REVIEW
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GENETICS OF MEDULLARY THYROID CARCINOMA

MICHAEL ARULDHAS[1] M, KALPANA, N, VENGATESH G, SAKHILA BANU K[2] and GOVINDARAJULU P.

Department of Endocrinology, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai- 600113,India.
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SUMMARY

Existing modes of diagnosis and therapy for medullary thyroid carcinoma (MTC) have limitations. Informations on the genetics of familial medullary thyroid carcinoma (FMTC), a constituent of multiple endocrine neoplasia II (MEN II) syndrome may help to understand the etiology of the disease and to design gene therapy. Association between specific mutations in *ret* protooncogene to specific phenotypes of MEN II subjects provide vital line of due for the diagnosis of the disease. Screening of suspected / potent carriers of *ret (rearranged during transformation)* mutations will help to decide about the time and course of treatment. Gene therapy for MTC appears to be imminent and this review attempts to provide a comprehensive account of informations from more than hundred research articles published in this area.

Key Words: Calcitonin; Familial medullary thyroid carcinoma; Gene therapy; Immuno therapy; Medullary thyroid carcinoma; Multiple endocrine neoplasia; *ret* protooncogene.

INTRODUCTION

Gene therapy has come to stay as the most effective mode of combating, not only monogenic diseases with genetic defects but also in the treatment of multifactor diseases like diabetes, obesity, hypertension and cancer. Gene therapy for endocrine disorders is in the incipience and the clinical trials are yet to actuate. The search of gene therapy for endocrine cancers may benefit from gene replacement strategies related to tumour suppressor genes, inhibition of oncogenes, anti-angiogenesis and suicide gene therapy or immunomodulatoin therapy (1). The success of any gene therapy depends on adequate basic research, which shall identify genes linked to specific biological functions or the specific gene mutation in a particular disease condition. The purpose of this review is to update the readers about the recent developments in the field of molecular pathology of thyroid cancer, so as to generate new research interest in the area of gene therapy for endocrine cancers, with particular interest to thyroid cancer, specifically to medullary thyroid carcinoma (MTC).

Cancer of the thyroid gland, the major endocrine cancer constitutes only about 1% of all malignancies (2). Papillary carcinoma (PTC), follicular carcinoma (FTC) and medullary thyroid carcinoma (MTC) are the main histological types of thyroid cancer. There are wonderful reviews on the clinical, biochemical and histo-pathological aspects of thyroid cancers (3-6), which may be referred by those who are interested. The present review will avoid details of those aspects, expect for a brief account of background information to the readers. Due to the limitation of space, all reports available in any specific aspect could not be included.

[1]*Corresponding author, email: aruldhasmm@ yahoo.com*
[2]*Present address : Ontogenie et Reproduction, CRBR, CHUL, 2705 Boul W Lauira, Laval University Stc Roy, que, Canada GIV 4G2*

EPIDEMIOLOGY

PTC appears to be the chief thyroid cancer (60-70%), followed by FTC (15-20%), anaplastic carcinoma (5-10%) and MTC (2-4%) as per epidemiological studies carried out in the United States of America, United Kingdom, Sweden, Japan, and Switzerland (5, 7, 8). However, the percentile distribution of different types of thyroid cancer keeps on change as newer data are being added. MTC appears to be the most dreadful among these as the average survival rate for MTC patients is lower than that for more common thyroid cancers like PTC and FTC, due to a high proportion of last-stage diagnosis, probably (7, 8).

While ~80% cases of MTC occur as sporadic, ~20% are familial (9-13). Familial MTC (FMTC) is clinically distinct with respect to incidence, genetics and age of onset in association with other diseases, and may not be associated with other endocrinoipathies (14). Unlike other type of thyroid cancers and diseases, gender bias in the incidence of MTC appears to be lacking. The tumour occurs around the fifth decade of life but the familial form can be detected much earlier. The influence of racial or environmental factors on the incidence of MTC is perceptible. Existence of a large family with many affected members in one area may also increase the frequency of the incidence MTC (15).

MARKERS

As there is consistent production of calcitonin (CT) in MTC subjects, measurement of serum CT may be a useful tool in the diagnosis, establishment of the presence of metastasis and in following the effects of treatment (16, 17). Increased serum CT titer can also be found in association with other tumours, particularly oat-cell carcinoma of lung, gastrinoma, carcinoid tumours, and carcinoma of the breast (6, 18-21). In general, MTC will be negative to thyroglobulin (22) whereas, a rare variant of MTC contained both CT and thyroglobulin, suggesting features of MTC and FTC (23). MTC may be associated with melanin production on rare occasions, and some tumour cells contain both melanin and CT (24-26).

CT mRNA is encoded by a multi-exonic gene located in chromosome 11p. CT gene related peptide (CGRP) is produced by alternate splicing of CT gene and is present in very low amount in normal C-cells when compared with CT. CGRP is predominant in the brain and other neural tissues, and is a highly potent vasoactive peptide with no known effect on calcium metabolism. In view of its vasoactive role, it is linked to the flushing noticed in some MTC patients (6, 18). Elevated CGRP may be linked to diarrhea episodes in some MTC subjects, along with vasoactive intestinal peptides, serotonin and prostaglandins (6, 27, 29, 30). Unlike in normal C-cells, both CT and CGRP are seen in MTC, though CT is the major marker (6, 31). Carcino embryonic antigen (CEA) is produced in large quantity by normal C-cells. An ominous sign of the differentiation of MTC will be increased production of CEA accompanied by a fall in CT (32). Some MTC cells express ACTH, which may be the reason for the development of Cushing's disease in a few MTC subjects. Chromogranin A, neuron-specific enolase, synaptophysin and neural cell adhesion molecule (NCAM) are also secreted by MTC (6, 18). Sialic acid in the NCAM is of significance as the degree of its modification may serve as the marker to distinguish FMTC from other types (33).

PATHOLOGY

The MTC is a slowly growing solid tumour with well circumscribed but not encapsulated cells (encapsulation may be seen rarely), and large vesicular nuclei (6, 18, 34, 35). Sheets of cells with abundant glandular cytoplasm and irregular masses of amyloid and much collagen can be seen under light microscope. Round cells separated by thin fibrovascular stroma is the classical pattern of growth (9, 18). The tumour may be with fusiform cells forming a whirling pattern, and rarely the most rapidly growing patterns of a reminiscent oat-cell carcinoma. MTC is a malignant neoplasm of the calcitonin (CT) producing light or para follicular or C-cells of the thyroid gland (13, 36-38) and has the clinical and histological features of a neuroendocrine tumour as C-cells originate from the embryonic neural crest (14, 40-43).

MOLECULAR PATHOLOGY

The development of MTC begins with the initial diffused C-cell hyperplasia, followed by the outgrowth of microscopic carcinoma and the macroscopic (gross) cancer. These specific stages are accompanied by tumour cell heterogeneity, loss of endocrine factors like CT secretion (6). Progression of the tumour in MTC may involve the expression of either tumour suppressor genes or oncogenes. FMTC is inherited as an autosomal dominant trait with a high degree of penetrance and is associated with the type II multiple endocrine neoplasia (MEN) syndromes (16, 43, 44). MEN IIA and MEN IIB (MEN III) are the two types of MEN II. Apart from MTC, 50% MEN IIA subjects have pheochromocytoma and 20% have hyperparathyroism. MEN IIB, which contributes only 5% of all MEN II cases is an aggressive variant and will be characterized by MTC, pheochromocytoma, mucosal neuromas, ganglioneuromatosis of the gut, bumpy lips and marfanoid habitus (7, 15, 45, 46). On the other hand, FMTC is characterized by MTC only (47, 48).

The milestone in the molecular biology research of MTC was the mapping and localization of MEN-IIA gene in chromosome 10q 11.2 (49-51) and the identification of gremline mutations of *ret* (*rearranged during transformation*) proto-oncogene (a gene, which has the potential to become an oncogene on specific mutations) (52, 59, 60). *Ret* mutations act as dominant oncogene mutations in MTC, and C-cell hyperplasia may be the result of a single mutated *ret* allele. Nevertheless, a perceptible percentage of MTC tumours are associated with loss of a portion in short arms of chromosome 1 and 22, though the exact genes involved are not known (61, 62). Recently, McGregor *et al.* (63) indicated over expression of the neurotrophin receptor TRKC and down regulation of TRKB during the progression of MTC tumour.

The *ret* proto-oncogene, which contains 21 exons and has a size of 55kb (59, 64) encodes a membrane receptor tyrosine kinase of 150 to 170 Kd (60).Tyrosine kinase receptor (TKR) encoded by *ret* has three structural domains i.e. the ligand-binding extracelluar domain having a cysteine-rich region, a hydrophobic transmembrane domain, and a carboxy terminal intracellular domain of tyrosine kinase with catalytic and substrate recognition sites (6, 60). This cell surface receptor transduces growth and differentiation signals in several developing tissues, including those derived from the neural crest like the thyroid C-cells, adrenal medulla,

developing brain and kidney (14, 43, 60). The highly conserved cystine rich domains of the RET protein is essential to maintain the secondary and tertiary structure of the extra cellular domain (65). The extra cellular domain with a calcium-binding cadherin-like region and a cysteine-rich region interacts with at least two ligands identified to date, neurturin (Ntn) and glial-cell derived neurotrophic factor (GDNF).The physiological function of GDNF is the promotion of neuronal survival (64). *gdnf* knock out mice presented surprising results similar to *c- ret* protooncogene deletions, in addition to severe defects in the neuronal development of the gastrointestinal tract as seen in Hirschsprung's disease (aganglionic megacolon) and kidney development (13, 66-68). Binding of GDNF to the TKR results in the formation of a multimeric receptor complex by interacting with a second protein called GDNFR α_1 or $NtnR_1$ and GDNFR α_2 or $NtnR_2$ (46, 60, 69-71).

The tyrosine kinase catalytic core in the intracellular domain activates downstream signaling events by phosphorylating and stimulating the proto-oncogene *src,* resulting in cell proliferation (60, 72-74). *ret* gene mutations are noticed in 95% of individuals with MEN IIA and MEN IIB, and in about 85% of individuals with FMTC, and MTC tumour tissues express normal and mutated *ret* alleles (75). Point mutations of *ret* proto-oncogene was identified about a decade back in subjects with MEN IIA, MEN IIB and FMTC in six closely located exons (12, 15, 51, 59, 67-69, 76). Approximately 92% of MENII variants are related to germ line missense mutations of *ret* proto-oncogene in the chromosome 10q 11.2 encoding TKR (77-79). RET mutations in most of the MEN IIA cases occur mainly in exon 10 and 11 (52, 53, 80). A large intragenic deletion spanning from exon 16, which affected the normal allele in a MEN IIA patient with heterozygous germline mutation (**C** 634 **R**) has come to the surface this year (80). Rarely cases with deletion of some segments of *ret* gene are reported. MEN II A cases with duplication of 9 or 12 bp in exon 11 were reported recently (55, 56). Deletion of specific portions in the extra cellular and transmembrane regions of the RET protein due to mutation or rearrangements of the *ret* gene may result in the formation of a constitutive tyrosine kinase (does not require ligand binding to get activated), with a potential to transform a normal cell into malignant (6).

In men II patients, these germ line mutations chiefly occur on two main functional domains in the RET protein in the extra cellular ligand binding domain, especially in MEN II A and in MEN IIB, and FMTC (52). The frequent mutation, which leads to FMTC (75-80%), occurs in codon 634 in exon 11 (15, 58). Mutation in codon 634 has been reported to be associated with three amino acid changes i.e., arginine, tyrosine and tryptophan (60). Disruption of a cysteine rich region (codons 609, 611, 618, 620 of exon 10 and codon 634 of exon 11) located near the c-terminus of the extra cellular domain is a common feature of *ret* gene mutations in MEN IIA (53). It is hypothesized that disruption of the cysteine rich region lead to abnormal intramolecular protein folding, steady state dimerization and autophosphorylation of the receptor and a mitogenic signal, in the absence of the ligand. This finding of Santoro *et al.* (81) was a breakthrough, which unearthed the ligand independent activation of TKR i.e. constitutive catalytic activity of TKR. This mutant receptor functions as an inherited dominant oncogene.

Majority of the mutations recorded till date in MEN IIA are in codon 634, followed by 620. Most kindred have mutations in codons 609, 611, 618, 620 and 634 (60). A study involving 477 MEN II families and 18 tertiary referral centers, mutations at codon 634 showed significant correlation with the presence of pheochromocytoma and hyperparathyroidism (79,

82). Among the known mutations of codon 634, **C634R** (TGC to CGC i.e. Cyst to Arg) mutation is predominantly associated with the presence of hyperparathyroidism, whereas no patient with FMTC had **C634R** mutation (38, 79, 83). Germ line mutations in the codon 634 engender a powerful activation of *ret* protooncogene leading to early onset of MTC and MEN IIA phenotypes (84).

A few cases of FMTC were shown to have mutations in the intracellular non-cysteine codons 768 of exon 13, 804 of exon 14, and codon 891 of exon 15 related to the intracellular kinase domain. The mutation of the intra cellular tyrosine kinase (codon 918) has no effect on receptor dimerization but causes enhanced phosphorylation of a different set of substrate proteins, which also results in cellular transformation (3). Two codons of the intracellular tyrosine kinase domain of *ret* are routinely screened for mutations in MEN IIA and FMTC. One is codon 768 in exon 13, the mutation of which is known to alter GAG (Glu) to GAC (Asp) (84, 85). The other is codon 804 in exon 14, where the GTG/TTG (Val/Leu) change was first reported in two unrelated FMTC families (86), and the GTG/ATG (Val/Met) change was reported subsequently (87).

Mutations involving the cysteine codons 609, 618 and 620 are associated with MEN IIA, FMTC and Hirscsprung's disease (73, 74, 88). Mutations in these codons were detected in about 10% of families with MEN IIA and 2 out of 3 families with FMTC. Mutations in these codons are associated with weak activation of *ret* (73). It is suggested that these mutations are associated with high transforming activity but may affect the transport of RET receptors to the cell surface (3). In another study involving two large families with cysteine codon 618 (exon 10) mutations, pheochromocytoma was found in only two of 60 patients (89), suggesting that FMTC associated with a *ret* gene exon 10 mutation constitutes a sub-type of MEN IIA with a low frequency of pheochromocytoma, rather than a separate clinical entity. Any *ret* mutations at codon 634 in exon 11 results in a higher incidence of pheochromocytoma and hyperparathyroidism (15, 89). Some mutations, such as those involving codons 618 and 620 in exon 10, may be associated with milder forms of the disease (61). However, heterozygous missense mutations were also identified in exon 13 (codons 790 and 791) in five families, four with FMTC and one with MTC and pheochromocytoma (90).

The mutation in codon 620 is of interest as the kindreds have a paradoxical association of MTC and Hirschsprung's disease (73-75, 78). While Hirschsprung's disease, which is characterized by the absence of ganglion cells in the hind gut region, is the result of an inactivating mutation of *ret* gene of TGC to CGC (Cys > Arg), MEN IIA is the result of a gain of function mutation (75, 79). Another explanation is that the classical *ret* mutation MEN IIA may be associated with other mutations, which may interfere with the expression of mutation at codon 609, 618 and 620. This report suggests that subjects with Hirschsprung's disease with MEN IIA mutations should be closely watched for the appearance of MTC and other tumours of MEN IIA (6). *In vitro* assays demonstrate that the transforming activity of codon 634 mutations is 3-fold to 5-fold higher than that of codon 609, 611, 618 or 620 mutations (91).

The disease-causing point mutation at codon 918 of exon 16,which is responsible for 95% of the MEN IIB phenotype, and lies within the catalytic core of the tyrosine kinase, results in the methinonine substitution and causes a constitutive activation (i.e., gain of function) to the TKR, independent of the normal ligand-binding and dimerization steps (57).

This mutation changes the substrate recognition pocket of the catalytic unit of the TKR and enhances its affinity to the ligand. The hallmark of this mutation is the early onset variant of MTC with metastasis to lymph nodes and other organs (79). Sporadic MTC may be associated with somatic mutations at the same location as germline mutations in FMTC. Mutation in *ret* codon 918 has been reported in 50% of sporadic MTC tumours, as in the case of MEN IIB, but confined to tumour cells. A few cases (4-6%) of MTC subjects had *ret* gene mutation, despite the absence of any family history for MEN II. This shall attest the need to screen all MTC patients for *ret* germline mutations (6). *ret* germline **M918T** mutation is only associated with MEN IIB, whereas somatic mutations at this codon are frequently observed in sporadic MTC (92).

Loss of functions of putative suppressor gene in MEN IIA subjects has been linked to the loss of heterozygosity on chromosomes 1,3,11,17 and 22 (50, 53, 93-97).A very recent report (80) provided evidence for the first time for the existence of a germ line mutation (**C** 634 **R**) with an additional somatic deletion spanning exon 4 to exon 16. These authors also showed that the deletion causes loss of heterozygosity of *ret*, extensively in metastases, thus indicating a probable role of this deletion mutation in tumour progression. The *ret* proto-oncogene has also been known to involve in approximately 25% of hPTC. Several studies have demonstrated that *ret* is activated through somatic rearrangement (98), which may be especially due to the impact of radiation.

USEFULNESS OF *RET* ANALYSIS IN THE DIAGNOSIS OF MTC

Stimulation of calcitonin secretion using pentagastrin (0.5mg/Kg body weight) or pentagastrin plus calcium (2mg/Kg body weight) over one minute has been the main diagnosis for FMTC for many years (6). However, *ret* DNA test has come to stay as the preferred diagnostic tool. Identification *ret* gene mutations in the germ line DNA help to identify the affected individuals in a family accurately. Subjecting the DNA material extracted from the WBC of the subjects to PCR amplification of *ret* gene exons 10, 11, 13, 14 and 16 is the current technique available. The amplified fragments will be subjected to mutation analysis by automated DNA sequencing, restriction enzyme analysis, and detection of DNA mismatches. These tests are highly sensitive and specific for the disease causing mutations. Mutations in exons 10 and 11 are common in majority of MEN IIA and FMTC kindreds, whereas mutation in exon 16 is common in families with MEN IIB. Since natural DNA polymorphism may cause failure of one *ret* allele to amplify leading to wrong identification, test results should be reconfirmed before informing the patients (6).

Screening of potent carriers of MTC for *ret* gene mutations help to identify subjects at high risk. As elaborated earlier, the major lesions of MEN-IIA subjects have MTC and C-cell hyperplasia followed by the penetrance of pheochromocytoma and hyperparathyroidism (9-13). MTC is the initial manifestation in most of the cases of MEN-IIA, followed by pheochromocytoma around 29years of age. Earlier, screening and classification of MTC has been carried out by using CT secretogogues calcium and penta-gastrin. There are some cases of cryptic FMTC individuals with negative family history. Though a majority of FMTC families turned to be true, a few members in some families classified as FMTC may develop

pheochromocytoma (6). However, there is no consensus about the exact timing of prophylactic surgery due to the inconsistency in phenotypes based on CT and penta-gastrin stimulation. Genetic screenings for carriers of *ret* proto-oncogene germ line mutations help to perform early prophylactic thyroidectomy to ensure definite cure. At present, surgery is advocated at 2, 5 or 6 years of age (99-102). The main objective of prophylactic surgery is to avoid malignant progression from C-Cell hyperplasia to MTC, and to eliminate lymph node metastasis as the latter will make chemotherapy a difficult task (103). A recent report (84) has given a ray of hope in this regard. These authors have demonstrated a significant correlation between genotype and phenotypes. They correlated *ret* genotypes of 63 FMTC subjects with age at diagnosis, sex, the TNM systems and basal CT levels. They have shown clear hierarchy of *ret* mutation with respect to the onset of FMTC. The existence of a positive correlation between mutation at codon 634 and the occurrence of pheochromocytoma and hyperthyroidism is known (79). **C 634 R** (TGC CGC; Cys Arg) mutations have a strong correlation with hyperparathyroidism (6). Though, no **C634R** mutation could be noticed in FMTC by a few groups (46, 79), Decker and peacock (104) reported the association of codon 634 mutation TGC-CGC) in 51% MEN II subjects studied.

Germ line mutation in codon 634 of exon 11 engendering a strong activation of *ret* proto-oncogene is responsible for the early onset of MTC and the MEN II A phenotype. On the other hand, germ line mutation in codon 609, 611, 618 and 620 (exon 10) may lead to less activation and appears to be responsible for malignant transformation (84). Mutations in non- cystine domain of exon 13 and 14 (codon 768, 790 and 804) afford a weaker activation, resulting in attenuated form with late-onset MTC and FMTC phenotype (86, 89). Nilsson *et al.* (105) reported a germline mutation in exon 14 **(V 804L)**, which is associated with pheochromocytoma and FMTC. Unlike MEN IIA, *de novo* mutations are relatively common in MEN IIB, at least in ~50% cases. Thyroid tumours in MEN IIB may be aggressive with widespread metastases at early age. Therefore, majority of investigators recommend prophylactic thyroidectomy before 3 years of age in those children in whom MEN IIB is recognized.

The recent study of Machens *et al.* (84) predicated three categories of MTC on the basis of their findings of correlation between *ret* oncogene mutation and the age of patient at diagnosis : (i) A high risk group with mutations in codon 634 and 618 with the youngest age of 3 and 7 years, respectively. An intermediate risk group with mutations in codon 790, 620 and 611, with youngest ages at 12, 34 and 42½, respectively and a low risk group with mutations in codon 768 and 804 with the young stages of 47 and 60 years, respectively. This classification may help to decide about the time of prophylactic thyroidectomy in potent carriers of the mutations. However, infants with high risk mutations below the age of 5 years may have to undergo prophylactic surgery even earlier, in the event of elevated basal or stimulated CT. However, one has to bear in mind that normal serum CT will not rule out MTC (99, 101). Therefore, genetic analysis may be more reliable than biochemical analysis. Gills *et al.*, (102) suggested screening of mutations to begin at the age of 1 year, basal on the finding of MTC and nodal metastases in a 5 year old girl and her 3 year old sister with MTC, both harboring C634R genotype. Therefore, it is advised to screen all subjects with MTC, periodically for the development of pheochromocytoma. Genetic screenings for carriers of *ret*

proto-oncogene germline mutations help to perform early prophylactic thyroidectomy to ensure definite cure. *ret* analysis is accurate and effective in identifying subjects at risk. Immediate prophylactic thyroidectomy is advocated in *ret* mutation positive subjects, though an alternative school of thought is to perform more frequent CT stimulation tests. Thyroidectomized subjects have to be screened as usually for pheochromocytoma. *ret* mutation analysis helps to avoid unnecessary surgery in suspected individuals who may not actually prone to develop MTC. The important advantage is that the *ret* negative subjects can be re-assured that no-further diagnosis is necessary.

PROSPECTIVE LINES OF THERAPY

Currently, there is no effective therapy available to treat patients with progressive MTC, and surgery is the primary mode of treatment (107). Chemotherapy and radiation have only a palliative role without any provision for cure and therefore, search for alternate therapies for disseminated MTC is a hot area of research (84). One direction of cancer treatment is the use of immunostimulation where in tumour regression is achieved by inducing T-cell or antibody mediated ***cytotoxicity against tumour*** specific antigens (107). Induction of cellular immunity and a clinical response in two patients with parathyroid carcinoma and a neuroendocrine pancreas carcinoma were reported recently (108, 109).

The use of antigen encoding plasmid **DNA vaccines** is a new approach to elicit cellular and humoral anti-tumour immune responses (110). The advantage of DNA vaccines is the development of effective antigen-specific cellular and humoral immunity as the protein produced *in vivo* can enter the MHC class I and II pathways (111). In a recent experimental study in mice (112), it was shown that DNA immunization with human pre-pro CT expression plasmid results in the induction of antigen specific cellular and humoral immune response.

Another approach of active immuno therapy is the use of **autologous dendritic cells pulsed with tumour antigen** (113,114), which result in the formation of a cytotoxic immuno response leading to the rejection of established tumours like melanoma (115-117). A very recent study (118) reported the use of this technique in developing a immunotherapy for MTC in seven patients with the metastasized MTC. 47 patients had sporadic MTC and 37 had FMTC. These authors demonstrated for the fist time, the induction of a T-cell dependent immunity with DC vaccination using CT and / or CEA peptide pulsed DC. 37 patients showed clinical response and one showed significant regression of liver and pulmonary lessons.

Genetic immuno therapy involving the infection of murine MTC cells with an adenoviral vector harboring the mouse IL-2 gene abrogated tumorigenecity of cancer cells and induced a sustained state of immunity in syngeneic BALB/C mice (119). *In vivo* studies in mice showed rejection and /or stabilization of pre-established tumours after the injection of the adenoviral rector without any appreciable toxicity to other organs (120, 121). All patients had reduction in CT and CEA levels within a few months. These results give hope that immunotherapy for MTC is possible.

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