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ORIGINAL ARTICLE

Elevated plasma miR-210 expression is associated with atypical genitalia in patients with 46,XY differences in sex development

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Abstract

Background: Differences of sex development (DSD) is a term used for conditions in which the chromosomal, gonadal or phenotypical sex is atypical. 46,XY DSD patients frequently present undervirilized external genitalia. The expression of different miRNAs in many organs of the male genital system has been reported, and these miRNAs have been associated with testicular function and its disorders, but no description has been related to DSD conditions. This study aimed to evaluate the plasma expression of miR-210 in 46,XY DSD patients who presented atypical genitalia at birth.

Methods: Eighteen 46,XY DSD patients who presented atypical genitalia (undescended testis and/or hypospadias, bifid scrotum or micropenis) at birth and 36 male control individuals were selected. Plasma levels of miR-210 and reference miR-23a were measured using RT-qPCR and the data were analysed by the $2^{-\Delta Ct}$ method.

Results: MiR-210 plasma levels were significantly higher in 46,XY DSD patients with atypical genitalia than in male control subjects ($p = 0.0024$). A positive association between miR-210 levels and the presence of cryptorchidism and hypospadias ($p = 0.0146$ and $p = 0.0223$) was found in these patients. Significantly higher levels of miR-210 were observed in patients with 46,XY DSD and cryptorchidism than in control subjects ($p = 0.0118$). These results are in agreement with previous literature reports, in which increased levels of miR-210 expression were observed in human testicular tissue from adult males with undescended testes in comparison with samples of descended testes.

Conclusion: Our study showed a positive association between the presence of atypical genitalia and plasma levels of miR-210 expression in the group of patients with 46,XY DSD of unknown aetiology studied. These findings contribute

to reveal a new perspective on the role of miRNAs in the development of male external genitalia and the broad spectrum of phenotypes presented by patients with 46,XY DSD.

KEYWORDS

atypical genitalia, cryptorchidism, differences in sex development, hypospadias, MicroRNA, miR-210, undescended testes

1 | INTRODUCTION

Differences of sex development (DSD) is a term used to describe some conditions in which chromosomal, gonadal and/or phenotypical sex development is atypical. Depending on the cause of DSD and when the change during genital development occurs, atypical genitalia may be present at birth. Atypical external genitalia is identified in approximately 1:4500 newborns (Hughes et al., 2006).

Complex interactions between different pathways contribute to the development of normal male external genitalia (Achermann et al., 2015; Mendonca et al., 2009). These pathways include an androgen-independent step and an androgen-dependent step in addition to other endocrine/environmental influences (Hanley & Arlt, 2006; Kim et al., 2006; MacLaughlin & Donahoe, 2004).

It is well known that the lack of or deficient production, secretion or action of male hormones can cause micropenis, abnormal (ventral) placement of the external urethral meatus (hypospadia) and the absence of one or both testicles in the scrotum (cryptorchidism) (Mendonca et al., 2010; Mendonca et al., 2016). These are features that are frequently identified in 46,XY DSD patients. The affected individual can present a spectrum of genital undervirilization signals (Arnhold et al., 2011; Domenice et al., 2016; Lee et al., 2006).

There are varying degrees of external genital atypia ranging from males with a micropenis to females with clitoromegaly. Different degrees of undervirilized external genitalia were observed in affected members of the same 46,XY DSD family, although a single deleterious allelic variant was identified in all the affected members (Batista et al., 2018).

Modulating factors may affect the expression of mutated proteins and might explain the phenotypical variability for the same allelic variant of one DSD gene (Robevska et al., 2018; Wisniewski et al., 2019). These findings provide evidence that other molecular mechanisms of gene expression regulation might participate in the sex determination and differentiation process as a novel cause of 46,XY DSD and/or as a DSD phenotype modulator. The role of epigenetic regulation has already been demonstrated in sex determination in mammals (Haller & Ma, 2019; Miyawaki & Tachibana, 2019).

Although miRNAs have been mostly studied in cancer and monogenic diseases, the role of miRNAs in DSD remains unknown. MiRNAs are small noncoding RNAs (sncRNAs) that consist of approximately 20 to 25 nucleotides (Bartel, 2004; Lee & Ambros, 2001). They interact with the 3′UTR segment of mRNAs by base complementarity in a process that involves the RISC protein complex. This process blocks protein translation and inhibits protein expression (Bartel, 2004).

Several miRNAs have been associated with testicular function and its disorders (Duan et al., 2016; Ibarra-Ramírez et al., 2020; Lian et al., 2009; Tang et al., 2018). Lian et al. described that miR-210 was upregulated in the testes of infertile men with maturation arrest (Lian et al., 2009). This same pattern of miR-210 expression was shown in the cryptorchid testes of adult men (Abu-Halima et al., 2014; Duan et al., 2016; Lian et al., 2009; Tang et al., 2018). The current study aimed to evaluate the plasma expression of miR-210 in patients with 46,XY DSD who had atypical genitalia at birth.

2 | METHODOLOGY

2.1 | Control group and 46,XY DSD patients group

This study was approved by the Ethical Committee of the Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (HC-FMUSP) (CAAE 90497318.0.0000.0068), and written informed consent was obtained from all patients and control individuals or their parents/legal guardians.

2.1.1 | Control group

Blood samples were collected from 36 male control individuals. The control group consisted of two subgroups: (i) 27 healthy male blood donors between 18 and 55 years of age (average age of 30.3 years; $SD \pm 10.8$) from the Pro-Sangue Foundation Blood Center of Sao Paulo, hereafter referred to as the postpubertal control group, and (ii) nine healthy males between 3 and 12 years old (average age of

5.7 years, *SD* ± 3.1) who underwent postectomy, hereafter referred to as the prepubertal control group. Controls were also subgrouped into age intervals of the following ranges: 1–12, 18–20, 21–30, 31–40 and 41–55 years old.

Upon examination of the external genitalia of the prepubertal control group, a testicular volume <3 ml and Tanner stage 1 were identified in all subjects. All male control individuals, patients, or their legal representatives were asked to fill out a questionnaire on previous diseases, use of drugs or hormonal therapies, and previous genital surgeries. These factors might interfere with the selected miRNA profile and affect the results.

Physical examination of the external genitalia of the postpubertal control individuals was not performed, and no previous or current treatment for urogenital diseases was mentioned in their questionnaire responses. The hormonal profiles of the control individuals were not evaluated.

2.1.2 | 46,XY DSD patients group

Blood samples were collected from a group of eighteen 46,XY DSD patients regularly attending our service. All patients were born with atypical external genitalia, and all underwent genitoplasty. All participants had a 46,XY karyotype without numerical or structural abnormalities (20 cells analysed), and underwent hormonal and molecular studies (customized panels of candidate DSD genes or whole-exome sequencing) (Table 1). A list of the gene panel used is provided in Appendix Table A1. In our group of patients, 16 out of 18 were classified as of unknown aetiology.

2.2 | Subgrouping of the 46,XY DSD patients

2.2.1 | Pubertal status

The 46,XY DSD patients were divided into the following subgroups: the postpubertal DSD subgroup, comprising 12 patients (average age of 31.1 years; *SD* ± 16), and the prepubertal DSD subgroup, comprising six patients (average age of 3.15 years; *SD* ± 2.8). The prepubertal classification was based on a testicular volume of <3 ml, Tanner stage 1, and/or basal testosterone levels of <20 ng/dl. Data from the first evaluation of the external genitalia of the selected patients conducted at the Development Endocrinology Unit of HC-FMUSP were retrospectively collected from medical records/charts (Table 2). All patients underwent genioplasty, and in those who presented with cryptorchidism, the testes were located in the scrotum or an orchiectomy was performed (if it was not possible to correctly position them or if female sex assignment was done).

2.2.2 | Atypical genitalia

The 46,XY DSD patients were grouped according to the following criteria (patients can present more than one): (I) testes position into scrotal testes when both testes were found to have descended into the scrotum, or cryptorchidical testes when at least one testicle was found undescended—8 patients presented with descended testes and 10 patients presented with cryptorchidical testes; (II) the presence or absence of hypospadias—16 patients presented with hypospadias (second- or third-degree hypospadias); (III) presence of complete scrotal fusion or bifid scrotum—10 patients presented with fused scrotum and eight patients presented with bifid scrotum; (IV) the length of a stretched flaccid penis: micropenis, which was indicated by a Penile Length (PL) less than 2.5 standard deviations (*SD*) below the mean for age or stage of sexual development (PL ≤ −2,5 SD), 10 patients presented with micropenis.

2.3 | Blood collection, processing, and storage

Peripheral venous blood was collected in vacutainer serum tubes that were spray-coated with K2EDTA (BD, North Ryde, NSW, Australia). The blood samples were then centrifuged at 2000*g*, 2500*g*, and 3000*g* for 15 min each to avoid haemolysis of red blood cells. The presence of haemolysis in the plasma was evaluated by nanophotometry at a wavelength of 414 nm. Samples were considered haemolysis-free when the absorbance levels remained below 0.2, as described previously by others (Blondal et al., 2013; Kirschner et al., 2011, 2013; Wong et al., 2006).

It is known that haemolysis in plasma may affect the expression of some miRNAs, as described by Kirschner et al. (2011, 2013) and Blondal et al. (2013). There were seven samples from the postpubertal control group and one sample from the prepubertal control group that showed a reddish colouration characteristic of haemolysis and/or absorption values at 414 nm higher than 0.2, and thus, they were excluded from the analysis (initially $n = 44$, then $n = 36$). When haemolysis was detected in the patient, they were recalled for a new blood collection. Only one sample out of 46,XY DSD patients (prepubertal group) had to be excluded and was not recollected (initially $n = 19$, then $n = 18$).

TABLE 1 Clinical data of 46,XY DSD patients included in the study, their conditions (age, hormonal therapy use), and results from exome or gene candidate panels at the time of the blood sample collection

Patient	Social sex	Patient age at the sample collection	Medication in use	Consanguinity	Affected family members	Birth weight	Molecular findings	ClinVar: Clinical significance (Landrum et al., 2014)	ClinGen: Polyfen/SiFT (predicted) (Rehm et al., 2015)
1 [gcp]	M	2y1m	No	No	No	AGA	nsf	–	–
2 [gcp]	M	1y11m	No	No	No	AGA	nsf	–	–
3 [gcp]	M	2y7m	No	No	No	AGA	nsf	–	–
4 [gcp]	M	1y10m	No	No	No	SGA	*BMP8B* (ENSG00000116985.6) p.Arg116Cys	Not reported	Probably damaging/ deleterious
5 [gcp]	M	1y9m	No	No	No	AGA	nsf	–	–
6 [gcp]	M	8y9m	No	No	1 Sibling	AGA	*DHX37* (ENSG00000150990.3) p.Ala737Thr	Not reported	Probably damaging/ deleterious
7 [gcp]	M	17y	No	No	No	AGA	nsf	–	–
8 [gcp]	F	33y	Estradiol	No	No	AGA	*AR* (ENSG00000169083), p.Ser343Ser	Not reported	Benign
9 [gcp]	M	24y	No	No	No	SGA	*AR* (ENSG00000169083), p.Glu213Glu	Not reported	Benign
10 [wes]	M	14y3m	No	No	Father and 1 sibling	AGA	nsf	–	–
11 [gcp]	M	15y	No	No	No	SGA	nsf	–	–
12 [wes]	M	64y3m	Testo	No	No	AGA	*NR5A1* (ENSG00000168066.16), c.1139-3C > A	Not reported	Probably damaging/ deleterious
13 [gcp]	F	34y6m	Estradiol	No	No	AGA	nsf	–	–
14 [gcp]	M	15y2m	No	No	No	AGA	nsf	–	–
15 [gcp]	M	48y	No	No	No	AGA	nsf	–	–
16 [gcp]	M	37y	Testo	No	No	AGA	*DHX37* (ENSG00000150990.3) p.Leu67Val	Not reported	Probably benign
17 [gcp]	F	40y	Estradiol	No	1 sibling	AGA	nsf	–	–
18 [gcp]	M	12y11m	No	No	No	AGA	nsf	–	–

Note: Genbank (Clark et al., 2016) gene ID: BMP8B [656], DHX37 [57647], NR5A1 [2516], AR [367].

Abbreviations: APA, appropriate for gestational age; F, female; gcp, gene-candidate panels; M, male; m, months; nsf, no substantial findings; SGA, small for gestational age; Testo, testosterone; wes, whole exome sequencing; y, years.

TABLE 2 External Masculinization Score (EMS) of the 46,XY DSD patients included in this study

Patient	Scrotum	Score	Penis: Length (*SD*)	Score	Urethral position	Score	Gonadal position (R/L)	Score	EMS
1	Bifid	0	−1.1	3	Perineal	0	Inguinal/Inguinal	1.0/1.0	5
2	Fused	3	0	3	Penile	1	Scrotal/Scrotal	1.5/1.5	10
3	Fused	3	−1.0	3	Penile	1	Scrotal/Scrotal	1.5/1.5	10
4	Bifid	0	−2.5	0	Perineal	0	Scrotal/Inguinal	1.5/1.0	2.5
5	Bifid	0	−2.3	3	Perineal	0	Inguinal/Inguinal	1.0/1.0	5
6	Fused	3	−3.9	0	Normal	3	Absent/Absent	0/0	6
7	Bifid	0	−2.3	3	Scrotal	1	Scrotal/Scrotal	1.5/1.5	7
8	Fused	3	−2.5	0	Perineal	0	Scrotal/Scrotal	1.5/1.5	6
9	Bifid	0	−1.7	3	Perineal	0	Inguinal/Scrotal	1.0/1.5	5.5
10	Bifid	0	−5.0	0	Perineal	0	Inguinal/Scrotal	1.0/1.5	2.5
11	Bifid	0	−3.3	0	Penile	1	Scrotal/Scrotal	1.5/1.5	4.5
12	Bifid	0	−3.6	0	Perineal	0	Inguinal/Inguinal	1.0/1.0	2
13	Fused	3	−3.4	0	Perineal	0	Inguinal/Inguinal	1.0/1.0	5
14	Fused	3	−5.5	0	Penile	1	Scrotal/Scrotal	1.5/1.5	7
15	Fused	3	−2.5	0	Perineal	0	Scrotal/Scrotal	1.5/1.5	6
16	Fused	3	−2.5	0	Normal	3	Absent/Absent	0/0	6
17	Bifid	0	−2,5	0	Perineal	0	Scrotal/Abdominal	1.5/0.5	2
18	Fused	3	−3	0	Perineal	0	Scrotal/Scrotal	1.5/1.5	6

Note: SD, standard deviation; R/L, right and left; EMS, external masculinization score as proposed by Ahmed et al. (2000).

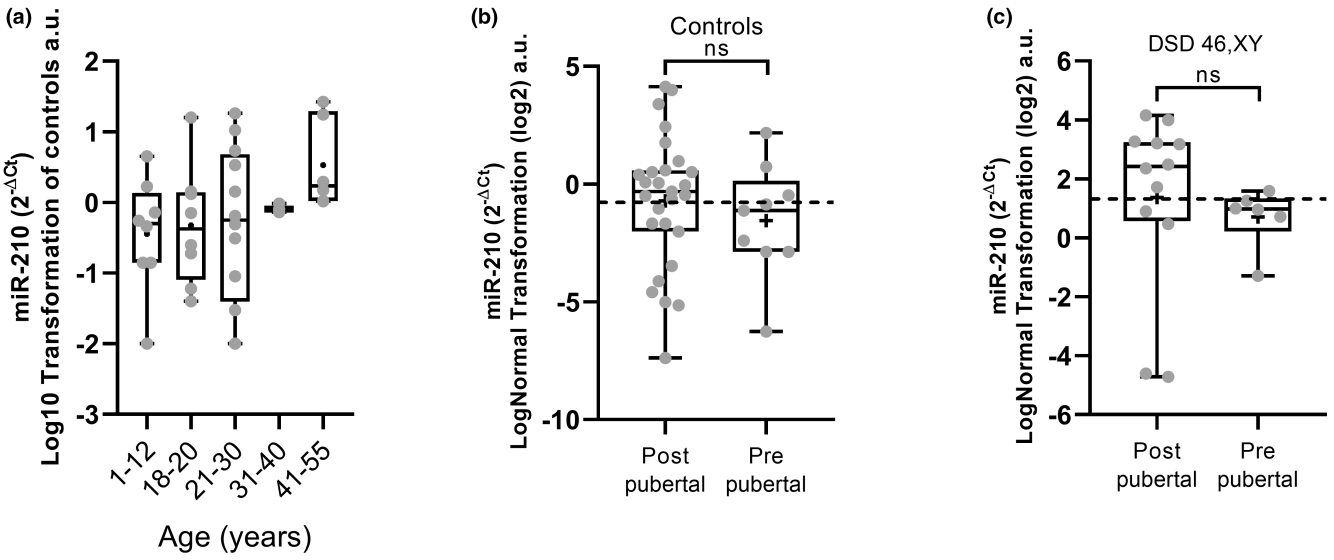

FIGURE 1 First, we questioned whether age or pubertal status could influence miR-210 expression. To test this hypothesis controls were sub-grouped by age into intervals (a), showing no statistical difference. Furthermore, controls (b) and 46,XY DSD patients (c) were sub-grouped into prepubertal and postpubertal status, again, showing no statistical difference in miR-210 expression values. RT-qPCR data were analysed by $2^{-\Delta Ct}$ and then transformed by log conversion, the miR-23a was used as a reference. Box and whiskers represent interquartile, minimum and maximum values; grey dots are the average of duplicates (control subjects) or triplicates (46,XY DSD patients); + and dashed lines represent the mean; a.u. = arbitrary units.

All blood samples were processed no more than 6 hours after collection. The plasma was stored at −80°C until RNA extraction and then kept at −80°C for long-term storage.

2.4 | RNA isolation

RNA was extracted from plasma using the MagMAX mirVana Total RNA Isolation Kit (Thermo Fisher, Waltham, Maryland, USA) and quantified with a Qubit 2.0 fluorometer and HS Assay RNA kit (Thermo Fisher). All steps were performed according to the manufacturer's instructions.

2.5 | Reverse transcription

Reverse transcription was performed using a TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher), and cDNA was quantified with a Qubit 2.0 fluorometer and dsDNA HS Assay Kit (Thermo Fisher). All steps were performed according to the manufacturer's instructions.

2.6 | Quantitative real-time PCR

RT–qPCR for miR-210 (chr11:568089–568,198, miRbase [Ambros et al., 2003] accession MI0000286/MIMAT0000267, 66-cugugcgugugacagcggcuga-87) and the reference miR-23a (chr19:13836587–13,836,659, miRBase [Ambros et al., 2003] accession MI0000079/MIMAT0000078, 45-aucacauugccagggauuucc-65) was performed using 1 ng of the preamplification reaction product and the TaqMan Advanced miRNA Assay kit (478532_mir/A25576, and 477970_mir/A25576) with Fast-Advanced Master Mix (Thermo Fisher) according to the manufacturer's instructions. miR-23a was selected as a reference gene based on the study by Blondal et al. (2013), which showed that this miRNA is not affected by haemolysis and has stable expression in plasma samples.

Reactions were carried out in an Agilent Aria-MX Real-Time System (Agilent, Santa Clara, California, USA). Fluorescence thresholds were set to 0.1 for all miRNAs, and all 40 cycles were considered. MiR-23a was selected as the reference gene, and the $2^{-\Delta Ct}$ method was used to compare the expression levels amongst the groups. A plate replicate and triplicate were used to control individuals and patients, respectively. Ct was set to 40 in samples that demonstrated no Cq for miR-210 in control individuals.

2.7 | Statistical analysis

The Anderson–Darling, D'Agostino's K^2, Pearson's chi-square, Kolmogorov–Smirnov, and Shapiro–Wilk tests were used to analyse the distribution of data. Data were transformed using the lognormal distribution by y = log(y). Group pairs were compared using unpaired Student's *t*-test (two-tailed) or the Mann–Whitney test. The *F* test was used to compare variances. To compare 3 or more groups, ANOVA was used with Tukey's post-test, or Kruskal–Wallis test with Dunn's posttest was used. Bartlett's test was used to compare variances. The results were considered statistically significant when $p < 0.05$.

3 | RESULTS

First, we examined the expression data to identify any differences between miR-210 in prepubertal and postpubertal subgroups. MiR-210 expression was similar in all groups, with a discrete elevation in the "40–55 years" subgroup. Interestingly, no significant differences in plasma miR-210 expression levels were observed in any of the subgroups of the control subjects at different ages (Figure 1a).

Additionally, no significant difference was observed when control individuals were compared according to their pubertal status (prepubertal vs. postpubertal control groups) (Figure 1b). Comparison between the prepubertal and postpubertal 46,XY DSD patient groups also showed no difference in miR-210 expression levels (Figure 1c). These findings support that the data from both the prepubertal and postpubertal groups could be analysed in conjunction.

Following such findings, the expression levels of plasma miR-210 were significantly higher in the 46,XY DSD patient group than in the control group (Figure 2a). Patients were then grouped according to the features of the External Masculinization Score (EMS), as described by Ahmed et al. (2000). We then sought to analyse the expression values of miR-210 in 46,XY patients by subgroups as described earlier.

A positive association between miR-210 levels and the presence of cryptorchidism was found, and significantly higher levels of miR-210 were observed in 46,XY DSD patients with cryptorchidism than in control subjects (Figure 2b). No significant difference was observed between 46,XY DSD patients with descended testis and control subjects.

Furthermore, 46,XY DSD patients with hypospadias showed significantly higher miR-210 expression

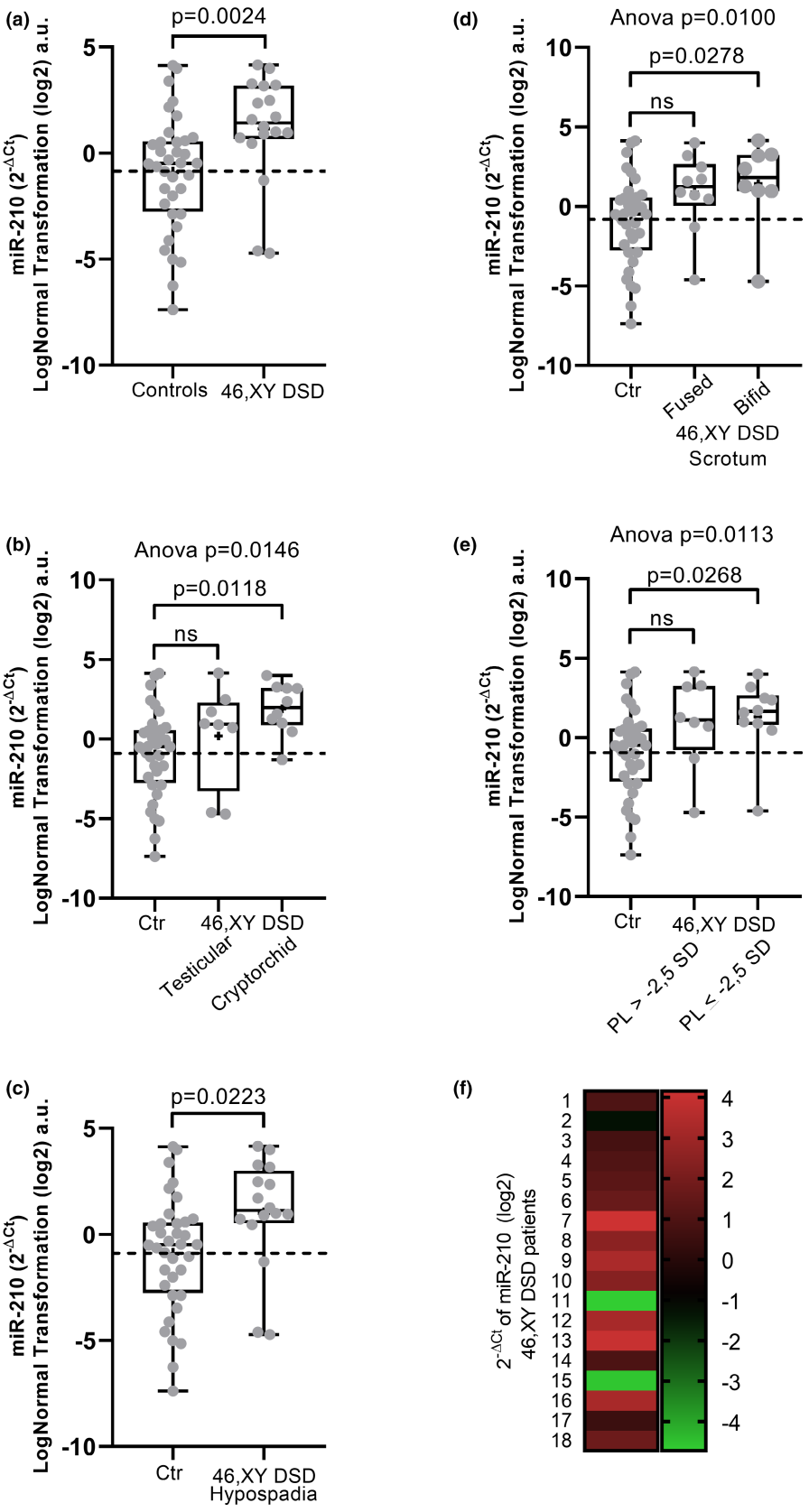

FIGURE 2 The expression of miR-210 was analysed in 46,XY patients and controls (a), showing higher levels of expression in patients. Following this finding, patients were grouped as per manifestations of atypical genitalia. (b) 46,XY DSD patients were grouped by testicular position. Then, patients were grouped by the presence of hypospadias (c), bifid scrotum (d) and penis length standard deviation (PL-SD) (e). Our data showed a positive association between such characteristics and the expression of miR-210. (f) miR-210 expression values (numbers on the right column) of the 18 46,XY DSD patients studied (left column) were compared to the mean of controls (mean = −0.92) and plotted as a heatmap. RT-qPCR data were analysed by $2^{-\Delta Ct}$ and then transformed by log conversion, the miR-23a was used as a reference. Box and whiskers represent interquartile, minimum and maximum values; grey dots are the average of duplicates (control subjects) or triplicates (46,XY DSD patients); + and dashed lines represent the mean; a.u. = arbitrary units. Ctr = controls.

compared with control subjects (Figure 2c). In this case, however, just two patients out of the initial 18 included in the study did not present with hypospadias. Therefore, we could not evaluate its significance against control individuals or 46,XY DSD patients with hypospadias.

Moreover, higher miR-210 expression values were observed in patients with bifid scrotum compared to

control subjects (Figure 2d). No significant difference was observed when comparing patients presenting with fused scrotums and control individuals. Patients with a micropenis (PL ≤ −2,5 SD subgroup) showed significantly higher miR-210 levels than control subjects, but no significant difference was observed between individuals in the PL > −2,5 SD subgroup and control subjects (Figure 2e).

Three patients were treated with oestradiol, and two patients were under testosterone treatment (Table 1). To determine whether oestradiol or testosterone could alter the miR-210 expression, a heatmap was constructed based on individual miR-210 levels against the mean of control subjects. No association was observed (Figure 2f). Furthermore, only two 46,XY DSD patients, showed lower levels of miR-210 than the mean expression levels of the control group. Two 46,XY DSD patients showed no difference, and fourteen 46,XY DSD patients had higher levels of miR-210 expression than the mean expression levels of the control group. These findings suggest that posttranscriptional mechanisms could play a role in regulating external genitalia development.

4 | DISCUSSION

Over the past years, miRNAs have been shown to influence numerous biological processes and have been associated with different disorders in humans (Mitchell et al., 2008; Saliminejad et al., 2019; Wang, 2017). The role of miRNAs in regulating primordial germ cell specification, spermatogenesis, oogenesis and gonadal tumorigenesis has been widely demonstrated (Panneerdoss et al., 2012; Robles et al., 2019). However, at present, their potential role in 46,XY DSD has rarely been discussed (Ibarra-Ramírez et al., 2020; Wisniewski et al., 2019).

Differential expression of several miRNAs linked to male reproductive disorders has been described, and miR-210 is included in this group (Robles et al., 2019). MiR-210 is ubiquitously expressed in a wide range of cells and has physiological roles in the inhibition of cell proliferation, inhibition of mitochondrial respiration, inhibition of DNA repair, vascular biology and angiogenesis. It is also known to be a major hypoxia-inducible miRNA (Zhang et al., 2012).

Placental hypertension and preeclampsia can lead to foetal hypoxia and affect newborns (Redman & Staff, 2015). A meta-analysis concluded that maternal hypertension diseases, including preeclampsia, were positively associated with hypospadias in human offspring (Sheriff et al., 2019). Moreover, Gunel et al. (2011) found that the expression of miR-210 was increased in the placentas and plasma samples of preeclamptic patients, and it could be detected prenatally with non-invasive methods.

In our study, patients #4 and #9 were considered small for gestational age (Table 1). This reduced foetal growth is a common finding that reaches one-third of infants of mothers with preeclampsia and may explain these patients' findings (Redman & Staff, 2015). Both patients #4 and #9 showed miR-210 expression values that were higher than the mean of the control individuals.

Previous studies have shown that miR-210 expression is upregulated in the testes of infertile men with maturation arrest (Barbu et al., 2021; Lian et al., 2009). The authors showed that IGF2 (GenBank gene ID: 3481) was directly targeted by miR-210 using in vitro experiments in NT2 cells. Therefore, these data indicated that miR-210 might be associated with spermatogenesis by targeting IGF2 in male infertility (Tang et al., 2018). The effects of the insulin-like growth factor system on testicular differentiation and function are well established (Cannarella et al., 2018; Pitetti et al., 2013). Insulin/IGF signalling is central in the regulation of the final number of Sertoli cells, testicular volume and sperm concentration (Pitetti et al., 2013).

Upregulated miR-210 expression was also demonstrated in human cryptorchidic testis and mouse testis subjected to experimental cryptorchidism (Duan et al., 2016). Analyses of human postcryptorchidopexy testicular tissue revealed 297 downregulated and 153 upregulated miRNAs compared with normal testicular tissue, but miR-210 expression was not significantly altered in these samples (Tang et al., 2018). The authors associated the difference in miRNA profiles with the different types of human cryptorchidic tissue analysed.

Interestingly, a recent systematic review by Jia and Hao (2021), which analysed dysregulated miRNAs in cryptorchidism, showed 21 potential miRNAs from the studies collected, but only miR-210, miR-449a, and miR-34c were dysregulated in both animal and human tissues from the 185 samples analysed.

Cryptorchidism is a common anomaly in the male genitalia (2% to 4% of male infants) and is frequently observed in 46,XY DSD patients (Barthold & González, 2003; Kalfa et al., 2019). In our group of 46,XY DSD patients with unknown aetiology and atypical genitalia, undescended testis was present in 60% of these patients. The expression of miR-210 in plasma was significantly higher in the DSD group than in the male control group.

Genetic variants were found in patients #4 and #16, but at this time it was not possible to fully clarify their aetiology based on those findings (Table 1), both patients had atypical genitalia, including inguinal (patient #4), and undescended testes (patients #16), and showed higher levels of miR-210 than the mean of controls. Patients #6 (da

Silva et al., 2019) and #12 (Gomes et al., 2022) had their aetiology clarified based on molecular studies (Table 1), and showed higher levels of miR-210 than the mean of controls, the same pattern observed in the group of patients ($n = 16$), whom besides molecular studies, were still classified as of unknown aetiology. These novel findings may help to clarify the phenotype of 46,XY DSD patients of unknown aetiology.

In addition, the real-time qPCR analysis revealed that our group of 46,XY DSD patients with undescended testis had higher levels of circulating miR-210 than male control individuals, which is in contrast to the 46,XY DSD patients with descended testis. Our results in these plasma samples are in accordance with the findings of Duan et al. (2016) in testicular samples. Unfortunately, testicular tissue samples from DSD patients were not available in our study, preventing the comparison between the expression profile of miR-210 in plasma and undescended testis tissue in DSD patients.

Our study showed a positive association between miR-210 expression and the presence of cryptorchidism and hypospadias, which are two characteristics frequently observed in the atypical genitalia of patients with 46,XY DSD. Deregulation of miR-210 expression might represent a secondary effect of a physiological pathway disorder or a novel aetiological factor in abnormal male sex development.

Findings in this small group of patients had strong statistical values, however further analysis in a large number of 46,XY DSD patients will be necessary to establish the role of miR-210 in their phenotype modulation, and deeper investigation must be done to establish the pathogenic potential of miRNA in 46,XY DSD.

5 | CONCLUSION

The present study showed a positive association between the presence of atypical genitalia and plasma levels of miR-210 expression in the group of patients with 46,XY DSD studied. Our findings contribute to opening a novel perspective on the possible role of miRNAs in the development of the male external genitalia and the broad spectrum of phenotypes presented by patients with 46,XY DSD, more importantly to those that besides molecular studies remain with unknown aetiology.

AUTHOR CONTRIBUTIONS

Designed the research: Elias FM, Mendonca BB, and Domenice S. Performed the research: Elias FM and Nishi MY. Analysed the data: Elias FM and Gomes NL. Provided patient care and collected the clinical data: Sircili MHP, Batista RL, Gomes NL, Ferrari MTM, Costa EMF, Denes FT, Mendonca BB, and Domenice S. Wrote the paper: Elias FM, and Domenice S.

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CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by the Ethical Committee of the Hospital das Clinicas of the University of Sao Paulo Medical School, University of Sao Paulo (CAAE 90497318.0.0000.0068), and written informed consent was obtained from all patients and control individuals or their parents/legal guardians.

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APPENDIX

TABLE A1 Gene candidate panel[a]

Genes									
ACBD7	ACVR1B	ACVRL1	AKR1C2	AKR1C3	AKR1C4	AMH	AMHR2	AR	ARL6
ARX	ATRX	BBS1	BBS10	BBS12	BBS2	BBS4	BBS5	BBS7	BBS9
BMP15	BMP8B	BMPR1B	CAMK1D	CBLN1	CHD7	CITED2	CITED4	CTNNB1	CUL7
CYP11A1	CYP17A1	CYP19A1	CYP26B1	DHCR7	DHH	DHX37	DMRT1	DMRT2	DNAJC15
DND1	ESR1	FAM189A2	FBLN2	FGD1	FGF9	FGFR2	FOXL2	FRAT1	FSHR
FST	GADD45G	GATA4	GATA6	GDF9	GDNF	GPR56	GPR83	GTF2F1	HHAT
HNF1B	HPGDS	HSD11B1	HSD17B3	HSD3B2	IGF1R	IHH	INHBB	IRX3	KATNAL1
KATNBL1	LEF1	LGR5	LHCGR	LHX1	MAMLD1	MAP3K1	MKKS	MSX1	NANOS2
NANOS3	NCOA1	NCOA2	NCOA3	NR0B1	NR3C1	NR5A1	PAPPA	PDGFA	PDGFRB
POR	PRKACG	PTGDS	PUM2	RSPO1	RSPO2	SIX1	SIX4	SMAD4	SMARCE1
SOX10	SOX13	SOX7	SOX9	SRA1	SRD5A2	SRY	STAG3	STAR	STIM1
SUPT3H	TCF21	TRIM32	TRIM37	UBE3A	USP34	WT1	WWOX	ZFPM2	

[a]AgilentSure Design 2.0 Software (Agilent Technologies), Illumina platform (Illumina Technologies).