Summary and conclusions
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The present study included a thorough genetic analysis of the sex reversal cases with diverse phenotypes to ascertain the phenotypic identification of each category and understand the genetic etiology. The AIS cases along with many others are categorized as primary amenorrhea; however, the genetic etiology behind this disorder remains elusive in a significant number of cases. Our study revealed that most of the cases classified as 'primary amenorrhea' were in fact androgen insensitivity patients. Pelvic ultrasound in AIS cases indicated testis in the abdomen or the inguinal canal, and the histology of the gonadal tissues revealed the presence of well-distinguished seminiferous tubules with various degrees of cell differentiation. This suggests that a careful clinical examination along with the gonadal biopsy can help distinguish these cases from the other disorders.

Another feature of AIS was the presence of androgens in the normal male range or even higher. Secondary sexual features developed up to different extents in these individuals, which therefore may not always help in distinguishing these patients from others with similar phenotype. Most of the patients included in this study presented late due to unawareness about the disorder, and the late presentation was responsible for most of the lesions observed in the gonadal tissue, which may even be life threatening due to chances of carcinogenesis. Consideration of the age at presentation and the extent of the gonadal lesion in our study suggested that the gonads should be removed before the age of 20 years, to avoid malignancy due to disturbed levels of testosterone, LH and FSH.
The identification of the mutations in AR gene in a significant percentage (~20%) of AIS cases could explain the genetic etiology of the disorder, and also indicated the frequent occurrence of AR mutations in these cases. Mutations in different individuals rendered AR non-functional up to different extents, resulting in an array of disorders represented on a scale between PAIS and CAIS. Most of the mutations were substitutions of the highly conserved amino acids with functionally similar or dissimilar residues, indicating a higher frequency of substitutions in comparison to the deletions/insertions in AR gene.

As indicated by the previous studies the deletions are more common in exons 1 and 2, while substitutions are more common in the exons 3-8. This may also be one of the reasons why we observed only substitution mutations in these cases. Structural-functional correlation helped to correlate the mutations with the phenotype in most of the cases.

We observed more number of mutations in the LBD in comparison to the DBD region; however this may not correlate with the frequency of mutation in these regions and may simply reflect the large size of the LBD. In vitro assays using mutant AR gene indicated the loss of ligand binding to the receptor or the loss of transactivation function of the ligand-AR complex. The mutations in the AR-LBD resulted in the partial to complete loss of ligand binding, which also resulted in the loss of transactivation function due to failure of complex formation between AR and the ligand. Similarly, most of the mutations in AR-DBD resulted in the loss of transactivation function but these mutations also affected the ligand-binding to certain extents. In either condition the extent of the loss of function could be correlated with the phenotype in most of the cases.
We studied complete coding and UTR regions of SRD5A2 gene, and the triplet repeats of AR gene to understand the genetic basis of the varying manifestation of AIS. No mutation was found in SRD5A2 gene, which could contribute to the variation in the phenotype, however, as evidenced by earlier studies the mutations in this gene may account for the variation in the phenotype in the affected siblings from the same family. We found putative role of AR-CAG repeat in affecting the overall AIS phenotype. On the basis of the smaller size of the CAG repeats among the PAIS cases in comparison to CAIS, we proposed that the loss of AR function, due to the mutations causing partial loss of AR function, may be compensated up to some extent by the presence of shorter CAG repeats in the background. Similarly, the presence of longer CAG repeats in the background may enunciate the overall extent of androgen insensitivity. However, the demonstration of the same in the familial cases with varying phenotype would be of further help to illustrate the concept.

Among the other category of the sex reversals were the gonadal dysgenesis cases. The sequencing of the major sex-determining genes in these cases could not explain the etiology of the disorder in 90% of the familial cases (9 out of 10). The genome wide scan on these families aimed at searching the additional genes involved in the sex reversal resulted in identification of a putative sex-determining novel locus on X-chromosome. We narrowed down to a region of 3.41 Mb with LOD score of 5.77. Unfortunately, in the highest density mapping, we observed no recombination in a stretch of 12 markers. Human genome database had 53 transcript entries for this region of X-chromosome.

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In silico analysis of this region indicated certain candidate genes namely UBE1, SSX2, MAGED1, GAGEC1, ZNF741, ZNF21 and WDR13, NUDT10, NUDT11. However, the sequencing of the later genes did not result in the identification of any mutation, which could underlie this disorder. The lack of the information about the expression of most of these genes, in the indifferent gonad was the major complicating factor in the selection of the candidate genes more precisely and hence identifying the underlying gene. To select the candidate genes more precisely, the information regarding the expression of these genes in the indifferent gonad is a must. Availability of such information in databases would be immensely useful for selecting the right candidate gene(s). The identification of the underlying gene may also be helped by the identification of more families with the sex reversal/gonadal dysgenesis, which may help to further narrow down the region of interest by a recombination in the stretch of 12 markers identified in this study.

The study of the unique cases of sex reversal included a 46,XX male individual, who was completely masculinized and had almost normal male genitalia despite the absence of SRY gene. The infertility could be explained by the absence of spermatogenic genes on the Y-chromosome, however the development of external genitalia and testicles despite the absence of SRY gene, indicated the existence of other potential candidates, which may initiate the testicular differentiation in the absence of SRY gene. These candidates may be the normal partners in the sex-determination, which when mutated, may give rise to male phenotype, despite the absence of SRY gene. It is also possible that the alternation of the dosage of a gene, which is normally upregulated by SRY gene, might have resulted in the initiation of the male pathway in this individual. In either condition, various possibilities
for the phenotype give clue to the existence of more sex-determining genes, which need identification to completely understand the process of sex reversal and sex-determination.

Another unique case was a SRY negative 46,XX individual with ambiguous genitalia. The presence of SRY gene in 46,XX individuals have been mentioned earlier in multiple studies, however ovary differentiation and regular menstruation observed in this individual was interesting. The histology of the gonads showed ovarian tissue in both the gonads and also the presence of few mature Graffian follicles in the ovaries. The individual consisted of the undifferentiated vaginal folds, an enlarged phallus, and separate vaginal and urethral openings. Extensive genetic analysis showed no mutation in any of the known sex-determining genes. We also analyzed several STR markers from X and Y-chromosomes. However, the genetic etiology could not be explained by mutation in any of these genes. The real-time PCR analyses for the gene copy number identified four copies of WNT4 gene in this individual. Taking into consideration this observation and the evidences from previous studies, we hypothesized that the WNT4 gene may be placed at the crossroads of the sex-determination pathway and the switch may be decided at the level of WNT4 gene.

In conclusion, our study on the genetic basis of sex reversal resulted in the identification of several pathogenic mutations in AR gene resulting in AIS. In vitro assays emphasized the pathogenic nature of AR mutations. Phenotypic variation in AIS cases could be attributed to the CAG repeats length variation up to certain extent. The mapping of the unexplained sex reversal phenotype identified a region on Xp11.21-11.23, which will fuel
the studies aimed at identification of sex-determining genes. Extensive genetic analysis of
the unique sex reversal cases also indicated the existence of more unknown sex-
determining genes, along with providing the clues to the genetic cascade of sex-
determination. Moreover, the identification of AR mutation was helpful in providing
genetic counseling to the affected individuals and carrier siblings to abandon the
transmission of the defective AR alleles to the coming generations.

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