Gene therapy is regarded as one of the most promising novel therapeutic approaches for hopeless cases of thyroid cancer and those not responding to traditional treatment. In the last two decades, many studies have focused on the genetic factors behind the origin and the development of thyroid cancer, in order to investigate and shed more light on the molecular pathways implicated in different differentiated or undifferentiated types of thyroid tumors. We, herein, review the current data on the main genes that have been proven to (or thought to) be implicated in thyroid cancer etiology, and which are involved in several well-known signaling pathways (such as the mitogen-activated protein kinase and phosphatidylinositol-3-kinase/Akt pathways). Moreover, we review the results of the efforts made through multiple gene therapy trials, via several gene therapy approaches/strategies, on different thyroid carcinomas. Our review leads to the conclusion that future research efforts should seriously consider gene therapy for the treatment of thyroid cancer, and, thus, should: (a) shed more light on the molecular basis of thyroid cancer tumorigenesis, (b) focus on the development of novel gene therapy approaches that can achieve the required antitumoral efficacy with minimum normal tissue toxicity, as well as (c) perform more gene therapy clinical trials, in order to acquire more data on the efficacy of the examined approaches and to record the provoked adverse effects.

The thyroid is a major human endocrine gland that controls heart rate, blood pressure, temperature and metabolism. Most thyroid tumors are benign, with primary malignant tumors accounting for fewer than 1% of all carcinomas (1). The incidence of thyroid cancer is increasing throughout the world and it is more frequent than ovarian, urinary bladder or pancreatic cancer. This fact could be due to the recorded enhancement in the detection of early thyroid cancer by the use of thyroid ultrasound, neck imaging and fine-needle aspiration biopsy; for the same reason, the 5-year relative survival rates for thyroid cancer have increased significantly from 93% in 1983-1985 to 97% in 1995-2001 (2, 3). In the United States of America (USA) alone in 2006, about 30,180 cases of thyroid cancer were diagnosed with an incidence of 25 per million per annum (4). Thyroid cancer can occur in any age group, with an incidence 3 times higher in women than in men (5), its aggressiveness increasing significantly in older patients. In spite of the progress in the treatment of thyroid malignant tumors by traditional methods (surgical and/or chemotherapy, radiotherapy), 10-20% of these patients die from advanced differentiated, poorly and undifferentiated thyroid tumors. Therefore, alternative therapies are of interest because of the possibility of developing selective targeting approaches, such as using therapeutic genes to target tumor cells by application of tissue-specific promoters such as thyroglobulin (TG), thyroid-stimulating hormone receptor (TSH-R), calcitonin (CT) and CT-related polypeptide alpha (CTRPα). These promoters have no or very limited expression elsewhere, thereby reducing extratumoral toxicity (e.g. in the liver or the lungs) even if the therapy leads to the destruction of all normal thyroid tissue (6).

Thyroid carcinomas arise from the two cell types present in the thyroid gland. The endodermally-derived follicular cells give rise to papillary, follicular, and probably anaplastic, carcinomas (PTC, FTC and ATC respectively), while the neuroendocrine-derived CT-producing C cells give rise to medullary thyroid carcinoma (MTC). PTC and FTC are well-differentiated thyroid carcinomas (WDTC), while ATC is characterized as undifferentiated. Moreover, poorly differentiated thyroid carcinoma is an intermediate class of malignant tumor between WDTC and ATC (7).
PTC is the most common form of thyroid cancer, accounting for about 75-80% of thyroid malignancies (1) with a 1% death rate and an approximately 5% recurrence rate (8). It represents the most common pediatric thyroid malignancy and most PTCs in adults occur between 20 and 50 years of age (3). The PTC patient has the highest 20-year survival (99%) in the low-risk group, whereas survival is only 43% in the high-risk group; distant metastases are uncommon but if present, they are lymphatic and the prognosis is poor.

FTC cases account for about 15% of all thyroid cancer cases. FTC is less common in younger adults and children than papillary cancer, while it is characterized by a 20-year survival of about 97% in the low-risk group and 49% in the high-risk group; it is more common among elderly patients. This type of thyroid cancer is more aggressive than PTC and tends to spread through the bloodstream to other parts of the body (vascular invasion is characteristic of follicular carcinoma), and therefore distant metastasis is more common (9, 10).

ATC is the least common (accounting for 5%-15% of primary malignant thyroid neoplasms) and the most deadly type of thyroid cancer. It is characterized by sudden, rapidly growing thyroid mass, mostly arising from a benign thyroid disease or a pre-existing differentiated thyroid carcinoma. It tends to occur most often among elderly women and in iodine-deficient areas, or in a pre-existing goiter, while it is also rapidly fatal with a mean survival of 6 months after diagnosis (11, 12). ATC is always refractory to conventional therapies including surgery, radioactive iodine and chemotherapy (13). Many tumors previously classified as ATC have now been recognized as lymphomas or poorly differentiated MTCs by means of immunohistochemistry (IHC) (14). Metastases to the neck lymph nodes are present in over 90% of cases at the time of diagnosis, while distant metastasis presents a very poor prognosis, even with aggressive therapy (11).

MTC is a neuroendocrine malignancy that secretes excessive amounts of CT, frequently metastasizes and has limited treatment. MTC accounts for 3 to 10% of all thyroid cancer cases, but bears significant mortality (15). Among MTCs, 25% are sporadic, whereas 75% are inherited as the predominant component of the multiple endocrine neoplasia type 2 (MEN2) syndromes (MEN2A, MEN2B and familial MTC) (16). MTC is less common and more aggressive than PTC or FTC, and tumor tends to spread to lymph nodes very early and to distant sites via the bloodstream with excessive CT production (16). This cancer is treated with difficulty and has a much lower cure rate than do the ‘well differentiated’ thyroid carcinomas (PTC and FTC) (9, 10), but cure rates are higher than they are for ATC. The recorded overall 10-year survival is about 60-70% (16, 17).

The etiology of most thyroid carcinomas is not well understood, but as in every type of cancer, they range from environmental or therapeutic (radiation) (18) iodine administration (19) and radioiodine exposure (20) etc., which have been shown to produce characteristic chromosomal translocations to genetic factors. The genetic abnormalities are considered as bases for the development of thyroid cancer and have been extensively explored over the last two decades due to their importance as potential targets for novel therapeutic approaches. The reaction of the thyroid gland to these factors differs according to age, sex, immunological status etc., in addition to the effects of hormones, cytokines and growth controlling factors (18).

The aim of this review is to provide an up-to-date summary of the genetic background of the main types of thyroid cancer and to focus on the gene-targeting approaches that have been developed (in order to act as potential gene therapy approaches, in the future).

The Main Genes Implicated in Thyroid Cancer Development

The genetic abnormalities involved include rearrangements, point mutations or other gene disruptions, each of which plays a role in disease pathogenesis; most of them have been used as diagnostic tools and molecular targets through the difference in expression according to the type of tumor and its histological subtype. The main and most common genes that are found to be mutated and expressed, underexpressed or totally unexpressed in thyroid malignancies are described below (for a synopsis, see Table I).

The V-raf murine sarcoma viral oncogene homolog B1 (BRAF) gene. The RAF proteins (ARAF, BRAF, and CRAF) are essential serine/threonine protein kinases; they play critical roles in cell proliferation, differentiation and apoptosis by signaling a complex kinase pathway that mediates cellular responses to growth signals [mitogen-activated protein kinase pathway (MAPK)] (21, 22). The BRAF protein is expressed at higher levels in hematopoietic cells, neurons, and testicles (23) and is the predominant isoform in thyroid follicular cells. Among the isoforms of RAF kinase, BRAF appears to be the most potent activator of the MAPK pathway and it is activated through multiple mechanisms (Figure 1). BRAF mutation is the most common genetic alteration in thyroid cancer, occurring in about 45% of sporadic PTCs, particularly in the relatively aggressive subtypes (24). A substantial body of data indicate that the BRAF mutational status is a significant predictor of poor clinical outcome, while a significant association of BRAF mutational status with extra-thyroidal invasion has been shown (25).

The RAS gene. The RAS oncogenes were the first to be associated with thyroid cancer. Members of the RAS family are signal-transducing proteins that share properties with the
G-proteins. Point mutations in H-RAS, K-RAS and N-RAS have been described in many tumor types. The complex RAS-initiated MAPK-terminated cascade aberrant signaling is regarded as crucial for the development of thyroid cancer. Mutation of the phosphatidylinositol-3-kinase catalytic alpha polypeptide (PIK3CA) [the product of which binds specifically with RAS and activates the phosphoinositide-3-kinase/Akt (PI3K/Akt) signaling pathway] also has an important role in thyroid tumorigenesis (26-28) (Figure 1).

The RET gene. There is substantial evidence for the involvement of the RET gene in thyroid cancer pathogenesis. Identified in 1985 (29), the RET gene is located on chromosome 10q11.2 and encodes a tyrosine receptor protein consisting of an extracellular domain with a ligand binding site, a transmembrane domain and an intracellular tyrosine kinase domain. The RET receptor is activated by interacting with a multicomponent complex that includes a soluble ligand family, the glial cell line-derived neurotrophic factors (GDNF), and also a family of cell surface bound coreceptors (30). Ligand binding results in receptor dimerization leading to autophosphorylation of the protein on tyrosine residues and initiation of the signaling cascade.

Table I. Malignant thyroid tumors and the main and most common genes that are usually found to be mutated and expressed, underexpressed or totally unexpressed in these tumors (for details, see text).

<table>
<thead>
<tr>
<th>Malignant thyroid tumor type</th>
<th>Associated genes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary thyroid carcinoma (PTC)</td>
<td>BRAF, RAS, RET, NTRK1, p53, APC**, PTEN**</td>
</tr>
<tr>
<td>Follicular thyroid carcinoma (FTC)</td>
<td>RAS, PAX8-PPARγ1, p53, PTEN, GRIM-1, mlDNA, APC**, PTEN**</td>
</tr>
<tr>
<td>Anaplastic thyroid carcinoma (ATC)</td>
<td>BRAF, RAS, p53, β-Catenin, PTEN</td>
</tr>
<tr>
<td>Medullary thyroid carcinoma (MTC)</td>
<td>RET, p53</td>
</tr>
</tbody>
</table>

*Full names of genes can be found within the text. **Familial syndrome-associated genes.

RET plays a critical role in cell differentiation, proliferation and cell survival in nerve cells (Figure 1). Subsequent studies have demonstrated a novel form of RET (RET/PTC) resulting from different gene rearrangements. The most common rearranged forms of the RET gene are RET/PTC1, RET/PTC2 and RET/PTC3 and there are more than ten additional types of RET rearrangements, occurring primarily in radiation-induced tumors (31). Many of these mutations, particularly those leading to the activation of the MAPK pathway, are being actively explored as therapeutic targets for thyroid cancer.

The neurotrophic receptor-tyrosine kinase 1 (NTRK1) gene. The NTRK1 gene is located on chromosome 1q22 and encodes the receptor for the nerve growth factor. Similar to RET, NTRK1 undergoes oncogenic activation by chromosomal rearrangement (32) (Figure 1).

The paired box gene 8-peroxisome proliferator activated receptor gamma (PAX8-PPARγ) gene. Over the last few years, significant PAX8-PPARγ gene fusion has been identified in FTC cases in association with the presence of a recurrent translocation [t(2;3) (q13;p25)] (33). The consequence of this translocation is the fusion of the DNA binding domains of the thyroid transcription factor PAX8 to domains A-F of the PPARγ1 (33, 34).
The p53 gene. The p53 gene encodes a nuclear transcription factor that plays a key role in cell cycle regulation, DNA repair, and apoptosis and acts as a tumor suppressor gene. Alterations of the p53 gene are a peculiar feature in ATCs (35-38). Moreover, the accumulation of p53 protein, frequently caused by p53 mutations, characterizes tumoral areas with a less differentiated phenotype (38, 39). Conversely, p53 gene mutations are rare in differentiated thyroid carcinomas (40). Accordingly, the introduction of mutated p53 genes into thyroid cells blocks the expression of thyroid differentiation markers and suppresses the thyroid transcription factor PAX-8 (41). Interestingly, p53 gene function is impaired in all thyroid carcinoma cell lines established from both differentiated and undifferentiated histotypes (42). All the above strongly suggest that p53 inactivation is required for the establishment of human thyroid carcinoma cell lines.

The beta-catenin (β-catenin) encoding gene. The β-catenin protein is encoded by the CTNNB1 gene (chromosome 3p22-21.3). This protein has a role in cell adhesion via its interaction with the cytoplasmic tail of cadherins (which are important transmembrane proteins) and in the Wnt signaling pathway (involving a complex network of proteins that play a crucial role in carcinogenesis) (43).

The adenomatous polyposis coli (APC) gene. The APC gene is a tumor suppressor gene that prevents the uncontrolled growth of cells that may result in cancerous tumors. The APC gene mutations seem to be associated with thyroid cancer development in a complex and mutated-codon-specific way (44).

The global receptor involved in myogenesis-1 (GRIM-1) gene. GRIM-1 is involved in the mitochondrial respiratory chain and in apoptosis (45).

The phosphatase and tensin homolog (PTEN) gene. The PTEN gene, which acts as a tumor suppressor gene, encodes a protein that helps in the regulation of cell division and plays a role in intracellular signaling. Mutations of this gene contribute to the development of a multitude of cancer types (46). It should be noted that PTEN acts in opposition to PIK3, therefore it is considered as a regulator for cell proliferation and apoptosis (47).

The mesenchymal epithelial transition factor (MET) gene. The MET gene encodes for a tyrosine kinase membrane receptor, which functions, primarily, as a receptor for hepatocyte growth factor/scatter factor (HGF/SF). Overexpression of MET has been reported in multiple human PTC cases.

The C-erbβ-2 gene. The protooncogene erb beta (erbβ) encodes two functional thyroid hormone receptors (β-1 and β-2). The C-erbβ-2 gene is overexpressed or underexpressed depending on the thyroid tumor type (48).

Epigenetic alteration. This is a widely described molecular mechanism involved in thyroid tumorigenesis. The aberrant methylation of tumor suppressor genes is an example of epigenetic alteration (49).

Gene Expression in Differentiated Thyroid Cancers (PTC and FTC)

Familial genetic predisposition for differentiated thyroid cancer such as an association of PTC and colorectal disease as well as FTC and breast disease have been described in at least two hereditary cancer syndromes, familial adenomatous polyposis (FAP) (50) and a hereditary hamartoma syndrome (51). The genes for the two syndromes are characterized by APC (52, 53) and PTEN gene mutations (54), respectively.

In PTC, BRAF mutations have been identified in approximately 40-70% of the tumors (55, 56), and can occur early (57). The BRAFV600E mutation is the most common genetic change in PTC (24). RET/PTC mutations are also regarded as one of the most common mutations in this type of cancer, while at least 15 types of RET/PTC rearrangements have been described (48, 58). The frequency of RET/PTC rearrangements in the adult population in the USA is approximately 30-40%. In recent studies, RET rearrangements have emerged as the second most common genetic abnormality found in PTCs. RET/PTC1 and RET/PTC3 account for 60-70% and 20-30% of cases, respectively, while RET/PTC2 has been implicated in approximately 10% of cases. RET rearrangements are particularly common in tumors in pediatric patients (50-60%) and in patients exposed to accidental/therapeutic radiation during childhood (60-70%) (59); thus ionizing radiation considered as the major risk factor for development of PTC, can directly induce RET recombination events. Several studies have emphasized the fact that RET rearrangements are associated with PTCs lacking evidence of progression to poorly differentiated or anaplastic carcinomas (60, 61). BRAFV600E (V600E) mutations are unique to PTC, not being found in any other form of well-differentiated follicular neoplasm or MTC (24). Practically, in PTCs, there seems to be no overlap between RET/PTC, BRAF or RAS mutations, which altogether were found to be present in 66% of cases (62). NTRK1 rearrangements are considerably less frequently found in PTCs than are RET rearrangements (63). The prevalence of NTRK1 rearrangements is approximately 3% in post-Chernobyl PTCs (64). RAS point mutations are uncommon in PTCs of conventional type, with an overall frequency of less than 10% (65, 66). Differentiated thyroid carcinomas (PTCs and FTCs) both seem to be largely unaffected by p53 gene mutations (36). Studies of transgenic
models have demonstrated that lack of functional p53 in RET/PTC mice promotes anaplasia and invasiveness of thyroid carcinomas (67). Gimm et al. (46), showed a 20-30% frequency of hemizygous deletions of the PTEN gene in PTCs.

In FTC, rearrangements of PAX8-PPARγ1 have been reported in about 53% of cases (33, 34, 68). In a comparative study of RAS mutations and PPARγ rearrangements, 49% of conventional FTC had RAS mutations, 35% had PAX8-PPARγ rearrangements, only 3% had both abnormalities (57), while 12% were negative for both, p53 mutations account for less than 10% (36). PTEN mutation is presented in about 20-30% of FTCs (46). Deletions and point mutations of mitochondrial DNA (mtDNA) are common in both neoplastic and normal oncocytic follicular cells, but the role of these abnormalities in the development of oncocytic tumors is unknown (69).

Mutations of the GRIM-1 gene have been implicated in the pathogenesis of oncocytic thyroid tumors (45).

Gene Expression in Poorly Differentiated and Undifferentiated Thyroid Cancer

The genes for p53 and β-catenin have been implicated in the progression of these tumors. Inactivating mutations of p53 occur in approximately 25% of poorly differentiated carcinomas and 60% of undifferentiated carcinomas (36, 38, 70). β-Catenin has a role in cell adhesion via its interaction with the cytoplasmic tail of cadherins and in the Wnt signaling pathway. Garcia-Rostan et al. (43), have demonstrated that CTNNB1 mutations and nuclear (rather than plasma membrane) localization of β-catenin are restricted to poorly differentiated or undifferentiated carcinomas. These findings highlight the important role of CTNNB1 mutations in the progression of thyroid cancer. BRAF and RAS mutations appear to confer a predisposition to the development of both poorly differentiated and undifferentiated carcinomas (25, 57).

Several studies have emphasized the fact that RET rearrangements are associated with PTCs that lack evidence of progression to poorly differentiated or ATCs (60, 61), while 60% of undifferentiated carcinomas had hemizygous PTEN deletions (46).

Gene Expression in MTC

RET point mutations are crucial for the development of MTC. Activating germline point mutations of RET are present in more than 95% of patients with MEN2A, MEN2B and familial MTC (71, 72). In sporadic MTC, somatic mutations are present in up to 70% of cases (73), while a statically significant correlation between the presence of somatic mutation and more advanced pathological TNM states has been recently observed (74).

Tumorigenecity Hypotheses

In order to explain the cascade of events that take place from the initiation until the development of a thyroid tumor, taking into consideration all the precipitating factors and their interaction with genetic susceptibility, two hypotheses have been developed.

The first hypothesis, known as the ‘multi-step carcinogenesis hypothesis’ was put forward in the 1980s. According to this hypothesis, cancer cells, including those of the thyroid, are considered to be derived from well-differentiated normal cells (such as thyrocytes) via multiple incidents of damage to their genome. Oncogenes or tumor suppressor genes, which accelerate proliferation or foster malignant phenotypes (such as the ability to invade the surrounding tissue or metastasize to distant organs) are mainly altered (75) (Figure 2). For example, under this hypothesis, PTCs are derived from some unknown precursor cells generated by normal thyrocytes by rearrangements of...
the RET gene (RET/PTC) or by BRAF mutations (76, 77), while FTCs are generated from thyrocytes via follicular adenomas by RAS mutations or the PAX8-PPARγ1 rearrangement (33, 34, 78). Poorly differentiated ATCs are generated by both FTCs and PTCs through genomic changes, such as p53 mutations (75, 79).

In 2000, a novel hypothesis of thyroid carcinogenesis was proposed, namely the ‘fetal cell carcinogenesis’, according to which cancer cells are derived from the remnants of fetal thyroid cells instead of thyrocytes. A considerable number of researchers have concluded that cancer cells derive from immature progenitor or stem cells, but not from well-differentiated cells (80, 81).

Takano (81) has explained well the clinical and biological features of this hypothesis and the recent molecular evidence that supports his novel hypothesis for thyroid carcinoma, according to which cancer cells derive from the remnants of three types of fetal thyroid cells, instead of normal thyroid follicular cells (Figure 2). This hypothesis suggests that thyroid cancer cells are generated from fetal cells by proliferation without differentiation, and that oncogenes play an oncogenic role by preventing fetal cells from differentiation. Therefore, any defect or inhibition of differentiation may lead to cancer cell formation. FTC, PTC and FTC derive from thyroid stem cells, thyroblasts and prothyrocytes, respectively. BRAF mutations and rearrangements of the RET (RET/PTC) prevent the differentiation of thyroblasts into prothyrocytes, resulting in the generation of PTCs, while the PAX8-PPARγ1 rearrangement prevents differentiation of prothyrocytes into thyrocytes, resulting in the generation of FTCs.

Different clinical and molecular features support one or the other hypothesis, but the exact mechanism of thyroid carcinoma development remains unclear. Therefore, in the etiology of thyroid carcinoma, it remains controversial as to whether thyroid tumors of different histologies share a common origin and whether they follow an adenoma-carcinoma progression.

**Gene Therapy Approaches in Thyroid Cancer**

The American Association of Clinical Endocrinologists (AACE) in collaboration with the American Association of Endocrine Surgeons (AAES) emphasized the Guidelines for the Management of Thyroid Carcinoma and the importance and prevalence of thyroid cancer as a “forgotten cancer” (1). Thyroid cancer occurs as frequently as other well-publicized types of cancer, and despite multimodality treatment for thyroid cancer (including surgical resection, radioiodine therapy, TSH-suppressive thyroxin treatment, and chemotherapy/radiotherapy), survival rates remained not improved over the last decade. Therefore, development and evaluation of novel treatment strategies, including gene therapy, are very important. Gene therapy has been regarded as a promising future therapeutic approach for any thyroid cancer, especially for types that do not respond to traditional treatment aiming to restrict the therapy to target tissues and to decrease extratumoral toxicity, by maintaining a high therapeutic index.

**Gene Therapeutic Strategies for Obtaining Efficient Targeting for Cancer**

Because of the non-selectivity of the available gene delivery systems, which renders cancer gene therapy strategies potentially toxic to normal cell populations, selectivity of gene therapy for tumor cells has been achieved either by direct intratumoral administration of recombinant vectors or by integration of tissue promoters and/or enhancers into gene delivery vectors that are activated in transformed, but not normal cells. However, cell-specific promoters, such as the CT or TG promoters, maintain a highly-regulated expression but are often weaker than nonspecific promoters, such as the commonly employed cytomegalovirus (CMV) promoter. In addition, these promoters can be modified to achieve more potent tissue specificity such as CALCA (the gene encoding RET) (82). Targeting viruses specifically to tumors would be very desirable and has been partially accomplished in some systems by changing viral tropism or employing an agent bound to the virus in order to direct it to a specific receptor on the tumor cell surface. For example, an antibody can be bound to the viral rod protein, and this antibody can be coupled with a tumor-targeting molecule (e.g. a growth factor or a second antibody) (83). A new strategy has been developed functioning through the attachment of target ligands to adenovirus (Ad) proteins or receptors by chemical or genetic modification of Ad particles. A recent example involves the identification of human MTC-specific peptide ligands (one of which has the potential to be covalently linked to Ad particles while maintaining effective cellular internalization properties) (84, 85). This strategy allows Ads to be retargeted to MTC cells with high transduction efficiency and no effect on the liver. Other interventions to achieve efficient targeting depend on the selection of viruses or on the use of inducible promoters and the use of the Cre-loxP system. The latter is a recently developed approach to target gene transcription in tumor cells and enhance the function of the thymidine kinase/ganciclovir (tk/GCV) system and the expression of tk under control of TG promoter to target expression (86). In general there are two main strategies for achieving efficient gene therapy treatment: (a) maintenance of tissue specificity, and (b) efficient transduction of target cells.

By considering the above two strategies, gene therapy can be effective and safe, but for each approach there is an
A minimal effect was observed in the other cell lines when GCV was added, more than 90% of the cells were killed; a retrovirus (Re) expressing HSV-tk under control of the TG promoter was given intravenously (i.v.) to rats treated with GCV, severe liver damage was induced. In contrast, when the same amount of Ad expressing tk under control of the TG promoter was given (i.v.), neither significant changes of serum transaminase levels and histologic liver abnormalities were observed, nor adverse effects (89). For enhancing tk expression in TG-expressing cells, Nagayama et al. (86), combined the function of the tk/GCV system with the Cre-loxP system. As a result, the cytotoxic effect was 5 to 10-fold higher than that with TG promoter alone.

These studies demonstrated the ability of the TG promoter to transcriptionally target therapeutic genes to thyroid carcinoma cells, despite the lower efficacy compared to constitutive viral promoters such as the CMV early promoter. On the other hand, the Re vector was expected to have effects distant from the injection site (bystander effects) which have an important role in antitumor therapy (86-88), while the Ad vector in rat MTC (rMTC) cells through the HSV-tk/GCV system was limited to a low bystander effect in vitro. The latter correlated well with the limited antitumor efficacy in vivo. Intratumoral application of an Ad carrying the HSV-tk gene under the control of the CMV promoter followed by GCV treatment resulted in the destruction or stabilization of smaller tumors without a therapeutic effect in larger tumors (90). The latter confirms that a significant bystander effect is an important advantage for effective suicide gene therapy.

Many scientists took advantage of this point using a virus expressing a cytokine gene (as a suicidal gene) for increasing antitumor immunity and making its effect persist for months or longer. Interferon-γ (IFNG), interleukin-2 (IL2), tumor necrosis factor-alpha (TNFA), IL12, granulocyte-macrophage colony-stimulating factor (CSF2), and lymphotactin (XCL1) are among the genes employed. This type of suicidal gene approach is enrolled within what the scientists called ‘immunomodulatory gene therapy’, which depends upon the principle of many types of cancer expressing tumor-associated antigens that can be recognized by the immune system (released from tumor cells either physiologically or after cytotoxic therapy). Mobilization of the immune system, by delivery of genes that enhance the immunogenicity of the tumors and the responsiveness of the immune system, is associated with a number of advantages including inherent specificity of the immune system (decreasing normal tissue toxicity), a systemic immunogenic effect, signal amplification, and permanent antitumor immunity because of inherent memory of the immune system. Local expression of certain cytokines is able to elicit an immune response against the tumor by stimulating surrounding immunocompetent cells, targeting cytotoxic T-cells and natural killer cells, thereby leading to a more efficient method with better results but less adverse effects. These approaches are summarized below (see also Table II).

### Introduction of a (Not Normally Present) Gene in Order to Confer a New Function to Cells or Tissues

Suicidal gene induction as a ‘cytoreductive gene therapy’ approach [such as prodrug approach, sometimes known as gene-directed enzyme prodrug therapy (GDEPT)] utilizes an agent which can be directly injected into the tumor to allow the anticancer prodrug to be converted to its cytotoxic form (Table III). This approach depends on the use of specific promoters for tumor cells or residual normal cells as in the case when tumor cells express herpes simplex virus thymidine kinase (HSV-tk, suicidal gene). In this case, the administration of GCV (prodrug), which is phosphorylated by HSV-tk and competes with deoxyguanosine triphosphate in DNA polymerization, results in the arrest of DNA synthesis and cell death (87). Braiden et al. (88), by transducing three thyroid cell lines, namely Fischer rat thyroid cell line-5 cells (FRTL-5), FRO cells (human ATC cell line) and FR3T3 cell line (a malignant cell line developed from FRTL-5 cells) with a retrovirus (Re) expressing HSV-tk under control of the TG promoter, achieved cell death in all cell lines except for the FRO cells, which do not express TG. It should be noted that this vector has to be given by direct injection into the tumor. In another study, an Ad with TG promoter driving expression of tk (Ad-TG-tk) was used to transduce FRTL-5 cells, and when GCV was added, more than 90% of the cells were killed; a minimal effect was observed in the other cell lines studied (HEPG2, COS-1, rat and human MTC, HeLa, GH3, P98G, and CA77). Moreover, when an Ad using nonspecific CMV promoter to express tk (Ad-CMV-tk) was given intravenously (i.v.) to rats treated with GCV, severe liver damage was induced. In contrast, when the same amount of Ad expressing tk under control of the TG promoter was given (i.v.), neither significant changes of serum transaminase levels and histologic liver abnormalities were observed, nor adverse effects (89). For enhancing tk expression in TG-expressing cells, Nagayama et al. (86), combined the function of the tk/GCV system with the Cre-loxP system. As a result, the cytotoxic effect was 5 to 10-fold higher than that with TG promoter alone.

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### Table II. Different gene therapy approaches related to thyroid carcinoma.

<table>
<thead>
<tr>
<th>No.</th>
<th>Approach</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Introduction of a (non-normally present) gene in order to confer a new function to cells or tissues.</td>
</tr>
<tr>
<td>2.</td>
<td>Replacement of a mutation-induced deactivated gene by reintroduction of a normal form of that gene into tumor cells.</td>
</tr>
<tr>
<td>3.</td>
<td>Production of a mutated protein (by a mutated gene) that blocks the function of an oncogenic protein.</td>
</tr>
<tr>
<td>4.</td>
<td>Reintroduction or amplification of a specific gene expression.</td>
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<tr>
<td>5.</td>
<td>Antisense approach.</td>
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<tr>
<td>6.</td>
<td>Use of siRNA.</td>
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<tr>
<td>7.</td>
<td>Use of RNAi.</td>
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<tr>
<td>8.</td>
<td>Use of replication-competent viral vectors (replication-selective, viral-mediated oncolysis).</td>
</tr>
<tr>
<td>10.</td>
<td>NIS-introducing or toxic gene therapy approach.</td>
</tr>
</tbody>
</table>

siRNA: Small interfering RNA; NIS: sodium iodide symporter gene.
inducing rejection of tumor cells. Zhang et al. (91, 92), used a replication-defective Ad harboring the IL2 gene for treatment of MTCs in mice and rats that resulted in tumor regression of smaller tumors and tumor stabilization of larger tumors, with low in vivo toxicity after systemic application of the Ad. The antitumor effect was shown to be dependent upon cytotoxic T lymphocyte activity against the tumor, which also prevented tumor growth after reinjection of tumor cells (indicating the development of long-term antitumor immunity); similar results have been obtained by Lausson et al. (93), supporting the possibility of a future vaccine production for those at risk of developing cancer. IL-12 is one of the antitumor cytokines used in different studies for the treatment of follicular cell-derived and MTC, with a significant therapeutic effect and long-term antitumor immunity (94-96). In order to express both these genes in one vector, various vectors have been developed (Re or Ad vectors) expressing both HSV-tk and IL2 or IL12 (human or murine), and these have been shown to have systemic and long-term antitumor immunity superior than that of each gene system alone (97-99). Similar results regarding the feasibility and efficiency of a combined immunomodulating and suicide gene therapy approach for thyroid carcinomas was obtained by Barzon et al. (100), who used a Re vector in differentiated and ATC cells with bystander effects both in vitro and in vivo. For the achievement of larger selectivity, a transcriptionally targeted Re vector was generated, replacing the viral enhancer with the enhancer sequence of the human TG gene, which allowed selective transgene expression and cell killing in differentiated thyroid tumor cells, but not in ATC cells or non-thyroid cells (101).

Table III. Suicidal gene approaches associated anticancer prodrug with or without immunomodulatory cytokines gene insertion in different gene therapeutic trials with different vectors and cell lines (for details and the meaning of the abbreviations used, see text).

<table>
<thead>
<tr>
<th>Study/Reference</th>
<th>System</th>
<th>Cell line type</th>
<th>Effects</th>
<th>Side-effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagayama et al. (86)</td>
<td>Re-TG-tk / Cre-loxP</td>
<td>FRTL-5, FRTC, FRO</td>
<td>Enhances the expression of TK in TG-expressing tissues</td>
<td>---</td>
</tr>
<tr>
<td>Nishihara et al. (87)</td>
<td>Re-CMV-tk</td>
<td>FRO, WRO</td>
<td>Cytotoxic</td>
<td>Extrathyroidal tissue toxicity</td>
</tr>
<tr>
<td>Braiden et al. (88)</td>
<td>Re-TG-tk</td>
<td>FRTL-5, FRTC, FRO</td>
<td>Bystander effect</td>
<td></td>
</tr>
<tr>
<td>Zang et al. (89)</td>
<td>Ad-TG-tk, Ad-CMV-tk</td>
<td>FRTL-5, HEPG2, COS-1, MTC, HeLa, GH3, P98G, CA77</td>
<td>Cytotoxic only to TG-expressing tissues</td>
<td>Not effective in the anaplastic form</td>
</tr>
<tr>
<td>Zang and DeGroot (90)</td>
<td>Ad-CMV-tk</td>
<td>Rat MTC</td>
<td>Bystander effect in vitro</td>
<td>No effect on large tumors</td>
</tr>
<tr>
<td>Zang et al. (91, 92)</td>
<td>Ad-CMV-mIL-2</td>
<td>Mouse MTC</td>
<td>Destruction of smaller tumors and stabilization of larger tumors</td>
<td>---</td>
</tr>
<tr>
<td>Lausson et al. (93)</td>
<td>---</td>
<td>Rat MTC</td>
<td>Tumor progressively rejected</td>
<td>---</td>
</tr>
<tr>
<td>Zhang and DeGroot (94, 96)</td>
<td>Ad-CMV-mIL-12</td>
<td>Rat MTC</td>
<td>Development of long-term antitumor immunity</td>
<td>---</td>
</tr>
<tr>
<td>Yamazaki et al. (95)</td>
<td>Ad-CALC1-mIL-12</td>
<td>Rat MTC</td>
<td>Development of long-term antitumor immunity</td>
<td>---</td>
</tr>
<tr>
<td>Soler et al. (97)</td>
<td>Re-CMV-tk-IL-2</td>
<td>MTC</td>
<td>Development of long-term antitumor immunity (superior to that of each system alone)</td>
<td>---</td>
</tr>
<tr>
<td>Zhang and DeGroot (98)</td>
<td>Ad-CMV-tk-IL-12</td>
<td>Rat MTC</td>
<td>Enhanced antitumor activity (superior to that of each system alone)</td>
<td>---</td>
</tr>
<tr>
<td>Yamazaki et al. (99)</td>
<td>Ad-TCP-tk+ Ad-TCP-mIL-12</td>
<td>Rat MTC</td>
<td>Enhanced antitumor activity (superior to that of each system alone)</td>
<td>Minimal toxicity</td>
</tr>
<tr>
<td>Barzon et al. (100, 101)</td>
<td>Re-TG-tk-IL-2</td>
<td>Human differentiated thyroid cancer cells and ATC</td>
<td>Bystander effects both in vitro and in vivo</td>
<td>Effective only in the differentiated cell line</td>
</tr>
<tr>
<td>Barzon et al. (102)</td>
<td>Re-tk-IL-2 (intra-tumor)</td>
<td>Human ATC</td>
<td>Activation of a local and systemic immune-inflammatory response</td>
<td>Transduction efficiency low with mild side-effects</td>
</tr>
</tbody>
</table>
The first report of gene therapy for ATC on humans was by Barzon et al. (102), where two patients with end-stage ATC were treated by direct intratumor injection of Re vector producer cells followed by GCV. The Re vector carried human IL2 gene and tk gene of herpes simplex virus type 1. Treatment was safe and associated with only mild adverse effects, while it increased the production of T helper type 1 cytokine expression in peripheral blood mononuclear cells with local tumor necrosis.

An alternative GDEPT approach is based on the Escherichia coli enzyme purine nucleoside phosphorylase (PNP), which converts a nontoxic prodrug, fludarabine phosphate, to 2-fluoroctadenine. The active metabolite is an adenosinetriphosphate (ATP) analog that inhibits ATP-dependent reactions; it kills both proliferating and non-proliferating cells by inhibition of protein and RNA synthesis. The PNP-fludarabine system also induces a very efficient local bystander effect which is under control of the modified CALCA (TSE2.CP1) promoter in Ad (103).

Replacement of a Mutation-induced Deactivated Gene by Reintroduction of a Normal Form of that Gene into Tumor Cells

p53 is a transcription factor mediating critical cellular responses, including cell cycle arrest and apoptosis, after exposure to DNA-damaging stimuli (104-106). Mutations of the thyroid p53 tumor suppressor gene are found in most poorly differentiated tumors (which have lost expression of normal p53) as part of the process of becoming unregulated carcinomas, while reintroduction of the wild-type p53 (wt-p53) has been used in a variety of experimental thyroid cancer models (Table IV). The expression of wt-p53 in a p53-null thyroid carcinoma cell line (FRO) resulted in reduced cell growth in vitro and in inhibition of tumorigenesis in vivo in nude mice (107). As a result of the p53-induced antiangiogenic effect, due to down-regulation of vascular endothelial growth factor (VEGF) and to up-regulation of thrombospondin (106), not only are p53-transduced cells killed but so are the surrounding non-transduced cells by the transduction of their neighbors, leading to a reduction in the level of transduction efficiency required for successful gene therapy. In ATC cell lines, p53 reintroductions resulted in inhibition of cell proliferation and restored a more differentiated phenotype retaining the ability to respond to TSH with an increased expression of thyroid-specific genes such as TG, thyroperoxidase and TSHR (108, 109). In order to enhance the apoptotic killing by p53 gene transfer in ATC cell lines (FRO and WRO), Imanishi et al. (110), used the histone deacetylase inhibitor (HDAC-I) depsipeptide, suggesting that this combinatorial treatment approach might also be useful in the treatment of undifferentiated thyroid carcinomas. This treatment also takes advantage of the fact that a replicating Ad (or a non-replicative form) is used in order to infect the tumor cells, while the expression of the p53 protein leads to apoptosis of the tumor cells. Apparently, the gene expression in normal cells, which already have an intact and expressed p53, causes less, or in some cases no, harm (111, 112). Nagayama et al. (113), re-expressed p53 in four human ATC cell lines harboring p53 mutations (ARO, FRO, NPA, WRO) and in normal human thyroid follicular cells in vitro and in vivo by the use a replication-defective Ad. The evaluation of the therapeutic efficacy of p53 expression resulted in a dose-dependent cell killing in both normal and carcinoma cells, but normal thyroid cells were relatively resistant to p53-mediated cell death despite their higher Ad infectivity. The mechanism of cell killing was shown to be apoptosis.

In addition, wt-p53 expression sensitized some of the cell lines to the chemotherapy and this approach clearly offers

Table IV. Gene therapy trials for reintroduction of the wild-type p53 gene (alone or with chemotherapy) in different neoplastic cell lines (for details and the meaning of the abbreviations used, see text).

<table>
<thead>
<tr>
<th>Study/Reference</th>
<th>Cell line type</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagayama et al. (107)</td>
<td>FRO</td>
<td>Cell growth inhibition in vitro, tumorigenicity inhibition in vivo.</td>
</tr>
<tr>
<td>Moretti et al. (108)</td>
<td>ARO</td>
<td>Inhibition of cell proliferation, restoration of a more differentiated phenotype, as well as TG, thyroid peroxidase and TSH-receptor expression increase.</td>
</tr>
<tr>
<td>Fagin et al. (109)</td>
<td>NPA, ARO, FRO, WRO</td>
<td>Re-expression of thyroid peroxidase, significant cell differentiation.</td>
</tr>
<tr>
<td>Imanishi et al. (110)</td>
<td>FRO, WRO</td>
<td>Increased transcriptional activity of p53 (by 10-100 times) with introduction of histone deacetylase inhibitor, enhanced apoptotic killing by p53 transfer.</td>
</tr>
<tr>
<td>Moretti et al. (111)</td>
<td>FRO, WRO</td>
<td>FRO: no effects in term of proliferation and activity. WRO: strong growth inhibition.</td>
</tr>
<tr>
<td>Narimatsu et al. (112)</td>
<td>FRO</td>
<td>Radiosensitivity enhancement, cell-growth inhibition.</td>
</tr>
<tr>
<td>Nagayama et al. (113)</td>
<td>ARO, FRO, NPA, WRO, FRTC</td>
<td>Dose-dependent killing, chemosensitivity enhancement.</td>
</tr>
<tr>
<td>Blagoskonny et al. (114)</td>
<td>BHT-101, SW-1736, KAT-4</td>
<td>Chemosensitivity enhancement.</td>
</tr>
<tr>
<td>Kim et al. (115)</td>
<td>NPA</td>
<td>Cell-growth inhibition, chemosensitivity enhancement.</td>
</tr>
</tbody>
</table>
potential for therapy, especially when there is difficulty in transducing all cells by direct tumor injection (86). In vitro, Blagosklonny et al. (114), found that there is an increased chemosensitivity of ATC to doxorubicin. In vivo experiments using FRO and NPA cell xenografts in nude mice showed inhibition of tumor growth following direct injection of the Ad expressing wt-p53. This effect was augmented by combination with doxorubicin, resulting in tumor regression (113), similar results were obtained by Kim et al. (115), when a retroviral p53 gene transfer into p53 mutant PTC cells (NPA) resulted in a dose-dependent inhibition of tumor cell growth and enhanced chemosensitivity to adriamycin (doxorubicin) in vitro and in vivo.

Production of a Mutated Protein (by a Mutated Gene) that Blocks the Function of an Oncogenic Protein

The human RET proto-oncogene encodes for a transmembrane receptor that consists of three functional domains: the extracellular ligand binding domain, the transmembrane segment and the intracellular domain formed by a tyrosine kinase. Mutations of the RET gene result in constitutive activation of RET tyrosine kinase with aberrant downstream signaling and initiation of tumor formation in many malignant tumors including thyroid malignancies. Drosten et al. (116, 117), demonstrated that more than 95% of MTCs harbor dominant activating mutations in the RET proto-oncogene, which play a central role in the development of tumor. Ad vectors expressing dominant-negative RET mutants were used in human MTC cells under the control of a CALCA gene-related promoter. Dominant-negative RET mutant proteins have amino acid changes in the extracellular domains and because of these changes, the glycosylation process is disturbed, resulting in hampered protein transport to the cell surface. The dominant-negative mutant protein dimerizes with oncogenic RET protein in the endoplasmic reticulum (ER), thereby preventing expression of both dominant-negative and oncogenic RET protein on the cell surface. A pronounced shift of endogenous oncogenic RET protein localization from the cell surface to the ER was demonstrated, resulting in strong inhibition of cell viability caused by induction of apoptosis in vitro. Furthermore, ex vivo infection of thyroid tumor cells with this C-cell-targeted dominant-negative RET mutant Ad resulted in suppression of subcutaneous tumor growth in nude mice, whereas direct injection of this vector into pre-established, subcutaneous, xenograft thyroid tissue tumors retarded tumor growth in only a limited number of mice. In contrast to p53 restoration, this approach requires high levels of ex vivo transduction efficiency, because it does not affect surrounding cells, thereby limiting its therapeutic efficacy in vivo.

Reintroduction or Amplification of a Specific Gene Expression

Most poorly differentiated and ATCs have lost the ability to express the TG gene, which is accompanied by loss of transcription factors [thyroid transcription factor-1 (TTF1), TTF2 or PAX8] interacting with the TG promoter. Non-TG-expressing ARO and WRO thyroid cell lines (which had low intrinsic TG) transfected with the TTF-1 and PAX-8 genes, resulted in increased function of the TG promoter (118). In addition, co-transduction of TTF1 with the TG promoter by Ad vectors reactivated the TG promoter in dedifferentiated thyroid cells but exhibited only little effect in non-thyroid cells (119). Another study depending on this approach has been conducted by Chung et al. (120), who provided an explanation for the significantly decreased levels of growth arrest and DNA damage 45 gamma (GADD45γ) RNA in ATC cells, compared to normal primary cultured thyrocytes and by Ad-mediated re-expression of GADD45γ in ATC cells (ARO, FRO, NPA). It should be noted that GADD45 family proteins have been implicated in a variety of growth-regulatory mechanisms, including DNA replication and repair, G0/G1 checkpoint control, and apoptosis. The above modifications resulted in a significant inhibition of the proliferation due to apoptosis. In addition to this, the expression of tyrosine phosphatase eta (PTPη) gene, which encodes a receptor-type tyrosine phosphatase protein with tumor-suppressor activity, had been used in several studies (121-123), resulting in cell growth inhibition by interfering with RET autophosphorylation. The latter reduces its kinase activity and leads to the inhibition of downstream Ras-ERK (Ras GTPase-extracellular signal-regulated kinase), and AKT-Pi3K signaling pathways (Figure 1), which, in turn, reduces oncogenic activity.

Antisense Approach

The antisense approach aims to turn off a mutated gene in a cell by targeting the mRNA transcripts copied from the gene in order to disrupt the transcription of the faulty gene. High mobility group 1 (HMGI) proteins are overexpressed in several human malignant tumors and by using an adenovirus carrying the HMGI(Y) gene in an antisense orientation (Ad-Yas) has led to the suppression of HMGI(Y) protein synthesis and prevention of thyroid cell transformation. Such virus-induced programmed cell death was successful in two human ATC cell lines (ARO and FB-1) and in a variety of other tumor cell lines, but not in normal thyroid cells (124). Another study by Hassan et al. (125), applied this approach by transfecting FTC cell line (FTC-133) cells with oligodeoxyribonucleotide phosphorothioates (ODNs) (mutant Tp53 knockout) and finally managed to impair VEGF secretion in the undifferentiated FTC-133 cell line.
Use of Ribozymes

Another approach having the same outcome as that with antisense uses ribozymes. The ability of ribozymes to recognize and cut specific RNA molecules makes them exciting candidates for human therapy. Among the characteristic examples of this method is a synthetic ribozyme that destroys the mRNA encoding a receptor of VEGF and also the ribozyme designed by Parthasarathy et al. (126), to cleave the sequence that results from the mutation of codon 634 from TGC to TAC in the RET proto-oncogene. Cleavage specificity was demonstrated by the observation that transformed NIH3T3 cells that expressed the active ribozyme (RETC634) no longer formed colonies in soft agar, unlike NIH3T3 cells that expressed wt-RET codon 634.

Use of Small Interfering RNA (siRNA)

This approach utilizes siRNA (similarly to the antisense approach) in order to prevent the production of the faulty protein. Overexpression of antiapoptopic genes, such as BCL2 (B-cell chronic lymphocytic leukemia/lymphoma 2) and BIRC5 encoding for survivin protein, was found in MTC tissues and MTC cell line. Targeting the BIRC5 gene with siRNAs reduced the expression of survivin protein, which led to an increase in apoptosis concomitant with suppression of in vitro MTC growth (127).

Antisense, ribozymes and siRNA approaches were introduced without bystander activity in non-targeted tissue, and therefore require high vector transduction efficiency to increase the efficiency of transduction.

Use of Replication-competent Viral Vectors (Replication-selective, Viral-mediated Oncolysis)

The development of an Ad system that can replicate exclusively in wt-p53-deficient cells by using a double infection approach has been achieved in order to infect the cells with two recombinant viruses. The recombinant virus contains an expression unit of the synthetic p53-responsive promoter and the Cre recombinase gene, and the other Ad contains two expression units: the first consists of the EIA gene flanked by a pair of loxP sites downstream of the constitutive CAG promoter, and the second consists of the EIB19K gene under the control of the CMV promoter. Co-infection of these two Ads into p53-expressing cells leads to expression of Cre recombinase gene, which then excises the EIA gene that is flanked by a pair of loxP sites, thereby stopping virus replication. However, in cells without p53 expression, Cre recombinase is not expressed, the EIA gene is not excised, and virus replication takes place, thereby causing cell lysis (128).

Another anticancer therapeutic approach is through achieving selective replication oncolytic viruses (virotherapy), which represents a novel targeted form by using the dl1520 (ONYX-015) virus was genetically engineered for replication-selectivity for treatment in cancer patients (129-130). Oncolytic viral therapy offers several advantages over conventional anticancer drugs, viruses can be rapidly modified by recombinant DNA technology, allowing for the rational creation of ‘designer viruses’. In addition to the ability of these viruses to self-replicate within the cancer cell and their unique pharmacokinetic properties that are distinct from conventional therapeutics, Ads and other viruses have been engineered for selective replication within neoplastic cells. The most common goal is the deletion of the viral gene whose product is necessary for replication in normal cells but expendable in cancer cells. dl1520 was expected to replicate selectively in a high percentage of human cancer types (where the p53 pathway is non-functional in about 50% of human neoplasia). Recently, it has been shown that EIB55K mediates late-viral RNA transport, and dl1520 tumor-selective replication is determined by a unique property of tumor cells to efficiently export late viral RNA in the absence of EIB55K. Thus, loss of E1B-55K-mediated late viral RNA export, rather than p53 degradation, is the major determinant of dl1520 selective replication in neoplastic cells (131). The use of dl1520 oncolytic virus as a monotherapy has demonstrated limitations in efficacy because of the multifunctional nature of many viral proteins; the gene deletion approach for viral selective replication frequently results in reduced replication and therapeutic potency. Moreover, the complexities of tumor biology and the heterogeneity of human tissues necessitate further investigation for the design of strategies in order to increase the tumor-eradicating potential of such a method (132). It is almost impossible to deliver the virus to all the tumoral cells; therefore, the uninfected tumoral cells will continue to grow and complete tumor regression will only be obtained by the association of ONYX-015 with chemotherapeutic drugs (133).

Recently, a sizable fraction of undifferentiated or poorly differentiated thyroid tumors were shown to contain mutations in β-catenin. A conditionally replicative Ad (named HILMI) has been developed, which replicates specifically in cells with an active Wnt/β-catenin pathway. As a result, several thyroid cancer cell lines derived from undifferentiated or anaplastic tissues and possessing an active Wnt/β-catenin pathway are susceptible to cell killing by HILMI. Furthermore, viral replication in ATC cells as xenograft tumors in nude mice was observed, and prolonged survival of mice with ATC tumors was observed following administration of the HILMI therapeutic vector (134).

ONYX-411 is a new virus that replicates selectively and induces cell death in eight human ATC cell lines. The cytopathic effect of the virus is specific to cells with retinoblastoma dysfunction, which appears to be frequent in
gene transfer in ATC. To use radioiodine therapy by sodium iodide symporter (NIS) promoter and that doxorubicin might be used as an adjuvant enhancing transgene expression under the control of the CMV promoter, findings by Kim enhance by the apoptotic effects of anticancer agents can be observed in ATC cell lines (113, 114). Enhanced sensitivity to chemotherapeutic regimens in ONYX-015-infected cells may depend on the E1A gene expression. In fact, E1A expression activates the cell cycle and increases cellular sensitivity to p53-independent apoptosis (136, 137). Therefore, the apoptotic effects of anticancer agents can be enhanced by the E1A Ad gene product (138). Recently, findings by Kim et al. (139) indicate that doxorubicin enhances transgene expression under the control of the CMV promoter and that doxorubicin might be used as an adjuvant to radioiodine therapy by sodium iodide symporter (NIS) gene transfer in ATC.

Increased efficacy of the dl1520 virus has been repeatedly shown following ionizing radiation. Portella et al. (140), showed that ATC ONXY-015-treated cells were very sensitive to radiation-induced apoptosis. Moreover, lovastatin in combination with dl1520 virus has also been used to increase oncolytic activity, since lovastatin is a drug that increases Ad replication and enhances the effects of oncolytic Ad in vitro and in vivo against ATC cells (141). Lovastatin (which is a 3-hydroxy-methylglutaryl-CoA reductase inhibitor), a drug used for the treatment of hypercholesterolemia, has also been reported to exert growth inhibitory activity in vitro and in vivo. Farnesyl-(FPP) and geranyl-geranyl-pyrophosphate (GGPP), intermediates in the cholesterol synthetic pathway, are needed for isoprenylation, a crucial step for membrane attachment of cellular proteins such as Ras, Rho, Cdc42 and Rac, and thus, by inhibiting protein isoprenylation, lovastatin induces a reduction of cell proliferation and apoptosis (142, 143). Moreover, it has been demonstrated that low doses of lovastatin induce apoptosis of ATC cells (144, 145). In addition, it was observed that lovastatin significantly enhanced the antineoplastic activity of dl1520 against ATC cells and its replication.

Another oncolytic herpes simplex virus, G207, presents a promising activity against human ATC, when combined with paclitaxel. A significant enhancement of paclitaxel effect (increase in antitumoral activity, including microtubule acetylation, mitotic block, and apoptosis) in addition to direct oncolytic effects of G207 was shown (146).

### NIS-Introducing or Toxic Gene Therapy Approach

Normally, iodide is accumulated in the thyroid gland in concentrations that reach 20 to 40-fold over the plasma levels (147) and this accumulation is under the control of NIS which is a transmembrane glycoprotein present in the basolateral pole of the thyroid follicular cells that were firstly cloned in rats (148) and then in humans (149). The entire process is under the control of TSH. It should be noted that a major limitation for the treatment of some thyroid carcinomas with radioactive iodide (150) has been attributed to the progressive reduction or loss in the ability of these carcinomas to take up radioactive iodine due to iodine metabolism abnormalities (such as defects in iodide uptake and/or organification) (151-153). Induction of hypothyroidism in order to elevate endogenous TSH or the administration of human recombinant TSH is used by physicians in such cases to enhance iodide uptake, proving that cancer cells still maintain a certain degree of differentiation and hormonal responsiveness. However, when thyroid cancer undergoes total loss of differentiation, no iodide uptake is observed, and the prognosis is clearly worse. Overall, low or an absence of NIS expression was thought to occur under these circumstances (153-155). Surprisingly, after the cloning of NIS, in some studies that were based on IHC and reverse-transcriptase PCR (RT-PCR), it was revealed that up to 70-80% of thyroid tumors expressed or even overexpressed NIS, and this expression was mainly cytoplasmatic and not targeted to the basolateral membrane (156-158). These observations pointed out that targeting of, and retention in, the plasma membrane are essential for NIS to be fully functional, explaining why iodide uptake is diminished in thyroid cancer.

The principle of introducing NIS gene for developing a novel cytoreductive or toxic gene therapy strategy in the treatment of thyroidal and extrathyroidal malignancies was based on the transfer of NIS gene followed by radioiodine therapy (159), either by transfection of NIS cDNA (148, 149, 160) or with Re or Ad vectors (161, 162). Iodide was, finally, uptaken by the transduced cells and by coupling with 131I administration as a treatment or with a recombinant Ad (Ad-NIS radioiodine administration), a procedure that appears to be a very promising strategy for treating tumors of various origins (163). Application of the NIS gene extends the diagnostic and therapeutic use of 131I in the management of differentiated thyroid cancer to the treatment of non-thyroidal cancer and dedifferentiated ATC and MTC. In addition, NIS gene transfection is associated with a bystander effect, because not only NIS-transduced cancer cells, but also surrounding non-transduced cells are destroyed by the crossfire effect of the β-emitter 131I (164). Early studies in malignantly transformed rat thyroid cells (FRTL-Tc) without iodide transport activity showed that transfection with rat NIS cDNA using electroporation is able to restore...
radiiodine accumulation both in vitro and in vivo (165). More recently, stable transfection of a NIS-defective follicular thyroid carcinoma cell line (FTC-133) with the NIS gene was able to re-establish iodide accumulation activity both in vitro and in vivo (166). Petrich et al. (167), transiently transfected a variety of PTC, FTC and ATC cell lines (B-CPAP, K1, WRO, 8505C, FTC-133) with the NIS gene, thereby inducing perchlorate-sensitive accumulation of $^{125}$I. These studies show that NIS gene delivery into thyroid cancer cells is capable of restoring $^{131}$I accumulation and might therefore represent an effective therapy for dedifferentiated and anaplastic thyroid tumors that lack iodide-accumulating capacity. Lee et al. (168), used a recombinant Ad to transduce a panel of human thyroid carcinoma cell lines (ARO, FRO, NPA) with the hNIS gene. They demonstrated significant iodide accumulation associated with rapid iodide efflux in vitro that may limit the therapeutic potential of hNIS/radiiodide-based treatment, but more recent trials still confirmed the potential of the hNIS-mediated $^{131}$I gene therapy for ATC (169).

There is a significant correlation between low NIS expression and more aggressive tumors (170), and BRAF-positive PTC correlated with high recurrent rate and impairment of NIS targeting to the membrane as assessed by IHC (171). In addition to the role of other important gene mutations such as those of RAS family genes and RET rearrangements, all of them contribute to the partial or complete loss of differentiation of thyroid cancers.

Conclusion

More understanding of the molecular pathways and tumorigenesity is remaining the only key for developing new gene therapy approaches to achieve the efficient and successful antitumoral efficacy with minimum normal tissue toxicity by taking into consideration the subsequent trials in each approach alone and then interpretation of all findings and modifications. The selection of the most promising approach must be based on the balance between therapeutic efficacy and unwanted effects in accordingly designed clinical trials.

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