MOLECULAR CYTOGENETIC CHARACTERISTICS OF SMALL SUPERNUMERARY MARKER CHROMOSOMES 15 AND 22 IN ASYMPTOMATIC CARRIERS

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Small supernumerary marker chromosomes (sSMC) are structurally abnormal chromosomes that cannot be identified unambiguously by standard cytogenetic methods. A comprehensive approach involving the use of molecular cytogenetic methods is required for the more thorough morphological assessment of such chromosomes, as well as for the development of strategy for genetic counseling of the patients being the sSMC carriers. It is widely accepted that the development of abnormal phenotype by the patients having sSMC in their karyotype is associated with the presence of euchromatic region material in the marker chromosome. Therefore, it results from the presence of relatively large DNA copy number variations (CNVs) in the form of duplication, triplication, and more increased copy numbers; which are localized in the pericentromeric region of the appropriate chromosome. Pericentromeric CNVs can be involved in the chromosome imbalance in asymptomatic carriers of sSMC as well, however, the boundaries of such imbalance have not been clearly identified. The study was aimed to acquire additional information about the genomic topology of the DNA regions insensitive to the genes copy number increase. FISH analysis with commercial and homemade DNA probes was performed in 18 carriers of sSMC 15 and 22 having no clinically significant phenotypic abnormalities. The molecular cytogenetic testing showed that pericentromeric euchromatic regions sized 1.2 Mb and 714 kb, respectively, were found in 33% of cases (6 out of 18). We assume that these regions comprise no potentially dosage-sensitive genes.

Keywords: sSMC, CNV, pericentromeric euchromatin, FISH, homemade DNA probe

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МОЛЕКУЛЯРНО-ЦИТОГЕНЕТИЧЕСКАЯ ХАРАКТЕРИСТИКА МАЛЫХ СВЕРХЧИСЛЕННЫХ МАРКЕРНЫХ ХРОМОСОМ 15 И 22 У АСИМПТОМАТИЧЕСКИХ НОСИТЕЛЕЙ

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Малые сверхчисленные маркерные хромосомы (мСМХ) — структурно аномальные хромосомы, которые невозможно однозначно идентифицировать с использованием методов стандартной цитогенетики. Для более детального анализа морфологии таких хромосом и разработки стратегии медико-генетического консультирования пациентов-носителей мСМХ необходим комплексный подход, включающий молекулярно-цитогенетические методы. Общепринят тот факт, что формирование аномального фенотипа у пациентов с мСМХ в кариотипе связано с наличием материала эухроматиновых районов, вовлеченных в состав маркерной хромосомы. И, как следствие, обусловлено присутствием в геноме протяженных вариаций числа копий участков ДНК (copy number variations, CNV) в виде дупликации, трипликации и большей копийности, которые локализованы в прицентромерном районе соответствующей хромосомы. Прицентромерные CNV могут быть вовлечены в хромосомный дисбаланс и у асимптоматических носителей мСМХ, однако границы такого дисбаланса окончательно не установлены. Целью исследования было получить дополнительные сведения о геномной топографии участков ДНК, не чувствительных к увеличению копийности генов. Был проведен FISH-анализ с коммерческими и несерийными ДНК-зондами у 18 носителей мСМХ 15 и 22 без клинически значимых аномалий фенотипа. Установлено, что в 33% (6 из 18) случаев присутствуют участки прицентромерного эухроматина размером 1,2 м.п.н. и 714 т.п.н. соответственно. Мы предполагаем, что эти регионы не содержат потенциально чувствительных к дозе генов.

Ключевые слова: мСМХ, CNV, прицентромерный эухроматин, FISH, несерийный ДНК-зонд

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Small supernumerary marker chromosomes (sSMC) represent a heterogeneous group of structurally abnormal chromosomes that cannot be identified unambiguously by standard cytogenetic testing due to their small size and the features of genetic makeup, specifically due to the submicroscopic copy number variation of DNA (Copy Number Variations, CNV) [1]. The share of sSMC carriers among newborns in the population is 0.044%, among them 70% have no apparent clinical manifestations [2, 3]. This sSMCs can originate from any one of the 24 human chromosomes and can have different shapes, such as inverted duplication (inv dup), ring (r), and minute (min) shapes [4, 5]. In people having the 47,XN,+mar karyotype, sSMC most often originate from chromosomes 15 (about 30%) and 22 (about 20%) [1]. Clinical manifestations associated with the presence of sSMC in the karyotype can vary considerably, from normal phenotype to significant disturbances of physical and psychomotor development. These manifestations depend on the chromosomes involved in their development, the presence of euchromatic regions, gene content, degree of mosaicism and uniparental disomy.

Phenotypically normal carriers of sSMC can have CNVs in the form of duplication/triplication located in the pericentromeric euchromatic regions [6]. This suggests that such CNVs comprise no dosage sensitive genes, so the increase in the number of copies do not results in major phenotype alterations. Over the past decade, there is growing evidence of the fact that the presence of CNVs involving rather long euchromatic regions, sized up to several million base pairs (Mb), does not cause phenotypic alterations in the carriers [7].

Thus, euchromatic sSMC in the karyotype of asymptomatic carriers can represent a perfect model for analysis of the length of human genome regions insensitive to the changes in the copy number of genes located in the pericentromeric regions. This will make it possible to more accurately define the boundaries, where the dosage-insensitive regions end and the genome regions, the changes in the copy number of which can result in abnormal phenotype and psychomotor development delay, begin [8]. Thorough assessment of each sSMC case and accumulation of additional data contribute to the expansion of knowledge about the mechanisms of formation and pathogenetic significance of CNVs associated with the presence of such supernumerary marker chromosomes in the genome. The study was aimed to acquire additional data on the genome topology of the DNA regions insensitive to the genes copy number increase.

METHODS

The study involved 18 peripheral blood samples collected from asymptomatic carriers of sSMC 15 (n = 9: 3 males, 6 females) and sSMC 22 (n = 9: 5 males, 4 females). Inclusion criteria: all individuals taking part in the assisted reproductive technology program.

Cytogenetic assessment of the GTG-banding chromosomes was carried out in accordance with the standard protocol [9]. The marker chromosomes were identified by FISH with the commercial DNA probes for the centromeric (pericentromeric) regions of chromosomes 15 (SE 15, Kreatech; Netherlands) and 22 (CCP22-Pericentromeric, CytoTest Inc.; USA), as well as for the regions 15q11.2 (LSI SNRPN, Kreatech; Netherlands) and 22q11.2 (LSI TBX1, Kreatech; Netherlands), in order to rule out the clinically significant euchromatic regions comprised in the marker chromosome. FISH analysis involving the use of commercial DNA probes was performed in accordance with the manufacturers' protocols (Kreatech, CytoTest Inc.; USA). Denaturation and hybridization were performed using the ThermoBrite hybridization system (StatSpin; USA). The analysis involved the use of the AxioImager M.1 epifluorescence microscope (Carl Zeiss; Germany) and the Isis software tool for digital image processing (MetaSystems; Germany)

A fundamental phase of the study involved the development of our own (homemade) DNA probes for the pericentromeric euchromatin of chromosomes 15 and 22 and FISH analysis aimed to identify CNVs potentially insensitive to the increase in the DNA region copy number. Primers were selected using the Primer-BLAST NCBI software [10] and the UCSC Genome Browser database [11]. The OligoAnalyzer™ Tool was used to test specificity of the selected primers [12]. Primers were synthesized by Evrogen (Russia). The nucleotide sequences of the DNA primers selected are provided in Table 1.

The sequences of the selected DNA primers were used to conduct Long-range PCR using the BioMaster LR HS-PCR (2x) kit (Biolabmix; Russia) in the GeneAmp PCR System 9700 (Applied Biosystems; USA) in accordance with the manufacturer's protocol [13]. The resulting amplicons were purified on the columns using the diaGene kit for the reaction mixture DNA purification (Dia-M; Russia) in accordance with the manufacturer's instructions, then the purified DNA products were combined in one tube in order to obtain a DNA probe with the size of 10–30 thousand base pairs (kb). Nick translation was used to introduce a fluorescent label into the DNA probe [14–16].

To perform FISH with homemade DNA probes, we denatured DNA of the chromosome preparation and DNA probe separately [14, 15, 17].

DAPI I (Abbott Molecular; USA) dissolved in the Vectashield solution (Vector Labs; USA) to a ratio of 1:20 was used for counterstaining of chromosomes. The images of metaphase chromosomes were analyzed using the Isis software tool for digital image processing (MetaSystems; Germany) and the AxioImager M.1 epifluorescence microscope (Carl Zeiss; Germany).

RESULTS

Conventional cytogenetics analysis revealed the karyotype with a supernumerary marker chromosome (47,XN,+mar) in all patients (n = 18). Mosaicism with high levels of abnormal clone (above 40%) was observed in all cases. FISH with commercial DNA probes for the centromeric regions of acrocentric chromosomes made it possible to identify sSMC as the one originating from chromosome 15 in 9 cases and as the one originating from chromosome 22 in 9 cases (Fig. 1A). Furthermore, the molecular cytogenetic analysis results confirmed the lack of clinically significant CNVs in the small supernumerary marker chromosomes in all the cases (Fig. 1B).

FISH analysis aimed to detect CNVs in the proximal euchromatic regions involved the chromosome preparations derived from cultured lymphocytes of all 18 asymptomatic carriers of the sSMC derived from chromosomes 15 and 22. The homemade locus specific DNA probes (hm) were developed for this purpose. When selecting localization of these DNA probes, we were guided by knowledge about the pericentromeric euchromatic regions of chromosomes 15 and 22 which are insensitive to changes gene dosage. It was 3 Mb for chromosome and about 100 kb for chromosome 22 [7, 18] Thus, two hm with the size of about 10–30 kb were designed for chromosome 15. The proximal DNA probe that was most close to the centromere (hm-15-prox) was located at a distance of 1.2 Mb from the pericentromeric heterochromatin, while the

Table 1. Nucleotide sequences of primers used in the study

Chromosome region	DNA probe location relative to centromere	Sequences of DNA primers	PCR product size (bp)
15q11.2	Proximal	F 5'-TACATCTTACACCCCACCCACCCAAACC-3' R 5'-TTTGCGGAAGGCATTAGTCCCCTTTGTT-3'	9882
	Distal	F 5'-TTAAAACGTGGGCTCTTCATTATCGCCT-3' R 5'-TGGACACCAGACAAAACAAAGGAGTCAA-3'	9323
		F 5'-TGACTCCTTTGTTTTGTCTGGTGTCCAA-3' R 5'-CTTATCCTTCCACACTCGCTGAGAACAG-3'	9140
		F 5'-CATGGTAATGTTGCGGTGTGTCTTTGTT-3' R 5'-CTATCTTTAGGCTGCTTGTCTGGTGCTT-3'	9676
22q11.2	Proximal	F 5'- CCCATATCCTTTCCCAAACTCAACACGA-3' R 5'-TTTTTCCCTCTGAACTGGTTTCTGCACT-3'	9441
		F 5'- AGTGCAGAAACCAGTTCAGAGGGAAAAA-3' R 5'-GAACCATCCACGAGGGAGAGTAGTTTTG-3'	9842
		F 5'-TCGCCATGTACTTCACTTTGTTCTGGTT-3' R 5'-GACTGGTCAAGGATGAGGATTTGTCAGG-3'	9600
	Median	F 5'-TCTTCCTTGCCTGGAGGTGGGATCTAGT-3' R 5'-GAGGAGGGAGGGTGCTGACAAAACTGAA-3'	9531
		F 5'-CAATGTCTAGGGGCAACAGAGGGCAGAT-3' R 5'-AGGGCAGGAAATGTGTTCGTCTCGCTTA-3'	9316
	Distal	F 5'-AGAGAGAGGAAGGGGTCGGCTCAAACTA-3' R 5'-TGTGGGGTGTTGGTGACATGGAGTATGG-3'	9718
		F 5'-CAATCCATGCCCACAACATACCAGCCAC-3' R 5'-TATCACTGCCACCCCATCCCCATTTCTG-3'	9862
		F 5'-CAGAAATGGGGATGGGGTGGCAGTGATA-3' R 5'-CAAGAGGCTGGGGCTTCTCTGGTCTTAG-3'	9761

distal DNA probe (hm-15-dist) was located at a distance of 2.2 Mb from the pericentromeric heterochromatin of chromosome 15 (Fig. 2A). The interval between two DNA probes was 1 Mb. Three homemade locus specific DNA probes (hm) with the size of about 30 kb were designed in order to assess the pericentromeric euchromatic region of chromosome 22. The proximal DNA probe, that was most close to the centromere (hm-22-proximal), was located at a distance of 478 kb from the pericentromeric heterochromatin, the median one (hm-22-median) was located at a distance of 714 kb from the pericentromeric heterochromatin, and the distal DNA probe (hm-22-distal) furthest from the centromere was located at a distance of 1.2 Mb from the pericentromeric heterochromatin of chromosome 22 (Fig. 2B).

The results of FISH analysis of the pericentromeric euchromatic regions of sSMC originating from chromosomes 15 and 22 involving the designed homemade DNA probes is provided in Table 2; a total of 30 metaphase chromosome spreads were assessed in all cases.

As shown in Table 2, no pericentromeric euchromatin was found in four patients with sSMC 15 and eight patients with sSMC 22, i.e. the marker chromosomes comprised heterochromatic regions only.

In five patients (№ 3, 6–9; Table 2), pericentromeric euchromatin was found in the sSMC originating from chromosome 15, however, only the proximal DNA probe hybridization signal was detected, i.e. the euchromatic region size did not exceed 1.2 Mb from the chromosome 15 pericentromeric heterochromatin (Fig. 3).

In one case (11 in Table 2), hybridization with two homemade DNA probes, specifically proximal and median, revealed pericentromeric euchromatin in the sSMC 22. Therefore, the

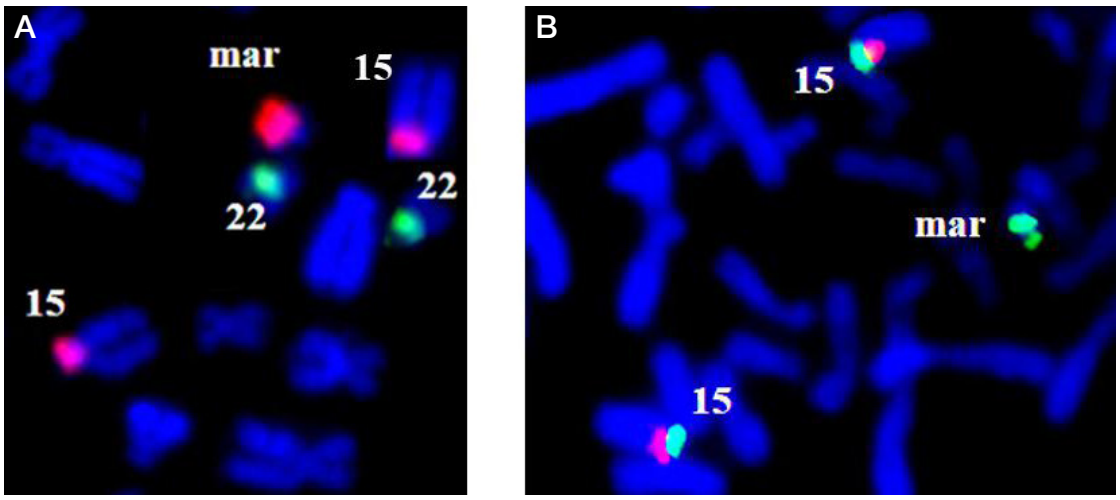

Fig. 1. Results of FISH with commercial DNA probes for chromosome 15. **A**. The marker chromosome originating from chromosome 15 in the form of inverted duplication (ish dic (15;15)(D15Z1+,D15Z1+)). Centromere of chromosome 15 (D15Z1) — red hybridization signal, pericentromeric region of chromosome 22 (CCP22-Pericentromeric) — green hybridization signal (control). **B**. The marker chromosome originating from chromosome 15 comprises no 15q11.2-q13 euchromatic region. Centromere of chromosome 15 (D15Z1) — green hybridization signal, LSI SNRPN — red hybridization signal

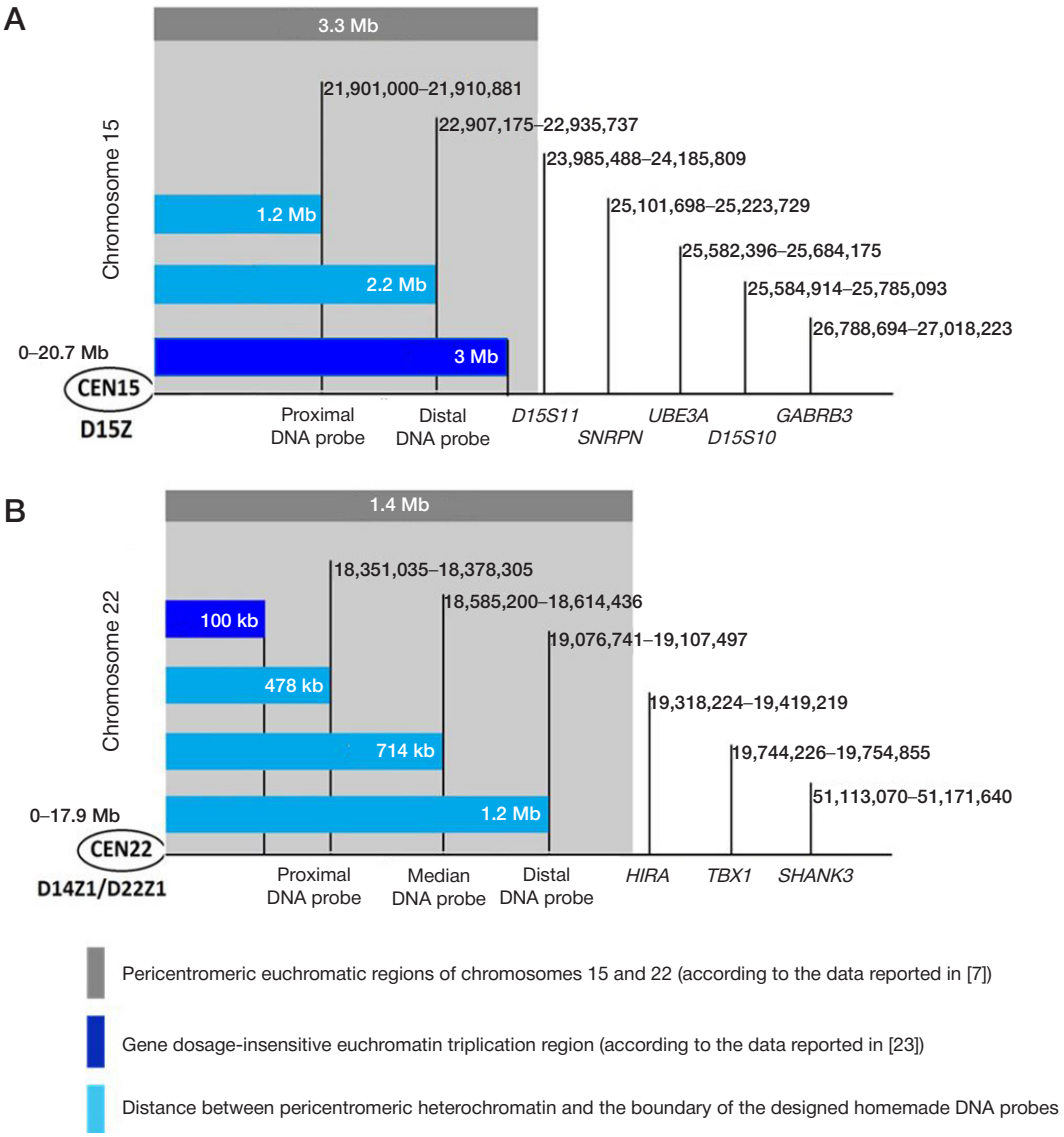

Fig. 2. A. Size and localization of homemade DNA probes within the pericentromeric euchromatic region of chromosome 15. **B**. Size and localization of homemade DNA probes within the pericentromeric euchromatic region of chromosome 22

overall size of the imbalance involved in the chromosomal rearrangement was 714 kb (Fig. 4).

Thus, in the carriers of the sSMC originating from chromosomes 15 and 22 having no apparent clinical manifestations, the marker chromosomes comprise heterochromatic regions only in 67% of cases (12/18); in 33% of cases, euchromatic regions were identified in the pericentromeric regions of the chromosomes 15 and 22 sized 1.2 Mb (chr15 [hg19]: 20,700,000–21,910,881) and 714 kb (chr22 [hg19]: 17,900,000–18,614,436), respectively.

DISCUSSION

Small supernumerary marker chromosomes represent a rare chromosomal abnormality, since these are both numerical and structural aberrations [18]. The clinical manifestations associated with sSMC vary, however, syndromal forms have been reported for some of those, such as cat eye syndrome (MIM#115470), Emanuel syndrome (MIM#609029), Pallister–-Killian syndrome (MIM#601803), and isochromosome 18p syndrome (MIM#614290) [19].

The clinical features can be most often largely explained by euchromatin involvement in the chromosome imbalance, i.e. the presence/absence of dosage-sensitive genes in the

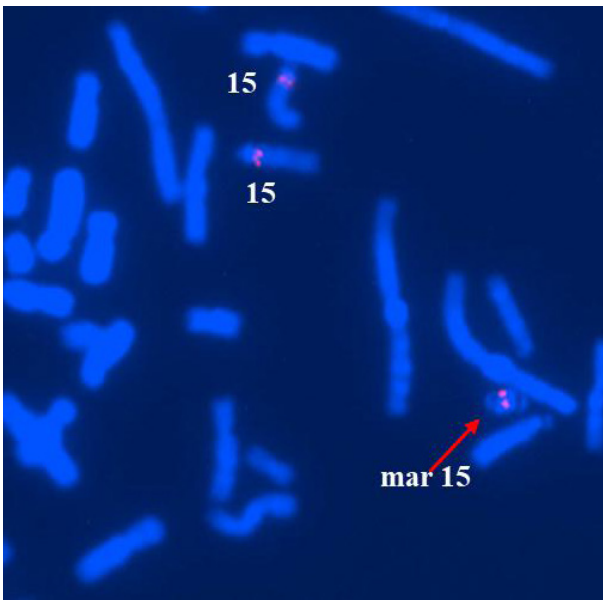

Fig. 3. Results of hybridization involving the homemade DNA probe for the chromosome 15 pericentromeric euchromatin — hm-15-prox

Table 2. Molecular cytogenetic characteristics of sSMC

№	Sex	FISH with commercial DNA probes	FISH with homemade DNA probes (hm)		
			Proximal	Median	Distal
1	M	ish dic(15;15)(D15Z1+,SNRPN-;SNRPN-,D15Z1+)	hm15-	Not provided for для sSMC 15	–
2	F	ish dic(15; 15)(D15Z1+,SNRPN-;SNRPN-,D15Z1+)	hm15-		–
3	F	ish dic(15; 15)(D15Z1+,D15S10-;D15S10-,D15Z1+)	hm15+		hm15-
4	F	ish dic(15; 15)(D15Z1+,SNRPN-;SNRPN-,D15Z1+)	hm15-		–
5	F	ish dic(15; 15)(D15Z1+,D15S10-;D15S10-,D15Z1+)	hm15-		–
6	F	ish dic(15; 15)(D15Z1+,D15S10-;D15S10-,D15Z1+)	hm15+		hm15-
7	M	ish dic(15; 15)(D15Z1+,D15S10-;D15S10-,D15Z1+)	hm15+		hm15-
8	M	ish dic(15; 15)(D15Z1+,D15S10-;D15S10-,D15Z1+)	hm15+		hm15-
9	F	ish dic(15; 15)(D15Z1+,D15S10-;D15S10-,D15Z1+)	hm15+		hm15-
10	F	ish dic(22; 22)(D14Z1/D22Z1+,TBX1-;TBX1-;D14Z1/D22Z1+)	hm22-	–	–
11	M	ish dic(22; 22)(D14Z1/D22Z1+,TBX1-;TBX1-;D14Z1/D22Z1+)	hm22++	hm22+	hm22-
12	M	ish r(22)(p13q11.1)(D14Z1/D22Z1+,TBX1-)	hm22-	–	–
13	F	ish dic(22; 22)(D14Z1/D22Z1+,TBX1-;TBX1-;D14Z1/D22Z1+)	hm22-	–	–
14	F	ish dic(22; 22)(D14Z1/D22Z1+,TBX1-;TBX1-;D14Z1/D22Z1+)	hm22-	–	–
15	M	ish i(22)(p10)(D14Z1/D22Z1+,acro-p++)	hm22-	–	–
16	F	ish dic(22; 22)(D14Z1/D22Z1+,TBX1-;TBX1-;D14Z1/D22Z1+)	hm22-	–	–
17	M	ish dic(22; 22)(D14Z1/D22Z1+,TBX1-;TBX1-;D14Z1/D22Z1+)	hm22-	–	–
18	M	ish dic(22; 22)(D14Z1/D22Z1+,TBX1-;TBX1-;D14Z1/D22Z1+)	hm22-	–	–

Note: hm (homemade) — homemade DNA probe.

pericentromeric euchromatic regions. However, no specific genes have been yet identified [7].

With the development of molecular cytogenetics methods, it became possible to determine the sSMC chromosomal origin and classify sSMC based on the presence or absence of euchromatic regions, thereby facilitating the analysis of the relationship between sSMC and clinical phenotypic abnormalities [6]. Laboratory approaches to the diagnosis and assessment of sSMC are well represented in the literature [20–22]. Fluorescent *in situ* hybridization and chromosomal microarray analysis are most often used to identify the structure and determine the gene content. The shortcomings of the latter include difficulties in interpreting the exact degree of mosaicism, which is a crucial point, given the high frequency of the sSMC mosaic forms [1]. It is more preferable to use FISH, however, the commercial DNA probes not always cover the chromosomal region of interest. Thus, the homemade DNA probes designed to solve specific problems become more and more important. In one of the studies, the possibility of using the PeCR-FISH kit for determination of pericentromeric euchromatin in sSMC developed by the authors is discussed; it has been shown that the locus specific homemade DNA probes based on the BAC clones can be used to accurately determine the sSMC size and the pericentromeric euchromatin involvement [7]. During the reported study, we have designed our own homemade oligonucleotide DNA probes for the pericentromeric euchromatin of chromosomes 15 and 22. The FISH analysis results demonstrate high quality of hybridization signals and, therefore, the possibility of using this approach to assess pericentromeric euchromatin in sSMC carriers.

Our data on the pericentromeric euchromatin size in asymptomatic carriers of sSMC 15 and 22 are consistent with the available literature data and complement these data. To date, there is a rather large number of reported cases of the presence of sSMC 15 in the karyotype of patients without phenotypic features, specifically 418 cases according to the marker chromosome database (ChromosOmics — Database, [23]), among which the majority of sSMC comprise heterochromatin only. The number of sSMC cases with euchromatin involvement, apart from prenatal cases, is small [18]. For example, it was found that in an asymptomatic carrier of sSMC15 with a history of infertility, the length of the gene dose-insensitive region is 3.8 Mb (15-Oq11.2/2-6 in the above database). It should be noted that high degree of mosaicism has been reported in the patient (74% based on the abnormal clone). The proximal pericentromeric euchromatic region revealed in five asymptomatic carriers of sSMC 15 in our sample turned out to be shorter (1.2 Mb) compared to the previously reported “non-critical” region, which makes it possible to significantly complement the database of the marker chromosome 15 region insensitive to the genes dosage.

The number of papers focused on studying sSMC 22 in asymptomatic carriers is much smaller, which correlates with the rate of this chromosomal abnormality [24]. To date, the data of 156 such patients have been reported (according to the website), among them the majority have sSMC comprising heterochromatin only [23]. It has been shown that the size of dosage-insensitive pericentromeric euchromatin region of chromosome 22 in case of triplication is about 100 kb. However, it is important to note that the reported case is unique, it is represented by detection of triplication in the fetus, i.e. identified during prenatal assessment, while normal phenotype has been reported after birth [7, 18]. In the case we have identified, the size of pericentromeric euchromatin of chromosome 22 in the sSMC 22 asymptomatic carrier was 714 kb, which far exceeded the reported size of the “non-critical” proximal region of the chromosome 22 q-arm. New data were obtained on the size of the proximal euchromatin of chromosome 22, which is insensitive to an increase in the dose of genes, may be important for the prenatal diagnosis of cases of the chromosome 22 genomic imbalances (duplication, triplication).

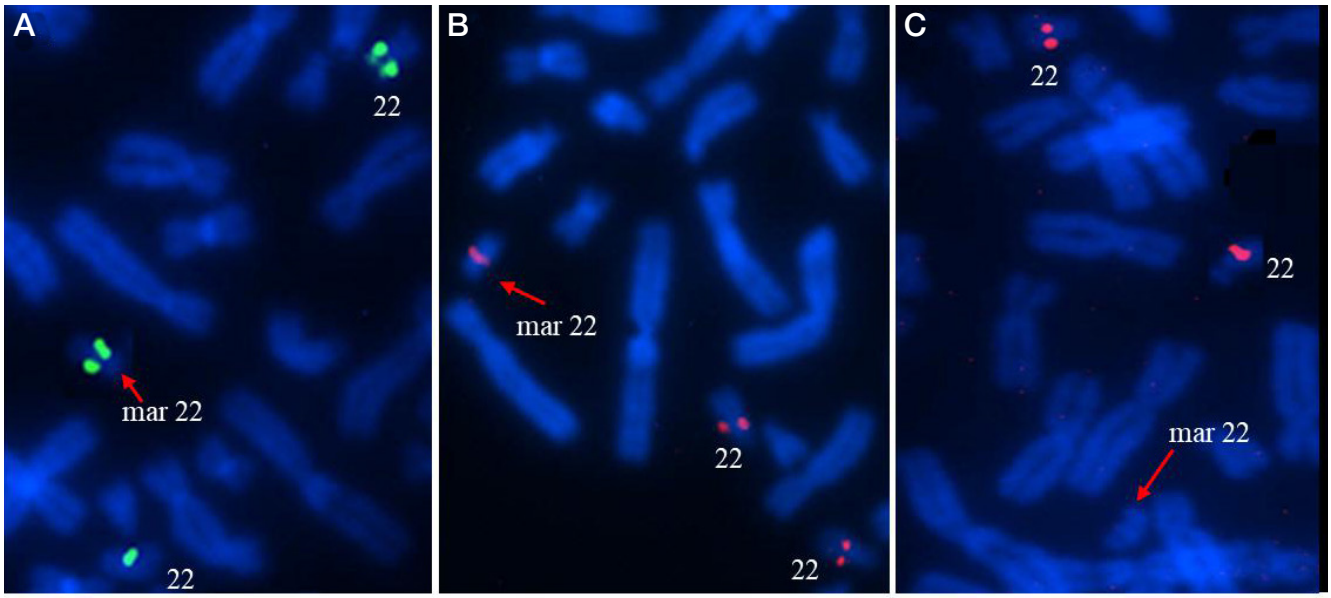

Fig. 4. Results of hybridization involving the homemade DNA probe for the chromosome 22 pericentromeric euchromatin. **A.** Proximal DNA probe. **B.** Median DNA probe. **C.** Distal DNA probe

CONCLUSIONS

Our research findings suggest that, in the pericentromeric euchromatin of the long arm of chromosome 15 (chr15(hg19): 20,700,000–21,910,881) and the long arm of chromosome 22 (chr22(hg19): 17,900,000–18,614,436), there is an apparent absence of genes that are potentially sensitive to an increase in the copy number of DNA sections. The genomic imbalance resulting from duplication/triplication of these regions does not appear to have an adverse impact on intellectual development or cause developmental abnormalities. Our developed approach utilizing non-serial DNA probes has demonstrated the high sensitivity and specificity of the FISH method for analyzing the pericentromeric euchromatin of chromosomes 15 and 22.

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