Mass-Spectrometric Analysis with Sequenom EpiTYPER of *GNAS* Methylation in Pseudohypoparathyroydism Type Ib Patients Reveals Overall Methylation Defects also for the Familial Cases

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Introduction

GNAS **(fig.1) encodes the stimulatory G-protein subunit, Gsalpha, an essential intermediate between receptor coupling and cyclic adenosine monophosphate generation. *GNAS* also combines elaborate patterns of imprinting with tissue- and developmental stage-specific expression to regulate growth and metabolism.**

Maternal inheritance of *GNAS* mutations lead to pseudohypoparathyroidism type Ia (PHPIa), while paternal inheritance of the same mutations leads to pseudopseudohypoparathyroidism (PPHP).

Pseudohypoparathyroidism type Ib (PHPIb) patients display epigenetic *GNAS* defects so far described as involving all the three *GNAS* Differentially Methylated Regions (DMRs) in the sporadic cases (NESP hyper- + XL and Ex1A hypomethylation), or affecting only the exon1A DMR (hypomethylation) in the familial cases (*Bastepe 2008*).

Figure 1. *GNAS* structure and localization of Sequenom amplicons used in the study

Genomic organization of the *GNAS* cluster. Features of the paternal (Paternal) and the maternal (Maternal) allele are shown above and below the line, respectively. The arrows show initiation and direction of transcription. The first exons of the protein coding transcripts are shown as black filled rectangles and the first exons of the noncoding transcripts are shown as gray filled boxes. The Nespas and Ex1A transcripts are noncoding. Paternally and maternally methylated regions (DMRs) are shown by + symbols above and below the line, respectively, so as the Imprinting Control Regions (ICR). Amplicons used in the study (NESP_4, NESP_28, XL_44, Ex1A_3 and Ex1A_13) are reported between green lines. The figure is not to scale (adapted from *Peters 2008*)

Materials and Methods

DNA bisulfite treatment. **Preliminary experiments were performed to compare DNA quality after bisulfite treatment with the MethylDetector™ bisulfite modification kit (Active Motif, Carlsbad, CA, U.S.A.) and another commercially available kit. The MethylDetector kit resulted to provide the best quality of DNA for further analises.**
Genomic DNA (1ug) extracted from leukocytes of a total of 8 PHPIb patients and 35 healthy controls was used for bisulfite treatment as described by the MethylDetector manufacturer.

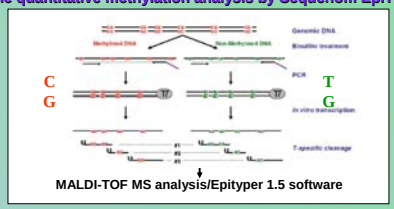
PCR-based assay followed by restriction digestion. **Different nested PCRs were performed to amplify the NESP, XL, and exon 1A regions using bisulfite-treated DNA, as described (*Freson 2008*). Amplified PCR products were digested with restriction enzymes that only recognize the non-converted (methylated) DNA sequence: *Ssi*I (Fermentas, St. Leon-Rot, Germany) for NESP55, *Sal*I (Fermentas) for XL, and *Hin*fI (Fermentas) for exon 1A.**

Sequenom EpiTYPER analysis. **Primers to amplify different amplicons in the three DMRs were designed using Sequenom EpiDesigner BETA (www.epidesigner.com). Sequenom MassARRAY methylation analysis was performed using the MassARRAY Compact System (Sequenom, Inc. San Diego, CA). This system is based on mass spectrometry (MS) analysis for qualitative and quantitative detection of DNA methylation using homogeneous MassCLEAVE (hMC) base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (*van den Boom 2009; Ehrich 2005*) .**
Spectra were elaborated by the Epityper software v1.0.5 (Sequenom) which provides methylation values of each CpG unit expressed as percentage. Those values result from the calculation of the ratio mass signals between the methylated and non-methylated DNA.

Statistical analysis. **A hierarchical cluster analysis was performed to be able to cluster patients on the basis of the average of methylation in each region of the GNAS cluster studied. For each amplicon studied, 3 clusters have been identified (table 1) and independent t-test was performed to study the statistical significance of each cluster compared to the group of the healthy controls. To take into account the problem of multiple comparisons, the significant level was fixed to P=0.017 (Bonferroni correction with n=3 contrasts).**

Figure 2. Base-specific cleavage process for cytosine quantitative methylation analysis by Sequenom EpiTYPER

Base-specific cleavage process for quantitative analysis of cytosine methylation by MALDI-TOF MS (Sequenom EpiTYPER). As a first step genomic DNA is bisulfite treated to introduce methylation-dependent sequence changes in the genomic DNA, which then can be amplified as a stable signal during PCR amplification. After PCR amplification, the PCR product is transcribed into a single-stranded RNA from the reverse. The RNA transcript is then cleaved base specifically by RNase A that cleaves at every C and U. Cleavage products are analyzed by MALDI-TOF MS. A difference in methylation will lead to an A/G sequence change within each of the cleavage products. The corresponding mass signals will therefore shift 16 Da (the mass difference between A and G) if a methylation event occurs (Adapted from *van den Boom 2009*).

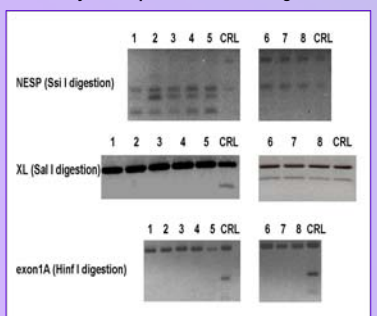


Results

PCR-based assay followed by restriction digestion

The methylation of the different *GNAS* DMRs was studied in 8 PHPIb previously described patients (*Freson 2002; Freson 2008*). As shown in figure 3, patients 1 to 5, described as having a sporadic form of the disease, displayed an overall *GNAS* methylation defect, while patients 6 to 8, with familial PHPIb, showed an exon1A-only methylation defect in combination with the STX16 deletion.

Figure 3. *GNAS* methylation analysis of PHPIb patients by PCR followed by methylation specific restriction digestion



***GNAS* methylation pattern of PHPIb patients (1-5 sporadic, 6-8 familial) and 1 control obtained by amplification of bisulfite-treated DNA of the NESP, XL and exon1A DMRs followed by methylation specific restriction digestion**

Sequenom EpiTYPER analysis

The study of *GNAS* methylation via Sequenom EpiTYPER showed a pronounced and highly significant methylation defect for all the DMRs for the sporadic PHPIb cases (patients 1 to 5).

In contrast to the original finding that familial PHPIb subjects have an exon1A-only methylation defect, overall methylation defects in the NESP and XL DMRs of these patients (6 to 8) was observed. These methylation differences are significantly different from the control subjects (n=35) but are milder than the methylation defects found in the sporadic PHPIb cases.

Table 1. *GNAS* methylation clusters after hierarchical cluster analysis

	Severe hypermethylation +++	Moderate hypermethylation ++	Moderate hypomethylation -	Severe hypomethylation - -	Normal methylation CrIs (n=35)
NESP_4	83 ± 9*	42 ± 5*	11 ± 2*	/	22 ± 9
NESP_28	84 ± 13*	39 ± 6*	20 ± 3*	/	27 ± 7
XL_44	84 ± 13*	/	19 ± 3*	9 ± 2*	30 ± 4
1A_3	/	56 ± 9*	30 ± 2*	14 ± 4*	35 ± 9
1_13	/	/	25 ± 2*	12 ± 3*	35 ± 8

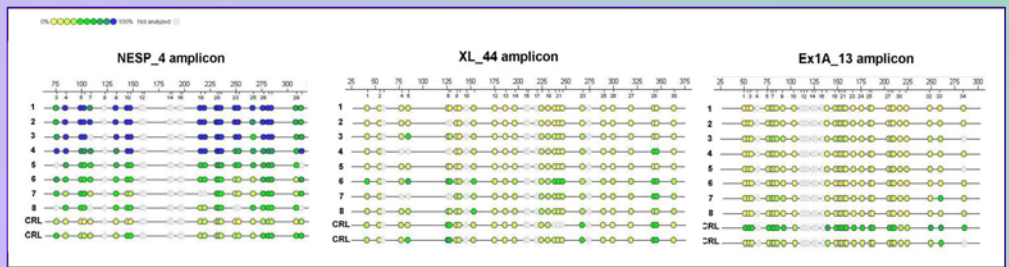
Data reported as mean (%) ± SD
* P=0.017 (Bonferroni correction with n=3 contrasts) vs crIs

Table 2. *GNAS* methylation patterns of PHPIb patients

Case	GNAS methylation				
	NESP_4	NESP_28	XL_44	1A_3	1A_13
1, F	+++	+++	- -	- -	- -
2, F	+++	+++	- -	- -	- -
3, F	+++	+++	-	NL	- -
4, M	++	++	-	- -	- -
5, F	+++	+++	- -	- -	- -
6, M	NL	++	NL	-	- -
7, M	+	NL	+	-	- -
8, M	+	NL	-	-	- -

GNAS methylation pattern of PHPIb patients
+++, ++, +, --, - : see table 1, NL: normal

Figure 4. Sequenom EpiTYPER epigrams of NESP_4, XL_44 and Ex1A_13 amplicons in PHPIb patients



***GNAS* methylation profile of PHPIb patients (1-5 sporadic, 6-8 familial) and 2 controls. The colored circles indicate the degree of methylation with yellow representing 0% methylation and blue representing 100%. Each circle corresponds to a CpG site from the different DMRs studied. Numbers indicate the CpG units that are used by the software to generate methylation percentages. Each CpG unit corresponds to one or more than one CpGs, and are created by the software on the basis of the sequences amplified and on the prediction of the fragments generated after T-cleavage of the transcripts.**

Conclusions

- **The Sequenom EpiTYPER resulted to be an efficient and sensitive methodology to study *GNAS* methylation in PHPIb patients -> 3 familial PHPIb cases are here for the first time reported as having an overall *GNAS* methylation defect that was not detectable with the PCR+digestion method.**
- **The MethylDetector kit (Active Motif), used for the bisulfite treatment of the DNA in both the methodologies here presented, provides good quality of DNA conversion and low rate of DNA fragmentation, critically important for the following DNA amplification.**
- **The application of the Sequenom EpiTYPER to the study of PHPIb patients showed its high sensitivity to identify even small DNA quantities affected by a methylation defect, thus revealing its potential as tool to study other imprinting diseases.**