Two nonsense somatic mutations in MEN1 identified in sporadic insulinomas
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Insulinomas are functional pancreatic neuroendocrine tumors that cause hypoglycemia and severe morbidity. The aim of our study was to identify gene mutations responsible for tumorigenesis of sporadic insulinoma. Whole exome sequencing analysis was performed on tumors and paired peripheral blood from three patients with insulinomas. After initial analysis, somatic mutations were obtained and a deleterious protein product was further predicted by various bioinformatic programs. Whole exome sequencing identified 55 rare somatic mutations among three insulinoma patients, including MEN1 gene nonsense mutations (c. 681C>G; p.Tyr227* in exon 4 of MEN1 and c. 346G>T; p.Glu116* in exon 2 of MEN1) in two different tumor samples. The mutations resulted in a significant truncation of the protein and a non-functional gene product, which was involved in defective binding of menin to proteins implicated in genetic and epigenetic mechanisms. Our results extend the growing list of pathogenic MEN1 mutations in sporadic cases of insulinoma.

Insulinoma is a rare and sporadically occurring neuroendocrine tumor that secretes an excess of insulin, resulting in symptoms of hypoglycemia in patients [1]. Additionally, the catecholamines released from insulinoma produce several symptoms including sweating, nausea, weakness, anxiety and palpitation [2]. Insulinoma is usually a benign neoplasm that is smaller than 2 cm in diameter, without signs of angiogenesis or metastases, and is easily curable by surgical resection [2]. However, an understanding of the mechanism of the pathology of insulinoma is still needed. The small number of disease cases and lack of suitable animal models and cell strains have limited the study of the pathogenesis of insulinoma. The risk factors and related molecular mechanisms of insulinoma remain unclear.

Exome sequencing, which allows study of the complete protein-coding regions in the genome, is valuable in searching for underlying genetic variation in disease. Moreover, extensive exome sequencing studies from human tumors have indicated that there are a large number of mutations in each tumor [3]. Accumulating evidence suggests that genetic change is specific for insulinomas, such as high loss of heterozygosity rates on chromosome 22q and gain of 9q34 [4,5]. Additionally, exome sequencing has revealed that there was a somatic mutation in the DNA-binding zinc finger of the transcription factor Yin Yang 1 in insulinoma [6–8].

In order to further screen for potential genetic alterations in insulinoma, we selected the tumor tissue and matched blood of patients with insulinoma and performed exome sequencing and analysis of deleterious effects on the protein. In this study, we obtained a total of 55 gene mutations in insulinoma. Among them, mutations in MEN1 were most related to the
pathology of insulinoma, which provides evidence for use in early disease screening and target treatment of insulinoma.

**Materials and methods**

**Insulinoma patient samples**

In our present study, three patients (INS1, INS2 and INS3) with primary insulinoma were enrolled from the Second Hospital of Hebei Medical University. The paired tumor and a peripheral blood sample for DNA extraction were collected from the patients after surgical removal. The insulinoma was diagnosed depending on current clinical guidelines and histopathological confirmation [9]. The diagnosis was established using the following six tight criteria: (a) documented blood glucose levels ≤ 2.2 mmol L⁻¹ (≤ 40 mg dL⁻¹); (b) concomitant insulin levels ≥ 66 µU L⁻¹ (≥ 36 pmol L⁻¹; ≥ 3 lU L⁻¹); (c) C-peptide levels ≥ 200 pmol L⁻¹; (d) proinsulin levels ≥ 5 pmol L⁻¹; (e) β-hydroxybutyrate levels ≤ 2.7 mmol L⁻¹; (f) absence of sulfonylurea (metabolites) in the plasma and/or urine. Written consent forms were obtained from the enrolled participants, and the research protocol was approved by the ethics committee of the Second Hospital of Hebei Medical University (2017-R086) and complied with the principles of the Declaration of Helsinki. Clinical information for the patients is shown in Table 1.

**Exome sequencing and data analysis**

Genomic DNA was isolated from blood and tissue and was controlled for quality by measuring its concentration using a Nanodrop 2000 (Illumina, San Diego, CA, USA) and measuring fragmentation by agarose gel electrophoresis. Qualified Genomic DNA was prepared for exome sequencing with an Agilent SureSelect Human All Exon 50 Mb Exon Kit (Agilent Technologies, Santa Clara, CA, USA). The genomic DNA of each sample was fragmented and captured for exome sequencing with the Illumina HiSeq 2500 Sequencer platform (Illumina). For each sample, sequencing reads with 125-bp paired-end and Q30 > 92% were generated.

After filtering the low quality and contaminating reads, sequence reads were mapped to the human genome sequence (hg19) using the Burrows–Wheeler alignment tool (http://bio-bwa.sourceforge.net/), which generated the sequence alignment/map file. The PCR duplicate reads were further removed using the PICARD software program.

**Single nucleotide variant detection and annotation**

To obtain the important candidate genes, the MUTECT software [10] was used to detect single nucleotide variants (SNVs). Variants were filtered for minimum genotype quality of 50 and minimum coverage depths of 10. Then, the software ANNOVAR (http://www.openbioinformatics.org/annovar/) was applied to annotate the qualified variants. Finally, the variants were obtained and the deleteriousness of variants was subsequently predicted by various bioinformatics programs (e.g. SIFT, POLYPHEN2, LRT, MUTATIONTASTER, MUTATIONASSESSOR, FATHMM, RADIALSVM, LR).

**Results**

**General characteristics of the patients**

We studied three patients with insulinoma, two female and one male. None had a family history of insulinoma. Moreover, we also excluded multiple endocrine neoplasia type 1 (MEN-1 syndrome) from non-tumor tissue. The WHO grading classification of pancreatic neuroendocrine tumors updated in 2010 includes neuroendocrine tumor G1, neuroendocrine tumor G2, neuroendocrine carcinoma G3 and mixed adenoneuroendocrine carcinoma [11]. Depending on the classification system [12], two patients (INS1 and INS3) were classified as Grade II and one was classified as Grade I in this study. All presented with signs and symptoms of hypoglycemia. The hypoglycemia was corrected in all cases after surgical removal. General pathological and demographic characteristics for the three patients are shown in Table 1.

**Genetic analysis**

Genomic DNA from insulinomas and matched blood samples was subjected to whole exome sequencing. After mapping of the human genome sequence (hg19), a total of > 86% of the exome region was covered

Table 1. Clinical information for the patients with insulinoma. INS1, INS2 and INS3 represent the three patients with insulinoma.

| Sample | Gender | Age at diagnosis (years) | Grade | Metastatic disease | Tumor size (cm) | Ki67 |
|--------|--------|--------------------------|-------|--------------------|-----------------|------|
| INS1   | Male   | 64                       | G2    | No                 | 0.6             | 2%   |
| INS2   | Female | 75                       | G1    | No                 | 0.8             | 2%   |
| INS3   | Female | 57                       | G2    | No                 | 1.0             | 2%   |
(Table 2). Exome sequencing analysis overall identified 40,210, 40,272 and 41,910 SNVs for tumor tissue, and 41,106, 40,050 and 41,451 SNVs for the matched blood samples. We identified 55 rare somatic mutations among the three patients, of which 39 were non-synonymous, four were nonsense and 12 were synonymous. An overview of detected somatic mutations after exome sequencing is provided in Table 3. MEN1 gene nonsense mutations occurred in two different tumor samples. A c. 681C>G; p.Tyr227* mutation was found in exon 4 of the MEN1 gene in INS1, and c. 346G>T; p.Glu116* mutation was found in exon 2 of the MEN1 gene in INS2. The mutations were not present in the corresponding leukocyte DNA. Additionally, these mutations were predicted to be damaging by SIFT or LRT.

**Mutation of MEN1 gene**

The p.Tyr227* mutation (SWLYLKGSYMRCDDRK MEV) and p.Glu116* mutation (VSSRELVKKVSD VIWNSL) both resulted in a significant truncation of menin, which may destroy the functional domain and affect its function. Amino acids of p.Tyr227* and p.Glu116* mutations are highly conserved across multiple species (Fig. 1A,B). Moreover, two nonsense mutations locate in the functional domain of MEN1, indicating that it may affect the binding of lysine methyltransferase 2A (Fig. 1C). In the current study, the nonsense mutations happened early in the sequence of MEN1, which may obviously result in a non-functional gene product (Fig. 1D).

**Discussion**

Insulinoma is a common neuroendocrine tumor with an incidence of four in every 1 million persons annually [13]. Moreover, most of the insulinomas arise sporadically. Although rare, it has the potential to produce profound metabolic derangements that require early recognition and treatment. In this study, exome sequencing was performed on three sporadic insulinoma cases to delineate genetic contributors to this rare endocrine tumor. Although the overall frequency of somatic mutations was low and predicted to be damaging, there were two nonsense variants that occurred in MEN1 in two of the three patients, namely a c. 681C>G; p.Tyr227* mutation in exon 4 of MEN1 and a c. 346G>T; p.Glu116* mutation in exon 2 of MEN1. Our result showed that the mutations in MEN1 may play an important role in the development of insulinoma. However, further functional research is needed to validate their roles.

MEN1 consists of 10 exons [9] and encodes a protein with 615 amino acids [14]; it is considered a putative tumor suppressor gene associated with neuroendocrine tumors [15]. Menin, the encoded protein of MEN1, is a typical GTPase stimulated by nm23. It is mostly found in the nucleus and can regulate gene expression in a positive or negative way, and it has been demonstrated to interact with transcription activators, transcription repressors, cell signaling proteins and various other proteins. In addition, it plays major roles in DNA repair, cell cycle regulation and chromatin remodeling. Generally, menin is considered as a transcriptional regulator and interacts with a number of nuclear and cytosolic proteins, which indicates that it may participate in various biological pathways of tumor formation [16–18]. Additionally, a number of sporadic endocrine tumors, including parathyroid adenomas, pancreatic insulinomas and pituitary prolactinomas, have somatic mutations of MEN1 alleles, suggesting that MEN1 may play a role in non-hereditary endocrine tumors [15,19,20]. Our results showed that MEN1 mutations were found in two of the three insulinoma patients, which provided further evidence that MEN1 might be an important factor in the pathological process of insulinoma.

It is noted that insulinomas can occur sporadically or in combination with MEN-1 syndrome. Moreover, the MEN1 gene is the first gene that has been identified as a candidate gene in the tumorigenesis of insulinoma. MEN-1 syndrome represents an autosomal dominant disorder related to mutations in the MEN1 gene mapped to chromosome 11q13 [21,22]. Generally, simple and local tumor enucleation of MEN-1 syndrome-associated insulinomas is not likely to be curative. Although genetic testing for MEN1 fails to detect mutation rate of 10–25%, it plays a vital role in identifying patients with hereditary insulinomas [23,24]. Therefore, genetic testing for MEN-1 syndrome is beneficial to clinical diagnosis. Okamoto et al. [25] suggested that a novel six-nucleotide insertion in exon 4

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**Table 2. Summary of sequencing data.** C1, C2 and C3: tissues of three patients with insulinoma; N1, N2 and N3: blood of three patients with insulinoma.

| Sample | Reads | Length (bp) | Q30 > (%) | Exome size (bp) | Exome coverage |
|--------|-------|-------------|-----------|----------------|----------------|
| C1     | 104,472,186 | 125 | 94.82 | 74,856,280 | 87.36% |
| C2     | 107,751,402 | 125 | 92.34 | 74,856,280 | 87.79% |
| C3     | 112,709,600 | 125 | 94.69 | 74,856,280 | 88.19% |
| N1     | 110,592,066 | 125 | 94.50 | 74,856,280 | 87.90% |
| N2     | 98,905,302  | 125 | 94.49 | 74,856,280 | 86.08% |
| N3     | 109,660,740 | 125 | 94.58 | 74,856,280 | 88.44% |
Table 3. Overview of the gene mutations found by exome sequencing in the three patients with insulinoma.

| Sample | Chr | Start (bp) | Ref | Alt | Gene     | Mutation type | AA change |
|--------|-----|------------|-----|-----|----------|---------------|------------|
| INS1   | 1   | $1.1 \times 10^8$ | G   | A   | PSRC1    | Nonsynonymous | p.Ala227Val |
| INS1   | 2   | 1 926 848   | A   | G   | MYT1L    | Synonymous    | p.Asn231Asn |
| INS1   | 2   | 2.38 $\times 10^8$ | T   | C   | COL6A3   | Nonsynonymous | p.Glu186Gly |
| INS1   | 3   | 1.47 $\times 10^8$ | C   | T   | ZIC4     | Nonsynonymous | p.Arg246His |
| INS1   | 3   | 1.89 $\times 10^8$ | C   | T   | TPRG1    | Synonymous    | p.Leu120Leu |
| INS1   | 5   | 1.46 $\times 10^8$ | G   | T   | PPP2R2B  | Nonsynonymous | p.Pro235Thr |
| INS1   | 6   | 32 548 632  | T   | A   | HLA-DRB1 | Nonsynonymous | p.Arg218Ser |
| INS1   | 7   | 1 542 657   | G   | A   | INTS1    | Nonsynonymous | p.Arg77Cys  |
| INS1   | 7   | 4 249 780   | T   | A   | SDK1     | Nonsynonymous | p.Leu329*   |
| INS1   | 7   | 1.48 $\times 10^8$ | A   | G   | CNTNAP2  | Nonsynonymous | p.Lys1166Glu |
| INS1   | 7   | 1.57 $\times 10^8$ | G   | A   | HLA-DRB1 | Nonsynonymous | p.Arg218Ser |
| INS1   | 9   | 99 157 190  | A   | G   | ZNF367   | Synonymous    | p.Cys202Cys |
| INS1   | 10  | 99 153 502  | C   | A   | RRP12    | Nonsynonymous | p.Leu157Ser |
| INS1   | 10  | 1.3 $\times 10^8$ | C   | G   | MKI67    | Nonsynonymous | p.Leu157Ser |
| INS1   | 11  | 64 575 141  | G   | C   | PPP2R2B  | Nonsynonymous | p.Pro235Thr |
| INS1   | 11  | 1.02 $\times 10^8$ | A   | G   | CEP126   | Nonsynonymous | p.Ile674Val |
| INS1   | 12  | 95 897 008  | G   | A   | ABCC4    | Synonymous    | p.Leu157Ser |
| INS1   | 14  | 74 968 287  | G   | T   | LTP2     | Nonsynonymous | p.Pro1726Gln|
| INS1   | 16  | 28 943 787  | G   | C   | CD19     | Nonsynonymous | p.Gly70Ala  |
| INS1   | 16  | 31 405 651  | C   | A   | ITGAD    | Nonsynonymous | p.Pro1726Gln|
| INS1   | 17  | 21 318 727  | A   | T   | KCN12    | Nonsynonymous | p.Leu329*   |
| INS1   | 17  | 57 761 285  | A   | G   | CLTC     | Nonsynonymous | p.His1462Arg|
| INS1   | 19  | 10 799 330  | G   | A   | ILF3     | Nonsynonymous | p.Gly7477Ala|
| INS2   | 1   | 22 332 006  | T   | C   | CEL3A3   | Nonsynonymous | p.Leu329*   |
| INS2   | 1   | 40 229 393  | G   | A   | CEPL26   | Nonsynonymous | p.Leu329*   |
| INS2   | 6   | 32 007 839  | G   | T   | CYP21A2  | Nonsynonymous | p.Leu329*   |
| INS2   | 8   | 52 733 231  | G   | A   | PCMTD1   | Nonsynonymous | p.Leu329*   |
| INS2   | 8   | 88 298 821  | T   | A   | CNBD1    | Nonsynonymous | p.Tyr322Asn |
| INS2   | 8   | 1.25 $\times 10^8$ | T   | C   | TMEM65   | Nonsynonymous | p.Leu329*   |
| INS2   | 9   | 1.25 $\times 10^8$ | T   | C   | TMEM65   | Nonsynonymous | p.Leu329*   |
| INS2   | 9   | 1.13 $\times 10^8$ | C   | A   | SVEP1    | Nonsynonymous | p.Leu329*   |
| INS2   | 11  | 64 577 236  | C   | A   | MEN1     | Nonsynonymous | p.Leu329*   |
| INS2   | 11  | 89 018 006  | C   | A   | TYR      | Nonsynonymous | p.Leu329*   |
| INS2   | 12  | 6 787 522   | G   | A   | ZNF384   | Nonsynonymous | p.Leu329*   |
| INS2   | 12  | 9 243 947   | A   | G   | A2M      | Nonsynonymous | p.Leu329*   |
| INS2   | 12  | 1.25 $\times 10^8$ | A   | G   | UBC      | Nonsynonymous | p.Leu329*   |
| INS2   | 17  | 7 671 513   | G   | A   | DNAH2    | Nonsynonymous | p.Leu329*   |
| INS2   | 17  | 7 834 438   | C   | G   | TRAPP1   | Nonsynonymous | p.Leu329*   |
| INS2   | 17  | 34 797 666  | G   | A   | TBC1D3B  | Synonymous    | p.Leu329*   |
| INS2   | 19  | 41 355 849  | A   | G   | CYP2A6   | Synonymous    | p.Leu329*   |
| INS2   | X   | 37 027 691  | T   | C   | FAM74C   | Synonymous    | p.Leu329*   |
| INS3   | 1   | 12 854 188  | T   | C   | PRAMEF1  | Nonsynonymous | p.Leu329*   |
| INS3   | 2   | 1.28 $\times 10^8$ | G   | A   | MYO7B    | Nonsynonymous | p.Leu329*   |
| INS3   | 4   | 1.52 $\times 10^8$ | C   | A   | RPS3A    | Nonsynonymous | p.Leu329*   |
| INS3   | 4   | 1.52 $\times 10^8$ | C   | A   | RPS3A    | Nonsynonymous | p.Leu329*   |
| INS3   | 5   | 1.4 $\times 10^8$ | T   | C   | PCDHA5   | Nonsynonymous | p.Leu329*   |
| INS3   | 6   | 99 850 428  | T   | C   | PNSR     | Nonsynonymous | p.Leu329*   |
| INS3   | 6   | 1.38 $\times 10^8$ | C   | T   | OLIG3    | Nonsynonymous | p.Leu329*   |
| INS3   | 8   | 33 449 689  | C   | A   | DUSP26   | Nonsynonymous | p.Leu329*   |
| INS3   | 10  | 21 903 830  | T   | G   | MLLT10   | Nonsynonymous | p.Leu329*   |
| INS3   | 11  | 1 093 437   | G   | C   | MUC2     | Nonsynonymous | p.Leu329*   |
| INS3   | 11  | 1.26 $\times 10^8$ | C   | T   | PUS3     | Nonsynonymous | p.Leu329*   |
| INS3   | 16  | 7 629 904   | C   | T   | RBFOX1   | Synonymous    | p.Leu329*   |
| INS3   | 17  | 79 667 512  | G   | A   | HGS      | Nonsynonymous | p.Leu329*   |
| INS3   | 19  | 19 030 142  | G   | A   | COPE     | Nonsynonymous | p.Leu329*   |
of the MEN1 gene might contribute to the familial insulinoma. A prior study indicated that MEN1 gene mutations were lacking in 27 sporadic insulinomas [26]. By evaluating a large family with malignant insulinoma and hyperparathyroidism, Hasani-Ranjbar et al. [27] found a novel MEN1 gene frameshift germ-line mutation, which was associated with malignant insulinoma. Moreover, a recent study found several novel pathogenic MEN1 mutations in sporadic cases of insulinoma [28]. Herein, we found mutations in MEN1 on chromosome 11 in patients with insulinoma, which had a damaging role in the function of the encoded protein. Our finding was consistent with other studies indicating the role of MEN1 mutation in human sporadic insulinomas [26,29–32], which provided a crucial clue in the treatment of insulinoma. Interestingly, Waldmann et al. [33] found the p.E116X mutation in exon 2 of the MEN1 gene in 21 patients with MEN-1 syndrome and adrenal lesions. In addition, Turner et al. [34] identified the p.Y227X mutation in exon 4 of the MEN1 gene in multiple endocrine neoplasia type 1. This further suggested that MEN1 was significantly associated with insulinoma.

Conclusions

In the current study, exome sequencing for three sporadic insulinomas identified two somatic nonsense mutations in MEN1 (c. 681C>G; p.Tyr227* and c. 346G>T; p.Glu116*), which might induce a non-functional gene product and contribute to the oncogenesis of sporadic insulinoma. However, there were some limitations in this study. The samples selected were small and larger samples are further needed to validate their roles in insulinoma. Further studies are needed to precisely explore the role of genetic mutations of MEN1 in clinical manifestations of these patients. In addition, validation of MEN1 mutations (such as by Sanger sequencing) and study of the underlying biological function of the MEN1 mutations is needed in a further study.

Author contributions

JD, QS and MW analyzed and interpreted the data. CQ was the major contributor in writing the manuscript. CY designed the project. All authors read and approved the final manuscript.
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