of Akt and Raf and by inducing apoptosis.<sup>56</sup>
- iii) Demethylating agents: Hypermethylation of DNA occurs frequently in the promoter

regions of genes, resulting in altered binding of cofactors leading to altered (usually reduced) gene expression. The enzyme methyltransferase transfers a methyl group to cytosine rings in CpG islands (sequences rich in cytosine and located in the 5' regulatory region of many genes), thereby reducing binding of transcription factors to the promoters of regulated genes. Blockers of methyltransferase have been used to induce reexpression of tumour suppressor genes and other genes important for facilitating therapy or reducing cell growth. 5-Azacytidine and 5-aza-2'-deoxycytidine are compounds that are incorporated into DNA and are able to inhibit methylation. In thyroid cancer cell lines, hyper methylation of the sodium-iodide symporter (NIS) gene promoter has been demonstrated, and treatment with 5-azacytidine restores NIS expression as well as the expression of several important thyroid cell transcription factors, resulting in enhanced iodide uptake in some cell lines.<sup>9</sup> The concept of inhibiting methyltransferase activity is attractive for thyroid cancer, particularly for patients with tumours that no longer effectively concentrate iodine.

- iv) Histone de acetylase inhibitors: Histones are small positively charged basic proteins that compose most of the protein structure of the chromosomes, binding tightly to the negatively charged DNA, forming a condensed protein-DNA complex. Relaxation of this dense structure via modification of histones by acetyl transferase and histone deacetylase (HDAC) is required to allow for transcription of DNA into mRNA. Disruption of this process using HDAC inhibitors has been demonstrated to induce cell cycle arrest and differentiation; however, the mechanisms mediating this activity remain unclear. In vitro, incubation of thyroid cancer cells with the HDAC inhibitor depsipeptide and Trichostatin A was shown to increase the expression of thyroglobulin and NIS, with

consequent intracellular iodine accumulation.<sup>57</sup> Also, HDAC inhibitors are able to induce apoptosis in several cancer cell lines, probably through derepression of specific cell death genes.

- v) Peroxisome proliferator-activated receptor gamma (PPARgamma) agonist: Genetic alterations in the gene for the peroxisome proliferator-activated receptor gamma (PPARgamma) have been described and PPAR agonists have been shown to redifferentiate thyroid cancers in animal models. Philips et al<sup>58</sup> performed a pilot study in five patients with thyroglobulin-positive and radioiodine scan-negative thyroid cancers using the PPARgamma agonist rosiglitazone. Treatment with rosiglitazone increased the production of thyroglobulin in some patients with thyroid cancers, but only rarely restored scintigraphically significant iodine trapping.

## CONCLUSION

De-differentiation of thyroid cancer poses a therapeutic challenge and needs alternative therapeutic strategies to deal with. Although, a plethora of theoretical options are available, most are untested and require methodical and rigorous evaluation. Gene therapy, particularly NIS gene therapy, and treatment with RA appear to be most promising. However, further clinical trials with larger number of patients are required before making any conclusive and valid inference.

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## COMMENTS:

Differentiated thyroid cancer is a potentially curable disease in majority of cases. TSH control and activation of the sodium iodide symporter (NIS) remains the cornerstone of both adjuvant and salvage treatment. At the same time we also know that it is difficult to destroy all metastatic sites with radio-iodine to an acceptable level even in radioiodine concentrating or the differentiated variety. This has been assumed mainly due to low residence time of radioactive iodine in the thyroid cancer cells resulting in delivery of low radiation doses. Iodine as a base of common medications and its dietary intake are two important causes. Different strategies have been attempted to increase intracellular residency time of radioiodine. On the other hand majority of deaths due to thyroid cancer occurs due to its inability to concentrate radioiodine and de-differentiation of cancer cells is supposed to be the prime cause for this effect. It has been seen here that thyroid cancer, like many other malignancies is also characterized by genetic alterations resulting in

dysregulated cell growth and various attempts and strategies are being put forward for attempting re-differentiation of such thyroid cancer cells. This will be of prime importance in case some of these attempts are translated into actual clinical practice which could re-differentiate the cancer cells putting them under TSH regulation again so that it could further concentrate radioiodine. The aim of any alternative strategy thus should be two fold : firstly there should be an attempt to re-differentiate cancer cells and secondly to increase residence time of radioiodine in the cells. Gene therapy, especially for restoration of iodine uptake in thyroid cancers by re-expression of the NIS and re-differentiating properties of retinoic acid derivatives are the only ones that hold some promise in thyroid cancer. Some other agents which could be a potential adjuvant to increase the effectiveness of radioiodine, based on increasing NIS expression, are histone deacetylase inhibitors, suberoylanilide, hydroxamic acid and demethylating agents. These have been found to