Dear Editor,

Pancreatic neuroendocrine tumors (PNETs/pNETs/p-NETs/PanNETs) are rare endocrine neoplasms that can be either functioning tumors that secrete hormones characteristic of their endocrine cell of origin or nonfunctioning tumors. The most common functioning PNETs are the insulin-secreting β-cell tumors (insulinomas) that are mainly sporadic, but may also occur in 10% of patients with the hereditary tumor syndrome multiple endocrine neoplasia type 1 (MEN1) (OMIM ID: 131100). Patients with the MEN1 syndrome carry a heterozygous germline inactivating mutation in the MEN1 tumor suppressor gene and specific somatic loss of the normal MEN1 allele, leading to endocrine tumors mainly of the parathyroids, pituitary and pancreas (PNETs). Whole genome sequencing (WGS) or whole exome sequencing (WES) of sporadic PNETs has revealed somatic MEN1 mutation in 37–44% of non-functioning PNETs (Jiao et al. 2011, Scarpa et al. 2017). However, four different WES studies of sporadic insulinomas found <2% with somatic MEN1 mutation, but rather they found a somatic heterozygous recurrent mutation in the Yin Yang 1 (YY1) gene (c.C1115G/p.T372R) in 30, 33 and 13% of tumor samples (Wang et al. 2014). Another study from India did not find this YY1 mutation in their insulinoma samples (Irshad et al. 2017). To determine the frequency and consequence of the recurrent YY1 mutation, we analyzed a cohort of 23 sporadic insulinomas.

This study was conducted under the approval of the Institutional Review Board of the National Cancer Institute, National Institutes of Health (NIH). Formalin-fixed paraffin-embedded (FFPE) tissue sections were obtained from insulinomas of patients (15 females and 8 males) who underwent surgery at the NIH Clinical Center between 2002 and 2011. The diagnosis of insulinoma was ascertained by a 48-h supervised fasting test with blood glucose, insulin, proinsulin and C-peptide measurements until symptoms of hypoglycemia occurred. The median duration of supervised fast was 16 h (range: 3–46 h), median fasting end glucose was 37.4 mg/dL (range: 15–49), median fasting end insulin was 29.5 µU/mL (range: 2.3–85), median fasting end proinsulin was 239.3 pmol/L (range: 30–1300) and median fasting end C-peptide was 4.0 ng/mL (range: 1.5–10.8). Inappropriately elevated insulin and proinsulin levels associated with fasting blood glucose values of <50 mg/dL established the diagnosis of insulinoma in all cases. Biochemical testing for other features of MEN1 was performed to rule out MEN1 and to confirm that the patients had sporadic insulinoma. Insulinoma was further confirmed by histopathology, and immunohistochemistry for insulin showing positive staining in all samples (Desai et al. 2014). DNA and RNA were isolated using the AllPrep RNA/DNA FFPE kit (Qiagen). DNase-treated tumor RNA was amplified using an mRNA amplification kit (Ambion), and processed for first-strand cDNA synthesis with the Superscript-III cDNA synthesis kit (Invitrogen). Standard PCR was used to amplify the YY1 region in exon-5 (Cao et al. 2013) or other SNPs on human chromosome 14q32 and analyzed by Sanger sequencing. The human YY1 cDNA-coding region was cloned into the pFlag-CMV4 vector (Sigma), and the p.T372R mutation was introduced using oligos CTTCAATTGCGCAGACATGTGCGAATC and GATTCGCACATGTcTGCGCAAATTGAAG with a site-directed mutagenesis kit (Agilent). All other primers sequences are available upon request. For RNA and protein expression, and cell proliferation assays, plasmids were transfected into different cell lines using Lipofectamine-2000 (Invitrogen). The cell lines used were from human embryonic kidney (HEK293) (ATCC), human cervical cancer (HeLa) (ATCC), rat insulinoma (INS-1E) (Merglen et al. 2004) and mouse insulinoma (MIN6-4N) (Desai et al. 2014). Both insulinoma cell lines tested negative for the YY1 p.T372R mutation. DNase-treated RNA was analyzed with the SYBR-green
one-step QRT-PCR kit (Agilent), and protein whole-cell extracts were assessed for YY1 expression by Western blot. Cell number was determined with a Cellometer Auto T4 Bright Field Cell Counter (Nexcelom).

The YY1 p.T372R heterozygous mutation was found in 2 of 23 sporadic insulinoma samples (8%), but not in the corresponding RNA of the two mutation-positive samples (Fig. 1). YY1 mRNA was abundantly expressed in the tumors with the mutation (Fig. 1B). Previously in the four studies that reported this mutation in sporadic insulinomas, only one study looked at and detected the mutant YY1 RNA (cDNA) sequence together with wild-type (WT) YY1 in mutation-positive tumors (Cromer et al. 2015). Absence of the mutation in our RNA samples could be potentially due the poor quality of RNA isolated from FFPE tissue sections. However, coding region SNPs in the WDR25 gene (located within 100 kb of YY1) were heterozygous in the DNA and RNA in the two mutation-positive tumor samples, which rules out allelic imbalance at 14q32 or poor quality of RNA (Fig. 1C, D and E). Also, immunohistochemistry of insulinomas with or without the YY1 mutation showed no significant difference in the expression of YY1 protein (Fig. 1F). Among four previous studies, one study reported that the YY1 mutation was found exclusively in females (Lichtenauer et al. 2015). Both our cases with this mutation were female, but the data were not statistically significant because there were twice as many females than males in our study cohort (15 females and 8 males).

Human YY1 encodes a 414 amino acid protein that is a ubiquitously expressed transcription factor with four C2H2-type zinc fingers and belongs to the GLI-Krüppel-related family of proteins (UniProtKB – P25490). YY1 functions as a transcriptional activator or repressor depending on the target promoter region sequence and the recruitment of chromatin-modifying proteins. The recurrent mutation p.T372R is located in a DNA-binding region, the third zinc finger of YY1, which could alter YY1 DNA binding for transcriptional activity. Among four previous studies, only one study examined the expression of transfected WT and mutant YY1 by Western blot in HEK293 cells (Cromer et al. 2015). Consistent with that study, we found similar protein expression of transfected WT and mutant YY1 in HEK293 cells (Fig. 2A). However, the expression of the mutant YY1 RNA and protein was compromised in HeLa cells and in two different rodent β-cell insulinoma cell lines (INS-1E and MIN6-4N) (Fig. 2B, C and D). Previously, the expression of YY1 was not examined in β-cell insulinoma cell lines. The expression of RNA from the Neor gene was not affected that is expressed from the plasmid in which the YY1-coding region was cloned, which rules out differences in transfection efficiency of the WT and mutant YY1 plasmid constructs (Fig. 2E). These observations suggest a cell type-specific effect on RNA and protein expression of the mutant YY1, perhaps resulting from abnormal synthesis or post-transcriptional processing of the mutant mRNA. In functional assays, YY1 p.T372R was considered as a gain-of-function mutation and oncogenic because WT and mutant YY1 slightly increased the proliferation of MIN6 cells (Cao et al. 2013). Another study reported that the mutant YY1 was not oncogenic and the mutation was not a gain-of-function because mutant YY1 target genes (ADCY1 and CACNA2D2) did not affect the proliferation of INS-1 cells (Cromer et al. 2015). Furthermore, ectopic expression of WT or mutant YY1 did not alter β-cell proliferation in human islets (Wang et al. 2017). Our analysis showed that there was no significant difference in the proliferation of two β-cell insulinoma cell lines upon transfection of WT or mutant YY1 (Fig. 2F and G). Insulinomas with mutant YY1 were reported to contain increased expression of YY1 target genes such as the mitochondrial genes UCP2 and IDH3A, and another YY1 target gene COL1A1 (Cao et al. 2013). However, a neomorphic effect of mutant YY1 p.T372R was also reported whereby its binding sequence in DNA was different from the binding sequence of normal YY1, and the presence of mutant YY1 in insulinomas affected the expression of a set of genes different from normal YY1-containing insulinomas (Cromer et al. 2015). The expression of these genes increased insulin secretion in INS-1 cells (Cromer et al. 2015). We did not study the direct effect of WT or mutant YY1 on these target genes because the expression of mutant YY1 RNA and protein was compromised in MIN6 and INS-1E cells.

Altered YY1 activity is predicted in various cancers due to increased YY1 RNA or protein expression (bladder, blood, bone, breast, cervix, colon, liver, lung, prostate, ovary and skin), or reduced YY1 RNA or protein expression (some melanomas, pediatric osteosarcomas and urothelial carcinomas) (Atchison et al. 2011). Although the frequency of the recurrent YY1 p.T372R mutation in our cohort of 23 sporadic insulinomas (8%) differs from previous studies (0–33%), these data indeed support the relevance of YY1 activity in the context of insulinomas. Further studies of insulinomas with the heterozygous YY1 p.T372R mutation can help to determine whether the mutation affects mutant mRNA synthesis or processing, whether it increases or decreases the activity of YY1 or whether it regulates novel target genes. A better understanding of
Figure 1
YY1 mutation analysis and YY1 protein staining in insulinomas. (A) Nucleotide sequence of the tumor DNA and RNA (cDNA) of insulinomas INS3 and INS8 showing the region where the YY1 p.T372R mutation is located (arrows). A heterozygous mutation was detected in the tumor DNA but the tumor RNA (cDNA) only showed normal sequence. (B) Agarose gel electrophoresis of YY1 RT-PCR products using RNA samples from the two tumors with YY1 mutation (INS3 and INS8), and the two insulinoma cell lines (INS-1E and MIN6-4N) that were used in this study. No RT indicates no reverse transcriptase control PCR reactions. Endogenous YY1 mRNA was abundantly expressed in all samples. (C, D and E) The two tumors with YY1 mutation do not show allelic imbalance for the expression of genes near YY1 on chromosome 14q32. UCSC browser image to show genes near YY1 with their orientation indicated by the arrows (C). Highly heterozygous coding region SNPs (heterozygosity ≥ 0.4) were identified in genes located near YY1 using the NCBI Gene resource. Such SNPs were seen in two genes: WARS: https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=7453, and WDR25: https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=79446. The reported heterozygosity for the SNPs used in this analysis were: rs9453 (p.L335L, TTG>CTG) (0.379), rs2273800 (p.H149R, CAT>CGT) (0.423), rs3742387 (p.P223P, CCA>CCG) (0.422), and rs13065 (p.Y523Y, TAT>TAC) (0.500). Primers were designed to amplify the specific regions containing the SNPs from DNA and RNA. Genomic DNA and RNA isolated from the two tumor samples with the YY1 mutation (INS3 and INS8) were used for PCR with each primer pair. The PCR products were purified and sequenced. Sequence traces are shown for each SNP. The SNP rs9453 in the WARS gene and the SNP rs2273800 in the WDR25 gene could not be analyzed at the RNA level because no PCR product was obtained by RT-PCR, which could be due to the lack of expression of WARS in pancreatic β-cells/insulinomas and due to the reported alternative splicing of WDR25 (data not shown). The other two SNPs in WDR25 (rs3742387 and rs13065) were informative for both the tumor DNA and tumor RNA to show that the heterozygous SNP observed in the DNA was also present in the respective RNA sample (D and E). (F) YY1 protein (top panels: nuclear) and insulin (bottom panels: cytoplasmic) staining in sections of the two insulinomas that tested positive for YY1 p.T372R (INS3 and INS8) and two insulinomas that tested negative for the mutation (INS7 and INS20). The YY1 protein staining was similar irrespective of the YY1 mutation status in the tumors. The antibodies used were YY1 (Abcam, catalog# ab109237) and insulin (Abcam, catalog# ab6753).
Figure 2
Expression and functional analysis of WT and mutant YY1. (A, B, C and D) RNA and protein expression: Four different mammalian cell lines were transiently transfected with empty vector (V) or plasmids expressing flag-tagged WT-YY1 (WT) or mutant YY1 p.T372R (MUT). QRT-PCR data are shown for the transfected YY1 mRNA expression using primers specific for the transfected YY1 (forward primer in the YY1 cDNA and reverse primer in the vector before the PolyA signal). QRT-PCR data for transfected YY1 mRNA normalized to endogenous Gapdh are shown relative to vector-transfected cells. Western blot analysis of YY1 protein is shown for each cell line next to the QRT-PCR graph, with P84 as the loading control. The antibodies used were: YY1 (Abcam, catalog# ab109237) and P84 (GeneTex, catalog# GTX 70220). The same RNA and protein expression results were obtained when the WT or mutant YY1 cDNA insert was subcloned into another plasmid and transfected into the four different cell lines. (E) QRT-PCR analysis of NeoR mRNA relative to endogenous Gapdh using RNA samples from the indicated cell lines that were used to detect transfected YY1 mRNA. The NeoR cDNA is located on the vector that was used for cloning WT and mutant YY1. Therefore, the level of NeoR mRNA could be used to assess the transfection efficiency of each plasmid construct. Similar level of NeoR mRNA was detected indicating that the difference in transfection efficiency does not account for the different level of transfected YY1 mRNA detected for WT and mutant YY1. (F and G) Cell proliferation assay: INS-1E and MIN6-4N cells were transiently transfected with empty vector or plasmids expressing Flag-tagged WT-YY1 or mutant YY1 p.T372R. Cell number was counted at 48, 96 and 144 h post transfection.
the genetic background and molecular events underlying the development and progression of insulinomas with or without YY1 mutation could help to enhance management and treatment options. These studies could also provide insights into the regulatory mechanisms associated with normal and abnormal β-cell proliferation.

References


Desai SS, Modali SD, Parekh VI, Kebebew E & Agarwal SK 2014 GSK-3beta protein phosphorylates and stabilizes HLXB9 protein in insulinoma cells to form a targetable mechanism of controlling insulinoma cell proliferation. Journal of Biological Chemistry 289 5386–5398. (https://doi.org/10.1074/jbc.M113.533612)


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