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CHROMOSOMAL ABERRATIONS AND MUTATIONAL ANALYSIS OF BMP-15 GENE IN AMENORRHEA

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ABSTRACT

Prevalence of amenorrhea is higher in Indian population with a potential social impact. The study of cytogenetic pattern and mutational studies in its correlation with other physiological factors will help for better understanding the cause and earlier diagnosis. BMP15 gene (Bone Morphogenetic Protein 15) Cytogenetic Location: Xp11.2. To find out the cytogenetic pattern and BMP-15 gene mutations among amenorrhea patients and to correlate with the phenotype and clinical findings among these cases. This study was performed on 200 women volunteers after taking an informed consent. Out of 200 cases 100 cases were grouped as controls and 100 cases as study group. Cytogenetic study was done by conventional karyotyping and other clinical investigations (hormonal assay, fasting blood glucose) were also carried out. Mutational analysis was performed on 197 women volunteers. Out of 197 cases 97 cases were grouped as controls and 100 cases as study group using PCR. In the present study the frequency of classic Turner's (25%) and mosaic Turner's (12.5%) among primary amenorrhea cases were analyzed. All Secondary amenorrhea cases were with normal chromosomal complement that is 0% frequency of chromosomal aberrations. One case with polycystic ovarian disease exhibited Premature Chromatid Separation (PCS). Mutational analysis showed 46% of the subjects among study group with mutated BMP-15 gene. Identification of known genetic causes could aid in development of effective treatments for women with amenorrhea, as well as earlier diagnosis which may allow for family planning before the onset of amenorrhea.

KEYWORDS: ***BMP-15, mutations, amenorrhea, Imperforate hymen, Mulleriandysgenesis, Premature chromatid separation, Turner's syndrome, X-chromosome, chromosomal aberrations.***

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INTRODUCTION

Onset of menstruation is termed as menarche and it marks the advent of reproductive period of women. Absence of menstruation is termed as amenorrhea. The incidence of menstrual irregularity among Indian women is 5%, indicating that menstrual disturbance is a persisting problem among them.[1] With a potential social impact and higher prevalence of amenorrhea in the Indian society, extensive evaluation is required to overcome anxiety. Integration of hormones from hypothalamus, pituitary and ovary is required for regular menstrual cycles. Normal menstruation requires anatomically normal reproductive tract and a genetically normal chromosomal compliment of 46, XX.[2] BMP15 gene (Bone Morphogenetic Protein 15) is also known as Gdf- 9B(Growth differentiation factor) which is located at Xp11.2 and holding a taxonomic identifier as 9606 with a sequence length of 392 AA . The molecular position on the X chromosome is from 50,653,734 to 50,659,640 base pairs. (NCBI Gene 2013). There is strong evidence to point out that BMP-15 is responsible for developing secondary follicles from primordial follicles from the ovary. The mechanism of action of bone morphogenic protein is thought to be either by direct stimulation of the ovary or influence of the specific growth differential factor.[3] BMP – 15 gene (MIM 300247) is expressed in oocytes present in ovary.[4] Studies have indicated that bone morphogenetic protein has a role to play in maturation of the developing oocyte and in the development of secondary ovarian follicles from primordial ovarian follicles. Defective bone morphogenetic protein results in defective ovary.[5] The present study is performed to find out cytogenetic pattern among amenorrhea patients and to correlate with the phenotype among these cases. The mutational correlation of BMP15 gene in cases of amenorrhea among amenorrhea cases was observed using PCR among women volunteers.

MATERIAL AND METHODS

This case-control study was performed on 200 women of 3 years. The volunteers were outpatients attending the OPD of Vinayaka Missions Medical College Hospital, Salem, Tamil Nadu, India. Ethical clearance and approval for the study were obtained from Institutional Ethics Committee and Institutional Review Board of Vinayaka Missions Medical College.

Cytogenetic analysis

Out of 200 cases 100 cases were grouped as controls and 100 cases as study group according to following criteria-

Inclusion criteria for study group: (ASRM, 2008)[6]

- Age: 16 to 40 years old females.
- Patients with primary amenorrhea due to non-attainment of menarche.
- Patients with secondary amenorrhea due to the following causes: anatomic defects of the genital tract, hypothalamic/pituitary causes, endocrinopathies, chronic oligomenorrhea or anovulation, polycystic ovarian syndrome, premature ovarian failure.

Inclusion criteria for control group

- Age: 16 to 40 years old females.
- Women with the history of regular menstrual cycles.
- Women with normal serum hormonal levels.

Exclusion criteria for both study and control groups

- Age: Women below 16 years and above 40 years.
- Pregnant and lactating mothers.
- Women undergoing any treatment with medication or drugs affecting menstrual cycle.
- Women having history of surgical treatment in relation to genital tract.

Diagnosing primary and secondary amenorrhea was carried out with the help of detailed history, physical examination and laboratory testing.[7]

Work flow of Karyotyping

AGT Cytogenetics Laboratory Manual [8]

Sample collection → Culture → Harvesting → Slide preparation → Staining → Interpretation.

Peripheral blood was collected in a sterile heparin coated vacutainers with aseptic precautions. Blood sample was inoculated in a culture tube containing RPMI 1640 culture medium, Phytohaemagglutinin (PHA) and Autologous plasma. The cultures were incubated in 370C for 72 hours. The cell division was arrested in metaphase stage of cell cycle by using Colchicine solution. Hypotonic solution treatment was done by using potassium chloride solution. The cells were fixed by using fixative and after three to four washes with fixative the slides were prepared by dropping two to three drops of cell suspension from 2 feet height over a cleaned slide. The staining was done by conventional GTG banding. The stained slide was analyzed based on [Fig-1]. Chromosomes were classified and interpreted.

Mutational analysis

The mutational analysis was performed on 197 women volunteers after taking an informed consent for a period volunteers and 3 women out of 200 were excluded with chromosomal aberrations so, out of 197 individuals 97 cases were grouped as controls and 100 cases as study group. Inclusion criteria for study and control groups for mutational analysis are similar as cytogenetic analysis and women with normal chromosomal complement are included. Exclusion criteria for study and control groups for mutational analysis are similar as cytogenetic analysis and women with abnormal chromosomal complement are excluded.

Steps involved in mutational analysis

- ***Isolation of DNA***

  The DNA was isolated from the whole blood using Helini Pure fast human blood genomic DNA minispin prep kit protocol.

- ***Primer design***
  Primer pair such as F4 primer sequence 5'-

  AGTGACGTCCCTTGGGCTTG-3 (sense) and R4: 5'- CAAAGCCTGACAGTAAACCC-3 (antisense) with amplification site GDF-9/BMP-15; exon 1 having an amplification length of 477 base pairs. [Table-1][9]

- ***PCR set up***
  For PCR the volume of the cDNA synthesis reaction mixture was not more than 1/10 of the total PCR reaction volume and 2.5µl of the RT mixture for 25µl PCR total volume was taken. [Table 2, 3]

- ***Agarose gel electrophoresis***
  Bands were observed over UV-Transilluminator.

STATISTICAL ANALYSIS

Based on the diagnosis the study group was categorized into four sub-groups: Individuals with- 1) Primary amenorrhea (PA) (n=8); 2) Secondary amenorrhea with unknown etiology (SA) (n=6); 3) Polycystic ovarian disease (PCOD) (n=82), Premature ovarian failure (POF) (n=4).

RESULTS

Cytogenetic analysis

Out of eight primary amenorrhea samples three samples were found to have an abnormal karyotype. Out of these three samples, two had classic Turner's syndrome with chromosomal complement 45, XO [Fig2] and one sample exhibited mosaicism 45, XO / 46, XXq-[Fig3]. In the present study normal chromosomal complement was found in all secondary amenorrhea (PCOD, POF, and secondary amenorrhea with unknown etiology) cases. But only one case with PCOD exhibited PCS. Karyotype wise distribution of subjects among the cases and controls is demonstrated in [Table- 4].Clinical features of patients with primary amenorrhea in the present study are depicted in [Table- 5]. In present study one secondary amenorrhea with PCOD case was found to have premature chromatid separation (PCS) [Fig- 4].

Mutational analysis of BMP-15 gene

In the present study one exon of BMP-15 gene with primer was amplified. The result is displayed in [Table-6]. In the study group 46% (n=47) of the subjects demonstrated the presence of bands which is indicative of having mutated BMP-15 gene. And two cases (2%) of the control samples showed positive to mutations **[**Fig-5].

DISCUSSION

Cytogenetic investigations are considered as the most valuable and fundamental investigation in the diagnosis of amenorrhea. Frequency of sex chromosomal anomalies among amenorrhea cases in different studies is shown in [Table- 7].[10-19] In the present study the frequency of classic Turner's (25%) and mosaic Turner's (12.5%) among PA cases is similar when compared with previous studies in which they were ranging from 7%-46%. But, the frequency of chromosomal aberrations in SA cases was varying widely in different studies. In the present study all secondary amenorrhea (PCOD, POF, and secondary amenorrhea with unknown etiology) cases were with normal chromosomal complement that is 0% frequency of chromosomal aberrations. Frequency of classic and mosaic form of Turner's syndrome among primary amenorrhea cases in various countries is shown in [Table-8][15,18-27]. The figures quoted in this table show a wide range of frequency distribution in different countries, where the range of classic Turner's is 2%-63% and the range of the mosaic Turner's is 15%-66%. In the present study the percentage of classic Turner's was 25% and mosaic Turner's was 12.5%. The phenotype presentation of the present case varied widely when compared with the previously reported cases on Xq deletion. [28] Prevalence of Xq deletion has been found to be a rare structural aberration among PA cases shown in [Table-9]. [10,12,14,16,19]. Mosaicism with Xq deletion was the most uncommon sex chromosomal aberration which was seen in one PA case of the present study. Premature chromatid separation is often confused with a similar term called Premature centromeric division which is characterized with a rod shaped X-chromosome and without a distinct centromeres and this is due ageing among women.[29] Errors of cell division lead to formation of gametes which have more chances of non disjunction which could be a cause for spontaneous abortion, recurrent abortions and Down's syndrome child.[30] The frequency of premature chromatid separation was 10% - 45% was observed among metaphase spreads in couples with a history of recurrent abortions.[31] All cases with premature chromatid separation were having normal chromosomal complement 46, XX in females and 46, XY in males.[32] In present study a case of woman of 25 years of age, having PCS[Fig-4] with a normal chromosomal complement was found with 20% of metaphase spreads were exhibiting PCS. Hormonal levels (FSH, LH, Prl & TSH) were within normal limits and exhibited a normal phenotype. The Fasting blood glucose level was also normal. Critical regions on X-chromosome were identified as Xq and Xp11.2-p22.1. These regions are functionally necessary for the development of ovary.[33-35] Pathogenesis and etiology of ovarian disorders are still unclear among the amenorrhea cases with normal chromosomal complement. BMP-15 gene (MIM 300247) is located at locus Xp11.2which is within the critical region of X-chromosome. Several reports confirmed that "BMP-15" gene is responsible for the development of ovary in animal models.[4,35,36] Among study population 46% (n=47) demonstrated the presence of bands which means having mutated BMP-15 gene and only 2% (n=2) of the control population showed positive to mutations [Fig- 5]. Distribution of mutational analysis is shown in [Table-6]. The difference between study and control groups was found to be highly significant with a *p* value of <.00001.As mutational analysis showed higher percentage of mutations among test samples not only when compared with the controls of present study but also increased mutational levels was observed when compared with the previous studies which proves that BMP-15 is a candidate gene. As very few studies were conducted in India this study explains the need of further

investigations on BMP-15 gene like Single Nucleotide Polymorphism, DNA sequencing among amenorrhea cases to note the exact site of mutations. Frequency of BMP-15 gene mutations in different countries reported by various authors is depicted in [Table-10]. This table proves the presence of a significantly higher percentage of mutations in the present study (46%) when compared with the previous studies. No study has been reported earlier related to mutational analysis of BMP-15 among women with PCOS and in the present study many subjects were with PCOS (82%).This study provides evidence of BMP-15 gene mutations in women with PCOS. Further the study can be extended to detection of single nucleotide polymorphism and DNA-sequencing of BMP-15 gene among women with PCOS to detect exact position of nucleotide replacement.

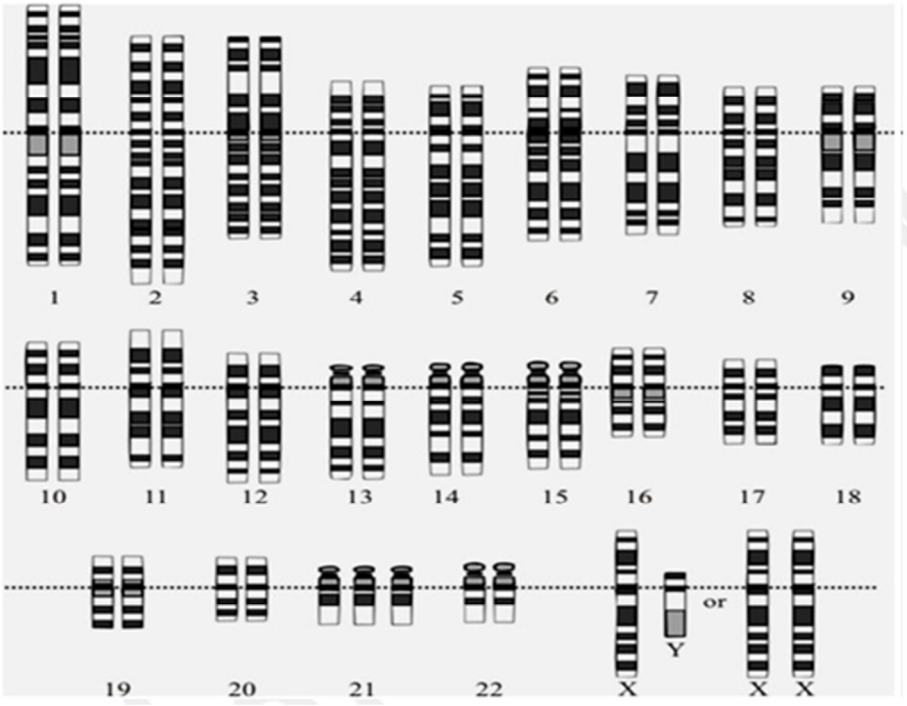


Figure 1
Normal banding patterns of Metaphase chromosomes

Table 1
Primer Design

Sl. NO	Sequence ID	Sequence (5'–3')	Length	Yield (OD)	Yield (ug)	Volume 100 pmol/µl	Tmp (^{0}C)	MW (g/mol)	GC content
1	CP23224	AGT GAC GTC CCT TGG GCT TG	20	10.7	353.1	575	55.9	6140	60%
2	CP23225	CAA AGC CTG ACA GTA AAC CC	20	10.7	353.1	582	51.8	6064	50

Table 2
Components for PCR and their volumes

Component	Volume
Helini 2X RedDye PCR Master Mix	20µl
Primer mix	7µl
cDNA- synthesis	3µl
Final volume	30µl

Table 3
Thermal profile Maintained in PCR

Step	Time	Temp
Initial denaturation	5-3 min	95 ^{0}C
35 cycles Denaturation	30 sec	95 ^{0}C
Annealing	30 sec	60 ^{0}C
Extension	30 sec	72 ^{0}C
Final extension	3 min	72 ^{0}C

Figure 2
Metaphase spread of a Turner's syndrome patient with chromosomal complement (45 XO).

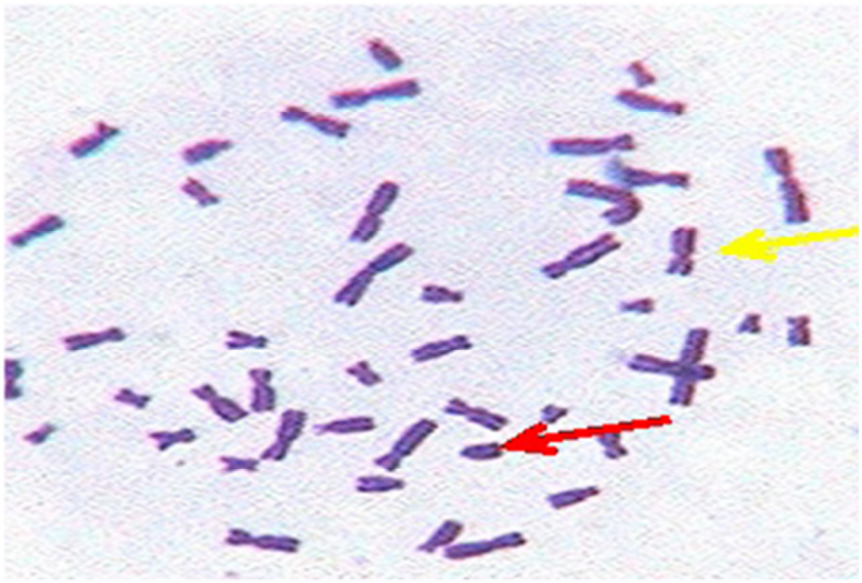


Figure 3
Metaphase spread showing Mosaicism and Xq deletion Normal X- chromosome (yellow arrow); X chromosome with q arm deletion (red arrow). (45,XO/46,XXq-)

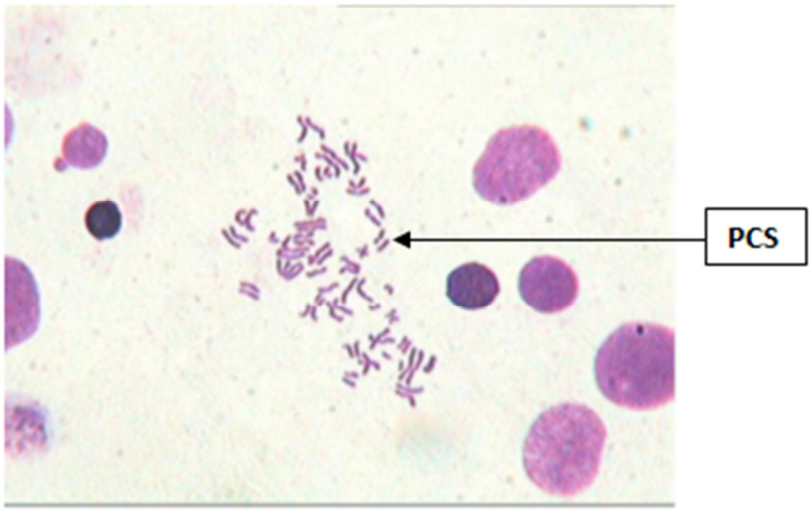


Figure 4
Metaphase spread displaying Premature chromatid separation (PCS)

Table 4
Karyotype wise distribution of subjects among the cases and controls

Karyotype	Cases	Controls	P value (*X*2 by applying Yate's correction)
46XX	97	100	<.0001(HS)
46XO	2	0	
45XO/46XXq-	1	0	
Total	**100**	**100**	

HS- highly significant

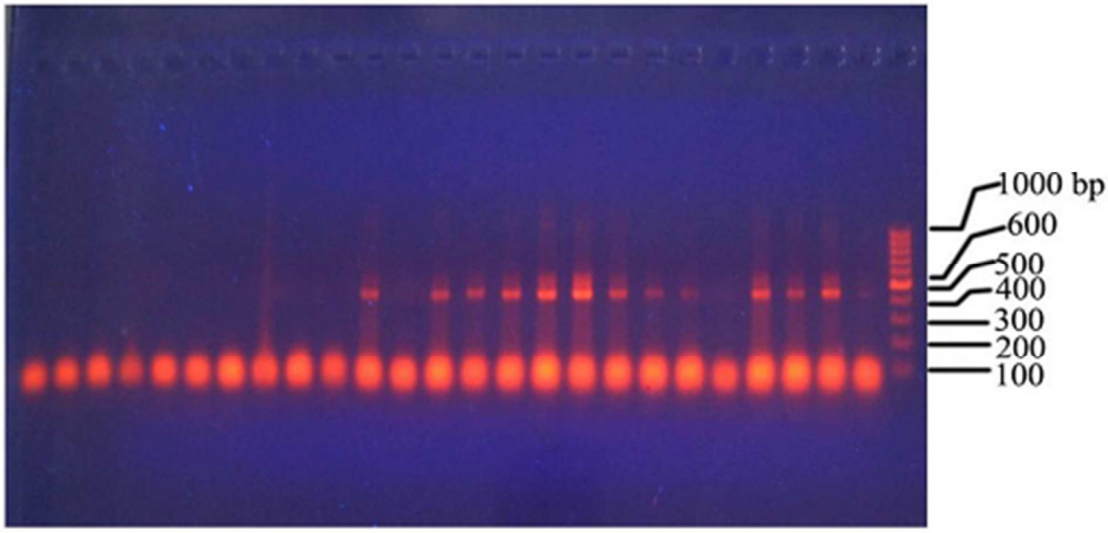
Table 5
Clinical features of patients with primary amenorrhea in the present study

Clinical features	Classic TS (45,XO)	Mosaic TS (45,XO/46, XXq-)	PA with normal karyotype (46, XX)	Total
No. of Cases	2	1	5	8
Short stature (<150 cm)	2	1	0	3
Webbed neck	0	0	0	0
Short neck	1	1	0	2
Breast developmental delay	2	1	0	3
Presence of Rudimentary uterus/ absence of uterus	2	1	4	7
Absence of ovaries/streak gonads	2	1	2	5
Raised FSH levels (>24ng/ml)	2	1	4	7
Raised LH levels (>16µg/L)	0	1	0	1

TS-Turner's syndrome, PA- Primary amenorrhea, FSH-Follicle stimulating hormone, LH-Luteinizing hormone

Table 6
Frequency distribution of BMP-15 gene mutations between cases and controls

Mutations	Cases (N=97)	Controls (N=100)	P value (by applying *X2* square test)
Present	47	2	<.00001(HS)
Absent	50	98	
Total	98	100	



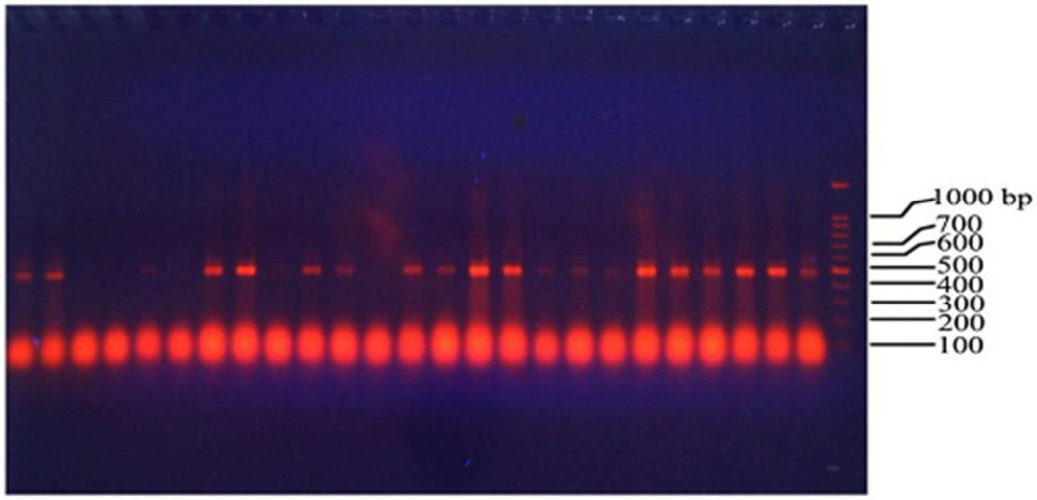


Figure 5
Showing bands over UV-trans illuminator in test samples near 500bp which implies presence of mutations in BMP-15 gene

Table 7
Frequency of sex chromosomal anomalies among amenorrhea cases in different studies

Author	Year	Total No. (N)	No. of PA (n)	No. of SA (n)	No. of PA with CA (%)	No. of SA with CA (%)
Goldman, et al	1982	107	63	44	10(6.3%)	1(0.4%)
Opitz, et al	1983	103	88	15	25(28%)	5(33%)
Ten, et al	1990	117	117	-----	36(31%)	------
Goud, et al	2006	58	58	-----	8(14%)	------
Rajangam, et al	2007	865	620	245	161(26%)	39(16%)
Xiao, et al	2008	131	131	------	48(36.6%)	------
Kalavathi, et al	2010	979	852	127	221(26%)	9(7%)
Vijayalakshmi, et al	2010	140	140	-----	39(27.8%)	------
Jouyan, et al	2012	354	354	------	163(46%)	------
Datta, et al	2013	637	251	28	132(20.7%)	
Present study	2016	100	8	92	3(37.5%)	0(0%)

PA- Primary amenorrhea, SA-Secondary amenorrhea, CA-Chromosomal aberrations, N- Total number of samples

Table 8
Frequency of classic and mosaic form of Turner syndrome among primary amenorrhea cases in various countries

Country	Classic Turner (%)	Mosaic Turner (%)	Reference
Brazil	29	53	Duarte, et al. 2004
Korea	2.1	50.8	Kim, et al. 1999
Italy	50	37	Nucaro, et al. 2008
Denmark	45	15	Nielsen, et al. 1991
Minnesota	42	48	Wiktor, et al. 2005
Tunisia	32	47	Kammoun, et al. 2008
Iran	34	66	Jouyan, et al. 2012
Kuwait	63	22	Abulhasan, et al. 1999
Singapore	57	---	Tan KB, et al. 2009
China	7	18.3	Zhao, et al. 2008
India	19	17	Datta, et al 2013
This study	25	12.5	---------

Table 9
Comparison of Mosaicism and Xq deletion among amenorrhea patients in different studies

Author	Year	Total No. of cases	No. of cases with 46, XXq- (%)	No. of cases with 45,XO/46, XXq- (%)
Goldman, et al	1982	150	1(0.6%)	0(0%)
Ten, et al	1990	117	1(0.8%)	0(0%)
Rajangam, et al	2007	865	4(0.4%)	0(0%)
Kalavathi, et al	2010	979	1(0.1%)	0(%)
Datta, et al	2013	637	3(0.4%)	2(0.3%)
Present study	2017	100	0(0%)	1(1%)

Table 10
Frequency of BMP15 gene variants in patients with amenorrhea and controls of different ethnicity

Origin	Size of cohort	Patients with mutations (%)	Size of control population	Controls with mutations (%)	References
Europe and North Africa	203	1.5	54	0	Laissue, et al. (2006)
India	202	8.9	197	0	Dixit, et al. (2006)
Italy and USA (Caucasian)	300	4.3	216	0	Rossetti, et al. (2009)
China	100	6	100	1	Wang, et al. (2010)
Europe, North Africa and Asia	50	12	214	1.9	Tiotiu, et al. (2010)
Europe and USA (Caucasian)	166	4.2	211	0	Di Pasquale, et al. (2006)
New Zealand	38	0	51	0	Chand et al. (2006)
Japan	15	0	–	----	Takebayashi, et al. (2000)
present study	100	46	100	2	-------

CONCLUSION

Identification of known genetic causes could aid in development of effective treatments for women with amenorrhea, as well as earlier diagnosis which may allow for family planning before the onset of amenorrhea. Eliciting a proper history along with a meticulous clinical examination and investigations for chromosomal aberrations will provide a solid foundation for treatment of women with amenorrhea leading to a fruitful reproductive life. The mutations of BMP15 gene level have a significant role to play in causing amenorrhea. Hence analysis of BMP15 gene should be preferably done in all cases of amenorrhea with subsequent DNA genome sequencing. This gene can be considered as a candidate gene in amenorrhea. Correlation of BMP-15 gene mutations in the present study has focused only on the relationship between a specific gene (BMP-15) and the cause of amenorrhea. The frequency of mutations was found to be very high among amenorrhea cases proving BMP-15 as a candidate gene in folliculo genesis. It is suggested that avenues are still open to further carryout specific analysis of cause and effect through sophisticated genetic studies.

AUTHOR CONTRIBUTION STATEMENT

Dr. V. Rajitha designed the project, conceptual ideas and proof outlined. Mrs G.Panneer Selvi helped in conducting the cell culture and interpretations. Dr. K.C. Shanthi assisted in statistical analysis and proof reading.

CONFLICT OF INTEREST

Conflict of interest declared none.

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