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Original article

Alteration of rRNA gene copy number and expression in patients with intellectual disability and heteromorphic acrocentric chromosomes

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ABSTRACT

Background: Intellectual disability (ID) is an important medical and social problem that can be caused by different genetic and environmental factors. One such factor could be rDNA amplification and changes in rRNA expression and maturation.

Aim of the study: The aim of the present study was to investigate rRNA levels in patients with heteromorphism of the p-arms of acrocentric chromosomes bearing nucleolus organizer regions compared to a healthy control group.

Material and methods: Frequencies of p-arms enlargements in patients with ID and in healthy people were analyzed by G-banding screening. rRNA gene copy numbers on affected acrocentric chromosomes in peripheral blood lymphocytes were evaluated in ID patients and healthy bearers using FISH, and in immortalized lymphocytes of one patient – using FISH and real time PCR. Simultaneously, levels of 18S, 28S and 5,8S rRNA in both groups by means of qRT-PCR were investigated.

Results: No difference in acrocentric chromosome heteromorphism frequency in patients versus the healthy group were found. However, we found an amplification of rDNA, a significant elevation in 28S and 5.8S rRNA expression and changes in the 28S/18S rRNA ratio in ID patients compared to healthy controls. At the same time, FISH appeared to be not reliable enough for copy number evaluation, but RT-PCR showed rDNA copy changes in heteromorphic cells compared to normal.

Conclusion: Our findings indicate a loss of the correct regulation of rDNA activity and processing after amplification. This could disturb the ribosomal apparatus and thus lead to intellectual disability via at least two mechanisms.

1. Introduction

ID is an important medical and social problem that is commonly accompanied with comorbidities, as well as psychological issues, social difficulties and often financial pressure for patients and their relatives. Its frequency is approximately 1% of the human population, and it can be caused by different genetic and/or environmental factors. To date, more than 700 genes related to ID have been described. Many types of ID are associated with chromosome abnormalities [1]. One genetic feature that could be related to ID is enlargement of the p-arms on acrocentric chromosomes. Although acrocentric chromosomes with NOR regions with substantially enlarged p-arms were reported since the 1970s, these can be accompanied by ID or be present in healthy people [2–4] and even pass through generations [5,6]. In these cases, substantial amplification of rDNA was rarely shown because very few reports investigated rDNA in enlarged p-arms of marker chromosomes. Moreover, few cases have investigated NOR activity, and there are no reports about rRNA levels measured quantitatively in bearers of NOR-chromosomes with enlarged p-arms. In addition, it

Abbreviations: FISH, fluorescent *in situ* hybridization; ID, intellectual disability; NOR, nucleolus organizer region; PHA, phytohemagglutinin; qRT-PCR, quantitative real-time polymerase chain reaction (PCR); rDNA, ribosomal DNA; rRNA, ribosomal RNA; UID, undifferentiated intellectual disability.

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should be noted that slight heteromorphism of p-arms in human acrocentric chromosomes is considered normal by the human chromosome atlas; moreover, these chromosomes are considered the most variable in the human karyotype [7]. It is important to note that changes and/or impairment in rRNA expression have been reported in neurodegenerative diseases, such as Alzheimer's disease [8,9] and Huntington disease [10,11], and increased NOR activity has been shown in lymphocytes and buccal epithelium of children with Down syndrome [12,13]. We propose that ID correlates with NOR activity and rRNA expression levels, and so rRNA levels could play a substantial role in the development of ID. Thus, the main aim of our present study was to examine rDNA amounts and rRNA expression levels and acrocentric chromosomes with p-arms enlargement in patients with ID and in healthy controls.

2. Ethics

The involvement of patients, their relatives and random donors in the study was strictly designed in accordance with international standards, which include the awareness of the subject and his or her informed consent to participate in the study in its entirety and guarantees of confidentiality. All studies conformed to ethical standards developed in accordance with the Helsinki Declaration of the World Medical Association, as amended in 2000. In addition, the studies were supervised by the Institutional Review Board.

3. Materials and methods

3.1. Investigated groups

A total of 88 blood samples from the IMCB SB RAS repository were studied. Fifty-six samples were from ID individuals and the remaining 32 were from healthy controls.

For the molecular investigation, 12 patients with UID and with heteromorphism of acrocentric chromosomes as determined by G-banding and without other genetic abnormalities were selected. Six of the 12 patients had diagnoses of mild intellectual disability, and the remaining patients had other diagnoses related to ID (hyperkinetic conduct disorder, infantile autism etc.). The average age of this group was 7.1 ± 1.8 years. The control group included 13 healthy persons without any known genetic diseases. The average age of this group was 27.4 ± 1.6 years. The differences in age between groups arised, because patients involved in the present study were children addressed to diagnostics due to ID, and healthy donors were their parents/sibs or randomly selected people. Healthy children had no need to be diagnosed and thus were not present in the study.

3.2. Metaphase preparation and staining

Blood samples were cultivated in RPMI-1640 medium (Gibco, USA) with 20% Fetal bovine serum (Gibco) and 1–3% PHA-M (Gibco) for 72 h. Incubation with 0.03 μg/ml of KaryoMax colcemid solution (Gibco) and 2.5 μg/ml ethidium bromide for 3 h was followed by hypotonic treatment for 25 min and fixation in Carnoy fixative (methanol/acetic acid – 3/1). Immortalized cell lines GM06895 (Coriell Institute Cell Repository, USA) and CPG148 (IMCB SB RAS cell repository) were cultivated in RPMI-1640 medium (Gibco) with 15% fetal bovine serum (Gibco) and 4 mM L-glutamine (Gibco). Metaphases were prepared by dropping the cell suspension. For G-banding [14], metaphases were treated with 0.25% trypsin solution for 1 min and staining with Giemsa stain for 2 min. Slides were analyzed on an Olympus BX-53 microscope with ×1000 total magnification. For image capture and analysis, VideoTest Karyo 3.1 (iMicroTec, Russia) software was used. For each case, no less than 12 metaphase spreads were analyzed.

3.3. Fluorescent in situ hybridization (FISH)

Plasmid pHr13 containing genes for 18S, 28S and 5.8S rRNA [15] was labeled with a BioNick DNA labeling system (ThermoFisher Scientific, USA). Before FISH, slides were pretreated with 100 μg/ml RNAse for 1 h at 40 °C and with 0.005% pepsin solution in 10 mM HCl for 10 min at 37 °C (if not G-banded before). FISH was performed with 0.06 ng of the labeled probe in 50% formamide at 40 °C overnight. Detection was performed with Alexa-555-streptavidin conjugate (ThermoFisher Scientific) or using FITC-conjugated avidin and anti-avidin antibody (both from New England Biolabs, USA). Chromosomes were counterstained with DAPI (0.08 μg/ml). Slides were analyzed on an Olympus BX-53 microscope with ×1000 total magnification. For image capture and analysis, VideoTest FISH 2.0 (iMicroTec, Russia) software was used.

3.4. RNA isolation, cDNA producing and qRT-PCR

Total RNA extraction from whole blood or blood plasma was performed using an Aurum Total RNA Mini Kit (BioRad, USA) or ExtractRNA reagent (Eurogen, Russia) followed by reverse transcription using an iScript Select cDNA Synthesis Kit (BioRad). All procedures were performed according to the manufacturers' protocols. Real-Time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (BioRad) in a C1000 Touch Thermal Cycler with a CFX 96 RealTime System (BioRad).

Primers for real-time PCR (Table 1) were designed using ref. seq NR_003286.2, NR_003287.2 and NR_003285.2 for 18S, 28S and 5.8S rRNA, respectively. Beta-actin (ref.seq. NM_001101.3) was used as a reference gene. A healthy male donor (CPG57 according to IMCB SB RAS nomenclature) was used as a control for the rRNA level evaluation in all cases. Each sample was measured in 3 analyses [16].

3.5. DNA isolation and RT-PCR

Genomic DNA from immortalized cell lines GM06895 and CPG148 was extracted using a Wizard Genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol. Real-time PCR was performed using the same primers as for 18S, 28S and 5.8S rRNA expression level estimation (see section above), but GAPDH was used as a single copy reference (ref.seq. NG_007073.2; for primer sequence see Table 1). Calibration curves

Table 1
Primers used for evaluation of the rRNA levels and rDNA copies.

Target	Forward primer	Reverse primer
18S rRNA	5′-GAGAAACGGCTACCACATCCAA -3′	5′-CCAATTACAGGGCCTCGAAAGA -3′
28S rRNA	5′-GGGTGGTAAACTCCATCTAAGG -3′	5′-GCCCTCTTGAACTCTCTCTTC -3′
5,8S rRNA	5′-GGTGGATCACTCGGCTCGT -3′	5′-CCGCAAGTGCGTTCGAAGTG -3′
Beta-actin	5′-CACGGCATCGTCACCAACTG -3′	5′-GCAACGTACATGGCTGGGG -3′
GAPDH	5′-CTGCAGGGCCTCACTCCTTTTGCAG-3′	5′-GGCAGGTTTTTCTAGACGGCAGGTCAG-3′

for all primers were created and corresponding rDNA copy was estimated using MS Excel software (Microsoft, USA).

3.6. Statistics

Statistical comparison of obtained data between groups was performed by Student's *t*-test using GraphPad software (http://www.graphpad.com/quickcalcs). We considered differences statistically significant if $p < 0.05$.

4. Results

4.1. Analysis of acrocentric chromosome p-arm heteromorphism

In the IMCB SB RAS, we have a collection of blood samples of patients with ID and their relatives. We performed G-banding screening of the patients to identify karyotype features that could be associated with ID. The NOR-chromosome p-arm was considered to be enlarged if it was comparable or longer than the p-arm of chromosome 18 [7]. First, we performed statistical evaluation of acrocentric chromosomes with NOR region p-arm enlargement of the 56 ID patients and the 32 healthy controls. In the ID group, 21 patient (37.5 ± 5.9%) had enlarged p-arms in acrocentric chromosomes. In the healthy group, p-arm heteromorphism was revealed in 11 patients (34.4 ± 8.4%). Thus, the difference in the frequency of acrocentric chromosomes with p-arm heteromorphism between the groups was not statistically significant. This finding shows that this feature itself is not directly connected with an increased probability of ID development, although it can be thought to represent a risk factor. Interestingly, chromosome 13 had more frequent p-arm enlargement compared to other chromosomes. We found 13p+ in 17 of 24 ID p+ cases (70.8 ± 9.3%) (Fig. 1, a). Among healthy bearers of acrocentric chromosomes with enlarged p-arms, 13p+ was also the most frequent, being observed in 8 of 12 cases (66.7 ± 13.6%) (Fig. 1, b). Thus, chromosome 13 tends toward preferential p-arm enlargement among all analyzed people with acrocentric chromosome heteromorphisms.

4.2. rRNA genes localization by FISH

Enlarged p-arms of acrocentric chromosomes contain rRNA genes (rDNA). To investigate if enlargement leads to rDNA amplification, we hybridized a plasmid, pHr13, which contains genes of 18S, 28S and 5.8S rRNA, to metaphase spreads of 12 ID patients with heteromorphic acrocentric chromosomes and 9 healthy controls with heteromorphic acrocentric chromosomes. There was substantial heterogeneity in signal size and shape within individual metaphase spreads of patients and of healthy controls. Although we observed signal enlargements on acrocentric chromosomes with enlarged p-arms (Fig. 2a), we often observed large signals on p-arms of acrocentric chromosomes that did not meet the

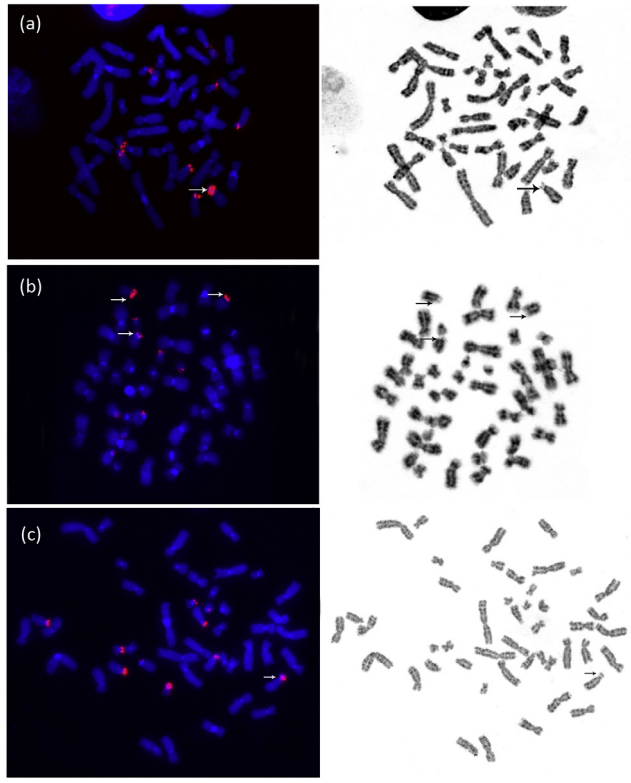

Fig. 2. Different types of rDNA signals on metaphase spreads with p-arm enlargements in acrocentric chromosomes. a – Enlarged signal on an affected chromosome (arrow) in a healthy control, b – Large signals on both affected and normal chromosomes (arrows) in an ID patient, c –Equally sized signals on both affected and normal chromosomes (arrow) in a healthy control. Left: FISH localization of dig-labeled rDNA (red); right: same G-banded metaphase.

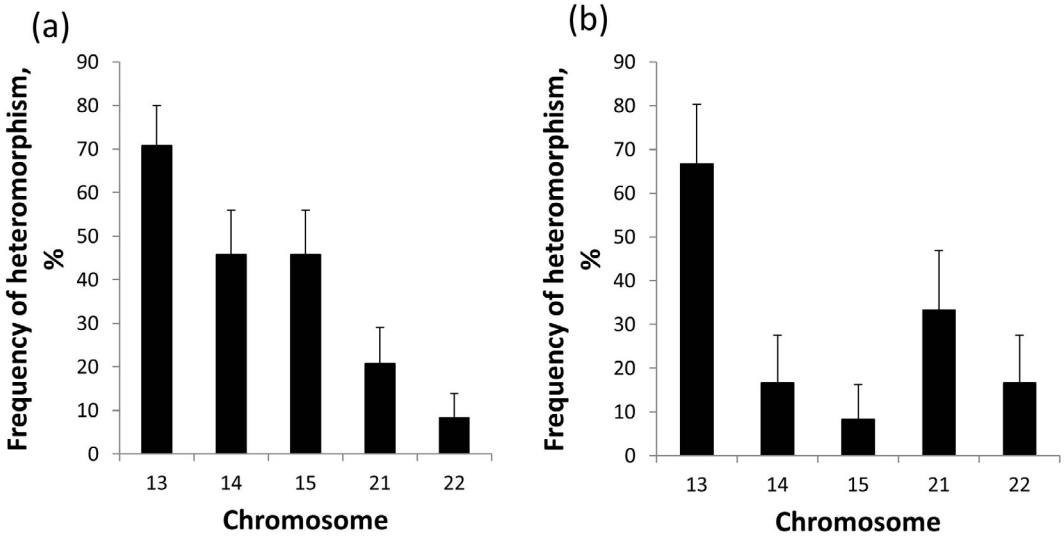

Fig. 1. Distributions of heteromorphism in acrocentric chromosomes in ID patients (a) and healthy controls (b).

enlargement criteria (Fig. 2b) based on phenotype alone. In some cases, the FISH signal on affected chromosomes was small and did not differ from homologs and normal acrocentric chromosomes (Fig. 2c). We did not observe any substantial difference in signal distribution between ID patients and healthy bearers of acrocentric chromosomes with enlarged p-arms. rRNA gene repetitive unit copy number is known to vary widely, not only between individuals but also between cells within one individual. Additionally, FISH signal size difference can result from hybridization efficiency on individual chromosomes. FISH analysis does not allow for an estimation of rDNA amplification.

4.3. Quantitative evaluation of rRNA genes in patient's DNA

Blood samples held in the IMCB SB RAS repository were collected in heparinized vacuum tubes to facilitate lymphocyte cultures. Heparin is known to inhibit PCR, making rDNA quantification by RT-PCR on collected samples unreliable. To study possible rDNA amplification we purified DNA from cell cultures of a patient with ID and heteromorphic chromosomes 13 and 15 (CPG148 according to IMCB SB RAS nomenclature). FISH analysis (Fig. 3) of this patient showed larger and brighter rDNA signals on both chromosomes with enlarged p-arms and a bright and large signal on chromosome 22. We estimated the rDNA copy number in these cells by real-time PCR relative to a control cell line, GM06895. Copy numbers of sequences corresponding to 18S and 5.8S rRNA in CPG148 are 19.60 ± 0.82 and 20.09 ± 0.13 times the level of these genes in GM06865, respectively, and for the 28S sequence it is 9.60 ± 0.15 times the level in GM06865.

4.4. rRNA gene expression

Finally, we examined 18S, 28S and 5.8S rRNA levels in blood samples of 12 ID patients with enlarged p-arms of acrocentric chromosomes and 13 healthy controls. The control group included samples with and without acrocentric chromosome heteromorphism. One healthy reference control with a normal karyotype was used for normalization in all cases. The ID patient group was heterogenic by rRNA gene expression levels in contrast to the control group. Nevertheless, the mean 28S rRNA and 5.8S rRNA levels were substantially and statistically significantly higher in ID patients ($p \leq 0.01$) compared with the control group, being 6.1 and 6.4-fold elevated, respectively, when normalized to a reference control (Fig. 4). The 18S rRNA did not exhibit a statistically significant difference between ID patients and healthy controls, although the value for ID patients tended to be higher (p = 0,12). All healthy controls showed no statistical significance in rRNA levels in relation to the presence of heteromorphic acrocentric chromosomes.

5. Discussion

A comparison of different population studies presented in the Atlas of human acrocentric chromosome heteromorphisms [7] shows some discrepancies due to racial and population (e.g. age distributions) differences, as well as variations in staining techniques and criteria used in the analyses. Moreover, all of these studies were performed before 1980. However, preferential heteromorphism segregation for chromosome 13, which was found in both ID patients and controls in our study, has been reported previously for other populations [17–19]. The most important finding in our study is an absence of a difference in acrocentric chromosome p-arm heteromorphism frequencies between ID patients and healthy controls. This indicates that acrocentric chromosome p-arm heteromorphism is not the main and direct cause of ID.

Traditionally, rRNA gene number in humans has been estimated to be approximately 300 copies per diploid human genome, varying from 100 to 400–600 (thus, 4–6-fold) between individuals [20,21]. However, the latest data from ribosomal repeat sequencing in the human genome shows a very high polymorphism in rDNA dosage, varying from 6.15 times for 18S to 10.85 times for 28S and to 46.78 times for 5.8S differences in the normal human diploid genome. Moreover, although the precursor 45S transcript arises from one single copy unit, meaning that the amount of all 3 corresponding rRNA sequences should be equal, at least approximately when considering methodological and computational errors, there can be differences between 18S, 28S and 5.8S sequence numbers in a single genome [22]. Thus, recombination in rDNA repeat areas is more difficult to determine than it should seem. In our research in CPG148, we found approximately equal amounts of 18S and 5.8S sequences but a different amount of 28S sequences. The amount of 18S corresponding sequences in CPG148 (which had bright and large FISH-signals on affected chromosomes) relative to GM06895 exceeded the normal variation, while 28S corresponding sequences were within the normal variation, and 5.8S did not differ. Thus, it could be supposed that the p-arm enlargement of chromosomes 13 and 15, at least in CPG148, is accompanied by changes in the rRNA genes and the corresponding

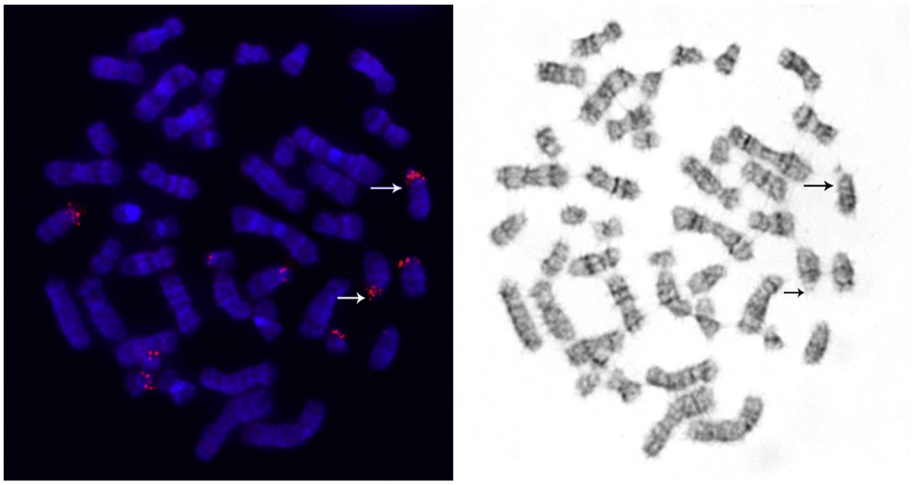

Fig. 3. Localization of dig-labeled rDNA (red) on chromosomes of patient CPG148. Arrows indicate chromosomes with enlarged p-arms. Left: FISH results; right: same G-banded metaphase.

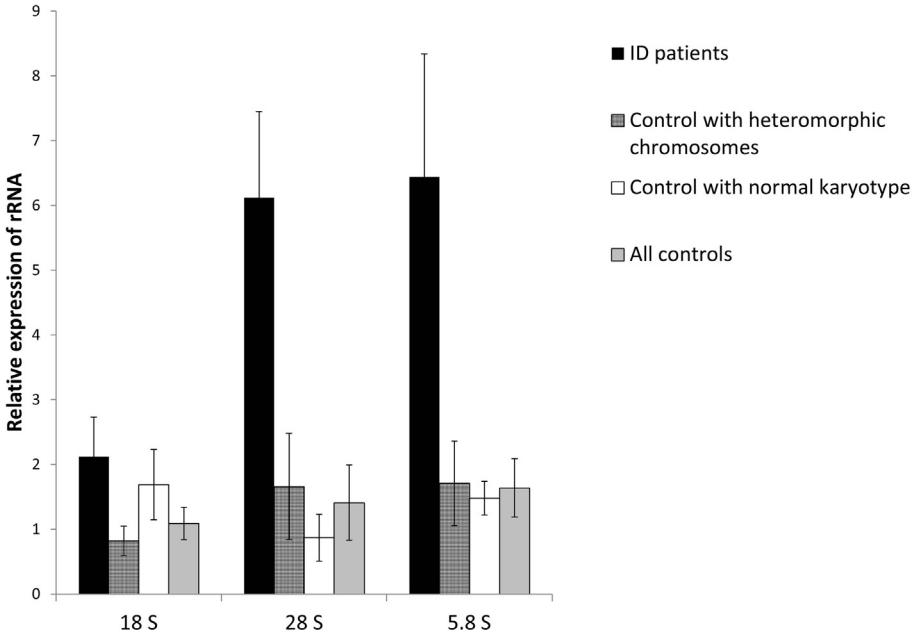

Fig. 4. rRNA levels in studied samples.

rRNA sequences numbers and that the real-time PCR results for CPG148 are consistent with the FISH results. Considering the data obtained in this study, it can be concluded that p-arm enlargement of acrocentric chromosomes with NOR regions is accompanied by rDNA over amplification, at least in some cases.

Correct ribosome assembly was shown to be essential for normal neural (dendritic tree) development [23], and perturbation of rRNA synthesis was shown to lead to neurodegeneration in mice [24]. Thus, rRNA level could play a substantial role in the development of ID. The obtained results support our proposed relationship between RNA levels and ID. Although we did not observe significant differences in 18S rRNA levels between patients and controls (although the mean 18S rRNA level in the group of patients tended to be higher), significantly elevated 28S and 5.8S rRNA levels were found in ID patients.

These results show that heteromorphism itself may not be the main cause of ID, although it may be thought to be a risk factor. We want to emphasize that the frequencies of p-arm enlargements in acrocentric chromosomes with NOR regions do not significantly differ between ID patients and controls; however, rRNA levels in patients are higher compared not only to healthy controls with normal karyotypes but also – and this is most important – to healthy people with enlarged p-arms of acrocentric chromosomes with NOR regions. Thus, excessive copies of rDNA repetitive units appear to not be crucial themselves. It was shown in chicken cell lines with excessive rDNA (tri- or tetrasomic for the NOR-bearing chromosomes) that rRNA expression levels did not differ from that of fully normal diploid cells [25,26]. Therefore, one of the possible mechanisms of ID development seems to be rDNA hyperactivity due to unrevealed causes, which could include disturbance of its regulation. Activity of rRNA genes is regulated by the very large amount of factors, and altered activity of any of them (i.e. due to mutation) could cause loss of dosage compensation, and the search of these factors is one of the next directions of our work.

However, elevated NOR activity is not the only factor playing a role. The 28S/18S rRNA ratio in ID patients also tends to be increased compared to healthy controls, but this is evidently the case only for a subset of the patients. Thus, changes in rRNA maturation processes may also be connected with ID development. Payao et al., upon observing a changed 28S/18S rRNA ratio in Alzheimer patients, proposed preferential degradation of 28S rRNA or, in some patients, all major rRNA subunits [8]. In ID patients with heteromorphism of acrocentric chromosomes with NOR regions, we also observed this ratio shift, but in the opposite direction. We did not observe an increased 28S/18S ratio in any patient. Thus, preferential 28S rRNA degradation and decreased rRNA levels may be a feature of neurodegeneration but not congenital ID development. In contrast, ID may be caused or accompanied by excessive activity of rDNA and, additionally, by 18S rRNA degradation (due to unknown causes and mechanisms that still have to be revealed) after splicing of the 45S primary transcript, and this might be associated with rDNA hyperactivity in some patients. In both cases, however, pathological changes in rRNA expression and/or maturation occur.

6. Conclusion

Our results show that acrocentric chromosome p-arm enlargements and excessive rDNA in them are not sufficient to cause ID. The crucial control point is rRNA expression and, namely, its correct regulation and dosage compensation. If amplified rDNA becomes hyperactive and/or processing is disturbed, problems can arise. Thus, at least two mechanisms may occur, by which development of ID in people with acrocentric chromosome heteromorphism is possible. Disturbance of ribosomal apparatus occurs, which could impair neural system development and crucial changes leading to ID. However, the triggers of these pathologic processes wait to be revealed.

Conflict of interests

The authors declare that they have no conflicts of interest.

Authors contribution

ISK – group statistics, FISH analysis, qRT-PCR, statistical analysis, article preparation.

NAL – cell culturing and fixation, cytogenetic screening (G-banding).

AAD – primer design and qRT-PCR optimization.

YVM and ARS – all clinical studies.

ASG – study concept.

EMG – FISH performance and analysis.

DVY – study concept, financial support, article preparation.

All authors read and approved the final manuscript.

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