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A Novel Somatic Mutation Implicates ATP6V0D1 in Proinsulin Processing

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Abstract

Context: Prohormone convertase 1/3 (PC1/3), encoded by protein convertase subtilisin kexin type 1 (PCSK1), converts inactive prohormones into biologically active peptides. Somatic mutations of insulinomas are associated with genetic defects interfering with control of insulin secretion from pancreatic beta cells. However, somatic mutations in proinsulinomas have not been described.

Objective: We report a case of a proinsulinoma, with suppressed insulin and C-peptide levels.

Methods: A 70-year-old woman presented with a 20-year history of "blackouts." During a 72-hour fast, blood glucose level dropped to 1.9 mmol/L with suppressed plasma insulin and C-peptide levels, but proinsulin levels were raised at 37 pmol/L (<10 pmol/L).

Results: Imaging revealed 3 distinct DOTATATE-avid pancreatic lesions. Laparoscopic spleen-preserving distal pancreatomy was performed. In view of discordant insulin, C-peptide, and proinsulin levels, whole exome sequencing analysis was performed on the tumor. In the somatic exome of the tumor, we found mutations in PCSK expression regulators, as well as a novel truncating somatic mutation in *ATP6V0D1*, a subunit of the ion pump that acidifies the β-cell compartments where the PCSKs act.

Conclusion: Appropriately suppressed insulin levels in the context of hypoglycemia do not always indicate the absence of a neuroendocrine islet cell tumor and proinsulin levels may be indicated to solidify the diagnosis. In the context of elevated proinsulin levels, low insulin and C-peptide levels might be explained by somatic mutations that likely implicate proinsulin processing within the tumor. Furthermore, we propose several mechanistic candidates, including *ATP6V0D1*. Experimental validation using cellular approaches may in future confirm pathomechanisms involved in this rare condition.

Key Words: proinsulin, pancreatic neuroendocrine tumors, hypoglycemia, whole exome sequencing, proinsulin processing

Insulinomas and proinsulinomas are pancreatic neuroendocrine tumors (pNET) that are diagnosed due to inappropriately unsuppressed or elevated insulin levels in the presence of hypoglycemia [1]. Dysregulated insulin secretion in hyperinsulinemic hypoglycemia (HH) drives glucose into insulin-sensitive tissues (eg, skeletal muscle, adipose tissue, and liver) and further exacerbates insulin-mediated inhibition of glycogenolysis, lipolysis, gluconeogenesis, and ketogenesis, predisposing the individual to hypoglycemic brain injury [2]. Clinical diagnosis can be challenging since symptoms of hypoglycemia mimic nonspecific symptomatology such as seizures, syncope, or psychiatric illness, resulting in delayed diagnosis

for approximately 4 years (range of 1-30 years) [1, 3, 4]. On the other hand, misdiagnosis can lead to unnecessary pancreatotomy [4]. While insulinomas account for ~20% to 30% of adult pNETs [4], proinsulinomas are exceedingly rare, with most of the available literature confined to case reports [5]. HH can also occur in congenital hyperinsulinemia (CHI) [6]. The incidence of CHI ranges from 1 in 40 000 to 1 in 50 000 in the general population and to 1 in 2500 in certain communities with high rates of consanguinity [6, 7]. Moreover, cases of CHI may be sporadic, with somatic mutations resulting in channelopathies (eg, *ABCC8*, *KCNJ11* loss-of-function mutations), defects in enzyme action (eg, mutations in

GLUD1), transcription factor defects (eg, HNF1A, HNF4A) or mitochondrial defects (eg, UCP2), eventually culminating in altered insulin secretion [6].

Proprotein convertases (PCs) are calcium-dependent serine endoproteases responsible for processing many inactive prohormone precursors, at the C terminus at the single or dibasic residue, into active hormones [8]. In normal pancreatic β cells, preproinsulin is processed within the lumen of the endoplasmic reticulum and undergoes co- and posttranslational translocation across the endoplasmic reticulum before it is eventually cleaved into proinsulin [8, 9]. Proinsulin is cleaved to proinsulin intermediates in the trans-Golgi network toward nascent secretory granules where C-peptide is excised to form mature granules for insulin secretion [10]. Prohormone convertase subtilisin/kexin types 1 and 2 (PCSK1 (PC1/3) and PCSK2) are exclusive to neuroendocrine and endocrine cells and cleave a range of targets including proinsulin, progonadotrophin-releasing hormone (pro-GnRH), proglucagon, prothyrotrophin releasing hormone, prosomatostatin, proghrelin and pro-growth hormone releasing hormone (pro-GHRH) [9]. Both convertases have an acidic pH optimum (pH 5-5.5), which requires acidification of these compartments to enable proinsulin processing. V-ATPase, a multisubunit proton pump whose components are highly expressed in beta cells likely plays an important role in this [11].

Individuals with inherited germline mutations of PCSK1 present with malabsorptive diarrhea, hyperinsulinemic hypoglycemia, central adrenal and thyroid defects and early-onset obesity [12]. Clinical features of PC deficiency are considerably heterogeneous, presumably reflecting the variable ability of the incompletely processed precursors to compensate for defective PC conversion and continue bioactivity at their cognate receptors [12].

Somatic mutations in insulinomas have been documented (eg, *ABCC8, KCNJ11, GCK, SCHAD, GLUD1, SLC16A1, HNF1A, HNF4A,* and *UCP2*) [6]. However, no cases of somatic mutations in proinsulinomas have been described thus far. Here we present the first case report of a somatic mutation of *ATP6V0D1* which, alongside other changes interfering with PCSK expression, may drive the production of proinsulin within the tumor.

Case Presentation

A 70-year-old woman presented with a 20-year history of syncopal episodes, occurring at a weekly basis. These episodes were sometimes preceded by diaphoresis, shaking, and transient blurred vision with no apparent triggering factors. She had first been investigated 16 years prior to her current presentation. A 72-hour fast at the time excluded significant hypoglycemia and was thought to be related to reactive hypoglycemia. A low-glycemic diet failed to improve her symptoms and she was re-referred to the Endocrinology team with syncopal episodes coinciding with blood glucose of 0.6 mmol/L. She had no other medical problems and denied the exogenous use of insulin or oral antidiabetic medications. Her body mass index was 19 kg/m² and physical examination was unremarkable. She did not gain additional weight throughout this time.

Investigations

The patient underwent a 72-hour supervised fast which revealed a low blood glucose level of 1.9 mmol/L (3.9-6.1 mmol/L) after 24 hours (Table 1) with appropriately suppressed insulin and C-peptide at 0.3 μIU/mL (1.4-14.0 μIU/mL) and 96 pmol/L

Table 1. 72-hour fast demonstrating blood glucose of 1.9 mmol/L at 24 hours following the fast

Time (hrs)	Blood glucose (mmol/L)
0	4.8
4	3.5
8	3.2
10	3.0
12	2.8
14	2.6
17	2.6
19	2.2
20	2.6
21	2.4
22	2.1
24	1.9
25	5.1

At that level, serum C-peptide is 96 pmol/L and proinsulin is 37 pmol/L.

(>200 nmol/L), respectively, therefore ruling out a typical insulin-producing adenoma (Table 2). As she displayed concomitant hypoglycemia with nonelevated insulin and plasma β-hydroxybutyrate levels, proinsulin from the time of the nadir glucose during the 72 hours fast was measured. Proinsulin was significantly elevated at 37 pmol/L (<10 pmol/L), thus suggesting a diagnosis of a proinsulin-secreting tumor.

Localization of the tumor was confirmed on magnetic resonance imaging (MRI), which showed a 13 mm diameter arterial-enhancing mass in the anterior aspect of the pancreas. Computed tomography (CT) scan (Fig. 1 A and 1B) and endoscopic ultrasound (EUS) confirmed the presence of 3 pancreatic lesions: the largest lesion was 42 mm in the tail of pancreas and 2 other lesions protruding from the distal body of pancreas measuring 13 mm each. A gallium 68 positron emission tomography (PET)-CT scan confirmed the lesions to be DOTATATE-avid (Fig. 1C and 1D).

Table 2. Summary of the other laboratory results

Biochemistry	Results	Reference range
IGF-1	4.2 nmol/L	4.8-21.6 nmol/L
IGF-2	43.2 nmol/L	—
IGF-2:IGF-1	10.3	—
IGF binding protein	3.2 mg/L	0.7-4.4 mg/L
Sulfonylurea screen	Negative	—
Plasma ketones	Negative	—
Glucagon level	19 pmol/L	0-50 pmol/L
Chromogranin A	222 pmol/L	0-60 pmol/L
Chromogranin B	65 pmol/L	0-150 pmol/L
Pancreatic polypeptide	188 pmol/L	0-300 pmol/L
Somatostatin	29 pmol/L	0-150 pmol/L
Vasoactive intestinal polypeptide	6 pmol/L	0-30 pmol/L

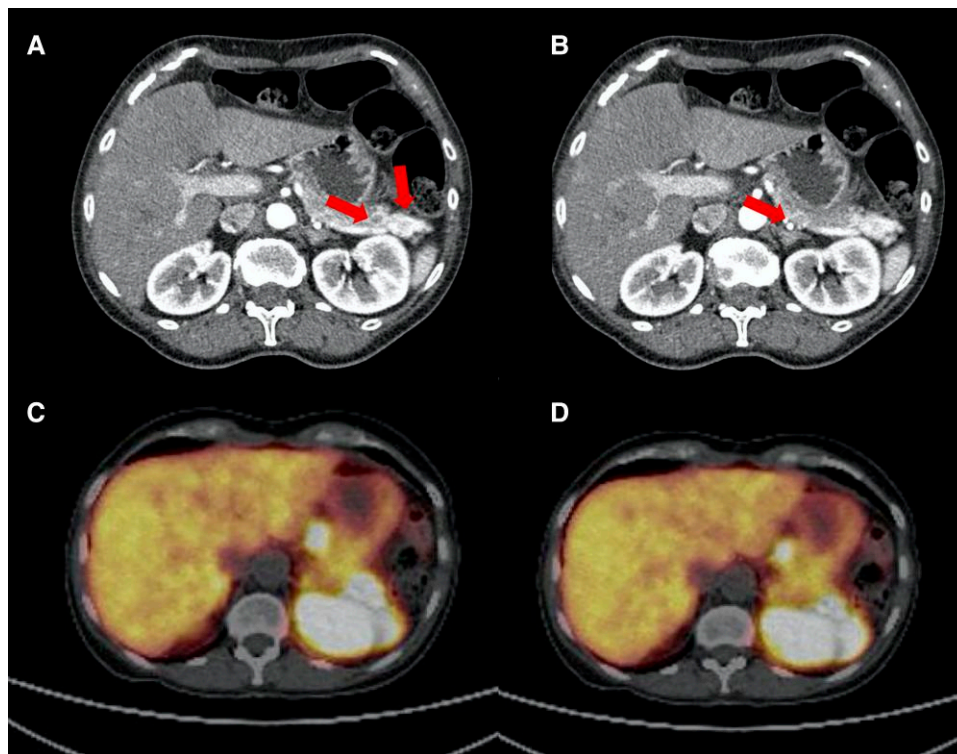Abbreviations: IGF-1, insulin-like growth factor 1; IGF-2, insulin-like growth factor 2.

Figure 1. Computed tomography (CT) abdomen in arterial phase shows 3 enhancing lesions in the pancreas representing (pro)insulinomata. (A) The largest lies in the tail of the pancreas measuring up to 4.2 cm in diameter, with an exophytic lesion measuring 12.7 mm protruding from the anterior aspect of the distal pancreas. (B) Just proximal to the exophytic lesion is a further 12.8 mm enhancing lesion. On gadolinium scan, these lesions are significantly avid, thereby confirming suspicion of neuroendocrine tumors (C and D).

Treatment

Following discussion in a neuroendocrine multidisciplinary meeting, the patient underwent an elective laparoscopic spleen-preserving distal pancreatectomy (SPDP), with resection of 9 cm × 4 cm × 1 cm body and tail of pancreas. Histology revealed well-differentiated grade 1 neuroendocrine tumors, the largest measuring 35 mm in the tail of pancreas along with 2 separate nodules (15 mm and 6 mm). Apart from these nodules, multiple scattered nests of neuroendocrine tumor were present in the pancreas and lymphovascular and perineural invasion were seen, including metastasis in 1 of the 4 peripancreatic lymph nodes. The tumor cells were positive for CD56, insulin (Ki67 < 1%). Immunohistochemical staining was not specific to distinguish between insulin and proinsulin, but given the unequivocal biochemical results, a diagnosis of proinsulinoma was made. The tumor staging score was pT2/3pN1pMx. The patient recovered well postoperatively. More than 6 years later, she remains symptom-free with stable weight and no further spontaneous hypoglycemic episodes.

Whole Exome Analysis

Samples were obtained from storage and sent for further analyses. A tumor sample was obtained from a paraffin block, and genomic DNAs were extracted using phenol-chloroform following a standard protocol [13]. As a germline control, we extracted genomic DNA from whole blood using QIAGEN's QIAamp DNA Blood mini kit (Cat. no. 51104). The tumor sample, as expected, was heavily degraded, precluding polymerase chain reaction (PCR) amplification of exon regions.

PCR amplification and sequencing (Genewiz, Agenta Life Sciences) of all *PCSK1* and *PCSK2* exons in blood sample showed no difference vs reference gene sequences. In order to identify mutation/deletions in the tumor samples, we performed whole exome sequencing (BGI https://www.bgi.com/global/home). Quality control was performed by the company and was deemed adequate for sequencing.

The whole exome sequence of tumor DNA was compared to that of the patient's germline (whole blood). Following enrichment with the Agilent_V6 kit, DNA was sequenced at a depth of 208×. A Phred quality score of >20 was achieved in 97.6% and 97.8% of reads for blood and tumor DNA, respectively. Reads were aligned by the Burrows-Wheeler algorithm and somatic variants called by GATK MuTect2 and annotated by GATK Funcotator.

We analyzed the resulting VCF files strictly for the purpose of explaining the proinsulin (as opposed to insulin) secretion, with no attempt to address a molecular tumorigenesis model. We found 4271 variants affecting protein sequence in the tumor that were absent in the germline. Of these, 3194 were missense, 138 were small insertions or deletions (indels) and 944 were larger copy-number variants (CNV) encompassing at least one exon. Mosaicism levels ranged from 0.05 to 0.73, with a median of 0.147 and a 0.115-0.193 interquartile range, indicating marked heterogeneity in the tumor, with multiple clonal cell lineages.

There were no protein-altering mutations in the insulin gene (*INS*) or *PCSK1* or *PCSK2* that could explain the lack of processing of the proinsulin. In a search for upstream mutations that could affect *PCSK1* or *PCSK2* expression, we searched the RegNetwork repository [14] for regulators that might be

mutated, at 4 levels up the regulatory network (Fig. 2). *ATF1*, the common regulator of both convertases, had no functional variants, and neither did *CRB1* or *EGR1*, which regulate *PCSK1* and *PCSK2* respectively. Several potentially functional mutations were found upstream. Notable are (1) a high-mosaicism splicing mutation of *RBL1*, a repressor of *MYC* which, in turn regulates *CRB1* and (2) a missense variant in *CEBPA* which regulates both *CRB1* and *EGR1*.

As it is impossible to know whether any of the above can explain the lack of proinsulin cleavage, we also searched for a mutation that could affect it independently of *PCSK1* or *PCSK2* expression. An obvious candidate was acidification of the compartments where this cleavage occurs. A very interesting finding in this respect, was a splice-site single-nucleotide variant in exon 2 of *ATP6V0D1* (ATPase6, V0 subunit D, isoform 1), an important component of V-ATPase, highly expressed in β-cells. The mutation (chr16:67453544 G>A in hg38) disrupts a donor site that should result in inclusion of intron 2 sequence in the transcript, with a stop 17 codons downstream, thus targeting the transcript for nonsense-mediated decay.

Discussion

Hypoglycemia due to proinsulinoma is infrequently reported [3, 15]. A review of 16 proinsulinoma cases reported a 2:1 female preponderance, with a mean age of diagnosis at 56 years [4]. The majority of cases presented with symptomatic hypoglycemia with normal or low insulin levels [16]. We herein report a case of a proinsulinoma, with truncating mutation of *ATP6V0D1* plus additional variants which may influence a network of downstream events to compromise the expression and activity of proinsulin processing enzymes.

The diagnosis of proinsulinoma was initially missed as she displayed suppressed insulin, C-peptide, and plasma β-hydroxybutyrate levels in the context of hypoglycemia. However, the persistence of symptoms prompted further investigations, which revealed elevated proinsulin levels in the repeat 72-hour fast, coupled with radiological findings that

indicated the diagnosis of proinsulin-releasing tumor. Immunohistochemical staining of the tumor was not specific to distinguish between insulin and proinsulin; however, this has been previously used to identify proinsulinomas [17].

Due to the rarity of this condition [1, 15], molecular mechanisms contributing to the pathomechanisms in proinsulinomas are rarely investigated. We carried out whole exome analysis and report a new case of truncating somatic mutation in *ATP6V0D1*, encoding one of the subunits of the V0 H + ATPase, which may explain the absence of proinsulin cleavage through failure to acidify the vesicles where it ought to have taken place. Although the level of mosaicism of this mutation was low (0.062), it can still explain the biochemical phenotype, if located in a tumor cell clonal line that had also lost the feedback mechanism of inhibiting secretion in the presence of hypoglycemia.

Proinsulin processing occurs in the maturing secretory granule at ∼ pH 5.3 [18]; the formation of higher order structures, involving the binding of insulin binding to Zn^{2+} and Ca^{2+}, occur as the pH is reduced further in the mature dense core granule [19]. Whereas PCSK2 activity (responsible for proinsulin and split proinsulin 31-32 cleavage at Lys64-Arg65) is substantially retained up to pH values above 7.0, that of PCSK1 (cleavage at Arg31-Arg32) is negligible at pH 7.5 [18]. Correspondingly, studies by Orci and colleagues using a fixable pH probe and electron microscopy [20] revealed that acidification was likely to be required for proinsulin processing, while other studies revealed that granule protonophores prevent prohormone processing in vitro [21]. Alkalinization of the granule resulting from the lack of active ATP6V proton pumps may therefore: (1) substantially impair processing, favoring (split) proinsulin accumulation; and (2) impact the normal storage within granules, and hence intracellular retention, of mature insulin. In this context, it should be noted that proinsulin-containing granules may be subjected to less rigorous (nutrient-dependent) control of exocytosis, potentially driving basal proinsulin hypersecretion [19]. Importantly, if confirmed by functional studies, including measurements of circulating split proinsulin 31,32 and 64,65, the present
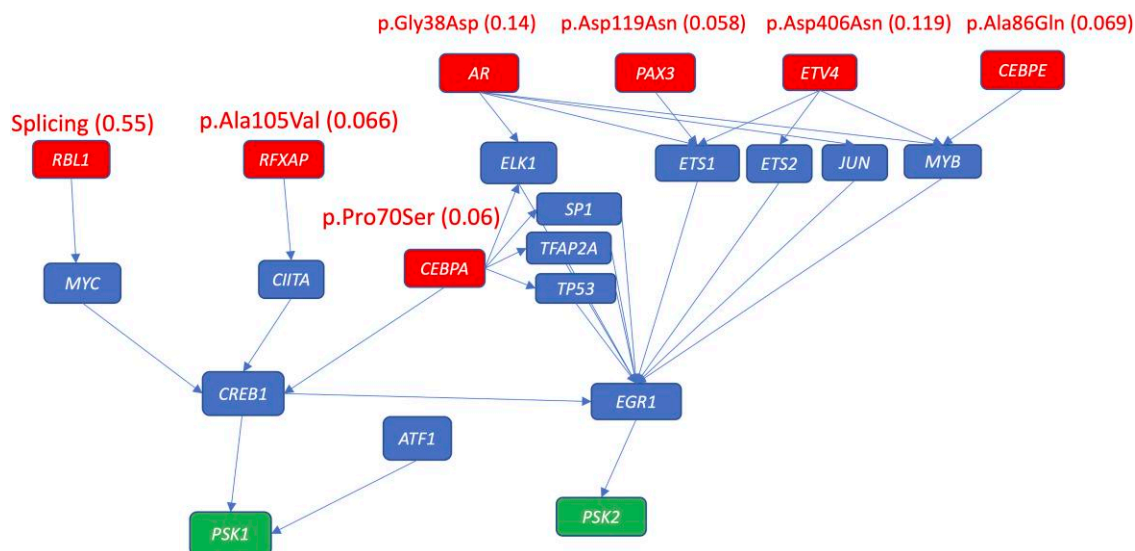

Figure 2. Regulators of *PCSK1* and *PCSK2* expression, from RegNetwork. Genes with somatic mutations in the tumor are highlighted in red. Amino acid change and mosaicism level indicated in parentheses. The BL1 variant substitutes the G (in the [−] strand) of the canonical AG acceptor site in exon 9, which should result in a frameshift skipping of exon 9 (of a total 22 exons).

findings would appear to provide genetic evidence for the presumed role of ATP6V as a critical guarantor of insulin granule acidification in humans [22]. However, we do not exclude the additional impact of changes in the expression of PCSK1 or 2 resulting from the upstream events illustrated in Fig. 2.

Conclusion

In conclusion, this case highlights the potential pathomechanisms of proinsulin-secreting pNETs. Low insulin levels per se in the presence of hypoglycemia do not exclude the presence of pNET, and proinsulin levels, and the specific cleavage products present, should be measured to confirm the diagnosis and potential molecular mechanisms. Somatic mutations leading to impaired proinsulin processing play a plausible role in driving proinsulin production in tumor cells.

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Author Contributions

P.A., P.C.E., R.C., and N.O. co-wrote the manuscript. M.H. performed DNA extraction and sample preparation for sequencing; N.P., C.P., and G.R. analyzed whole exome sequencing data and co-wrote the manuscript. D.S. operated on the patient as part of her clinical management. F.W. was in charge of the patient's clinical management and co-wrote the manuscript. All authors reviewed and contributed to the manuscript.

Disclosures

All other authors report no conflicts of interest. The views expressed are those of the authors and not necessarily those of the above-mentioned funders, the UK National Health Service (NHS), the NIHR, or the UK Department of Health.

Data Availability

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Patient Consent

Consent was obtained from the patient and signed.

References

1. Goldin SB, Aston J, Wahi MM. Sporadically occurring functional pancreatic endocrine tumors: review of recent literature. *Curr Opin Oncol*. 2008;20(1):25-33.

2. Hussain K, Aynsley-Green A. Hyperinsulinism in infancy: understanding the pathophysiology. *Int J Biochem Cell Biol*. 2003;35(9):1312-1317.

3. Kriger AG, Berelavichus SV, Kaldarov AR, *et al*. Proinsulin-secreting neuroendocrine tumors of the pancreas: a single-centre experience. *Gastrointest Tumors*. 2019;6(3-4):64-70.

4. Cryer PE, Axelrod L, Grossman AB, Heller SR, Montori VM, Seaquist ER. Evaluation and management of adult hypoglycemic disorders: an Endocrine Society Clinical Practice guideline. *J Clin Endocrinol Metab*. 2009;94(3):709-728.

5. Murtha TD, Lupsa BC, Majumdar S, Jain D, Salem RR. A systematic review of proinsulin-secreting pancreatic neuroendocrine tumors. *J Gastrointest Surg*. 2017;21(8):1335-1341.

6. Senniappan S, Shanti B, James C, Hussain K. Hyperinsulinaemic hypoglycaemia: genetic mechanisms, diagnosis and management. *J Inherit Metab Dis*. 2012;35(4):589-601.

7. Mathew PM, Young JM, Abu-Osba YK, *et al*. Persistent neonatal hyperinsulinism. *Clin Pediatr (Phila)*. 1988;27(3):148-151.

8. Ramzy A, Kieffer TJ. Altered islet prohormone processing: a cause or consequence of diabetes? *Physiol Rev*. 2022;102(1):155-208.

9. Chen YC, Taylor AJ, Verchere CB. Islet prohormone processing in health and disease. *Diabetes Obes Metab*. 2018;20(Suppl 2):64-76.

10. Liu M, Wright J, Guo H, Xiong Y, Arvan P. Proinsulin entry and transit through the endoplasmic reticulum in pancreatic beta cells. *Vitam Horm*. 2014;95:35-62.

11. Sun-Wada GH, Toyomura T, Murata Y, Yamamoto A, Futai M, Wada Y. The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta-cells. *J Cell Sci*. 2006;119(Pt 21):4531-4540.

12. Stijnen P, Ramos-Molina B, O'Rahilly S, Creemers JWM. PCSK1 Mutations and human endocrinopathies: from obesity to gastrointestinal disorders. *Endocr Rev*. 2016;37(4):347-371.

13. Pikor LA, Enfield KS, Cameron H, Lam WL. DNA Extraction from paraffin embedded material for genetic and epigenetic analyses. *J Vis Exp*. 2011;26(49):2763.

14. Liu ZP, Wu C, Miao H, Wu H. RegNetwork: an integrated database of transcriptional and post-transcriptional regulatory networks in human and mouse. *Database (Oxford)*. 2015;2015:bav095.

15. Celli R, Tang LH, Cai G, *et al*. Proinsulin expressing neuroendocrine tumors of the pancreas: an underrecognized entity. *Pancreas*. 2019;48(1):55-59.

16. Striano P, Striano S. Insulinoma presenting as refractory late-onset epilepsy. *Epilepsia*. 2006;47(2):452.

17. Al Mukaddam M, Baloch ZW, Rickels MR. Case 33-2012: a woman with altered mental status after childbirth. *N Engl J Med*. 2013;368(5):486-487.

18. Davidson HW, Rhodes CJ, Hutton JC. Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases. *Nature*. 1988;333(6168):93-96.

19. Omar-Hmeadi M, Idevall-Hagren O. Insulin granule biogenesis and exocytosis. *Cell Mol Life Sci*. 2021;78(5):1957-1970.

20. Orci L, Ravazzola M, Amherdt M, Madsen O, Perrelet A, Vassalli JD. Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles. *J Cell Biol*. 1986;103(6 Pt 1):2273-2281.

21. Rhodes CJ, Lucas CA, Mutkoski RL, Orci L, Halban PA. Stimulation by ATP of proinsulin to insulin conversion in isolated rat pancreatic islet secretory granules. Association with the ATP-dependent proton pump. *J Biol Chem*. 1987;262(22):10712-10717.

22. Suckale J, Solimena M. The insulin secretory granule as a signaling hub. *Trends Endocrinol Metab*. 2010;21(10):599-609.