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Circulating miR-30b levels increase during male puberty

Running title: mir-30b increase during puberty

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ABSTRACT

OBJECTIVE: The role of microRNAs as endocrine regulators is emerging, and microRNA mir-30b has been reported to repress Mkrn3. However, the expression of miR-30b during male puberty has not been studied.

DESIGN AND METHODS: Circulating relative miR-30b expression was assessed in sera of 26 boys with constitutional delay of growth and puberty (CDGP), treated with low-dose testosterone (T) (n=11) or aromatase inhibitor letrozole (Lz) (n=15) for 6 months and followed up to 12 months (NCT01797718). The associations between the relative expression of miR-30b and hormonal markers of puberty were evaluated.

RESULTS: During the 12 months of the study, circulating miR-30b expression increased 2.4 ± 2.5 (SD) fold (p=0.008) in all boys, but this change did not correlate with corresponding changes in LH, testosterone, inhibin B, FSH, or testicular volume (p=0.25-0.96). Lz-induced activation of the hypothalamic-pituitary-gonadal (HPG) axis was associated with more variable miR-30b responses at 3 months (P<0.05), whereas those treated with T exhibited significant changes in relative miR-30b levels in the course the study (p<0.01-0.05).

CONCLUSIONS: Circulating miR-30b expression in boys with CDGP increases in the course of puberty, and appears to be related to the activity of the HPG axis.

INTRODUCTION

Paternally inherited loss-of-function mutations in *MKRN3*, a gene encoding a putative ubiquitin E3 ligase, cause central precocious puberty (1). The role of microRNAs as endocrine regulators of even distant tissues is rapidly emerging (2). Heras *et al* demonstrated that *in vitro* microRNA mir-30b repressed Mkrn3 by binding to its 3'UTR (3). Additionally, hypothalamic mir-30b expression increased during puberty in male and female rats (3). Inhibition of this binding during the juvenile period delayed the onset of puberty in female rats, in which neonatal estrogen exposure enhanced hypothalamic Mkrn3 expression and suppressed miR-30b (3). The expression pattern of miR-30b during human puberty is unknown.

Herein, we investigated longitudinal changes in circulating mir-30b levels in boys with constitutional delay of growth and puberty (CDGP), who were treated with low-dose testosterone (T) or aromatase inhibitor letrozole (Lz), a blocker of estrogen synthesis (4). We hypothesized that miR-30b levels increase in the course of puberty, and that the levels are differentially modified by changes in the hormone milieu induced by the two treatment modalities used to expedite male puberty.

METHODS

The study included 26 boys with CDGP who participated in a randomized controlled trial between 2013 and 2017 (4). At the start of the study, all the boys showed early signs of puberty (4). During the study, the boys received either Lz (2.5mg/vrk) (n=15) or intramuscular T (1mg/kg every 4 weeks) (n=11) for 6 months. The study visits were at 0, 3, 6, and 12 months. At each visit, the boys were physically examined, and a morning serum sample was obtained and stored at -80ºC (4). Serum hormone values and clinical parameters

were from our previous study (4,5). At the start of the study, the mean age, bone age, height, and weight of the participants were 14.7±0.6 years, 12.3±1.1 years, -2.1±0.9 SDS, and 48.3±14.2 kg, respectively.

Micro RNA was extracted (mirVana PARIS Kit, Invitrogen) from 400ul of serum aliquots, and reverse transcribed using TaqMan MicroRNA RT Kit (Applied Biosystems). For quantitative RT-PCR (conditions available upon request), predesigned assays, hsa-miR-30b, U6 snRNA (Applied Biosystems), were used; samples of each boy were analysed in the same assay run. Three-month sample was missing in four boys and six-month sample in two. The expression of miR-30b was adjusted by the expression of the reference gene (U6). U6 is one of the most common reference genes for circulating miRNAs analysis (6), and its hypothalamic expression in rats appears to remain stable in puberty (3). The adjusted relative expression at different time points (0, 3, 6, and 12 mo) was calculated by dividing the corresponding adjusted expression level by the adjusted expression level at 0 mo; thus, at 0 mo the relative miR-30b expression in each boy was 1.

The study was registered with ClinicalTrials.gov (NCT01797718). The Finnish National Committee on Medical Research Ethics and the Finnish Medicines Agency approved the study protocol, and a written consent was obtained from all participants after full explanation of the purpose and nature of all procedures used.

Data analyses were performed with SPSS statistic for Windows. The changes in the relative expression of miR-30b was analyzed with paired sample t test. Between-group comparisons were performed with independent samples t test. The homogeneity of variances were tested with F-test. Correlations were evaluated with Spearman’s rank correlation. Statistical significance level was set to a p value < 0.05.

RESULTS

The hormonal and clinical characteristics of the participants during the study period is demonstrated in Table 1. We first examined whether the relative miR-30b expression at 12 months, *i.e.* when puberty had progressed in all boys, was higher than at the beginning of the study. The overall patterns of miR-30b levels in both treatment groups are shown in Figure 1. Indeed, between 0 and 12 months, the relative circulating miR-30b increased 2.4±2.5 (SD) fold ($p=0.008$, $n=26$). However, this change did not correlate with absolute or relative changes in parameters shown in Table 1. On the other hand, the boys treated with Lz exhibited clear activation of the HPG axis at 3 months, whereas the opposite occurred in those who received T (Table 1, ref. 4). Intriguingly at 3 months, the boys treated with Lz also exhibited higher variance in relative miR-30b levels ($F(1,20)=4.9$, $p=0.038$) than those in the T group, although the mean relative miR-30b levels at 3, 6, or 12 months did not differ between the groups. The longitudinal changes in the relative miR-30b levels in the T group were significant between 0-12 months, 3-6 months, 6-12 months (in all $p<0.05$) and 0-6 months ($p=0.008$); in the Lz group the only significant longitudinal change occurred at 6-12 months ($p<0.05$) (Figure 1).

DISCUSSION

Considering that hypothalamic mir-30b expression increased during puberty in male and female rats (3), we set out to investigate circulating miR-30b in humans. Indeed, this pilot study shows that circulating miR-30b expression in boys with CDGP increases in the course of puberty. Although miR-30b has been extracted from serum and urine (7), its source in circulation is unclear. For example, peripheral blood leukocytes are known to express it (8), whereas miR-30b is also highly expressed in mouse testis, and patients with Sertoli cell only syndrome exhibit reduced testicular miR-30b expression (9, 10). Subsequently, the relationship between circulating and hypothalamic miR-30b expression, if any, remains unknown. The changes in miR-30b expression and hormonal or clinical markers of puberty between 0 and 12 months did not correlate, suggesting that circulating miR-30b levels do not reflect its hypothalamic expression, or that exocytotic vesicle-mediated regulation of male puberty does not immediately translate to basal reproductive hormone levels. On the other hand, in female rats, hypothalamic miR-30b expression at the age of 35 days was suppressed by neonatal estrogen exposure (3). In our work, estrogen depletion by Lz induced HPG axis activity (4), and was associated with more variable miR-30b levels than was brought about by exogenous T, suggesting that peripheral miR-30b levels are related to the activity of the HPG axis also in humans. It should be noted that our study subjects had CDGP and exhibited already early signs of puberty and were treated with two different medications for the initial six months. Further, the results are based upon exploratory analyses, and to confirm them, the corresponding hypotheses have to be tested in further confirmatory studies[11]. Regardless of these limitations, circulating miR-30b may represent a new non-classical endocrine signal that participates in the regulation of male puberty.

DISCLOSURES

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FIGURE LEGEND

Figure 1. The relative expression (mean+SEM) of serum miR-30b levels in 26 boys with constitutional delay of growth and puberty who were treated for six months *(grey area*) with letrozole (Lz) (n=15) or intramuscular low-dose testosterone (T) (n=11). Between 0 and 12 months, the relative miR-30b expression increased 2.4±2.5 (SD) fold in all boys (P=0.008). The longitudinal changes in the relative miR-30b levels in the T group were significant between 0-12 months, 3-6 months, 6-12 months (in all $p<0.05$) and 0-6 months ($p=0.008$); in the Lz group the only significant longitudinal change occurred at 6-12 months ($p<0.05$).