CLINICAL SCIENCE

Glucose-dependent insulinotropic peptide receptor overexpression in adrenocortical hyperplasia in MEN1 syndrome without loss of heterozygosity at the 11q13 locus

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BACKGROUND: The molecular mechanisms involved in the genesis of the adrenocortical lesions seen in MEN1 syndrome (ACL-MEN1) remain poorly understood; loss of heterozygosity at 11q13 and somatic mutations of *MEN1* are not usually found in these lesions. Thus, additional genes must be involved in MEN1 adrenocortical disorders. Overexpression of the glucose-dependent insulinotropic peptide receptor has been shown to promote adrenocortical tumorigenesis in a mice model and has also been associated with ACTH-independent Cushing syndrome in humans. However, to our knowledge, the status of glucose-dependent insulinotropic peptide receptor expression in adrenocortical lesions in MEN1 has not been previously investigated.

OBJECTIVE: To evaluate glucose-dependent insulinotropic peptide receptor expression in adrenocortical hyperplasia associated with MEN1 syndrome.

MATERIALS/METHODS: Three adrenocortical tissue samples were obtained from patients with previously known *MEN1* germline mutations and in whom the presence of a second molecular event (a new *MEN1* somatic mutation or an 11q13 loss of heterozygosity) had been excluded. The expression of the glucose-dependent insulinotropic peptide receptor was quantified by qPCR using the ΔΔCT method, and *β-actin* was used as an endogenous control.

RESULTS: The median of glucose-dependent insulinotropic peptide receptor expression in the adrenocortical lesions associated with MEN1 syndrome was 2.6-fold (range 1.2 to 4.8) higher than the normal adrenal controls ($p = 0.02$).

CONCLUSION: The current study represents the first investigation of glucose-dependent insulinotropic peptide receptor expression in adrenocortical lesions without 11q13 loss of heterozygosity in MEN1 syndrome patients. Although we studied a limited number of cases of MEN1 adrenocortical lesions retrospectively, our preliminary data suggest an involvement of glucose-dependent insulinotropic peptide receptor overexpression in the etiology of adrenocortical hyperplasia. New prospective studies will be able to clarify the exact role of the glucose-dependent insulinotropic peptide receptor in the molecular pathogenesis of MEN1 adrenocortical lesions.

KEYWORDS: MEN1; adrenocortical hyperplasia; *GIPR*; expression.

Costa MHS, Domenice S, Toledo RA, Lourenço DM Jr, Latronico AC, Pinto EM, et al. Glucose-dependent insulinotropic peptide receptor overexpression in adrenocortical hyperplasia in MEN1 syndrome without loss of heterozygosity at the 11q13 locus. Clinics. 2011;66(4):529-533.

Received for publication on October 17, 2010; **First review completed on** December 8, 2010; **Accepted for publication on** December 8, 2010

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INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1) is a familial tumor syndrome associated with heterozygous germline mutations in the *MEN1* gene, which encodes a 610-amino-acid nuclear protein named MENIN.[1,2] Tumors of the parathyroid glands, the anterior pituitary and the endocrine pancreas are the lesions more frequently observed in MEN1 syndrome patients; however, more than twenty different neoplasias in endocrine and non-endocrine tissues have been associated with MEN1 syndrome.[3] A recent study that used endoscopic ultrasound has shown that up to 73% of MEN1 syndrome patients may have small, benign and non-functioning adrenocortical lesions.[4] Bilateral adrenocortical macronodular hyperplasias or adenomas may also occur in MEN1 syndrome patients, while non-functioning carcinomas, aldosterone-secreting tumors or pheochromocytomas are rare.[4-8]

The *MEN1* gene is a typical tumor suppressor gene, and in accordance with the two-hit hypothesis of Knudson,[9] virtually all MEN1 tumors contain a heterozygous germline mutation of the *MEN1* gene and inactivation of the normal allele. The combination of the two molecular hits determines the development of the tumor lesions in MEN1 syndrome.[10,11] Recent *in vitro* studies have demonstrated that MENIN acts as a transcription factor that can regulate numerous genes potentially involved in cell cycle control, including those that encode cyclin-dependent kinase inhibitors (CDKIs), such as *p27Kip1* and *p18INK4C*.[12-14]

Nonetheless, the molecular mechanism of the adrenocortical hyperplasia in MEN1 syndrome seems to be different from the classical MEN1 tumorigenesis, as maintenance of heterozygosity at the 11q13 locus has been observed.[15-17]

Associations between adrenocortical lesions and pancreatic endocrine tumors (PET) in MEN1 syndrome patients have been observed and may indicate that unknown growth factor(s) secreted by PETs could play roles in MEN1 adrenocortical lesion development.[7,15,17-19] The gastric inhibitory polypeptide or glucose-dependent insulinotropic peptide (GIP) might be a candidate to participate in this process.

Overexpression of glucose-dependent insulinotropic peptide receptor (*GIPR*) has been demonstrated to be capable of promoting adrenocortical tumorigenesis in a mice model.[20] In addition, *GIPR* overexpression has also been associated with Cushing syndrome resulting from ACTH-independent macronodular hyperplasia.[21-24] However, the status of *GIPR* in ACL-MEN1 remains unknown.

SUBJECTS AND METHODS

Patients and tissue specimens

This study was approved by the Ethics Committee of the Hospital das Clinicas, São Paulo, Brazil, and written informed consent was obtained from all patients and healthy controls. Three unrelated MEN1 syndrome cases (2 females and 1 male, with an age range of 37 to 59 years) were studied (Table 1). All cases presented with primary hyperparathyroidism, enteropancreatic tumors and adrenocortical disorder. Case 3 also presented with prolactinoma and the typical clinical features of hypercortisolism, whereas patients 1 and 2 had no clinical features of adrenal hyperfunction. All patients presented a family history compatible with MEN1 syndrome. The diagnosis of MEN1 syndrome was established according to the NIH MEN Consensus and molecular findings.[1]

Adrenal tumor samples from cases 1 and 2 were obtained by cutting a biopsy and from case 3 after a bilateral adrenalectomy, which is recommended for Cushing syndrome treatment. Adrenal tissue fragments were immediately stored in liquid nitrogen until RNA extraction. Histological data from the adrenal lesions were compatible with adrenocortical hyperplasia in all cases. Eight normal human adrenal tissue samples were obtained during surgical treatment for kidney tumors, and they were used as normal controls.

MEN1 sequencing

DNA extraction from peripheral leukocytes and tissues was performed according to standard procedures. For the synthesis of cDNA, total RNA was isolated from frozen tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was performed using total RNA from each sample and Multiscribe from a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The protocols for PCR and sequencing analysis of germline DNA have been previously reported.[25] Exonic primers were used to sequence the entire *MEN1* coding region in the cDNA (sequences available upon request).

LOH analysis

Five *MEN1*-flanking polymorphic markers (D11S4191, PYGM, D11S987, D11S527 and D11S937) located at centromeric and telomeric regions of the *MEN1* gene and covering the entire 11q12.1-11q14.1 region were used to evaluate loss of the *MEN1* wild-type allele. The LOH analysis protocol has been previously reported.[26]

Forward primers were labeled with fluorescent dyes (FAM or VIC) that were different from those used in the internal size standard (TAMRA or ROX-350; Applied Biosystems). GeneScan software (Applied Biosystems) was used to analyze the results. When comparing the heights of the allele peaks in the endocrine tissues (adrenocortical, parathyroid glands and pancreas) and in the blood samples, an allelic imbalance ratio of <0.5 or >2.0 was defined as LOH.

GIPR expression analysis

Quantitative real-time PCR (qPCR) was performed using a 7000 real-time PCR System (Applied Biosystems) according to the manufacturer's instructions. TaqMan gene expression assays were used for *GIPR* (ID Hs006092_m1) and β-actin (assay ID-4326315E). The reactions consisted of 12.5 μl of 2X TaqMan Universal PCR master mix, 1.25 μl of each 20X assay on demand, 1.5 μl of cDNA and water to obtain a 25 μl final volume. The PCR parameters were 50℃ for 2 min, 95℃ for 10 min and 50 cycles at 95℃ for 15 sec

Table 1 - Clinical, histological and molecular data from 3 patients with MEN1 syndrome and adrenal disorders.

Patient (n)	Age (yr)/Gender	Clinical MEN1 syndrome presentations	Clinical adrenal presentations	Histological adrenal analysis	*MEN1* mutation
1	59/M	Hyperparathyroidism, pancreatic tumor, bronchopulmonary carcinoid	Non-functioning	Hyperplasia	308delC
2	37/F	Hyperparathyroidism, pancreatic tumor	Non-functioning	Hyperplasia	W183X
3	37/F	Hyperparathyroidism, pancreatic tumor, prolactinoma	Cushing's syndrome	Hyperplasia	893+1G>A

F- female; M- male.

Table 2 - *GIPR* expression in adrenal tissue from 3 patients with MEN1 syndrome as compared with the normal human adrenal gland pool (median).

Patient (n)	1	2	3	Normal adrenal tissue
GIPR expression	1.2	2.6	4.8	0.08 to 1.4
Median		2.6		0.7

and 60℃ for 1 min. Validation experiments were performed to confirm that the amplification efficiency of the controls was similar to that of the target genes.

A cycle threshold (CT) value in the linear range of amplification was selected for each sample in triplicate and normalized to β-actin expression levels. The relative expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method,[27] where the $\Delta\Delta CT$ is the difference between the selected ΔCT value of a particular sample and the ΔCT of a pool in 61 normal adrenal glands from autopsies (Clontech, Palo Alto, CA, USA). The mean expression of the target genes in the normal adrenal controls was assigned an expression value of 1.0, and the fold increases in the expression levels were determined by comparison.

Statistical Analysis

The expression results were compared using the Mann-Whitney test. The data are presented as the median and range for each group. The Spearman test was used to establish correlations between receptor expression, patient clinical aspects and hormone levels. A p-value <0.05 was considered significant.

RESULTS

Status of the *MEN1* gene in MEN1 tumors

At least 2 out of the 5 fluorescent polymorphic markers used in this study were informative for the analysis of the 11q13 LOH in each tumor. Loss of the remaining *MEN1* allele was observed in the pancreatic tumors used as controls, while no loss was observed in the adrenocortical tumors of the three patients. cDNA sequences from the ACTs showed no additional changes (Supplementary Tables 1 and 2).

GIPR expression in MEN1-ACTs

Adrenocortical neoplasias from patients 1, 2 and 3 had 1.2-, 2.6- and 4.8-fold (median 2.6, ranging from 1.2 to 4.8) higher expression of *GIPR* as compared with the normal human adrenocortical tissue (median 0.7, ranging from 0.1 to 1.4) (Figure 1). This finding represents a significantly increased expression of the *GIPR* gene in MEN1 adrenocortical neoplasias ($p = 0.02$).

DISCUSSION

Despite the high frequency of adrenocortical lesions in MEN1 syndrome patients, knowledge of the molecular pathogenesis of these lesions remains limited. Our data support the previous findings that, unlike MEN1-related tumors, ACL-MEN1 tumors do not present loss of heterozygosity at the *MEN1* tumor suppressor gene locus, 11q13. In addition, the possibility of inactivation of the *MEN1* wild-type gene in the hyperplastic adrenal tissue by a *de novo* somatic mutation was excluded because no other pathological alteration was found by sequencing of the *MEN1* cDNA.

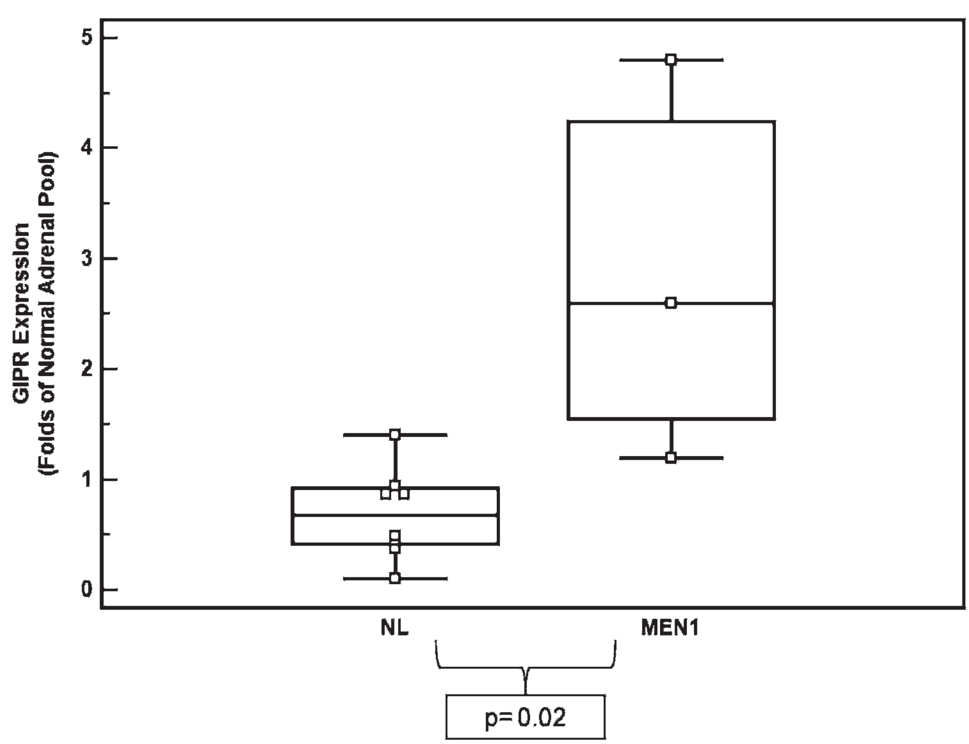

Figure 1 - *GIPR* expression in adrenal tissue from 3 patients with MEN1 syndrome as compared with normal human adrenal gland (median). *GIPR* expression in all analyzed tissues was 2.6-fold higher than that observed in normal adrenal controls.

The frequent association of adrenocortical lesions and PETs in patients with MEN1 syndrome suggests a common physiopathological link between both disorders in MEN1 syndrome.[7,15,17,18] Several lines of evidence have led us to hypothesize that GIP, which stimulates insulin release by pancreatic β cells, may play a role in MEN1 adrenal tumorigenesis.

GIP is synthesized and released by the K cells, located in the duodenum and small intestine after food intake, and plays an important role in regulating the proliferation and fate of pancreatic cells.[28,29] Overexpression of *GIPR* in the adrenal cortex has been previously demonstrated in patients with ACTH-independent Cushing syndrome resulting from food-dependent adrenal cortisol secretion.[21,24,30] *GIPR* overexpression has also been identified in adrenocortical adenomas with androgen or aldosterone hyperproduction and less frequently in adrenocortical adenomas.[23,31] Recently, one study showed that the aberrant expression of a non-mutated *GIPR* gene was sufficient to initiate the formation of benign adrenocortical tumors and hyperplastic adrenal tissue using an *in vivo* cell transplantation model in mice.[20]

The potential involvement of the *GIPR* gene in the etiology of adrenocortical hyperplasia occurring in MEN1 syndrome might be supported by this evidence. Although a small number of adrenal samples were analyzed, our data demonstrated a previously unknown overexpression of this gene in ACL-MEN1. Using normal human adrenal tissue as a reference for the expression assays, our results indicated a significantly higher expression of GIPR in all three samples of the MEN1 adrenal tissue; on average, it was 2.6-fold higher than in normal adrenal glands.

As the GIPR is a transmembrane receptor coupled to the adenyl cyclase/cAMP signaling cascade, its overexpression may lead to disruption of the cAMP pathway. Deregulation of cAMP has been reported in sporadic adrenocortical tumors and attributed to mutations in the *PRKAR1A*, *GNAS1*, *PDE11A* and *PDE8B* genes,[32-35] but not in the *GIPR*.[36] Unfortunately, we were not able to perform cAMP measurements in the adrenocortical tissues that overexpressed *GIPR*.

In vitro, overexpression of the GIPR agonist (GIP) has been associated with both hormonogenesis and cell proliferation because it increases cAMP production and the synthesis of DNA in the GIP-dependent, cortisol-secreting adenoma cells.[37] Our data indicate that, as in ACTH-independent macronodular adrenal hyperplasia, *GIPR* may play a role in the cellular proliferation of adrenocortical hyperplasia occurring in MEN1 syndrome.

Because the investigative protocol was developed after adrenal surgery, we do not have data regarding the cortisol response to food intake in these MEN1 syndrome patients.

In conclusion, knowledge regarding the genesis of the frequent adrenal lesions observed in MEN1 syndrome patients remains limited. The current study represents the first investigation of *GIPR* in MEN1 human adrenocortical lesions without 11q13 LOH and suggests a potential role of *GIPR* overexpression in the development of the adrenocortical lesions associated with MEN1 hyperplasia. New prospective studies will be able to clarify the exact role of *GIPR* in the molecular pathogenesis of ACL-MEN1.

ACKNOWLEDGMENTS

We thank the staff of the Laboratorio de Hormonios e Genetica Molecular LIM/42 of the Hospital das Clinicas da faculdade de Medicina da Universidade de São Paulo.

FUNDING: This work was supported in part by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP Grants 03/07449-1 to Marcia Helena Soares Costa and 04/15046-7 to Berenice Bilharinho Mendonca) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ Grants 300825 to Berenice Bilharinho Mendonca and 300469/2005-5 to Ana Claudia Latronico). Delmar Muniz L. Junior and Sergio Pereira A. Toledo were supported in part by the Fundação Faculdade de Medicina.

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Supplementary Table 1- Allelic distribution of the five microsatellite markers used in the LOH study of MEN1 syndrome patients.

Patients	Tissues	D11S4191	PYGM	D11S987	D11S527	D11S937
1	Blood	89/91	169/171	120/120	142/159	152/160
	Adrenal	89/91	169/171	120/120	142/159	152/160
2	Blood	97/119	167/173	120/122	147/157	160/160
	Adrenal	-	167/173	120/122	-	160/160
	Pancreatic	97	167	120	147	160/160
3	Blood	95/107	175/183	116/116	143/145	156/160
	Adrenal D	95/107	175/183	116/116	143/145	156/160
	Adrenal E	95/107	175/183	116/116	143/145	156/160
	Pancreatic	95	183	116/116	143	156

(-) blank spaces indicate unavailable sample material.

Supplementary Table 2 - *MEN 1* mutations identified in different tissues of MEN1 syndrome patients.

Patient	*MEN1* mutation	Peripheral blood	Adrenal Tissue	Pancreatic Tissue
1	308delC	308delC	308delC	NA
2	W183X	W183X	W183X	W183X
3	893+1G>A	893+1G>A	893+1G>A	893+1G>A

NA, not available.