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Mestrado e Doutorado

Carcinoma Medular de Tireóide Hereditário:

Aspectos Moleculares, Clínicos e Oncológicos

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Orientadora: Profa. Dra. Ana Luiza Maia

Porto Alegre, Dezembro de 2005

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Tese apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, para obtenção do título de Doutor

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Capítulo I

Carcinoma Medular de Tireóide:

Aspectos Moleculares, Clínico-Oncológicos e Terapêuticos

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Sinopse

O Carcinoma Medular de Tireóide (CMT) pode ocorrer na forma esporádica ou na forma familiar. O CMT hereditário é parte das síndromes de Neoplasia Endócrina Múltipla (NEM) 2A e 2B, Carcinoma Medular de Tireóide Familiar (CMTF) ou outras formas. Mutações de linhagem germinativa do proto-oncogene *RET* causam a forma hereditária da neoplasia e os testes genéticos atualmente disponíveis formam a base para o manejo adequado da hereditariedade do tumor visto que o diagnóstico precoce melhora significativamente o prognóstico no indivíduo afetado e nos carreadores. O diagnóstico molecular do carcinoma medular de tireóide foi implementado no Serviço de Endocrinologia do Hospital de Clínicas de Porto Alegre em 1997, e desde então tivemos a oportunidade de analisar diferentes famílias com um grande número de afetados. Nós observamos uma grande variabilidade na apresentação clínica, mesmo entre indivíduos da mesma família. No presente artigo, revisamos os avanços nos mecanismos moleculares, diagnóstico e tratamento, bem como relatamos a nossa experiência no manejo dessa forma rara de neoplasia tireoidiana.

Unitermos: CMT, proto-oncogene *RET*, MEN 2A, MEN 2B, CMTF

Summary

Medullary Carcinoma of the Thyroid (MTC) may be sporadic or may occur on a hereditary basis. Hereditary MTC can occur either alone – familial MTC (FMTC) – or as the thyroid manifestation of multiple endocrine neoplasia type 2 (MEN 2) syndromes (MEN 2A and MEN 2B) or others. Germline mutations in *RET* cause MEN 2 and genetic testing now available forms the basis for MTC screening procedures. Our group established a protocol for molecular analysis of hereditary MTC in 1997 and since then we have had the opportunity to study large kindred with this disease. We observed a wide spectrum in clinical presentation and natural course of the disease even among genetically-related individuals. Here we described the recent advances in understanding the molecular mechanisms, diagnose and treatment of this rare form of thyroid cancer.

Keywords: MTC, *RET* proto-oncogene, NEM 2A, NEM 2B, FMTC

Introdução

O Carcinoma Medular de Tireóide (CMT) é uma neoplasia das células C ou parafoliculares da tireóide, correspondendo 5 – 8% dos tumores malignos da glândula. O CMT apresenta-se como tumor esporádico (75-80%) ou na forma hereditária (20-25%) (1). Na forma familiar é um dos componentes de uma síndrome genética de herança autossômica dominante, apresentando-se isoladamente, na forma de Carcinoma Medular de Tireóide Familiar (CMTF) ou como um dos componentes das síndromes de Neoplasia Endócrina Múltipla (NEM) 2A ou 2B ou outras formas hereditárias (2,3).

O proto-oncogene *RET* é o responsável pela forma hereditária da neoplasia (4). Os testes moleculares, atualmente disponíveis formam a base para o manejo adequado da hereditariedade do tumor, pois o diagnóstico e, consequentemente o tratamento precoce melhoram significativamente o prognóstico no indivíduo afetado e nos carreadores assintomáticos (5).

O diagnóstico molecular do carcinoma medular de tireóide foi implementado no Serviço de Endocrinologia do Hospital de Clínicas de Porto Alegre em 1997, e desde então o nosso serviço se tornou Centro de Referência para o screening genético desse carcinoma. No presente artigo, revisamos os avanços nos mecanismos moleculares, diagnóstico e tratamento, bem como relatamos a nossa experiência no manejo dessa forma rara de neoplasia tireoidiana.

Epidemiologia, Classificação e Apresentação Clínica

O CMT é responsável por 5 a 8% das neoplasias malignas da tireóide, sendo mais freqüente na forma esporádica (75-80%) do que na hereditária (20-25%) (1).

Na forma hereditária apresenta-se como um dos componentes das síndromes clínicas de neoplasia endócrina múltipla tipo 2 (NEM2), sub-classificada como Neoplasia Endócrina Múltipla Tipo 2A (NEM 2A), 2B (NEM 2B), Carcinoma Medular de Tireóide Familiar (CMTF) e outras formas hereditárias (2,3) (Tabela 1).

Carcinoma Medular Esporádico

Na forma esporádica, o CMT se apresenta como um tumor unifocal e unilateral, cujo diagnóstico ocorre na quinta ou sexta décadas de vida (5). Clinicamente, o tumor se caracteriza como nódulo único ou massa tireoidiana associada à linfadenopatia cervical ou a outros sintomas locais. Raramente pode estar associado a diarréia, rubor ou doença metastática (1,5).

Carcinoma Medular Hereditário

O CMT hereditário manifesta-se clinicamente como um nódulo ou massa cervical e, freqüentemente, os pacientes já apresentam comprometimento em linfonodos cervicais ao diagnóstico. As metástases à distância e os sintomas paraneoplásicos são eventos mais tardios na doença (5,6). O CMT hereditário é usualmente precedido por hiperplasia celular e apresenta com maior freqüência uma distribuição multifocal e multicêntrica. O pico de incidência ocorre na terceira e quarta décadas de vida nas formas de NEM 2A e CMTF e mais precocemente na NEM 2B, sendo diagnosticado na infância (2,3,6).

A síndrome genética NEM 2A se caracteriza por CMT (95%), feocromocitoma (30 – 50%) e hiperparatireoidismo (10 – 20%) (2,3). A doença adrenomedular é

usualmente multicêntrica e bilateral, geralmente detectada após o aparecimento de CMT e com taxa de malignidade inferior a 10% (2,3,6,7). O hiperparatireoidismo ocorre em aproximadamente 10 a 20% dos indivíduos com NEM 2A, acometendo geralmente todas as glândulas paratireóides (2,3,6). A lesão histológica mais comumente observada nos estágios iniciais da doença é a hiperplasia da glândula, porém se a doença é diagnosticada mais tardiamente a lesão adenomatosa se superpõe à hiperplasia (1,6). A síndrome NEM 2A foi subdivida em três subtipos fenotípicos, baseando-se na apresentação clínica (tabela 1): a) NEM 2A (1), que consiste nos indivíduos que apresentam os três componentes da síndrome (CMT, feocromocitoma e hiperparatireoidismo); b) NEM 2A (2), que incluí indivíduos que apresentam CMT e feocromocitoma, sem hiperparatireoidismo; c) NEM 2A (3), que está relacionado a indivíduos com CMT e hiperparatireoidismo, sem feocromocitoma (2,3). Outras associações raras da NEM 2A incluem a associação com uma lesão pruriginosa da região escapular caracterizada pela deposição de amilóide, conhecida como líquen amilóide cutâneo (CLA) e a doença de Hirschsprung (8-10).

A síndrome NEM 2B caracteriza-se por CMT (90%), feocromocitoma (45%), ganglioneuromatose (100%) e hábitos marfanóides (65%) (2,3). Essa síndrome caracteriza-se por um fenótipo único que incluí ganglioneuromatose difusa da língua, lábios, olhos e do trato gastrointestinal (2,3,11). As fácies características são precocemente reconhecidas durante a infância (neuromas da mucosa) (2,3,11). O envolvimento gastrointestinal pode causar diarréia e constipação intermitente, dor abdominal, megácolon e ocasionalmente obstrução intestinal (2,3,11). Outro aspecto fenotípico da NEM 2B é o hábito marfanóide com dedos e extremidades longas, hiperextensão de articulações e anormalidades epifisárias (2,3,11).

O CMTF consiste na presença de CMT isolado em pelo menos quatro membros da mesma família e as outras formas de CMT hereditário, consistem no acometimento de dois ou três membros da mesma família com CMT, sem a presença de feocromocitoma ou hiperparatireoidismo (2,3).

Aspectos Bioquímicos

O CMT é um tumor cujas células C produzem uma variedade grande de substâncias, incluindo: calcitonina (CT), *calcitonin gene-related peptide* (CGRP), antígeno carcinoembrionário (CEA), amilóide, somatostatina, hormônio adrenocorticotrófico (ACTH), peptídeo intestinal vasoativo (VIP), prostaglandinas, serotonina e outras (12,13). A CT é o marcador mais importante sendo utilizado na detecção, no manejo pós-cirúrgico dos indivíduos com CMT e na avaliação de indivíduos afetados ou com risco de apresentar a doença. Visto que alguns indivíduos apresentam níveis normais de CT, as vezes são necessários testes provocativos para avaliar a sua secreção (12,13). Os testes de estímulo podem ser realizados com a infusão de cálcio ou pentagastrina e mais recentemente com omeprazole (12-14). Esses testes apresentam algumas dificuldades de realização, bem como uma baixa especificidade e sensibilidade (falso-positivos e falso-negativos podem ser observados de 5 a 18% dos casos) (5,6,12). Além da CT, outras substâncias podem ser avaliadas nestes pacientes como o CEA e o CGRP plasmático.

Aspectos Moleculares

Gene envolvido e Mutações

Em 1970, iniciaram os primeiros estudos para identificação da mutação genética causadora do CMT (14-16). No entanto somente em 1993 foi identificado o proto-oncogene *RET* como o gene causador da neoplasia (4). O proto-oncogene *RET* apresenta 21 exons e codifica um receptor tirosino-quinase expresso nas células derivadas da crista neural, incluindo tumores neuroendócrinos originados dessas células (17). A proteína RET é constituída por 3 domínios: um domínio extracelular que contêm o peptídeo sinalizador com regiões *cadherin-like* e regiões ricas em cisteínas; um domínio transmembrana e uma porção intracelular contendo dois domínios tirosino-quinase (TK1 e TK2) (Figura 1) (18,19).

O ligante do RET foi identificado em 1996, um peptídeo da superfamília do TGF-β (*transforming growth factor*), denominado *glial neurotrophic derived factor* (GNDF), atuando via receptores α-GDNF (20). O GNDF-α acoplado ao seu receptor específico liga-se à porção extracelular do RET, causando a dimerização do receptor com posterior autofosforilação dos resíduos tirosina-quinase, liberando fosfato, um substrato importante na cadeia do crescimento e diferenciação celular. Mutações no gene determinam uma ativação permanente do RET desencadeando o processo neoplásico (21-23).

Mutações do tipo *missense* originárias da linhagem germinativa celular são responsáveis pelo carcinoma medular de tireóide hereditário. Os exons mais comumente afetados são os exons 10, 11 e 16, no entanto, mutações nos exons 13, 14 e 15 também foram descritas (24-29) (tabela 1). As mutações mais

frequentemente encontradas no CMTF e NEM 2A ocorrem nos resíduos de cisteína do exon 10 (códon 609, 611, 618, 620) e do exon 11 (códon 634) (24-27). Nos pacientes com CMTF as mutações estão distribuídas homogeneamente entre os códons 618, 620 e 634, ao contrário dos pacientes com NEM 2A, cuja mutação mais comum ocorre no códon 634 (24-27). Uma mutação específica no códon 918 (M918T), exon 16, está associada a 95% dos casos de NEM 2B (29). Recentemente foi identificada uma nova mutação no exon 8 (1597G→T) correspondendo a uma substituição glicina → cisteína no domínio extracelular do RET associado a CMTF (30).

Correlações Clínicas e Moleculares

Nos últimos anos diferentes estudos têm sido realizados com o objetivo de avaliar possíveis correlações entre mutações específicas e as diferentes apresentações clínicas (2,3). Diferenças na intensidade da indução da dimerização do receptor constitui uma explicação razoável na determinação dos diferentes fenótipos resultantes de mutações nas diferentes cisteínas. De fato, em estudo multicêntrico de mutações no *RET* que avaliou 477 famílias com NEM 2, observou-se que mutações códon-específica do *RET* se correlacionavam com os diferentes os fenótipos da NEM 2 (2). Mutações no códon 634, por exemplo, foram associadas à presença de feocromocitoma e hiperparatireoidismo, sendo que o tipo de mutação que ocorre de modo mais freqüente na NEM 2A, C634R, não foi detectada em nenhum caso de CMTF. Mutações nos códons 768 e 804 foram identificadas unicamente em casos de CMTF e no códon 918 especificamente na NEM 2B (2). Nesse estudo, a síndrome de NEM 2A foi a mais freqüente e o CMTF foi

diagnosticado somente em 10% dos casos. No entanto, em um estudo similar francês a prevalência de CMTF foi de aproximadamente 60% (31), sugerindo que freqüência de determinadas mutações pode variar de acordo com o *background* genético. A nossa casuística indica que a maioria das famílias brasileiras afetadas apresenta o fenótipo 2A (32).

Alguns autores têm sugerido uma classificação de risco de acordo com a localização das mutações, sendo que os códons 634 e 618 seriam considerados de elevado risco de transformação neoplásica, os códons 790, 620 e 611 de risco intermediário e os códons 804 e 768 de baixo risco de malignidade (6). Outros estudos, no entanto, têm chamado a atenção para a ampla variabilidade clínica e agressividade tumoral associadas a mutações no *RET* em códons classicamente descritos como de baixa atividade (ex. 804), indicando que mutações idênticas podem se comportar de modo diferente em um grupo com mesmo *background* genético (33-36). Em estudo recente realizado em nosso serviço observamos que pacientes com mutações no códon 634, consideradas de alto risco, também apresentam uma grande heterogeneidade clínica da NEM 2A (32). Nesse estudo também observamos que indivíduos com a mutação C634R apresentavam significativamente mais metástases a distância que indivíduos com o genótipo C634Y, sugerindo que trocas específicas de nucleotídeos nesse códon podem alterar a evolução natural da doença na NEM 2A. Visto que a disfunção do gene está presente desde o nascimento, ou seja, os indivíduos nascem com essa alteração genética, assumimos que a idade do indivíduo ao diagnóstico indicaria o período de exposição. A análise através de curvas de Kaplan-Meier quanto à presença de metástases locais e à distância ao diagnóstico, comparando a troca de aminoácido C634R e C634Y, demonstrou uma diferença significativa entre os 2

genótipos (Figura 2) (32). No entanto, recentemente um estudo multicêntrico avaliando apenas carreadores idade inferior a 20 anos não encontrou diferenças na progressão da hiperplasia das células C para carcinoma medular entre as diferentes trocas de aminoácidos no códon 634 (3).

Um outro aspecto interessante descrito é o fenômeno denominado "antecipação" da doença, ou seja, o aparecimento do CMT em indivíduos cada vez mais jovens através das gerações, sugerindo a participação de outros eventos moleculares no inicio do processo neoplásico. De fato, embora as mutações no *RET* estejam diretamente implicadas no processo neoplásico na NEM 2, o motivo pelo qual apenas um pequeno grupo celular no órgão afetado adquire o potencial oncogênico ainda não foi elucidado (37). Outros mecanismos moleculares como a trissomia do cromossoma 10 com duplicação do alelo mutante *RET* ou perda do alelo *wild-type* têm sido sugeridos como co-responsáveis (38,39). Recentemente, rearranjos do *RET* através de translocações, inversões ou alterações genômicas extensas, com aumento na expressão do *RET* mutante também foi associado ao processo neoplásico no CMT (40). Outros estudos sugerem a associação de determinados polimorfismos como G691S (exon 11) e S904S (TCC-TCG, exon 15), ao diagnóstico mais precoce do carcinoma hereditário (41).

Carcinoma Medular Esporádico

Os processos moleculares envolvidos na etiologia do carcinoma medular de tireóide esporádico permanecem pouco compreendidos. Cerca de 50% dos CMT esporádicos apresentam a mutação somática M918T (42-46). Esta mutação não parece ser uniforme entre as várias subpopulações de células dentro de um mesmo

tumor ou das metástases, sugerindo que o CMT esporádico possa ter uma origem policlonal ou que as mutações do proto-oncogene *RET* não sejam eventos iniciais na tumorigênese do carcinoma (26,43).

Polimorfismos (variações genômicas que ocorrem em mais de 1% da população) do *RET* foram identificados em pacientes com CMT esporádico e doença de Hirschsprung (39,47-49). Gimm e cols. investigando variações genéticas que levassem ao CMT esporádico, encontraram uma frequência significativamente maior do polimorfismo no códon 836 (S836S; AGC/AGT) nos pacientes com CMT esporádico com a mutação somática M918T comparada à população controle (47). Mais tarde, Ruiz e colbs. confirmaram estes achados na população de origem espanhola com CMT, encontrando um risco 2 a 3 vezes maior da neoplasia quando a sequência variante S836S estava presente (48). Borrego e cols. observaram nos pacientes com doença de Hirschsprung uma frequência maior dos polimorfismos A45A e L769L comparada à população normal (49). Em adição, Wiench e cols. observaram que o polimorfismo L769L era mais frequente nos pacientes jovens (< 30 anos) com CMT esporádico do que em paciente mais idosos (36 vs. 15%, respectivamente), entretanto a relevância dos resultados para esta população não foi determinada visto que o estudo não avaliou a frequência em um grupo de indivíduos controle (40).

Apesar da série de estudos demonstrando a associação de tumores com mutações somáticas do proto-oncogene *RET*, a dúvida quanto à gênese tumoral permanece já que a mutação ocorre em apenas uma parcela dos casos. Embora o CMT hereditário tenha os mecanismos moleculares bem definidos, o diagnóstico do câncer parece ocorrer em idades mais precoces a cada geração, sugerindo que

fatores ambientais ou uma segunda alteração genética possam estar envolvidos com este processo.

Rastreamento

A aplicação do *screening* genético para o manejo adequado da hereditariedade do CMT possibilita o diagnóstico precoce e é de fundamental importância, já que determina a conduta terapêutica e o prognóstico da doença no indivíduo afetado e em seus familiares. Além disso, apresenta baixo custo e não possuí efeitos colaterais como os observados com os testes provocativos como elevado índice de falso-positivo e falso-negativo.

Um estudo comparativo entre o *screening* clínico e a análise de DNA em famílias com NEM 2 concluiu que a diagnóstico molecular é superior na identificação dos indivíduos carreadores e em risco para o desenvolvimento da síndrome (13). O teste genético deve ser indicado em indivíduos afetados com a neoplasia, independente da idade ao diagnóstico. Em caso de identificação da mutação os ascendentes e descendentes diretos desse indivíduo devem ser analisados. Os indivíduos *RET* negativos estão dispensados do acompanhamento médico, não sendo necessário realizar *screening* para feocromocitoma e/ou hiperparatireoidismo. Nos indivíduos testados positivamente para mutações no *RET*, está indicado a tireoidectomia total (vide abaixo) e a avaliação bioquímica para o feocromocitoma e hiperparatireoidismo.

No nosso Serviço foram detectadas mutações em 28 indivíduos carreadores assintomáticos no total de 184 indivíduos analisados no período 1997-2003, sendo identificadas mutações em todos aqueles com diagnóstico clínico e histopatológico

de CMT. A avaliação molecular é indicada também nos casos de CMT esporádico, no sentido de excluir doença familiar, já que, segundo alguns relatos, o CMT hereditário pode existir em contexto aparentemente esporádico. De fato, dos 17 probandos identificados no nosso Serviço, três (18%) foram encaminhados como portadores de carcinoma esporádico. Esses casos ilustram a necessidade do rastreamento molecular nos casos de CMT aparentemente esporádico, confirmando dados da literatura que demonstram que a análise genética pode identificar mutações em até 25% dos casos esporádicos.

Aspectos Terapêuticos

Cirurgia:

A cirurgia é o procedimento de escolha no tratamento das doenças relacionadas à NEM 2A. A possibilidade de cura do carcinoma medular de tireóide, única neoplasia maligna da síndrome, depende principalmente do estadio tumoral ao diagnóstico e da ressecção completa do tumor.

Carcinoma Medular de Tireóide: O tratamento primário recomendado é a tireoidectomia total com dissecção dos linfonodos cervicais, compartimento central (nível VI e VII) e cadeias cervicais bilaterais (níveis II, III, IV, V) (50,51). Os linfonodos quando abordados de maneira meticulosa elevam as taxas de cura bioquímica melhorando o prognóstico (52).

A recorrência da doença, ou seja, uma elevação nos níveis de calcitonina, é um problema freqüente no acompanhamento destes pacientes. O quadro clínico

associado é o que melhor define a conduta nestes casos: (1) pacientes sintomáticos ou com doença cervical progressiva mas sem evidências de metástases à distância são candidatos a um novo procedimento cirúrgico; (2) pacientes com curso indolente da doença, tratamento cirúrgico inicial adequado e métodos de imagem negativos podem ser acompanhados de maneira conservadora (53).

Os familiares de pacientes com NEM 2 e carreadores da mutação devem realizar tireoidectomia total com exploração da região cervical. No entanto, a linfadenectomia do compartimento central não é consenso. O procedimento deve ser indicado o mais precocemente possível na NEM 2B, sendo recomendado antes dos 6 meses de vida (54). Nos indivíduos com NEM 2A, a indicação da tireoidectomia depende do tipo de mutação (códon/nucleotídeo). Os indivíduos carreadores de mutações nos códons 634 e 618, consideradas mais agressivas e diagnosticadas mais precocemente, devem ser tireoidectomizados entre 5 - 7 anos (6,54-57). Para mutações de risco intermediário (códons 611, 620 e 790) o procedimento é indicado antes dos 14 anos, enquanto para as de baixo risco (códons 768 e 804) antes dos 20 anos de idade (6,54-57). Para o tratamento profilático, consideramos também o tipo de troca de nucleotídeo ocorrida no códon 634, já que determinadas substituições de aminoácidos podem determinar uma alteração no curso da doença (32).

Feocromocitoma: A adrenalectomia bilateral é o procedimento mais recomendado, porém existem algumas divergências quanto à conduta cirúrgica mais adequada em pacientes com feocromocitoma associado à NEM tipo 2 (54,58,59). Alguns autores preconizam a adrenalectomia bilateral devido ao elevado número de recorrências (5). No entanto, a adrenalectomia unilateral diminui a necessidade e o

tempo de reposição de corticosteróides. Uma nova abordagem terapêutica é a ressecção da medula com preservação do córtex adrenal, com resultados promissores (60).

Hiperparatireoidismo: No hiperparatireoidismo associada a NEM 2A geralmente ocorre um acometimento difuso das paratireóides (5). Não existe consenso quanto à melhor técnica cirúrgica nestes casos. Os procedimentos freqüentemente empregados são a paratireoidectomia total com autotransplante, a paratireoidectomia subtotal preservando uma parte bem vascularizada de uma das glândulas *in situ* ou a ressecção de uma única paratireóide (61).

Outros Tratamentos

Quimioterapia:

A quimioterapia apresenta resultados limitados no tratamento do carcinoma medular de tireóide. Os estudos descritos na literatura, séries de casos, mostram que os agentes quimioterápicos não alteram a sobrevida destes pacientes (62). Os melhores resultados são descritos em termos de estabilização de doença, geralmente durante períodos curtos, ou de resposta parcial, com taxas em torno de 15 a 30% (63-65). Desse modo a quimioterapia tem sido recomendada, com restrições, a poucos pacientes com doença metastática rapidamente progressiva. Uma variedade de drogas já foi utilizada nos protocolos de tratamento, incluindo

doxorrubicina, cisplatina, ciclofosfamida, bleomicina, vincristina, paclitaxel, 5-fluorouracil e dacarbazina (63-65).

Radioterapia:

A resposta ao tratamento com radioterapia externa também é considerada insatisfatória nos pacientes com carcinoma medular de tireóide. Alguns indivíduos com tumores inoperáveis, especialmente aqueles com metástases ósseas, podem se beneficiar com o tratamento radioterápico (66). Recentemente, Brierley e colbs. avaliando pacientes de alto risco (doença residual microscópica, envolvimento de linfonodos ou invasão extra-glandular) observaram uma menor freqüência de recidiva local entre os tratados quando comparados com os não tratados com radioterapia no pós-operatório (67). Possíveis complicações da radioterapia externa sobre a região cervical incluem fibrose cervical, traqueíte actínica, disfagia crônica e paraplegia (5).

Radiofármacos:

O emprego de ^{131}I MIBG e ^{90}Y-DOTA-D-Phe[1]-Tyr[3]-octreotídeo no tratamento do carcinoma medular de tireóide tem demonstrado efeitos limitados (62,68). A radioimunoterapia é uma nova modalidade terapêutica, na qual anticorpos monoclonais anti-antígeno carcinoembriônico são utilizados para o tratamento do CMT (68,69). Em um estudo de fase I, delineado para avaliar toxicidade de doses

escalonadas da droga, doze pacientes receberam ^{131}I anti-CEA Mab, sendo a remissão parcial observada apenas em 1 e estabilização da doença em 10 pacientes (69).

Modificadores de resposta biológica:

O octreotídeo e o α-interferon são utilizados em pacientes com doença metastática avançada com o objetivo de reduzir os níveis de calcitonina e melhorar os sintomas relacionados aos níveis elevados do hormônio, tais como o rubor e a diarréia. Não foram observadas alterações no tamanho tumoral com o uso destas drogas (62).

Terapia gênica:

Ainda em fase experimental com modelos animais, a terapia gênica abre uma perspectiva promissora para o tratamento do CMT (70). Distintas abordagens têm sido utilizadas: introdução de genes supressores tumorais; transferência de genes que determinam a ativação de drogas para formas tóxicas (genes suicidas); transferência de genes que aumentam a resposta imunológica contra o câncer (imunização gênica) e terapias combinadas (70-72).

Seguimento

As dosagens séricas de calcitonina e antígeno carcinoembrionário devem ser obtidas em torno de 2 meses após a tireoidectomia, devido à meia-vida longa destes marcadores na circulação sanguínea (52). Os níveis normais de calcitonina são excelentes indicadores de uma ressecção curativa, enquanto que níveis elevados desse marcador indicam a necessidade do rastreamento de metástases (6,12).

A ultrassonografia / tomografia computadorizada está indicada quando existir suspeita de recidiva cervical. Disseminação local e metástases à distância podem ser avaliadas por tomografia computadorizada ou ressonância nuclear magnética. Cintilografia é recomendada na investigação de metástases ósseas. Uma variedade de radioisótopos também são empregados, incluindo ^{131}I-MIBG, ^{111}In-octreotídeo, ^{99}Tc-DMSA, ^{131}I anti-CEA e anti-calcitonina (52,62). No entanto, nenhum dos exames de imagem tem demonstrado sensibilidade na localização da doença oculta.

As técnicas diagnósticas mais invasivas parecem apresentar melhores resultados. Dosagens de calcitonina obtidas através de cateterização seletiva são úteis para orientar a remoção do tecido tumoral oculto (62). As micro-metástases hepáticas têm sido demonstradas pela laparoscopia em muitos pacientes que apresentam tomografia computadorizada e ressonância nuclear magnética normais (53).

Os pacientes com NEM 2A devem ser investigados periodicamente para doença adrenomedular e hiperparatireoidismo. Em nosso serviço, estes pacientes além da dosagem de calcitonina e antígeno carcinoembriônico semestral, são avaliados para o hiperparatireoidismo através das determinações séricas do cálcio e PTH anualmente. Determinações das metanefrinas / catecolaminas urinárias, assim

como tomografia computadorizada de tórax, abdome e região cervical são realizadas anualmente. O rastreamento com metaiodobenzilguanidina é indicado para pacientes com níveis elevados de calcitonina e cuja doença não foi localizada pelos exames radiológicos.

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Agradecimentos

Tabela 1 - Classificação, incidência e mutações associadas ao Carcinoma Medular de Tireóide.

Fenótipo	Incidência	Mutações Germinativas no proto-oncogene RET (Exon/códon)	Apresentação Clínica
CMT esporádico	80%		CMT
NEM 2ª		11 / 634	
2A(1)	4%		CMT, feocromocitoma e hiperparatireoidismo
2A(2)	4%		CMT e feocromocitoma
2A(3)	1%		CMT e hiperparatireoidismo
NEM 2B	3%	16 / 918	CMT, feocromocitoma e ganglioneuromas
CMTF	1%	10,13,14,15/ 609,611,618, 620; 768,790,791; 804,883; 891.	CMT (pelo menos em 4 membros)
Outros	7%		CMT (em 2 ou 3 membros)

Adaptado [3]

Figura 1 - Proto-oncogene *RET*.
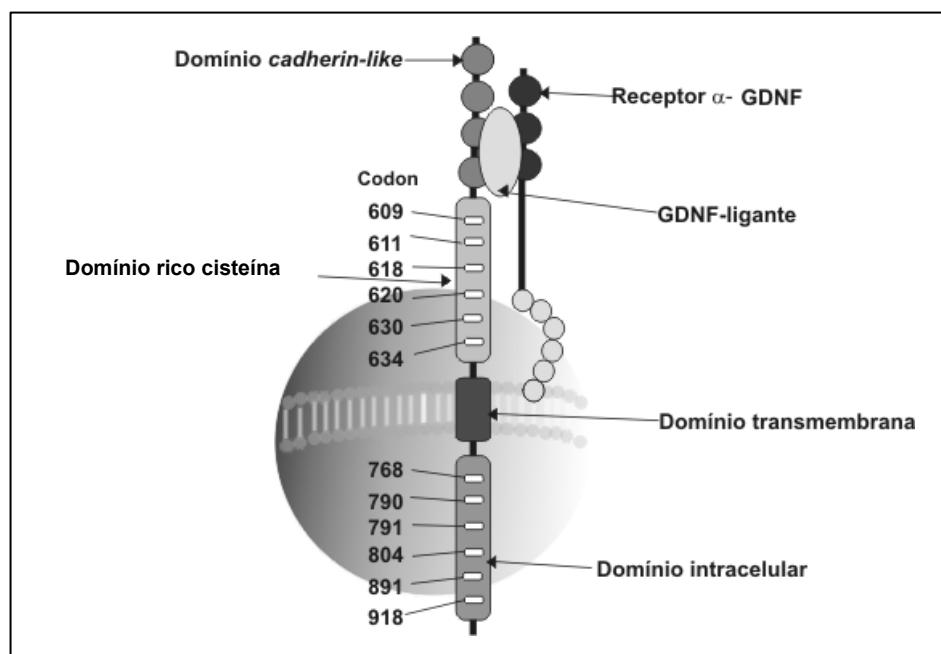
Figura 2 - Proporção estimada de pacientes com mutações específicas no códon 634 e metástases à distância ao diagnóstico. O teste *log rank* foi utilizado para comparar as curvas (p = 0,001).
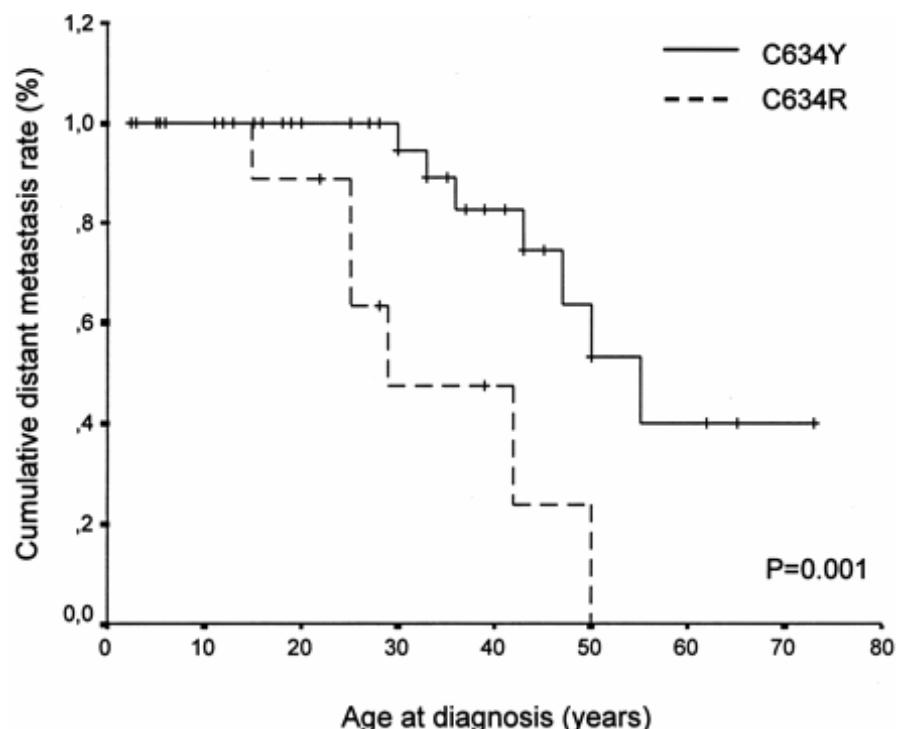
Capítulo II

RET Codon 634 Mutations in Multiple Endocrine Neoplasia Type 2:

Variable Clinical Features and Clinical Outcome

RET Codon 634 Mutations in Multiple Endocrine Neoplasia Type 2:
Variable Clinical Features and Clinical Outcome

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Running title: *RET* proto-oncogene and MEN 2

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Abstract

Since the establishment of a protocol for molecular analysis of hereditary medullary thyroid carcinoma (MTC) in southern Brazil, in 1997, 17 independent families with *RET* germline mutation have been identified. Because neither molecular diagnosis nor the pentagastrin test were available before the establishment of this protocol, we had the opportunity to observe a large number of patients in whom the disease has evolved naturally without medical intervention, namely prophylactic thyroidectomy. We observed a wide spectrum in terms of clinical presentation and natural course of the disease even among genetically-related individuals. Sixty-nine individuals from 12 different families presented a codon 634 mutation, the most prevailing missense mutation in our series. The specific mutations identified were C634Y (n=49), C634R (n=13), and C634W (n=7). Individuals with the C634R mutation presented significantly more distant metastases at diagnosis than subjects with the C634Y or C634W mutations (54.5% vs. 19.4% vs. 14.3%, respectively, P=0.03). Further analysis of the estimated cumulative frequency of lymph node and/or distant metastases by Kaplan-Meier curves showed that the appearance of lymph nodes and metastases occurred later in patients with C634Y than in those with C634R (P=0.001). Our results suggest that specific nucleotide and amino acid exchanges at codon 634 might have a direct impact on tumor aggressiveness in MEN 2A syndrome.

Key words: MEN 2A, MEN 2B, CMTF, *RET* Proto-oncogene.

Introduction

Medullary thyroid carcinoma (MTC), a tumor of the parafollicular C cells of the thyroid, may occur sporadically or as part of three clinically distinct dominantly inherited cancer syndromes. In patients with familial MTC (FMTC) only the thyroid is affected. Patients with multiple endocrine neoplasia (MEN) 2A develop MTC, pheochromocytoma (pheo) and/or primary hyperparathyroidism (HPT) (1). In contrast, MEN 2B patients have MTC, pheo, ganglioneuromas of the digestive tract, mucosal neuromas and/or skeletal abnormalities (1).

The *RET* proto-oncogene is the susceptibility gene for hereditary MTC (2). Germline mutations in MEN 2A and FMTC syndromes have been described in exons 10,11, 13, 14 and 15 of *RET*, while a single germline mutation in exon 16 has been found in >95% of unrelated MEN 2B cases (1,3). Genetic testing for germline mutations in the *RET* proto-oncogene has become available and today forms the basis for MTC screening procedures. Molecular biology now allows early identification of carriers of *RET* proto-oncogene germline mutations who will develop MTC later in life. In these patients, early prophylactic thyroidectomy must be considered to ensure definitive cure. In fact, early thyroidectomy may decrease the mortality from hereditary MTC to less than 5% (4).

In the past few years, several genotype-phenotype correlations have focused on the relationship between specific mutations and different MEN 2 syndrome variants (5-7). The international *RET* mutation consortium analysis, which studied 477 independent MEN 2 families, found a statistically significant association between the presence of any mutation at codon 634 and presence of pheo and HPT (5). Contrariwise, mutations at codons 768 and 804 are thus far associated with FMTC,

while codon 918 mutations are MEN-2B-specific (5). It is interesting to note that while the international *RET* mutation consortium analysis reported that only 10% of FMTC families have germline mutations in the intracellular domain of the *RET* gene, the French Calcitonin Tumors Study Group found that this kind of mutation is present in about half of FMTC families (6), suggesting that the frequency of specific *RET* mutations in MEN 2A phenotype may be influenced by the genetic background of the studied population.

The international *RET* mutation consortium analysis did not include any Brazilian families, but in 1997 our group established a protocol for molecular analysis of MTC in southern Brazil. Until then, neither molecular diagnostic tools nor the pentagastrin test had been available, and therefore we had the opportunity to observe the natural evolution of the disease, without prophylactic interventions, in large families harboring the codon 634 *RET* mutation. These observations have allowed us to study the heterogeneity in phenotype and disease presentation associated with this mutation and motivated us to describe our findings. Therefore, the present report has two aims: first, to describe the frequency of the *RET* proto-oncogene in a sample of Brazilian kindred with hereditary MTC; and second to describe the natural course of the disease in 69 heterozygotes from 12 independent families presenting the *RET* codon 634 mutation.

Materials and Methods

Patients

Patients with a diagnosis of medullary thyroid carcinoma attending the Endocrine Division at Hospital de Clínicas de Porto Alegre were invited to participate in the study. Our division is a reference center for molecular testing of germline *RET* mutation in Brazil, and therefore patients referred to us by other Brazilian centers for molecular investigation were also invited to participate.

A total of 88 patients with germline mutation of the *RET* proto-oncogene and / or and immunohistochemistry diagnosis of MTC were identified. This sample encompassed 17 index cases and 61 affected members of families with hereditary MTC, plus 10 individuals with sporadic tumors. Before undergoing genetic testing, all patients and/or their legal guardians gave their written informed consent, as required by the institution's Ethics Committee.

MEN 2A cases were classified following the International Consortium of MEN Syndromes (5). Briefly, families with MTC, Pheo, and HPT were classified as MEN 2A(1); families with MTC and Pheo as MEN 2A(2); and families with MTC and HPT, as MEN 2A(3). The classification of FMTC refers to families with a minimum of 4 members with MTC. Families with fewer than 4 members affected by MTC were classified under the category others. The data collected for each family included the clinical features of family members (association of other endocrine neoplasias), the presence and type of *RET* mutations, and information on atypical features noted, such as Hirschsprung's disease or cutaneous lichen amyloidosis (CLA).

Patients with positive genetic screening underwent a complete clinical examination, laboratory tests [levels of basal calcitonin (Calcitonin IRMA - DSL7700, Diagnostic Systems Laboratories, Inc., Webster, TX, reference range < 10 pg/ml), plasma calcium and parathyroid hormone (PTH) (Immulite 2000 Intact PTH, Diagnostic Products, Los Angeles, CA)], and extensive diagnostic imaging investigation that included cervical ultrasonography, cervical, thorax, and abdominal computed tomography (CT). Selected patients were submitted to whole-body metaiodobenzylguanidine (MIBG) scintigraphy to rule out pheo and/or local and distant metastasis. Also, a punch biopsy of the skin was performed in selected patients suspected of having CLA in an area clinically affected by a characteristic lesion. Biopsy specimens were fixed in 10% formalin and stained with hematoxylin and eosin, crystal violet, and congo red.

The regular follow-up of hereditary MTC in our Division consists of basal calcitonin, serum calcium and PTH determinations every 6 months and of a yearly abdominal and chest CT. We advocate a preventive total thyroidectomy for gene carriers older than 5 years, associated with a standard systematic central cervical lymph node dissection in those with suspected MTC or C cell disease on the basis of increased calcitonin level. Study participants with pheo or HPT underwent specific surgery. Tumor staging was performed according to the current UICC TNM classification (8).

DNA Extraction and PCR amplification

Genomic DNA was prepared from white blood cells according to standard protocols. Oligonucleotide primers for amplification of different *RET* exons were

designed on the intronic sequences flanking exons 10 (5' AGGCTGAGTGGGCTACGTCTG 3' / 5' GTTGAGACCTCTGTGGGGCT 3'), 11 (5' ATGAGGCAGAGCATACGCAGCC 3' / 5' CTTGAAGGCATCCACGGAGACC 3'), 13 (5' AACTTGGGCAAGGCGATGCA 3' / 5' AGAACAGGGCTGTATGGAGC 3'), 14 (5′ AAGACCCAAGCTGCCTGA 3′ / 5′ GCTGGGTGCAGAGCCATAT 3′), 15 (5′-GACCGCTGTGCCTGGCCAT 3′ / 5′-GCAGGCAGTCCTTGGGAAGC 3′) and 16 (5' AGGGATAGGGCCTGGGCTTC 3' / 5' TAACCTCCACCCCAAGAGAG 3'). PCR reactions were run in a final volume of 50 μL using 100 or 200 ng genomic DNA, containing 20mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 unit of Taq polymerase and 1 μM of specific primer. Genomic DNA was denatured for 3 min at 94°C prior to 35 cycles at 94, 65 and 72°C for 1 min at each temperature followed by a 5 min 72°C step in a programmable thermal controller (MJ Research, Inc). Following PCR, the amplicon sizes were analyzed in 1.5% agarose gel and the products visualized by ethidium bromide staining.

Single strand conformational polymorphism analysis, restriction enzyme analysis and direct sequencing

For single strand conformational polymorphism (SSCP) analysis of exons 10, 11, 13, 14 and 15, the amplified DNA fragments were denatured in formamide and cooled in ice before gel loading. Separation was carried out in a vertical electrophoresis apparatus in an 8-12% polyacrylamide-0.8% bis-acrylamide gel at 8°C, 45°C, 30°C or at room temperature for exons 10, 11, 13 and 14-15 respectively, at 200-240 mV for 2-4h (9,10). DNA bands were visualized by silver staining according to standard procedures (11). The bands presenting altered migration were

further analyzed by differential restriction enzymes (12) for 2h. The product was examined on a 2.5% agarose gel and the bands were visualized by ethidium bromide staining. Amplicons of exon 16 were directly screened for mutations by restriction enzyme analysis with Fok I (12). Whenever necessary, the presence of the mutation was confirmed by direct sequencing of the PCR product using the Sanger method in an automated sequencer, according to the manufacturer's instructions (Alf Express, Amersham Pharmacia Biotech, etc).

Statistical Analysis

Results are expressed as mean ± SD unless otherwise specified. Baseline characteristics were compared using the χ^2 test or Fisher's exact test for qualitative variables, or the Student's t-test or Mann-Whitney's U-test for quantitative variables. The differences in cumulative lymph node and/or distant metastasis rates among groups were tested by Kaplan Meier curves; comparisons between curves were performed using the Log Rank test. The Statistical Package for the Social Sciences 7.5 (SPSS, Chicago, IL) was used for the statistical analysis. P values of less than 0.05 were considered as statistically significant.

Results

Sample description

We analyzed the *RET* proto-oncogene from 160 individuals, 150 from members of 17 separate MEN 2 families and 10 from patients with apparently sporadic medullary thyroid carcinoma. A total of 78 individuals with hereditary MTC were enrolled in this study. Fifty-four of these individuals were identified based on clinical signs of thyroid neoplasia and familial thyroid cancer or endocrine related neoplasias. In addition, molecular screening identified another 24 individuals without clinical evidence of disease but at risk because of an affected relative. A mutation was identified in all kindred patients with documented germline transmission of MTC.

RET proto-oncogene mutations and disease phenotype

Table 1 summarizes the clinical and molecular data of the families with MEN 2. Of the 17 families with hereditary MTC analyzed, 8 were diagnosed with MEN 2A; 4 with MEN 2B; 3 with the rare syndrome of MEN 2A associated with CLA; 1 with FMTC; and 1 was categorized as "others." MEN 2A patients were further subclassified into 3 operational categories based on the combination of disease features identified (6).

We observed a wide spectrum of clinical presentation and natural course of the disease among MEN 2A individuals. The presence of pheo/HPT ranged from 12.5 – 100%. Age at diagnosis also showed ample variation in both individuals diagnosed based on the presence of palpable thyroid nodule (9 - 63 yr.) and in those

22 identified by molecular screening (2.5 – 73 yr.). Of the 8 families classified as MEN 2A, all but one had a mutation at codon 634, exon 11. The identified mutations were TGC→CGC (Cys→Arg, 42.8%), TAC (Cys→Tyr, 42.8%) and TGG (Cys→Trp, 14.2%).

Three families presented the rare syndrome of MEN 2A associated with CLA and all of them presented a germline mutation at codon 634. Because of the stringent operational definition of FMTC (2), only 1 family fell into this category. A mutation at codon 634 was identified in the index case and in 4 other family members. One family was included in the category "others." The proband, a 43-yr. old male who denied having a family history of thyroid cancer presented a TGC→TAC (Cys→Tyr) change at codon 634 and 1 of his 2 offspring was also diagnosed with MTC at age 25 yr. The most prevalent mutation in our series was observed at codon 634, accounting for 93% of cases.

Four patients with MEN 2B syndrome were identified. All individuals presented the characteristic phenotype and *de novo* mutation at codon 918, exon 16, resulting in the substitution of a methionine residue by threonine (M918T). As expected, these patients presented very aggressive tumors, with cervical or distant metastases at the time of diagnosis. One patient died at the age of 18 yr. as a consequence of gastrointestinal bleeding.

RET 634 mutation heterozygotes

In view of the large number patients with a codon 634 mutation, we analyzed the individual clinical and oncological features of these patients. Sixty-nine individuals from 12 unrelated families were found to harbor the germline *RET* 634 mutation

(table 1). In 47 (68.1%) subjects, including index patients, the diagnosis was based on clinical evidence through evaluation of a thyroid nodule (table 1). All of these individuals presented elevated basal serum calcitonin. Molecular screening identified another 22 (31.9%) patients without clinical signs of thyroid cancer. Serum basal calcitonin was determined in 19 of these, and was elevated in 8 (42%). As expected, the mean age at diagnosis was significantly lower in these individuals than in patients with clinical evidence of disease (21.7 ± 21.6 vs. 29.8 ± 11.6 yr., P<0.04), although both groups presented a wide age range (table 1).

In the group of individuals diagnosed by *RET* screening, we were surprised to identify as gene carriers 3 women with ages 62, 65 and 73 years in a MEN 2A + CLA kindred (family # 11, table 1). They had no clinical complaints and their thyroid physical examination was considered normal. Thyroid ultrasonography displayed 1 or more nodules (varying in diameter from 0.3 – 2 cm) and guided-fine needle aspiration confirmed MTC. Serum basal calcitonin was elevated in all 3 patients (880 pg/ml, 1100 pg/ml and 37.0 pg/ml, respectively, reference range < 10 pg/m). The 65-year old patient underwent surgery, and the histopathologic examination revealed a 2cm nodule on each thyroid lobe, with C cell hyperplasia and MTC. No metastasis was found in a total of 62 lymph nodes removed. The two other patients refused surgery. None of them presented distant metastases.

The frequency of pheo in the group of patients with clinical disease was 38.3% (n=18). Nine individuals (19.1%) presented HPT; in 7 of these patients, HPT was associated with pheo and MTC while in 2 patients it was associated to MTC only. All patients except for one presented MTC as the first disease manifestation.

A total of 50 patients underwent surgery, 43 with clinical disease and 7 gene carriers. All patients presented C cell hyperplasia and/or MTC at histopathology. In

the group of patients with clinical disease (table 2), lymph node and distant metastases were present in 45.8 and 25% of individuals, respectively. Only 1 out of 7 gene carriers presented lymph node metastases – a 27-year-old woman with a C634Y germline mutation. Seven patients died of MTC, and all of them had disseminated disease at diagnosis. Neither sex (P=0.109) nor associated endocrine neoplasia, pheo (P=0.174) or HPT (P=0.92), were associated with mortality. In contrast, age at diagnosis (40.9 ± 10.4 *vs*. 28.6 ± 10.5, P=0.007) and stage of disease (P=0.001) were significantly associated with death.

We also analyzed the clinical and oncological features of 47 patients identified based on clinical evidence, grouped by nucleotide and amino acid exchange at codon 634 (table 2). The specific mutations were C634Y (n=49), C634R (n=13), and C634W (n=7). In these patients, we did not find significant differences in age at diagnosis (P=0.46), frequency of pheo (P=0.62) or HPT (P=0.61), and lymph node metastasis (P=0.19) among individuals with the 3 genotypes analyzed. However, the presence of distant metastases at diagnosis was significantly higher in C634R heterozygotes (P=0.03).

Natural history of MEN 2A in codon 634 mutation heterozygotes

Based on the finding of a significant association between the C634R mutation and the presence of distant metastases at diagnosis, we speculated that specific changes in cysteine substitution at codon 634 could affect natural history of disease in MEN 2A. As gene dysfunction is present since birth, we assumed that the individual age at diagnosis would indicate the period of exposure, and thus we performed additional analyses using the Kaplan Meier model. Indeed, Kaplan Meier

estimates of cumulative lymph node metastasis rate in the 50 patients who underwent surgery yielded distinct curves for C634R and C634Y genotypes (P=0.027). The presence of distant metastases at diagnosis as a function of age was also analyzed. Kaplan Meier estimates of distant metastasis rates yielded significantly different curves for C634R and C634Y heterozygotes (P=0.001) (Fig. 1). Both events, lymph nodes and distant metastases, occurred earlier in individuals harboring the C634R mutation. The youngest patient with lymph nodes and distant metastases (a 15-year-old girl) presented a C634R germline mutation. On the other hand, distant metastases were not diagnosed before age 30 year in individuals with the C634Y mutation. Individuals with the C634W mutation were not analyzed because of the small number patients/ events.

Discussion and Conclusions

We showed the frequency profile of *RET* proto-oncogene mutations in a sample of 17 unrelated Brazilian families with hereditary MTC. Because of the lack of genetic or clinical screening until recently, we had the unique opportunity to observe the natural history of MEN 2A in a large number of individuals harboring codon 634 mutations, classically described as high risk. We observed a wide variance in disease phenotype, age at onset and tumor behavior in different families. Individuals with the C634R genotype had significantly more distant metastases than those with the C634Y or C634W mutations, despite similar ages at diagnosis. Accordingly, Kaplan-Meier estimates of cumulative lymph node and/or distant metastasis rates demonstrated that these events occurred earlier in individuals harboring the C634R mutations, indicating that nucleotide and amino acid exchange might have a direct impact on tumor aggressiveness in MEN 2A syndrome.

The *RET* proto-oncogene is expressed in cells of neuronal and neuroepithelial origin and encodes a receptor tyrosine kinase (13). Approximately 92% of the three variants of MEN 2 are related to germline mutations of *RET* (3). Mutations on the highly conserved extracellular cysteine ligand-binding domain encoded by exons 10 and 11 induce constitutive tyrosine kinase activity due to aberrant homodimerization (14,15). The transforming capacity of the c-*RET* examined in transfected NIH-3T3 cells has been shown to be dependent on specific mutated codons with the C634R (TGC→CGC) mutant showing a 3-to 5-fold higher transforming activity compared with any exon 10 Cys mutants (16). Although the three-dimensional structure of the *RET* extracellular domain is still unknown, these cysteines likely form intramolecular disulfide bonds in the wild-type receptor, and the mutation results in an unpaired

cysteine, which forms an activating intermolecular bridge (17). Differences in dimerization induction intensities are a reasonable explanation for the phenotypes resulting from mutations of the different cysteines. In fact, the international *RET* mutation consortium analysis studied 477 MEN 2 families from 18 tertiary referral centers, which did not include any kindred from Brazil, and demonstrated that specifically mutated *RET* codons correlate with MEN 2 variants (2).

Differences in the frequency of specific *RET* mutations in MEN 2A phenotypes have been found in series from different countries, suggesting that the occurrence of these mutations may be influenced by genetic background (5-7,18-20). In our series, the most frequent phenotype was the MEN 2A syndrome with codon 634 mutation, in agreement with the results of the International *RET* mutation consortium analysis. In that study, this kind of mutation was found in 86% of all cases of MEN 2A(1) and MEN 2A (2). One of our MEN 2A (2) families presented a C618R mutation, which was observed in only 4% of the families in the *RET* consortium. The family with FMTC presented the C634Y mutation, the most prevalent codon 634 specific mutation associated with this phenotype in the *RET* consortium.

In general, there is an agreement to recommend total thyroidectomy in MEN 2 carriers. However, no universal consensus exists as to the optimal timing and extent of prophylactic surgery in these patients. A recent study (7) has proposed a division of hereditary MTC into three risk groups, based on age at disease onset and genotype: high risk group, codon 634 and 618 mutations; intermediate risk group, codon 790, 620 and 611 mutations; and low risk group, codon 768 and 804 mutations. However, some reports have also called attention to the clinical variability and aggressiveness associated with *RET* mutation at codons that are classically described as having weakly activation, such as codon 804. Such reports indicate that

identical *RET* mutations behave differently, even in the same genetic background (21,22).

We studied 47 patients with codon 634 mutation in whom the disease has naturally evolved without medical interference (prophylactic or therapeutic thyroidectomy) and we have also observed a wide spectrum in the clinical presentation. Particularly, we studied a family harboring a C634Y mutation in which we identified, by molecular screening, members ages 62, 65 and 73 yrs. who were not aware of their condition and presented no clinical signs of disease, except for a thyroid nodule measuring less than 2 cm in diameter detected by ultrasonography. An interesting aspect was that the 65-year old patient – who was submitted to surgery – had elevated basal serum calcitonin and MTC at histopathological examination, but no lymph node or distant metastases, despite the advanced age, indicating low tumor aggressiveness. Although we do not have histopathological data about lymph node metastases for the 62 and 73 year-old patients because they have refused surgery so far, both also seem to have an indolent disease. The observation of such unexpected clinical course of MCT in patients harboring the classically described high-risk 634 mutation suggested to us that nucleotide and amino acid exchange at this codon could have an impact on the oncological features of MEN 2A.

Indeed, patients harboring the C634R mutation presented significantly more distant metastases at diagnosis than subjects with C634Y or C634W, notwithstanding similar age at diagnosis. Accordingly, Kaplan Meier estimates of cumulative lymph nodes and distant metastasis rates yielded distinct curves, indicating that these events occur earlier in individuals with the C634R genotype. These findings probably explain the significant association of this genotype with mortality in our series. Differences in oncological features nowadays are often difficult

to detect since gene carriers have thyroidectomy even before MTC has emerged. Ours results suggest that there might be differences in the type of nucleotide and amino acid exchange at codon 634 that affect the pace of malignant progression and that may ultimately lead to widespread metastatic MTC. In agreement with our findings, the youngest patients with hereditary MTC and lymph node metastasis reported in the literature outside a MEN 2B setting was a 5-year old girl with the C634R (Cys→Arg) missense change at codon 634 (23). Recently, the presence of MTC has been reported in a prophylactic thyroidectomy specimen obtained from a 17-month old girl harboring the same mutation (24). The latter study also identified a 75 year-old gene-carrier with the C634Y genotype.

In our series, we identified three kindreds with the rare syndrome of MEN 2A associated with CLA. CLA was first associated with MEN 2A by Gagel et al in 1989 (25), although Nunziata and colleagues had had previously reported the presence of a pruritus in affected members of a particular kindred (26). So far, this association has been reported in a total of only 19 families (3, 25-29). As in our study, these families were distributed along the operational phenotypic categories, with an apparent excess of MEN 2A(2) cases, all of which presented 634 mutations.

In conclusion, our results showed the frequency profile of proto-oncogene *RET* mutations of MEN 2A in 17 Brazilian families. In addition, we have demonstrated that families with hereditary thyroid carcinoma exhibit a highly variable disease presentation and that even high-risk mutations, such as those at codon 634, could present an indolent course depending on the type of nucleotide and amino acid substitution. Individuals harboring C634R, the most prevailing missense change at codon 634, seem to have a more aggressive disease, as demonstrated by more frequent distant metastases at diagnosis. They also seem to develop lymph nodes

and distant metastasis at an earlier age, according to Kaplan Meier analyses. In contrast, the C634Y genotype appears to have an indolent behavior, with low potential for spreading the disease in some individuals. Based on these results, we suggest that the timely prophylactic thyroidectomy advocated for codon 634 heterozygotes should take into account specific amino acid exchanges. The most significant drawback of our observations is the limitation in the number of patients/families. Considering the relatively small number of families with each genotype studied, and the fact that a large number of the individuals analyzed as unit-genotype belong to the same kindred, we cannot rule out the possibility of interference of other hereditary molecular events in our conclusions. Finally, it is our opinion that other information, in addition to the *RET* mutation, is still needed to allow understanding of disease mechanisms and to clarify the process leading to development of the full syndrome phenotype. Until such information is available, the best therapeutic approach in gene carriers of hereditary medullary thyroid carcinoma is still a matter of debate.

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Acknowledgments

Table 1 - Clinical characteristics and *RET* mutations in families with Multiple Endocrine Neoplasia Type 2.

Phenotype	(%)	N families	Individuals analyzed/ affected	Codon/ Amino acid substitution	Age of proband at diagnosis (yr)	Pheo	HPT	Age range	
								Individuals with clinical disease (n)	Individuals without clinical disease (n)
MEN 2A	47.0								
MEN 2A (1)		1	13/8	C634Y	19	(1)	(2)	18 - 50 (6)	2.5 - 43 (2)
		2	29/12	C634Y	36	(6)	(1)	11 - 36 (9)	7 - 20 (3)
		3	5/2	C634R	25	(1)	(1)	25 (1)	5.5 (1)
		4	12/7	C634W	36	(3)	(1)	9 - 37 (7)	
		5	5/3	C634R	42	(2)	(1)	35 - 42 (1)	8 (1)
MEN 2A (2)		6	3/3	C634Y	45	(1)	(-)	27 - 46 (2)	7 (1)
		7	6/2	C634R	29	(2)	(-)	29 (1)	5 (1)
		8	6/5	C618R	34	(1)	(-)	34 - 63 (3)	7 - 12 (2)
MEN 2A+CLA	17.6	9	3/2	C634R	25	(-)	(-)	15 - 25 (2)	
		10	7/4	C634R	28	(1)	(-)	22 - 50 (4)	
		11	36/20	C634Y	43	(1)	(-)	16 - 43 (7)	3 - 73 (13)
FMTC	5.8	12	7/4	C634Y	55	(-)	(-)	19 - 55 (4)	
Other forms	5.8	13	3/2	C634Y	43	(-)	(-)	25 - 43 (2)	
MEN 2B	23.5	14	4/1	M918T	14	(1)	(-)		
		15	4/1	M918T	11	(-)	(-)		
		16	3/1	M918T	21	(-)	(-)		
		17	4/1	M918T	14	(-)	(-)		

MEN 2A(1): families with MTC, Pheo, and HPT; MEN 2A(2): families with MTC and Pheo; FMTC: families with a minimum of 4 members with MTC; Others: families with fewer than 4 individuals with MTC; CLA: cutaneous lichen amyloidosis. Histological proof of MTC, pheo, and HPT was required.

Table 2 - Clinical and oncological features of Multiple Endocrine Neoplasia 2A grouped by nucleotide / amino acid exchange.

	634	C634W	C634Y	C634R	P
N (total)	47	7	30	10	
Sex (% female)	55.3	85.7	43.3	70.0	0.07
Mean age (yr.)	29.8 ± 11.6	24.3 ± 12.1	30.6 ± 11.8	31.0 ± 10.5	0.46
Age range (yr.)	5 – 65	9 - 37	11- 55	15 - 50	
% Pheo	38.3	42.9	33.3	50	0.62
% HPT	19.1	14.3	16.7	30	0.61
PN1 (%)	51.2	42.9	44.4	77.8	0.19
PM1 (%)	31.1	14.3	24.1	66.7	0.03*

Figure 1 - Kaplan Meier estimates of the proportion of patients with specific codon 634 mutation and distant metastases at diagnosis. The log rank test was used to compared curves (P=0.01).
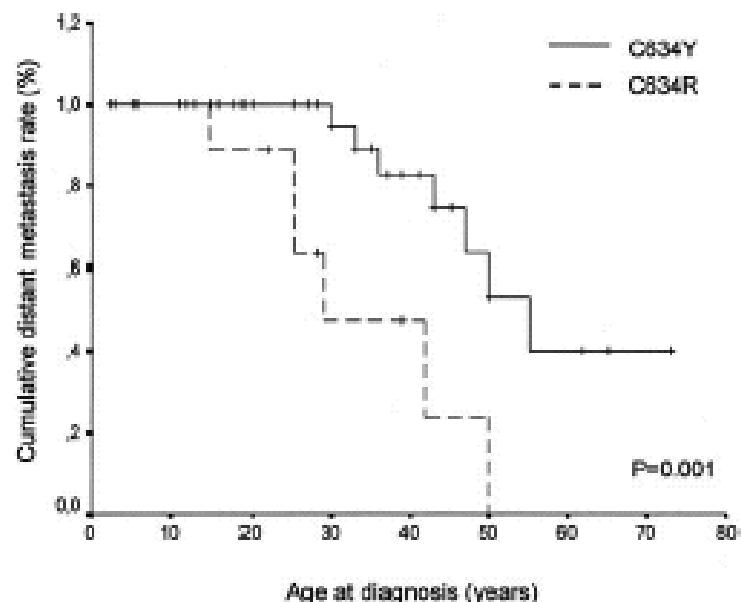
Capítulo III

Malignant Progression of Hereditary Medullary Thyroid Carcinoma in Children and Young Adults

Artigo submetido à publicação

Malignant Progression of Hereditary Medullary Thyroid Carcinoma in Children and Young Adults

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Running title: *Gene Carriers and Thyroidectomy*

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Abstract

Medullary thyroid carcinoma (MTC) may occur sporadically or as component of the Multiple Endocrine Neoplasia Type 2 syndrome. The DNA-based *RET* genotype analysis enables the identification of gene carriers at risk of developing MTC. However, no universal consensus exists as to the optimal timing of the prophylactic procedure. Here we described the clinical and oncology features of 51 Brazilian children and young adults under 25 years of age harboring *RET* mutations. In 22 patients, the diagnosis was established based on clinical evidences of MTC while *RET* screening was able to identify other 29 asymotomatic gene carriers. Forty-two patients underwent total thyroidectomy, 22 therapeutically and 20 prophylactically. The mean age at surgery was significantly higher in the group of patients with clinical disease than in those identified by *RET* analysis (17.9 ± 5.5 vs. 13.5 ± 5.8 years, P=0.018). C-cell hyperplasia associated to MTC was observed 69% at thyroidectomy specimens. The group of patients who underwent therapeutic surgery presented more advanced disease based on the TNM classification than those in the prophylactic group (P<0.001). A positive correlation between age at surgery and TNM stages was observed (P=0.0013). In the therapeutic group, after a follow up period of 10.4 years, 11% died and 22% have persistent disease. In contrast, all patients of the prophylactic surgery are considered cured based on basal serum calcitonin. In conclusion, our data showed a time-dependent MTC progression, further demonstrating the importance of early diagnosis and intervention on the management of this hereditary neoplasia.

Key words: *RET* Proto-oncogene, Gene carriers. Prophylactic Thyroidectomy

Introduction

Medullary thyroid carcinoma (MTC) may occur sporadically or as a manifestation of an autossomal-dominant inherited syndrome Multiple Endocrine Neoplasia Type 2 (MEN 2), characterized by the presence of various endocrine tumors in variable clinical expression (1). Features of MEN 2A include MTC, pheochromocytoma (PHEO), and hyperparathyroidism (HPT). MEN 2B presents a specific phenotype that encompasses MTC, neuromas of lips, tongue, and gastrointestinal tract, marfanoid habitus and/or skeletal anomalies. The presence of isolated MTC in at least 4 members characterizes the familiar medullary thyroid carcinoma (FMTC) (2-5). MEN 2A can also be associated with the rare syndrome of cutaneous lichen amyloidosis (CLA) and Hirschsprung disease (5-8).

The malignant transformation of the C-cell begins very early in life among the hereditary form of MTC. Patients with MEN 2B generally develop MTC earlier and presents a more aggressive tumor than MEN 2A. FMTC usually having a later age at onset and exhibits a less aggressive behavior (9-11). Nearly 100% of hereditary forms of MTC are associated with germline mutation of the *RET* proto-oncogene (*RET*) (2,3,10,12). The various mechanism of *RET* activation might determine the pace of malignant transformation from C-cell hyperplasia to MTC, the first and most commonly fatal neoplasm among *RET* gene carriers (13,14).

The DNA-based *RET* genotype analysis gained worldwide acceptance and the identification of asymptomatic gene carriers at risk of developing hereditary MTC has allowed early prophylactic thyroidectomy (10,14-17). Perhaps the most important consideration related to genetic testing is when to perform total thyroidectomy in asymptomatic gene carriers (18). Early prophylactic procedure will most likely obviate

the need for the potentially more radical approach to MTC, which requires systematic dissection of the central cervical lymph-node compartment (10,18,19). Most of the authors agree that *RET* carriers of mutations at codon 918 and 922 should undergo prophylactic thyroidectomy preferably within the first 6 months of life while surgery can be deferred until the age of 5 years for asymptomatic carriers of the other *RET* mutations (10,14-17). Exceptions may include specific carriers of mutations at codons 630 and 634 who may require earlier surgical intervention (20). In other mutations, thyroidectomy may be performed between the ages of 5 to 10 years old.

Meanwhile, when to perform an additional central lymph node dissection is still controversial. The lag period between the appearance of node-negative MTC and the evolution of lymph node metastases is estimated to be of 6.6 years for carriers of *RET* mutations at codon 634 (10). Nodal metastases are uncommon before the age of 10 years in 630 and 634 mutations and before the age of 20 years in 609, 611, 618, 620, 768, 790, 791, 804, and 891 mutations (10,15). Nevertheless, the unpredictability of the malignant transformation has prompted to offer prophylactic surgery on asymptomatic *RET* gene carriers despite normal basal and stimulated calcitonin levels (10,21-24).

Close surveillance of patients prophylactically thyroidectomized during childhood will be of a great help in order to define the best time to perform and for evaluating the effectiveness of this procedure. A recent study has reported a lower incidence of persistent or recurrent disease in children who underwent total thyroidectomy before 8 years of age and who had no metastases to cervical lymph nodes (25).

Here we described the clinical presentation, time-dependent MTC progression, and oncology features of 51 Brazilian children and young adults harboring *RET* mutations.

Patients and Methods

Subjects

Patients with the diagnosis of MCT attending the Endocrine Division at Hospital de Clínicas de Porto Alegre were invited to participate in the study. Our division is a reference center for molecular testing of germline *RET* mutation in southern Brazil and patients from other Brazilian medical centers referred to us for molecular investigation were also invited to participate. A total of 130 patients with germline mutation of the *RET* proto-oncogene and/or histological and immunohistochemistry diagnosis of MTC were included. This sample was initially formed from index cases and affected members of 22 families with hereditary MTC. Before undergoing genetic analysis, all patients and/or their legal guardians had given informed consent in accordance with institutional Ethics Committee.

MEN 2A classification used in this study was according to the International Consortium of MEN 2 Syndromes (3). Patients with MTC, pheochromocytoma (PHEO), and hyperparathyroidism (HPT) were classified as MEN 2A(1), families with MTC and PHEO as MEN 2A(2), and families with MTC and HPT, as MEN 2A(3); FMTC correspond to families with a minimum of 4 members with MTC and others, families with fewer than 4 individuals with MTC. Data provided from each family include clinical features of family members (association of other endocrine neoplasias), presence and type of *RET* mutations, and information of atypical features noted, such as Hirschsprung's disease or cutaneous lichen amyloidosis (CLA).

Patients with positive genetic screening underwent a complete clinical examination, laboratory tests (Until 2004, basal calcitonin (VR. 10 pg/dl, Calcitonin IRMA - DSL7700, Diagnostic Systems Laboratories, Inc., Webster, TX, USA) and after, January 2004 (VR. Male < 12.0 pg/dl and female < 6.0 pg/dl, Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA), carcinoembrionary antigen (cea), plasma calcium and parathyroid hormone (PTH) levels (Immulite 2000 Intact PTH, Diagnostic Products, Los Angeles, CA, USA), and extensive diagnostic imaging investigation that included cervical ultrasonography, cervical, thorax, and abdominal computed tomography (CT), whole-body metaiodobenzylguanidine (MIBG) scintigraphy (selected patients) to rule out PHEO and/or local and distant metastasis. In selected patients suspected to have CLA a punch-biopsy of the skin was performed in an area clinically affected by a lesion. Biopsy specimens were fixed in 10% formalin and stained with hematoxylin and eosin, crystal violet, and congo red.

The standard follow up of MTC in our Division consists of the determination of basal serum calcitonin, cea, serum calcium and PTH (every 6 months) and, abdominal and chest CT (every year). We advocate a preventive total thyroidectomy for gene carriers older than 5 years, associated with a standard systematic central cervical lymph node dissection in those with suspected medullary thyroid carcinoma or C-cell disease on the basis of increased calcitonin level. Patients with PHEO or HPT underwent specific surgery. Tumor staging was performed according to the current UICC TNM classification (26).

DNA Analysis

Genomic DNA was prepared from peripheral blood leukocytes by standard procedures (27). *E*xons 10, 11, 13, 14, 15 and 16 were amplified using specific primers by polymerase chain reaction (PCR) as described previously (28). *RET* mutations were screened by single-strand conformational polymorphism (SSCP) and/or restriction enzyme digestion and/or direct sequencing, according to the manufacturer's instructions (Alf Express, Amersham Pharmacia Biotech, etc). All PCR reactions were performed in 50 μL volume using 100 or 200 ng genomic DNA, containing 20mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of Taq polymerase and 1 μM of specific primer. Genomic DNA was denatured for 3 min at 94°C prior to 35 cycles at 94, 65 and 72°C for 1 min at each temperature followed by a 5 min 72°C step in a programmable thermal controller (MJ Research, Inc). Following PCR, the amplicon sizes were analyzed in 1.5% agarose gel and the products visualized by ethidium bromide staining.

Statistical Analysis

Results are expressed as mean ± SD unless otherwise specified. Baseline characteristics were compared using the χ^2 test or Fisher's exact test for qualitative variables, or the Student's t-test or Mann-Whitney's U-test for quantitative variables. The Statistical Package for the Social Sciences 12.0 (SPSS, Chicago, IL) was used for the statistical analysis. P values of less than 0.05 were considered as statistically significant.

Results

Sample Description

We analyzed *RET* proto-oncogene of 181 members of 22 independent MEN 2 families, and a total of 97 individuals with hereditary MTC were enrolled. Fifty-eight of these individuals were identified based on clinical signs of thyroid neoplasia and familial thyroid cancer or endocrine related neoplasias. In addition, molecular screening identified 39 individuals as *RET* gene carriers. Mutations were identified in all patients with documented germline transmission of MTC.

MCT progression in patients under 25 years-old

The clinical and oncological features of 51 individuals 25 years of age or younger from 21 independent MEN 2 families are summarized in Table 1. Of them, 10 kindred were classified as MEN 2A (26 individuals), 3 with MEN 2A associated CLA (14 individuals), 1 MEN 2A associated to Hirschsprung disease (1 individual), 6 with MEN 2B (9 individuals), and 1 family was classified as others (1 individual).

Twenty-two individuals were identified based on clinical signs of thyroid neoplasia and further confirmed by *RET* analysis and twenty-nine individuals identified as gene carriers, being all diagnosed by molecular screening, except 1 who was identified by pentagastrin stimulated testing at the age of 5 years (Table 1). The identified *RET* mutations were as follows: C634Y (27 individuals), C634R (8 individuals), C634W (4 individuals), C618R (3 individuals) and M918T (9 individuals).

Three families presented the rare syndrome of MEN 2A associated to CLA (30 individuals) and all of them presented a germline mutation at codon 634, 2 harboring the genotype C634R and one C634Y. One family was classified as MEN 2A associated to Hirschsprung disease. The index patient was a 35 year-old woman who harbor a C618R mutation and one of 4 siblings also presented *RET* mutation, without any clinical of Hirschsprung disease. Only 1 family was included in the category of "others", being 2 individuals affected with isolated MTC.

Six patients with MEN 2B syndrome were identified. Five individuals presented the characteristic phenotype and *de novo* mutation at codon 918 in exon 16, resulting in the substitution of a methionine residue by threonine (M918T). As expected, these patients presented very aggressive tumors, with cervical or distant metastases at the time of diagnosis. One patient died at the age of 18 years, as a consequence of metastatic MTC and gastrointestinal bleeding. Only 1 family with MEN 2B presented 6 affected individuals. The index case was a 9-year-old boy who complained of ganglioneuromatosis of the oral mucosa and was attended at the genetic division of our hospital because oral neuromas. His mother (26-year-old), 3-year-old sister, and aunt (18-year-old) were then diagnosed by molecular screening. Clinical examination revealed neuromas at oral mucosa and tongue in all of them. Past medical history revealed that his grandmother underwent total thyroidectomy for MTC (T2N0M0) at the age of 31 years old and abdominal surgery for unilateral left pheochromocytoma. Another aunt died at the age of 16 years old, 2 years after a diagnosis of metastatic MTC associated to unilateral pheochromocytoma.

The mean age at diagnosis of the 51 patients was 13.7 ± 6.2 years (from 1.75 to 25 years of age). Patients diagnosed by molecular screening were younger than those with clinical signs of MTC (11.2 ± 6.4 vs.16.9 ± 4.4 years, $P < 0.001$).

Pheochromocytoma was diagnosed in 5 individuals (4 unilateral and 1 bilateral), 3 with MEN 2B and 2 MEN 2A. Adrenal disease was the first manifestation in 1 patient. Three individuals presented hyperparathyroidism, 2 with histological examination demonstrating adenoma of the parathyroid gland and one an apparently normal gland but elevated intact PTH levels.

Serum calcitonin was determined in 32 patients. Twenty-one patients (65.6%) presented elevated serum basal calcitonin levels at diagnosis and 2 individuals showed abnormal pentagastrin test. Of note, all patients with normal basal calcitonin (9 individuals) that underwent thyroidectomy were classified as stage I and presented thyroid nodules less than 1.0 cm at histological examination.

Thyroid ultrasound (US) was performed in 43 patients and demonstrated the presence of one or more nodule in 32 (74.4%). US failed to detect thyroid nodules in 11 individuals (25.6%) who presented histological diagnosis of medullary carcinoma associated or not to C-cell hyperplasia. All these patients were classified as stage I and presented tumors less than 1.0 cm at histological examination. Fine needle aspiration biopsy was performed in 24 cases. The results were as follows: MTC in 17 (70.8%), suspicious of MTC in 4 (16.6%), and not satisfactory in 3 (12.5%) cases.

Therapeutic vs. Prophylactic Thyroidectomy

Forty-two patients underwent total thyroidectomy (Table 2). The mean age at the surgery was 15.8 ± 6.0 years, ranging from 5.0 to 29.5 years. Patients who underwent therapeutic thyroidectomy (22 individuals) were significantly older than patients (20 individuals) who underwent prophylactic procedure (17.9 ± 5.5 vs. 13.5 ± 5.8 years, P=0.018).

C-cell hyperplasia associated to MTC was observed in 29 of 42 patients (69%) at thyroidectomy specimens (table 2). Twelve individuals (28.6%) presented multifocal MTC, most of them in the group of individuals submitted to therapeutic surgery (10 patients, 83%). Only 2 patients in the group diagnosed by *RET* analysis presented multifocal disease. Interesting, 1 patient despite unilateral and unique thyroid nodule associated to C-cell hyperplasia.

In the group of patients who underwent therapeutic thyroidectomy, metastases to regional nodes were found in 4 patients with MEN 2A (mean age of 21.4 ± 1.6 years) and 1 associated to CLA (29.5 years). Distant metastases were identified in 2 patients (15 and 25 years at diagnosis) with MEN 2A associated to CLA and in 3 individuals with MEN 2B. No patients identified by molecular screening presented local or distant metastases. No patient younger than 15 years of age, excluding those with MEN 2B, presented metastatic disease at diagnosis.

The TNM classification in the group submitted to therapeutic thyroidectomy was as follows (Figure 1): 4 of 22 patients (18.2%) were classified as stage I (13.4 ± 4.4 years); 8 individuals (36.4%) as stage II (17.3 ± 4.7 years), 5 (22.7%) as stage III (22.7 ± 4.3 years) and 5 (22.7%) as stage IV; (18.0 ± 6.2 years). Three of 5 patients classified as stage IV presented MEN 2B syndrome. Tumoral sizes in these patients were ≤ 1.0 cm in 4 individuals (18.2%). Fourteen individuals (63.6%) presented tumor sizes > 1.0 and ≤ 4.0 cm. Four individuals (18.2%) presented tumor sizes larger than 4.0 cm, one of them associated to MEN 2B syndrome.

The TNM classification in the group submitted to prophylactic thyroidectomy was significantly different from that in the therapeutic group (P<0.001). Most patients were classified as stage I (17 of 20, 85%; mean age 12.5 ± 5.8 years). Three patients (15%)

were at stage II (18.9 ± 4.7 years). No patient in this group was classified as stage III or IV. Tumoral sizes in these patients were ≤ 1.0 cm in 17 patients (85%) and > 1.0 and ≤ 4.0 cm in 3 individuals. No patient diagnosed by molecular screening had tumor larger than 4.0 cm.

As expected, we observed a positive correlation between age at surgery and TNM stages (r2= 0.33, P=0.0013). Indeed, the ages at thyroidectomy was significant different among both groups (P= 0.018). The mean age at surgery at each stage was similar in both groups.

Data on follow up and outcome are available in 18 of 22 patients who underwent therapeutic thyroidectomy. The mean follow up period was 10.4 ± 7.1 years (from 1 to 29 years). Two individuals with MEN 2B died at 16 and 21 years of age, 2 and 7 years after the diagnosis, respectively. The postoperative serum calcium levels were evaluated at least once year postoperatively in all patients who underwent total thyroidectomy. Twelve individuals out of 16 are biochemically cured and 4 have persistent disease (25%). Six patients (37.5%) developed permanent hypoparathyroidism, requiring calcium and vitamin D supplements to maintain the serum calcium levels within or near the normal.

In the group of patients submitted to prophylactic procedure, the mean follow up period was 3 years (from 5 months to 13 years). None of them died. All patients all biochemically cured, based on serum normal basal calcitonin. One patient (9 year-old girl) developed permanent hypoparathyroidism.

Discussion and Conclusion

In the present study, we report the molecular, clinical and oncological features of 51 children and young adults with hereditary MTC. In 22 of these individuals, the diagnosis was established based on clinical evidences of disease and therapeutic thyroidectomy was performed. Molecular screening was able to identify other 29 children and young adults without any clinical manifestation of MTC and prophylactic thyroidectomy indicated. The observed differences in the TNM stages between these 2 groups of patients, mainly determined by the age at surgery, are overwhelming to demonstrate the importance of the early diagnosis and intervention in the management of this aggressive hereditary disease.

The Multicenter European Multiple Endocrine Neoplasia (EUROMEN) study confirmed preliminary data from large institutions of an age-dependent and codon-specific progression of early medullar carcinoma (10). A significant age-related progression from C-cell hyperplasia to MTC and, ultimately, nodal metastasis in patients grouped by extracellular and intracellular domain mutations was observed (10). The authors reported that malignant progression from C-cell hyperplasia to MTC may occur during the first years of life in asymptomatic carriers of germ-line 634 codon mutations in *RET* and nodal metastasis occur approximately 6.6 years after the malignant transformation, being a very rare event before the age of 14 years (10). As a substantial lag interval exists before medullar carcinoma progresses from C-cell hyperplasia, genetic diagnosis of *RET* mutations is unique in offering the opportunity of preemption by codon orientated prophylactic thyroidectomy before medullar carcinoma arises and codon orientated prophylactic surgery marks the transition from curative to truly prophylactic surgery, ushering in the new era of DNA-based

management of MEN 2. The earlier medullary thyroid carcinoma is kept from progressing along the pathway of tumor cell dissemination, the better are the chances of surgical cure (25). Total thyroidectomy and node resection is the only curative therapeutic method of MTC (14,15,28,29). Survival rates are reported to be about 65% in 10 year of follow up and are directly associated to the age at onset, presence of lymph node disease, metastases at presentation and the extent of the thyroid surgery (31,32).

Data about the experience with MEN 2 syndromes in South America are limited (23,28,33). Recently, the evaluation of histological findings and prophylactic thyroidectomy in MEN 2 Chilean population were reported (32). In agreement with other studies, the authors observed that the tumor is often preceded by biochemical detection of the disease and that the prophylactic procedure should be done early in life because the age-dependent progression from C-cell hyperplasia to MTC. Here we described the molecular and clinical data from 51 patients with 25 years of age or younger from 21 unrelated MEN 2 kindred (Table 1). Genetic screening identified 29 asymptomatic gene carriers and 22 individuals were diagnosed based on clinical signs of medullary thyroid carcinoma. Mutations were identified in all patients with documented germline transmission of MTC. Serum basal calcitonin and/or pentagastrin test was elevated in about 75% of patients. Interestingly, all patients with normal basal calcitonin were classified as stage I at histological specimens, with thyroid nodules less than 1.0 cm, demonstrating the correlation between tumor size and calcitonin levels. Thyroid ultrasound was able to identify one or more nodules in 74.4% of the cases but failed in 11 cases, all of them with tumor sizes less than 1.0 cm. Fine needle aspiration biopsy was able to establish the diagnosis in about 71% of the cases and was suspicious for MTC in other 20%.

In our series, 42 individuals underwent thyroidectomy, 22 of them therapeutically and 20 prophylactically. As expected, the mean age at surgery was significantly lower in the group of patients identified on basis of molecular diagnosis than in those with clinical disease (Table 2). Most of the patients with clinical disease presented multifocal medullary thyroid carcinoma at histological examination compared to only 4.8% of patients diagnosed by molecular screening. In agreement with the EUROMEN study, no patient younger than 15 years of age presented nodal or distant metastases. However the owing to unpredictability of the timing of somatic "hits" required for malignant progression, there may be a small risk of earlier progression to medullary thyroid carcinoma beyond data delineated in the different series of patients reported.

The most important consideration to take account related to genetic testing is the ideal timing of total thyroidectomy in asymptomatic carriers (30). Systematic dissection of the central cervical lymph-node compartment and a more aggressive approach of MTC could be avoided by an early prophylactic procedure (10,18,19,33). In fact, we observed that the TNM stages in the group of patients who underwent therapeutic thyroidectomy were significantly different from those observed in the prophylactic group (Table 2). In agreement with the above statements, the age at the procedure appeared to be the most determinant factor. There was a time-dependent progression of MTC, with an advance of age according to the progression of TNM stages. It is interesting to note that the mean age at surgery at each stage was similar between the groups (Figure 1).

A recent study evaluated the effect of early thyroidectomy in young patients identified by direct DNA analysis as carriers of a *RET* mutation characteristic of MEN 2A (25). The authors reported that after a mean follow up period of 5 years of the

prophylactic surgery, no evidence of persistent or recurrent MTC were found in patients submitted to thyroidectomy before 8 years of age (25). Although, it should be stressed that a longer period of evaluation is still necessary to confirm that they are cured, these results indicate an extremely high chance of cure when early thyroidectomy is performed. In our sample, we had the opportunity to compare the effect of the intervention timing between 2 groups of children and young adults. The only difference between these children was the availability of the molecular screening allowing pre-clinical diagnosis. The results on disease outcome reflect the importance of early intervention. In the group of patients submitted to therapeutic surgery 2 individuals died (9%) and 4 (18%) have persistent disease after a mean follow up period of 10 years. In contrast, all patients who underwent prophylactic thyroidectomy are considered cured based on serum basal calcitonin. However, we should call attention for the relatively short follow up period (mean of 3 years, ranging from 5 months to 13 years) and for the fact that a pentagastrin testing was not performed in these individuals.

In our division, we advocate total thyroidectomy in MEN 2 carriers harboring *RET* mutations at codons described as the most aggressive, represented mainly by codon 918 and associated to MEN 2B, as soon as possible. While, in patients harboring mutations at extracellular domain especially mutations at codon 634, at the age of 5 years. And, those harboring mutations at the intracellular domains and associated to FMTC or MEN 2A, presenting a less aggressive behavior, between 5 to 10 years.

In conclusion, the data analysis from 42 thyroidectomized children and young adults harboring *RET* mutations demonstrate that early diagnosis and thyroidectomy are essential to the hereditary MTC prognosis. The understanding of other mechanisms associated to tumorigenesis and *RET* activation to clarify the process

involved in the pace of malignant progression and that may ultimately lead to widespread metastatic MTC, will help to define the best therapeutic approach, namely the ideal timing to perform the prophylactic thyroidectomy.

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Table 1 - Clinical characteristics and *RET* mutations in 51 Children and Young Adults with Multiple Endocrine Neoplasia Type 2:

	Age at Diagnosis (y)	Surg	Age at Surgery (y)	Diagnosis Screening	Clinical Diagnosis	PHEO	HPT	PTH	Ca	CT	CT (R.V)	CEA	US Nodule	Tumor size	Local Meta	Distant Meta	Tumor Stage	Lymph Ressec	Mutation	Phenotype
1	1,75	No	.	Yes	No	No	No	.		3,9	NL	.	No	.	.	.	.		C634R	MEN 2A
2	2,5	Yes	5,75	Yes	No	No	No	29,44		17	NL	0,32	No	1	0	0	1	9-/9	C634Y	MEN 2A
3	3	Yes	6,5	Yes	No	No	No	36,2		69	High	2,18		1	0	0	1	11-/11	C634Y	CLA
4	3	No	.	Yes	No	No	No	.		16	High	3,89		.	.	.	.		M918T	MEN 2B
5	5	Yes	5	Yes	No	No	No	.		30	PG	.	No	1	0	0	1	No	C634R	MEN 2A
6	5,5	Yes	6,5	Yes	No	No	No	.	9,0	37,5	High	.	No	1	0	0	1	No	C634R	MEN 2A
7	6	No	.	Yes	No	No	No	.		.	.	.		.	.	.	.		C634Y	MEN 2A
8	6,75	Yes	9	Yes	No	No	No	36,2	8,9	9,5	NL	1,08	No	1	0	0	1	19-/19	C634Y	CLA
9	7	No	.	Yes	No	No	No	.		.	.	.		.	.	.	.		C634Y	MEN 2A
10	7	No	.	Yes	No	No	No	.		.	.	.		.	.	.	.		C634R	MEN 2A
11	7	Yes	9,75	Yes	No	No	No	.		0,7	NL	.	No	1	0	0	1	9-/9	C618R	MEN 2A
12	8	No	.	Yes	No	No	No	.		.	.	.		.	.	.	.		C634Y	MEN 2A
13	9	Yes	9	No	Yes	No	No	.		.	.	.		1	0	0	1		C634W	MEN 2A
14	9	Yes	9,5	Yes	No	No	No	.		3	NL	.	No	1	0	0	1		C634R	MEN 2A
15	9,25	Yes	11,75	Yes	No	No	No	28,08		28	NL	6,07	No	1	0	0	1		C634Y	CLA
16	9,25	Yes	10	Yes	Yes	No	No	-	9,2	47	NL	1,97	Yes	1	0	0	1	17-/17	M918T	MEN 2B
17	11	Yes	11	No	Yes	No	No	.		100	High	.	Yes	1	0	0	1	No	C634Y	MEN 2A
18	11	Yes	11	No	Yes	Yes	No	.		1200	High	.	Yes	2	0	0	2		M918T	MEN 2B
19	12	Yes	15,75	Yes	No	No	No	.		12,9	NL	.	Yes	1	0	0	1	11-/11	C618R	MEN 2A
20	12,5	Yes	13,25	Yes	No	No	No	.	9,4	9,98	High	.	Yes	1	0	0	1	13-/13	C634Y	CLA
21	12,75	Yes	13,75	Yes	No	No	No	.		28	High	0,86	No	1	0	0	1	25-/25	C634Y	CLA
22	13	Yes	13,5	No	Yes	No	No	.		.	.	.	Yes	2	0	0	2		C634W	MEN 2A
23	13,25	Yes	14,75	Yes	No	No	Yes	75,8	*4,3	39	High	1,53	No	1	0	0	1	20-/20	C634Y	CLA
24	13,75	Yes	14,75	Yes	No	No	No	25,6	9,5	55	High	3,6	No	1	0	0	1	9-/9	C634Y	CLA
25	14	Yes	14	No	Yes	No	No	.		.	.	.	Yes	2	0	0	2		C634W	MEN 2A
26	14	Yes	16	No	Yes	Yes	No	.	9,2	642,3	High	.	Yes	2	1	1	4		M918T	MEN 2B
27	14	Yes	14	No	Yes	Yes	No	.		.	.	.	Yes	.	1	1	4		M918T	MEN 2B
28	14,8	Yes	15	No	Yes	No	No	.		685	High	.	Yes	2	1	1	4	3+/36	M918T	MEN 2B
29	15	Yes	15	No	Yes	No	Yes	.		540	High	.	Yes	1	0	0	1	9-/9	C634Y	MEN 2A
30	15	Yes	16	No	Yes	No	No	.		3500	High	.	Yes	2	1	1	4		C634R	CLA

31	15	Yes	15	No	Yes	No	No	.		.	.	Yes	2	0	0	2		M918T	MEN 2B
32	16	No	.	Yes	No	No	No	.		.	.		.	.	.	.		C634Y	MEN 2A
33	16	Yes	17,25	Yes	No	No	No	29,57	10,0	518	High	7,15	Yes	2	0	0	2	21-/21	C634Y	CLA
34	16,5	Yes	17,5	Yes	Yes	No	No	27,3	*4,0	410,9	High	.	Yes	2	0	0	2	17-/17	C634Y	CLA
35	18	Yes	19,75	No	Yes	No	Yes	.		30	PG	.	Yes	2	0	0	2		C634Y	MEN 2A
36	18	Yes	18	No	Yes	No	No	.		.	.	.	Yes	4	1	0	3	2+/36	C634Y	MEN 2A
37	18	No	.	Yes	No	No	No	.		.	.	.		.	.	.	.		C618R	HIRS
38	18,5	No	.	Yes	No	No	No	.		.	.	.		.	.	.	.		M918T	MEN 2B
39	19	Yes	19	No	Yes	No	No	.		80	High	.	Yes	1	0	0	1	No	C634Y	MEN 2A
40	19	Yes	19	No	Yes	No	No	.		.	.	.	Yes	2	0	0	2		C634W	MEN 2A
41	19	Yes	29,5	Yes	Yes	Yes	No	.	7,0	882,5	High	.	Yes	2	1	0	3	2+/36	C634Y	CLA
42	19	Yes	22,75	No	Yes	No	No	.		.	.	24,4	Yes	2	1	0	3	3+/36	C634Y	MEN 2A
43	20	Yes	21,25	Yes	No	No	No	.		788	High	.	Yes	1	0	0	1		C634Y	MEN 2A
44	20	Yes	20,75	Yes	No	No	No	52,9	*4,6	77	High	2,79	Yes	1	0	0	1	28-/28	C634Y	CLA
45	20	Yes	20	No	Yes	No	No	.		3579	High	.	Yes	3	0	0	3		C634Y	MEN 2A
46	21	Yes	21	No	Yes	No	No	.		.	.	.	Yes	2	0	0	2		M918T	MEN 2B
47	21,75	Yes	22	Yes	Yes	No	No	14,8	9,5	2200	High		Yes	2	0	0	2	20-/20	C634R	CLA
48	23	Yes	23,25	No	Yes	No	No	.		.	.	.	Yes	.	1	0	3		C634Y	MEN 2A
49	25	Yes	25	No	Yes	Yes	No	.		46	High	.	Yes	2	0	0	2		C634Y	MEN 2A
50	25	Yes	25	Yes	Yes	No	No	.		.	.	.	Yes	1	0	0	1		C634Y	Others
51	25	Yes	29	No	Yes	No	No	.		1600	High	.	Yes	2	1	1	4		C634R	CLA

Surg; Surgery, PHEO: pheochromocytoma, HPT: hyperparathyroidism, PTH: intact paratohormone, Ca: total serum calcium and *ionic calcium, CT: basal calcitonin, CT (V.R): calcitonin reference value, NL: normal, PG: pentagastrin stimulated testing, CEA: carcinoembrionary antigen, US Nodule: presence of thyroid nodule at ultrasound, Local Meta: presence of local metastasis, Distant Meta: presence of distant metastasis, Lymph Ressec: number of lymph nodes ressected during surgery and number of positive or negative lymph/total lymph ressected.

Table 2 - The histological findings of 42 patients who underwent total thyroidectomy:

	Age at Therapeutic Thyroidectomy (n)	Age at Prophylactic Thyroidectomy (n)
Extracellular and Intracellular Mutations	17.9 ± 5.5y (22)	13.5 ± 5.8y (20)
MEN 2A extracellular Mutations **Codon 618 and 634**	**(16)**	**(19)**
C-Cell Hyperplasia + Multifocal MTC	18.5 ± 6.4y (10)	14.0 ± 6.0y (17)
Multifocal MTC	19.8 ± 5.5y (6)	5.75 y (1)
C-Cell Hyperplasia + Unilateral MTC	(0)	15.75y (1)
MEN 2B intracellular Mutations **Codon 918**	**(6)**	**(1)**
C-Cell Hyperplasia + Multifocal MTC	15 y (2)	(0)
Multifocal MTC	15.5 ± 4.2y (4)	10y (1)

Figure 1 - Correlation between age at surgery and TNM stage of patients who underwent therapeutic or prophylactic thyroidectomy.