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Elli FM; De Sanctis L; Peverelli E; Bordogna P; Pivetta B; Miolo G; Beck-Peccoz P; Spada A; Mantovani G.. Autosomal Dominant Pseudohypoparathyroidism type Ib: a novel inherited deletion ablating STX16 causes Loss of Imprinting at the A/B DMR.. THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM. 99 (4) pp: E724-E728. DOI: 10.1210/jc.2013-3704

Autosomal Dominant Pseudohypoparathyroidism type Ib: a novel inherited deletion ablating *STX16* causes Loss of Imprinting at the A/B DMR.

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Abbreviated title: A novel STX16 deletion causing PHP-Ib.

Key terms: Pseudoipoparathyroidism type Ib; GNAS; STX16; deletion; MS-MLPA.

Word count: Text: 1953, Abstract: 211, Tables: 0, Figures: 2.

Disclosure statement: The authors have nothing to disclose.

Supporting grant: This work was supported by a grant from the Italian Ministry of Health to G.M. (GR-2009-1608394.

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Abstract

Pseudohypoparathyroidism type Ib (PHP-Ib) is a rare imprinting disorder characterized by end-organ resistance to parathyroid hormone and thyroid-stimulating hormone. PHP-Ib familial form, with an autosomal dominant pattern of transmission (AD-PHP-Ib), is typically characterized by an isolated loss of methylation at the A/B DMR, secondary to genetic deletions disrupting the upstream imprinting control region in the STX16 locus.

However, deletions described up to nowadays failed to account for some cases of patients with a methylation defect limited to the A/B DMR, thus it is expected the existence of other still unknown rearrangements, undetectable with conventional molecular diagnostic methods. Hence the need to use different methods to search for novel pathogenetic defects in PHP-Ib patients, such as methylation specific-multiplex ligation-dependant probe amplification (MS-MLPA).

In the present study we report the clinical, biochemical and molecular analysis of a AD-PHP-Ib patient with a novel *STX16* deletion that overlaps with previously identified 3.3 kb and 4.4 kb *STX16* deletions, but that, unlike these genetic defects associated with AD-PHP-Ib, goes unnoticed with commonly used first level diagnostic techniques.

Our work highlights the importance of performing accurate investigations in PHP-Ib patients with methylation defects, as in case of deletions the segregation ratio is about 50% and the disease phenotype is transmitted in an autosomal dominant fashion via the mother.

Introduction

Pseudohypoparathyroidism type Ib (PHP-Ib; MIM # 603233) is a rare imprinting disorder characterized by end-organ resistance to parathyroid hormone (PTH) and thyroid-stimulating hormone (TSH), without physical features of Albright hereditary osteodystrophy (AHO), such as short stature, rounded face, brachydactyly, ectopic ossifications and mental retardation, that are instead typical of PHP type Ia (PHP-Ia; MIM # 103580).

PHP-Ib has been associated with methylation defects in the *GNAS* imprinted cluster (MIM #139320), a complex locus that generates 5 transcripts using alternative first exons. Gs alpha, the alpha subunit of the heterotrimeric stimulatory G protein (Gsα), is the best characterized *GNAS* product (Figure 1A) (1-6).

Contrarily to the other GNAS products, Gsα promoter is not differentially methylated and Gsα expression occurs from both alleles in most tissues, albeit in some tissues, including proximal renal tubules, pituitary, gonads and thyroid, this gene is transcribed predominantly from the maternal allele (7-13). Gsα tissue-specific imprinting is assumed to be controlled by the upstream A/B differentially methylated region (DMR), that likely has cis-acting, methylation-sensitive and tissue-specific negative regulatory elements for the Gsα promoter, as methylation defects affecting the maternal A/B DMR lead to biallelic expression of the A/B transcript and the loss of Gsα expression in renal proximal tubules, but have little effect in tissues where Gsα is biallelically expressed (4,14-17). The A/B DMR epigenetic status is maintained by an imprinting control region (ICR) located in the upstream syntaxin-16 gene *(STX16;* MIM # 603666*)*.

PHP-Ib is often sporadic (spor-PHP-Ib) but it may occasionally present as familial, with an autosomal dominant and maternally inherited pattern of transmission (AD-PHP-Ib) (18). No clinical differences have been observed between the sporadic and the familial form (19). Sporadic PHP-Ib cases show broad GNAS imprinting abnormalities involving multiple DMRs, with no known underlying genetic lesion (20,21). AD-PHP-Ib cases are typically caused by an isolated loss of methylation (LoM) at the A/B DMR, secondary to genetic deletions disrupting the STX16 ICR or, less frequently, by loss of all the maternal GNAS imprints due to deletions removing the NESP55 DMR (22-26) (Figure 1A).

A 3-kb deletion removing *STX16* exons 4-6 is the most typical genetic defect found in the AD-PHP-Ib form (22), while a 4.4-kb deletion overlapping the smaller one and removing *STX16* exons 2-4 has been detected in few familial cases, confirming that *STX16* contains an important regulatory element involved in PHP-Ib pathogenesis (24). Overall, the finding that the majority of patients share a common sized deletion confirms that this chromosomal rearrangement arises through a specific mechanism involving the DNA sequence.

However, routine molecular and cytogenetic diagnostic methods (i.e. Sanger sequencing and routine or high-resolution karyotyping) fail to detect these deletions in a minority of PHP-Ib patients carrying methylation defects limited to the A/B DMR, suggesting the existence of other unknown genetic rearrangements within the *STX16* gene or elsewhere in the genome (27).

In the present study we report the clinical, biochemical and molecular analysis of a PHP-Ib patient with a novel *STX16* deletion of about 29.6 kb. This new deletion overlaps with previously identified *STX16* deletions (22, 24), but, unlike classical genetic defects associated with AD-PHP-Ib, it is undetectable with commonly used molecular diagnostic methods. Our work highlights the importance of performing accurate investigations in apparently sporadic PHP-Ib patients with GNAS methylation defects, in order to give appropriate genetic counseling to familial cases.

Patient and Methods

Patient

The proband, a 21-yr-old Italian male, was diagnosed as affected by Pseudohypoparathyroidism at the age of 13-ys-old, after he was hospitalized for transient loss of consciousness and brief titanic crisis. Laboratory findings showed a phospho-calcium metabolism disorder (calcaemia: 4.7 mg/dL, normal range: 9-10.5 mg/dL; phosphataemia: 9.5 mg/dL, normal range: 2.8-4.5 mg/dL) consistent with the diagnosis of hypocalcaemic tetanus, whose correction led to a significant clinical improvement. The brain CT scan showed the presence of rough calcifications at the level of the basal ganglia and of the corticomedullary junction. Hypocalcemia,

hyperphosphatemia and raised serum PTH levels (PTH: 654.2 pg/mL, normal range: 10-65 pg/mL) in the absence of vitamin D deficiency were suggestive of Pseudohypoparathyroidism.

His past clinical history was non-significant, he did not show signs of AHO and there was no familial history of phospho-calcium metabolism disorders.

Methylation Specific - Multiplex Ligation-dependent Probe Amplification assay (MS-MLPA) of STX16 and GNAS loci

Dosage of allele segments and methylation analyses of STX16 (MIM#603666) and GNAS loci were performed by MS-MLPA using the SALSA MLPA ME031 GNAS probemix (MRC-Holland, Amsterdam, The Netherlands). The protocol was implemented following the manufacturer's recommendations. MS-MLPA PCR products were separated by capillary electrophoresis using ABI3130xl Genetic Analyzers (Perkin-Elmer Corp.) with an internal size standard GeneScan 500LIZ (Applied Biosystems, Foster City, CA). Data analysis was performed using GeneMapper software (Applied Biosystems, Foster City, CA) and Coffalyser v9.4 (MRC-Holland, Amsterdam, The Netherlands).

COBRA of GNAS DMRs

Differential methylation of GNAS DMRs was initially assessed on genomic DNA by COmbined Bisulfite-treated Restriction Analysis (COBRA), as previously reported (22). In brief, 360 µg of DNA was treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol, and was amplified by PCR with primers specific for GNAS DMRs (27). Amplified fragments of AS, XL and A/B DMRs were digested with BstUI and NESP DMR was digested with AciI (New England Biolabs, Ipswich, GB), according to manufacturer's recommendations.

Investigation of STX16 3-kb and 4.4-kb deletions

We investigated the presence of known *STX16* deletions on genomic DNA by multiplex PCR, as previously described (12). Briefly, multiplex PCR was performed using two pairs of primers for both 3-kb and

4.4-kb *STX16* deletions giving raise to PCR products of different length according to the presence of the deleted allele.

Southern Blot analysis of STX16 locus

To confirm the *STX16* deletion found by MS-MLPA we performed Southern Blot analysis of patient and wild-type control DNAs. As first step, restriction endonucleases SacI and AlwI (New England Biolabs, Ipswich) were used to cut high-molecular-weight DNA strands into smaller fragments. Digested DNA was resolved by 0.5% agarose electrophoresis, in parallel with the Biotinylated 2-Log DNA Ladder (0.1-10 kb) (New England Biolabs, Ipswich), and then blotted by alkaline transfer method to positively charged nylon membrane Hybond N (GE Healthcare, Piscataway, NJ, USA). The membrane was baked at 80°C to immobilize DNA, then was exposed to 60 ng of biotinylated hybridization probe 5'-BITEG-GGCAGGGCCTCAGCAGGTTCAGACAAGTCCCCAGAAGGGAGCTGCAGGCGCCCTCGAGT-3' (Eurofins MWG Operon, Ebersberg). The pattern of hybridization was visualized with streptavidin-horseradish peroxidase conjugate using North2South chemiluminescent nucleic acid hybridization and detection kit (Pierce, Rockford, USA), chemiluminescence was detected using the ChemiDoc-IT Imaging System (UVP, Upland,CA) and analyzed using the image analysis program NIH ImageJ.

Semiquantitative PCR (sq-PCR) and long range PCR (LR-PCR) of STX16 locus

As a first step toward the delimitation of the *STX16* deletion, a semiquantitative amplification of specific sequences in the region spanning from STX16 and NESP loci and in *STX16* intron 1 was performed (primers available upon request). Briefly, serial dilutions of wild-type DNA were amplified by non-saturated PCR (20 cycles) together with patient DNA and, after agarose electrophoresis, were analyzed by densitometric scanning of photographs of gels with NIH ImageJ software. Finally, to locate breakpoints at nucleotide level, long range PCRs with primers flanking the deleted sequence and direct sequencing of amplicons were performed (conditions and primers available upon request).

Results and Discussion

Pseudohypoparathyroidism type Ib is caused by the disruption of long-range imprinting control regions regulating GNAS cluster methylation status and is often sporadic, but it may occasionally present as familial, with an autosomal dominant pattern of transmission (AD-PHP-Ib). Hormonal resistance develops only after maternal inheritance of the disease, while paternal inheritance of the same defect is not associated with endocrine abnormalities (28). No phenotypic differences have been observed between the sporadic and the familial form (19).

About 10 years ago the AD-PHP-Ib form was linked to a region on 20q13.3 and Bastepe et al. isolated the first causative genetic defect, a 3-kb at STX16 locus, that is the predicted chromosomal location for the ICR controlling the A/B DMR imprinting (22). During the last decade, different Research Units reported few other causative STX16 and GNAS deletions, demonstrating the need to investigate for these less common genetic defects associated to PHP-Ib (23-26).

Nevertheless, the deletions described up to nowadays do not account for some cases of PHP-Ib patients carrying a methylation defect limited to the A/B DMR, thus it is expected the existence of other still unknown rearrangements, undetectable by routinely used diagnostic techniques for PHP-Ib.

To this purpose, in the present study, we extensively investigated a patient showing a clinical PHP-Ib phenotype and an isolated imprinting defect at GNAS A/B DMR but no evidence of classical *STX16* deletions.

A powerful tool for evaluating such subtle genetic abnormalities in PHP patients is methylation specific-multiplex ligation-dependant probe amplification (MS-MLPA), a multiplex PCR method that allows, at the same time, a targeted investigation of deletions/duplications affecting GNAS and STX16 loci and of the methylation status of GNAS DMRs.

Methylation analysis of GNAS DMRs by COBRA demonstrated LoM limited to the A/B DMR and an emimethylated status at NESP, AS and XL DMRs (Figure 1B), but multiplex PCR failed to detect known 3.3-kb and 4.4-kb *STX16* deletions in our patient (Figure 1C). Subsequent MS-MLPA analysis confirmed LoM at the

A/B DMR associated with the presence of an extended loss of diploidy affecting the STX16 locus, which pointed for the presence of a large deletion removing *STX16* exons 3-8 (Figure 2, upper panel).

To confirm this finding we performed Southern Blot analysis and we analyzed parent's DNA. Southern Blot confirmed a 50% signal reduction in the index compared to a wild-type control (Supplementary Figure 1). Moreover, we found that also his unaffected mother carried the same deletion (data not shown), demonstrating the maternal inheritance of the defect.

As a first step toward the delimitation of this novel *STX16* deletion, we made a semiquantitative amplification of specific sequences in the region spanning from STX16 and NESP loci and within *STX16* intron 1 (data not shown). These experiments allowed the raw localization of upper, between chromosome positions 57'229'525 and 57'236'686, and lower breakpoints, between 57'257'525 and 57'290'623.

Finally, to locate breakpoints at nucleotide level, we performed long range PCRs using primers flanking the deleted sequence followed by direct sequencing, revealing a 29′591 bp deletion (g.57′235′162_57′259′753del) (Figure 2, lower panel). Based on this data, the deletion, starting in intron 1, removes *STX16* exons from 2 up to 8, overlapping with previously described rearrangements (22, 24).

This non-recurrent deletion shares a common genomic region-of-overlap with previously identified 3.3 kb and 4.4 kb *STX16* deletions, that encompasses the critical region containing the putative *cis*-acting ICR necessary for the maintenance of the methylation imprint at the exon A/B.

In conclusion, in the present work we have identified and refined the boundaries of a novel *STX16* deletion, resulting in a switch from a maternal to a paternal epigenotype at the exon A/B DMR, as a cause of AD-PHP-Ib. Our finding further confirms the parent-of-origin-specific transmission of the disease, the possibility that mothers carrying a disease-causing deletions may be clinically unaffected, and the existence of control elements within the *STX16* region crucial for keeping across generations imprinting at the A/B DMR. Moreover, our work stresses the importance of performing additional investigations in PHP-Ib patients with apparently sporadic methylation defects to allow precise genetic counseling, as in case of submicroscopic structural mutations the disease phenotype is transmitted in an autosomal dominant fashion via the mother.

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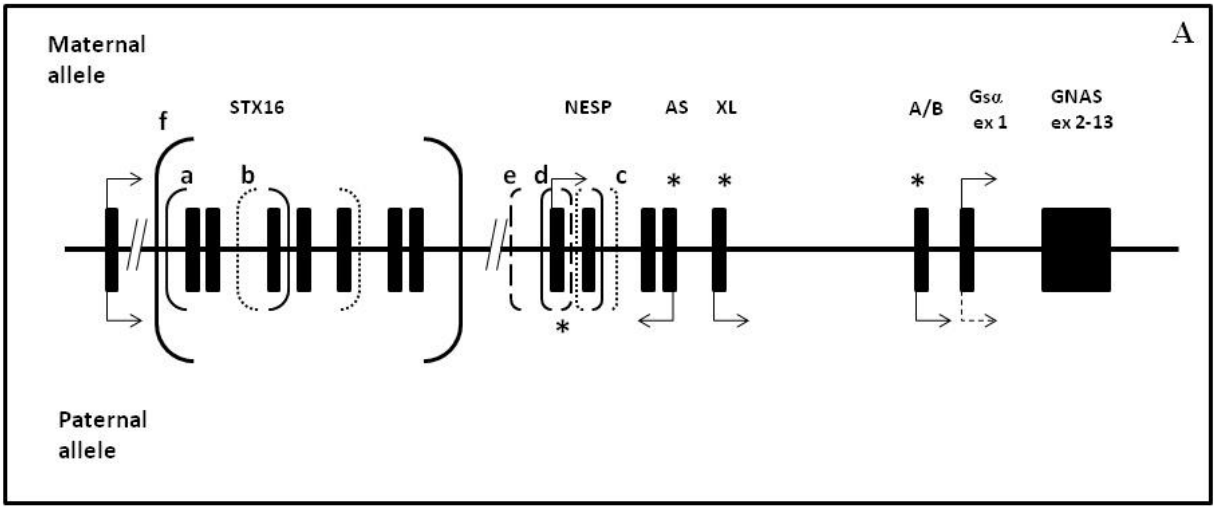
Legend to figures

Figure 1. Panel A shows a schematic graph of general organization and imprinting at *GNAS* and *STX16* loci. The diagram is not drawn to scale. Exons are indicated as black rectangles, the four differentially methylated regions (DMRs) are highlighted with an asterisk on the methylated allele and the allelic origin of transcription as arrows on the paternal or the maternal allele. *STX16* and *NESP-AS* deletions are surrounded by brackets: a, ref. 24; b, ref. 22; c, ref. 25; d, ref. 23;e, ref. 26; f, the novel deletion identified in the present paper. Panel B shows methylation analysis of *GNAS* DMRs by COBRA. Wild-type NESP, AS, XL and A/B DMRs are digested by the enzyme used into two bands, one corresponding to the unmethylated allele and one to the methylated allele. The absence of the digested band at A/B DMR indicates loss of methylation on the maternal allele. Our index resulted to have a loss of methylation restricted to the A/B DMR. Panel C shows *STX16* deletion analysis performed by multiplex PCR. The normal control shows two PCR products (1496 and 966 bp), whereas the deleted sample shows an additional 1387-bp band amplified on the deleted allele. Our index did not carry *STX16* known deletions; interestingly, the 966 bp fragment appeared faint, as the deleted positive control.

Figure 2 Upper panel. This figure shows a summary chart of the data analysis for copy number determination performed by MS-MLPA. On the X-axis MLPA probes are shown according to their chromosome location, while on the Y-axis the final probe ratio, or ploidy status, is shown. Probes specific to *STX16* exons 3-8 (black bars) give a 35-50% reduced signal during capillary electrophoresis, suggesting the presence of a heterozygous deletion. Lower panel. Characterization of the novel *STX16* deletion breakpoints. This figure shows the chromatogram of the boundaries of the deletion (g.57′235′162 - 57′259′753del) obtained by Sanger sequencing of long range PCR.

Supplementary Figure 1 *STX16* Southern Blot image showing the index and a wild-type control (left panel). The graph of the densitometric quantification shows a marked decrease in the index (black bar), confirming the presence of a deletion removing the recognition sites for SacI and AlwI endonucleases (right panel).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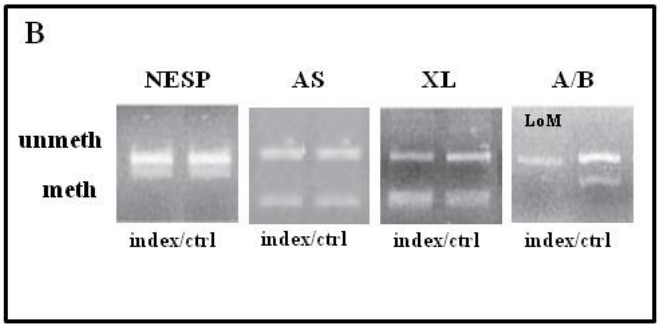
NESP
AS
XL
A/B
Gsα ex 1
GNAS ex 2-13
f
a
b
e
d
c
Paternal allele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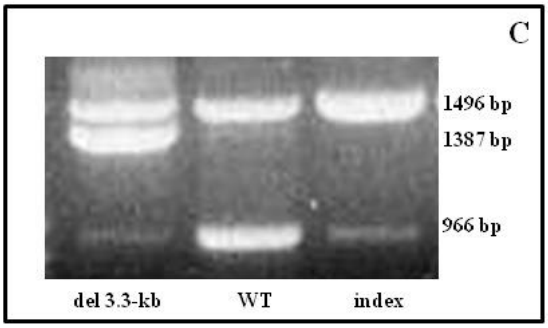
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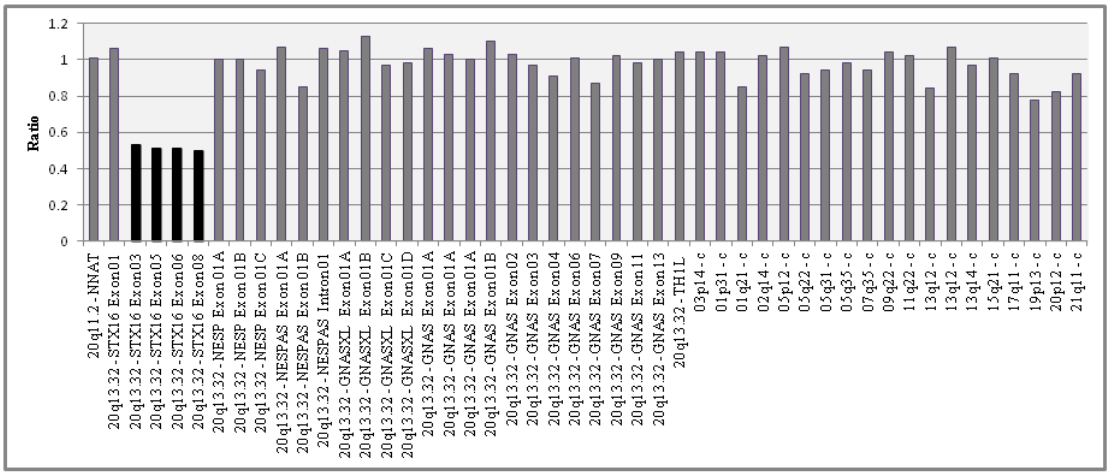
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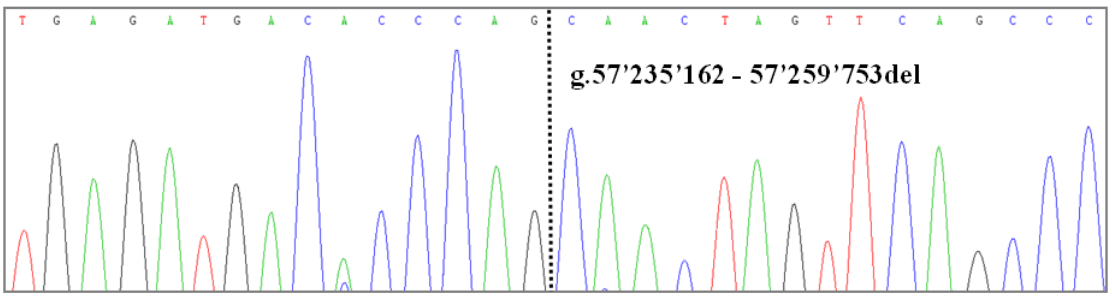
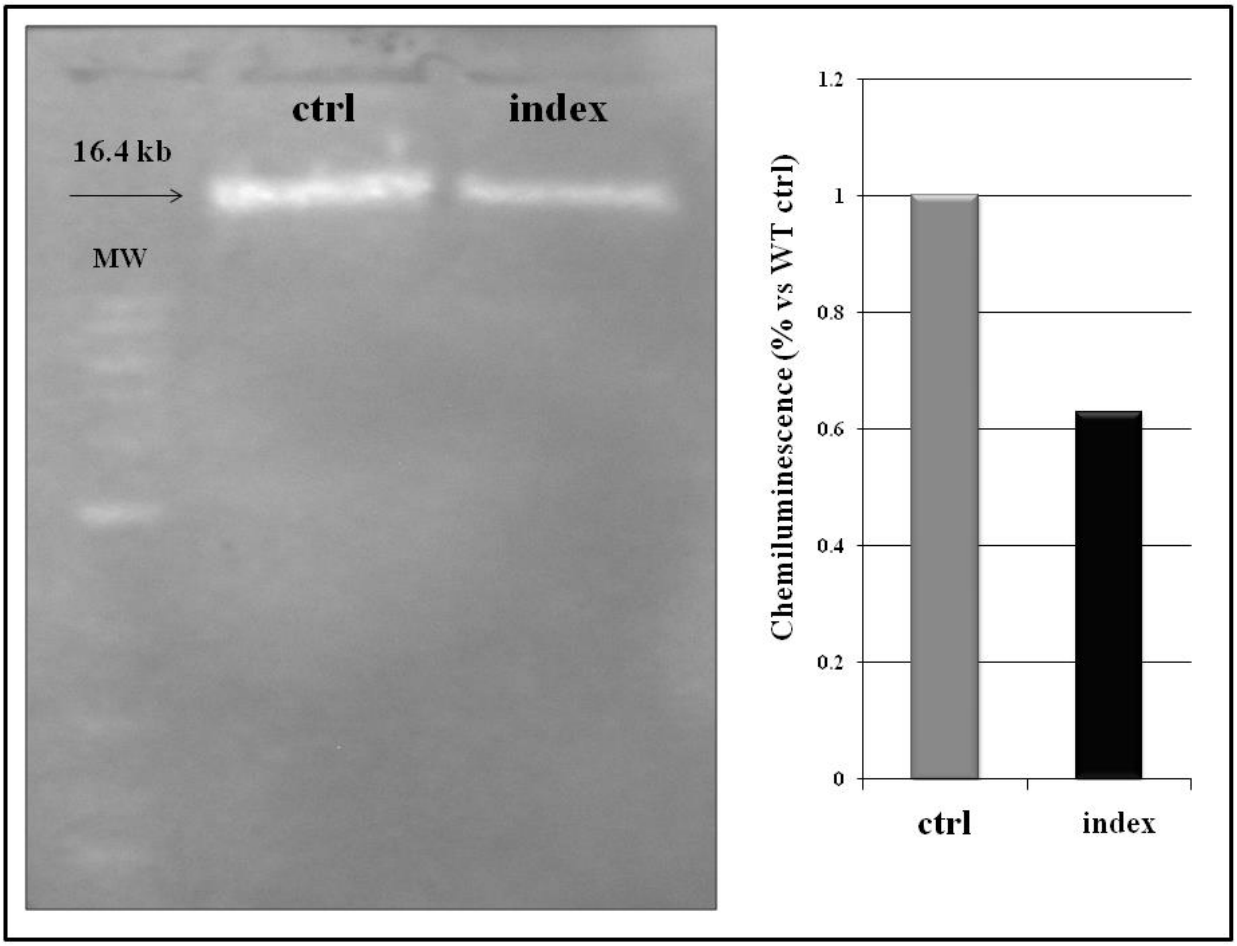

g.57'235'162 - 57'259'753del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