A cumulative effect involving malfunction of the *PTH1R* and *ATP4A* genes explains a familial gastric neuroendocrine tumor with hypothyroidism and arthritis

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Abstract

Background Type I gastric neuroendocrine tumors (gNETs) classically arise because of hypergastrinemia and involve destruction of parietal cells, which are responsible for gastric acid secretion through the ATP4A proton pump and for intrinsic factor production.

Methods By whole exome sequencing, we studied a family with three members with gNETs plus hypothyroidism and rheumatoid arthritis to uncover their genetic origin.

Results A heterozygous missense mutation in the *ATP4A* gene was identified. Carriers of this variant had low ferritin and vitamin B₁₂ levels but did not develop gNETs. A second heterozygous mutation was also uncovered (*PTH1R* p.E546K). Carriers exhibited hypothyroidism and one of them had rheumatoid arthritis. Gastrin activates parathyroid hormone like hormone/parathyroid hormone 1 receptor (*PTH1R*) signaling, which is involved in gastric cell

homeostasis. Activation of parathyroid hormone/*PTH1R*, which is upregulated by thyrotropin in the thyroid, is also involved in *RANKL* expression, which regulates bone homeostasis. Thyrotropin and *RANKL* expression were deregulated in *PTH1R* mutation carriers, suggesting a link between the *PTH1R* gene, hypothyroidism, rheumatoid arthritis, and gastric disease. Only patients with both mutations developed gNETs plus hypothyroidism and rheumatoid arthritis.

Conclusion Both mutations suggest that a collaborative mechanism is operative in this family, in which mutations in these genes affect the function and viability of parietal cells and lead to the achlorhydria that drives hypergastrinemia and the formation of gNETs.

Keywords Gastric neuroendocrine tumor · *PTH1R* · *ATP4A* · Hypothyroidism · Rheumatoid arthritis

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Introduction

Type I gastric neuroendocrine tumors (gNETs) arise from gastric enterochromaffin-like cells. They have a benign course and a late age of onset (mean 66 years). Classically, hypergastrinemia in patients who have autoimmune atrophic gastritis causes hyperplasia of gastric enterochromaffin-like cells that can progress to type I gNETs [1]. Tumor progression is associated with the destruction of parietal cells, which are responsible for gastric acid and intrinsic factor secretion, and leads to hypochlorhydria and vitamin B₁₂ malabsorption anemia caused by a lack of gastric acid and intrinsic factor respectively [2]. In normal conditions, gastrin directly regulates acid secretion of parietal cells, the proliferation, development, and

homeostasis of which are coordinated by the acidity of the stomach and different growth factors (Fig. 1a).

Parathyroid hormone like hormone (PTHLH) has been identified as one of these gastric growth factors, and responds to gastrin/cholecystokinin B receptor 2 (CCKR2) activation [3]. PTHLH interacts with the parathyroid hormone 1 receptor (PTH1R) to activate different growth factors involved in gastric homeostasis and proliferation (Fig. 1b). In *Cckbr* (gastrin receptor) knockout mice, PTHLH is downregulated [4].

PTH1R is also found in the thyroid gland, and responds to parathyroid hormone (PTH) activation, which is regulated by thyrotropin (Fig. 1b). PTH/PTH1R activation is involved in osteoclast production and bone development [5]. Both osteopenia/osteoporosis and thyroid malfunction were related with chronic atrophic gastritis, hypergastrinemia, and hypoacidity [6, 7]. In particular, malabsorption of vitamin B₁₂ (anemia due to a lack of intrinsic factor) has been reported in several hypothyroidism disorders. However, although various bone and thyroid diseases are clinically related to gastric disease, a common genetic origin has not been elucidated.

Recently, we studied a consanguineous family (family 1) with five members affected by type I gNETs (Table 1) [8]. We identified the deleterious p.R703C mutation in homozygosis in the *ATP4A* gene, which explained the lack of acid secretion and ferropenic anemia and the origin of hypergastrinemia and gNET development instead of a classic tumorigenesis process [8, 9].

In the present study we performed Whole exome sequencing of a second family from Navarra (Spain) (family 2) with 11 siblings, including three members with gNETs (45 years old on average at diagnosis); these patients had classical clinical features, including low levels of ferritin and vitamin B₁₂-deficiency anemia (Fig. 2a), but also had hypothyroidism and high thyrotropin levels (Table 1). Rheumatoid arthritis was also diagnosed in affected member II.10 at an age of 38 years. The mother also had hypothyroidism and rheumatoid arthritis.

Materials and methods

Whole exome sequencing and bioinformatics pipeline

Genomic DNA was isolated from peripheral blood lymphocytes with a FlexiGene DNA kit (QIAGEN). Exomes from selected DNA samples were fully captured, enriched, and sequenced (see the electronic supplementary material).

Immunohistochemistry

Paraffin-embedded tissue samples were obtained from total gastrectomy specimens and monitoring biopsies (Fig. 2a). Anti-intrinsic factor antibody was produced at the Spanish National Cancer Research Center (Table S1).

Fig. 1 Regulation of acid and intrinsic factor secretion and gastric homeostasis. **a** Gastrin acts directly on enterochromaffin-like (ECL) cells and parietal cells through cholecystokinin B receptor 2 (CCKR2). Histamine from ECL cells also positively regulates the activity of parietal cells through H₂ receptors. **b** *Left* Activation of gastrin/CCKR2 regulates parathyroid hormone like hormone

(PTHLH)/parathyroid hormone 1 receptor (PTH1R) activation, which is involved in gastric cell homeostasis and proliferation. *Right* Thyrotropin (TSH) promotes parathyroid hormone (PTH) secretion in the thyroid gland. Activation of PTHLH/PTH1R is involved in bone homeostasis through receptor activator of nuclear factor κ B ligand (RANKL) regulation. IF intrinsic factor, PC parietal cell

Table 1 Mean age at onset and average serum concentrations of gastrin, vitamin B₁₂, ferritin, and thyrotropin of members of families 1 and 2 with gastric neuroendocrine tumors (gNETs) and *ATP4A* p.Q680L carriers

Family	gNET members/total members	Mean age at diagnosis (years)	Gastrin (0–100 pM)	Vitamin B ₁₂ (210–920 pg/mL)	Ferritin (25–28 ng/mL)	Thyrotropin (0.3–5.5 IU/mL)
Family 1	5/10	35	484 pM	444.25 pg/mL	6.25 ng/mL	0.75 IU/mL
Family 2	3/11	45	1819 pM	105.00 pg/mL	13.50 ng/mL	9.62 IU/mL

Normal levels are shown in *parentheses*

Fig. 2 **a** Pedigree of family 2. Age of onset [gastric neuroendocrine tumor (gNET) cases] is shown in *parentheses*. Segregation for the *ATP4A* p.Q680L and *PTH1R* p.E546K mutations found in this work is also shown. The genotype of the parents is inferred. An *asterisk* indicates that formalin-fixed and paraffin-embedded (FFPE) tissue was available. **b** Hybridization with anti-intrinsic factor antibody to normal stomach wild-type (wt). Lower staining with anti-intrinsic factor was observed in II.4, who carries only the *ATP4A* mutation.

One gNET individual (II.7) is also shown as an example. Normal tissue (N) and tumor tissue (T) from gNET individuals were evaluated separately. **c** Reverse transcription PCR studies of *RANKL* expression in serum. The members studied are grouped according to *PTH1R* p.E546K mutation. Student's *t* test was used for the statistical analysis of normally distributed values. *RANKL* expression with normalized using *GAPDH* messenger RNA levels as a control. F1 family 1

Real-time quantitative PCR

Real-time quantitative PCR was performed with complementary DNA to test the levels of expression of *PTH1R* messenger RNA. Complementary DNA was obtained by reverse transcription PCR of 1200 ng of total RNA from peripheral blood cells with use of a high-capacity complementary DNA reverse transcription kit (Applied Biosystems catalog no. 4368814) following the manufacturer's instructions. PCR was performed with complementary DNA at approximately 25 ng/mL and Power SYBR Green PCR master mix (Applied Biosystems catalog no. 4367659). Expression levels were evaluated with

the $\Delta\Delta C_t$ method [10] and normalized with use of *GAPDH* quantification as a standard.

Results

We first studied the whole *ATP4A* gene by Sanger sequencing (Table S2). A heterozygous deleterious mutation (*ATP4A* p.Q680L; rs61729956) was found in the three affected members with gNETs (Fig. 2a, Table 2). The p.Q680 position is located in the same phosphoryl-binding pocket as the *ATP4A* p.R703C mutation that caused the gNETs in family 1 [8]. This mutation may affect the

Table 2 Variants found in the *ATP4A* and *PTH1R* genes for families F1 and F2

Family	Gene	Exon	Complementary DNA	Protein	Damage prediction	Inheritance	dbSNP	Frequency	
								1000 Genomes	ExAc
Family 1	<i>ATP4A</i>	14	c.2107C>T	p.R703C	Deleterious	Homozygous	Calvete et al. [8]	ND	ND
Family 2	<i>ATP4A</i>	14	c.2039A>T	p.Q680L	Deleterious	Heterozygous	rs61729956	0.003	0.003
	<i>PTH1R</i>	14	c.1636G>A	p.E546K	Deleterious	Heterozygous	rs77048718	0.008	0.016

ND not described

ATP4A protein in a similar way as the mutation in family 1 and affect the effectiveness of gastric acid production [9]. Two other siblings who did not have gNETs (II.1 and II.4) also carried this variant (Fig. 2a). Carriers of this mutation have an intermediate phenotype; these individuals had low ferritin (20.7 ng/mL) and vitamin B₁₂ (187.9 pg/mL) levels, although these levels were not as low as in the gNET patients (Table 1) and were not sufficiently low for them develop gNETs.

Whole exome sequencing was performed in two affected siblings with type I gNETs (II.7 and II.10), looking for a second gene. A deleterious missense mutation (rs77048718) in the *PTH1R* gene (p.E546K) was uncovered (Table 2). *PTH1R* encodes the transmembrane receptor for PTH and PTHLH, which is related to the development of parietal cells and the stomach (Fig. 1b). By in silico studies we compared the putative *PTH1R* p.E546K protein with the wild-type *PTH1R* (Fig. S1). Putative binding sites were annotated with the standard score given by PredictProtein (threshold greater than 20) (Table S3) [11]. Five of 15 putative binding sites were reduced (threshold less than 20) in the putative abnormal protein, including an RNA binding site (Fig. S1). In addition, three affected members had hypothyroidism and at least one of them also had rheumatoid arthritis (II.10). Although we were not able to study the genetic condition of the parents, the mother also had hypothyroidism and rheumatoid arthritis.

Sanger sequencing confirmed that the three members with gNETs shared two mutations (*ATP4A* p.Q680L and *PTH1R* p.E546K) (Fig. 2a). Both mutations, which belong to the same regulation pathway for parietal homeostasis (Fig. 2b), have an expected frequency of being together of 0.0024%.

Immunohistochemistry studies

Anti-intrinsic factor, which stains parietal cells, was hybridized to the stomach of *ATP4A* p.Q680L mutation carriers and the gNET patients. Lower immunoreactivity was observed for individuals carrying this mutation compared with normal (wild-type) stomachs (Fig. 2b). The less

intense staining of parietal cells is in agreement with low ferritin and vitamin B₁₂ levels, and demonstrates the deleterious effect of this mutation on gastric acid secretion and homeostasis of the stomach. Positive staining was not observed with anti-intrinsic factor in the tumor tissue of the affected members from family 2 (Fig. 2b). Lower staining of gNETs compared with tissue from carriers of the *ATP4A* p.Q680L mutation alone suggests a cumulative effect with the *PTH1R* p.E546K mutation.

RANKL expression

PTH1R is also involved in the regulation of calcium and bone formation. Thyrotropin regulates activation of PTH/*PTH1R* to maintain the osteoclast/osteoblast equilibrium through receptor activator of nuclear factor κ B ligand (RANKL) (Fig. 1b). Therefore, high thyrotropin levels found in gNET patients (Table 1) might be explained by the lack of negative regulation through PTH/*PTH1R* activation of this stimulating hormone (Fig. 1b). To evaluate the putative effect of the *PTH1R* p.E546K mutation, RANKL expression levels were also studied by real-time quantitative PCR in peripheral blood, as *PTH1R* regulates RANKL expression. Relative RANKL messenger RNA levels were increased in the three gNET individuals of family 2 (Fig. 2c). RANKL expression was also increased in the member carrying the *PTH1R* mutation alone and who did not have gNETs (II.9). No different expression of RANKL was observed in these gNET members from family F1; this suggests that the altered expression depends on the mutation of *PTH1R* and not on the achlorhydria or any other clinical factor of gNETs. Importantly, thyrotropin levels were never found increased in gNET members of family 1 (Table 1).

Discussion

Heterozygous carriers of the *ATP4A* p.Q680L mutation (II.1 and II.4) exhibited slightly decreased gastric acid secretion. On the other hand, *PTH1R* is activated by

PTHLH in the stomach and directly regulates gastric mucosa development (Fig. 1b) [3]. *PTHLH* expression depends on transcription factors activated by gastrin–CCKR2, since it is downregulated in *Cckbr*-knockout mice, which lack gastric acid secretion [4]; this demonstrates that PTHLH/PTH1R activation is involved in gastric chlorhydria homeostasis. Therefore, carriers of only one mutation had fewer functional parietal cells and an intermediate phenotype. Importantly, none of the members who carried only one of the *ATP4A* p.Q680L or *PTH1R* p.E546K mutations developed gNETs. Both heterozygous mutations partially contribute to parietal cell malfunction in a collaborative manner, which would accumulate the malfunction of the two genes (Fig. 1b). Members of this family with gNETs had both pathogenic mutations in the *ATP4A* and *PTH1R* genes. Although the mutations were annotated in two different genes, both are related to the viability and function of parietal cells and gastric homeostasis (Fig. 2a) that correlate with a lack of chlorhydria and intrinsic factor secretion, which would explain iron-deficiency anemia and vitamin B12-deficiency anemia.

PTH also activates PTH1R in the thyroid gland (Fig. 1b). Carriers of the *PTH1R* p.E546K mutation from family 2 had increased levels of thyrotropin (Table 1), which regulates parathyroid cell activity and RANKL expression (Fig. 2c), and which also depends on PTH/PTH1R activation. The expression of RANKL was also found to be altered when a short hairpin RNA was used to reduce *PTH1R* expression in cells from a murine osteosarcoma model [12], suggesting that in our case the altered expression of RANKL was correlated with the *PTH1R* mutation. High levels of thyrotropin and RANKL have been described in nongastric diseases such as Hashimoto thyroiditis [13] and rheumatoid arthritis [14] respectively. These altered thyrotropin and RANKL levels can explain the hypothyroidism and rheumatoid arthritis respectively observed in the family. The frequency of the *PTH1R* mutation is 0.008 (Table 2), and we cannot discard incomplete penetrance regarding rheumatoid arthritis and thyroid malfunction. The risk of developing rheumatoid arthritis throughout life in adults is 3.6% for women and 1.7% for men [15]. In addition, rheumatoid arthritis has a late age of onset (65 years on average), which also might hide its real prevalence.

Whole clinical data and DNA from the parents were not available. Both diseases (Hashimoto thyroiditis and rheumatoid arthritis) were observed in the mother. Therefore, we can speculate that *PTH1R* p.E546K mutation segregated from the mother. As far as we know, none of the parents developed gNETs. Only combination with the mutation *ATP4A* p.Q680L mutation started the tumorigenesis process in this family. Therefore, the *ATP4A* p.Q680L allele would segregate from the father.

All these data suggest a cumulative mechanism involving two genes in gNETs in this family and for the first time involving the *PTH1R* gene. Therefore, lack of chlorhydria leads to hypergastrinemia and gNETs that differ from the classic description of tumorigenesis of gNETs, where hypergastrinemia leads to the destruction of parietal cells that results in hypochlorhydria [2]. Mutation in the *PTH1R* gene also explains hypothyroidism and arthritis. Various bone and thyroid diseases have been clinically related to gastric disease [6, 7], but this is the first time that these different diseases have been related to a common genetic background.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

Informed consent Informed consent was obtained from all patients included in the study.

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