

Research Article

Partial *SHOX* duplications associated with various cases of congenital uterovaginal aplasia (MRKH syndrome): A tangible evidence but a puzzling mechanism

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Abstract

The Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome is the most severe form of congenital malformation of the inner female reproductive tract. It is diagnosed as such when the uterus, the upper vagina and optionally the Fallopian tubes are absent. It accounts for approximately 1 in 5000 live-born females and has been classified in two subtypes: type 1 in the presence of isolated uterovaginal aplasia and type 2 when associated in various combinations with extragenital malformations of the kidneys, skeleton, heart and auditory system. Most cases of MRKH syndrome are sporadic, although a significant number of many familial cases have been reported to date. Despite numerous studies, the genetics of the syndrome remains largely unknown and appears to be heterogeneous: chromosomal abnormalities and some candidate gene variants appear to be associated with a few cases; others have been suggested but not yet confirmed. To date, mainly the *GREB1L* gene appears to be a serious candidate. Among the remaining hypotheses, the controversial contribution of partial duplications of the *SHOX* gene is still puzzling, as the deficiency of this gene is a major cause of skeletal dysplasia syndromes. We have attempted to resolve this controversy in a study of 60 MRKH cases. Our results tend to show that *SHOX* duplications can be the origin of a genetic mechanism responsible for MRKH syndrome.

Introduction

Among congenital malformations of the female genital tract (FGT), aplasia of the uterus and vagina, generally referred to as Mayer-Rokitansky-Küster-Hauser syndrome (MRKHS) (OMIM #277000), is the most severe form [1,2] with an incidence estimated to about 1 in 5000 newborn females [3]. However this syndrome is not as easy to diagnose as such in the first instance, since the aplasia of the uterus may be total or partial, that of the vagina is of various length, and Fallopian tubes affection ranges from total aplasia to almost complete structures. Attempts to classify FGT malformations illustrate the difficulties to reach a consensus [4,5,6,7]. The only common feature appears to be a primary amenorrhea in phenotypically female subjects (normal development of secondary sexual characteristics [1] and normal external

genitalia), indicating the presence of functional ovaries with no sign of hyperandrogenism [8], in an otherwise normal XX chromosomal background. At this first stage, a differential diagnosis can already be undertaken to avoid an initial wrong direction [1]. The syndrome is defined as MRKH type 1 when a complete aplasia of the uterus and vagina is observed without any associated malformations, but where Fallopian tubes are most often present. On the other hand, MRKH type 2, includes hypoplastic to aplastic uterus, upper vagina aplasia and variable affection of Fallopian tubes, the whole being associated with a wide range of malformations, themselves found with various degrees of frequency [9,10,11]. They are mainly renal (unilateral agenesis, ectopic or horseshoe kidney), skeletal (scoliosis, Klippel-Feil anomalies, hemivertebrae) and to a lesser extend auditory or cardiac. During this last decade,

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thorough analysis of large cohorts, has allowed to gather consistent data on the nature and frequency of associated malformations. In spite of some yet unexplained geographic variations [10,12], isolated uterovaginal aplasia (MRKH type 1) and association of renal or skeletal malformations (MRKH type 2) remain the most frequent forms encountered worldwide with around 60% of type 1 and 40% of type 2 [11].

Once the genetic origin of the syndrome was admitted [13,14,15], the approaches to find the cause have followed technological developments to currently lead to whole exome/genome sequencing (WES/WGS), via hypothesis-based mutational analysis of candidate genes, comparative genomic hybridization (CGH) array, or the search for deletions or duplications of targeted chromosomal regions by multiplex ligation-dependent PCR assay (MLPA) [16]. To the best of our knowledge, none of the hypothesized candidate genes could formally account for the syndrome [9,11]. Numerous chromosome imbalances, mainly segmental deletions, have been associated with MRKHS using CGH array, but their pathogenicity has not been established for the vast majority of them [9]. However two recurrent genomic rearrangements mapping to 17q12 [17,18,19] and 16p11.2 [18] were found in several unrelated cases of MRKHS of both types. These two loci respectively include the *LHX1* and *TBX6* genes which were considered as strong candidates based on their documented role during development of the female genital tract. Subsequent mutational analysis of both genes evidenced this role, but pathogenic variants were found in a very limited number of cases, in *LHX1* [20,21,22,23], and in *TBX6* [22,24]. Nowadays, the third generation sequencing allows very fine linkage analysis through WGS and sequencing of (almost) all coding regions of the genome (WES). These technologies have recently been applied to the genetics of MRKHS, first on a selected group of MRKH type 1 patients, which led to unveil new candidate genes (*PIK3CD*, *SLC4A10* and *TNK2*) bearing putatively pathogenic variants [25]. Two other teams using a WES approach, identified different *GREB1L* gene variants from a total of 5 families and 6 sporadic cases [26,27]. Data from these latter studies also suggest that *GREB1L* is the first major gene involved in MRKHS. Indeed its involvement in familial cases of uterovaginal aplasia associated with renal dysplasia, together with other sporadic cases of isolated uterovaginal aplasia (type 1) or associated with skeletal or facial malformations (type 2), reinforces this assumption. This also suggests that, beyond its involvement in kidney [28] and probably female genital tract development [29], *GREB1L* could play a more pleiotropic role during development and be involved in other malformative syndromes.

Despite the discovery of this new gene, there are still many unexplained cases of MRKHS for which genetic studies have not been completely successful. This applies to the various chromosomal rearrangements involving genes not yet characterized as well as to the controversial association of various partial heterozygous duplications of the *SHOX* gene

with MRKHS. Indeed, non-overlapping duplications of this gene were described using the MLPA technique, in cases of MRKHS type 1, familial (father-transmitted) or sporadic [30]. This study was later contradicted through analysis of a larger cohort by mean of the same technique [31], but this result remains puzzling, especially considering the relatively high frequency of these duplications within the MRKH cohort of patients analyzed (5/30) [30]. It is even more puzzling that the only role assigned to the *SHOX* gene, is its involvement in some skeletal dysplasia syndromes such as Léri-Weill dyschondrosteosis (LWD, MIM 127300), Langer mesomelic dysplasia (LMD, MIM 249700), idiopathic short stature (ISS, MIM 300582) or its contribution to Turner syndrome, all attributable to various deletions, duplications or mutations within the coding or regulatory sequences of the *SHOX* gene [32-34]. This is why we wanted to contribute to this debate by studying a cohort of 60 patients using the same MLPA technique. We found four duplications of various lengths, within or adjacent to the *SHOX* gene, some similar to those described previously, and some new ones, in four sporadic cases. The association of these genetic events with MRKHS seems then relevant and we discuss it in technical and mechanistic terms.

Patients and methods

Patients

We studied a cohort of 60 women who had utero-vaginal aplasia diagnosed by clinical examination and transabdominal ultrasonography and/or magnetic resonance imaging (MRI) or celioscopy. All patients had a normal karyotype 46, XX. They were examined for associated malformations using renal ultrasound, spinal X-ray, echocardiogram and audiogram, as necessary. Twenty (~33%) had isolated uterovaginal aplasia (MRKH type I). The remaining women (~67%) had a type 2 syndrome with renal, spinal or other skeletal malformations (including Klippel-Feil sequence, Sprengel deformity, digit defects such as clinodactyly, brachydactyly or syndactyly), cardiac anomalies or hearing impairment. All the subjects were enrolled through a French national multicentric research program, called PRAM (Programme de Recherche sur les Aplasies Müllériennes). This study has been approved by the local institutional review board (Project # 05/16-543), and is registered with the French Ministry of Health (DGS # 2005/030). Informed consent was obtained from all subjects.

The four patients in whom we found partial duplications of the *SHOX* gene, showed MRKH syndrome type 2 (Table 1). There were no other affected siblings, nor was there any family history to report.

Methods

Genomic DNA was extracted from whole blood of patients or healthy control subjects, using the QIAamp DNAKit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol.

MLPA analysis was performed using the commercial



Patient	MRKH type	Uterus	Fallopian tubes	Associated malformations	Location and maximal size of the duplication (probes involved)	Analogy with cases previously described by Gervasini et al.
PRAM-19	2	Total aplasia	Present	Spina bifida occulta	<i>SHOX</i> exon 5 - 3.3 kb (L19676)	Partial overlap with patients II-3, II-4 (sisters) and II-2 (father) carrying exon 4 to exon 6 duplication
PRAM-42	2	Total aplasia	Present	Scoliosis + pelvic solitary kidney	<i>SHOX</i> exons 1 to 6 - 94,5 kb (L20651, L00702, L06220, L00802, L15501, L19676, L00911, L20178, L15503)	Patient 3 with the whole gene duplicated
PRAM-51	2	Severe hypoplasia	Present	Scoliosis	<i>SHOX</i> exons 1,2 and 3 - 16,5 kb (L00702, L06220, L00802)	Partial overlap (probes L00702 and L06220 = exons 1 and 2) with patient 1
PRAM-76	2	Hypoplasia	Absent	Klippel-Feil malformation, scoliosis, facial dysmorphism, ovarian dysgenesis, hearing impairment	Adjacent (downstream) to <i>SHOX</i> - 468,4 kb (L19679 and L19677)	No

Table 1: Diagnosis and main clinical features of patients with MRKH syndrome heterozygous for partial *SHOX* gene duplications. Genetic similarities with the cases previously described by Gervasini and collaborators [30]. All patients showed normal size, weight and BMI for their age.

Table 2: Gene location and sequences of primers used in the DP/LC experiments. Different combinations of *HMBS/SHOX* primers were used according to the best compatibility calculated by the Primer Premier 5 software which also provides the best ΔG (kcal/mol) value for each oligonucleotide in a given mix of *HMBS/SHOX* sens and antisens primers. Consequently, the *HMBS* exon 14 primer pair was used in combination with 3 different *SHOX* primer pairs (upper part of the table) and the *HMBS* exon 15 primer pair with 4 different other *SHOX* primer pairs (lower part of the table).

Gene	Location	Sens primer (5'-3')	Antisens primer (5'-3')	PCR size
<i>HMBS</i>	Exon 14	TAGACGGCTCAGATAGCATAACAAG	AGGTGGGATTTGGTGAGAACA	132 nt
<i>SHOX</i>	4 kb upstream	CGGCACAAATTAGGCCATTA	CATTGCCTGTCCGGGTGAAA	147 nt
<i>SHOX</i>	Exon 5	GAGCCTTACGGATGCCTTTC	CAGGATGCCGCAGCAAAT	107 nt
<i>SHOX</i>	Intron 6	GAGAAGCCGGTTAAGGAATGTA	CGGGACCTGCACGTACAAT	113 nt
<i>HMBS</i>	Exon 15	AGACCATGCAGGCTACCATCC	GGTCATCCTCAGGGCCATCT	148 nt
<i>SHOX</i>	Exon 1	GGTGCAAAGGCGAGGAGA	AATGCCAGGGTGTGAC	166 nt
<i>SHOX</i>	Exon 2	TCCAGGACATCACGGAGGG	CCGGAGCGCAAAGGAACT	132 nt
<i>SHOX</i>	Exon 3	GCGTCAAAGCGCATTGGT	TCCTCGTCTCCGACTTCA	170 nt
<i>SHOX</i>	Exon 6	CGCCCTACCTGATGTTCCC	GGTCGGCGATGCTGGAAT	129 nt
<i>SHOX</i>	Exon 7	TCCTGGGCTCAAGCAATCC	CCTCAGCAGCAAAGCAAGATC	124 nt

SALSA MLPA kit P018-F1 *SHOX* (MRC-Holland, Amsterdam, Netherlands) in accordance with the manufacturer's instructions. P018 *SHOX* probe mix contains probes for each exon of the *SHOX* gene and some other targeting upstream and downstream regions of the gene, meant to be involved in its transcriptional regulation. More precisely, this kit includes 48 probes, six of which target the exons 1 to 6, two the intron 6, thirteen its regulatory or flanking regions and seven target other genes within the pseudoautosomal region 1 (PAR1) of the X and Y chromosomes where the *SHOX* gene locates. Eight other probes serve as internal control for the X chromosome outside the PAR1 region and 12 are external to this chromosome, of which 2 are specific for the Y chromosome. Finally, two extra probes provide amplification and denaturation controls. MLPA reactions were achieved from 200 ng of genomic DNA and PCR products were afterwards subjected to capillary electrophoresis using an ABIPRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Electrophoretic patterns were analyzed using the GeneMarker software (Softgenetics, State College, PA, USA) which uses the internal control probes to normalize peak heights and then compares tests samples to a normal control panel. This latter consists

in five normal genomic DNAs which are individually MLPA-processed and for which the peak areas of each amplification are averaged and then compared to each peak area of the DNA of the patient under study. Each patient was thus analyzed at least twice independently.

SHOX duplications were confirmed by another semi-quantitative technique using the multiplex PCR/liquid chromatography (MP/LC) method [35] in a duplex assay (DP/LC) that we had already proven in two previous studies [36,37]. Briefly, two target genomic sequences, from an internal control *HMBS* gene (hydroxymethylbilane synthase gene located at 11q23.2-qter) and from the region found duplicated, were amplified using unlabeled specific primers in the same PCR reaction. Primers (Table 2) were designed using the Primer Premier Software (Premier Biosoft International, Palo Alto, CA, USA) and in order to amplify genomic sequences overlapping or adjacent to those targeted by the MLPA probes. Experiments were achieved in parallel from a pool of five genomic DNAs of control subjects and from DNA of a patient of interest. PCR products were then separated by high-performance liquid chromatography (HPLC), and quantitated

by fluorescent detection using a post-column intercalation dye. Navigator™ Software (Transgenomic, Omaha, NE, USA) was used for data analysis and the HMBS peak was used for normalization; relative peak intensity for each *SHOX* amplicon directly reflected genomic copy number.

Results and discussion

Partial heterozygous duplications within or in the vicinity of the *SHOX* gene were once described in association with the MRKH syndrome in four independent cases (3 isolated and 2 sisters with the same phenotype). These results were obtained through a genetic analysis by MLPA and were confirmed by haplotyping and pyrosequencing [30]. However, they were challenged by the study of a larger MRKH cohort where no relevant duplication of *SHOX* was found using the same commercial SALSA MLPA kit P018-E1 *SHOX* [31]. Since then, no further studies have been reported to try to resolve this controversy, except for the recent description of a new case of MRKH showing a heterozygous duplication of a distant regulatory element of the *SHOX* gene [38]. In the present

study, we analyzed a cohort of 60 MRKH patients and found various *SHOX* duplications in four independent patients (Figure 1). These duplications involved different exons of the *SHOX* gene for three of them and a region close to the gene for the fourth (Table 1). The duplications were also confirmed by another gene dosage technique, DP/LC (Figure 2). These results raise then several questions: 1) are duplications found in MRKH patients significant and thus, can they be associated with the syndrome? 2) If so, can this syndrome be attributable to defects in the *SHOX* gene? 3) If not, what is the genetic link between these duplications and MRKH syndrome?

Significance of SHOX duplications and association with MRKH syndrome.

First, it seems that the heterozygous *SHOX* duplications found by MLPA are not experimental artifacts as they were corroborated by another method, DP/LC, allowing to measure the copy number of genomic sequences overlapping or adjacent to those targeted by the MLPA probes. Moreover, the fact that duplications of contiguous regions were found

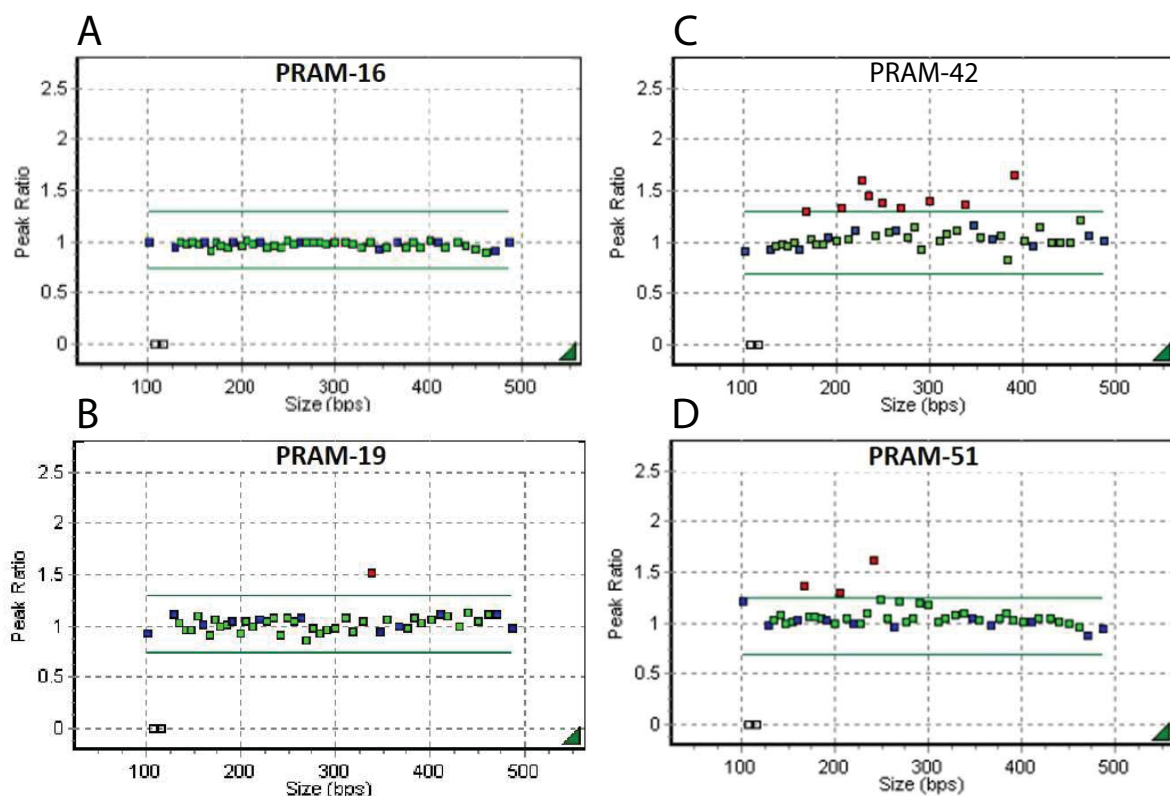


Figure 1: *SHOX* gene dosage in four independent MRKH patients (PRAM-16, -19, -42 and -51) assessed by the MLPA kit P018-F1 *SHOX*. In the diagrams, MLPA probes are represented along the x-axis (size of PCR products) and the fluorescent intensity ratio is represented on the y-axis. Each probe is represented by a square (green for *SHOX* gene and surrounding regions, blue for internal controls, grey for the Y chromosome). The correspondence between the probes size and their respective location is shown on **supplementary data S1**. The upper and lower arbitrary borders are shown respectively as a green upper and lower line. Probe ratios crossing the upper or the lower border are respectively indicative for a duplication or a deletion. Thus, a ratio of 1.5 (3:2) indicates the presence of an additional copy (heterozygous duplication) of a DNA stretch of a gene present in two copies in the genome, while a ratio of 0.5 (1:2) would indicate a heterozygous deletion. The detailed analysis of MLPA experiments is also included in **supplementary data S1**. (A) Results of patient PRAM-16 showing no copy number variations. (B) Results of patient PRAM-19 showing a heterozygous duplication of 1 probed region (*SHOX* exon 5). (C) Results of patient PRAM-42 showing the duplication of 9 contiguous probed regions (4.7 kb upstream of the *SHOX* gene up to end of intron 6, 1.4 kb upstream of exon 7) and (D) Results of patient PRAM-51 showing the duplication of 3 contiguous probed regions (*SHOX* exons 1, 2 and 3).

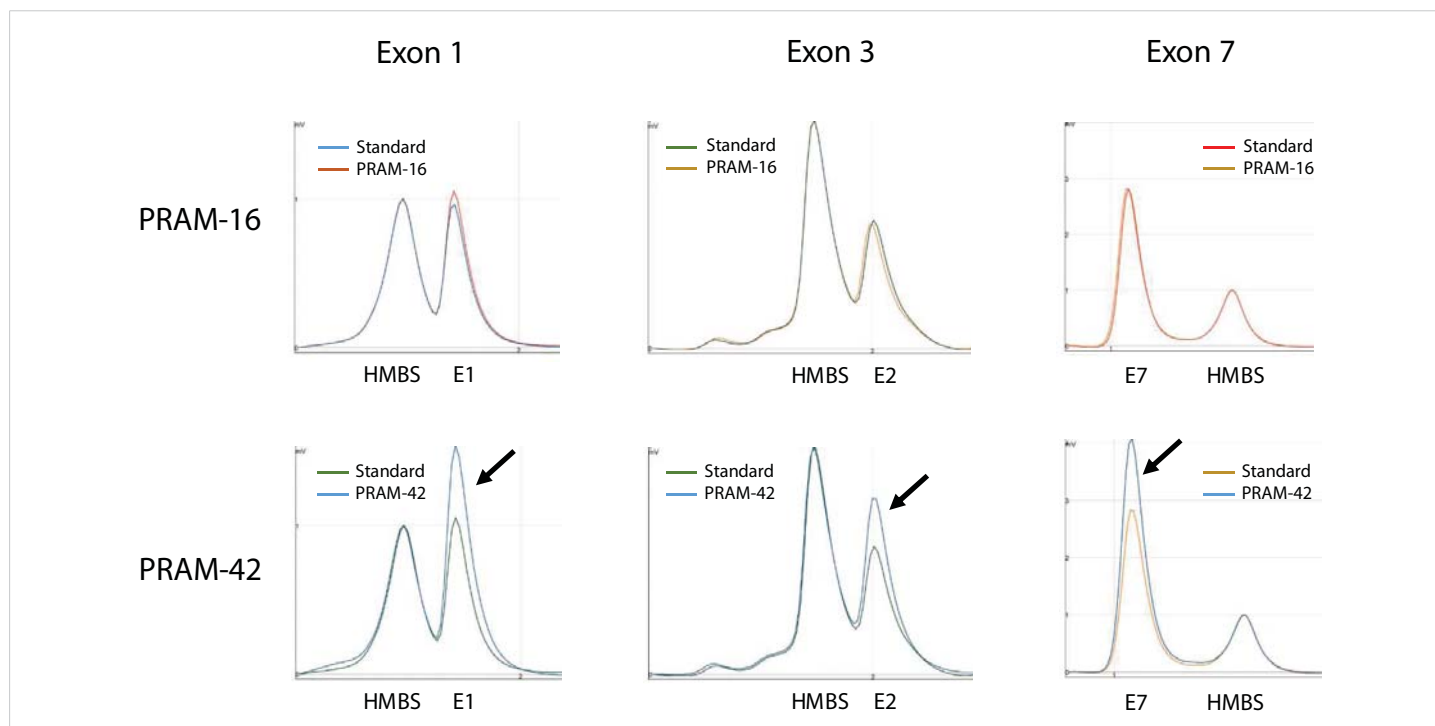


Figure 2: DP/LC chromatograms for three different duplexes used in *SHOX* analysis. x-axis: retention time in min; y-axis: fluorescence intensity. Example of results obtained for quantification of copy number of *SHOX* exons 1, 3 and 7 in patients PRAM-16 and PRAM-42. In these experiments, a pool of 5 independent genomic DNAs was used as standard and the hydroxymethylbilane synthase (HMBS) gene, located at 11q23.2-qter, was used as an internal control. Profiles are superimposed and then normalized using the control amplicon for HMBS. Black arrows show the triple dosage of *SHOX* exons 1, 3 and 7 in patient PRAM-42. Primers sequences and location are summarized in Table 2.

when, in the technique, there is no collinearity between the size of the corresponding PCR products and their position on the gene (typically the case for *SHOX* exon probes), attests to the absence of artifacts. Are these duplications then simple copy number variants (CNV) or are they associated with MRKH syndrome? In the work of Gervasini and colleagues [30], in ours as well as in other investigations using the same commercial MLPA kit [33,39,40], generally no CNV or at least no likely pathogenic variants were found in the control DNAs, whereas Sandbacka and colleagues [31] found as many in each patient and control group. In this latter study, the so-called genomic aberrations (duplications but also deletions) often concerned the same *SHOX* areas (downstream of the gene) in both groups, which is rather suggestive of a defective standardization. In addition, it appears that no alternative technique was used to verify the copy number of the regions found aberrant. Finally the use of genomic DNAs prepared with two very remote techniques (Puregene DNA Isolation kit and phenol-chloroform method) despite the instructions from the MPLA kit supplier, also may provoke artifacts. This seems all the more plausible since the variants described by Sandbacka and colleagues [31] are listed in the Database of Genomic Variants (DGV) while those described by Gervasini and colleagues [30] as well as those of our present study, are not and involve inner parts of the gene, reflecting probably an underlying relationship with MRKH syndrome.

Can SHOX duplications be responsible for MRKH syndrome?

The *SHOX* (short stature homeobox-containing gene on chromosome X) gene locates on the pseudoautosomal region (PAR1) on the short-arm tips of both X (Xp22.33) and Y (Yp11.3) chromosomes and escapes X-inactivation [41]. It encodes a transcription factor which plays a pivotal role in bone elongation and two copies of the gene are required for normal skeletal development [42]. It is the major gene involved in different skeletal dysplasia syndromes such as Léri-Weill dyschondrosteosis (LWD), Langer mesomelic dysplasia (LMD), idiopathic short stature (ISS), and it contributes to the phenotypic manifestations of Turner syndrome. Intragenic *SHOX* deletions and duplications of various size, mutations or deletions within the *SHOX* coding or regulatory sequences, or monosomy X0 account for the skeletal defects in these syndromes [32,34,43,44]. By contrast, *SHOX* overdosage either by experimental overexpression [45] or due to structural or numerical abnormalities of the sex chromosomes (mainly X trisomy), can lead to long limbs and tall stature [42]. When limited to the entire *SHOX* gene and neighboring (regulatory) sequences, the associated phenotype is also restricted to height, with normal or tall stature [46,47,48]. Surprisingly, about as many partial deletions as partial duplications have been reported in large-scale studies of subjects with skeletal dysplasia syndrome [49,44]. Furthermore, partial *SHOX* duplications, as well as those encompassing *SHOX* transcription enhancers, have appeared to be highly penetrant alleles in respect to LWD/ISS [40,49,50] and have more deleterious effects in regards to skeletal dysplasia and height gain, than



complete *SHOX* duplications. However, while partial *SHOX* deletions have always been associated with skeletal defects [33,40,49], partial *SHOX* duplications have been identified in patients with variable and non-overlapping phenotypes, as different as LWD and ISS, autism spectrum disorders and related neurodevelopmental conditions [51], or MRKH syndrome [30], as already pointed out [44]. The hypothesis that the variable clinical manifestations associated with partial or total duplications of *SHOX* are probably dependent on the physical location of the duplicated sequence [40,44], then seems the most plausible.

What could be the genetic/mechanistic link between partial SHOX duplications and MRKH syndrome?

This question is all the more relevant since there is occasional overlap between the numerous duplications associated with LWD/ISS and those identified in MRKH patients, even though these two types of syndromes do not have common phenotypic manifestations. It is in the answer to this question that the hypothesis stated above takes on its full meaning. Indeed, haploinsufficiency of the *SHOX* gene, when mutated, partially or totally deleted, or deprived of its regulatory sequences, is a major cause of skeletal dysplasia [34]. The numerous cases of LWD/ISS associated with partial heterozygous duplications of *SHOX* can then only be explained by the intragenic insertion of the duplicated sequences interrupting the reading frame of the gene or interfering with its transcriptional regulation. This pathogenic mechanism was already suggested through the cytogenetic localization of the duplicated *SHOX* sequences in patients with LWD or ISS [40,49] or by DNA sequencing at the fusion junction of the duplication showing integration of the duplicated sequence at a genomic region adjacent to the original position [49]. On the other hand, *SHOX* duplications, when associated with MRKHS, cannot have been inserted within the sequences of *SHOX* without manifesting as LWD or ISS. This proposal is furthermore supported by the absence of the phenotypic characteristics of LWD or ISS syndromes in the biggest cohorts of MRKH patients previously studied worldwide [3,9,10,23,52,53] and by the absence of uterine malformations in LWD or ISS patients [34,54]. This raises the question of whether similar duplications can be inserted either in the vicinity of their homologous sequences (intrachromosomal duplication) or within another chromosome (interchromosomal duplication). During meiosis and especially in males, recombination between highly similar duplicated sequences (non-allelic homologous recombination) can occur and generate deletions, duplications, inversions and translocations throughout the genome, and it is responsible for genetic diseases known as 'genomic disorders', most of which are caused by altered copy number of dosage-sensitive genes [55]. This is particularly the case for the obligatory exchanges that occur between Xp/Yp pseudoautosomal regions (PAR1). Crossovers in this 2.6 Mb chromosomal segment, create a male-specific recombination 'hot domain' with a recombination rate that is about 20 times

higher than the genome average [56]. More specifically, the 231 kb interval encompassing the *SHOX* gene, shows a 27-fold increase recombination relative to the genome average rate [57]. This characteristic may be due to the presence of numerous repeated sequences, Alu-like elements or rich A-T regions identified at certain breakpoints, such as in intron 3 [44] or downstream of the *SHOX* gene [33]. These various sequences are then likely to promote non-allelic homologous recombination by unequal intra- or inter-chromosomal crossing-over, thus generating deletions and duplications (nonrecurrent CNVs) [58] underlying LWD/ISS and MRKHS. The relative small size of microdeletions (~3 to 374 kb) and microduplications (~3 to 571 kb) identified in both LWD/ISS and MRKHS in previous studies [30,40,44] and in the present one (Table 1), seems to reflect the high density of recombinogenic sequences found in the PAR1 region and even more in the interval comprising the *SHOX* gene. Moreover, it appears that multiple combinations are possible to generate deletions or duplications from these active sequences, some being used predominantly [33,39,44,49,59]. Various genetic outcomes for duplication CNVs have been demonstrated, including the pathogenicity of some intergenic duplications [60]. The insertion of duplicated *SHOX* sequences into another locus, resulting in the disruption of the reading frame and the loss of function of a host gene, is therefore the sole explanation for associating MRKH syndrome with these duplications. Thus the inactivation of a dosage-sensitive host gene could take place while keeping intact the original template *SHOX* gene at its locus. The identification of such host gene by next-generation approaches [60], would certainly lead to the characterization of new genetic cause(s) for MRKHS.

To conclude, the absence of phenotypic overlap between LWD/ISS and MRKHS suggests, in the case of *SHOX* duplications, different mechanisms of generation and insertion of these duplications, the study of which could be beneficial to medical genetics. The data obtained in this study tend to demonstrate that the *SHOX* gene is not involved in MRKH syndrome and encourages future investigations to identify the gene(s) that the insertion of *SHOX* duplications involve in this syndrome.

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