Glucagon receptor gene mutations with hyperglucagonemia but without the glucagonoma syndrome

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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 recently been 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 years 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 and 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,
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 cholecystitis on abdominal ultrasound. Upon referral to our centre in 2011, extensive investigations including upper and lower intestinal endoscopy, computerised tomography and magnetic resonance imaging (MRI) were carried out. Apart from the previously diagnosed cholecystitis, 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. 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 and the patient remained asymptomatic with a normal MRI result. Serum glucagon was 10 pmol/L and insulin was 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.

Most patients present with abdominal pain and increased serum glucagon levels but fail to exhibit the characteristics of the glucagonoma syndrome (necrotic migratory erythema, diabetes mellitus, stomatitis and weight loss)\(^1\). While macroscopic tumors are evident on imaging in some, numerous microadenomas scattered throughout the pancreas and enlarged islets are the findings in others\(^1\). 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. Yu et al\(^2\), Zhou et al\(^6\) 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.

Key words: Hyperglucagonemia; Glucagon receptor gene; Mutation; Adenomatosis; Pancreas
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).

\[ \text{MEN1} \] sequencing was carried out on gDNA from the peripheral blood. PCR amplification of exons 2-10 of \[ \text{MEN1} \] 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.

\[ \text{VHL} \] sequencing was carried out on gDNA from the peripheral blood. PCR amplification of exons 1-3 of \[ \text{VHL} \] 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 \[ \text{MEN1} \] or \[ \text{VHL} \].

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). Polymerase chain reaction (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\(^9\). 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\(^9\). Bioedit software was used to analyse the sequencing results\(^9\). 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).

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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. to postulate that diffuse glucagon cell hyperplasia might represent a precursor from of glucagon cell neoplasia. In the case described by Yu et al., 60%-80% of the hyperplastic islet cells stained positive for glucagon but negative for insulin. A similar trend was noted by Henopp et al. 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 hyperplastic 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. 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. 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 lifelong 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 et al\[^{[3,6]}\] 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 adenylyl cyclase activation\[^{[19]}\]. Neuroendocrine cells undergoing hyperplastic changes is particularly relevant for MEN1 conditions however they probably also occur in sporadic cases. Very recently Klöppel et al\[^{[10]}\] 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\[^{[19]}\].

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

### Table 3  Hyperglucagonemia without the glucagonoma syndrome-review of the literature

<table>
<thead>
<tr>
<th>Patient</th>
<th>Origin</th>
<th>Clinical symptoms</th>
<th>Serum Glucagon (pmol/L)</th>
<th>Imaging</th>
<th>Octreoscan[^a]</th>
<th>Localization</th>
<th>Pancreatic pathology</th>
<th>GCGR</th>
<th>Other Genes</th>
<th>Relatives GCGR</th>
<th>Present case</th>
</tr>
</thead>
<tbody>
<tr>
<td>54, M</td>
<td>Abdominal pain</td>
<td>Elevated</td>
<td>Negative</td>
<td>Negative</td>
<td>No focal abnormality</td>
<td>α-cell hyperplasia, large cystic tumor and small solid tumors, microadenomas</td>
<td>Homozygous gDNA point mutation</td>
<td>Heterozygous gDNA deletion 5 point mutations</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>43, F</td>
<td>Abdominal pain</td>
<td>Elevated (25-fold)[^{[3]}]</td>
<td>Positive</td>
<td>Negative</td>
<td>Tail</td>
<td>-</td>
<td>-</td>
<td>Normal pancreatic morphology on standard H and E staining</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60, F</td>
<td>Abdominal pain</td>
<td>Abdominal pain</td>
<td>Positive</td>
<td>Negative</td>
<td>Uncinate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>36, F</td>
<td>Abdominal pain</td>
<td>Abdominal pain</td>
<td>Constipation</td>
<td>Negative</td>
<td>Tail</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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; EUS: Endoscopic ultrasound; GCGR: Glucagon receptor.

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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 \textit{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.

\textbf{COMMENTS}

\textbf{Case characteristics}

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

\textbf{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.

\textbf{Differential diagnosis}

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

\textbf{Laboratory diagnosis}

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

\textbf{Imaging diagnosis}

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

\textbf{Pathological diagnosis}

Histology (H and 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.

\textbf{Treatment}

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

\textbf{Related reports}

This is a very rare disease entity. Genetic lesions in the glucagon receptor (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.

\textbf{Experiences and lessons}

The authors have identified novel GCGR mutations which appear to be associated with hyperglucagonemia without the glucagonoma syndrome. Physicians dealing
REFERENCES


