Genome-wide copy number variation study in anorectal malformations

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EDITOR'S COMMENTS:

One issue jumps out -- it seems highly implausible that the PLINK p values for individual regions are at the level of 5x10-5 after correction for multiple testing, given the sample sizes and the number of CNVs in each region (7:0, 5:0 as best as I could tell -- the data are not clearly presented and the Supplementary Tables referred to at this point in the text do not contain the relevant data). As this is one of the remaining significant results in the manuscript, (the other main result is the overall excess of rare duplications, and is unreplicated) this point needs to be clarified. If the results are really that significant, I would then worry about a systematic bias where there are more CNVs being called in general. What does the QQ plot look like for all of the genomic regions analyzed by PLINK? What is the inflation factor for this analysis? What is the distribution of non-copy number 2 calls among the cases and among the controls (excluding trisomy 21 patients)? Are there some cases that are outliers?

RESPONSES:

In the manuscript we presented separate analyses for genic and non-genic regions. The significant CNVs were in non-genic regions. The tests presented were chi-squares and permutations were performed to obtain empirical *p*-values. There were **457 non-genic regions**, so the permutation corrected for 457 regions. However, given that the most significant CNV was present in 7 cases and no controls while most other CNVs were less frequent, most CNVs were unable to produce such extreme *p*-values in the simulation and in effect the simulation was approximately equivalent to a Bonferroni correction for approximately 24 CNVs, since only those CNVs that are present 7 or more times can produce a *p*-value as small as the one obtained.

We believe this test is valid, since it is based on the actual distribution of CNVs obtained, but also recognize that to be fair, **the simulation should have included both all genic and non-genic regions,** so we have amended the paper accordingly. The three CNV regions remain genome-wide significant. Therefore, the permutation empirical *p*-values reported (initially corrected for 457 non-genic regions) have been replaced with *p*-values **corrected for 2439 total CNV regions** (1982 genic and 457 non-genic regions). One may argue that this test is still too liberal, since all CNVs with lower frequency cannot contribute to the correction, so we also now include the *p*-values from the more appropriate for low counts **Fisher's exact test, and include a Bonferroni correction for 2439 total CNV regions** (1982 genic and 457 non-genic regions). In this case, two of the three CNV regions remain genome-wide significant. However, this approach may be too conservative.

The following table lists the initially reported results of the top three rare CNV regions, and results from the permutation test and Fisher's exact test performed on and corrected for the 2439 CNV regions (1982 genic regions and 457 non-genic regions). We now include a new table in the main text showing the results of the top three rare CNV regions from both the permutation

test and Fisher's exact test (Table 3 – *pasted below*), and the QQ plots of these tests in the supplementary materials (Supplementary Figures 9a and b – *pasted below*).

Table: The initially reported results of the top three rare CNV regions, and results from the permutation test and Fisher's exact test performed on and corrected for the 2439 CNV regions (1982 genic regions and 457 non-genic regions)

Chr.	Starting position (in hg18)	Ending position (in hg18)	Number of cases	Number of controls	Permutation test **(initially reported)**		Permutation test **(now included)**		Fisher's exact test	
					Empirical *p*-values	Empirical *p*-values corrected for all **non-genic regions** (N=457)	Empirical *p*-values	Empirical *p*-values corrected for all **genic and non-genic regions** (N=2439)	*p*-values	*p*-values after Bonferroni correction
7	38285115	38330273	7	0	1.00×10^{-06}	6.59×10^{-5}	2.00×10^{-06}	0.000147	3.20×10^{-06}	0.00779436
14	21937715	22009307	6	0	0.000139	7.16×10^{-4}	0.000134	0.001372	1.98×10^{-05}	0.048239486
1	40794563	40804646	7	3	0.000228	0.011	0.000240	0.022894	0.0002435	0.594073571

Genic and non-genic regions are defined as those that harbour at least one CNV in cases or controls. These three regions do not harbor any genes, i.e. non-genic regions. Permutation tests (1,000,000 iterations) were initially performed on 457 rare non-genic regions and corrected for these regions. According to PLINK, the empirical *p*-values corrected for all the tests were calculated by comparing each observed test statistic against the maximum of all permuted statistics (i.e. over all regions) for each single replicate.

Now we replaced the reported values with the results from permutation test performed on 2439 rare CNV regions (1982 genic regions and 457 non-genic regions) by using PLINK, and corrected for all these 2439 regions. We also performed the Fisher's exact test on these regions, and included a Bonferroni correction for 2439 total CNV regions.

We have also modified the text in the **supplementary materials and methods** under the section "***Rare copy number variation regions (CNVRs) analysis - genic and non-genic regions***" as below:

"Rare CNVRs were divided into two categories: genic CNVRs and non-genic CNVRs. Genic CNVRs were genic regions (based on RefSeq genes extended by 5kb upstream and downstream) in which a CNV resides. Non-genic CNVRs are defined by regions that result from the union of overlapping CNVs that do not intersect any genic CNVRs (Supplementary Figure 5). Simple permutation-based (1-sided) test of association was performed for ~~each~~ all genic and non-genic CNVRs together (with 1,000,000 iterations) by using PLINK to test if there are significantly more cases that have CNV in the defined region. The empirical p-values corrected for all the tests were also calculated by comparing each observed test statistic against the maximum of all permuted statistics (i.e. over all regions) for each single replicate.

One may argue that this test is still too liberal, since all CNVs with lower frequency cannot contribute to the correction, so we also performed the more appropriate for low counts Fisher's exact test by using R, and included a Bonferroni correction for 2439 total rare CNV regions (1982 genic and 457 non-genic regions)."

Changes were also made accordingly in the **Results section in the main text** under the heading *"**Rare CNVRs that are statistically associated to ARMs**":*

"Following the global rare CNVs enrichment observed in the patients, rare CNV regions (CNVR; genic: N=457 and non-genic: N=1982) were defined (see Supplementary Figure 5) and individually evaluated for their associations with ARMs by using permutation tests. The number of CNVs within each defined CNVR was compared between patients and controls. We also performed the more appropriate for low counts Fisher's exact test, and included a Bonferroni correction for 2439 total rare CNV regions.

Using permutation tests, we identified 3 non-genic CNV regions that were statistically associated with ARMs on 7p14.1 (corrected empirical p-value = 0.000147; p-value from Fisher's exact test after Bonferroni correction = 0.00779), 14q11.2 (corrected empirical p-value = 0.00137; p-value from Fisher's exact test after Bonferroni correction = 0.0482), and 1p34.2 (corrected empirical p-value = 0.0229; p-value from Fisher's exact test after Bonferroni correction = 0.594) ~~(Supplementary Figures 8a, b and c)~~ (Table 3). All were hemizygous deletions.

Deletions on 7p14.1 (a 45kb region) were observed in 7 ARM-patients (6 isolated; 1 with bifid scrotum) but in none of the controls. This region (5.3kb upstream of TARP (TCR gamma alternate reading frame protein) overlaps a 411bp CpG island and a transcription factor binding site (Supplementary Figure 8a). Deletions on 14q11.2 (a 73kb region) were observed in 5 ARMs cases (3 isolated; 1 with bifid scrotum, 1 with Down syndrome) but not in our controls (Supplementary Figure 8b). The 1p34.2 ARMs-associated deletion was detected in 7 ARM-patients (6 isolated; 1 with heart and kidney anomalies) and in 3 control individuals (Supplementary Figure 8c)."

Regarding the total number of rare CNVs in cases and controls, we now include 3 supplementary figures (Supplementary Figures 10 a, b and c – *pasted below*) showing the distributions of them. **Coinciding with the global burden test performed on rare CNVs (as shown in Table 1), there are generally more rare CNVs in cases than in controls, as the whole distribution of cases is on the right of that of controls (Supplementary Figures 10a). There are no outlier cases.** In one of our QC procedures, samples with genome-wide LRR standard deviation greater than 3.5 or with more than 500 CNVs called were already excluded from the analysis (patients=5; controls=17). Those individuals with CNVs in the three top non-genic regions were also examined for their total number of rare CNVs. A new supplementary table (Supplementary Table 14 – *pasted below*) is now included.

The median of the observed chi-square statistics for these genic and non-genic regions is 0 while the expected median should be around 0.456 (i.e. inflation factor is equal to 0). The test statistics are deflated as many of the CNVs are present only once (N=1238) (no chi-square can be computed). Therefore, correcting for 2439 total rare CNV regions (Bonferroni correction) may be over conservative, yet the association is still statistically significant for the 7p14.1 and 14q11.2 regions.

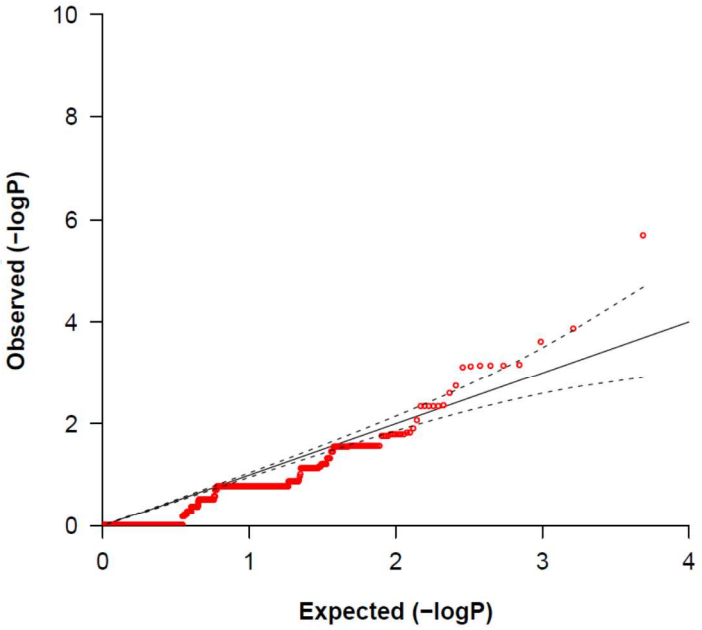
Table 3: Results of the top three rare CNV regions from the permutation test and Fisher's exact test.

Chr.	Starting position (in hg18)	Ending position (in hg18)	Number of cases	Number of controls	Permutation test		Fisher's exact test	
					Empirical *p*-values	Empirical *p*-values corrected for all tests	*p*-values	*p*-values after Bonferroni correction
7	38285115	38330273	7	0	2.00×10^{-06}	0.000147	3.20×10^{-06}	0.00779436
14	21937715	22009307	6	0	0.000134	0.001372	1.98×10^{-05}	0.048239486
1	40794563	40804646	7	3	0.000240	0.022894	0.000243573	0.594073571

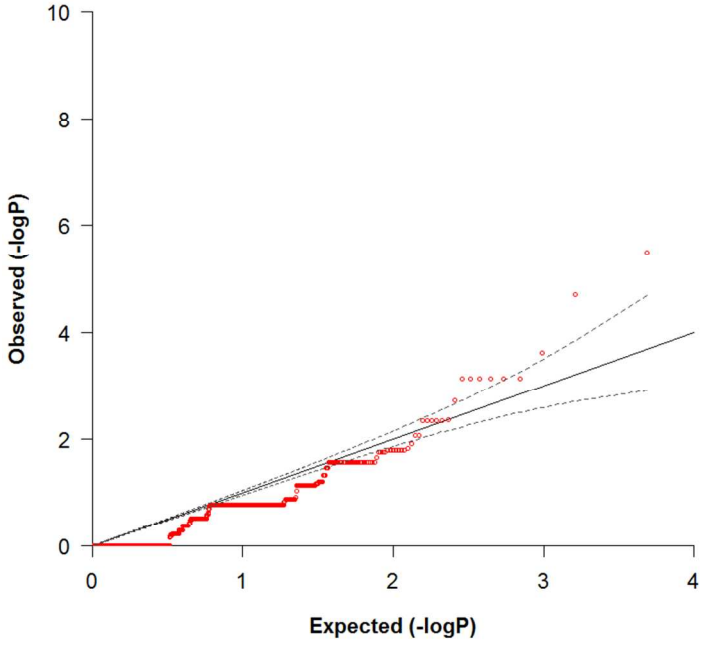
Genic and non-genic regions are defined as those that harbour at least one CNV in cases or controls. These three regions do not harbor any genes, i.e. non-genic regions. Permutation tests (1,000,000 iterations) were performed on 2439 rare CNV regions (1982 genic regions and 457 non-genic regions) by using PLINK. According to PLINK, the empirical *p*-values corrected for all the tests were calculated by comparing each observed test statistic against the maximum of all permuted statistics (i.e. over all regions) for each single replicate. We also performed the Fisher's exact test on these regions, and included a Bonferroni correction for 2439 total CNV regions.

Supplementary Figure 9: QQ-plot of the association tests performed on the 2439 rare CNV regions (1982 genic regions and 457 non-genic regions)

(a) QQ-plot of the permutation tests (1,000,000 iterations) results that were generated based on the 2439 rare CNV regions by using PLINK



(b) QQ-plot of the Fisher's exact test results that were generated based on the 2439 rare CNV regions

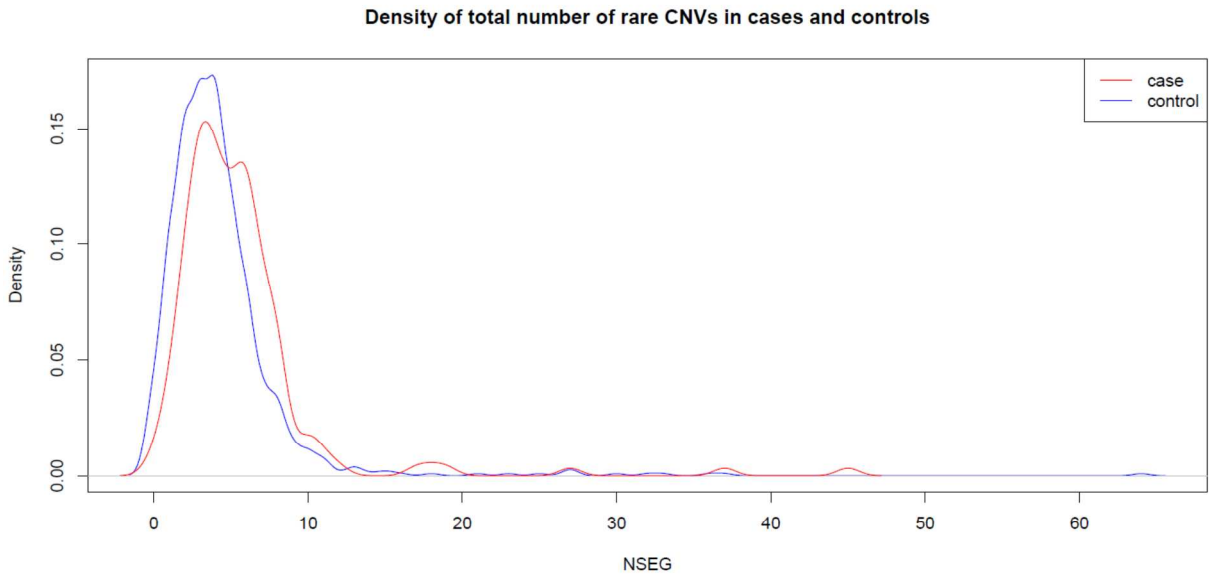


Supplementary Figure 10: Distribution of the total number of rare CNVs in cases and controls

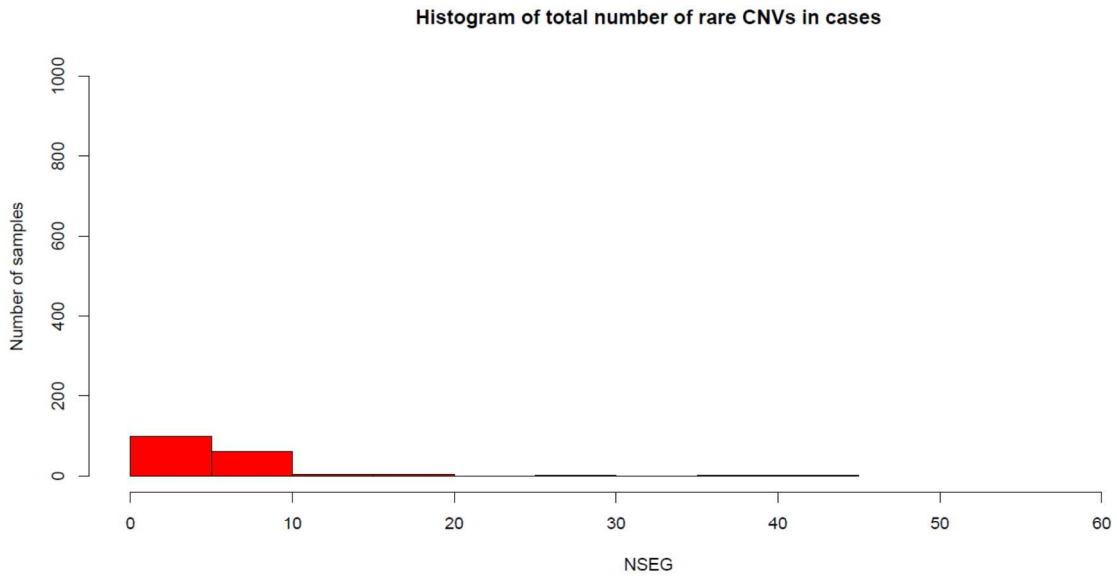
Coinciding with the global burden test performed on rare CNVs (as shown in Table 1), there are generally more rare CNVs in cases than in controls, as the whole distribution of cases is on the right of that of controls (a). The distribution of the total number of rare CNVs in cases is displayed in red, while the distribution in controls is displayed in blue. No outlier case is observed. Histograms are also shown alongside with the one after Kernel density smoothing.

NSEG: total number of rare CNVs

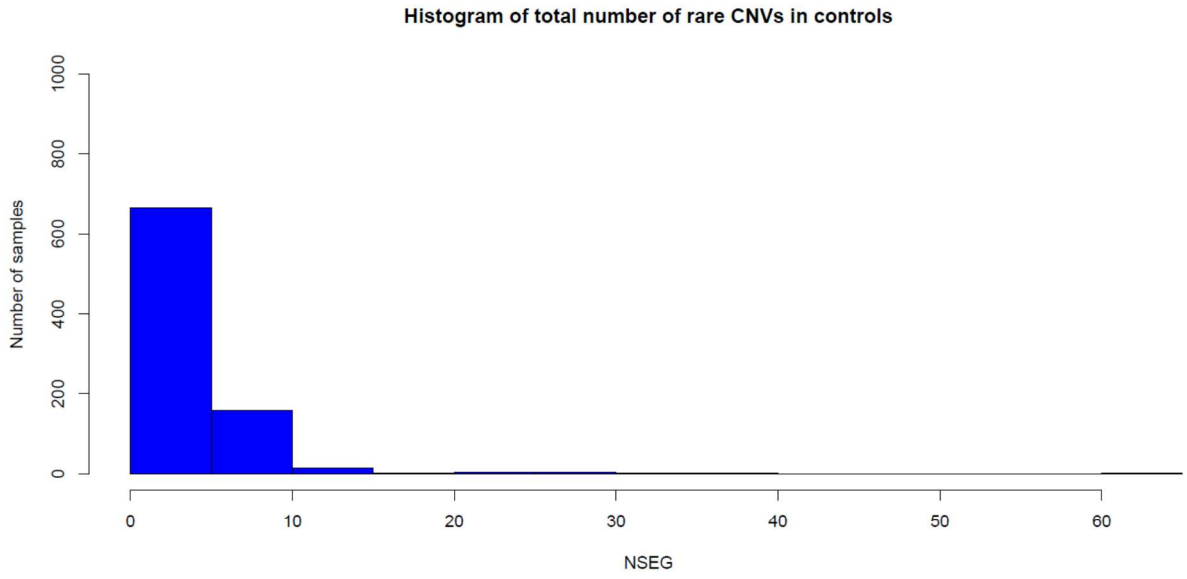
(a) Kernel density smoothing of the histogram, which allows better visual comparison of the two distributions.



(b) Histogram of the total number of rare CNVs in cases



(c) Histogram of the total number of rare CNVs in controls



Supplementary Table 14: Total number of rare CNVs in those individuals who have CNVs in the three top non-genic regions

Chr.	Starting position (in hg18)	Ending position (in hg18)	Sample ID	Affection status	Total number of rare CNVs
7	38285115	38330273	MG-IA218C	Case	7
			MG-IA105C	Case	8
			MG-IA98C	Case	8
			MG-IA179C	Case	8
			MG-IA220C	Case	8
			MG-IA95C	Case	7
			MG-IA381C	Case	7
14	21937715	22009307	MG-IA179C	Case	8
			MG-IA126C	Case	5
			MG-IA105C	Case	8
			MG-IA220C	Case	8
			MG-IA98C	Case	8
			MG-IA105C	Case	8
1	40794563	40804646	MG-IA158C	Case	8
			MG-IA161C	Case	5
			MG-IA338C	Case	8
			MG-IA344C	Case	3
			MG-IA365C	Case	4
			MG-IA327C	Case	3
			MG-IA106C	Case	3
			AK2858	Control	1
			AK6029	Control	4
			HTP529	Control	6

TITLE: Genome-wide copy number variation study in anorectal malformations

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ABSTRACT

Anorectal malformations (ARMs, congenital obstruction of the anal opening) are among the most common birth defects requiring surgical treatment (2-5/10,000 live-births) and carry significant chronic morbidity. ARMs present either as isolated or as part of the phenotypic spectrum of some chromosomal abnormalities or monogenic syndromes. The etiology is unknown. To assess the genetic contribution to ARMs, we investigated single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) at genome-wide scale. A total of 363 Han Chinese sporadic ARM-patients and 4,006 Han Chinese controls were included. Overall, we detected a 1.3-fold significant excess of rare-CNVs in patients. Stratification of patients by presence/absence of other congenital anomalies showed that while syndromic-ARM patients carried significantly longer rare duplications than controls (p=0.049), non-syndromic patients were enriched with both rare deletions and duplications when compared to controls (p=0.00031). Twelve chromosomal aberrations and 114 rare-CNVs were observed in patients but not in 868 controls nor 11,943 healthy individuals from the Database of Genomic Variants (DGV). Importantly, these aberrations were observed in isolated-ARM patients. Gene-based analysis revealed 79 genes interfered by CNVs in patients only. In particular, we identified a *de novo DKK4* duplication. *DKK4* is a member of the *WNT* signaling pathway which is involved in the development of the anorectal region. In mice, *Wnt* disruption results in ARMs. Our data suggest a role for rare-CNVs not only in syndromic but also in isolated-ARM patients and provide a list of plausible candidate genes for the disorder.

INTRODUCTION

Anorectal malformations (ARMs, congenital obstruction of the anal opening) are among the most common birth defects requiring surgical treatment (2-5/10,000 live-births (1)) and carry a significant chronic morbidity. The condition is attributed to a defect in the proliferation of the embryonic rudiments that will form the distal end of the gut,, and it is probably due to disorders in the expression of pattern determining genes. The spectrum of ARMs ranges from anal stenosis to imperforated anus with/without anal fistula to persistent cloaca, in which the intestinal and genitourinary tracts remain a common channel. ARMs might appear as part of the phenotypic spectrum of many chromosomal abnormalities (2-4) or monogenic syndromes (5, 6).

The etiology of ARMs remains unknown. While environmental factors are not to be dismissed, several lines of evidence indicate that there is a genetic component (7). Indeed, even though ARMs appear mostly sporadically (no affected relatives), they also segregate within families with patterns of inheritance ranging from autosomal-dominant, X-linked, to autosomal-recessive (8-10). Moreover, higher risk of anal atresia/stenosis has been associated with consanguinity (11).

The approach currently being taken towards the discovery of genes involved in ~~isolated~~ ARMs in humans is that of the analysis of candidate genes selected according to the data provided by (i) their role in syndromes that include ARMs as part of their spectrum; (ii) mutant mice/rat studies (12, 13) as in most cases, mutations in the human orthologs give rise to similar or related phenotypes. However, while mice mutant for *Shh, Gli2*, or *Gli3* display different congenital defects that include ARMs as a common feature, point mutations in the human orthologs (*SHH*, *GLI3*) are associated with syndromes or genetically heterogeneous disorders in

which the ARMs phenotype is not always the norm. Failure to identify human genes underlying ARMs so far may be attributed to the reason that there is no single major gene, suggesting genetic and phenotypic heterogeneity.

To explore the genetic contribution to the pathogenesis of this condition, we investigated at genome-wide scale, single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) on Han Chinese ARM patients. Patients were also stratified into those presenting ARMs as an isolated feature and those presenting ARMs together with associated anomalies and analyzed accordingly.

RESULTS

Common variants do not contribute to the isolated-ARMs phenotype

Common single nucleotide polymorphisms (SNPs)

After stringent quality control (QC) on genome-wide association study (GWAS) genotypes, 489,243 SNPs (average call rate 99.85%) that were successfully genotyped in 175 ARMs cases and 2,971 controls were tested for association to ARMs under additive, dominant and recessive models. After correcting for the first 12 principal components (i.e. correcting for population stratification), 48 SNPs reached association *p*-values $<10^{-4}$ under the additive model (Supplementary Table 3). No SNP reached genome-wide significance ($p<10^{-8}$). QQ-plot and Manhattan plot of the association test results were shown in supplementary Figure 2 and 3 respectively. The genomic inflation factor after principal components analysis (PCA) correction did not deviate from 1 (λ=1.0093) indicating that chances of spurious associations due to the population substructure were minimal.

As PCA had revealed that two subpopulations of Han Chinese (Northern and Southern) existed in our dataset (see Supplementary Figure 1), we then performed association tests separately to assess if susceptibility loci differed between the two subpopulations. When the Northern Chinese patients (N=103) were tested for ARMs association against Northern Chinese controls (N=1411), we identified 44 SNPs with association *p*-values $<10^{-4}$ under the additive model. As for the Southern Chinese (patients: N=72; controls: N=1560) we identified 42 SNPs with *p*-values$<10^{-4}$. Similar results were obtained when patients were stratified according to the presence/absence of additional anomalies or syndromes (data not shown).

We then proceed to replicate the signals detected in the discovery phase. After QC, 110 SNPs were successfully genotyped on 167 patients affected with isolated ARM (Northern: N=81,

Southern: N=86) and 174 normal controls (Northern: N=88, Southern: N=86). Meta-analysis of all Han Chinese revealed that none of the combined *p*-values for the SNP tested reached genome-wide significant level.

Copy Number Polymorphisms (CNPs)

Global burden of CNPs were examined in all cases and controls in terms of length, frequency and number of genic regions overlapped. None of the global burden test results was statistically significant (see Supplementary Table 6), thus suggesting that there is no enrichment of common CNVs in cases compared with controls. Similar results were obtained when the analysis was performed on stratified patients (isolated or syndromic patients with ARMs).

Lack of ARMs association with common susceptibility loci may also imply that the disorder results from variants whose frequency in the population is lower than 1% (rare variants). Using the same data set, we proceeded to study the contribution of rare CNVs to the ARMs phenotype as described in the following sections.

Rare CNVs contribute to ARMs

Global burden analysis: ARM patients are enriched with rare CNVs

Rare CNVs are known to play a more significant role in disease susceptibility than CNPs (common CNVs) (15, 16). **Thus, global burden** of rare CNVs (defined as CNVs that are observed in less than 1% samples of the dataset) was compared between cases and controls **using permutation tests (1,000,000 iterations)**. Patients were enriched with rare deletions and rare duplications by 1.3 fold each. **Rare CNVs were classified into two groups in terms of size (length<100kb or length>100kb) and their distributions in patients and controls are**

tabulated in Table 1. Globally, more duplications were identified in ARM patients (CNV with length < 100kb: empirical *p*-value = 0.007344; CNV with length >100kb: empirical *p*-value = 0.002307) when compared to controls.

Significant enrichment of rare duplications (CNV with length < 100kb: empirical *p*-value = 0.0187; CNV with length >100kb: empirical *p*-value = 0.0064) and deletions (CNV with length < 100kb: empirical *p*-value = 0.0102; CNV with length >100kb: empirical *p*-value = 0.0181) were observed in non-syndromic patients (isolated ARMs, N=126), while interestingly, syndromic patients (N=44, among which 15 have Down syndrome) were only modestly enriched with long duplications (length > 100kb, empirical *p*-value = 0.0490). The results were tabulated in supplementary Tables 7a and b. Although the association tests are significant, it would be important to replicate the excess in deletions and duplications in an independent group of patients and controls of all ancestries available.

Chromosomal aberrations

Within the set of long CNVs, we examined closely those CNV longer than 1Mb which are referred to as chromosomal aberrations. Global burden analysis revealed that, overall, ARM-patients have 3 fold more chromosomal aberrations (defined as longer than 1Mb) than controls (p=0.0368 for deletions and p=0.00614 for duplications) even after excluding patients with Down syndrome (around 9% of our ARM-patients) (see Supplementary Tables 8a and b). The chromosomal aberrations in ARM-patients also spanned more genic regions than those in controls. This applied to either deletions (empirical *p*-value=0.00038) or duplications (empirical *p*-value=0.000226).

Importantly, we identified 12 chromosomal aberrations (besides trisomy 21) that were unique to ARM patients as they were not identified in controls or in the normal individuals of the Database of Genomic Variants (DGV) (Table 2). Seven chromosomal aberrations encompassed genes that were not disrupted in controls. The chromosomal aberrations observed were checked against the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER) which is a database of submicroscopic chromosomal imbalances and clinical information for 6,169 patients with developmental disorders. There were five aberrations that had also been found in DECIPHER patients with similar or ARM-related symptoms.

Interestingly, a 2.5Mb heterozygous deletion on chromosome 22q11.21 was identified in one affected female (MG-IA162C, isolated-ARM). Deletions involving 22q11.21 had been reported in a patient with VACTERL syndrome, which includes ARMs as part of the spectrum (17), and in 5 other syndromic ARM patients (18-20). Chromosomal aberrations on chromosome 22q11.21 were also reported in five DECIPHER patients with hindgut problems (Table 2), including **1** patient with isolated-ARM and 1 syndromic patient with sacrum and kidney anomalies. Thus, it would appear that chromosomal aberrations involving this region are not only involved in syndromes with ARMs as part of the phenotypic spectrum but also in the isolated ARMs phenotype (21). CNVs that overlap known critical regions are likely to be pathogenic in nature. Importantly, these chromosomal aberrations were identified in patients with the ARM-isolated phenotype.

Rare CNVRs that are statistically associated to ARMs

Following the global rare CNVs enrichment observed in the patients, rare CNV regions (CNVR; genic: N=457 and non-genic: N=1982) were defined (see Supplementary Figure 5) and individually evaluated for their associations with ARMs by using permutation tests. The number of CNVs within each defined CNVR was compared between patients and controls. We also performed the more appropriate for low counts Fisher's exact test, and included a Bonferroni correction for 2439 total rare CNV regions.

Using permutation tests, we identified 3 non-genic CNV regions that were statistically associated with ARMs on 7p14.1 (corrected empirical *p*-value = 0.000147; *p*-value from Fisher's exact test after Bonferroni correction = 0.00779), 14q11.2 (corrected empirical *p*-value = 0.00137; *p*-value from Fisher's exact test after Bonferroni correction = 0.0482), and 1p34.2 (corrected empirical *p*-value = 0.0229; *p*-value from Fisher's exact test after Bonferroni correction = 0.594) ~~(Supplementary Figures 8a, b and c)~~ (Table 3). All were hemizygous deletions.

Deletions on 7p14.1 (a 45kb region) were observed in 7 ARM-patients (6 isolated; 1 with bifid scrotum) but in none of the controls. This region (5.3kb upstream of *TARP* (TCR gamma alternate reading frame protein) overlaps a 411bp CpG island and a transcription factor binding site (Supplementary Figure 8a). Deletions on 14q11.2 (a 73kb region) were observed in 5 ARMs cases (3 isolated; 1 with bifid scrotum, 1 with Down syndrome) but not in our controls (Supplementary Figure 8b). The 1p34.2 ARMs-associated deletion was detected in 7 ARM-patients (6 isolated; 1 with heart and kidney anomalies) and in 3 control individuals (Supplementary Figure 8c).

These 3 non-genic regions overlapped with CNVs listed in the Database of Genomic Variants (DGV). Yet, given their associations with the ARMs phenotype it is tempting to speculate that they affect regulatory sites and that may contribute to disease in conjunction with additional altered loci.

Rare CNVs unique to ARM patients

As rare-CNVs are more likely to be pathogenic if they involve gene-rich regions and are only found in affected individuals, we proceeded with the identification of rare-CNVs that were exclusive to ARM patients (Supplementary Figure 6). This yielded 433 CNVs of which 342 were observed only once (non-recurrent) and 91 CNVs (distributed in a total of 35 CNVR–regions-) were observed in more than one patients (recurrent; Supplementary Table 10). We then filtered these CNVs against the DGV and this resulted in 114 CNVs that not only were exclusive to ARM patients, but also were absent in control individuals in the DGV. While 9 were recurrent (distributed in 4 CNVR-regions-), 105 were non-recurrent (Supplementary Table 11). These CNVs were subsequently classified according to their genic content (genic and non-genic CNVs).

Gene-based analysis: genes of the WNT and SHH signaling pathways are disrupted in ARM patients

As the pathogenicity of a genic CNV may be linked to not only the number of genes included but also to the biologically plausibility of the gene in relation to the phenotype under study, genes intersected by the CNVs were carefully scrutinized and prioritized. We then performed a gene-based analysis in which all CNVs were included. We identified 496 genes that were not

disrupted in controls (472 unique -disrupted in one patient-; 24 recurrent, Supplementary Table 12). After filtering against the CNVs of 11,943 healthy controls from the DGV, 79 genes (Supplementary Table 13) were found to be uniquely interfered by CNVs in our patients (Supplementary Figure 7).

Given the relevance of the *WNT* and *SHH* interrelated signaling pathways in embryonic development, together with the human ARM-reminiscent phenotypes displayed by animal models for those pathways (22, 23), we explored those CNV events that overlap with gene members of the *WNT/SHH* signaling pathways. We identified 2 patients (MG-IA349C-isolated-; MG-IA78C with Down syndrome) with a 34.4kb heterozygous deletion spanning exon 5 to exon 8 of *INTU* (4q28.1; Figure 1a), and one patient (MG-IA147C: isolated imperforate anus and diagnosed with autism at the age of 6) with a duplication (3 copies) of the whole *DKK4* gene (8p11.21; Figure 1b). These computationally predicted CNVs were validated using Taqman® copy number assays (Figures 2a, b). Importantly, the duplication of *DKK4* was *de novo* (Figure 2c). The inherited or *de novo* nature of the *INTU* deletion could not be established as parental DNA was not available.

INTU (inturned planar cell polarity effector homolog) encodes a structural protein that controls ciliogenesis and the organization of the cytoskeleton (governing the apical actin assembly and controlling the orientation of ciliary microtubules) and its disruption is associated with the failure in planar cell polarity (PCP) and hedgehog signaling pathways (24, 25). Many hedgehog pathway components, including the *Gli* family of transcription factors, localize to cilia and proper *Intu* expression is required for their ciliary translocation to the nucleus (26-29). Mutations in *Intu* cause loss of *Shh* signaling (*Gli1* protein) in the mouse posterior spinal cord, and mice die at E9.5(26). Importantly, defects in *SHH* (i.e. mutation in *Gli2* and/or *Gli3*) or

planar cell polarity (PCP) signaling lead to the ARM phenotype in mice (22, 23). The effect is dosage-dependent, i.e. more severe phenotypes are observed when two copies of the mutated genes are defective (22). Yet no coding region mutations in these genes have been identified in humans affected with isolated ARMs. *GLI3* coding sequence mutations are associated with Pallister-Hall syndrome (OMIM # #146510) which includes imperforate anus its phenotypic spectrum.

Excess of DKK4 leads to ARMs

DKK4 encodes a secreted protein member of dickkopf (*DKK*) family of *WNT* regulators. *DKK*s, together with *WNT* secreted proteins play an important role in antero-posterior axial patterning, limb development, somitogenesis and eye formation(30). During development, *DKK4* competes with *WNT* ligands for the co-receptors, thus antagonizing *WNT* signaling pathway. In mice, defects in *Wnt* signaling pathway lead to anorectal malformations (12, 13, 30, 31).

From all of the above, it would appear that deregulation of the *WNT* pathway by overexpression of *DKK4* may further impair *WNT* signaling and lead to ARMs. We then tested this hypothesis in a mouse anorectum organotypic culture. The urogenital sinus and the hindgut are connected at the cloaca at E12 in mouse embryo (Figure 3a). By E13.5, the cloaca is being separated by a sheet of mesenchyme called urorectal septum, which has elongated and descended towards the cloaca membrane (Figure 3b), and at the same time, the genital tubercle has grown distally due to the proliferation of the rostral mesoderm of the genital tubercle. This process compartmentalizes the cloaca into two cavities from which the anal opening and urethra opening will originate respectively. In control culture, the genital tubercle has grown distally after 36 hours. The urorectal septum has already elongated and reached the cloaca membrane (Figure 3c).

In contrast, treatment with Dkk4 protein (Figure 3d) perturbed the growth of the urorectal septum and resulted in the lack of cloaca compartmentalisation. The hollow space resembled the phenotype of persistent cloaca as shown in the mid-sagittal section depicted in Figure 3b. However, the distal growth of the genital tubercle appeared unaffected by the addition of Dkk4 protein. This experiment proves that excess of *DKK4* may lead to anorectal malformations. Therefore, it would appear that *DKK4* is a candidate gene for ARMs.

However as *Intu* is a cytoplasmatic protein, we could not test the effect of deletion directly by employing the same experiment design. Mutations in *Intu* had been reported before that they cause loss of *SHH* signaling (*Gli1* protein) in the mouse posterior spinal cord, and mice die at E9.5(26). Therefore, **remaining support for selecting *INTU* as a possible candidate gene is the fact that it is within a rare CNV and that it is involved in *SHH* signaling.**

DISCUSSION

CNVs are abundant and can be functionally influential. Their importance in human diseases has become increasingly apparent over the past five years. With the advancement in detection resolution and genome coverage of genotyping arrays, detection of copy number variations at genome-wide scale is possible. Based on the intensity of SNP and CNV probes in the array, CNVs can be predicted and analyzed for their association to the disease. Several large-scale studies have reported that CNVs, especially rare CNVs, may account for a significant proportion of human phenotypic variation, including disease susceptibility (32, 33). Data from the latest CNV studies indicate that disease status is more likely to be caused by an accumulation of rare CNVs rather than by differences in CNP loads (33). We now appreciate that at least 15% of human neurodevelopmental diseases are due to rare and large copy number changes which lead to local dosage imbalance for dozens of genes. Large CNVs, both inherited and *de novo*, have been implicated in the etiology of autism, schizophrenia, kidney dysfunction, and congenital heart disease (34). Studies of the general population suggest that collectively, rare CNVs are quite common and are under strong purifying selection. This implies that a significant fraction of the human population carries an unbalanced genome and such individuals, may be sensitized by the effect of another variant interacting with these CNVs in a digenic manner.

One of the major challenges in CNV discovery is to discriminate between benign and pathological variants. The rarer or longer the CNV, the more likely it is to be pathogenic. CNV is also more likely to be pathogenic when the genetic event is *de novo,* when CNVs are found only in patients and when the genes encompassed or disrupted by the CNV belong to a pathway known to contain genes associated with a similar phenotype under study (35, 36). Indeed, many

of the CNVs identified in this study meet some of the above criteria. Besides the 114 rare CNVs exclusive to ARM patients, rare CNVs were overall in excess in the patients. Moreover, some CNVs not only intersected with gene members of pathways (i.e. *SHH* and *WNT*) that are involved in the development of the anorectal region, but also contained genes associated with similar or related phenotypes in mice and humans (12-14, 22, 31, 37). Importantly, we could prove that the *DKK4* duplication was *de novo*. Interestingly, while mice mutant for *SHH* gene members (*Shh, Gli2*, or *Gli3*) displayed congenital defects that include ARMs as a common feature, point mutations in the human orthologs (*SHH*, *GLI3*) are associated with syndromes or genetically heterogeneous disorders in which the ARM phenotype is not always the norm.

As any other developmental disorders, rare chromosome aberrations (CNV longer than 1Mb) have been reported in 4.5–11% of the patients, mostly with syndromic ARMs (21). Indeed, ARMs can be part of the phenotypic spectrum of many chromosomal anomalies such as trisomy 13, 18, 21 or 22 to mention a few (21, 38). Here, we identified 12 chromosomal aberrations (besides trisomy 21) that were unique to isolated-ARM patients, indicating a role for those aberrations in isolated-ARMs.

Developmental disorders are notoriously associated with a myriad of rare chromosomal aberrations and CNVs, and their rarity makes clinical interpretation problematic and genotype-phenotype correlations uncertain. A genomic rearrangement shared by patients with phenotypic features in common surely implies greater certainty in the pathogenic nature of the CNV. Comparison of the chromosomal aberrations unique to ARM patients with those reported in patients of DECIPHER (Table 2) or in The European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA) has revealed the presence of the 22q11.2 deletion in the later of 6 cases with syndromic ARMs (21). A similar deletion was detected in

one of our patients with isolated-ARMs. Likewise, our patients shared rearrangements with DECIPHER patients with related phenotypes.

Surely, rare CNVs implicate several novel disease candidates genes since there is a multitude of ways in which gene function can be altered by these structural variations (alter gene dosage, disrupt coding sequences, or affect gene regulation and consequently may lead to disease). Indeed, our initial analyses showed an excess of rare CNVs in ARM-patients when compared to controls, with 79 genes disrupted by CNVs uniquely in patients, providing a wealth of putative disease candidate genes, in particular *DKK4* and *INTU*. Common genetic variants (SNPs and CNPs) have been found to have little contribution to the condition as ARMs, being phenotypically heterogeneous, are likely to result from rare mutations in a variety of genes. As in many other congenital diseases, several genes acting in different tissues and at different developmental stages may be involved in ARMs. Mutations in any of these genes could lead to the phenotype. Because each gene and its product are subject to complex regulation at every stage, the reach of a mutational event will depend on the gene implicated. Thus, the complexity of these molecular events would explain both the genetic heterogeneity and phenotypic variability of the condition. Our data suggest that the condition is likely caused by rare variants (CNV or single point mutation) in any of the genes implicated in the developmental processes. This would be in line with the lack of association signals for common genetic variants and the manifestation of the disease. Thus, rare DNA variations in any of the developmental genes implicated could not only lead to the phenotype but also explain its variability on the grounds mentioned above.

MATERIALS AND METHODS

Subjects and ethics statement

The overall study was approved by the institutional review board of The University of Hong Kong together with the Hospital Authority (IRB: UW 07-321). Blood samples were drawn from all participants after obtaining informed consent (parental consent in newborns and children below age 7).

ARM patients

A total of 363 Chinese sporadic ARM patients (isolated or with additional associated anomalies) had prospectively been collected throughout Hong Kong and Mainland China. All patients included in this study went through renal ultrasound, lumbosacral radiography and ECHO cardiography. Patients were initially grouped into discovery phase by genome-wide scan (185 patients) and replication series (178 non-syndromic patients). The overall male-to-female ratio was approximately 1.4:1. Phenotypic characteristics of the patients are summarized in Supplementary Table 1. Patients were defined as syndromic if associated anomalies were observed in addition to ARMs (see Supplementary Table 1b). In the discovery phase, we included 46 syndromic ARM patients, among whom 15 had Down syndrome.

Controls

As controls, we used the DNA sample from a total of 3,249 Chinese individuals (discovery phase: N=3,072, replication phase: N=177) whom also recruited throughout Hong Kong and Mainland China. For the discovery phase, we included 3,072 individuals who were either phenotypically normal (N=1,421) or affected with conditions other than ARMs (N=1,651).

These 1,651 individuals ("shared" controls) had been included in other GWAS conducted in our institution (i.e. patients affected with schizophrenia, hypertension(39), epilepsy(40) or systemic lupus erythematosus(41)). Details on the characteristics of the shared controls can be found in Supplementary Table 2. Individuals affected with other conditions were used as controls because: i) disease-specific effect in the controls can be diluted if it consists of balanced disease samples; ii) sharing samples from different projects can detect differential errors due to different DNA preparation and genotyping; iii) cost is reduced for collecting phenotype and genotype data from additional control samples; iv) power increases with the number of controls used. For the SNP replication phase, 177 phenotypically normal individuals were recruited as controls. For the CNV analysis, we included 868 individuals who are phenotypically normal from other studies (111 controls from hypertension study and 757 individuals from osteoporosis study (42)).

Discovery phase

Whole-genome scan

The whole-genome scan was performed at deCODE Genetics (Reykjavik, Iceland) using Illumina Human 610-Quad BeadChips which assay 599,011 SNPs across the genome and 21,890 intensity-only CNV probes. SNP calls were provided by deCODE. SNP quality control and association tests together with the results are detailed in the supplementary material and methods.

CNVs: predictions

CNV segments were predicted by two programs, PennCNV (43) and QuantiSNP (44), the two most efficient and publicly available CNV calling algorithms for Illumina data (45). Both

programs implement hidden Markov models (HMM) while PennCNV integrate additional information in CNV prediction (i.e. population allele frequency and distance between adjacent SNPs) when compared to QuantiSNP.

CNVs: quality controls

In spite of the advancement in CNVs detection using genome-wide SNP arrays and better CNV prediction algorithms, the concordance of CNVs called by different algorithms is still low (<50%) (46). This implies a high false positive rate in CNV predictions. To obtain high-confidence calls, we only used the overlapping region of CNVs called by PennCNV and QuantiSNP. Before selecting the overlapping CNV regions, quality controls were done separately for the CNV predicted by two programs.

For both PennCNV and QuantiSNP callings, CNVs shorter than 1kb or called with fewer than 3 probes were removed. In addition to these filtering criteria, we also remove CNVs with maximum Bayes factor less than 10 for the predictions by QuantiSNP. In the analysis, only those regions intersected by CNVs called by both programs were included. Samples with genome-wide LRR standard deviation greater than 3.5 or with more than 500 CNVs called were excluded from the analysis (patients=5; controls=17).

CNVs might be artificially split by either of the calling programs. To circumvent this issue, adjacent CNVs of the same type (i.e. duplication or deletion) were merged if the length of gap in between was shorter than half of total length of the two consecutive CNV segments.

After quality control, 170 ARM cases (Northern Chinese: 98, Southern Chinese: 72) and 851 controls (Northern Chinese: 37, Southern Chinese: 784) with 4,129 and 21,027 CNVs respectively in total were analyzed for the discovery of disease-associated CNV regions.

CNVs Analysis

Common copy number polymorphisms (CNPs) analysis, CNV replication and CNV validation are detailed in the supplementary material.

Rare CNVs

Rare CNVs are defined as CNVs that are observed in less than 1% of samples in the dataset (i.e. observed in less than or equal to 10 samples in this study). We firstly compared the global burden of rare CNVs between cases and controls. Then, we defined rare copy number variation regions (rare CNVRs; supplementary material) and analyzed each of them individually.

Global burden

Global burden tests were performed in terms of CNV length, number of CNVs and genes overlapped. Permutations tests conducted by PLINK were used to determine the statistical significance (1,000,000 permutations for each burden test). The global burden tests were used to examine the possible differences in terms of common or rare CNVs enrichment between (i) ARM-patients and controls (empirical 1-sided *p*-values are reported); and (ii) Northern Chinese controls and Southern Chinese controls (empirical 2-sided *p*-values are reported). The enrichment of long CNVs (defined as those CNVs with length longer than 1Mb) between ARM-patients and controls was also examined (empirical 1-sided *p*-values are reported).

Gene-based CNV analysis

With the overlapped CNV calls from pennCNV and quantiSNP, we selected those gene regions that were only found to be disrupted in the ARMs cases, but not in the 868 controls or the 11,943 unique normal individuals from the Database of Genomic Variants (DGV). We then examined the developmental genes that were only disrupted in ARMs cases.

Mouse anorectum organotypic culture

Timed pregnant mice (strain ICR) at embryonic day E12 were sacrificed. The embryonic anorectums were treated and cultured as described previously (47). Different treatments were applied to simulate different conditions: (i) as control: control culture was treated with PBS containing 0.1%BSA; (ii) excess of *Dkk4* protein (secreted protein): recombinant mouse *Dkk4* (R&D) proteins were added to the culture medium at a concentration of 1.5μg/ml.

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CONFLICT OF INTEREST STATEMENT

None declared

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LEGENDS TO FIGURES

Figure 1: UCSC Genome Browser showing the two ARMs implicated genes: (a) *DKK4* and (b) *INTU*, and the CNVs interfering with the gene regions.

(a) UCSC Genome Browser showing the gene region of *DKK4* and the part of the 1.32Mb-duplication (chromosome 8p12-q11.21, 3 copies) observed in an isolated ARM patient (MG-IA147C). The duplication is represented by the blue bar. There is no CNV observed in the normal samples submitted to the Database of Genomic Variants (DGV).

(b) UCSC Genome Browser showing the gene region of *INTU* and the 34kb hemizygous-deletions in 4q28.1 observed in two ARM patients (MG-IA349C with isolated-ARM; MG-IA78C with Down syndrome). The deletions are represented by the red bars. There is no CNV observed in the normal samples submitted to the Database of Genomic Variants (DGV).

Figure 2: **Validation of CNVs interfering with ARMs implicated genes: (a) *DKK4* and (b) *INTU***

(a) Validation of the duplication (3 copies) spanning the whole *DKK4* gene in 1 ARMs case (MG-IA147C) and of the normal copy number (2 copies) in other GWAS ARMs cases. MG-IA147C (the sixth bar from the left) has 3 *DKK4* copies while the rest of samples tested had 2 copies.

(b) Validation of the deletion (1 copy) spanning *INTU* in 2 ARMs cases (MG-IA78C and MG-IA349C) and of the normal copy number (2 copies) in other GWAS ARMs cases. MG-IA78C and MG-IA349C (the two rightmost bars) have 1 copy while other GWAS subjects had 2 copies.

(c) Validation of the duplication (3 copies) spanning the whole *DKK4* gene in 1 ARMs case (MG-IA147C) and proofing it to be a *de novo* event by validating the normal copy number in the parents (MG-IA147A and MG-IA147B) of this ARMs case.

Figure 3: Excess of Dkk4 protein led to ARMs as shown by mouse embryonic anorectum culture. Mid-sagittal sections of the anorectums of E12 (a) and E13.5 (b) ICR mouse embryos were shown. Organ culture of E12 anorectums from ICR mice treated for 36 hours in culture with (c) 1% BSA as control (Ctrl), and (d) 1.5 mg/ml of Dkk4 protein (+DKK4) were processed and sectioned. Mid-sagittal sections of cultured anorectums were shown. In control culture, the genital tubercle has grown distally after 36 hours. The urorectal septum has elongated and reached the cloaca membrane (c). In contrast, treatment with Dkk4 protein (d) perturbed the growth of the urorectal septum and resulted in the lack of cloaca compartmentalisation. The hollow space resembled the phenotype of persistent cloaca as shown in the mid-sagittal section depicted in Figure 3b. However, the distal growth of the genital tubercle appeared unaffected by the addition of Dkk4 protein.

Abbreviations: cl, cloaca; cm, cloaca membrane; hg, hindgut; GT, genital tubercle; ugs, urogenital sinus; urs, urorectal septum. Bar 0.05mm. Dotted lines demarcate the hindgut.

TABLES

Table 1: **Global burden of RARE CNVs in ARMs cases and controls**

		Rare CNVs with length <100kb			Rare CNVs with length >100kb		
		Cases (n=170)	Controls (n=851)	Empirical *p*-values	Cases (n=170)	Controls (n=851)	Empirical *p*-values
Total number of segments							
	Deletion	538	2165		66	216	
	Duplication	218	888		126	374	
Number of rare CNVs per sample							
	Deletion	3.165	2.544	**0.03945**	0.3882	0.2538	**0.021955**
	Duplication	1.282	1.043	**0.007344**	0.7412	0.4395	**0.002307**
Proportion of samples with one or more rare CNVs							
	Deletion	0.9059	0.8261	**0.00484**	0.2647	0.2162	0.100801
	Duplication	0.6941	0.6381	0.093875	0.4294	0.3467	**0.025701**
Total length of rare CNVs spanned per sample (in kb)							
	Deletion	92.21	84.2	0.2479	314.6	268	0.234714
	Duplication	69.67	57.38	**0.01022**	505.2	373.9	0.060274
Number of genic regions spanned by rare CNVs per sample							
	Deletion	1.247	1.905	0.9369	0.4882	0.3384	0.15943
	Duplication	0.8588	0.6616	**0.026837**	1.176	0.8461	0.063724
Number of genic CNVs per sample							
	Deletion	0.5941	0.5347	0.09023	0.1118	0.1234	0.704262
	Duplication	0.4529	0.3643	**0.018863**	0.3118	0.2526	0.068167

Statistical significance was inferred using permutation with 1,000,000 iterations. Rare CNVs are defined as CNVs that are observed in less than 1% samples of the dataset. ARM cases (isolated: N=126; syndromic: N=44) are enriched with rare deletions and rare duplications by 1.3 fold each. Separate global burden tests were also performed by stratifying the isolated and syndromic ARM patients. Details are presented in the supplementary Table 7.

Table 2: Chromosomal aberrations that were only observed in ARM-patients

Chr	Chromosomal aberrations	Patient ID	Isolated (I) or Syndromic (S)	Genes uniquely disrupted in cases	Patients with related symptoms listed in DECIPHER (patient record, type of CNV, phenotype)
1	1.3Mb Duplication in 1q21.1	MG-IA201C	I	*ACP6*	record 983: DUP, absent uterus, fused labia, vaginal atresia
1	2.3Mb Deletion in 1q42.3-q43	MG-IA195C	I	*RBM34, MTR*	
1	8.1Mb Deletion in 1q43-q44	MG-IA87C	I	*FAM152A*	record 249405 [b]: DUP and DEL, megacolon/Hirschsprung syndrome, general abnormalities in heart
2	1.5Mb Deletion in 2q37.3	MG-IA33C	I		
5	3.7Mb Deletion in 5p15.33	MG-IA41C	I	*ISL1*	record 4119 [b]: DUP, megacolon/Hirschsprung syndrome, general abnormalities in heart
5	1.1Mb Duplication in 5q11.2	MG-IA147C	I[a]		record 1946: DEL, intestinal malrotation
7	1.4Mb Duplication in 7p21.3-p21.2	MG-IA370C	I		
7	6.7Mb Duplication in 7p11.1-7p11.21	MG-IA360C	I		
8	13.2Mb Duplication in 8p12-q11.21	MG-IA147C	I[a]	*ZNF703, ERLIN2, PROSC, BRF2, RAB11FIP1, GOT1L1, ADRB3, ASH2L, STAR, DDHD2, PPAPDC1B, FGFR1, TACC1, C8orf4, GOLGA7, GINS4, AGPAT6, NKX6-3, AP3M2, **DKK4**, C8orf40, CHRNA6, THAP1, CEBPD, SNAI2*	
11	8.4Mb Duplication in 11q14.3-11q22.1	MG-IA152C	I	*JOSD3, C11orf54, MED17, LOC390243, GPR83, PIWIL4, AMOTL1, CWC15, JMJD2D, SFRS2B, ENDOD1, FAM76B, MTMR2, CCDC82, JRKL*	
15	1.6Mb Duplication in 15q13.2-q13.3	MG-IA230C	I		

22	2.6Mb Deletion in 22q11.21	MG-IA162C	I	*SERPIND1, SNAP29*	record 252033 [b]: DUP, anal atresia/stenosis, general abnormalities in sacrum and kidneys; record 2366 [b]: DEL, anal atresia/stenosis, general heart abnormalities; record 249397 [b]: DEL, megacolon/ Hirschsprung; record 622 [b]: DEL, absent uterus, general abnormalities in kidneys; record 1645 [b]: DEL, malformed uterus, renal agenesis, fusion of vertebrae

Chromosomal aberrations are defined as CNVs with length longer than 1Mb. The patient IDs are listed to show the number of patients harboring the chromosomal aberrations. The chromosomal aberrations were also checked against the DECIPHER database for any other patients with similar or ARM-related symptoms. [a] patient was diagnosed with autisms at the age of 6; [b] this is the only chromosomal aberration observed in this DECIPHER patient

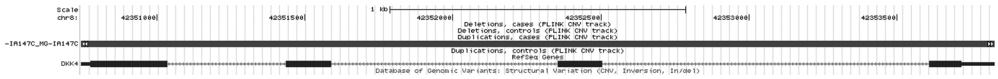
Table 3: Results of the top three rare CNV regions from the permutation test and Fisher's exact test.

Chr.	Starting position (in hg18)	Ending position (in hg18)	Number of cases	Number of controls	Permutation test		Fisher's exact test	
					Empirical *p*-values	Empirical *p*-values corrected for all tests	*p*-values	*p*-values after Bonferroni correction
7	38285115	38330273	7	0	2.00×10^{-06}	0.000147	3.20×10^{-06}	0.00779436
14	21937715	22009307	6	0	0.000134	0.001372	1.98×10^{-05}	0.048239486
1	40794563	40804646	7	3	0.000240	0.022894	0.000243573	0.594073571

Genic and non-genic regions are defined as those that harbour at least one CNV in cases or controls. These three regions do not harbor any genes, i.e. non-genic regions. Permutation tests (1,000,000 iterations) were performed on 2439 rare CNV regions (1982 genic regions and 457 non-genic regions) by using PLINK. According to PLINK, the empirical *p*-values corrected for all the tests were calculated by comparing each observed test statistic against the maximum of all permuted statistics (i.e. over all regions) for each single replicate. We also performed the Fisher's exact test on these regions, and included a Bonferroni correction for 2439 total CNV regions.

Abbreviations

Anorectal Malformation	ARM
Copy Number Polymorphism	CNP
Copy Number Variation Region	CNVR
Copy Number Variation	CNV
Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources	DECIPHER
Database of Genomic Variants	DGV
Dickkopf homolog 4 (Xenopus laevis)	DKK4
The European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations	ECARUCA
Inturned planar cell polarity effector homolog	INTU
Planar Cell Polarity	PCP
Principal Components Analysis	PCA
Quality Control	QC
Single Nucleotide Polymorphism	SNP



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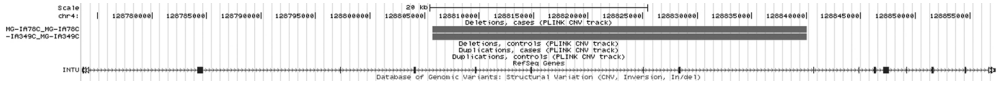

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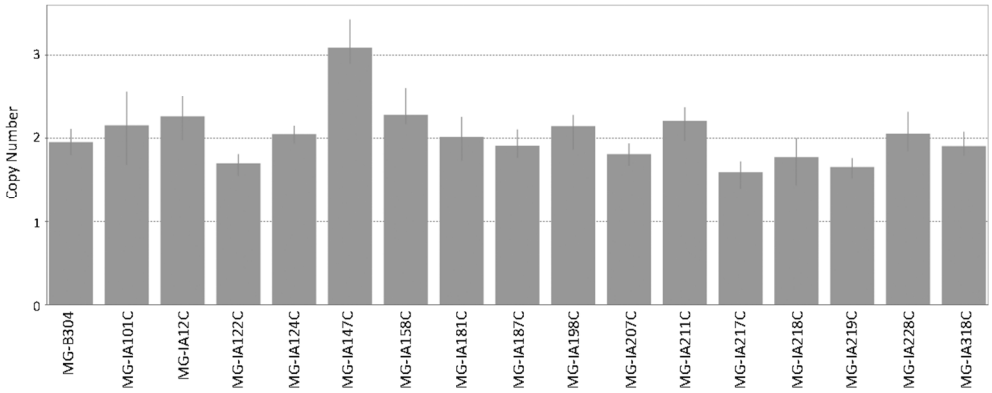

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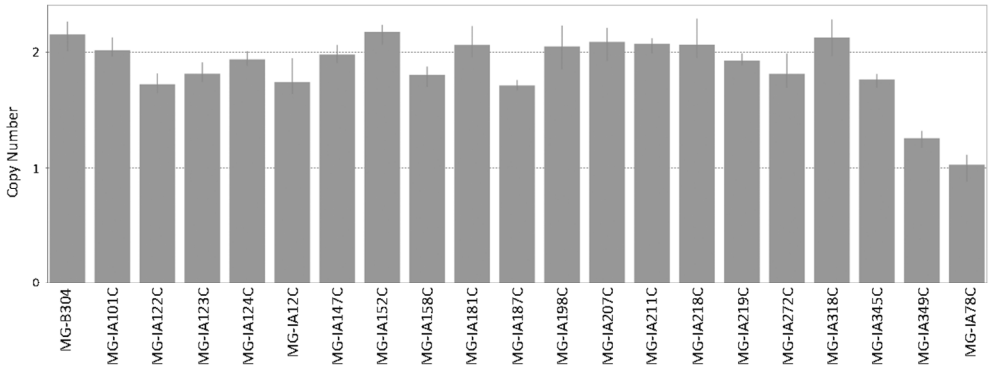

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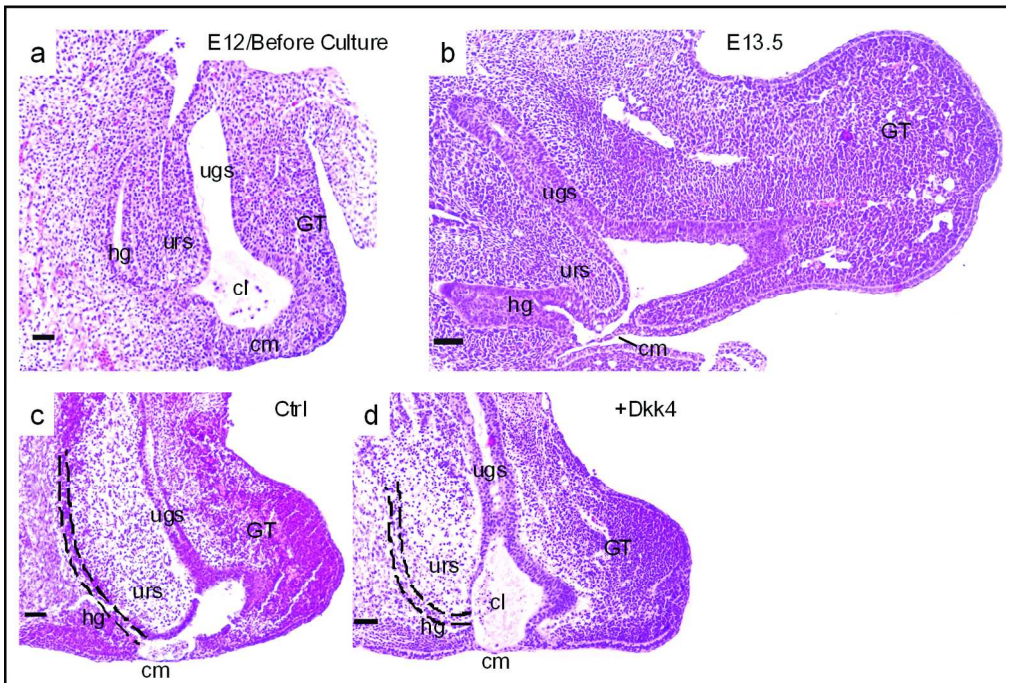

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