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**Glucagon receptor gene mutations with hyperglucagonemia but without the glucagonoma syndrome**

Miller HC *et al*. Hyperglucagonemia without glucagonoma

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**Abstract**

Pancreatic neoplasms producing exclusively glucagon associated with glucagon cell hyperplasia of the islets and not related to hereditary endocrine syndromes have been recently described. They represent a novel entity within the panel of non-syndromic disorders associated with hyperglucagonemia. This case report describes a 36 year old female with a 10 year history of non-specific abdominal pain. No underlying cause was evident despite extensive diagnostic work-up. More recently she was diagnosed with gall bladder stones. Abdominal ultrasound, computerised tomography and magnetic resonance imaging revealed no pathologic findings apart from cholelithiasis. Endoscopic ultrasound revealed a 5.5 mm pancreatic lesion. Fine needle aspiration showed cells focally expressing chromogranin, suggestive but not diagnostic of a low grade neuroendocrine tumor. OctreoScan® was negative. Serum glucagon was elevated to 66 pmol/L (normal: 0-50 pmol/L). Other gut hormones, chromogranin A and chromogranin B were normal. Cholecystectomy and enucleation of the pancreatic lesion were undertaken. Postoperatively, abdominal symptoms resolved and serum glucagon dropped to 7 pmol/L. Although H&E staining confirmed normal pancreatic tissue, immunohistochemistry was initially thought to be suggestive of alpha cell hyperplasia. A count of glucagon positive cells from 5 islets, compared to 5 islets from 5 normal pancreata indicated that islet size and glucagon cell ratios were increased, however still within the wide range of normal physiological findings. Glucagon receptor gene (*GCGR*) sequencing revealed a heterozygous deletion, K349\_G359del and 4 missense mutations. This case may potentially represent a progenitor stage of glucagon cell adenomatosis with hyperglucagonemia in the absence of glucagonoma syndrome. The identification of novel *GCGR* mutations suggests that these may represent the underlying cause of this condition.

**Key words**: Hyperglucagonemia; Glucagon receptor gene; Mutation; Adenomatosis; Pancreas

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**Core tip:** We identify novel mutations in the glucagon receptor gene in a patient with hyperglucagonemia but no glucagonoma syndrome. Physicians dealing with pancreatic disorders should be aware of this unusual condition.

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**INTRODUCTION**

Glucagon cell adenomatosis has been reported by Henopp *et al*[1] as an independent previously unrecognised disease of the endocrine pancreas. Multiple pancreatic neoplasms exclusively producing glucagon, associated with glucagon cell hyperplasia of the islets and unrelated to multiple endocrine neoplasia (MEN) type 1 (MEN 1), p27 MEN or von Hippel-Lindau (VHL) syndromes, are the hallmarks of the condition[2]. To date very few such patients have been reported[1,3,4].

Most patients present with abdominal pain and increased serum glucagon levels but fail to exhibit the characteristics of the glucagonoma syndrome (necrolytic migratory erythema, diabetes mellitus, stomatitis and weight loss)[5]. While macroscopic tumors are evident on imaging in some, numerous microadenomas scattered throughout the pancreas and enlarged islets are the findings in others[1,3,4]. Malignancy has not been identified in any cases reported to date. The underlying cause of glucagon cell hyperplasia and consequent development of glucagon cell neoplasia without the glucagonoma syndrome remains unknown. Zhou *et al*[3,6] have proposed that malfunction of the glucagon receptor (GCGR) and/or glucagon may be responsible for the disease after detection of a homozygous missense mutation, c.256C>T (P86S) in the *GCGR* of a patient.

We present another example of hyperglucagonemia without morphological evidence of neoplasia or the glucagonoma syndrome in which we identified *GCGR* mutations which may represent the underlying pathogenic cause of the condition.

**CASE REPORT**

A 36 years old Caucasian female with no previous medical or known family history was referred to us in 2011 with a 10 year history of non-specific diffuse abdominal pain. She repeatedly underwent complete gastrointestinal diagnostic work-up over a period of 8 years which revealed no pathologic results. In 2009, she had been diagnosed with cholelithiasis on abdominal ultrasound. Upon referral to our centre in 2011, extensive investigations including upper and lower intestinal endoscopy, computerised tomography (CT) and magnetic resonance imaging (MRI) were carried out. Apart from the previously diagnosed cholelithiasis, no other pathology was evident. Endoscopic ultrasound (EUS) confirmed calculi in the gallbladder and a mild dilatation of the distal common bile duct. In addition, a 5.5 mm hypoechoic lesion with irregular margins was detected in the pancreatic tail. Fine needle aspiration (FNA) revealed cells focally expressing chromogranin A (CgA). The features were suggestive but not diagnostic of a low grade neuroendocrine tumor. Somatostatin receptor scintigraphy showed no foci of increased uptake. While serum gastrin, vasoactive intestinal polypeptide, somatostatin, and pancreatic polypeptide were within the normal range, glucagon was elevated to 66 pmol/L (normal: 0-50 pmol/L). Serum fasting and postprandial glucose was normal. Neuroendocrine tumor markers chromogranin A and chromogranin B were not elevated. At laparotomy, a sub-centimeter lobulated lesion was found at the inferior margin of the pancreatic tail corresponding with the lesion identified on EUS. No further lesions were identified in the remaining pancreas after meticulous bimanual exploration and intraoperative ultrasound. There were no enlarged peripancreatic lymph nodes. The pancreatic tail lesion was enucleated and cholecystectomy performed. A grade 1 pancreatic fistula developed postoperatively and resolved within 2 wk. The further course was uneventful and the patient was entirely asymptomatic. Moreover, she reported that the abdominal pain she experienced over the last decade had completely disappeared. Serum glucagon was assessed 1 mo postoperatively after the pancreatic morphology returned to normal on imaging. It was found to have decreased to 7 pmol/L. Serum glucagon was monitored at regular intervals (see Table 1). At the last follow-up, 31 mo after surgery, the patient remained asymptomatic with a normal MRI result, serum glucagon was 10 pmol/L and insulin was within the normal range.

Histology (H&E) showed features of normal pancreatic tissue. Immunohistochemical examination for glucagon and insulin was undertaken using the technique of Henopp *et al*[1]. Approximately 20% of the islet cells were glucagon positive and 80% insulin positive. Glucagon cell hyperplasia was initially considered (Figure 1). In order to investigate this further, glucagon cell counts were done with 5 islets from 5 normal pancreatic controls and compared to 5 islets from the patient (Table 2). The counts showed that the average islet size and the average number of glucagon positive cells per islet were increased in the patient, however still within the wide range observed in normal pancreatic tissue.

***Methods of genetic investigation***

A peripheral blood sample was obtained from the patient, her daughter and a healthy individual as a normal control (informed consent obtained). Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit according to the protocol (Qiagen, catalogue number: 9506). PCR amplification of exons 2-13 (and most of exon 14) of *GCGR* and the intron:exon borders was carried out using previously described primers[6]. Purified PCR products were sequenced by the W.M. Keck Biotechnology Resource Laboratory at Yale University, New Haven, United States using an automated Applied Biosystems 373A Stretch DNA sequencer (Perkin-Elmer, Norwalk, United States). PCR products were sequenced using forward primers. If ambiguous peaks were evident, the sequence was confirmed with the reverse primers[7]. Bioedit software was used to analyse the sequencing results[8]. Sequencing products were compared to the control sample and the national centre for biotechnology information (NCBI) reference sequences for the human *GCGR*, DNA (NG\_016409.1), mRNA (NM\_000160.3) and protein (NP\_000151.1).

*MEN1* sequencing was carried out on gDNA from the peripheral blood. PCR amplification of exons 2-10 of *MEN1* was undertaken using previously described primers[9,10]. The DNA extraction, sequencing and analysis were carried out using the same technique as for *GCGR*. The reference sequence used was NCBI GenBank: U93237.1.

*VHL* sequencing was carried out on gDNA from the peripheral blood. PCR amplification of exons 1-3 of *VHL* was undertaken using primers previously described[11]. The DNA extraction, sequencing and analysis were carried out using the same technique as for *GCGR*. The reference sequence used was NCBI GenBank: NM\_000551.3.

***Results of genetic analysis***

A heterozygous deletion of 33 nucleotides in exon 11 of the *GCGR* was detected. This corresponded to a K349\_G359del in the GCGR with the loss of the following 11 amino acids, KSTLTLIPLLG. There were also 5 heterozygous point mutations including 4 missense mutations, E362K, V368L, K381E, S389N and 1 synonymous mutation (Figure 2). There were no mutations in *MEN1* or *VHL*. No mutations were detectable in the daughter.

**DISCUSSION**

This case report represents the second case of hyperglucagonemia which has been associated with a specific genetic lesion in the *GCGR.* The case could potentially represent a progenitor stage of an entity leading to glucagon cell adenomatosis.

To date 8 individuals exhibiting characteristics of glucagon cell adenomatosis with hyperglucagonemia but without glucagonoma syndrome have been reported in the literature[1,3,4,6,12,13]. It is a matter of debate whether all cases cited completely fulfil the criteria of glucagon cell adenomatosis as defined by Henopp *et al*[1]. For example, the individual described by Yu *et al*[3] had not only raised serum glucagon levels but also pathologic values of pancreatic polypeptide. The patient reported by Balas *et al*[13] in 1988 had normal serum glucagon levels; however immunohistological findings in the resected pancreas were consistent with glucagon cell adenomatosis. In our patient, although the morphology of the resected pancreatic islets was within the broad range of findings reported in unaffected pancreata, we speculate that a cluster of hyperfunctioning cells might potentially be responsible for the development of hyperglucagonemia. Functional studies would be needed to confirm this theory.

The majority of individuals had glucagon cell adenomatosis, but were asymptomatic with respect to evidence of the glucagonoma syndrome. The results of imaging ranged from no pathologic findings to diffuse pancreatic enlargement associated with multiple tumors of various sizes. Abdominal pain is present in most individuals as was the case in our patient (Table 3). While the case we present exhibited normal uptake on somatostatin receptor scintigraphy, diffusely increased uptake was reported on OctreoScan® in a patient with diffuse pancreatic enlargement and multiple tumors by Henopp *et al*[1]. In our patient the positive staining for chromogranin on FNA was thought to be suggestive of a neuroendocrine tumor. This might reflect the small number of cells obtained from the FNA, with a sampling error leading to a higher proportion of chromogranin positive cells (*e.g.,* if FNA sampling comprised an islet). In comparison to two reported cases which had highly elevated serum glucagon levels, our patient had only slightly increased serum glucagon (Table 3). The lack of standardised serum glucagon reporting in the majority of cases and the small number of patients means it is difficult to tell if the levels in our patient were truly lower than average.

The majority of previously reported patients demonstrated numerous microadenomas expressing almost exclusively glucagon and/or glucagon cell hyperplasia. This observation prompted Henopp *et al*[1,14] to postulate that diffuse glucagon cell hyperplasia might represent a precursor from of glucagon cell neoplasia. In the case described by Yu *et al*[3], 60-80% of the hyperplastic islet cells stained positive for glucagon but negative for insulin. A similar trend was noted by Henopp *et al*[1]. In our patient, the pancreatic morphology was unusual, nevertheless still within the wide range of physiological findings. Approximately 20% of the islet cells expressed glucagon while 80% expressed insulin. Based on this observation and only mildly increased serum glucagon, we hypothesize that the disease might have been diagnosed at a very early stage prior to evidence of hyperplasic transformation and development of overt morphological evidence of neoplasia/s. While a subcentimeter nodule at the pancreatic tail was evident on EUS and confirmed intraoperatively, standard histology showed regular findings. This scenario resembles a report by Martignoni *et al*[4] of hyperglucagonemia but no microadenomas.

Both of the two previously reported patients for whom follow-up data was available showed increased serum glucagon levels after pancreatic resection in the presence of negative imaging results[1,3]. These findings underline the presumption of disease persistence. Our patient however, had normal serum glucagon levels at 31 mo after surgery (10 pmol/L) (Table 1). Due to the genetic predisposition of the disease we cannot exclude the possibility that at some point in the future the disease may recur therefore our patient requires life-long follow up. Any future increases in serum glucagon levels could potentially represent the emergence of alpha cell hyperfunction consistent with the concept of a residual genomic lesion representing a diffuse alpha cell abnormality in the remaining pancreatic islets.

The GCGR is a member of the class B G protein-coupled receptor family, glucagon binding triggers downstream signalling, allowing glucagon to regulate blood glucose levels by stimulating glycogenolysis[15,16]. The knockout mouse for *GCGR* expresses high glucagon levels associated with pancreatic enlargement, glucagon cell adenomatosis and microglucagonomas or glucagonomas at 10-12 mo when compared to their heterozygous littermates[17,18]. Based on these observations, Yu and colleagues[3,6] sequenced *GCGR* and the glucagon gene in their patient with hyperglucagonemia, alpha cell hyperplasia and microglucagonoma. They detected a homozygous c.256C>T (P86S) mutation in *GCGR* resulting in lower binding affinity of GCGR P86S to glucagon and hypothesized that this mutation was responsible for the alpha cell hyperplasia and hyperglucagonemia. They showed *in vitro* that the GCGR P86S localized to the plasma membrane but bound glucagon with less avidity than wild type GCGR; a greater glucagon concentration was thus needed to trigger downstream signalling via adenylate cyclase activation[6]. Neuroendocrine cells undergoing hyperplastic changes is particularly relevant for MEN1 conditions however they probably also occur in sporadic cases. Very recently Klöppel *et al*[14] identified 3 further patients with germline *GCGR* mutations and glucagon cell adenomatosis unrelated to MEN1 or VHL syndromes. The genetic lesions present in the *GCGR* were not described, however a further 3 patients had glucagon cell adenomatosis in the absence of any *GCGR* mutation[14].

Our case represents the second case with genetic lesions described in the *GCGR* associated with hyperglucagonemia in the absence of the glucagonoma syndrome. The heterozygous K349\_G359del and E362K, V368L, K381E, S389N mutations could potentially represent a loss of function mutation in the *GCGR.* Functional studies would be needed to show if these mutations might be the cause of the hyperglucagonemia observed in our patient. All mutations were in exon 11 towards the C terminal end of GCGR. The point mutations appear to represent rather conservative amino acid changes in terms of hydrophobicity. Lysine and glutamate have a positively and a negatively charged R group respectively and the serine to asparagine change represents an alteration from a hydroxyl R group to a carboxamide R group. Site directed mutagenesis studies have noted that D385 is relevant to the specificity of glucagon/GCGR binding[19]. Since this residue is close to the K381E mutation site and adjacent to the glucagon binding site, the alteration in R group may affect glucagon binding. However in the absence of high resolution crystal structure data for the human glucagon receptor (except for the extracellular N terminal domain) and site directed mutagenesis studies for these sites, the effects of these genetic changes cannot be directly inferred[15].

The K349\_G359del falls within the 6th transmembrane domain of GCGR, therefore the 11 amino acid deletion could prevent GCGR from inserting into the plasma membrane. This would prevent GCGR binding to glucagon[20]. In structural studies where COS-1 cells were transfected with the rat glucagon receptor gene, truncation mutants lacking any of the different transmembrane domains, were not localized to the plasma membrane suggesting that all 7 transmembrane domains are needed for correct membrane insertion[21]. The K349\_G359del mutation is predicted by membrane topology prediction software to prevent the GCGR from properly inserting into the plasma membrane[22] (Figure 3). If this was the case, then the GCGR would be miss-localized preventing glucagon binding. This would however need to be confirmed by *in vitro* protein localization studies and assays to check glucagon binding efficiency in the presence of the deletion. In addition, since the mutation in the *GCGR* is heterozygous, there would still be a normal gene copy present which might allow sufficient glucagon signalling via the remaining receptors to give normal function. However, the clinical pathology evident in the presence of hyperglucagonemia seems to suggest that this may not be the case.

The phenotype could potentially represent incomplete dominance leading to the modest elevation of serum glucagon in our patient. Alternatively, it is possible that this individual might have a second mutation in the other copy of the *GCGR* within some of the pancreatic alpha cells which could potentially be causing them to become hyperfunctional.

It has been previously suggested that incretin treatment is associated with the development of alpha cell hyperplasia since pancreata from autopsies of incretin treated persons exhibit alpha cell hyperplasia (and beta cell hyperplasia) and some had glucagon expressing microadenomas[23,24]. A possibility exists that as incretin usage increases, alpha cell hyperplasia may become more prevalent.

In conclusion, we have identified a novel heterozygous K349\_G359 deletion and 4 missense mutations in the *GCGR* which appear to be associated with hyperglucagonemia without the glucagonoma syndrome. Physicians dealing with pancreatic disorders should be aware of this very unusual condition. Further study leading to a better understanding of this disease entity would be of benefit to patients. The further usage of *GCGR* sequencing in such individuals should be undertaken to provide additional information on the breadth of the spectrum of mutational abnormalities associated with alpha cell transformation and excess glucagon production.

**COMMENTS**

***Case characteristics***

36 year old patient with a 10 year history of non-specific diffuse abdominal pain.

***Clinical diagnosis***

A sub-centimeter lobulated lesion was found at the inferior margin of the pancreatic tail, no further lesions were identified in the remaining pancreas after meticulous bimanual exploration and intraoperative ultrasound.

***Differential diagnosis***

Fine needle aspiration (FNA) revealed cells focally expressing CgA. The features were suggestive but not diagnostic of a low grade neuroendocrine tumor.

***Laboratory diagnosis***

Serum glucagon was elevated to 66 pmol/L (normal: 0-50 pmol/L). Other gut hormones were within the normal range.

***Imaging diagnosis***

Endoscopic ultrasound (EUS) identified a 5.5 mm hypoechoic lesion with irregular margins in the pancreatic tail.

***Pathological diagnosis***

Histology (H&E) showed features of normal pancreatic tissue. Glucagon cell hyperplasia was initially considered based on glucagon immunohistochemistry. Further investigation revealed that the average islet size and the average number of glucagon positive cells per islet were increased in the patient, however still within the wide range observed in normal pancreatic tissue.

***Treatment***

At laparotomy, a sub-centimeter lobulated lesion was found at the inferior margin of the pancreatic tail and was enucleated.

***Related reports***

This is a very rare disease entity. Genetic lesions in the *GCGR* have only been described in one individual in the literature in the context of glucagon cell adenomatosis with hyperglucagonemia but without glucagonoma syndrome. Several additional cases exhibiting the characteristics of glucagon cell adenomatosis with hyperglucagonemia but without glucagonoma syndrome have been published however their *GCGR* mutation status remains unknown.

***Experiences and lessons***

We have identified novel *GCGR* mutations which appear to be associated with hyperglucagonemia without the glucagonoma syndrome. Physicians dealing with pancreatic disorders should be aware of this very unusual condition.

***Peer-review***

This is an interesting case of an entity not described before.

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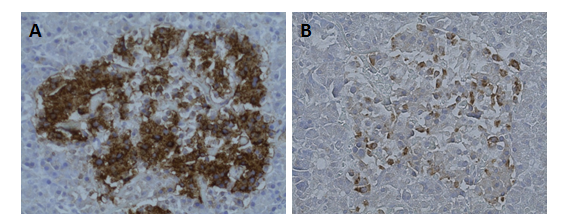
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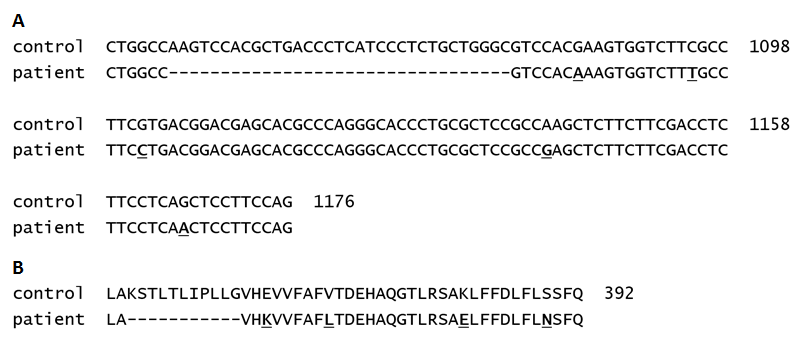
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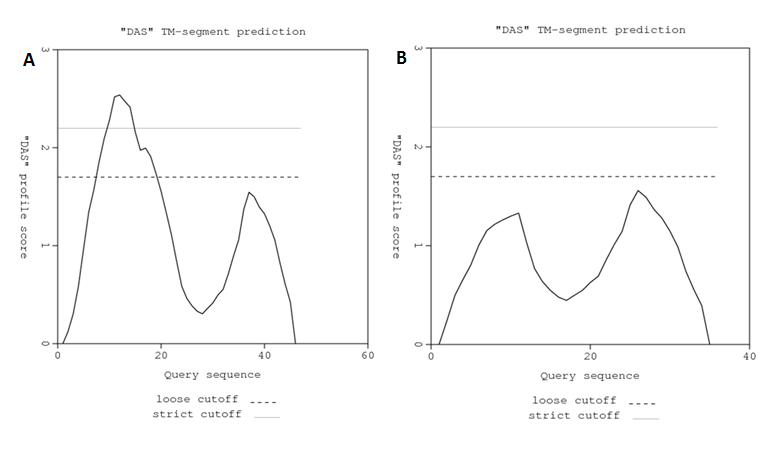
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**Figure 1 Islet from patient.** Immunohistochemistry for A: Insulin; B: Glucagon, x 200 magnification (600μm at maximum diameter).



**Figure 2 Genetic findings**. A: Sequencing results showing a heterozygous 33 nucleotide deletion and 5 point mutations in exon 11 of the *GCGR*; B: Amino acid sequence showing K349\_G359del, E362K, V368L, K381E, S389N in the GCGR. Alignments done using Clustal W multiple sequence alignments software[25]. Numbers indicate the position of the last residue shown along the GCGR cDNA/protein.



**Figure 3 Membrane topology prediction.** The loss of 11 amino acids from the GCGR 6th transmembrane domain is predicted to prevent the insertion of GCGR into the plasma membrane. A: Predicted normal GCGR membrane topology; B: Predicted K349\_G359del GCGR membrane topology. Software used: MetaTM[22]; input amino acid sequence shown in Figure 2B (DAS: Distributed Annotation System).

**Table 1 Serum glucagon levels**

|  |  |
| --- | --- |
| **Time** | **Serum glucagon (pmol/L)**  **(normal range: 0-50 pmol/L)** |
| Pre-surgery  2 mo | 66 |
| Post-surgery  1 mo | 7 |
| 5 mo | 28 |
| 6 mo | 6 |
| 17 mo | 15 |
| 20 mo | 29 |
| 31 mo | 10 |

**Table 2 Islet size and number of glucagon positive cells in the current case compared to 5 normal pancreata**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Average1**  **islet size** | **Average1 number**  **glucagon positive cells** | |
|  | **(μm)** | **Count 1** | **Count 2** |
| Patient | 192 | 29.2 | 28.8 |
| Control 1 | 256 | 59.8 | 61.8 |
| Control 2 | 186 | 25.6 | 25.2 |
| Control 3 | 255 | 32.2 | 31.6 |
| Control 4 | 260 | 52.4 | 53.4 |
| Control 5 | 190 | 44.6 | 42.6 |

1Average of 5 pancreatic islets.

**Table 3 Hyperglucagonemia without the glucagonoma syndrome – review of the literature**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Martignoni *et al***[4] | **Henopp *et al***[1]  (patient 2) | **Yu *et al***[3]**,**  **Zhou *et al***[6] | **Present case** |
| Patient | 54, M | 43, F | 60, F | 36, F |
| Origin | - | - | Persian | Caucasian |
| Clinical symptoms | Abdominal pain  Diarrhea1 | Abdominal pain | Abdominal pain  Constipation | Abdominal pain |
| Serum Glucagon (pmol/L) | Elevated | Elevated (25-fold)2 | 170113 | 66 |
| Imaging | Negative | Positive | Positive | Negative (positive on EUS) |
| OctreoScan® | Negative | - | Negative | Negative |
| Localization | No focal abnormality | Tail | Uncinate | Tail |
| Pancreatic pathology | α-cell hyperplasia  nesidioblastosis | α-cell hyperplasia, large cystic tumor and small solid tumors, multiple microadenomas | α-cell hyperplasia non-functioning pancreatic NET  microglucagonoma  microadenoma | Normal pancreatic morphology on standard H&E staining |
| *GCGR* | - | - | Homozygous  gDNA point mutation | Heterozygous gDNA deletion 5 point mutations |
| Other Genes | - | Negative for *MEN1/VHL* gDNA mutations | - | Negative for *MEN1/VHL* gDNA mutations |
| Relatives  *GCGR* | - | - | Brother  Negative | Daughter  Negative |

1Mild diabetes was initially suspected but then found to be unlikely; 2The glucagon levels were only measured postoperatively; 3Glucagon levels converted to pmol/L from pg/mL. M: Male; F: Female.