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A genome-wide screen for copy number alterations in an adolescent pilot cohort with Müllerian anomalies

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Abstract

Objective—To examine whether pathogenic CNCs can be identified in DNA from females with different classes of Müllerian anomalies.

Design—We conducted array-based CNV analysis using an oligonucleotide array from DNA in 12 adolescent females with different types of Müllerian anomalies.

Setting—University-affiliated tertiary care institution

Patients—20 adolescent females with clinically confirmed Müllerian anomalies

Interventions—array-based CNV analysis

Main Outcome Measure(s)—Copy number changes and/or regions with absence of heterozygosity

Results—192 copy-number variants that we identified in these samples were previously annotated as polymorphic. Three copy number changes that were identified in regions with minimal to no overlap with annotated polymorphisms failed significance criteria with detailed

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Author contribution

X.S. enrolled subjects and collected the samples. X.W. performed the DNA isolation and microarray experiments. J.M. performed the data analysis, prepared tables, and wrote the draft of the manuscript. J.E.D. and I.B.V. were responsible for project design, oversight of experiments and final manuscript preparation.

inspection. One subject harbored a 5.1 Mb region of absence of heterozygosity at Xq23 that is of unknown significance.

Conclusions—In conclusion, we did not identify pathogenic CNCs in this small pilot cohort of patients with various Müllerian anomalies, but larger studies will be needed to further investigate more comprehensively whether CNCs are associated with all classes of Müllerian anomalies.

Keywords

Müllerian anomalies; copy-number change; array-based copy number analysis; absence of heterozygosity

Introduction

Müllerian anomalies are a heterogeneous group of congenital anomalies of the female genital tract that result from lack of development, defective fusion, or flawed resorption of the Müllerian ducts (1). The most widely accepted classification system of different Müllerian anomalies was established by the American Fertility Society (AFS) in 1988. This classification chart divides anomalies into the following seven classes: (I) hypoplasia/agenesis, (II) unicornuate uterus, (III) uterine didelphys, (IV) bicornuate uterus, (V) septate uterus, (VI) arcuate uterus, and (VII) diethylstilbestrol-related (2, 3). The estimated prevalence in females is between 0.16 to 10% (4-7). Depending on the type and severity of the Müllerian anomaly, affected women may present at different ages and with varying symptoms, including dysmenorrhea, chronic pelvic pain, amenorrhea, or pregnancy complications (7).

The causes of most isolated Müllerian anomalies are unknown, but it is thought that many have a genetic basis. Thus far, genetic studies have focused on identifying mutations or chromosomal abnormalities associated with Mayer-Rokitansky-Küster-Hauser Syndrome (MRKH), the most severe of Müllerian anomalies, characterized by absence of the uterus and upper vagina (8-10). Patients with MRKH usually have a normal female karyotype and have normal development of secondary sex characteristics. MRKH can be described as isolated (type I) but is more frequently associated with vertebral, renal, and less frequently with auditory or cardiac defects (MURCS association or type II) (11). While most cases are sporadic, familial clustering occurs and supports a genetic etiology. In those families, the observed inheritance pattern is consistent with incompletely penetrant autosomal dominant inheritance (11). In a subset of MRKH with associated hyperandrogenism, germline mutations in the *WNT4* gene have been reported (12, 13).

Array-based DNA copy-number analysis, often referred to as chromosomal microarray analysis (CMA) can detect gains and losses of DNA, such as aneuploidy and deletions or duplications that are below the resolution of a karyotype. CMA has been highly successful for the discovery of the genetic basis for numerous inherited or sporadic developmental disorders. Many newly found chromosomal deletions and duplications have now been associated with birth defects and/or intellectual and neurobehavioral disabilities through research as well as clinical diagnostic use of CMA (14). Because the diagnostic yield is much higher, CMA has now replaced traditional karyotyping as a first-line genetic

diagnostic test for intellectual and developmental disabilities of unknown etiology in the pediatric and adult population (15) and more recently also for prenatal diagnosis in the presence of major fetal abnormalities on a prenatal ultrasound exam (16-20). Recently, CMA has been used to search for copy number changes (CNCs) in DNA from patients with MRKH, resulting in detection of recurrent copy number variants (CNVs), but their causal role in MRKH remains incompletely understood (8-10). The more frequently described copy number changes associated with Müllerian aplasia/MRKH include deletions in 1q21.1, 16p11.2, 17q12, and 22q11.2, some of which encompass known recurrent CNVs (8-10). In addition, duplications involving Xp22 have been described in Type I MRKH subjects (21).

It is currently unknown if these reported CNVs or other CNCs are associated with other types of Müllerian anomalies. The objectives of this study were to search for DNA copy number changes using array-based genomic analysis in a pilot cohort of adolescent females with different classes of Müllerian anomalies and to validate and further characterize any detected CNCs.

Materials and Methods

Subjects and sample collection

Eligible subjects were identified from the patient population of the Texas Children's Hospital Pediatric and Adolescent Gynecology clinic. Uterine malformations were classified according to the American Society for Reproductive Medicine (ASRM) Müllerian anomaly classification system. Adolescent females with clinically confirmed Müllerian anomalies by MRI, and their available family members were recruited and enrolled in this study under a protocol approved by the Baylor College of Medicine Institutional Review Board for Human Subject Research. Subjects with sexual differentiation disorders or cloacal malformations were excluded. All participants provided written informed consent. Clinical history and examination information relevant to their diagnosis was recorded and venous whole blood samples were collected from 30 subjects to extract DNA from peripheral blood leukocytes (PBLs) and to generate EBV-transformed B-lymphoblastoid cell lines (LCLs) at the Baylor College of Medicine Tissue Culture Core. When blood samples could not be obtained because some subjects declined having their blood drawn, saliva was collected using an Oragene kit from ten study participants for whom a blood sample could not be obtained (22).

DNA preparation

Total genomic DNA was extracted from PBLs and/or LCLs using the Puregene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol. Total genomic DNA was extracted from saliva using the Oragene DNA extraction kit (Oragene, DNA Genotek Inc., Ottawa, Canada). Reference total genomic human female DNA (NA12878) was obtained from Coriell Cell Repositories (Coriell Institute for Medical Research) for the purpose of co-hybridization to the arrays. Information provided by Coriell, indicates that this reference DNA is from is made from peripheral blood leukocytes of a Caucasian female from a Utah Mormon population.

CGH/SNP Array Hybridization

Total DNA extracted from blood was submitted to the Baylor College of Medicine Genomic and RNA Profiling Core (GARP) for sample quality assessment and array hybridization. DNA extracted from patients and the reference DNA were co-hybridized to an Agilent SurePrint G3 Human 2x400K combined CGH+SNP microarray (Agilent Technologies, Inc., Santa Clara, CA; Product Number G4842A) (Design ID 028081) which contains ~300,000 CGH probes and 120,000 SNP probes with median spacing of 7 kb.

Briefly, genomic DNA (~1.5 ug each) from the patient and reference female DNA, NA12878, was digested by *Alu* I and *Rsa* I restriction enzymes for 2 h at 37°C. The digested products were labeled with Cy3-dUTP and Cy5-dUTP fluorochromes using the Genomic DNA ULS Labeling Kit (Agilent Technologies). The labeled products were purified, hybridized and washed according to Agilent protocols. Each slide was scanned on an Agilent DNA microarray scanner at the GARP, and data extraction was conducted using Feature Extraction software (v1.5.1.0 FE version) design files as the template for automated gridding with the (CytoCGH_0105_May11) protocol to assign spot-intensity signals and ratios for each extraction set. Data were displayed as log2 ratios.

Data Analysis

Data was analyzed with Agilent's Genome Workbench (V6.5 Lite software package using the following parameters: aberration algorithm ADM-2, threshold 6.0, fuzzy zero, centralization, moving average window 2 kb, GC correction, 5-point aberration filter, intra-array replicates were combined to increase confidence of calls). For this study we classified as copy-number changes any aberrant signals that included five or more consecutive probes. Any such changes that were not listed or were infrequently described as polymorphic in the Database of Genomic Variants (available at <http://projects.tcag.ca/variation/>) were validated by real-time quantitative PCR. Absence of heterozygosity (AOH), also referred to as copy-number neutral loss of heterozygosity, was analyzed using the single nucleotide polymorphism copy number setting (confidence level at 0.95). Absence of heterozygosity was set to a threshold of 6.0. The threshold used to call a region of AOH, was a segment size

5 Mb. All identified copy number changes and regions of AOH were annotated according to hg19 (NCBI build 37) human genome build by referring to their coordinates using the University of California, Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu/>). Information regarding probe coverage and segmental duplication at the region of interest were also considered for interpretation. Custom tracks with identified copy number changes were generated through creation of browser extensible data (bed) files that were uploaded into the UCSC Genome Browser. We used these tracks containing information regarding copy number coordinates, size, location and gene content to compare findings in this cohort to the literature, International Collaboration for Clinical Genomics (<http://www.iccg.org/>), DECIPHER (<http://decipher.sanger.ac.uk/>) and Online Mendelian Inheritance in Man (OMIM) (<http://www.ncbi.nlm.nih.gov/omim>). CNVs previously identified in the reference DNA were compared to CNVs identified in this cohort. (http://www.sanger.ac.uk/research/areas/humangenetics/cnv/global_assess.html).

Copy Number Validation by Quantitative Real-Time PCR

Twenty nanograms of genomic DNA were amplified with PCR primers designed with Primer Express software (Applied Biosystems, Foster City, CA) and are listed below. All reactions were done in triplicate. Each target region was amplified with the Applied Biosystems Step One Real Time PCR system (Applied Biosystems, Foster City, CA) for quantitative real-time PCR (qPCR) with SYBR-Green chemistry. Data were normalized to an endogenous reference gene (*RNaseP*) and a melting curve analysis was performed to verify PCR product specificity. Primer sequences for *RNaseP* are as follows: Forward primer (5'-CCGGAGCTTGGAACAGACT-3') Reverse primer (5'-GTAGTCTGAATTGGGTTATGA-3'). Primer sequences for *Preferentially expressed antigen in melanoma (PRAME)* are as follows: Forward primer (5'-GACTCCGCCCTGCTTTCC-3') Reverse primer (5'-AACAGGTTTGTATTGGCGACAA-3'). Relative gene copy number was determined by the comparative threshold cycle method (C_t) after standard curves with serial dilutions were obtained for each amplification reaction as previously described (23).

Results

Twenty adolescent females with clinically confirmed Müllerian anomalies and twenty unaffected available family members enrolled in this study after providing the appropriate assent or consent. The mean age of subjects was 15.4 ± 3.0 years (12-25 years). All diagnoses in these subjects were previously confirmed using magnetic resonance and/or ultrasound imaging. Of the study participants, six subjects had uterine/vaginal atresia/agenesis (ASRM Class I), ten subjects had uterus didelphys (ASRM Class III), and four subjects had uterus bicornuate (ASRM Class IV). Ten subjects were Caucasian, seven were Hispanic, two were African-American, and one was of Asian descent. Associated conditions for enrolled subjects included scoliosis (n=3), aortic/pulmonic stenosis (n=1), absent kidney (n= 9), and low renal position (pelvic kidney) (n=1). Of the analyzed 12 subjects whose DNA passed quality control requirements for CMA, seven had concurrent renal anomalies and one had a cardiac anomaly (see Table 1).

Of the twenty total subjects, five subjects had previously undergone genetic testing. Three of the analyzed subjects were previously determined to have normal chromosomes by G-banded karyotype or had a normal result on a clinically performed chromosomal microarray analysis. One subject had a previously identified deletion of 10q23 but no additional details were available for this subject. Another subject had a homozygous *MTHFR* 677C>T mutation and was Factor V Leiden mutation negative. Technical limitations in extraction of DNA samples reduced the number of analyzed subjects to 12 (24, 25). Of the 12 subjects whose extracted DNA was of sufficient quality for CMA for analysis, one was previously shown to have normal chromosomes by G-banded karyotyping. The other subject had the homozygous *MTHFR* 677 C>T mutation.

Family history reviewed for subjects revealed endometriosis, hypothyroidism, hypertension, lupus, breast cancer, and diabetes. Due to the nature of the multi-specialty clinic, some family members were not available, deceased, or parents were divorced. Enrolled family members of affected subjects included fifteen mothers, three female siblings, one father, and

one maternal grandmother. For fifteen subjects, only one parent was available. For one subject, only a grandparent was available. For four subjects, no family members could be enrolled. Information about subjects and enrolled family members can be found in Table 1. Both blood and saliva samples were obtained from thirteen subjects and sixteen family members. Six subjects and four family members only provided a saliva sample after declining to provide a blood sample and one subject provided only a blood sample. Of the 12 analyzed subjects, ten had available family, while for two subjects there was no family participation.

Copy Number Analysis

For twelve of the twenty subjects, extracted DNA from blood samples passed minimum quality requirements and was further processed for array hybridization. We identified 192 copy number changes in total across all 12 analyzed subjects (Supplemental table S1). One benefit from using the well-characterized reference DNA, NA12878, was the ability to disregard 62 false-positive copy number changes (1q44, 3q29, 6p21.32, 10q23.2, 12q24.33, 14q11.2, 16p11.2, 17q21.31, and 22q11.22) that were likely due to overlapping copy number changes present in the reference DNA. The remaining 130 copy number changes were compared to the Database for Genomic Variants (DGV). Of these, 104 fully overlapped with presumably benign annotated variants and were excluded from further analysis. After this filtering process, 26 variants remained for potential further analysis. In order to identify potentially novel and pathogenic CNCs, we prioritized four CNCs of interest with minimal to no annotation in the Database for Genomic Variants, for further analysis and confirmation: a 44 Kb loss at 2p23.1 (Mul25), a 180 Kb gain at 2p25 (Mul6), a 33 kb loss at 10q24.33 (Mul1), a 52 kb loss at 10q26.3 (Mul6), but were not confirmed upon detailed inspection. We compared whether identified CNCs of interest overlapped with curated CNVs in the ISCA, DECIPHER, and OMIM databases, to determine if they were enriched for CNVs previously associated with uterine or renal anomalies, but did not find any significant enrichment.

Due to the lack of enrichment of potentially pathogenic copy number changes in this cohort, we next examined whether recurrent CNCs that were previously reported to be enriched in subjects with Müllerian aplasia, such as del/dup 1q21.1, del 16p11.2, del 17p12, del/dup 22q11.2, del Xp22.33 and dup Xq21.31, were present in the affected subjects in this study. None of the subjects had CNCs at 17p12 or Xp22.33. No individuals had duplications in Xq21.31. We identified one individual (Mul34) with a copy number gain at 1q21.1 (678 kb) that partially overlapped with reported copy number losses previously reported to be associated with Müllerian anomalies. However, it did not encompass *RBM8A*, the candidate gene in this region (26). Furthermore, detailed inspection of this region did not show consecutive probes altered, and this copy number change failed to meet the criterion. There were five apparent copy number gains in multiple subjects at the 16p11.2 locus, but they were excluded from analysis because they overlapped with a reciprocal copy number loss present in the reference DNA, such that the actual change in subjects was copy-number neutral. Thus, there was no evidence in any of the samples for a CNC in the previously reported Müllerian anomaly-associated candidate regions in 16p11.2 containing *TBX6* and *LHX1* (27). Nine out of twelve subjects also had an apparent gain at 22q11.22. This gain was

of interest, because it overlapped with previously reported Müllerian anomaly-associated copy number gains and losses and was therefore also selected for further detailed analysis (8-10, 28). We first determined from analyzing publicly available data that the reference DNA, NA12878, harbored a 39 Kb loss embedded within this region. To further examine whether the larger copy number gain in 22q11.22 was enriched in subjects in this cohort, we performed copy-number analysis by qPCR using primers outside the 39 Kb interval that was deleted in the reference DNA, and confirmed the copy number gain in eight subjects. Testing of single parental DNA, where available for ten of the twelve analyzed subjects, revealed that this CNV was inherited in four of the eight individuals, but unavailability of DNA from both parents in all cases, prevented confirmation of inheritance of this CNC in the other four.

Absence of Heterozygosity Analysis

We sought to identify whether large regions with copy number-neutral loss of heterozygosity were enriched in this cohort, as such regions could represent uniparental disomy or potentially unmask autosomal recessive mutations. All identified regions with absence of heterozygosity (AOH) are listed in Supplemental Table S2. In total, we identified sixteen regions with absence of heterozygosity (AOH) in ten of the twelve analyzed subjects, ranging in size from 0.6 Mb to 5.1 Mb (Table 2), but due to the limited SNP-probe density in the design of the array, we set a threshold for significance at >5 Mb for final interpretation (Table 2). Applying this cut-off, we found one region of AOH of 5.1 Mb at Xq23 (Mul27) that contained multiple genes. There were two additional regions of AOH, just below the 5Mb-threshold that also contained many genes: a 4.4 Mb region at 8q11.21 (Mul31) and a 4.4 Mb region 4q21.1-q21.22 (Mul36).

Discussion

We initiated a pilot study to begin investigating if Müllerian anomalies have a genetic basis and whether the findings are related to those previously reported in MRKH. In a small group of twelve affected individuals, we were not able to identify any clinically significant CNCs. Even though some observed changes overlapped with regions previously reported to be associated with MRKH, we found that in this small study, all detected CNCs were likely polymorphic and not causal. We also found one 5Mb region of absence of heterozygosity that is currently of uncertain significance. Our study was limited by its small size, missing parental samples in some cases and we did not yet analyze samples for point mutations in candidate genes.

Müllerian anomalies comprise a spectrum of developmental defects of Müllerian-duct derived components of the female reproductive tract. Although some limited progress has been made in the understanding the genetic basis of isolated and syndromic Müllerian aplasia or MRKH syndrome, a genetic cause is not defined in a large percentage of cases, and it is currently unknown what the genetic basis is of the less severe forms of Müllerian anomalies. Some of the findings are interesting from the perspective of interpretation of copy number changes in the 1q21.1, 16p11.2, 17p12, 22q11.2, Xp22.33 and Xq21.31

regions, that have been previously associated with MRKH or multiple malformation syndromes that also include Müllerian anomalies(8-10, 21, 28).

We did not find deletions in 1q21.1 in this group of affected individuals. Previously described deletions affecting the *RNA binding motif protein 8A (RBM8A)* gene in this locus have been associated with Thrombocytopenia-Absent Radius Syndrome, or TAR Syndrome (26). This condition is primarily associated with platelet deficiency and absence of the radius, but associated malformations in the renal and urogenital system have been reported. Deletions of this locus have been associated with MRKH.

We also excluded deletions in 16p11.2, a locus that contains a candidate gene *TBX6* that encodes *T-box transcription factor 6* with a role in the fate determination of axial stem to become either neural or mesodermal (27). Deletions of this region can present with highly variable phenotypes with autism or congenital malformations that can include Müllerian aplasia/MRKH (9, 27).

There were no copy number changes in 17q12, deletions of which have been associated with Müllerian duct aplasia, Unilateral renal agenesis, and Cervicothoracic-Somite anomalies (MURCS) syndrome (8-10). Relevant candidate genes in this locus include *LHX1*, encoding *LIM Homeobox 1* and *HNF1 β* , encoding *hepatocyte nuclear factor 1-beta*. *Lhx1*-null mouse models have absence of Müllerian ducts and mutations in *LHX1* have been shown to be associated with urogenital developmental abnormalities and MRKH (29). Mutations in *HNF1 β* cause renal cysts and diabetes syndrome.

Finally, we investigated the 22q11.22 locus, which is deleted in Velocardiofacial Syndrome (VCFS), a condition that can present with highly variable phenotypes, including renal and Müllerian anomalies. It has been suggested that uterovaginal aplasia may be a feature of the broad phenotypic spectrum of DiGeorge Syndrome (DGS) (28, 30). Eight of the twelve subjects appeared to have gains ranging in size from 696kb to 854kb at 22q11 that fully overlapped with CNVs previously associated with MRKH (10) and partially overlapped with another described CNC(8) 2.6 Mb distal to the DGS critical region, but the identified CNCs were not confirmed in validation studies.

We also evaluated loci implicated in Müllerian anomalies as well as other genitourinary malformations associated with DGS-like phenotypes at 4q34.2-qter, 8p23-pter, 10p14-p15, 17p13 or 18q21 (28), but no CNCs were identified in these loci.

Finally, we evaluated regions containing *WNT* genes, because a subset of women affected by MRKH with hyperandrogenism have mutations in *WNT4* (12, 13), but did not find any CNCs affecting genes that encode members of the *WNT* signaling pathway.

Conclusions

In conclusion, although this was a small pilot study and more numbers will need to be studied in the future. To our knowledge, this study was the first to investigate different types of anomalies in an adolescent female cohort. To date, we did not find an association between distinct ASRM classes of anomalies and specific CNCs. Our study was small and focused on

constitutional mutations. It is likely that more patients will need to be studied in the future to more fully understand the mutational spectrum underlying Müllerian developmental abnormalities. This will include whole exome sequencing to identify point mutations and smaller insertions and deletions in coding genes. Future analysis of affected tissues will be needed to address whether the defects result from somatic mutations. It is also possible that epigenetic differences could play a role in the etiology of subjects in this cohort. While addressing this question was beyond the scope of this pilot study, it is also a valid follow-up experiment we will consider in the future, considering our negative data. Our future work will include recruitment of additional subjects for CNC analysis and these other mutation screening approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Capsule

We sought to characterize whether pathogenic copy number changes (CNCs) were associated with different classes of Müllerian anomalies in a cohort of adolescent females. No pathogenic CNCs were observed.

Table 1

Summary of main clinical features for analyzed subjects

Subject ID	Ethnicity	ASRM Class	Clinical Features	Co-occurring anomalies	Number of CNVs	Clinical history	Enrolled family and medical history
Mul1	Caucasian	III	Ut. Didelphys	Renal agenesis	25	None	Mother-hypothyroidism, mitral valve prolapse, polycystic ovarian syndrome, for 2nd daughter/Glucophage; sister-asthma
Mul6	Hispanic	III	Ut. Didelphys	R Pelvic Kidney, scoliosis	17	asthma	Father-hypertension
Mul14	Hispanic	III	Ut. Didelphys	Absent L Kidney;	17	hypothyroidism	None
Mul17	Hispanic	III	Ut. Didelphys	Absent R Kidney	15	None	None
Mul21	Caucasian	III	Ut. Didelphys	Absent L Kidney	12	None	Mother-none
Mul23	Asian	I	Lower vaginal atresia	None	11	Grave's disease	Mother-none
Mul25	Hispanic	I	Uterine/vaginal agenesis	Solitary Right Kidney; scoliosis	20	None	Mother-none
Mul27	Hispanic	IV	Bicornuate uterus	None	19	Bipolar; ADHD; MTHFR homozygous 677C>T	Mother-Systemic Lupus erythematosus hyperthyroid
Mul31	Caucasian	IV	Bicornuate uterus	Solid mass in right adnexal region	13	pseudopapillary tumor of pancreas	Mother-Progesterone taken prenatally
Mul34	Hispanic	IV	Bicornuate Uterus	Aortic/pulmonic stenosis	15	None	Grandparent-hypertension, thyroid, hypercholesterolemia
Mul36	Hispanic	III	Ut. Didelphys	Absent R Kidney	12	None	Mother-none
Mul38	Caucasian	I	Müllerian agenesis	Unknown	16	None	None

Table 2

Borderline AOH findings for analyzed subjects

Subject ID	ASRM Class	Cytoband	Hg19 Coordinates	Genes	Size (Mb)
Mul27	IV	Xq23	ChrX:109788728-114909139	CHRD1, PAK3, CAPN6, DCX, ALG13, TRPC5, ZCCHC16, LHFPL1, AMOT, HTR2C, SNORA35, MIR764, MIR1912, MIR1264, MIR1298, MIR1911, MIR448, IL13RA2, LRCH2, RBMXL3, LUZP4, PLS3	5.1 Mb
Mul31	IV	8q11.21	Chr8:48217908-50510897	KIAA0146, CEBPD, PRKDC, MCM4, UBE2V2, EFCAB1, SNAI2, C8orf22...	4.4 Mb
Mul36	III	4q21.1-q21.22	Chr4:78376428-82820608	CXCL13, CNOT6L, MRPL1, FRAS1, ANXA3, BMP2K, PAQR3, NAA11, GK2, GDEP, ANTXR2, PRDM8, FGF5, C4orf22, BMP3, PRKG2, RASGEF1B	4.4 Mb