Gene Expression Profile of Patients with Mayer-Rokitansky-Küster-Hauser Syndrome: New Insights into the Potential Role of Developmental Pathways

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Abstract

Mayer-Rokitansky-Küster-Hauser syndrome (MRKHS) is a rare disease characterized by congenital aplasia of uterus and vagina. Although many studies have investigated several candidate genes, up to now none of them seem to be responsible for the aetiology of the syndrome. In our study, we identified differences in gene expression profile of in vitro cultured vaginal tissue of MRKHS patients using whole-genome microarray analysis. A group of eight out of sixteen MRKHS patients that underwent reconstruction of neovagina with an autologous in vitro cultured vaginal tissue were subjected to microarray analysis and compared with five healthy controls. Results obtained by array were confirmed by qRT-PCR and further extended to other eight MRKHS patients. Gene profiling of MRKHS patients delineated 275 differentially expressed genes, of which 133 downregulated and 142 upregulated. We selected six deregulated genes (MUC1, HOXC8, HOXB2, HOXB5, JAG1 and DLL1) on the basis of their fold change, their differential expression in most patients and their relevant role in embryological development. All patients showed upregulation of MUC1, while HOXB2 and HOXB5 were downregulated, as well as Notch ligands JAG1 and DLL1 in the majority of them. Interestingly, HOXC8 was significantly upregulated in 47% of patients, with a differential expression only in MRKHS type I patients. Taken together, our results highlighted the dysregulation of developmental genes, thus suggesting a potential alteration of networks involved in the formation of the female reproductive tract and providing a useful clue for understanding the pathophysiology of MRKHS.

Introduction

Mayer-Rokitansky-Küster-Hauser syndrome (MRKHS) is a rare disease characterized by congenital aplasia of the uterus and the upper two-thirds of the vagina, occurring in 1 out of 4500 female births. Normal secondary sexual characteristics and karyotype (46, XX) have been shown in MRKHS women. This syndrome may occur as isolated (type I), or associated with other malformations, mainly renal, skeletal and hearing defects, and, to a lesser extent, cardiac or digital defects (type II), indicated as malformations, mainly renal, skeletal and hearing defects, and, to a lesser extent, cardiac or digital defects (type II), indicated as MURCS association (Mullerian duct aplasia, Renal dysplasia and Cervical Somite anomalies) [1,2]. At the present, the aetiology and pathogenesis of this syndrome remain to be clarified.

The development of the female reproductive tract consists in a sequence of events governed by various transcription factors and signalling molecules. While the absence of androgens induces regression of the mesonephric duct (Wolffian duct) in females, in males the Mullerian duct degenerates through an active process mediated by the Anti-Mullerian Hormone (AMH) and its type II receptor (AMHR2) [3]. It has been proposed that inappropriate production of AMH in female embryos might induce a partial regression of the Mullerian duct, thus causing vaginal agenesis [4]. To date no mutations of AMH gene have been detected in MRKHS patients [5]. However, Rall et al. [6] hypothesized that an embryonic exposure to AMH signalling, caused by high levels of oestrogens or by deregulated expression of related genes, might occur in these patients.

MRKHS, initially considered to be sporadic, is now defined as a syndrome with autosomal-dominant inheritance with reduced penetrance and variable manifestation, consistent with a polygenic or multifactorial origin [7,8]. However, mutations of WNT genes detected in MRKHS patients were indicated not responsible for the arising of the syndrome [9,10]. Among them, heterozygous mutations of WNT4 detected in patients with absence of uterus and vagina classify this subgroup into the so-called WNT defects for the presence of hyperandrogenism [9].

In contrast, the analysis of HOX1 genes and hormones regulating HOX expression has yielded no mutations [13], although it has been described an hypomethylation and overex-
pression of HOXA5 and HOXA9 [6] and, in some MRKHS patients, a duplication of SHOX, member of the paired-related HOX family [14].

Interestingly, some of the malformations observed in type II MRKHS are also found in the clinical spectrum of the DiGeorge syndrome (DGS), and in DGS-like phenotypes. Consistent with these observations, whole genome analysis in MRKHS patients revealed critical chromosomal deletions in known DGS or DGS-like loci [15–17].

Although the genetic basis of MRKHS has been extensively investigated using different approaches, we are still far from reach a consensus on pathogenetic basis of the MRKH syndrome. The results obtained from previous studies showed a wide genetic heterogeneity among MRKHS patients. The reported discordance in monozygotic twins suggested that this syndrome might result from the combinations of transcriptional factors and epigenetic regulation other than genetic predisposition [18].

With this background, we decided to use a whole genome approach in order to identify a potentially altered gene profile in MRKHS patients. Among 2006 and 2011, we collected autologous in vitro cultured vaginal tissue from a series of 16 women with MRKHS that underwent reconstruction of neovagina and from 5 healthy controls, in order to analyze differential gene expression. Moreover, we investigated the potential relationship between gene expression profile and patients phenotype. We believe that the identification of relevant differentially expressed genes in cultured vaginal tissue may be used to better understand the molecular basis of vaginal agenesis.

Methods

Ethics Statements

The use of clinical samples (vaginal biopsies) for gene profiling conformed to the guidelines of the 1975 Declaration of Helsinki, as revised in 2008, and was approved by the Institutional Review Board of the Department of Gynecologic-Obstetrical and Urologic Sciences of Sapienza University of Rome. Written consent was obtained from all subjects prior to inclusion in the study. On behalf of the minors enrolled in the study, written informed consent was obtained from parents.

Patients

We analysed sixteen women aged 16–48 years who presented utero-vaginal aplasia diagnosed by clinical examination, trans abdominal and pelvic ultrasonography, nuclear magnetic resonance (NMR) and/or vaginoscopy. All patients had a normal 46, XX karyotype. Patients underwent a check-up to search for associated malformations, including renal ultrasonography, spine radiography and echocardiography. Ten of them presented isolated utero-vaginal aplasia (MRKHS type I). The other six women displayed also other malformations, such as kidney defects (unilateral kidney agenesis, ectopic kidney) or skeletal malformations (Table 1).

Patients were analysed for follicle-stimulating hormone (FSH), luteinizing hormone (LH), 17 beta-estradiol (E2), dehydroepiandrosterone sulphate (DHEAS), progesterone and testosterone. All hormonal indicators were consistent with physiological levels with respect to age of the patients.

Samples obtained from vaginal tissue biopsy of eight out of sixteen patients (mean age 24.0 years; range 16–48 years) were subjected to microarray analysis, whereas samples from all patients (mean age 23.7 years; range 16–48 years) were analysed through quantitative Real Time PCR (qRT-PCR). Specimens from five healthy women (mean age 31.8 years; range 27–40 years) were used as control.

Cell Cultures

Primary cultures of human vaginal mucosa cells (HVMs) were established from 1 cm² full-thickness mucosal biopsy of the vaginal vestibule of MRKHS patients and vagina of healthy controls. Following enzymatic dissociation, cells were seeded onto collagen IV [10 mg/ml]-coated culture plates and maintained in chemical defined Keratinocyte Growth Medium (KGM; Lonza Milano S.r.l., Milan, Italy), with medium change twice a week.

Primary culture of buccal mucosa cells from one MRKHS patient enrolled in this study was also obtained with the same procedure.

Cell cultures were examined by immunofluorescence and western blot analysis as described in Data S1 and their morphology was evaluated with a phase contrast microscopy. All cell cultures consisted of epithelial cells as they exhibited the cobblestone-like appearance. Moreover the expression of specific epithelial markers (K14 and K19) and lack of vimentin confirmed their epithelial origin (Fig. S1).

RNA Preparation

Total RNA from cultures derived from vaginal and buccal mucosa was extracted using TRIzol reagent (Invitrogen, Milan, Italy) following the manufacturer’s instructions. RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and evaluated for degradation using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The samples were selected for gene expression array analysis on the basis of compliance to the following criteria: RIN value >7, absorption ratio 260:280 ≥1.9, and ratio of the ribosomal bands 28S:18S >1.5.

Gene Expression Assay

Expression profiling was accomplished using the HumanHT-12 v3 whole-genome gene expression direct hybridization assay (Illumina, Inc., San Diego, CA, USA), with six arrays on each single BeadChip. Each array is comprised of >48,000 probes derived from human genes in the NCBI RefSeq and UniGene databases. A total of thirteen separate samples (eight patients and five healthy controls) were interrogated on the BeadChip arrays, with no pooling of samples within groups, in order to evaluate sample-to-sample variability in gene expression both within and across each group.

Initially, 100 ng of total RNA was converted to cDNA, followed by an in vitro transcription step to generate labelled cRNA using the Ambion Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. The labelled probes were then mixed with hybridization reagents and hybridized overnight to the HumanHT-12 v3.0 BeadChips. Following washing and staining, the BeadChips were imaged using the Illumina BeadArray Reader to measure fluorescence intensity at each probe. The intensity of the signal corresponded to the amount of the respective mRNA in the original sample.

Microarray Expression Analysis

BeadChip data files were analysed with Illumina’s GenomeStudio gene expression module (and Bioconductor package) to determine gene expression signal levels. Briefly, the raw intensity of Illumina Human HT-12 v3.0 gene expression array was scanned and extracted using BeadScan, with the data corrected by background subtraction in GenomeStudio module.
Table 1. Summary of MRKHS patients.

| Patient | Phenotype | Analysis | Genital system                                      | Renal system                      | Skeleton | Heart                        | Hearing                  | Other                                      |
|---------|-----------|----------|-----------------------------------------------------|-----------------------------------|----------|------------------------------|--------------------------|--------------------------------------------|
| 1       | Type I    | Array    | Vaginal agenesis, uterus hypoplasia                 | Normal                            | Normal   | Diastolic Dysfunction grade I| Normal                   | None                                       |
| 2       | Type I    | Array    | Vaginal agenesis, uterus aplasia                    | Normal                            | Normal   | Normal                       | Normal                   | Inguinal and umbilical hernia              |
| 3       | Type II   | Array    | Vaginal agenesis, uterus aplasia                    | Unilateral kidney agenesis, renal ectopia | Occipital meningocele, Spina Bifida, Arnold-Chiari type I, cleft palate, C2-C3 fusion | Normal | Normal                       | Inguinal hernia                          |
| 4       | Type II   | Array    | Rudimentary uterus, vaginal agenesis                | Unilateral kidney agenesis        | Normal   | Normal                       | Normal                   | None                                       |
| 5       | Type I    | Array    | Vaginal agenesis, rudimentary uterus                | Normal                            | Normal   | Normal                       | Normal                   | None                                       |
| 6       | Type I    | Array    | Unilateral salpingectomy                             | Normal                            | Normal   | Normal                       | Not determined            | None                                       |
| 7       | Type I    | Array    | Vaginal agenesis, uterus aplasia                    | Normal                            | Normal   | Normal                       | Normal                   | None                                       |
| 8       | Type II   | Array    | Vaginal agenesis                                    | Normal                            | Normal   | Normal                       | Normal                   | None                                       |
| 9       | Type I    | qRT-PCR  | Vaginal agenesis                                    | Normal                            | Normal   | Normal                       | Normal                   | None                                       |
| 10      | Type I    | qRT-PCR  | Vaginal agenesis, uterus aplasia                    | Normal                            | Normal   | Normal                       | Inguinal hernia           |
| 11      | Type I    | qRT-PCR  | Rudimentary uterus, vaginal agenesis                | Normal                            | Normal   | Normal                       | Normal                   | Notta’s syndrome                          |
| 12      | Type I    | qRT-PCR  | Vaginal agenesis                                    | Normal                            | Normal   | Normal                       | Normal                   | None                                       |
| 13      | Type I    | qRT-PCR  | Vaginal agenesis                                    | Normal                            | Normal   | Normal                       | None                     |
| 14      | Type II   | qRT-PCR  | Vaginal agenesis, uterus aplasia                    | Ectopic kidney                    | Normal   | Dextrocardia                 | Unilateral lung agenesis, cleft lip and palate |
| 15      | Type I    | qRT-PCR  | Vaginal agenesis                                    | Normal                            | Normal   | Not determined               | None                     |
| 16      | Type II   | qRT-PCR  | Half-uterus, unilateral salpingectomy                | Unilateral kidney agenesis        | Normal   | Interventricular septal defect | Not determined            | Oesophageal atresia                        |

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Figure 1. Gene expression profile of MRKHS patients. A) Clustering analysis of gene expression profiles of MRKHS patients and healthy controls. The distance on the X-axis represents the similarity relationships among samples. The label CTR (1–5) represents for healthy controls, the label P (1–8) represents for MRKHS patients. B) Assignment of differentially expressed genes to biological process categories. The y-axis indicates the number of differentially expressed genes within each biological process category. C) Venn diagram of similarities and differences in type I and type II MRKHS profiles. Each of the circles depicts the number of different transcripts identified as statistically significant in the comparison of the two sample groups (type I, red; type II, blue) with healthy control (CTR). Overlapping differences shared among the two sample groups are represented in the area of intersection between the two circles (violet). D) Heatmap of the expression profile of each MRKHS patient. The cut-off for inclusion in the heatmap was the alteration of gene expression in more than half of patients.
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The data obtained from the two populations were analysed for differences in expression. Up or down regulated transcript lists were obtained using average normalization, error model Illumina Custom, selected by p<0.00001, Diff Score>20 and fold change >2.

Heatmap was generated through the web interface Matrix2png, version 1.2.2 (http://www.chbli.ubc.ca/matrix2png/) [19].

Area-proportional Venn diagram for the comparison and visualization of biological lists of patients affected by type I or type II MRKHS was generated through the web application BioVenn (http://www.cmibi.rni.nl/cdd/biovenn/) [20].

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE52609 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52609).

Pathway Analysis
Differentially expressed genes were subjected to systematic network analysis to determine the biological processes and pathways associated with each gene using WebGestalt (WEB-based GEnec SCAnalysis Toolkit) (http://bioinfo.vanderbilt.edu/webgestalt/, Nashville, TN). Only genes that were significantly increased or decreased were included in the pathway analysis. The assignment of all differentially expressed genes to functional groups was performed through the Gene Ontology Slim Biological Process classification and reported as a graph.

Quantitative Real Time PCR (qRT-PCR)
1μg of total RNA was reverse transcribed using High Capacity RNA to cDNA Kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. cDNA was diluted 1:5 and then used for amplification of MUC1, HOXC8, HOXB2, HOXB5, JAG1 and DLL1 using the appropriate TaqMan gene expression assay kits (Applied Biosystems). A total of 2 μl/well of template was added to the sample wells along with Taqman Universal PCR master mix at a concentration of 1x and water to a volume of 25 μl/well. Assays were conducted in triplicate on an ABI 7500 Real Time instrument (Applied Biosystems) using the following conditions: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min, repeated 40 times. Relative quantification was performed using GAPDH mRNA as an endogenous control.

Statistical Methods
Statistical analysis for significance was performed using Student t-test. P values less than 0.05 were considered statistically significant.

Results
Cluster Analysis and Identification of Differentially Expressed Genes
Total RNA obtained by cell cultures derived from vaginal mucosa of eight MRKHS patients, five with type I MRKHS and three with type II MRKHS (Table 1, patients numbered from 1 to 8), and from vaginal mucosa of five healthy controls were analysed by microarray. The hierarchical clustering analysis (Fig. 1A) revealed that only two out of five control RNAs (CTR1–5) formed an independent cluster away from the patients groups, while the other controls were distributed within the three sub-clusters formed by most of patients’ samples. Two of the eight patients (P2 and P5) formed an independent cluster away from the others, which formed three sub-clusters (P1 and P6; P4, P7 and P8; P3) related each other with subsequently decreased similarity. This pattern indicated that patients were heterogeneous and therefore this might be potentially due to the multifactorial aetiology of the syndrome.

To identify differentially expressed genes in a statistically significant manner, we performed fold-change data analysis. The gene list obtained from microarray was filtered to identify candidate genes for which the expression levels differed by at least 2 fold between the two groups. After statistical testing procedures, removal of the transcripts with no Entrez gene ID and fold change cut-off (Fold≥2), we identified a total of 277 differentially expressed genes of which 133 downregulated and 142 upregulated.

The differentially expressed genes in MRKHS patients were then categorized by biological process. A large proportion of genes were mainly involved in functions relevant to tissue patterning and organogenesis, cell proliferation/differentiation, cell communication, cellular motility, endocrine and reproductive disorders, and others (Fig. 1B).

Pairwise comparisons between type I and type II groups were carried out, differentially expressed genes were identified for each comparison and are illustrated in a Venn diagram (Fig. 1C). The higher number of differentially expressed genes, detected in the comparison of the type II group versus CTR with respect to the type I group versus CTR (172 and 88 genes, respectively), was consistent with the severity of type II MRKHS, which clinical features imply the involvement of a higher number of organs and systems.

The overlapping region (violet) contained 46 transcripts that were significantly different in both the comparisons, thus indicating their potential role in MRKHS aetiology. These genes are listed in Table 2.

Heat map was provided to visualize the differentially expressed genes in each patient (Fig. 1D). We considered only genes deregulated in more than half of patients (>4).

Among the deregulated genes, we analysed HOX family in detail. MRKHS patients showed downregulation of HOXA13 (−2.0 fold), HOXB2 (−3.4 fold), HOXB4 (−4.2 fold), HOXB5 (−4.1 fold) and HOXB7 (−4.2 fold), and upregulation of HOXC6 (6.8 fold) and HOXC8 (38.2 fold). In particular we focused on HOXB2 and HOXB5, since they were included in the list of type I and type II overlapping genes (Table 2) and they were highly decreased in seven out of eight patients (Fig. 1D). HOXC3 also came to our attention despite the fact that it was altered in just three out of eight patients (Fig. 1D), since its overexpression was particularly high (38.2 fold increase). Characteristically, its deregulation was observed only in type I MRKHS patients.

We found also interesting the downregulation of JAG1 and DLL1 genes, encoding for Notch ligands. They were significantly decreased (−3.4 fold and −2.2 fold, respectively) in six and five out of eight patients, respectively, and in both type I and type II MRKHS patients; based upon these findings and their role in development we decided to further investigate them.

Among overexpressed genes, MUC1 and MSLV, coding for glycoproteins, were found to be significantly upregulated in all patients analysed by microarray (6.7 and 4.2 fold, respectively) (Fig. 1D). We focused in particular on MUC1, which is known to have a key role in the stabilization of β-catenin, an essential mediator of canonical Wnt signalling involved in female tract development [21,22].

Validation of Array-based Gene Expression Profiles by Quantitative Real Time PCR (qRT-PCR)
qRT-PCR validation of differentially expressed mRNA was performed on all MRKHS patients tested for microarray analysis.
| Probe set ID | Gene title | Gene symbol |
|--------------|------------|-------------|
| ILMN_1780806 | Homo sapiens ankyrin repeat domain 36B (ANKRD36B), mRNA. | ANKRD36B |
| ILMN_1767113 | Homo sapiens aldehyde oxidase 1 (AOX1), mRNA. | AOX1 |
| ILMN_1656248 | Homo sapiens adipocyte-specific adhesion molecule (ASAM), mRNA. | ASAM |
| ILMN_2206746 | Homo sapiens biglycan (BN), mRNA. | BGN |
| ILMN_2341611 | Homo sapiens CUB domain containing protein 1 (CDCP1), transcript variant 2, mRNA. | CDCP1 |
| ILMN_2146418 | Homo sapiens cysteine rich transmembrane BMP regulator 1 (chordin-like) (CRIM1), mRNA. | CRIM1 |
| ILMN_1689200 | Homo sapiens dihydriodil dehydrogenase (dimeric) (DHDH), mRNA. | DHDH |
| ILMN_1743373 | Homo sapiens delta-like 1 (Drosophila) (DLL1), mRNA. | DLL1 |
| ILMN_1812666 | Homo sapiens DnaJ (Hsp40) homolog, subfamily C, member 15 (DNAJC15), mRNA. | DNAJC15 |
| ILMN_1677466 | Homo sapiens dual specificity phosphatase 6 (DUSP6), transcript variant 1, mRNA. | DUSP6 |
| ILMN_1743145 | Homo sapiens endoplasmic reticulum aminopeptidase 2 (ERAP2), mRNA. | ERAP2 |
| ILMN_1774602 | Homo sapiens fasciculation and elongation protein zeta 1 (zygin l) (FEZ1), transcript variant 2, mRNA. | FEZ1 |
| ILMN_1768260 | Homo sapiens fibulin 2 (FBLN2), transcript variant 2, mRNA. | FBLN2 |
| ILMN_1746085 | Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2, mRNA. | IGFBP3 |
| ILMN_1747408 | Homo sapiens insulin-like growth factor binding protein 7 (IGFBP7), mRNA. | IGFBP7 |
| ILMN_1774874 | Homo sapiens interleukin 1 receptor antagonist (IL1RN), transcript variant 4, mRNA. | IL1RN |
| ILMN_1691376 | Homo sapiens jagged 1 (Alagille syndrome) (JAG1), mRNA. | JAG1 |
| ILMN_1705814 | Homo sapiens keratin 80 (KRT80), transcript variant 1, mRNA. | KRT80 |
| ILMN_3236021 | PREDICTED: Homo sapiens hypothetical protein LOC100133923 (LOC100133923), mRNA. | LOC100133923 |
| ILMN_1725750 | PREDICTED: Homo sapiens hypothetical LOC644695 (LOC644695), mRNA. | LOC644695 |
| ILMN_3200140 | PREDICTED: Homo sapiens misc_RNA (LOC645638), miscRNA. | LOC645638 |
| ILMN_1694778 | PREDICTED: Homo sapiens similar to Keratin, type I cytoskeletal 18 (Cytokeratin-18) (Keratin-18) (K18) (LOC646723), mRNA. | LOC646723 |
| ILMN_1697377 | PREDICTED: Homo sapiens similar to protein immuno-reactive with anti-PTH polyclonal antibodies (LOC649841), mRNA. | LOC649841 |
| ILMN_1667932 | PREDICTED: Homo sapiens similar to ankyrin repeat domain 36 (LOC652726), mRNA. | LOC652726 |
| ILMN_1663575 | PREDICTED: Homo sapiens similar to Six transmembrane epithelial antigen of prostate (MGC87042), mRNA. | MGC87042 |
| ILMN_1723516 | Homo sapiens mesothelin (MSLN), transcript variant 2, mRNA. | MSLN |
| ILMN_1677314 | Homo sapiens mucin 1, cell surface associated (MUC1), transcript variant 1, mRNA. | MUC1 |
| NM_1526731 | Homo sapiens mucin 20, cell surface associated (MUC20), transcript variant 5, mRNA. | MUC20 |
| ILMN_1658356 | Homo sapiens peptidase domain containing associated with muscle regeneration 1 (PAMR1), transcript variant 1, mRNA. | PAMR1 |
| NM_0806723 | Homo sapiens phosphatase and actin regulator 3 (PHACTR3), transcript variant 3, mRNA. | PHACTR3 |
| ILMN_2384745 | Homo sapiens pregnancy specific beta-1-glycoprotein 4 (PSG4), transcript variant 1, mRNA. | PSG4 |
| ILMN_1785699 | Homo sapiens parathyroid hormone-like hormone (PTHLH), transcript variant 3, mRNA. | PTHLH |
| ILMN_1744210 | Homo sapiens succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA), nuclear gene encoding mitochondrial protein, mRNA. | SDHA |
| ILMN_1651429 | Homo sapiens selenoprotein M (SELM), mRNA. | SELM |
| ILMN_1732410 | Homo sapiens solute carrier family 16, member 9 (monocarboxylic acid transporter 9) (SLC16A9), mRNA. | SLC16A9 |
| ILMN_2060115 | Homo sapiens sortilin-related receptor, LIDCL class A) A repeats-containing (SORL1), mRNA. | SORL1 |
| ILMN_1713496 | Homo sapiens ST3 beta-galactoside alpha-2,3-sialyltransferase 5 (ST3GAL5), transcript variant 2, mRNA. | ST3GAL5 |
| NM_004605.2 | Homo sapiens sulfotransferase family, cytosolic, 2B, member 1 (SULT2B1), transcript variant 1, mRNA. | SULT2B1 |
| NM_003359.1 | Homo sapiens transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase) (TGM1), mRNA. | TGM1 |
| ILMN_1759787 | Homo sapiens thrombomodulin (THBD), mRNA. | THBD |
| ILMN_1799028 | Homo sapiens tetraspanin 5 (TSPAN5), mRNA. | TSPAN5 |
| NM_145652.2 | Homo sapiens WAP four-disulfide core domain 5 (WFDC5), mRNA. | WFDC5 |

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Six genes (\textit{MUC1}, \textit{HOXC8}, \textit{HOXB2}, \textit{HOXB5}, \textit{JAG1}, \textit{DLL1}) were selected on the basis of their fold change, their differential expression in the majority of patients and/or their embryologically relevant role in the development of the female genital tract (Table 3).

All selected genes were confirmed to have significant alterations in their expression in MRKHS patients; \textit{MUC1} and \textit{HOXC8} were strongly upregulated (11.4 and 6.4 fold, respectively, \textit{P}<0.01), while \textit{HOXB2}, \textit{HOXB5}, \textit{JAG1} and \textit{DLL1} were downregulated (0.26, 0.05, 0.32 and 0.30 fold, respectively, \textit{P}<0.01) (Fig. 2A). Moreover, qRT-PCR results (Fig. 2B, grey columns) showed 100% validation efficiency in comparison to the expression data of microarray experiments (Fig. 2B, white columns).

Therefore, qRT-PCR results validated the microarray findings, thus confirming the modulation of all selected genes in the eight MRKHS patients.

### qRT-PCR in an Extended Cohort of MRKHS Patients

Expression analysis of the six selected genes was then conducted in an extended cohort of patients, comprising other eight MRKHS patients, five with type I MRKHS and three with type II MRKHS (Table 1, patients numbered from 9 to 17). We performed qRT-PCR analysis of sixteen patients in order to verify the trend of selected genes (Fig. 2C).

In the total cohort of patients, \textit{MUC1} and \textit{HOXC8} mRNA levels resulted to be significantly overexpressed (11.7 and 13.7 fold, respectively, \textit{P}<0.01). The hypothesis of an involvement of \textit{MUC1} in the pathogenesis of MRKHS is sustained by the observation that all patients (100%) showed upregulation of this mucin through microarray analysis, and fifteen out of sixteen MRKHS patients (93.8%) showed a significant overexpression of this gene also by qRT-PCR. In the case of \textit{HOXB2} and \textit{HOXB5}, we confirmed the decreasing trend with respect to healthy controls (0.42 and 0.49 fold, respectively), despite their very low basal expression levels.

As concerning Notch ligands, both \textit{JAG1} and \textit{DLL1} were confirmed to be significantly downregulated (0.43 and 0.44 fold, \textit{P}<0.01) also extending the number of patients.

Highly significant downregulation (\textit{P}<0.05) of \textit{JAG1} was observed in a total of ten MRKHS patients (62.5%), while \textit{DLL1} expression was significantly deregulated in all patients (100%). These data pointed out a strong role of Notch signalling in MRKHS pathogenesis.

\textit{HOXC8} was significantly upregulated (\textit{P}<0.05) in seven out of sixteen MRKHS patients (47%) (Fig. 2C). These patients showed very high levels of \textit{HOXC8} mRNA, ranged from 3.8 to 47.1 fold.

Data obtained from qRT-PCR from buccal mucosa of one MRKHS patient confirmed this trend indicating that alterations in the gene expression are not only confined to cells derived from vaginal mucosa but are also extended to other tissues although with a lower fold increase and limited sample number (Fig. S2).

Finally, we compared the transcript levels of the analysed genes between patients with type I and type II MRKHS. Microarray analysis pointed out the existence of a series of 46 genes differentially expressed in both type I and type II MRKHS patients, as reported in Fig. 1C and Table 2. Five out of the six selected genes were included in this category, and their fold change with respect to healthy controls did not significantly differ in the two groups (Fig. 3A). As concerning \textit{HOXC8}, however, microarray data indicated a differential expression of this gene only in type I MRKHS patients (Fig. 3A). Data obtained from qRT-PCR on all patients (ten type I and six type II MRKHS) confirmed this trend, with significantly different expression levels between the two groups (Fig. 3B).

### Discussion

During embryogenesis, specific signals are required to regulate proper female tract development different genes involved in such signals have been proposed as candidates responsible for the arising of MRKHS. At the present, however, the aetiology of this syndrome is still poorly understood and MRKHS is considered a multifactorial disorder.

Several studies have investigated mutations in developmental genes, without being able to prove a direct link with the onset of MRKHS [3,9–12]. Recent findings based upon discordance between monozygotic twins suggested the involvement of epigenetic factors [18]. In this context, the analysis of gene expression became a feasible approach, even if it could be performed only on adult tissues.

A recent study analysed gene expression profile of uterine tissue of MRKHS patients [6]. Combining whole-genome and methyl-ation arrays, the authors detected a series of hypermethylated and downmodulated or hypomethylated and overexpressed genes.

In the present study, we considered an alternative approach, carrying out for the first time gene expression profiling on cultured cells harvested from vaginal vestibulum biopsy avoiding the use of uterine or other derivation tissues in order to place all samples in the same conditions.

We identified 275 differentially expressed genes, 142 downregulated and 133 upregulated. Among them, we focused our attention on six genes (\textit{MUC1}, \textit{HOXC8}, \textit{HOXB2}, \textit{HOXB5}, \textit{JAG1}, \textit{DLL1}).

\textit{MUC1}, a heavily glycosylated transmembrane protein expressed in most of secretory epithelia, resulted overexpressed in all
Figure 2. Validation of differential gene expression by Quantitative Real Time PCR. 

A) qRT-PCR analysis of mRNA expression levels of the six selected genes in the eight patients subjected to microarray analysis. For each gene, relative mRNA levels of patients are shown as fold value of the levels of five healthy subjects (controls). Each experiment was performed in triplicate, and mRNA levels were normalized to GAPDH mRNA expression. Error bars represent standard deviations (*P<0.01).

B) Expression of selected genes in MRKHS patients compared to controls, tested by microarray analysis (left, white columns) and qRT-PCR (right, grey columns). Array data are shown as log fold change of patients divided by controls, qRT-PCR data are shown as log expression ratio of patients divided by controls. Bars indicate the measurement error (*P<0.01).

C) qRT-PCR analysis of mRNA expression levels of the genes of interest in sixteen MRKHS patients. For each gene, relative mRNA levels of patients are shown as fold value of the levels of five healthy subjects (controls). Each experiment was performed in triplicate, and mRNA levels were normalized to GAPDH mRNA expression. Error bars represent standard deviations (*P<0.01).

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Figure 3. Expression levels of the genes of interest in type I and type II MRKHS patients. 

A) Expression of selected genes in type I (white columns) and type II (grey columns) MRKHS patients tested by microarray analysis. Array data are shown as log fold change of patients divided by controls. Bars indicate the measurement error (*P<0.01).

B) qRT-PCR analysis of mRNA expression levels of the selected genes in type I (white columns) and type II (grey columns) MRKHS patients. For each gene, relative mRNA levels of patients are shown as fold value of the levels of five healthy subjects (controls). Each experiment was performed in triplicate, and mRNA levels were normalized to GAPDH.

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MRKHS patients analysed by microarray. Moreover, MUC1 mRNA has been found significantly deregulated in 93.4% of MRKHS patients assessed by qRT-PCR analysis. These results suggested for the first time the potential involvement of a mucin in the pathogenesis of MRKHS.

Several studies showed that the cytoplasmic domain of MUC1 specifically interacts with β-catenin [21,22], an essential mediator of canonical Wnt signalling that plays a critical role in female tract development through its direct and indirect action on AMH and its receptor [23,24]. Since MUC1 overexpression stabilizes β-catenin and enhances its nuclear translocation [21], we hypothesized that its overexpression during foetal development might induce improper AMH-AMHR2 activation, thus causing a partial Mullerian duct regression.

Our results pointed out also a potential alteration of Notch signalling pathway through the downregulation of its specific ligands JAG1 and DLL1. In particular, we found deregulated JAG1 in a high percentage of MRKHS patients (62.5%), and DLL1 even in all patients (100%). It is known that Notch signalling influences differentiation of many tissues, such as heart and kidney [25], but its potential role in the development of the reproductive tract has never been fully investigated. Turner et al. [26] proposed a role for Notch in normal testosterone-mediated development of the Wolffian duct. In humans, mutations in the JAG1 gene cause the Alagille syndrome, characterized by severe cardiac malformation and developmental anomalies in several organs, including kidney [27,28]. Perturbations in Notch signalling also contribute to the aetiopathology of Klippel-Feil syndrome [29] and spondylocostal dysostosis [30], skeletal disorders often observed in association with vaginal agenesis [31,32]. Such data led us to hypothesize a role for this gene family in the aetiopathogenesis of type II MRKHS. Nevertheless, we did not observe any statistically significant difference in JAG1 or DLL1 expression among type I and type II MRKHS patients. Notch ligands deregulation presumably acts in concert with genetic background, other specific gene mutation-dysregulation and/or developmental insults in the pathogenesis of MRKHS. The crosstalk between Notch and Wnt pathways has been described in different types of tumour cells [33] and in intestinal epithelial cell fate decision [34]. Similar interactions might be also required in the development of female reproductive organs. Although no mutations were found in WNT gene family in previous studies, it is conceivable that alteration in Notch pathways might also affect Wnt pathway. In this regard, it has been reported that Notch1 counteracts Wnt/β-catenin signalling through epigenetic modifications [35]. Interestingly, our microarray analysis revealed a downregulation of WNT3A, WNT7A and WNT7B in some MRKHS patients (data not shown).

Consistently with a previously published microarray study on MRKHS patients in which Rall et al. [6] reported a deregulation of HOXB genes, we observed a significant downregulation of HOXB2 and HOXB5 in most of MRKHS patients. At the same time, we observed a strong overexpression of HOXB3, which is known to be involved in correct patterning of the axial skeleton along the antero-posterior axis during early embryogenesis [36] and in kidney differentiation [37], only in type I MRKHS patients.

In light of these considerations, we can hypothesize that a consistent overexpression of HOXB3 might be a compensatory mechanism to offset the decreased expression of other developmental genes, which can limit the onset of other malformations, especially those involving renal system, and then produce a milder phenotype (type I MRKHS).

Taken together, our results suggest a potential dysregulation of a network of developmental genes that may disrupt Mullerian duct formation. Those genes may represent key players that, acting in a complex network, contribute to the arising and pathogenesis of MRKHS. Future studies will contribute to a better understanding of the biological role of these factors in this syndrome.

Supporting Information

Figure S1 Characterization of primary cell cultures obtained from vaginal biopsy (HVMa). A) Representative morphology of cell cultures. Images were assessed by phase contrast microscopy. Scale bar 100 μm. B) Representative immunofluorescence expression of K14 and K19 in HVMs. Scale bar 100 μm. C) Representative western blot analysis of K14, K19 and Vimentin in HVMs. HeLa, MCF7 and HF were used as positive or negative control. Anti-Tubulin antibody was used as loading control. The images are representative of at least three independent experiments. (TIF)

Figure S2 Comparison of expression levels of selected genes in vaginal mucosa and buccal mucosa of one MRKH patients. A) qRT-PCR analysis of mRNA expression levels of the six selected genes, in cell culture from vaginal mucosa of one MRKH patient. For each gene, relative mRNA levels of patients are shown as fold value of the levels of one healthy subject (control). Each experiment was performed in triplicate, and mRNA levels were normalized to GAPDH mRNA expression. Error bars represent standard deviations (*P<0.01). B) qRT-PCR analysis of mRNA expression levels of the six selected genes in cell culture from buccal mucosa of one MRKH patient. For each gene, relative mRNA levels of patient are shown as fold value of the levels of one healthy subject (control). Each experiment was performed in triplicate, and mRNA levels were normalized to GAPDH mRNA expression. Error bars represent standard deviations (*P<0.01). (TIF)

Data S1 Supplementary Material and Methods. (DOC)

Author Contributions

Conceived and designed the experiments: CN SC CM. Performed the experiments: MG MC SD EV. Analyzed the data: CN SC MG MC FR AA. Contributed reagents/materials/analysis tools: DM FB PBP. Wrote the paper: CN SC CM. Critically revised the manuscript: SD EV DM FB PBP FR AA. Approved the final version of the manuscript: CN SC MG MC SD EV DM FB PBP FR AA CM.

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