UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

O PAPEL DOS MARCADORES DE ANGIOGENESE NO FEOCROMOCITOMA

CARLA VAZ FERREIRA

Porto Alegre, junho de 2013

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

O PAPEL DOS MARCADORES DE ANGIOGENESE NO FEOCROMOCITOMA

CARLA VAZ FERREIRA

Dissertação apresentada ao curso de Pós-Graduação em Ciências Médicas: Endocrinologia, UFRGS como requisito parcial para obtenção do grau de Mestre.

Orientadora: Profa. Dra. Ana Luiza Maia

Porto Alegre, junho de 2013

CIP - Catalogação na Publicação

Ferreira, Carla Vaz
O papel dos marcadores de angiogênese no feocromocitoma / Carla Vaz Ferreira. -- 2013.
52 f.

Orientadora: Ana Luiza Silva Maia.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Porto Alegre, BR-RS, 2013.

1. Medicina. 2. Endocrinologia. 3. Feocromocitoma. 4. Marcadores de angiogenese. I. Maia, Ana Luiza Silva , orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

AGRADECIMENTOS

À minha orientadora Profa. Dra. Ana Luiza Maia, pela sua contribuição para o meu crescimento profissional e pessoal, pelo profissionalismo, dedicação, confiança e orientação a mim dedicados ao longo destes anos.

À colega e amiga Débora Siqueira, pela imprescindível colaboração para que este trabalho fosse concretizado, pela disponibilidade sempre que precisei de ajuda, e pela inestimável amizade.

A todos os colegas do Grupo de Tireóide. Especialmente as amigas Mírian Romitti, Lucieli Ceolin e Simone Wajner, pelos ensinamentos, paciência, amizade e palavras de incentivo nos momentos difíceis.

Aos alunos de iniciação científica, em especial às amigas, Helena Rohenkohl, Silvana Cavalcante e Rafaela Pinto Ribeiro, pela amizade, carinho e ajuda nas horas de necessidade.

À bolsista Shana Weber, pela amizade, dedicação e ajuda na rotina do laboratório.

As médicas patologistas Dra. Luise Meurer e Dra. Beatriz Assis Brasil pela disponibilidade e ajuda, dedicados a este trabalho.

Aos profissionais do Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre – Laboratório de Patologia que contribuíram com a realização deste trabalho.

Aos meus pais, pelo carinho e confiança a mim dedicada a cada nova escolha que faço, e acima de tudo pela família especial que formamos.

Ao meu noivo Vinícius, por toda compreensão, companheirismo e pelo constante incentivo para o meu crescimento, sempre me apoiando nas minhas escolhas.

À todas as pessoas e instituições que contribuíram direta ou indiretamente para a conclusão desta dissertação.

Esta Dissertação de Mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de dois manuscritos:

- Artigo de revisão: Advanced medullary thyroid cancer: pathophysiology and management; publicado no Cancer Management and Research 2013:5 57–66

- Artigo original referente ao trabalho de pesquisa: The role of angiogenesis markers in pheochromocytoma*

  *Trabalho premiado como melhor pôster clínico no XV Latin American Thyroid Congress, 2013.

Além dos artigos já citados, ao longo do período do mestrado foram desenvolvidos os seguintes trabalhos:

- Additive effect of RET polymorphisms on sporadic medullary thyroid carcinoma susceptibility and tumor aggressiveness. Ceolin L, Siqueira DR, **Ferreira CV**, Romitti M, Maia SC, Leiria L, Crispim D, Ashton-Prolla P, Maia AL. European Journal of Endocrinology 2012; 166 (5):847-54.

- Molecular Basis of Medullary Thyroid Carcinoma: The Role of RET Polymorphisms. Ceolin, Lucieli, Siqueira, Débora R., Romitti, Mírian, **Ferreira, C. V.**, Maia, Ana Luiza. International Journal of Molecular Sciences (Online). , v.13, p.221 - 239, 2012.

- Signaling pathways in follicular cell-derived thyroid carcinomas (Review). Romitti, M., Ceolin, L., Siqueira, D. R., **Ferreira, C. V.**, Wajner, S. M., Maia, A. L. International Journal of Oncology. , v.1, p.1 - 10, 2012.

LISTA DE ABREVIATURAS E SIGLAS

CEA - Carcinoembryonic antigen

c-kit - Stem cell-factor receptor

EGFRs - Epidermal growth factor receptors

FDA - US Food and Drug Administration

Flt3 - FMS-like tyrosine kinase 3

FMTC - Familial medullary thyroid carcinoma

HPT - Hyperparathyroidism

IHC - Immunohistochemistry analysis

MAPK - Mitogen-activated protein kinase

MEN - Multiple endocrine neoplasia

mg - Milligrams

mm- Millimeter

mm^2 - Square millimeter

mRNA - Messenger RNA

MTC - Medullary thyroid carcinoma

MVD - Microvessel density

NF1 - Neurofibromatosis type 1

PDGFR - Platelet-derived growth-factor receptor

PHEO - Pheochromocytoma

PI3K - Phosphatidylinositol 3-kinase

RET - Re arranged during Transfection; proto-oncogene

RTK - Receptor tyrosine kinase

SD - Stable disease

sPHEO - Sporadic pheochromocytoma

TK - Tyrosine kinase

TKIs - Tyrosine kinase inhibitors

VEGFA - Vascular endothelial growth factor-A

VEGF-R1; Flt-1 - Vascular endothelial growth factor receptor 1

VEGF-R2; KDR; Flk-1 - Vascular endothelial growth factor receptor 2

VEGFRs - Vascular endothelial growth factor receptors

VHL - Von Hippel-Lindau disease

SUMÁRIO

PARTE I

ADVANCED MEDULLARY THYROID CANCER: PATHOPHYSIOLOGY AND MANAGEMENT

ADVANCED MEDULLARY THYROID CANCER: PATHOPHYSIOLOGY AND MANAGEMENT

Carla Vaz Ferreira, Débora Rodrigues Siqueira, Lucieli Ceolin, Ana Luiza Maia.

Thyroid Section, Endocrine Division, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Grant support: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS), Fundo de Incentivo a Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE) and Programa de Apoio a Núcleos de Excelência (PRONEX), Brazil.

Correspondence: Ana Luiza Maia, M.D., Ph.D.
Serviço de Endocrinologia
Hospital de Clínicas de Porto Alegre
Rua Ramiro Barcelos 2350
90035 –003 Porto Alegre, RS, Brazil

Phone: 55-51-3359-8127; Fax: 55-51-3331-0207; E-mail: almaia@ufrgs.br

Abstract

Medullary thyroid carcinoma (MTC) is a rare malignant tumor originating from thyroid parafollicular C cells. This tumor accounts for 3-4% of thyroid gland neoplasias. MTC may occur sporadically or inherited. The hereditary MTC is part of syndromes of multiple endocrine neoplasia (MEN) 2A and 2B, familial medullary thyroid carcinoma (FMTC). Germline mutations of the *RET* (REarranged during Transfection) proto-oncogene cause hereditary form of cancer, whereas somatic mutations can be present in sporadic form of the disease. The *RET* gene encodes a receptor tyrosine kinase involved in the activation of intracellular signaling pathways leading to proliferation, growth, differentiation, migration and survival. Nowadays, the only possibility of cure for MTC patients consists of total thyroidectomy associated with lymph node dissection. Based on the knowledge of the pathogenic mechanisms of MTC, new drugs have been developed in attempt to control metastatic disease. Of these, the small-molecule tyrosine kinase inhibitors (TKIs) represent one of the most promising agents for MTC treatment and clinical trials have shown encouraging results. Hopefully, the cumulative knowledge about the targets of action of these drugs as well as TKI-associated side effects will help on choosing the best therapeutic approach in order to enhance its benefits.

Keywords: Medullary Thyroid Carcinoma, protooncogene *RET*, tyrosine kinase inhibitors.

Introduction to medullary thyroid cancer

Medullary thyroid carcinoma (MTC) is a rare malignant tumor originating from parafollicular C cells of the thyroid first described by Hazard *et al*. [1] This tumor accounts for 3-4% of all thyroid gland neoplasias.[2] Calcitonin, the main secretory product of MTC, is a specific and highly sensitive biomarker for C-cell disease. The carcinoembryonic antigen (CEA) is also produced by neoplastic C cells. These molecules are widely used as prognostic markers during the follow-up of MTC patients.[3, 4] The reported 10-year mortality rate for patients with MTC varies from 13.5-38%.[5, 6]

MTC may occur sporadically (75% of cases), or as part of the inherited cancer syndrome known as multiple endocrine neoplasia type 2 (MEN 2).[7, 8] The term MEN was introduced by Steiner et al. in 1968 to describe disorders that include a combination of endocrine tumors. The Wermer syndrome was designed as MEN 1 and the Sipple syndrome as MEN 2.[9] Later, MEN 2 was subdivided into three distinct syndromes: MEN 2A, MEN 2B and familial medullary thyroid carcinoma (FMTC). The hereditary MTC is usually preceded by C-cell hyperplasia (CCH) and these tumors are generally bilateral and multicentric. The mean age at diagnosis is around 45 years.[6, 10]

The MEN 2A subtype constitutes approximately 70–80% of cases of MEN 2 and is characterized by the presence of MTC (95%), pheochromocytoma (30–50%) and hyperparathyroidism (10–20%). Adrenomedullary disease is usually multicentric and bilateral (65-78%), generally detected after the onset of MTC.[11, 12] Two rare variants of MEN 2A have been described, one with cutaneous lichen amyloidosis (CLA), a pruriginous lesion of the scapular region characterized by amyloid deposition, and the other with Hirschsprung's disease, caused by the absence of autonomic ganglia in the terminal hindgut which results in colonic dilatation, obstipation and constipation.[13, 14] The clinical course of MTC in patients with MEN2A is variable and the disease progression is associated with codon-specific mutations.[11, 15]

The MEN 2B syndrome accounts for about 5% of the cases of MEN 2. MEN 2B is characterized by a single phenotype, which includes diffuse ganglioneuromatosis of the tongue, lips, eyes and gastrointestinal tract, and marfanoid habitus. MEN 2B patients present MTC (>90%), pheochromocytoma (45%), ganglioneuromatosis (100%) and marfanoid habitus (65%).[11, 12] MTC in the setting of MEN 2B develops earlier and has a more aggressive course, compared with MTC in other MEN 2 subtypes.[6, 16]

The FMTC subtype constitutes 10-20% of the cases of MEN 2.[11] MTC is the only manifestation of FMTC, thereby it is necessary to demonstrate the absence of a pheochromocytoma or hyperparathyroidism in two or more generations of the same family or the identification of related mutations to confirm the diagnosis. The clinical presentation of MTC occurs later and the prognosis is more favorable compared to the other forms of MTC.[17]

Sporadic MTC generally presents as a palpable thyroid nodule or cervical lymph node. Diagnosis tends to be late, generally in the fifth or sixth decade of life.[18] Lymph node metastases are detected in at least 50% of these patients, at diagnosis while distant metastases occur in around 20% of cases.[19, 20] A minority of patients with MTC present systemic manifestations which include diarrhea, flushing, or painful bone metastases.[16]

Epidemiology, etiology and pathophysiology of familial and sporadic medullary thyroid cancer

MTC represents approximately 3-4% of malignant thyroid gland neoplasias.2 The Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute, showed that MTC patients had a median age of 50 years at diagnosis and were white in the majority of the cases. There is no difference in the frequency of this tumor between genders.[21, 22]

Hereditary MTC affects approximately 1 in 30,000 individuals and is associated with germline mutations in the *RET* (REarranged during Transfection) proto-oncogene, an autosomal dominant disease with a high penetrance and variable phenotype. *RET* point mutations affect mainly exons 10, 11 and 16. Less common mutations occur in exons 5, 8, 13, 14 and 15.[21, 23]

The *RET* gene encodes a receptor tyrosine kinase (RTK), expressed in the cells derived from the neural crest: thyroid parafollicular cells (C cells), parathyroid cells, chromafin cells of the adrenal medulla and enteric autonomic plexus. The RET protein is constituted by three domains: an extracellular, a transmembrane and an intracellular. The extracellular domain includes regions homologous to the cadherin family of cell adhesion molecules and a large region rich in cysteine residues that performs the transduction of extracellular signals of cell proliferation, differentiation, migration, survival and apoptosis. The intracellular domain encloses 2 tyrosine kinase subdomains, TK1 and TK2 which contain the tyrosine residues involved in the activation of the

signaling intracellular pathways. The *RET* gene is subject to alternative splicing of the 3' region generating three distinct protein isoforms with 9 (RET9), 43 (RET43) and 51 (RET51) amino acids in the carboxy-terminal tail downstream from glycine 1063. RET9 and RET51, consisting of 1072 and 1114 amino acids, respectively, are the main isoforms.[24, 25]

The majority of families with MEN 2A (>90%) present point mutations in the *RET* proto-oncogene (missense type), involving codons located in the extracellular region: 609, 611, 618 and 620 (exon 10) and 634 (exon 11). The most frequent mutations are located in codon 634, occurring in more than 60% of all genetically identified MTC.[11, 17, 21, 26] Codon 634 mutations have been associated with the presence of pheochromocytoma and hyperparathyroidism,[27] and rarely with CLA.[28] Nevertheless, we observe a variety of phenotypic expressions in families with the same *RET* mutation.[11, 29-32] Patients harboring the genotype C634R (TGC/Cys → CGC/Arg, exon 11) present significantly more distant metastases at diagnosis than groups C634W (Cys/TGC → Trp/TGG, exon 11) and C634Y (Cys/TGC → Tyr/TAC, exon 11), suggesting that a change of specific amino acids may modify the natural development of the disease.[32] *RET* C634W mutation is associated with high penetrance for MTC and pheochromocytoma.[26] The risk profiles and penetrance estimations in MEN 2A caused by germline *RET* exon 10 mutations were recently analyzed by Frank-Raue et al (2011) in a large multicenter study that included 340 subjects from 103 families. It was observed that mutations affect mainly the cysteine codons 609, 611, 618 and 620 and 50% penetrance was achieved by the age of 36 years for MTC, by 68 years for pheochromocytoma, and by 82 years for hyperparathyroidism.[30]

MEN 2B occurs, in approximately 95% of the cases, through a specific M918T mutation (exon 16), resulting in the structural change of the intracellular domain of the RET protein. The genotype A883F (GCT → TTT, exon 15) accounts about 2%–3% of cases and[33, 34] a double mutation V804M/Y806C at codon 804 (Val/GTG → Met/ATG, exon 14) and 806 (Tyr/TAC → Cys/TGC) in the same allele was described in a patient with MEN 2B. Patients presenting with "atypical" MEN 2B harboring the germline double point mutation in codons 804 and 904 (V804M and S904C) were also reported.[35, 36] Mutations in codons 883 and 918 are associated with younger age of MTC onset and higher risk of metastases and disease-specific mortality.[10, 11, 37]

In FMTC, germline mutations are distributed throughout the *RET* gene, approximately 86-88% of FMTC families present mutations in exon 10 (codons 609, 611, 618, 620) and exon 11 (codon 634) of *RET*.[31, 38] Substitutions in the intracellular domain of *RET* in exon 13 (codon 768, 790, 791), exon 14 (codon 804 and 844) and exon 15 (codon 891) are less common. Interestingly, the most frequent mutation observed in MEN 2A, C634R, has not been described in FMTC families.[11, 38-41]

On the other hand, the molecular mechanisms involved in the sporadic MTC have not yet been clarified. About 50–80% of the cases present a somatic *RET* mutation M918T (Met/ATG → Thr/ACG, exon 16).[42, 43] Somatic mutations in codons 618, 603, 634, 768, 804 and 883 and partial deletion of the *RET* gene have been identified in few tumors.[19, 20] However, the mutation does not appear to be uniform among the various cell subpopulations in the tumor or in the metastases, suggesting that sporadic MTC might be of polyclonal origin, or that the mutations in the *RET* proto-oncogene are not initial events in MTC tumorigenesis.[42, 44]

The presence of somatic *RET* (M918T) mutation correlates with higher probability of persistent disease and lower survival rate in a long-term follow up.[19, 20]

In recent years, the presence of *RET* variant sequences or polymorphisms have been associated with susceptibility for the development or progression of MTC. Several studies have described an increased prevalence of the *RET* polymorphisms in individuals with hereditary or sporadic MTC when compared with the population.[43, 45-49]

Overview of current therapeutic strategies

Surgery is the only curative treatment for MTC.[10, 16, 50] There are no effective therapeutic options for distant metastatic disease, since chemotherapy and external beam radiation therapy for metastatic or cervical recurrent disease have limited response rates.[51, 52] A large study of an American cohort of MTC patients demonstrated that age at diagnosis, stage of disease and extent of surgery are important predictors of survival. Patients with tumor confined to the thyroid gland had a 10-year survival rate of 95.6%, whereas patients with regional stage disease or distant metastasis at diagnosis had an overall survival rate of 75.5% and 40%, respectively.[22]

The main challenge in the management of MTC is patients with advanced and progressive disease, because the conventional therapeutic options have poor results for

these individuals. Nevertheless, in the last few years, several studies have upgraded our knowledge on the molecular events associated with MTC. RTKs, such as RET and vascular endothelial growth factor-A (VEGFA) involved in proliferation and cell survival, play an important role in the tumorigenesis process. In response to binding of extracellular ligands, such receptors are phosphorylated and activated downstream signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways and many signalling effectors, like β-catenin and nuclear factor- kappa B (NF-κB).[53, 54] Thus, the molecules involved in these processes serve as potential therapeutic targets for new drugs.[55]

Review of therapies targeting receptor tyrosine kinase

The cumulative knowledge on the distinct signaling pathways and multiple genetic abnormalities involved in the pathogenesis of cancer has allowed the development of targeted molecular therapies. The protein kinases regulate the processes of cell proliferation, differentiation, migration and anti-apoptotic signaling. Protein kinases are characterized by their ability to catalyze the phosphorylation of tyrosine amino acid residues in proteins and thus activate various intracellular signaling pathways. Therefore, inhibitors tyrosine kinase (TKIs) may act as therapy for cancer by blocking the tyrosine kinase – dependent oncogenic pathways. TKIs can be specific to one or several tyrosine kinase receptors - most designed to inhibit multiple signaling pathways.[55, 56]

Thyrosine kinase activation plays a key role in the development of MTC and, therefore, the small-molecule TKIs represent one of the most promising agents for MTC treatment and clinical trials have shown encouraging therapeutic results. The objective index called RECIST (Response Evaluation Criteria in Solid Tumors) has been used to evaluate the tumor response and classified as follows: complete response (the disappearance of all target lesions), partial response (at least a 30% decrease in the sum of the longest diameter of target lesions), progressive disease (at least a 20% increase in the sum of the longest diameter of target lesions and stable disease (neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease).[57] Interestingly, the reduction in serum levels of tumor markers (calcitonin and CEA) observed with these medications occurs independently of

radiological response.[58, 59] Another relevant question concerns the different responses of the parenchymal target lesions (for example, metastasis to lung, liver, bone) vs nonparenchymal target lesions (metastasis in lymph nodes); one possible explanation is that the parenchymal lesions are better perfused.[60]

The most studied TKIs drugs for MTC treatment are vandetanib, cabozantinib, motesanib, sorafenib, sunitinib, axitinib, and imatinib (Tables 1 and 2).

Table 1 Summary of clinical trials with tyrosine kinase inhibitors in medullary thyroid carcinoma

Drug	Targets	N° of patients	Partial response (%)	Stable disease (%)	Reference
Clinical trials phase I and II					
Vandetanib (ZD6474)	VEGFR-1, VEGFR-2,	30	20	53	64
	VEGFR-3, RET, EGFR	19	16	53	65
Cabozantinib (XL 184)	VEGFR-2, RET, MET	37	29	41	69
Motesanib (AMG 706)	VEGFR-1, VEGFR-2, VEGFR-3,	91	2	48	73
	c-Kit, RET, PDGFR	5	40	40	77
Sorafenib (BAY 43-9006)	VEGFR-2, VEGFR-3,	16	6	50	58
	c-Kit, RET	15	25	–	78
Sunitinib (SU 11248)	VEGFR-1, VEGFR-2,	7	28[a]	46[a]	86
	VEGFR-3, RET, c-Kit	15	33.3	26.7	87
Axitinib (AG-013736)	VEGFR-1, VEGFR-2,	11	18	27	89
	VEGFR-3, c-Kit				
Imatinib (STI571)	RET, c-Kit, PDGFR	9	0	55	91
		15	0	27	92
			PFS drug vs placebo (months)	**Hazard ratio**	
Clinical trials phase III					
Vandetanib (ZD6474)	VEGFR-1, VEGFR-2,	331	30.5 vs 19.3	0.46	66
	VEGFR-3, RET, EGFR				
Cabozantinib (XL 184)	VEGFR-2, RET, MET	330	11.2 vs 4.0	0.28	70

Note: [a]Results for the total number of patients with advanced thyroid cancer, not only MTC patients.
Abbreviations: PFS, progression-free survival; VEGFR, vascular endothelial growth-factor receptor; EGFR, epidermal growth-factor receptor; PDGFR, platelet-derived growth-factor receptor.

Table 2 Summary of most common reported adverse events of tyrosine kinase inhibitors*

Adverse events (%)	Vandetanib (ZD6474)	Carbozantinib (XL 184)	Motesanib (AMG 706)	Sorafenib (BAY 43-9006)	Sunitinib (SU 11248)	Axitinib (AG-013736)	Imatinib (STI571)
Gastrointestinal disorders							
Diarrhea	47–77	15.9–57	41	71	26	48	43
Dyspepsia	–	–	–	10	–	–	30
Nausea	16–63	43	26	14	9	33	–
Vomiting	14–40	24	–	14	9	13	–
Oral pain	–	19	–	62	–	–	–
Stomatitis	–	–	–	48	–	25	–
Abdominal pain	14	–	–	29	–	–	–
Skin events							
Acne	20	–	–	–	–	–	–
Alopecia	–	–	–	48	–	–	–
Dry skin	15	12	–	76	–	–	–
Hand-foot-skin reaction	–	16.6	–	76	26	15	–
Pruritus	–	–	–	33	–	–	–
Rash	45–67	26	–	67	–	15	–
Respiratory disorders							
Cough	10	–	–	–	–	–	
Epistaxis	–	–	–	14	–	–	–
Cardiovascular disorders							
Hypertension	33–41	7.9–16	27	43	3	28	–
QTc prolongation	22	–	–	–	–	–	–
Blood system							
Leucopenia	–	–	–	52	31	–	–
Neutropenia	–	–	–	33	34–60	–	8
Thrombocytopenia	–	–	–	58	3–70	–	7
Anemia	–	–	–	38	6–10	–	–
Others							
Fatigue	24–63	9.3–55	41	5	26	50	70
Anorexia	16–43	–	27	8	3	30	31
Headache	26–47	–	–	7	–	13	–
Proteinuria	–	–	–	–	–	18	–

Notes: *Irrespective of causality; –, not reported.

Vandetanib (ZD6474, Zactima)

Vandetanib is an agent that selectively targets RET, vascular endothelial growth factor (VEGFR) and epidermal growth factor (EGFR) receptors.[61, 62] In human MTC cell lines, vandetanib inhibited the cell proliferation and phosphorylation of RET receptor, EGFR and MAPK pathways.[63]

The activity profile of this drug made it a good choice as a treatment for patients with unresectable MTC. A phase II clinical trial assessed the efficacy of vandetanib (300 mg once-daily) in patients with advanced hereditary MTC. A total of 30 patients were enrolled; a partial response was achieved in 20% of these patients and durable

stable disease for ≥ 24 weeks was reported in 53 % of the patients. Therefore, disease control rate was 73% and serum calcitonin levels decreased 50% or more in 80% of the patients.[64] Similar results were described in 19 patients with metastatic hereditary MTC receiving 100 mg/day vandetanib, where the disease control rate was 68%.[65] However, no direct comparison of the efficacy at each dose level - 100 or 300 mg/day - has been conducted.

More recently, in a large trial, 331 adults with metastatic MTC (90% with sporadic disease) were randomized to receive either vandetanib at a dose of 300 mg daily or placebo. A significant improvement in progression-free survival was observed for patients randomized to receive vandetanib (hazard ratio = 0.46, 95% CI 0.31–0.69). The rate of mortality in two-year follow-up was 15%. A subgroup analysis of the progression-free survival, in sporadic MTC patients, suggested that *RET* M918T mutation–positive patients had a higher response rate to vandetanib compared with *RET* M918T mutation–negative patients.[64, 66]

Based on these results, the U.S. Food and Drug Administration (FDA) approved vandetanib for the treatment of symptomatic or progressive medullary thyroid cancer in patients with unresectable locally advanced or metastatic disease - www.fda.gov.[67] Meanwhile, it is important to emphasize that preclinical studies have evidenced that *RET* activating mutations at codon 804 (V804L, V804M) cause resistance to some TKIs, such as vandetanib.[68]

Cabozantinib (XL184)

XL184 is a potent inhibitor of MET, VEGFR 2 and RET. A phase I study of XL184 (maximum-tolerated dose 175 md daily) was conducted in 37 patients with MTC. Overall, 68% of patients had a stable disease for 6 months or longer or confirmed partial response.[69] Data about a phase III study with XL184 in metastatic MTC, presented at the Annual Meeting of the American Society of Clinical Oncology 2012, demonstrated in an interim analysis that the XL184 treatment resulted in prolongation of progression free survival when compared wih placebo (11.2 vs 4.0 months, respectively).[70] The XL184 was also recently approved by the U.S. FDA for the treatment of MTC, in November 2012.

Motesanib (AMG 706)

Motesanib is a multi-kinase inhibitor that targets VEGFR 1, 2 and 3, platelet-derived growth factor receptor (PDGFR) and stem cell factor receptor (c-Kit). In a previous study, this compound potently inhibited angiogenesis in a variety of in vivo models and it was able to induce regressions of large established tumor xenografts.[71]

Recently, the effects of motesanib on wild-type and mutant RET activity in a mouse model of MTC were described. Treatment with motesanib resulted in substantial inhibition of RET tyrosine phosphorylation and VEGFR2 phosphorylation in TT tumor cell xenografts.[72]

A single-arm study, phase II, investigated the efficacy of motesanib (125 mg once daily) in 91 patients with advanced MTC. Eighty-one percent of patients had stable disease (SD) and 48% had durable SD (≥24 weeks); however, the overall response rate observed was only 2%. The clinical benefit rate was 51% (objective response and durable SD).[73] Another study found that changes from baseline in serum placental growth factor (PIGF) and soluble VEGFR 2 levels, after initiation of therapy with motesanib, predicted the therapeutic response in patients with metastatic medullary thyroid cancer.[74]

Sorafenib (BAY-43-9006)

Sorafenib is a multikinase inhibitor with potent activity against RAF and RTKs. Sorafenib inhibits oncogenic RET kinase activity in NIH3T3 cells while induces growth arrest in TT cells (C634R *RET* mutation–positive MTC cell line). Moreover, sorafenib inhibits the growth of cells carrying *RET* V804L or *RET* V804M, both mutants that are resistant to others TKIs.[75] In cell-based assays, sorafenib exhibits potent inhibition of several RTKs involved in tumor angiogenesis and is able to block autophosphorylation of VEGFR-2, VEGFR-3, PDGFR, FMS-like tyrosine kinase 3 (Flt-3), and c-Kit.[76]

A small observacional study, investigated the efficacy of sorafenib in 5 patients with progressive MTC; after 6 months, 2 patients showed a partial response and 2 patients exhibited stable disease.[77] In a phase II trial, 21 patients with metastatic or locally advanced MTC, hereditary or sporadic form, were enrolled to receive 400mg orally twice daily of sorafenib. The hereditary arm of the study was prematurely closed and it was therefore not possible to conclude on the effect of sorafenib; in the sporadic

MTC group, 50% of the patients demonstrated durable stable disease ≥ 15 months and only one partial response (6%). Eleven patients had a decrease in calcitonin and CEA.[58] More recently, another phase II trial examined a total of 15 patients with metastatic MTC treated with sorafenib. The radiological response rate was achieved for 25% of patients.[78]

To investigate the hypothesis that combinations of drugs with different therapeutic targets are synergistically effective and thereby it could be a better option to treat thyroid malignancies, the combination of sorafenib and tipifarnib - a selective oral farnesyltransferase inhibitor - was employed in a phase I trial. Of the 35 patients studied, 13 had MTC and 22 differentiated thyroid cancer. The MTC partial response rate was 38% and the stable disease rate, of at least 6 months, was 31%.[79] More recently a synergistic effect of sorafenib and AZD6244 (a MEK inhibitor) was demonstrated in the inhibition of human MTC cells, in vitro.[80] Despite the limitations in comparing different studies, it seems that combined treatment offers higher rates of partial response than the use of sorafenib only. Sorafenib is currently approved by U.S FDA for renal cell and hepatocellular carcinomas.

Sunitinib (SU11248)

Sunitinib is a small molecule that inhibits members of the RTKs family including the VEGFR-1, VEGFR-2, PDGFR, c-Kit and RET.[81-83]

Recently, two patients with metastatic MTC received sunitinib (50 mg/d, for 28 days, followed by 14 days of no treatment) with a satisfactory response.[84, 85] In a phase II study, thirty-five patients with advanced thyroid cancer – seven of them with MTC- received sunitinib at a dose of 37.5 mg daily. The objective response included 1 complete response (3%), 10 partial responses (28%) and 16 patients (46%) with stable disease.[86]

Currently ongoing, a phase II trial aims to determine the efficacy of sunitinib in patients with locally advanced or metastatic thyroid cancer. The partial results of the 15 patients with MTC show 33.3% partial response and 26.7% stable disease for ≥12 weeks.[87]

The U. S. FDA has approved sunitinib for treatment of advanced renal cell carcinoma and gastrointestinal stromal sarcomas.

Axitinib (AG-013736)

Axitinib is an oral tyrosine kinase inhibitor with a selectivity and potency against VEGFR-1, VEGFR-2 and VEGFR-3.[88] A multicenter, open-label phase II study of the 60 patients with advanced thyroid cancer, of whom 18% had MTC, was conducted using 5 mg daily of axitinib. In MTC patients only, the confirmed partial response rate was 18% and stable disease rate was 27%.[89]

Imatinib (STI571)

Imatinib is a TKIs used to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors. In MTC-derived cell lines expressing mutant RET receptors, imatinib inhibited RET Y1062 phosphorylation and induced cell-cycle arrest and apoptoptic cell death. However, the IC 50 of imatinib (the concentration that causes 50% growth inhibition) necessary to inhibit RET in vitro is higher than other small-molecule kinase inhibitors of RET activity.[90] An open-label trial evaluated nine patients with unresectable and progressive MTC treated with imatinib (600 mg daily) for 12 months. A complete or partial response was not seen; after 6 months five patients had stable disease and after 12 months only one.[91] Similar results were found in another clinical trial with imatinib in the same doses. Of the 15 patients with disseminated MTC treated for up to 12 months, 4 patients had stable disease over 24 months.[92]

A recent study compared the effect of four TKIs (axitinib, sunitinib, vandetanib, and XL184) on cell proliferation, RET autophosphorylation, and ERK activation in three cell lines: MZ-CRC-1 (M918T *RET* mutation), MTC-TT (C634W *RET* mutation) and TPC-1 (RET/PTC-1 rearrangement) cells. The results showed that all four TKIs were capable of reducing cell proliferation, yet XL184 was the most efficient inhibitor for MEN2A and vandetanib was the most potent inhibitor for MEN2B.[93] These data suggest that the use of specific treatments for each mutation could provide additional benefits in the management of metastatic MTC.

Safety and tolerability of receptor tyrosine kinase inhibitor therapies and implications for disease management

The tyrosine kinase inhibitors are used as chronic therapies, and therefore it becomes important to understand the profile of adverse effects. Generally, these effects are tolerable and the majority of patients have manageable toxicity. However, TKI-

related serious adverse events leading to death have been also observed and include aspiration pneumonia, respiratory arrest, respiratory failure, sepsis, staph infection, and acute heart failure and arrhythmia.[66, 94, 95]

Common adverse events associated with TKIs are diarrhea, rash, fatigue and nausea. Others adverse events related to use of TKIs included hypertension, neutropenia, leucopenia, hand-foot syndrome, stomatitis, proteinuria, abdominal pain, facial edema, thrombocytopenia, malaise, laryngeal mucosal swelling, QTc prolongation, among others (see Table 2).[58, 64-66, 69, 73, 77, 78, 86, 87, 89, 91, 92] Endocrine dysfunctions are often a side effect of TKIs treatment. The most frequent is hypothyroidism, which required an increase in thyroid replacement dose in approximately 50% of patients. However, the mechanism of hypothyroidism induction is still unclear.[96]

Conclusions and future directions

Until recently, patients with advanced or metastatic MTC receive only palliative care to relieve disabling symptoms, since the chemotherapy and radiotherapy have unsatisfactory results. In recent years, the cumulative knowledge of molecules and intracellular signaling pathways involved in the pathogenesis of MTC has allowed the use of new targeted therapies. Different TKIs have been studied in the management of metastatic MTC. The results demonstrated that TKIs are able to induce partial responses or stabilization of tumor growth. However, it is important to remember that TKIs also interact with physiological functions causing a number of highly toxic side effects. Moreover, most of the clinical trials were performed on a small number of patients with a brief follow-up period, since tumor growth is very slow in MTC. Therefore, caution is essential on identifying patients who will benefit for such therapies. Gathering information about the targets of action of these drugs as well as TKI-associated side effects will help on choosing the best therapeutic approach in order to enhance its benefits.

References

1. Hazard JB, Hawk WA, Crile G, Jr. Medullary (solid) carcinoma of the thyroid; a clinicopathologic entity. *J Clin Endocrinol Metab.* Jan 1959;19(1):152-161.

2. Davies L, Welch HG. Increasing incidence of thyroid cancer in the United States, 1973-2002. *JAMA.* May 10 2006;295(18):2164-2167.

3. Giraudet L, Al Ghulzan A, Auperin A, et al. Progression of medullary thyroid carcinoma: assessment with calcitonin and carcinoembryonic antigen doubling times. *Eur J Endocrinol.* Feb 2008;158(2):239-246.

4. Meijer JA, le Cessie S, van den Hout WB, et al. Calcitonin and carcinoembryonic antigen doubling times as prognostic factors in medullary thyroid carcinoma: a structured meta-analysis. *Clin Endocrinol (Oxf).* Apr 2010;72(4):534-542.

5. Girelli ME, Nacamulli D, Pelizzo MR, et al. Medullary thyroid carcinoma: clinical features and long-term follow-up of seventy-eight patients treated between 1969 and 1986. *Thyroid.* Jun 1998;8(6):517-523.

6. Modigliani E, Cohen R, Campos JM, et al. Prognostic factors for survival and for biochemical cure in medullary thyroid carcinoma: results in 899 patients. The GETC Study Group. Groupe d'etude des tumeurs a calcitonine. *Clin Endocrinol (Oxf).* Mar 1998;48(3):265-273.

7. Gilliland FD, Hunt WC, Morris DM, Key CR. Prognostic factors for thyroid carcinoma. A population-based study of 15,698 cases from the Surveillance, Epidemiology and End Results (SEER) program 1973-1991. *Cancer.* Feb 1 1997;79(3):564-573.

8. Saad MF, Ordonez NG, Rashid RK, et al. Medullary carcinoma of the thyroid. A study of the clinical features and prognostic factors in 161 patients. *Medicine (Baltimore).* Nov 1984;63(6):319-342.

9. Steiner A, Goodman A, Powers S. Study of kindred with pheochromocytoma, medullary thyroid carcinoma, hyperparathyroidism and Cushings disease: multiple endocrine neoplasia, type 2. . *Medicine (Baltimore).* 1968;47:371-409.

10. Pelizzo MR, Boschin IM, Bernante P, et al. Natural history, diagnosis, treatment and outcome of medullary thyroid cancer: 37 years experience on 157 patients. *Eur J Surg Oncol.* May 2007;33(4):493-497.

11. Eng C, Clayton D, Schuffenecker I, et al. The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. *JAMA.* Nov 20 1996;276(19):1575-1579.

12. Mulligan LM, Marsh DJ, Robinson BG, et al. Genotype-phenotype correlation in multiple endocrine neoplasia type 2: report of the International RET Mutation Consortium. *J Intern Med.* Oct 1995;238(4):343-346.

13. Decker RA, Peacock ML, Watson P. Hirschsprung disease in MEN 2A: increased spectrum of RET exon 10 genotypes and strong genotype-phenotype correlation. *Hum Mol Genet.* Jan 1998;7(1):129-134.

14. Gagel RF, Levy ML, Donovan DT, Alford BR, Wheeler T, Tschen JA. Multiple endocrine neoplasia type 2a associated with cutaneous lichen amyloidosis. *Ann Intern Med.* Nov 15 1989;111(10):802-806.

15. Machens A, Gimm O, Hinze R, Hoppner W, Boehm BO, Dralle H. Genotype-phenotype correlations in hereditary medullary thyroid carcinoma: oncological features and biochemical properties. *J Clin Endocrinol Metab.* Mar 2001;86(3):1104-1109.

16. Kebebew E, Ituarte PH, Siperstein AE, Duh Q, Clark OH. Medullary thyroid cancer. *Curr Treat Options Oncol.* Oct 2000;1(4):359-367.

17. Farndon JR, Leight GS, Dilley WG, et al. Familial medullary thyroid carcinoma without associated endocrinopathies: a distinct clinical entity. *Br J Surg.* Apr 1986;73(4):278-281.

18. Heshmati HM, Gharib H, van Heerden JA, Sizemore GW. Advances and controversies in the diagnosis and management of medullary thyroid carcinoma. *Am J Med.* Jul 1997;103(1):60-69.

19. Elisei R, Cosci B, Romei C, et al. Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *J Clin Endocrinol Metab.* Mar 2008;93(3):682-687.

20. Moura MM, Cavaco BM, Pinto AE, et al. Correlation of RET somatic mutations with clinicopathological features in sporadic medullary thyroid carcinomas. *Br J Cancer.* Jun 2 2009;100(11):1777-1783.

21. Mulligan LM, Kwok JB, Healey CS, et al. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature.* Jun 3 1993;363(6428):458-460.

22. Roman S, Lin R, Sosa JA. Prognosis of medullary thyroid carcinoma: demographic, clinical, and pathologic predictors of survival in 1252 cases. *Cancer.* Nov 1 2006;107(9):2134-2142.

23. Da Silva AM, Maciel RM, Da Silva MR, Toledo SR, De Carvalho MB, Cerutti JM. A novel germ-line point mutation in RET exon 8 (Gly(533)Cys) in a large kindred with familial medullary thyroid carcinoma. *J Clin Endocrinol Metab.* Nov 2003;88(11):5438-5443.

24. Myers SM, Eng C, Ponder BA, Mulligan LM. Characterization of RET proto-oncogene 3' splicing variants and polyadenylation sites: a novel C-terminus for RET. *Oncogene.* Nov 16 1995;11(10):2039-2045.

25. Takahashi M, Asai N, Iwashita T, Isomura T, Miyazaki K, Matsuyama M. Characterization of the ret proto-oncogene products expressed in mouse L cells. *Oncogene.* Nov 1993;8(11):2925-2929.

26. Milos IN, Frank-Raue K, Wohllk N, et al. Age-related neoplastic risk profiles and penetrance estimations in multiple endocrine neoplasia type 2A caused by germ line RET Cys634Trp (TGC>TGG) mutation. *Endocr Relat Cancer.* Dec 2008;15(4):1035-1041.

27. Frank-Raue K, Hoppner W, Frilling A, et al. Mutations of the ret protooncogene in German multiple endocrine neoplasia families: relation between genotype and phenotype. German Medullary Thyroid Carcinoma Study Group. *J Clin Endocrinol Metab.* May 1996;81(5):1780-1783.

28. Verga U, Fugazzola L, Cambiaghi S, et al. Frequent association between MEN 2A and cutaneous lichen amyloidosis. *Clin Endocrinol (Oxf).* Aug 2003;59(2):156-161.

29. Donis-Keller H, Dou S, Chi D, et al. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet.* Jul 1993;2(7):851-856.

30. Frank-Raue K, Rybicki LA, Erlic Z, et al. Risk profiles and penetrance estimations in multiple endocrine neoplasia type 2A caused by germline RET mutations located in exon 10. *Hum Mutat.* Jan 2011;32(1):51-58.

31. Mulligan LM, Eng C, Healey CS, et al. Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. *Nat Genet.* Jan 1994;6(1):70-74.

32. Punales MK, Graf H, Gross JL, Maia AL. RET codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. *J Clin Endocrinol Metab.* Jun 2003;88(6):2644-2649.

33. Gimm O, Marsh DJ, Andrew SD, et al. Germline dinucleotide mutation in codon 883 of the RET proto-oncogene in multiple endocrine neoplasia type 2B without codon 918 mutation. *J Clin Endocrinol Metab.* Nov 1997;82(11):3902-3904.

34. Smith DP, Houghton C, Ponder BA. Germline mutation of RET codon 883 in two cases of de novo MEN 2B. *Oncogene.* Sep 4 997; 15(10): 1213-1217.

35. Menko FH, van der Luijt RB, de Valk IA, et al. Atypical MEN type 2B associated with two germline RET mutations on the same allele not involving codon 918. *J Clin Endocrinol Metab.* Jan 2002;87(1):393-397.

36. Miyauchi A, Futami H, Hai N, et al. Two germline missense mutations at codons 804 and 806 of the RET proto-oncogene in the same allele in a patient with multiple endocrine neoplasia type 2B without codon 918 mutation. *Jpn J Cancer Res.* Jan 1999;90(1):1-5.

37. Zedenius J, Larsson C, Bergholm U, et al. Mutations of codon 918 in the RET proto-oncogene correlate to poor prognosis in sporadic medullary thyroid carcinomas. *J Clin Endocrinol Metab.* Oct 1995;80(10):3088-3090.

38. Hofstra RM, Fattoruso O, Quadro L, et al. A novel point mutation in the intracellular domain of the ret protooncogene in a family with medullary thyroid carcinoma. *J Clin Endocrinol Metab.* Dec 1997;82(12):4176-4178.

39. Bolino A, Schuffenecker I, Luo Y, et al. RET mutations in exons 13 and 14 of FMTC patients. *Oncogene.* Jun 15 1995;10(12):2415-2419.

40. Dabir T, Hunter SJ, Russell CF, McCall D, Morrison PJ. The RET mutation E768D confers a late-onset familial medullary thyroid carcinoma -- only phenotype with incomplete penetrance: implications for screening and management of carrier status. *Fam Cancer.* 2006;5(2):201-204.

41. Eng C, Smith DP, Mulligan LM, et al. A novel point mutation in the tyrosine kinase domain of the RET proto-oncogene in sporadic medullary thyroid carcinoma and in a family with FMTC. *Oncogene.* Feb 2 1995;10(3):509-513.

42. Eng C, Mulligan LM, Healey CS, et al. Heterogeneous mutation of the RET proto-oncogene in subpopulations of medullary thyroid carcinoma. *Cancer Res.* May 1 1996;56(9):2167-2170.

43. Siqueira DR, Romitti M, da Rocha AP, et al. The RET polymorphic allele S836S is associated with early metastatic disease in patients with hereditary or sporadic medullary thyroid carcinoma. *Endocr Relat Cancer.* 2010;17(4):953-963.

44. Romei C, Elisei R, Pinchera A, et al. Somatic mutations of the ret protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. *J Clin Endocrinol Metab.* Apr 1996;81(4):1619-1622.

45. Ceolin L, Siqueira DR, Romitti M, Ferreira CV, Maia AL. Molecular Basis of Medullary Thyroid Carcinoma: The Role of RET Polymorphisms. *Int J Mol Sci.* 2012;13(1):221-239.

46. Elisei R, Cosci B, Romei C, et al. RET exon 11 (G691S) polymorphism is significantly more frequent in sporadic medullary thyroid carcinoma than in the general population. *J Clin Endocrinol Metab.* Jul 2004;89(7):3579-3584.

47. Gimm O, Neuberg DS, Marsh DJ, et al. Over-representation of a germline RET sequence variant in patients with sporadic medullary thyroid carcinoma and somatic RET codon 918 mutation. *Oncogene.* Feb 11 1999;18(6):1369-1373.

48. Robledo M, Gil L, Pollan M, et al. Polymorphisms G691S/S904S of RET as genetic modifiers of MEN 2A. *Cancer Res.* Apr 15 2003;63(8):1814-1817.

49. Rocha AP, Magalhães PR, Maia AL, Maciel LZ. Polimorfismos Genéticos: Implicações na Patogênese do Carcinoma Medular de Tireóide. *Arq Bras Endocrinol Metab.* 2007;5(51):723-730.

50. Scollo C, Baudin E, Travagli JP, et al. Rationale for central and bilateral lymph node dissection in sporadic and hereditary medullary thyroid cancer. *J Clin Endocrinol Metab.* May 2003;88(5):2070-2075.

51. Brierley J, Tsang R, Simpson WJ, Gospodarowicz M, Sutcliffe S, Panzarella T. Medullary thyroid cancer: analyses of survival and prognostic factors and the role of radiation therapy in local control. *Thyroid.* Aug 1996;6(4):305-310.

52. Nocera M, Baudin E, Pellegriti G, Cailleux AF, Mechelany-Corone C, Schlumberger M. Treatment of advanced medullary thyroid cancer with an

alternating combination of doxorubicin-streptozocin and 5 FU-dacarbazine. Groupe d'Etude des Tumeurs a Calcitonine (GETC). *Br J Cancer.* Sep 2000;83(6):715-718.

53. Ichihara M, Murakumo Y, Takahashi M. RET and neuroendocrine tumors. *Cancer Lett.* Feb 20 2004;204(2):197-211.

54. Vieira JM, Santos SC, Espadinha C, et al. Expression of vascular endothelial growth factor (VEGF) and its receptors in thyroid carcinomas of follicular origin: a potential autocrine loop. *Eur J Endocrinol.* Nov 2005;153(5):701-709.

55. Broekman F, Giovannetti E, Peters GJ. Tyrosine kinase inhibitors: Multi-targeted or single-targeted? *World J Clin Oncol.* Feb 10 2011;2(2):80-93.

56. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell.* Oct 13 2000;103(2):211-225.

57. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst.* Feb 2 2000;92(3):205-216.

58. Lam ET, Ringel MD, Kloos RT, et al. Phase II clinical trial of sorafenib in metastatic medullary thyroid cancer. *J Clin Oncol.* May 10 2010;28(14):2323-2330.

59. Licitra L, Locati LD, Greco A, Granata R, Bossi P. Multikinase inhibitors in thyroid cancer. *Eur J Cancer.* Apr 2010;46(6):1012-1018.

60. Machens A, Dralle H. Parenchymal versus nonparenchymal target lesion response in clinical trials for metastatic medullary thyroid cancer. *J Clin Oncol.* Oct 1 2010;28(28):e534: author reply e535-536.

61. Carlomagno F, Vitagliano D, Guida T, et al. ZD6474, an orally available inhibitor of KDR tyrosine kinase activity, efficiently blocks oncogenic RET kinases. *Cancer Res.* Dec 15 2002;62(24):7284-7290.

62. Wedge SR, Ogilvie DJ, Dukes M, et al. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res.* Aug 15 2002;62(16):4645-4655.

63. Vitagliano D, De Falco V, Tamburrino A, et al. The tyrosine kinase inhibitor ZD6474 blocks proliferation of RET mutant medullary thyroid carcinoma cells. *Endocr Relat Cancer.* Feb 2011;18(1):1-11.

64. Wells SA, Jr., Gosnell JE, Gagel RF, et al. Vandetanib for the treatment of patients with locally advanced or metastatic hereditary medullary thyroid cancer. *J Clin Oncol.* Feb 10 2010;28(5):767-772.

65. Robinson BG, Paz-Ares L, Krebs A, Vasselli J, Haddad R. Vandetanib (100 mg) in patients with locally advanced or metastatic hereditary medullary thyroid cancer. *J Clin Endocrinol Metab.* Jun 2010;95(6):2664-2671.

66. Wells SA, Jr., Robinson BG, Gagel RF, et al. Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. *J Clin Oncol.* Jan 10 2012;30(2):134-141.

67. Deshpande H, Roman S, Thumar J, Sosa JA. Vandetanib (ZD6474) in the Treatment of Medullary Thyroid Cancer. *Clinical Medicine Insights: Oncology.* 2011;5 213-221.

68. Carlomagno F, Guida T, Anaganti S, et al. Disease associated mutations at valine 804 in the RET receptor tyrosine kinase confer resistance to selective kinase inhibitors. *Oncogene.* 2004;23:6056–6063.

69. Kurzrock R, Sherman SI, Ball DW, et al. Activity of XL184 (Cabozantinib), an oral tyrosine kinase inhibitor, in patients with medullary thyroid cancer. *J Clin Oncol.* Jul 1 2011;29(19):2660-2666.

70. Schoffski P, Elisei R, Müller S, et al. An international, double-blind, randomized, placebo-controlled phase III trial (EXAM) of cabozantinib (XL184) in medullary thyroid carcinoma (MTC) patients (pts) with documented RECIST progression at baseline. *J Clin Oncol.* 2012;30:suppl; abstr 5508.

71. Polverino A, Coxon A, Starnes C, et al. AMG 706, an oral, multikinase inhibitor that selectively targets vascular endothelial growth factor, platelet-derived growth factor, and kit receptors, potently inhibits angiogenesis and induces regression in tumor xenografts. *Cancer Res.* Sep 1 2006;66(17):8715-8721.

72. Coxon A, Bready J, Kaufman S, et al. Anti-tumor activity of motesanib in a medullary thyroid cancer model. *J Endocrinol Invest.* Feb 2011;35(2):181-190.

73. Schlumberger MJ, Elisei R, Bastholt L, et al. Phase II study of safety and efficacy of motesanib in patients with progressive or symptomatic, advanced or metastatic medullary thyroid cancer. *J Clin Oncol.* Aug 10 2009;27(23):3794-3801.

74. Bass MB, Sherman SI, Schlumberger MJ, et al. Biomarkers as predictors of response to treatment with motesanib in patients with progressive advanced thyroid cancer. *J Clin Endocrinol Metab.* Nov 2010;95(11):5018-5027.

75. Carlomagno F, Anaganti S, Guida T, et al. BAY 43-9006 inhibition of oncogenic RET mutants. *J Natl Cancer Inst.* Mar 1 2006;98(5):326-334.

76. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res.* Oct 1 2004;64(19):7099-7109.

77. Frank-Raue K, Ganten M, Kreissl MC, Raue F. Rapid response to sorafenib in metastatic medullary thyroid carcinoma. *Exp Clin Endocrinol Diabetes.* Mar 2011;119(3):151-155.

78. Ahmed M, Barbachano Y, Riddell A, et al. Analysis of the efficacy and toxicity of sorafenib in thyroid cancer: a phase II study in a UK based population. *Eur J Endocrinol.* Aug 2011;165(2):315-322.

79. Hong DS, Cabanillas ME, Wheler J, et al. Inhibition of the Ras/Raf/MEK/ERK and RET kinase pathways with the combination of the multikinase inhibitor sorafenib and the farnesyltransferase inhibitor tipifarnib in medullary and differentiated thyroid malignancies. *J Clin Endocrinol Metab.* Apr 2011;96(4):997-1005.

80. Koh YW, Shah MH, Agarwal K, et al. Sorafenib and Mek inhibition is synergistic in medullary thyroid carcinoma in vitro. *Endocr Relat Cancer.* Feb 2012;19(1):29-38.

81. Broutin S, Ameur N, Lacroix L, et al. Identification of soluble candidate biomarkers of therapeutic response to sunitinib in medullary thyroid carcinoma in preclinical models. *Clin Cancer Res.* Apr 1 2011;17(7):2044-2054.

82. Chow LQ, Eckhardt SG. Sunitinib: from rational design to clinical efficacy. *J Clin Oncol.* Mar 1 2007;25(7):884-896.

83. Schueneman AJ, Himmelfarb E, Geng L, et al. SU11248 maintenance therapy prevents tumor regrowth after fractionated irradiation of murine tumor models. *Cancer Res.* Jul 15 2003;63(14):4009-4016.

84. Cleary JM, Sadow PM, Randolph GW, et al. Neoadjuvant treatment of unresectable medullary thyroid cancer with sunitinib. *J Clin Oncol.* Aug 10 2010;28(23):e390-392.

85. Kelleher FC, McDermott R. Response to sunitinib in medullary thyroid cancer. *Ann Intern Med.* Apr 1 2008;148(7):567.

86. Carr LL, Mankoff DA, Goulart BH, et al. Phase II study of daily sunitinib in FDG-PET-positive, iodine-refractory differentiated thyroid cancer and metastatic medullary carcinoma of the thyroid with functional imaging correlation. *Clin Cancer Res.* Nov 1 2010;16(21):5260-5268.

87. Ravaud A, de la Fouchardiere C, Asselineau J, et al. Efficacy of sunitinib in advanced medullary thyroid carcinoma: intermediate results of phase II THYSU. *Oncologist.* 2010;15(2):212-213; author reply 214.

88. Inai T, Mancuso M, Hashizume H, et al. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol.* Jul 2004;165(1):35-52.

89. Cohen EE, Rosen LS, Vokes EE, et al. Axitinib is an active treatment for all histologic subtypes of advanced thyroid cancer: results from a phase II study. *J Clin Oncol.* Oct 10 2008;26(29):4708-4713.

90. de Groot JW, Plaza Menacho I, Schepers H, et al. Cellular effects of imatinib on medullary thyroid cancer cells harboring multiple endocrine neoplasia Type 2A and 2B associated RET mutations. *Surgery.* Jun 2006;139(6):806-814.

91. Frank-Raue K, Fabel M, Delorme S, Haberkorn U, Raue F. Efficacy of imatinib mesylate in advanced medullary thyroid carcinoma. *Eur J Endocrinol.* Aug 2007;157(2):215-220.

92. de Groot JW, Zonnenberg BA, van Ufford-Mannesse PQ, et al. A phase II trial of imatinib therapy for metastatic medullary thyroid carcinoma. *J Clin Endocrinol Metab.* Sep 2007;92(9):3466-3469.

93. Verbeek HH, Alves MM, de Groot JW, et al. The effects of four different tyrosine kinase inhibitors on medullary and papillary thyroid cancer cells. *J Clin Endocrinol Metab.* Jun 2011;96(6):E991-995.

94. Lenihan DJ, Cardinale DM. Late cardiac effects of cancer treatment. *J Clin Oncol.* Oct 20 2012;30(30):3657-3664.

95. Sheffel R, Dora JM, Siqueira DR, Burttet LM, Cerski MR, Maia AL. Toxic Cardiomyophaty leading to fatal acute cardiac failure related to vandetanib: a case report with histopathological analysis. (Submitted)

96. Torino F, Corsello SM, Longo R, Barnabei A, Gasparini G. Hypothyroidism related to tyrosine kinase inhibitors: an emerging toxic effect of targeted therapy. *Nat Rev Clin Oncol.* Apr 2009;6(4):219-228.

PARTE II

THE ROLE OF ANGIOGENESIS MARKERS IN PHEOCHROMOCYTOMA

THE ROLE OF ANGIOGENESIS MARKERS IN PHEOCHROMOCYTOMA

Carla Vaz Ferreira[1], Débora Rodrigues Siqueira[1], Mírian Romitti[1], Lucieli Ceolin[1], Beatriz Assis Brasil[2], Luise Meurer[2], Clarissa Capp[1], Ana Luiza Maia[1].

[1]Thyroid Section, Endocrine Division, [2]Pathology Department, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Grant support: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS), Fundo de Incentivo a Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE) and Programa de Apoio a Núcleos de Excelência (PRONEX), Brazil.

Running title: Angiogenesis markers in pheochromocytoma

Correspondence: Ana Luiza Maia, M.D., Ph.D.

Serviço de Endocrinologia

Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos 2350

90035-003 Porto Alegre, RS, Brazil

Phone: 55-51-3359-8127; Fax: 55-51-3331-0207; E-mail: almaia@ufrgs.br

Abstract

Background: Pheochromocytoma (PHEO), a rare catecholamine producing tumor arising from the chromaffin cells, may occurs sporadically (76-80%) or as part of inherited syndromes (20-24%). The malignant form is observed in about 14-35% of cases. Several studies have demonstrated that angiogenesis is a fundamental step in tumor proliferation and vascular endothelial growth factor (VEGF-A) seems to be central to this process. Accordingly, VEGF-A inhibitors are now widely used as anticancer agents. The role of the angiogenic markers in PHEO is not fully understood. Objectives: Evaluate the expression of VEGF-A and its receptors in PHEO and correlate to clinical and laboratory parameters. Methods: Twenty-two tumor specimens of PHEO (10 MEN2 and 12 sporadic, including three with malignant disease) were evaluated for VEGF-A, VEGFR-1, VEGFR-2 and microvessel density (MVD) expression by immunohistochemistry. Results: The mean age of patients with PHEO was 36.1 ±13.8 years. VEGF-A immunohistochemical staining was detected in 90.9%, VEGFR-1 in 45.5% and VEGFR-2 in 54.6% of PHEO samples. No statistically significant correlation was detected between VEGF-A, VEGFR-1 and VEGFR-2 expression and age at diagnosis ($p=0.87$, $p=0.06$ and $p=0.67$, respectively) or tumor size ($p=0.76$, $p=0.57$ and $p=0.44$, respectively). However, a significant difference was found on VEGFR-1, VEGFR-2 and MVD expression between benign and malignant PHEO ($p=0.027$, $p=0.029$ and $p=0.024$, respectively). Conclusions: These data demonstrated high expression of the VEGF-A and its receptors in PHEO and suggest that target therapy for these factors can be considered in unresectable or metastatic tumors.

Keywords: Pheocromocytoma; VEGF-A; microvessel density

Introduction

Pheochromocytoma (PHEO) is a rare catecholamine producing tumor arising from the chromaffin cells of the adrenal medulla (85% of cases) or extra-adrenal paraganglia (1, 2). The clinical picture usually results from excess secretion of catecholamines. Metastases may be present in about 14-35% of cases (3-5). The tumor may occur sporadically or, in approximately 20-24% of cases, as part of the inherited syndrome. The hereditary PHEO appears as a component of multiple endocrine neoplasia type 2 (MEN 2), von Hippel-Lindau disease (VHL), neurofibromatosis type 1 (NF1) or familial paraganglioma syndromes (caused by mutations of *SDHD* and *SDHB* genes) (6-8).

The tumor development and your survival depends on an adequate supply of oxygen and nutrients (9). Angiogenesis, the development of new blood vessels from established vasculature, provides growth and hematogenous dissemination of the cancer cells (10). Several pro-angiogenic and anti-angiogenic molecules are involved in the regulation of this process (11). Of them, the vascular endothelial growth factor (VEGF; VEGF-A) is the most well-characterized angiogenic factor (9).

VEGF-A, a cytokine that exerts a critical role in both pathologic and physiologic angiogenesis, binds and activates two tyrosine kinase receptors: vascular endothelial growth factor receptor 1 (VEGFR-1; Flt-1) and vascular endothelial growth factor receptor 2 (VEGFR-2; KDR; Flk-1) (12); on binding to its receptors, VEGF-A initiates a cascade of signaling events resulting in the activation of downstream proteins, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (13, 14). Several studies have demonstrated that VEGF-A mRNA is up-regulated in different human tumors, including lung, breast, gastrointestinal tract and kidney (15). Furthermore, VEGF-A expression has been associated with poor prognosis in human tumors (16-18).

Pheochromocytomas are well-vascularized tumors but the role of VEGF-A and its receptors is poorly understood. Takekoshi et al observed increased levels of VEGF-A and its receptors in 11 tumor specimens and suggested that upregulation of these molecules may be important in PHEO pathogenesis (19). Moreover, associations between the intensity of VEGF-A expression and microvessel density (MVD) in tumor tissue and malignant phenotype have been reported (20, 21). Others studies confirmed the relationship between VEGF-A expression and malignancy, but found no association

with MVD (20, 22, 23). However, these studies evaluated the expression of VEGF in PHEO samples classified according the malignancy, but not according the hereditable characteristic. In addition, other markers of angiogenesis as VEGFR-1 and VEGFR-2 were not evaluated.

The PHEO-associated MEN 2 syndrome includes two clinically distinct forms: MEN 2A and MEN 2B. Patients with MEN 2A develop medullary thyroid carcinoma (MTC), PHEO, and/or primary hyperparathyroidism (HPT). MEN 2B patients have MTC, PHEO, ganglioneuromas of the digestive tract, mucosal neuromas, and/or skeletal abnormalities (24). The *RET* proto-oncogene, the susceptibility gene for MEN 2, encodes a receptor tyrosine kinase (25). In MEN 2-associated MTC samples, an overexpression of the VEGF-A and its receptors was demonstrated, suggesting that these molecules might be implicated in tumor progression (26).

Surgery is the treatment of choice for the majority of patients. However the procedure might be a challenge in patients with large tumors and malignant disease. Chemotherapy and radiotherapy have limited response rates for those malignant cases the management of advanced disease is a major challenge (27, 28). Currently, molecular targeted therapies are considered as the most promising strategies for metastatic disease. These therapies are designed to target a specific molecule, such as VEGF-A and RET receptors. Here, we analyzed the expression of VEGF-A, VEGFR-1 and VEGFR-2 and MVD by immunohistochemistry in samples of sporadic, malignant and MEN 2-associated PHEO.

Material and Methods

Patients

We have studied tumor specimens from 22 patients with a diagnosis of PHEO obtained from patients attending the Endocrine Division at Hospital de Clínicas de Porto Alegre (a tertiary care, university-based teaching hospital). Our Institution is a referral center for molecular diagnosis of hereditary MTC. Ten patients had MEN 2-associated PHEO, whereas twelve had apparently sporadic PHEO (sPHEO). All patients with MEN2-associated PHEO harbor a RET germline mutation, identified by standard procedures described previously (29). The diagnosis was established based on the absence of known *RET* germline mutation, family history of PHEO and/or clinical

phenotype of a specific syndrome. Patients with PHEO metastases at non-chromaffin sites distant from the primary neoplasm (such as bone, liver or lungs) or extensive invasion of surrounding structures were considered as having malignant PHEO (28).

The data collected for each individual included the clinical and histopathological characteristics of PHEO, the association of another endocrine neoplasia, the presence of affected family members and the presence of *RET* germline mutations.

All patients and/or their legal guardians had given written consent in accordance with the institutional Ethics Committee.

Immunohistochemistry analysis (IHC)

IHC was performed on thin sections (3 mm) of previously formalin-fixed and paraffin-embedded tissues. The antibodies used were polyclonal rabbit antihuman VEGF-A (clone VG1; M7273 Dako Cytomation, Carpinteria, CA), monoclonal mouse anti-human VEGFR-1 (Flt-1/EWC, ab9540; Abcam Inc., Cambridge, MA), and monoclonal mouse antihuman VEGFR-2 (A-3: SC-6251; Santa Cruz Biotechnology, Santa Cruz, CA). Sections representing PHEO were submitted to routine immunohistochemical technique, which comprises deparaffination and rehydration, antigenic recovery, inactivation of endogenous peroxidase, and blockage of unspecific reactions. Primary antibodies were incubated overnight at a temperature of 4ºC, at dilutions of 1:400 (VEGF-A), 1:200 (VEGFR-1), and 1:200 (VEGFR-2), followed by application of streptavidin horseradish peroxidase conjugate (LSAB; Dako Cytomation), and diaminobenzidine tetrahydrochloride (Kit DAB; Dako Cytomation). Sections of human tissue were used as a positive control (skeletal muscle tissue for VEGF-A, placenta for VEGFR-1 and intestinal tumor for VEGFR- 2), and omission of the primary antibody as a negative control.

The intensity of VEGF-A, VEGFR-1, and VEGFR-2 staining in each lesion was determined and quantified as grade 0 (absent, -), grade 1 (weak, +), grade 2 (moderate, ++), and grade 3 (strong, +++) based on the staining characteristics of most of the tumor. The slides were read independently by two blinded and experienced pathologists (L.M. and B.A.B.) who were not aware of the respective clinicopathological data. When the two experts differed in their interpretations, a third investigator (C.C.) solved any disagreement.

Microvessel density assessment

For evaluation of MVD as a measure of angiogenesis, samples were prepared for IHC, as described above, using primary anti-CD31 antibody (clone JC7OA, M0823; DakoCytomation), at dilutions of 1:200. Sections of human lung carcinoma were used as positive control and omission of the primary antibody as a negative control.

The Chalkley point technique was used for assessment of vascular density internationally acknowledged as the criterion standard for the evaluation of MVD (30). The densest vascular areas (known as hot spots) were determined at low magnification (x100). For each tissue sample, two or three hotspots were selected, depending on the size of the tumor. The mean of the counts for the most angiogenic areas (hot spot) was recorded at x 400 magnification. The Chalkley point count was performed by two observers independently. The final MVD was the mean value of the two independent counts.

The results of MVD are expressed per one mm^2 (x 400; 0.14 mm^2 per field).

Statistical analysis

Results are expressed as median and interquartile intervals. Spearman's coefficient test was used to assess the correlation between expression of the angiogenic markers (VEGF-A, VEGFR-1, or VEGFR-2) and MVD with age at surgery and tumor size. Mann–Whitney's U-test was used to compare angiogenic markers and the number of MVD between patients with sporadic or hereditary disease. Also Mann-Whitney's U-test was used to investigate an association between expression of angiogenic markers or MVD and the presence of metastasis. The Statistical Package for the Social Sciences 18.0 (SPSS Inc., Chicago, IL) was used and $P<0.05$ was considered as statistically significant.

Results

Patients

The clinical features and immunohistochemical staining properties of the 22 patients with PHEO are summarized in Table 1. Ten patients had a MEN2-associated PHEO. The median age was 45 ±13 years and 70% were women. Bilateral PHEO occurred synchronously or metachronously in 3 (30%) of the patients. The identified *RET* germline mutations in MEN2-associated PHEO patients were as follows: 50% C634Y, 30% C634R, 10% C618R and 10% M918T.

Twelve patients have sporadic disease. The median age was 28.6 (±9.7) years and 83.3 % were women. All patients denied family history of PHEO, present unilateral disease and lack clinical features suggestive of known PHEO-associated syndromes. Three patients had metastatic disease: one patient had regional lymph node metastasis and two showed extensive invasion of adjacent structures.

Expression of VEGF-A, VEGFR-1 and VEGFR-2 in Pheocromocytomas

Twenty (90.9%) out of 22 PHEO specimens revealed VEGF-A immunoreactivity. Immunohistochemical staining for VEGFR-1 was present in 10 (45.5%) and VEGFR-2 in 12 of the 22 (54.6%) (Figure 1). Samples of the normal adrenal medulla display a weak expression of VEGF-A, but were negative for VEGFR-1 and VEGFR-2. In tumoral tissue, positive staining of VEGF-A, VEGFR-1, and VEGFR-2 were detectable in adrenal cells.

The VEGFR-1 staining was found to be positively correlated with VEGFR-2 immunoreactivity ($r = 0.72$, $p = 0.001$). No statistically significant correlation was detected between VEGF-A, VEGFR-1 or VEGFR-2 expression and age at diagnosis ($p = 0.87$, $p = 0.06$ and $p = 0.67$, respectively) or tumor size ($p = 0.76$, $p = 0.57$ and $p = 0.44$, respectively). However, patients with malignant PHEO had significant higher levels of VEGFR-1 and VEGFR-2 expression than patients with the benign form of the disease ($p= 0.027$ and $p= 0.029$, respectively; Table 2). Nevertheless, the levels of VEGF-A were similar between the groups.

MVD assessment

The median MVD for all samples was 53.6 microvessels/mm^2 (41.1-85.7). We observed a significant correlation between MVD and VEGF-A ($r = 0.45$ $p = 0.03$). VEGFR-1 and VEGFR-2 were not correlated with MVD ($p = 0.18$ and $p = 0.51$, respectively). The analysis of clinicopathological findings did not reveal a correlation between age at diagnosis or tumor size and MVD ($p = 0.21$ and $p= 0.07$). However, MVD was found to be significantly higher in patients with a malignant PHEO as compared with patients with benign PHEO [92.8 (85.7-92.8) vs. 50.0 (35.7-78.6), $p=0.024$, Table 2].

Sporadic and hereditary PHEO tumors

The expression of VEGF-A, VEGFR-1, VEGFR-2 and MVD were similar in sporadic and hereditary PHEO ($p = 1.0$, $p = 0.657$, $p = 0.820$, $p = 0.412$, respectively; Table 3). The malignant pheochromocytoma samples were excluded of these analyses.

Discussion

In this study, we have evaluated the expression of angiogenic markers in samples of hereditary or sporadic PHEO. We found expression of VEGF-A in the majority of the PHEO tissue samples, whereas VEGFR-1 and VEGFR-2 expression was present in approximately half of the samples. None of these molecules were associated with the clinical presentation of PHEO. However, we observed a significantly different expression of VEGFR-1 and VEGFR-2 between benign and malignant PHEO.

Patients with PHEO often experience debilitating symptoms and may have a poor quality of life, because of excess catecholamine secretion. The main symptoms and signs of PHEO include hypertension, palpitations, headache, sweating and pallor. According to the degree of catecholamine excess, patients may present with myocardial infarction, arrhythmia, stroke or other vascular manifestations (27, 28). Surgery is the treatment of choice for PHEO. However, in metastatic disease curative resection is seldom possible. In these cases, chemotherapy and radiotherapy have been proposed; but, unfortunately, both have limited value (27, 31).

The better understanding of different signaling pathways and multiple genetic abnormalities involved in the pathogenesis of cancer has allowed the development of targeted molecular therapies. Among the novel agents are the protein tyrosine kinase inhibitors. These are small molecules that inhibit different tyrosine kinase receptors, including VEGFR-1, VEGFR-2. The few reports on the use of sunitinib in patients with malignant PHEOs and paragangliomas that report complete or partial responses (32, 33). Interestingly, it was suggested that VEGFR-2 can be involved in the direct cytotoxic effects of sunitinib in PHEO cells (34). These results associated with our findings of increased expression of VEGF-A and its receptors in PHEO, also confirmed by others investigators, support the tyrosine kinase inhibitors as a rational therapeutic target in tumors that develop from chromaffin cells.

Considering the differences between the pathogenesis of hereditary and sporadic PHEO, mainly the presence of germline mutation in inherited forms, it is reasonable to investigate if these issues could interfere with tumoral angiogenesis. In patients with MTC-related MEN2, the analysis of angiogenic factors found that patients with hereditary form display increased expression of VEGFR-1, whereas higher expression of MVD was observed in sporadic MTC (26). A recent study in PHEO patients, evaluated the mRNA expression level of VEGF-A and its receptors in a large cohort of PHEO (102 patients with sporadic PHEO and 86 with hereditary form); the samples were separated into two groups depending on the presence of activation of the pseudo-hypoxic pathway. It was observed that PHEOs associated with *SDHx* mutations and VHL disease had a markedly increase in the expression of major angiogenic molecules than those associated with NF1 disease, MEN 2 syndrome or sporadic tumors. This finding suggests that angiogenesis may be different according to the cause of the tumor (35). Interestingly, another study demonstrated a higher expression level of VEGF-A in hereditary paraganglioma associated with inactivation of the SDHD gene than in sporadic tumors (36). As far as we know, we were the first to compare sporadic with MEN 2-associated PHEO. However, no differences were observed in the intensity of VEGF-A and its receptors or MVD between sporadic and hereditary patients.

The diagnosis of malignancy in PHEOs is difficult because of the lack of reliable histological or molecular markers; according to the World Health Organization, the only criterion to diagnose malignant PHEO is the presence of metastasis at non-chromaffin sites. For patients with metastatic disease, the 5-year overall survival rate ranges from

40% to 77% (37, 38). Thus, the identification of clinical, histopathological or immunohistochemical parameters able to determine early a potentially malignant tumor, even before the onset of clinically evident metastases, could increase the chances of cure. Previous studies showed increased expression of VEGF-A and its receptors in PHEO and demonstrated a correlation between VEGF-A expression and malignant phenotype; these findings suggest that these molecules play an important role in the adrenal tumor pathogenesis. (19-21). Our results confirm that the expression of VEGF receptors and MVD are potential candidates to help in the identification of PHEO with malignant behavior.

In the same way as the VEGF-A expression, tumoral vascular density has been studied as prognostic factor. Several studies have associated a high number of vessels in human tumors with the presence of metastasis (39-41). We did not demonstrate an association between MVD and clinical or pathological features of PHEO, but our findings showed an association between malignant PHEO and vascular density. Previous studies on MVD in PHEO described discrepant results. First, Liu et al correlated the number of tumor blood vessels with the invasive behavior (42). Later, this correlation was not confirmed by Ohji et al (23). More recent reports confirmed our results where we demonstrated higher MVD in malignant PHEO samples (21, 22, 43). Among the possible explanations for these divergences are the use of different techniques for MVD counting and differences among the populations studied.

PHEOs are rare tumors of the adrenal medulla; for this reason it is difficult to perform studies with large samples. Therefore, an important limitation of our study is the small sample size, which may explain the absence of significant findings between VEGF-A and its receptors and clinical and oncological features (type 2 error). In addition, we excluded the presence of other hereditary forms of PHEO only through clinical data, physical examination and family history; unfortunately, we did not have material available for genotyping all the germline mutation in the susceptibility genes of the PHEO. We performed a genetic test just to investigate protooncogene *RET* mutation.

In conclusion, we did not observe any difference in the expression of angiogenic molecules between sporadic and hereditary forms of PHEO. However, we found a higher expression of VEGF receptors and MVD in malignant PHEOs. These results confirm previous findings that described the importance of VEGF-A and its receptors in

the pathogenesis of PHEO. The molecules involved in the tumorigenesis process, such as VEGF-A and its receptors, serve as potential therapeutic targets for new drugs. Thus, current knowledge about the role of tumor angiogenesis in PHEO suggests that tyrosine kinase inhibitors are a promising therapeutic option for patients with metastatic PHEO.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Fundação de Amparo Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brazil), Fundo de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE, Brazil) and Programa de Apoio a Núcleos de Excelência (PRONEX, Brazil).

Declaration of interest

Carla Vaz Ferreira, Débora Rodrigues Siqueira, Mírian Romitti, Lucieli Ceolin, Beatriz Assis Brasil, Luise Meurer, Clarissa Capp, have nothing to declare. Ana Luiza Maia served as consultant/advisor for AstraZeneca within the past 2 years.

References

1. Lenders JW, Eisenhofer G, Mannelli M, Pacak K 2005 Phaeochromocytoma. Lancet. **366**:665-675.

2. Waguespack SG, Rich T, Grubbs E, et al. 2010 A current review of the etiology, diagnosis, and treatment of pediatric pheochromocytoma and paraganglioma. J Clin Endocrinol Metab. **95**:2023-2037.

3. De Toma G, Letizia C, Cavallaro G, et al. 2002 [Malignant pheochromocytoma. Personal experience, review of the literature]. Ann Ital Chir. **73**:413-418; discussion 418-419.

4. Harari A, Inabnet WB, 3rd 2011 Malignant pheochromocytoma: a review. Am J Surg. **201**:700-708.

5. Whalen RK, Althausen AF, Daniels GH 1992 Extra-adrenal pheochromocytoma. J Urol. **147**:1-10.

6. Neumann HP, Bausch B, McWhinney SR, et al. 2002 Germ-line mutations in nonsyndromic pheochromocytoma. N Engl J Med. **346**:1459-1466.

7. Opocher G, Schiavi F, Conton P, et al. 2003 Clinical and genetic aspects of phaeochromocytoma. Horm Res. **59 Suppl 1**:56-61.

8. Plouin PF, Duclos JM, Soppelsa F, et al. 2001 Factors associated with perioperative morbidity and mortality in patients with pheochromocytoma: analysis of 165 operations at a single center. J Clin Endocrinol Metab. **86**:1480-1486.

9. Papetti M, Herman IM 2002 Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol. **282**:C947-970.

10. Folkman J 1990 What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst. **82**:4-6.

11. Bergers G, Benjamin LE 2003 Tumorigenesis and the angiogenic switch. Nat Rev Cancer. **3**:401-410.

12. Shibuya M, Claesson-Welsh L 2006 Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res. **312**:549-560.

13. Petrova TV, Makinen T, Alitalo K 1999 Signaling via vascular endothelial growth factor receptors. Exp Cell Res. **253**:117-130.

14. Zeng Y, Zhao GF, Qian J, et al. 2001 [Effect of oxLDL on the uptake and clearance rate of cholesterol in vascular smooth muscle cells originated from human apoAI transgenic mice]. Zhongguo Yi Xue Ke Xue Yuan Xue Bao. **23**:328-332.

15. Ferrara N 1999 Molecular and biological properties of vascular endothelial growth factor. J Mol Med (Berl). **77**:527-543.

16. Gasparini G, Toi M, Gion M, et al. 1997 Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst. **89**:139-147.

17. Maeda K, Chung YS, Ogawa Y, et al. 1996 Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. Cancer. **77**:858-863.

18. Salven P, Teerenhovi L, Joensuu H 1997 A high pretreatment serum vascular endothelial growth factor concentration is associated with poor outcome in non-Hodgkin's lymphoma. Blood. **90**:3167-3172.

19. Yano A, Takekoshi M 2004 Transgenic plant-derived pharmaceuticals - the practical approach? Expert Opin Biol Ther. **4**:1565-1568.

20. Salmenkivi K, Heikkila P, Liu J, et al. 2003 VEGF in 105 pheochromocytomas: enhanced expression correlates with malignant outcome. APMIS. **111**:458-464.

21. Zielke A, Middeke M, Hoffmann S, et al. 2002 VEGF-mediated angiogenesis of human pheochromocytomas is associated to malignancy and inhibited by anti-VEGF antibodies in experimental tumors. Surgery. **132**:1056-1063; discussion 1063.

22. Favier J, Plouin PF, Corvol P, Gasc JM 2002 Angiogenesis and vascular architecture in pheochromocytomas: distinctive traits in malignant tumors. Am J Pathol. **161**:1235-1246.

23. Ohji H, Sasagawa I, Iciyanagi O, et al. 2001 Tumour angiogenesis and Ki-67 expression in phaeochromocytoma. BJU Int. **87**:381-385.

24. Ceolin L, Siqueira DR, Romitti M, et al. 2012 Molecular Basis of Medullary Thyroid Carcinoma: The Role of RET Polymorphisms. Int J Mol Sci. **13**:221-239.

25. Kouvaraki MA, Shapiro SE, Perrier ND, et al. 2005 RET proto-oncogene: a review and update of genotype-phenotype correlations in hereditary medullary thyroid cancer and associated endocrine tumors. Thyroid. **15**:531-544.

26. Capp C, Wajner SM, Siqueira DR, et al. 2010 Increased expression of vascular endothelial growth factor and its receptors, VEGFR-1 and VEGFR-2, in medullary thyroid carcinoma. Thyroid. **20**:863-871.

27. Adler JT, Meyer-Rochow GY, Chen H, et al. 2008 Pheochromocytoma: current approaches and future directions. Oncologist. **13**:779-793.

28. Chen H, Sippel RS, O'Dorisio MS, et al. 2010 The North American Neuroendocrine Tumor Society consensus guideline for the diagnosis and management of neuroendocrine tumors: pheochromocytoma, paraganglioma, and medullary thyroid cancer. Pancreas. **39**:775-783.

29. Punales MK, Graf H, Gross JL, Maia AL 2003 RET codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. J Clin Endocrinol Metab. **88**:2644-2649.

30. Vermeulen PB, Gasparini G, Fox SB, et al. 2002 Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. Eur J Cancer. **38**:1564-1579.

31. Ayala-Ramirez M, Feng L, Habra MA, et al. 2011 Clinical benefits of systemic chemotherapy for patients with metastatic pheochromocytomas or sympathetic extra-adrenal paragangliomas: Insights from the largest single-institutional experience. Cancer. **118**:2804-2812.

32. Hahn NM, Reckova M, Cheng L, et al. 2009 Patient with malignant paraganglioma responding to the multikinase inhibitor sunitinib malate. J Clin Oncol. **27**:460-463.

33. Joshua AM, Ezzat S, Asa SL, et al. 2009 Rationale and evidence for sunitinib in the treatment of malignant paraganglioma/pheochromocytoma. J Clin Endocrinol Metab. **94**:5-9.

34. Saito Y, Tanaka Y, Aita Y, et al. 2011 Sunitinib induces apoptosis in pheochromocytoma tumor cells by inhibiting VEGFR2/Akt/mTOR/S6K1 pathways through modulation of Bcl-2 and BAD. Am J Physiol Endocrinol Metab. **302**:E615-625.

35. Favier J, Igaz P, Burnichon N, et al. 2012 Rationale for anti-angiogenic therapy in pheochromocytoma and paraganglioma. Endocr Pathol. **23**:34-42.

36. Gimenez-Roqueplo AP, Favier J, Rustin P, et al. 2001 The R22X mutation of the SDHD gene in hereditary paraganglioma abolishes the enzymatic activity of

complex II in the mitochondrial respiratory chain and activates the hypoxia pathway. Am J Hum Genet. **69**:1186-1197.

37. Nomura K, Kimura H, Shimizu S, et al. 2009 Survival of patients with metastatic malignant pheochromocytoma and efficacy of combined cyclophosphamide, vincristine, and dacarbazine chemotherapy. J Clin Endocrinol Metab. **94**:2850-2856.

38. Chrisoulidou A, Kaltsas G, Ilias I, Grossman AB 2007 The diagnosis and management of malignant phaeochromocytoma and paraganglioma. Endocr Relat Cancer. **14**:569-585.

39. Weidner N, Carroll PR, Flax J, et al. 1993 Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol. **143**:401-409.

40. Weidner N, Semple JP, Welch WR, Folkman J 1991 Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med. **324**:1-8.

41. Macchiarini P, Fontanini G, Dulmet E, et al. 1994 Angiogenesis: an indicator of metastasis in non-small cell lung cancer invading the thoracic inlet. Ann Thorac Surg. **57**:1534-1539.

42. Liu Q, Djuricin G, Staren ED, et al. 1996 Tumor angiogenesis in pheochromocytomas and paragangliomas. Surgery. **120**:938-942; discussion 942-933.

43. Rooijens PP, de Krijger RR, Bonjer HJ, et al. 2004 The significance of angiogenesis in malignant pheochromocytomas. Endocr Pathol. **15**:39-45.

Table 1. Clinical features and VEGF-A and VEGF receptors expression in PHEO patients (n=22)

Case	Phenotype	RET germline mutation	Age (ys)[1]/sex	Tumor size[2]	VEGF-A[3]	VEGFR-1[3]	VEGFR-2[3]
1	MEN 2 A	C634Y	62 /F	3.50	2	0	0
2	MEN 2 A	C618R	38 /F	7.50	2	1	0
3	MEN 2 A	C634Y	61 /M	N/A	3	0	1
4	MEN 2 A	C634R	34 /F	5.50	3	1	1
5	MEN 2 B	M918T	49 /F	N/A	2	0	1
6	MEN 2 A	C634Y	44 /F	1.00	3	0	0
7	MEN 2 A	C634Y	49 /M	1.20	0	2	3
8	MEN 2 A	C634Y	55 /M	N/A	3	0	2
9	MEN 2 A	C634R	37 /F	6.40	2	0	0
10	MEN 2 A	C634R	21 /F	1.70	1	0	0
11	Sporadic[4]	-	14 /F	6.50	3	3	3
12	Sporadic[4]	-	30 /F	8.20	3	1	3
13	Sporadic[4]	-	23 /M	13.0	3	3	2
14	Sporadic	-	20 /F	5.50	2	2	2
15	Sporadic	-	18 /M	6.00	2	0	0
16	Sporadic	-	34 /F	11.5	1	0	0
17	Sporadic	-	45 /F	4.00	3	3	3
18	Sporadic	-	39 /F	5.50	3	0	0
19	Sporadic	-	23 /F	3.50	2	1	0
20	Sporadic	-	23 /F	8.50	3	3	3
21	Sporadic	-	35 /F	11.0	0	0	1
22	Sporadic	-	38 /F	7.50	2	0	0

N/A, no available; [1]Age at diagnosis of PHEO ; [2] Greatest tumor diameter; [3] grade 0 (absent, -), grade 1 (weak, +), grade 2 (moderate, ++), and grade 3 (strong, +++) based on the staining characteristics of most of the tumor; [4] Malignant PHEO

Table 2. VEGF-A, VEGR-1 and VEGR-2 expression and MVD and oncological features in PHEO patients

	Benign (n=19)	**Malignant** (n=3)	p-value
VEGF-A			
Absent	2	0	
+	2	0	0.063
++	8	0	
+++	7	3	
VEGFR-1			
Absent	12	0	
+	3	1	0.027
++	2	0	
+++	2	2	
VEGFR-2			
Absent	10	0	
+	4	0	0.029
++	2	1	
+++	3	2	
MVD	50.0 (35.7-78.6)[1]	92.8 (85.7- 92.8)[1]	0.024

PHEO, pheochromocytoma
MVD, microvessel density
[1] Median (percentile 25 –75)

Table 3. VEGF-A, VEGR-1 and VEGR-2 expression and MVD in sporadic or hereditary (MEN 2-associated) PHEO

	Hereditary (n=10)	**Sporadic** (n=9)	p-value
VEGF-A			
Absent	1	1	
+	1	1	1
++	4	4	
+++	4	3	
VEGFR-1			
Absent	7	5	
+	2	1	0.657
++	1	1	
+++	0	2	
VEGFR-2			
Absent	5	5	
+	3	1	0.820
++	1	1	
+++	1	2	
MVD	46.4 (14.3-78.6)[1]	50 (42.9-64.3)[1]	0.412

PHEO, pheochromocytoma
MVD, microvessel density
[1] Median (percentile 25 –75)

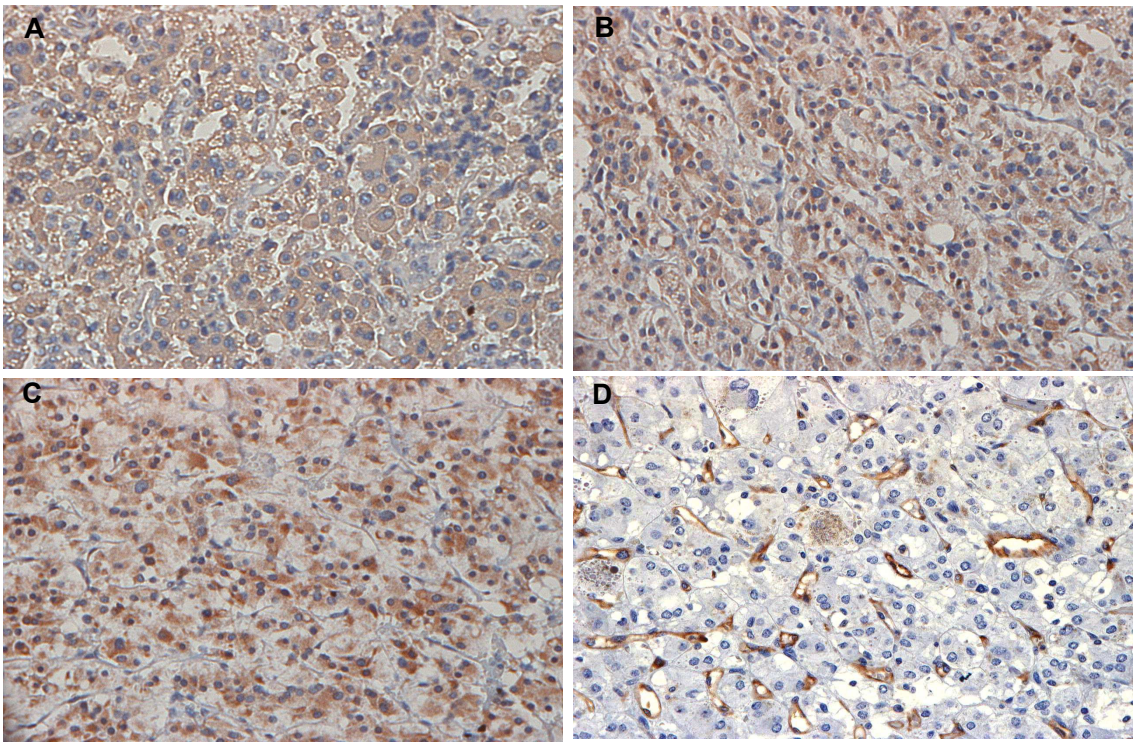

Figure 1 Immunohistochemical detection of VEGF-A (A), VEGFR-1 (B), VEGFR-2 (C) and microvessels (D) in tumor cells from PHEO samples (400x).