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CLINICAL, BIOLOGICAL, GENETIC AND IMUNO-HYSTOCHEMICAL PROGNOSTIC FACTORS IN THE MEDULLARY THYROIDIAN CARCINOMA

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RESUME

Le carcinome thyroïdien est le néoplasme le plus fréquent du système endocrinien, avec environ 10% des cancers thyroïdien représenté par le carcinome thyroïdien médullaire (MTC), qui peut en termes être sporadique ou familial. L'étude vise à conclure sur l'évolution du MTC, établissant les significations pronostiques des niveaux du CEA et de calcitonine, comme d'autres facteurs cliniques, biologiques ou génétiques, qui pourraient probablement être impliqués dans l'évolution de ces conditions, comme les réactions possibles que les patients peuvent avoir en ce qui concerne les thérapies. L'étude suit un modèle rétrospectif et a été effectuée sur les patients à l'institut national d'endocrinologie de « C.I.Parhon » qui ont été diagnostiqués avec CMT familial et sporadique par les examens histopathologiques, qui ont recueilli des données démographiques, des données sur fond médical/chirurgical des patients, les niveaux du CEA et de calcitonine, l'histoire des résultats du moment du diagnostic, l'histoire des examens de CT afin de déterminer le statut de la maladie, le verdict des répétitions ou de la métastase certaines aussi bien que leur taille, la détermination de l'état actuel de la maladie - du point de vue biologique et de langage figuré, aussi bien qu'une étude génétique.

MOTS CLES*: Le carcinome thyroïdien médullaire, MEN, calcitonine, RAT*

1. Introduction

The thyroidian carcinoma is the most frequent neoplasm of the endocrine system; most of the cases (*85-95%)* are well-differentiated papillary or follicular tumours, well-responsive to the treatment, the rest not being differentiated – either anaplastic or with a reserved diagnosis. The medullary thyroidian carcinoma (MTC) stands for around 10% of the well-differentiated thyroidian cancers, and it can be sporadic or familial, the latter being more wide-spread [1-3]:

-familial medullary carcinoma with no other associated endocrine affections (FMTC);

-MEN 2A associated with the MTC, pheochromocytoma and hyperparathyroidism;

- MEN 2B which combines the MTC with the pheochromocytoma and multiple mucous neuromas.

Therefore, the MTC is part of certain autosomally transmitted hereditary syndromes, characterised by a distinct phenotype and genotype. Moreover, compared to the MTC, half of the patients suffering from MEN 2A develop pheochromocytoma and one third develop hyperparathyroidism; the pheochromocytomas are often bilateral and seldomly malignant, while the hyperparathyroidism is caused by the hyperplasia of the parathyroid glands. The classic thyroidian lesion at the patients suffering from MEN 2 is the hyperplasia of the parafollicular cells producing the calcitonine, which can act as precursors of the meddulary thyroidian carcinoma. In patients diagnosed with MEN2, these tumours are usually multicentral and concentrated in the superior two thirds of the thyroid, thus reflecting the normal distribution of the parafollicular cells. MEN2 is associated with several phenotypical characters, such as: the cutaneous lichen amyloidosis, the intestinal ganglioneuromatosis, the presence of mucous neuromas and the marfanoid habitus [4-8].

The MTC tends to act more aggressively in the MEN2B cases (an earlier debut and a more rapid progression) than in the MEN2B or the sporadic ones. The aggressiveness of the MTC is even greater than the one of the papillary or follicular neoplasm, yet not as strong as the undifferentiated one. The MTC is the most frequent cause of death in patients suffering from MEN2 or FMTC, the tumour not being very responsive to the conventional radiotherapy doses and the standard chemotherapy regimes. These patients can only be cured by total thyroidectomy and only if the disease remains within the thyroid gland's surface, thus new treatment forms being required for the inoperable patients, that is the recurrent tumours or the metastatic MTC.

Because of the decisive role of the surgical resection in MTC patients, an early diagnosis of the disease is essential. The MTC has been associated with excessive levels of calcitonine – a polypeptidic hormone of 32 amino acids normally secreted by the parafollicular thyroidian cells. The calcitonine exerts its biological effects on three target organs: the bone, the kidneys and the gastro-intestinal tract; regarding the bone, it inhibits the osteoclastic resorbtion, concerning the kidneys, it increases the renal calcium excretion, inhibits the phosphorus resorbtion and increases the natriuresis; the abnormally high calcitonine levels are associated with the symptomatic diarrhea. The calcium and the pentagastrine are strong secretagogues of the calcitonine, determining a rise in its levels in a period of 30 to 300 seconds after the intravenous administration of the two combined agents. This is important due to the fact that in the post-thyroidectomy period, monitoring the calcitonine level is extremely relevant to the detection of the recurrent or residual MTC. Althought the thyroidectomy can prove to be curative, due to the incomplete surgical resection, the calcitonine levels can still appear high after the surgery. Also, the MTC can initially result in metastasis of the regional lymphatic ganglions and may also spread to more remote areas, such as the liver, lung or the bones.

The MTC cells have an increased biosynthesis activity, secreting various biogenic amines and carcino-embryonary antigen (CEA). Just as the calcitonine, the CEA is also progressively increasing following the direct association with the tumour's dimensions and can prove to be an unspecific tumoral marker. Limited studies have shown that patients with increased calcitonine and abnormal seric CEA levels are more prone experience a progression of the illness as well as metastasis, compared to the patients with normal CEA levels. These facts suggest that the CEA estimation can bring useful information when deciding on the prognostic of the patients affected by this condition. Due to the availability of the genetic tests, many carriers of the RET gene mutation in the MEN syndrome undergo the full thyroidectomy

before developing the medullary thyroidian carcinoma. However, the basal and stimulated calcitonine's plasmatic level is yet another useful index for the MTC screening and monitoring before and after the surgical intervention.

The RET gene, discovered by Takahashi in 1984, has 21 exons, covering more than 60 kb of the genomic DNA; it codifies a tyrosine kinasic receptor primarily expressed in the neural crest's cells, comprised of three domains:

- the extracellular one, involved in the intracellular signalling, which contains a region rich in cysteine, very important to the dimerziation of the receptor;
- the transmembranary one;
- the tyrosine kinasic intracellular one.

The MTC associates punctiform mutations of the RET proto-onctogene, both in the extracellular domain rich in cysteine and in the intracellular tyrosine kinasic one, displaying remarkable genotype-phenotype correlations, with progressive and therapeutic evolutions through the correlation between the type and the location of the mutation and the aggressiveness of the tumour or its response to the therapy. Concerning the connection between the structure and the function, the relevant fact is that the MEN2A mutations are limited to an extracellular cysteinic residues group, localised near the transmembranary domain, and can generate various amino acid substitutions; the rupture of the disulphide bonds between the cysteines results in damage to the proteic molecule in the absence of the ligand. Unlike the normal receptor, the mutant gene of MEN2A is able to trigger the malignant transformation when introduced in fibroblasts cultures, resulting that the mutant receptor acts in these families as an inherited dominant oncogene, yet has a reduced spontaneous dimerization and auto-phosphorilation potential. In the sporadic MTC form, the somatic mutations have the same location as the germinative ones have in the familial forms, yet there is insufficient data to prove that a patient suffering from the sporadic form of MTC displaying a RET mutation has a different prognostic than a MTC case without the RET mutation.

There are various mechanism through which the RET transformation determines the tumorigenesis – bringing cysteinic residues to the extracellular domain, this having an important role in the receptor's dimerization; the expression of a mutant RET receptor results from the dimerization in the absence of the ligand, combined with the activity of the signalising tracts;

- the second mechanism is based on the mutations of the tyrosine kinase domain, at the 918 codon level, resulting in the auto-phosphorilation of the kinase domain. The activation of RET by intragenic activator mutations determines the auto-phosphorilation of the tyrosine of the 1015 and 1062 codons, which will in terms lead to the activation of the transformation and effectory tracts.
- the third mechanism results from fusions of the regulative elements of the RET with other kinds of genes, most frequently from the thyroidian follicular cells, which will lead to the RET expression in the cells where it is no longer normally expressed, resulting in the pomotability of the dimerization.

There is already an international consensus regarding the total thyroidectomy made for a prophylactic reason at patients with RET gene mutations, due to the correlation between the affected codons and the clinical aggressiveness of the MTC proven by several studies, consequently describing three risk categories:

- the 1st category – very high risk – concerns the patients suffering mutations of the 883, 918 and 922 codons, usually found in MEN2B. Due to the fact that they can result in metastasis in children suffering from MTC from the age of 1, a total lymphatic dissection thyroidectomy is recommended in the first six months of life, preferably in the first month.

- the 2nd category – high risk - it concerns the patients suffering mutations of the 609, 611, 618, 620 and 634 codons, in which cases the thyroidectomy is recommended in the first 5 years of life.
- the 3rd category – intermediate risk – the mutations are found in the 768, 790, 791, 804 and 891codons and tend to produce less aggressive tumours; there is no consensus regarding the age when the prophylactic radical therapy would be advisable, yet most hypothesis range from 5 to 10 years. There is however a consensus regarding the necessity of a careful monitoring should the thyroidectomy not have been carried out at an early stage, by determining the basal calcitonine levels and running stimulative tests with Pentagastrine or calcium.

The sporadic form of the MTC, which is associated with 25%of the patients with somatic mutations of the RET proto-oncogene, ought not to be neglected either. Recent studies show that up to 6% of the patients with an apparently sporadic MTC have germinative mutations, concluding that all the MTC patients, no matter the form, must undergo tests in order to discover any eventual RET germinative mutations. This is of the utmost importance, due to the possibility of diagnosing the familial cases which were not suspected of carrying RET mutations, leading to a considerable increase in the early-diagnosis and prophylactic thyroidectomy curative therapy possibilities.

The negative family background does not clearly exclude a hereditary illness (the familial form of the TMC), thus the analysis of the germinative RET line being important in order to exclude any possible hereditary factor. Should the RET analysis prove to be negative, the hereditary possibility is 99% excluded, while the hypothesis of the families of the sporadic MTC patients having a hereditary illness is less than 1% probable.

Of all the MTC patients, at the time of the diagnosis, 80% are at the TNM II or III stage. The tests carried out on the mutational status of the RET proto-oncogene, especially on the MEN or FMTC patients led to an early diagnosis in the curable stage, reaching to the prophylactic thyroidectomy as early as possible after birth, though being dependent on the location of the mutation.

The studies show that the angiogenesis plays an important part in the tumor growth and in the extension of the of the blood flow in the tumour, being considered an independent prognostic indicator. In spite of the significant progress made lately in the use of chemotherapeutic agents in the treatment of solid tumours, many of them, including the MTC, still continue to be unresponsive to the most active chemotherapy combinations[9].

In the angiogenesis, the most important roles can be attributed to:

-VEGF — Vascular Endothelial Growth Factor;

-EGFR — Epidermal Growth Factor Receptor.

-FGF – Fibroblast Growth Factor.

VEGF is a pro angiogenetic factor which plays an important part in the tumour growth, stimulating the mitogeneis of the endothelial cells and increasing the vascular permeability [10].

The demonstration of the VEGF production in the thyroid carcinomas can prove to be an important marker of the tumoural aggressiveness and may be a useful predictor of the metastatic potential and the extension of the tumoural mass. VEGFR2 is a potential quantitative biomarker for the tumoural angiogenesis.

The over-expression of the VEGF and of its receptors, especially the VEGFR2, leads to a possible correlation with the tumoural growth rate, density and the microvascular proliferation, the tumour's metastatic potential and the pessimistic prognostic of the patient in a variety of malignities, possibly extendable to the thyroidian carcinoma.

EGFR belongs to the Erb B tyrosine kinasic receptor family; these transmembranar proteins are

activated consequent to the bonding of the peptidic growth factors of the EGF protein family. It has been suggested that EGFR is involved in the pathogenesis and progression of various types of carcinomas. The EGFR and the EGF-like peptides are often over-expressed in human carcinomas and both in vivo and in vitro studies have shown that these proteins are able to induce the cellular proliferation. The growth in the tumoural level of the EGFR appears to be associated with the local advanced or metastatic illness, resisting chemotherapy and the pessimistic prognostic at patients with carcinomas [11].

FGF has mitogenic, angiogenic and hormonal regulative functions. In particular, the FGF2 and its receptor, FGFR1, appear to be over-expressed in the human thyroidian carcinoma. The increase in the FGF2 expression has been associated with the distance and ganglionar metastasis; certain studies have suggested that the FGFR signaling may be an important element for the pharmacologic therapy of an inoperable MTC, however the data is so far insufficient. FGF may be a resistance marker in the VERGFR inhibitors therapy, the increased levels being associated with a pessimistic prognostic. The orientation towards the blood vessels, which sustain the tumour’s growth, may be regarded as a new perspective of the neoplastic treatment and the study of these growth factors may be a progress in the evaluation and observation of the patients suffering from medullary thyroidian carcinoma.

2. Patients and Methods

The study follows a retrospective pattern and has been carried out on patients at the “C.I.Parhon” National Endocrinology Institute who have been diagnosed with both familial and sporadic CMT by histopathological examinations, which gathered:

-Demographic data, the medical/surgical background of the patients.

-The determination of the CEA and calcitonine levels, the history of the results from the moment of the diagnosis; the history of the levels from the moment of the diagnosis.

-The history of the CT examinations in order to determine the status of the illness, the verdict of eventual recurrences or metastasis as well as their size.

-The determination of the current status of the illness – from both the biologic and imagery point of view, as well as a genetic study.

-The RET gene mutational analysis in a family of two sisters and their descendents (three children), diagnosed with MEN2 [12].

3. Results

1. The medullary thyroidian carcinoma type (figure 1):

- sporadic forms – 70% of the cases;
- familial forms – 30% of the cases, of which– 66% - MEN2;

– 34% familial MTC (FMTC).

The initial stage of the familial MTC has been diagnosed through:

- pheochromocytoma – 75% of the cases – bilateral suprarenal tumours.
- medullary thyroidian carcinoma – 25% of the cases.

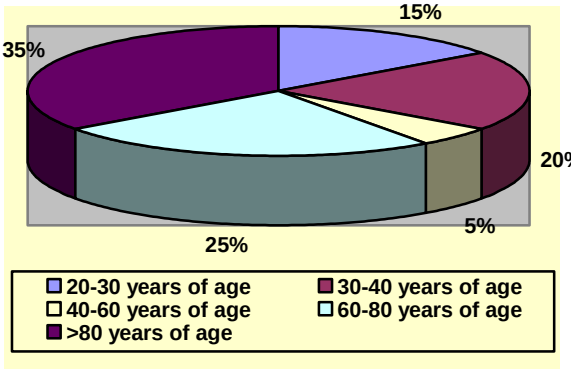

Figure 1. *At the time of the diagnosis, the age of the patients had the following distribution*

2. The period from the time of the diagnosis to the recurrence (in years):

- 1 to 5 years – 72% of the patients;
- 5 to 10 years – 16% of the patients;
- more than 10 years – 12% of the patients.

10% of the patients (with familial forms) had no local or distance recurrence due to the early diagnosis or had a shorter recurrence period.

3. Metastasis at the time of the evaluation:

- patients without metastasis – 75%;
- patients with metastasis – 25%
- of which – 42% – pulmonary metastasis.

-28% – hepatic metastasis.

– 15% – bone metastasis.

– 15% – multiple metastasis.

The period of time from the moment of the diagnosis until the first recurrences varies from a minimum of 1 year to a maximum of 15 years, while the histopathologic examination revealed vascular microinvasion in patients suffering from metastasis.

4. The initial tumour type:

- micro polynodular goiter – 80% of the cases;
- sole thyridian nodule – 20% of the cases.

The size of the thyridian nodule varies from 2 cm to 6.5 cm, while the size of the goiter varies from reduced to large with retrosternal extension.

5. At the time of the diagnosis, the patients had (figure 2):

- a MTC strictly limited to the thyroid , 55% of the patients;
- laterocervical adenopathies – 25% of the patients;
- mediastinal adenopathies – 20% of the patients.

6. At the time of the diagnosis, the calcitonine level was increased in all the patients, excepting one familial MTC patient, where the diagnosis was based on the calcium stimulation test, thus the calcitonine level increasing to nearly thrice the normal values.

At the time the postoperatory recurrences appear, the values of the previously low calcitonine increase again and tend to align to the initial values.

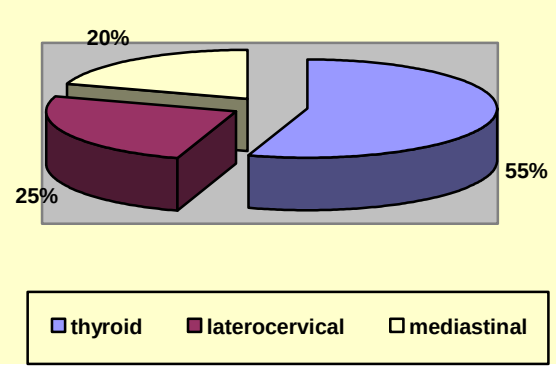

Figure 2. *Diagnosis of the patients*

The increase in the calcitonine levels can be considered to be significant, nonetheless at the time the recurrences appear, its level can be compared to the basic readings.

The CEA is significantly correlated to the moment of the tumour recurrence, when the levels are high in all the patients (at the time of the diagnosis, no patient has his CEA level investigated).

7. At the time of the diagnosis, all the patients had undergone a total thyroidectomy, further interventions to remove recurrences being needed in:

- 50% of the cases – 1 recurrence;
- 20% of the cases – 2 interventions;
- 30% of the cases – 2 interventions.

It can be observed that at the time of the evaluation, regardless of the total thyroidectomy, the patiens have at least cervical micro adenopathies.

All the patients having taken part in the study have been administered radio-iodine, generally right after the surgery (excepting 2 of the cases, who have been administered iodine 3 to 4 years after the surgery).

20% of the patients underwent chemotherapy in associations of various cytostatic agents.

The calcitonine and the CEA are not influenced by the radioactive iodine and the chemotherapy agents, their levels remaining high throughout the therapy. Generally, a decrease of these levels has been observed after the surgery – post-

thyroidectomy or lymphadenectomy in case of recurrences

The genetic analysis of the RET proto-oncogene in the MEN2 syndrome family:

In order to mutationally analyze the RET gene, we have proceeded to the extraction of DNA from the blood of 5 subjects collaborating with the faculty of the Biochemistry Department of the UMF "Carol Davila" Medical School, using a Promega protection kit.

Following the DNA extraction, the study went on to the preparation phase, in order to be able to start the RET gene analysis. The DNA has been purified and undergone a chain polymerization reaction in order to amplify several fragments belonging to the 10 and 11 exons, using specific polymers (table 1).

Table 1. *Primer oligonucleotides utilized (Bio Basic Inc.):*

Exon	Primer direction	Primer opposite direction
Exon 10	5'-GCCTATGCTTGCGACACCAGTTG-3'	5'-GATGTGCTGTTGAGACCTCTGTG-3'
Exon 11	5'-CATGAGGCAGAGCATACGCA- 3'	5'-GACAGCAGCACCGAGACGAT- 3'

The PCR conditions have been the following: the initial 10 minutes cycle at 95°C come is preceded by 30 cycles at 95°C – 60 sec, 64° C – 60 sec (for the 10 exon) and 62 °C – 30 sec (for the 11 exon), 72 °C – 30 sec and in the end, 7 minutes at 72°C; the device used was a BioRad thermocycler. The primers used allowed the obtaining of a 300bp product for the 10 exon and 161 bp for the 11 exon (figure 3).

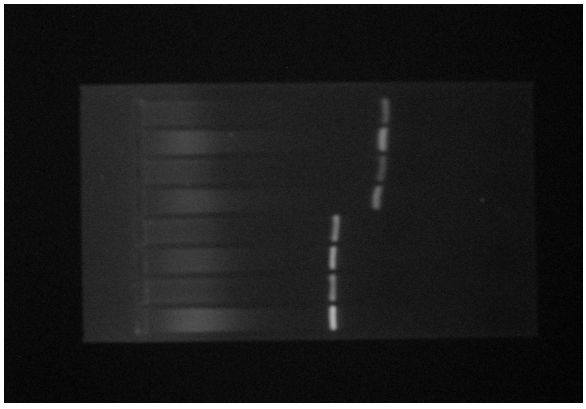

Figure 3. *The result of the aragose gel migration of the amplification product of the 10th (superior) and 11th (inferior) exons in 4 patients*

The final product undergone an electrophoretic migration, along with the 25 bp DNA ladder, at 80 mV in an agarose gel environment of 1,5% (agarose 1,5 mg + TBE 100 mL + ethidium bromate 100 μL) for a 30 minutes period, being visualized with an UV transiluminator and photographed.

In the end, the fragments have been sequentially distributed to the five patients in order to highlight the specific mutation.

The *DNA sequencing* has been carried out within the Molecular Biology Multiple Users Research Facility of the Biology College (the faculty being led by Prof. Dr. Marieta Costache), the statement of work being the usual one of the laboratory (Cătălina Luca, biochemist). A Big Dye R Terminator v 3.1 cycle sequencing kit has been used, the device being an ABI Prism 310 Genetic Analyser type sequenciator, while the treatment of the data has been carried out with the Sequencing Analysis 5.1 programme. This made the visualisation of the patients' DNA sequences possible, their comparison to the normal gene corresponding fragment in order to trace the punctiform mutations, using the BioEditor programme which allowed the alignment.

The sequencing of the amplification products of the 10th and 11th exons carried out on the five

subjects revealed the presence of a punctiform mutation at the 634 codon of the 11 exon in all the five cases. Once the genes responsible for triggering MEN have been identified, specific mutations also triggering the disease have been discovered in many MEN2 families. Without underestimating the importance of the other factors (ethnic background, the environment), most of the scientists agree that it is also important to stress upon the potential benefit of the patients, the families and the entire society derived from the identification of the carriers of these mutations.

The biochemical screening is accompanied by the falsely-positive results (which can lead to abusive thyroidectomies), but also by falsely-negative results, which can increase the risk concerning the patient. Moreover, following the identification of the specific mutations of the illness, the screening of the other members is somewhat easy, because the target is known and therefore simple techniques can be applied. The screening must be initiated at an earlier age than the one reported as being the debut of the syndrome's symptoms. Nonetheless, some authors suggest that concerning the MEN2 and the FMTC cases, the patients must undergo the thyroidectomy surgery until the age of five, especially in case of a MEN 2B, where the MTC is more aggressive and appears at a much earlier age. The advantage of the genetic screening is thus obvious: concerning the MEN2 and FMTC cases, the prophylactic thyroidectomy prevents the methastasis and therefore eliminates the patient's main pain source. Regarding the other manifestations of the MEN2, periodic monitoring can prevent any severe clinical manifestation.

4. Conclusions

The study revealed positive correlations between the calcitonine's value, the one of the CEA and the presence of the MTC diagnosis, as well as the presence of the recurrence and metastasis. The most frequent metastasis are usually discovered at the cervical and mediastinal lympho-ganglions, as well as the more distant ones at the hepatic and pulmonary level, especially in patients with vascular microinvasion at the histopathological exam. The tumour is relatively unresponsive to the conventional radiotherapy doses and to the standard chemotherapy regimes, the majority of the patients being at present at the metastatic or advanced local MTC stage.

These patients can only be cured if they undergo a total thyroidectomy and only if the illness is strictly located at the thyroid gland's level, thus new forms of treatments being required for the patients that cannot undergo a surgical resection, suffering from recurrent tumours or MTC metastasis. Due to the important part of the surgical resection in MTC subjects, an early diagnosis of the illness is crucial.

Therefore, the determination of the mutational status of the RET proto-oncogene is highly important, both for the early diagnosis of the affection and for the evaluation of the evolution, the prognostic of the illness and last but not least when considering the eventual responses to the therapy oriented towards this stage.

The studies show that the angiogenesis plays an important part in the tumour's growth and in the extension of the of the blood flow in the tumour, being considered an independent prognostic indicator. In spite of the significant progress made lately in the use of chemotherapeutic agents in the treatment of solid tumours, many of them, including the MTC, still continue to be unresponsive to the most active chemotherapy combinations.

This particular thyroidian carcinoma type being nonresponsive to the standard radiotherapy and chemotherapy regimes lead to an urgent need of new therapeutic forms, being comprised of inhibiting

tyrosine-kinasic agents targeting the RET and the tumoural angiogenesis – PKI 166, AEE 788, ZD6474 – which act on the RET and VEGFR (vascular endothelial growth factor receptor), AMG 706 – which acts on the RET, VEGFR and EGFR (epidermal growth factor receptor) and Kit.

Although the studies are only at the beginning, they have already shown two important aspects:

- the 804 position’s Valine is essential to the RET bond – agent, while the Valine’s mutations at this position result in resistance to the treatment;

- the metionine/ leucine are associated with the resistance to the therapy with inhibating tyrosin-kinasic agents, while the Glicine is associated with an increased susceptibility.

This is yet another proof of the necessity of the RET protooncogene’s analysis, in the appreciation of the response to the new therapeutic agents types still being tested for their clinical effectiveness.

Discovering the genetic defect at the offspring of a patient diagnosed with the MEN syndrome is only made possible by the prophylactic intervention – that is, the thyroidectomy – as to prevent the formation of the medullary thyroidian cancer. The partial prevention of this type of cancer is therefore achieved, by modifying the natural history of the disease and improving the abnormal gene carriers’ life quality.

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