Exome sequencing identifies ATP4A gene as responsible of an atypical familial type I gastric neuroendocrine tumour

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Abstract

Gastric neuroendocrine tumours (NETs) arise from enterochromaffin-like cells, which are located in oxyntic glands within the stomach. Type I tumours represent 70–80% of gastric NETs and are associated with hypergastrinaemia, chronic atrophic gastritis and achlorhydria. Gastrin is involved in the endocrine regulation of gastric acid production. Most type I gastric NETs are sporadic, have a good prognosis and their genetic basis are unknown. We performed an exome sequencing study in a family with consanguineous parents and 10 children, five of whom were affected by type I gastric NET. Atypical clinical traits included an earlier age of onset (around 30 years), aggressiveness (three had nodal infiltration requiring total gastrectomy and one an adenocarcinoma) and iron-deficiency rather than megaloblastic anaemia. We identified a homozygous missense mutation in the 14th exon of the ATP4A gene (c.2107C>T), which encodes the proton pump responsible for acid secretion by gastric parietal cells. The amino acid p.Arg703Cys is highly conserved across species and originates a change of one of the transmembrane domains that avoids the liberation of protons from cells to stomach. This is consistent with the achlorhydria that was observed in the affected individuals. No germline or somatic mutations in the ATP4A gene were found in sporadic gastric NET patients. Based on the results of this large family, it seems that this atypical form of gastric NET has an earlier age of onset, behaves more aggressively and has atypical clinical traits that differentiated from other studied cases.

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Introduction

Gastric neuroendocrine (also known as carcinoid) tumours (NETs) occur with an estimated frequency of 2 per 100 000 in the general population (1). The biological behaviour and prognosis vary considerably according to the type of gastric NET (2).

Type I gastric NETs usually arise in patients who have autoimmune atrophic gastritis and in whom the number of acid secreting gastric parietal cells is reduced (3). This leads to hypochlorhydria and hypergastrinaemia as a result of the gastric antral G cell hyperplasia. Hypergastrinaemia causes hyperplasia of gastric enterochromaffin-like (ECL) cells and in some patients this progresses to type I gastric NETs. Atrophy of fundic glands and parietal cells results in gastric hypochlorhydria. These tumours are often small and multiple, have a low Ki67 proliferative index (typically <2%) (4), rarely metastasize and are generally associated with a good prognosis (5). Until now, no familial forms have been described.

Type II gastric NETs are also associated with hypergastrinaemia, but gastrin is produced by a gastrinoma and most cases are not associated with hypergastrinaemia (7). They often have a more aggressive behaviour as result of a higher proliferative index.

Gastric luminal acidification (pH 2–3) is achieved through 

\[ \text{H}^+ / \text{K}^+ - \text{ATPase} \]

proton pumps which are located in parietal cells (8). Acidification is induced by signalling pathways involving gastrin (from G cells) and histamine (from ECL cells), and inhibited by somatostatin (SST) (from D cells). Parietal and ECL cells both express SST receptors (predominantly SSTR2). Parietal cells also produce the intrinsic factor (IF) glycoprotein, which is required for the absorption of vitamin B12 in the small intestine. The parietal cell atrophy which is usually associated with type I gastric NETs frequently results in the development of megaloblastic anaemia as a result of vitamin B12 malabsorption. Other risk factors for the pathogenesis of type I gastric NETs remain relatively poorly understood, but are likely to include genetic factors although none have been described to date.

The management of gastric NETs remains controversial, but is influenced by tumour type, grade and stage. Small localized type I gastric NETs can often be safely left untreated. However, larger polyps may require treatment with endoscopic polypectomy (2), SST analogues (9), removing the source of hypergastrinaemia by antrectomy (10) or potentially by using gastrin/CCK-2 receptor antagonist drugs such as netazepide (11).

In the present study, we used whole exome sequencing (WES) to study a consanguineous Roma/Gypsy family from Majorca Island (Spain), in which five of 10 siblings were affected by an atypical and aggressive gastric type I NET. We found a homozygous missense mutation in the ATP4A gene in all affected family members.

Results

Whole exome sequencing

We performed a WES of three siblings with type I gastric NET (II-3, II-7 and II-9) and their healthy parents (I-1 and II-2) (Fig. 1). The first variant calling of the five exomes generated 266 269 variants (Supplementary Material, Fig. S1). Only 1354 variants not present in Ensembl were found in common within the five sequenced exomes of the family. Taking into account the consanguinity of the parents, we considered a recessive model where the affected offspring had the responsible variant in homozygosity. Three variants annotated on chromosome 19 fitted our assumption: G.36046392>A (ATP4A), TGAA.48950001>T (GRWD1) and C.34291328>T (KCTD15), but only the first one fulfilled the different filtering criteria of deleteriousness (Condel: 0.945; Sift: 0; and Polyphen: 1).

The ATP4A gene (Chr19: 36040945–36054560, reverse strand) encodes the catalytic subunit of a gastric proton pump that uses the hydrolysis of ATP to generate an acid environment in the stomach. The canonical transcript of the ATP4A gene (22-exons) encodes a 1035 amino acids protein. Variant G.36046392A (reverse and complementary: c.2107C>T) is a missense mutation entailing the change p.Arg703Cys in exon 14 of the canonical transcript. The reference allele (C/C) was highly conserved along the phylogeny. The GERP conservation score was 2.5700 (values >2 are considered highly conserved) and the Grantham score was 180 (values >151 are considered radically conserved).

Sanger sequencing confirmed that the variant was present in heterozygosis in the healthy parents (C/T) and in homozygosis (T/T) in the three exome-sequenced affected offspring. The variant was also found in homozygosis (T/T) in the other two affected siblings (II-1 and II-8). We extended the study to other healthy members of the family and confirmed their status as either heterozygous (C/T) or normal genotype (C/C) (Fig. 1).

We tested the variant in three control populations (581 Spanish individuals, 116 Spanish Roma/Gypsy individuals and 494 individuals from Majorca Island), but we only found a single heterozygous case in the first group.

Figure 1. Pedigree of studied family with complete segregation of the G.36046392 A ATP4A variant (c.2107C>T).
In silico studies

Independence amino acid substitution at p.R703 position is not tolerated according with SNAP2 PredictProtein (Supplementary Material, Fig. S2) (12).

Putative transmembrane helices of ATP4A protein were predicted by PHDhtm algorithm (13). In wild type ATP4A, dehalogenase domain is annotated between fourth and fifth transmembrane helices (Supplementary Material, Fig. S2) of the protein and has cytosolic orientation. However, putative location of dehalogenase domain was annotated between the third and fourth transmembrane helices in the mutated ATP4A protein (when C was used instead of R residue at p.703 position). This putative new transmembrane helices location might alter the orientation of the dehalogenase domain from cytosol to gastric lumen and avoid the exit of protons out the cell.

ATP4A gene study in other cases with type I gastric NET

We looked for the ATP4A mutation in a set of 14 sporadic cases (B1-B14) and another 3 cases from UK (1–3) who had classical type I gastric NETs (with achlorhydria and hypergastrinemia) (Table 1). All cases had a late age of onset and megaloblastic anaemia, except sporadic case B12 who had an iron-deficiency anaemia. None of these individuals showed the mutation found in our studied family (Table 1). We sequenced the entire ATP4A gene in this group of cases, but no other putative pathologic variants were found using different predictors (Supplementary Material, Table S1). In addition, we could sequence the whole gene in DNA from paraffin-embedded tissues and fixed tissue slides of 4 (T1–T4) sporadic tumours (Table 2), and once again, no mutations were found (Supplementary Material, Table S1).

Immunohistochemistry studies

Proliferation index (Ki67) values for tumour tissues ranged between 3 and 4% in all affected family members (Table 2) that is within the range of most type I gastric NETs (Ki67 2–20%) (4).

Staining was quantified considering normal and tumour tissue areas (Table 2). A similar pattern of antibody binding was observed for the five siblings who had gastric NETs. The staining pattern of NET case II-8 is shown as an example in Figure 2. No positive staining was observed with anti-ATP4A (because parietal cells are absent in type I NET), and anti-SST antibodies in either the tumour or normal gastric corpus. However, strong anti-SSTR2 staining was observed in normal and tumour tissues from these

Table 1. Characteristics of familial and sporadic gastric NET type I cases used in the present study

<table>
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<th>Case</th>
<th>Name</th>
<th>Sex</th>
<th>Gastric NET</th>
<th>Number of polyps</th>
<th>Age/age at diagnosis (years)</th>
<th>ATP4A Mutation</th>
<th>Gastrin (pM)</th>
<th>Anaemia</th>
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<td>B12</td>
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Hz_ref, homozygous for the reference; Fe, iron-deficiency anaemia; U, unknown; Hz_var, homozygous for the variant; B12, megaloblastic anaemia; NT, not tested; Hz_ref, heterozygous; F, female; M, male.

*Members of generation III in family 1 ranged in age from 14 to 23, gastrin levels were normal and no presented anaemia.
patients compared with controls. SSTR2 staining was also found to be increased in endoscopic biopsy samples obtained from heterozygous carriers (P = 0.012) The ATP4A signal was also increased in heterozygous carriers (III-1 and III-4) who appeared to have higher numbers of parietal cells. The expression level by quantitative RT-PCR of ATP4A was found to be significantly increased in heterozygous subjects compared with homozygous reference cases (P = 0.006) while the expression level of SST was not significantly different (P = 0.41).

The increased SSTR2 hybridization signal along the higher expression of ATP4A in heterozygous subjects could suggest that heterozygous individuals might need higher ATP4A production to achieve normal levels of gastric acid secretion.

Paraffin-embedded tissue samples from the gastric fundus of four sporadic gastric NET cases (T1–T4) were available for analysis. The staining pattern was very similar to familial cases: no anti-SST and ATP4A hybridization signals were observed in tumoural tissue, while strong immune reactivity with anti-SSTR2 was observed (Fig. 2).

Clinical characteristics of the studied patients

High serum gastrin concentrations were found in all cases, ranging from 200 to 3500 pM (Table 1) (normal gastrin levels in serum are quoted as being <40 pM) (14). The studied family presented some specific clinical characteristics: (i) tumour aggressiveness: three out of five patients showed nodal infiltration and one of them had a synchronous focus of gastric adenocarcinoma (T1b N0); (ii) early age of onset: the mean age at diagnosis was 31.8 years old compared with 56.7 in the other studied cases (Table 1); (iii) type of anaemia: patients from the affected family presented with iron-deficiency anaemia, whereas most of other gastric NETs were associated with a megaloblastic anaemia. Interestingly, a correlation was found between age of onset and anaemia type. The mean age of onset for patients with iron-deficiency anaemia was 32.5 years while patients with megaloblastic anaemia were on average 52.6 years old at diagnosis (P = 0.002) (Fig. 3).

In order to clarify the origin of the different types of anaemia and the relation with the age of onset, we selected gastric NETs from our studied family who had iron-deficiency anaemia, and compared them with four paraffin-embedded tissue blocks from patients who had sporadic type I gastric NETs and megaloblastic anaemia, and performed IHC using an anti-IF that labels parietal cells. Staining (58.97%) in normal gastric tissue from a patient undergoing bariatric surgery was used as a positive control. Staining in gastric NETs was quantified considering normal and tumour tissue separately. No positive staining was observed with anti-IF in the tumoural (0.31% in average) or normal (0.97% in average) tissues from the four sporadic type I gastric NET cases. However, in the familial cases who had iron-deficiency anaemia, positive IF staining was found in the non-tumoural gastric tissue (21.66% on average). This was statistically different compared with normal gastric tissue from patients with sporadic type I gastric NETs who had megaloblastic anaemia (P = 0.021) (Fig. 4).

Discussion

We found a novel damaging missense variant (p.Arg703Cys) in the ATP4A gene in a consanguineous Roma/Gypsy family with five siblings who were affected by type I gastric NETs. All five affected members were homozygous for the variant, whereas the healthy family members were heterozygous or wild-type (Fig. 1). This is the first description of a hereditary type I gastric NET that follows a recessive model of inheritance.

ATP4A is the catalytic α subunit of a heterodimeric proton pump located in parietal cells. A mutation in this gene could
inhibit gastric acid production and explains the achlorhydria and hypergastrinaemia that were associated with the development of type I gastric NET tumours in the affected family. The variant was found in heterozygosis once in a group of 581 normal Spanish individuals. The variant was, however, detected neither in 116 Roma/Gypsy individuals nor in 494 individuals from Majorca Island, ruling out a possible founder mutation. We extended the study of this variant to other 21 sporadic gastric NET (17 in blood and 4 in tumour tissue), but we did not find any other case with this mutation. We then sequenced the entire ATP4A gene, but no other germline or somatic mutations were found. These results and the different clinical traits regarding the studied family suggest that the disturbance in gastric acid secretion and consequent deregulation of gastrin secretion that leads to hyperplasia of ECL cells may therefore have a heterogeneous origin, even within type I NETs.

Figure 2. IHC for selected antibodies in control tissue (from stomach reduction surgery), gastric NET type I (T/T), endoscopy sample from heterozygous members (C/T) of the studied family and sporadic gastric NET type I (T1). Normal (N) and tumour (T) tissue were considered separately.
The variant (p.R703Cys) is located within the conserved phosphoryl binding pocket that stabilizes the transition states during phosphoryl transfer within the P-domain between the segments. This arginine residue is conserved throughout the ATPases of eutherians (data not shown), presents high GERP and Grantham scores of conservation and has very low tolerance to amino acid substitution in this position (Supplementary Material, Fig. S2).

Anti-IF staining was negative in tumour tissue samples from gastric NET cases although it was observed in the non-tumoural gastric tissue of affected individuals in the studied family (Fig. 4). This suggests that some parietal cells remain within the stomach of the affected members of the family, although they do not stain for anti-ATP4A. Similar SST and SSTR2 staining patterns were observed for familial and other sporadic type I gastric NET cases. In heterozygous individuals (who had normal serum gastrin concentrations and normal gastric acid production), a moderate increase in ATP4A and SSTR2 staining was observed (Table 2), probably due to the need for larger number of parietal cells to achieve normal levels of gastric acid production in these individuals. This hypothesis was corroborated by RT-qPCR results. These results might suggest that heterozygous individuals have a single functional copy of ATP4A that increases its expression in order to achieve normal acid secretion, while in homozygous individuals, the presence of two abnormal ATP4A copies results in achlorhydria first and hypergastrinaemia later.

Figure 3. Age of diagnosis (Dx) and origin of anaemia (iron or vitamin B12 deficiency) for samples included in Table 1. Significance of the difference was calculated with the non-parametric Mann-Whitney test.

Regarding treatment, in humans, gastric acid secretion is blocked by administering proton pump inhibitor drugs (PPIs). Acid suppressing medications remain remarkably safe and effective in the vast majority of persons and are used to treat peptic ulcer disorders and gastroesophageal reflux disease (15). However, a possible relationship between PPI-induced achlorhydria and gastric NET development in humans remains controversial (16). It has also been described that fundic gland polyps develop in patients on long-term PPI therapy (17) and solitary well-differentiated NETs have been described in the oxyntic mucosa of two patients who were on long-term PPI treatment (18). On the other hand, studies in mice have demonstrated that absence of H+/K+-ATPase activity resulted in chronic loss of acid secretion, hypergastrinaemia, severe mucocystic hyperplasia (19) and iron-deficiency anaemia (20).

Genetic and clinical heterogeneity of type I gastric NETs

A significant correlation (P = 0.002) was found between age of onset of type I gastric NET and type of anaemia (Fig. 3). Deficiencies in iron absorption (due to achlorhydria) were observed in the affected members from the studied family and in one sporadic type I gastric NET case with an early age of onset (Table 1), whereas a deficiency of vitamin B12 (due to the lack of the IF glycoprotein) was found in the other studied cases. Anaemia is usually associated with gastric NETs type I as a consequence of the complete loss of parietal cells from autoimmune atrophic gastritis. This fact conducts to an absence of IF and achlorhydria, leading to vitamin B12 and iron malabsorption, respectively. However, in this family, achlorhydria directly arose from altered proton pump activity instead of from the actual loss of parietal cells during the long-term period of hypergastrinaemia (21). This results in iron-deficiency alone. On the other hand, IHC with anti-IF showed that some parietal cells (non-acid-secreting) remained within the stomachs of these individuals, suggesting that vitamin B12 could be still absorbed normally. Therefore, positive anti-IF staining and iron-deficiency anaemia alongside an early age of onset of gastric NET might be markers that would suggest an inherited abnormality of gastric acid production as described in the family outlined earlier. In addition, gastric NETs type I rarely behave in a malignant fashion. However, in the family described in this paper, three of five NETs presented with nodal infiltration and one of the patients had a synchronous focus of gastric adenocarcinoma. In fact, the treatment of all five affected members involved total gastrectomy.

Based on the clinical traits and genetic characteristics of the family, it is suggested that deregulation of the proton pump and achlorhydria from birth are probably responsible for the
very large number of observed polyps, nodal infiltration, earlier age of onset and tumour aggressiveness (Table 1). Lack of iron absorption has given rise to an iron-deficiency anaemia, while the normal production of IF has prevented the development of megaloectatic anaemia. The hypergastrinaemia observed in the other studied gastric NET cases did not arise as a result of mutations in ATP4A gene, but from the achlorhydria that is associated with the lack of parietal cells. Therefore, these sporadic type I gastric NETs developed in later life (consistent with the later age of acquisition of autoimmune atrophic gastritis) and were associated with a megaloblastic anaemia as a result of IF deficiency.

In summary, therefore, we have identified ATP4A as being responsible for an atypical familial recessive form of gastric type I NET. The early identification of these cases by IHC (using the IF biomarker) and/or iron-deficiency anaemia might justify a more aggressive surgical approach (such as total gastrectomy) and may help to guide new treatments for these gastric tumours in order to improve long-term prognosis. However, more studies in additional series of patients are necessary to confirm these preliminary results.

Materials and Methods

Patients

Family of study

A consanguineous Roma/Gypsy family (the parents were cousins) from Majorca Island (Spain) with 10 siblings was studied. Five of them with ages around 30 years were diagnosed with type I gastric NETs (II-1, II-3, II-7, II-8, II-9). Three showed nodal infiltration (II-1, II-3 and II-7) and one (II-3) had a synchronous focus of gastric adenocarcinoma without nodal infiltration (T1b N0) (Fig. 1). All patients were treated with total gastrectomy, were negative in MEN1 gene mutation studies by Sanger sequencing and for H. pylori. Both parents (I-1 and I-2) and siblings II-3, II-7 and II-9 were selected for WES. Blood samples from the remaining healthy individuals in the family were also obtained for further study of the segregation of the variants (Fig. 1) (Table 1).

Other studied cases

Blood samples from 14 sporadic type I gastric NET cases (B1–B14) were collected from various Spanish hospitals for the ATP4A (proton pump) gene study (Table 1). Blood samples from three type I gastric NETs cases (59, 53 and 48 years old at diagnosis) from UK were also collected. All samples were negative for MEN1 mutations by Sanger sequencing and H. pylori tests.

Controls

A group of 581 normal DNA samples from controls with no antecedents of cancer were used as a general Spanish control population for the study of the ATP4A mutation. In addition, 116 DNA samples from the Roma/Gypsy population and 494 individuals from Majorca Island were used as controls for the mutation study in order to confirm or rule out a possible founder mutation.

Paraffin-embedded tissue samples

Paraffin-embedded tissue samples of gastric NETs from affected and healthy heterozygous members of the family were obtained from total gastrectomy specimens. Gastric fundus biopsies were also collected from healthy family members I-1, II-4 and III-4. Paraffin-embedded tissue samples of the gastric fundus from four sporadic (T1-T4) gastric NETs were also studied (Table 2). All samples were negative for MEN1 mutations and H. pylori tests except T3, which was classified as a type II gastric NET (MEN1 positive). Normal paraffin-embedded gastric fundus and antrum tissues were also collected as controls (sample N1) from a patient undergoing bariatric surgery by stomach reduction. Written informed consent was obtained from all individuals included in this study.

DNA was extracted from paraffin-embedded tissue and from fixed tissue slides (micro dissection) using the DNeasy® Blood & Tissue Kit (Cat. No. 69504, Qiagen) following the manufacturer’s instructions.

Whole exome sequencing and bioinformatic pipeline

Genomic DNA was isolated from peripheral blood lymphocytes using the FlexiGene DNA Kit (Qiagen), and DNA concentration was determined using PicoGreen dsDNA quantification reagent (Invitrogen).

Exomes from selected DNA samples were fully captured, enriched and sequenced using the SureSelect Human All Exon Kit for 71 Mb (Agilent Technologies). WES was achieved using the SOLID 5500XL sequencing platform. ‘Paired end’ reads of 101 nt long were generated. A summary of the bioinformatic pipeline is shown in Supplementary Material, Figure S1. Reads were mapped against the Human reference genome GRCh37/hg19 with Bioscope v1.3 (www.bioscope.com). Coverage metrics were computed and target enrichment was evaluated using custom scripts. PCR duplicates, low quality mapping or multimapping reads (quality score of 0) were removed from the final alignment file. Filtering processes were performed using Picard-tools (http://picard.sourceforge.net/) and Samtools (22).

Variant calling was performed by combining three different algorithms: bioscope version 1.3 (www.bioscope.com), VarScan (23) and GATK (24). Identified variants were annotated using the Ensembl (release 64) database (25). Variants not described in the Ensembl database (dbSNP130, HapMap and 1000 Genomes) and known variants with a minimal allele frequency (MAF) of <0.01 and an MAF of <0.05 were considered for a dominant and a recessive hypothesis, respectively.

The overall sample coverage distribution along targeted regions was evaluated at this point. Variant effect prediction was tested using a modification of variant effect predictor script package (including Sift, Polyphen and Condel damage predictors) from Ensembl (26). A Heatmap representation of the tolerance to independent amino acid substitutions was predicted with the SNAP2 (implemented in PredictProtein software) that assesses the potential functional impact of the variants (12). We assessed the potential splice sites alteration with Alamut®-Mutation Interpretation Software version 2.3.2 (available at www.interactive-biosoftware.com) and SplicePort (27).

Variants were considered heterozygous with allele frequencies spanning from 0.2 to 0.8. Variants with frequencies over 0.8 were taken as homozygous.

Validation studies

Candidate variants were validated by Sanger sequencing, followed by segregation studies among the individuals of the studied family. Sanger sequencing of the candidate variant and sequencing of the 22 exons of ATP4A was also performed in the other gastric NET type I cases. Primers are shown in Supplementary Material, Table S2.

Real-time quantitative PCR

Real-Time-quantitative PCR (RT-qPCR) was performed with cDNA to test the levels of expression of ATP4A mRNA. cDNA was
obtained from reverse transcription of 1200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368814) following the manufacturer’s instructions. RNA was extracted from blood cells and the concentration quantified using Nanodrop ND-1000 (Wilmington).

Quantification was performed using Sequence Detection System 7900HT (Applied Biosystems). PCR was carried out with ~25 ng/µl of cDNA and POWER SYBR® green PCR Master Mix (Applied Biosystems #4367659).

Expression levels were evaluated with the ΔΔCt method (28) normalizing with GAPDH quantification as a standard. Significance of the differences in expression of the ATP4A and SST genes among individuals was assessed using 2−ΔΔCt values of each triplicate for each sample (28). Normal expression controls were included in all PCR series and assays were carried out in triplicate for each sample (28). Normal expression controls were performed to determine normal distribution of values. Student’s t-test was used for statistical comparison of normally distributed values.

dHPLC

The candidate variant was genotyped in the three control population groups by dHPLC as described (29). Heteroduplex was amplified with ATP4A exon 14 primers (which encompass the candidate variant).

Immunohistochemistry studies

Gastric tumour and mucosal histopathology were independently assessed in haematoxylin and eosin stained sections by two independent observers (WHO-revised classification of gastric tumours). Immunohistochemistry studies for ATP4A, SST and SSTR2. Anti-Ki67 IHC was also performed to determine the proliferation index of the tissue samples (Table 2). Details of the antibodies, incubation conditions and antigen retrieval methods are given in Supplementary Material, Table S3. Anti-IF antibody was homemade produced here at CNIO (30).

Normal tissue (sample N1) was used for control incubations and for determining normal score values. Quantification of the signal was achieved with Axiowiew software. Training (discrimination between positive and negative staining) was performed in triplicate using N1 normal tissue. Both the absence of staining and excess non-specific staining were considered as negative staining. Staining signals were quantified in triplicate and compared between individuals. Two strategies were performed for quantification. ATP4A and SSTR2 staining patterns were quantified considering the stained area relative to the total area (as both are membrane proteins), whereas Ki67, IF and SST staining patterns were quantified as the number of stained cells relative to the total number of cells (as both proteins are expressed intracellularly). Within samples from patients with gastric NETs, staining was quantified separately in normal (N) and tumour (T) tissue. Statistical comparison of staining scores was performed with a non-parametric Kruskal–Wallis test for K independent samples. Exact significance was considered for P-value acceptance.

Supplementary Material

Supplementary Material is available at HMG online.

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