Deleting STX16 exon 4 to understand the genetic mechanisms underlying pseudohyoparathyroidism-1B and GNAS imprinting

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INTRODUCTION

Autosomal dominant pseudohyoparathyroidism type B is characterized by renal parathyroid hormone resistance, with resultant hypocalcemia and hyperphosphatemia. This disorder is associated with an isolated loss of methylation at GNAS exon Aβ and most patients carry maternal microdeletions in the neighboring STX2A gene. (Fig. 1) The shortest deletion overlap is a 1.2-kb region spanning STX2A exons 4 and 5. (Fig. 2) To further understand the patient findings and no functional data exists supporting the association between the STX2A gene and GNAS imprinting, the Aβ deletion is established in the female germline and maintained throughout the development of somatic cells. The aim of the study was to investigate the role of the 1.2-kb STX2A region in the maintenance of Aβ methylation.

METHOD

- Cell Line: HCT116 cells, Human Colorectal Cancer Cell Line (ATCC), were cultured in McCoy's at 37°C. DNA was extracted by using QIAamp DNA Kit.
- CRISPR/Cas: Cas9 and guide RNAs were introduced to HCT116 cells by lentiviral vectors and transduced cells were enriched by blasticidin (cas9) and puromycin (sgRNA). www.porttal.madrid.unidad.org sgRNA tool was used for guide-RNA design. The CRISPR/Cas editing results were analyzed on the TIDE tool (Tracking of Indels by Decamers/ers).
- TOP10 Straining Kit: Bacteriophage Sequencing
- PCR cloning kit was used to sequencing of the PCR products containing the deletion. Sequenc Sequencing was done at MGH DNA Core.
- Bisulfite Treatment: 32 DNA Methylation-Gold® Kit was used for bisulfite conversion of gc rich Cas9 DNA.
- Methylation Status Analysis: MS-MPLA: Multiple Ligation-dependent probe amplification is obtained from MRC Holland.

RESULTS

HCT116 cells, a near-diploid human cell line derived from colorectal carcinoma, were employed as a somatic cell line. To delete 1.2-kb region, we used CRISPR/Cas9 and suitable guide-RNAs targeting upstream and downstream of exon 4. (Fig. 2) We first generated HCT116 cells stably expressing Cas9 and introduced the guide-RNAs into those cells by lentiviral delivery and selected the highest efficiency guide-RNAs, upstream 1 and downstream 4 by using TIDE analysis (Fig. 3A). After antibiotic resistance of the transduced cells, the presence of a 1.2-kb deletion spanning STX2A exons 4 and 5 linking intronic regions was confirmed by PCR (Fig. 2), followed by TIDE cloning and Sanger sequencing of the products (Fig. 3).

Table 1. TIDE Analysis of sequences from the targeted regions. 

<table>
<thead>
<tr>
<th>Primer</th>
<th>Number</th>
<th>GNAS Exon 5-6</th>
<th>GNAS Exon 3-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP1</td>
<td>6</td>
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<td>0.1</td>
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<tr>
<td>UP2</td>
<td>45</td>
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<td>0.1</td>
</tr>
<tr>
<td>UP3</td>
<td>60</td>
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<td>0.1</td>
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<tr>
<td>UP4</td>
<td>90</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>DOWN1</td>
<td>120</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>DOWN2</td>
<td>180</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Figure 2A. Forward and Reverse WF & Deletion Primers

Figure 2B.

1. 18bp DNA Laddier
2. U2DA + Deletion Primers
3. HCT116-cad+ Deletion Primers, 250bp
4. CRISPR
5. HCT116-cad+U1D4 WT Primers, 851bp
6. HCT116-cad+ WT Primers, 220bp

Figure 3. UCSC Database, STX2A Exons Aβ, TOP10 Straining Kit: Bacteriophage Sequencing

Figure 4A. MS-MPLA, Copy Number, human 3′-kb STX2A deletion

Figure 4B. MS-MPLA, Copy Number, HCT116 cells stably expressing Cas9

Figure 4C. MS-MPLA, Copy Number, HCT116-cad+U1D4 cells

SINGLE CELL CLONING

STX16-cad+U1D4 cells were sorted by FACs to obtain single cell colonies. The cloned cells were screened by PCR for the presence of the 2.1-kb deletion, revealing two clones in which a robust PCR amplicon was amplified (Fig. 5). Sanger sequencing of the products was performed to examine the deletion breakpoints (Fig. 7A/7B). To determine HCT116 cells have the homogenous or heterogeneous deletion, we designed WT primers targeting the non-deleted allele. (Fig. 7C)

REFERENCES & ACKNOWLEDGEMENT

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