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Clinical Investigations

CLINICAL AND MOLECULAR-CYTGENETIC STUDIES OF CRYPTIC CHROMOSOME ABERRATIONS IN INDIVIDUALS WITH IDIOPATHIC MENTAL RETARDATION AND MULTIPLE CONGENITAL MALFORMATIONS

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ABSTRACT
Cryptic chromosome aberrations are a common cause of idiopathic mental retardation and multiple congenital malformations syndromes (MR/MCM).

MATERIAL AND METHODS: This study describes results and compares three methods for detection of submicroscopic chromosome aberrations in 76 children with MR/MCM and normal routine G-banded karyotype.

RESULTS: Cryptic chromosome aberrations were detected in 15 patients (19.7%); in 3 of 19 patients (15.8%) by subtelomeric fluorescent in situ hybridization (FISH), in 5 of 47 patients (10.6%) by Multiplex Ligation Dependent Probe Amplification (MLPA) and in 7 of 23 patients (30.4%) by array-Comparative Genome Hybridization (array-CGH). Seven deletions, four duplications and four complex rearrangements have been diagnosed in the present study. Six were de novo and 2 were inherited from a parent carrier of balanced translocation.

DISCUSSION: We observed a slightly higher imbalance incidence compared to the literature. Among these aberrations there were well known syndromes as well as some rare variants.

CONCLUSION: This study confirms the utility of molecular-cytogenetic screening in patients with MR/MCM. We suggest array-CGH as the most reliable technique with a high diagnostic yield.

Key words: cryptic chromosome aberrations, mental retardation, multiple congenital malformations, array-CGH

INTRODUCTION
Cryptic chromosome aberrations are a common cause of idiopathic mental retardation and multiple congenital malformations syndromes (MR/MCM). Routine high-resolution karyotype is limited to aberrations larger than 5-10 Mb. In 5-10% of cases with idiopathic MR/MCM various rearrangements of subtelomeric regions have been detected. Described imbalances were mainly deletions. The detected regions are gene rich and have a high degree of sequence similarity which explains that relatively small deletions can frequently cause MR/MCM. The light color of subtelomeric regions in standard G-banded karyotype makes it hard to detect them, even impossible. However, defects located anywhere along the chromosome may cause MR or/and MCA. A considerably higher detection rate is now possible after the advent of molecular-cytogenetic...
methods as Fluorescent in situ Hybridization (FISH), Comparative Genome Hybridization (CGH), array-CGH and some other methods as Multiplex Ligated Dependent Probe Amplification (MLPA) and Quantitative-Real Time Polymerase Chain Reaction (Q-PCR). In general, submicroscopic chromosome aberrations were detected in 6-20% of individuals with MR and normal karyotype. Subtelomeric FISH revealed abnormalities in 4-9%. Schouten et al. and Koonen et al. used subtelomeric MLPA and found subtelomeric aberrations in 6.7% of individuals with unexplained MR. Palomares et al. compared MLPA to multiprobe FISH and found a high degree of concordance between these two techniques. However, MLPA was suggested to be a rapid, accurate, reliable, and cost-effective technique compared to FISH. Recently, it has been shown that array-CGH is the method of choice in the study of MR/MCM. This technique makes a whole genome screening and was also called molecular karyotyping. The detection rate of array-CGH in screening individuals with MR/MCM has been found to be 10-25%. AIMS OF THE STUDY

1. To find a reliable technique for screening cryptic chromosome aberrations in individuals with MR/MCM and to find criteria to include and exclude patients in screening programs.

2. To determine the incidence of cryptic chromosome aberrations as a cause of MR/MCM.

3. To help the diagnostic process in cases of unclear syndrome with multiple malformations.

4. To analyze genotype-phenotype correlations in cryptic chromosome aberrations.

5. To develop programs for prevention of MR in affected families by means of prenatal diagnosis.

PATIENTS AND METHODS

This study included 76 patients with MR/MCM of unknown etiology. Informed consent was obtained by at least one of the parents. The study was approved by the Ethical Committee of Medical University, Plovdiv. The patients were recruited from the individuals referred to the Medical Genetic Counseling Service, Department of Medical Genetics, University Hospital, Plovdiv. Each subject followed unique diagnostic algorithm in order to exclude single gene disorders, chromosomal aberration on standard banding techniques, teratogenic or perinatal causes:

1. Clinical examination included special attention to dysmorphic features, IQ evaluation by psychologist and anthropometry.

2. A genealogical study with special attention for family members affected by congenital malformations or mental retardation, infertility, miscarriages, sterility; consanguinity.

3. In selected patients examination by neurologist, endocrinologist, cardiologist, hematologist, child surgeon or neonatologist was done.

4. CNS imaging studies (transfontanel ultrasonography, CT scan, MRI), EEG, abdominal ultrasonography, X-rays, vision and hearing evaluation, blood analysis, hormonal status, metabolic screening.

5. Routine high resolution G-banded karyotype, FISH.

MR/MCM patients with normal results on these investigations and with a positive family history were invited to participate in the study. According to these criteria the study comprised 76 individuals, aged between 0 and 18 years. 37 of them were females and 39 were males. As a first step selected clinical material was screened for subtelomeric aberrations by subtelomeric multiprobe FISH and MLPA as described below. If such rearrangement was not detected, whole genome screening by array-CGH was performed (described below). If an abnormality was detected by one of these techniques, locus-specific FISH or Q-PCR was performed to confirm the aberration. After confirmation, parents were tested by locus-specific FISH with the same probes or by Q-PCR done with identical primers. In families with inherited submicroscopic aberrations, genetic counseling, prenatal diagnosis by FISH and ultrasound studies were performed. DNA extraction and leukocyte suspension preparation: Genomic DNA was extracted from blood samples using NucleoSpin® blood kit (Macherey-Nagel GmbH and Co KG, Germany) according to the manufacturer’s protocol. Leukocyte suspension was prepared by adding 0.5 ml blood with heparin to 2 ml lymphochrome (Bio-Whittaker, Cambrex Bio Science, Verviers, Belgium), 2.5 ml RPMI 1640 (Bio-Whittaker, Cambrex Bio Science, Verviers, Belgium), 1 ml fetal bovine serum (Sigma Aldrich Company LTD, Irvine, UK), 0.5 ml phytohemaglutinin (Sigma Aldrich Company LTD, Irvine, UK) and incubated at 37°C for 68 hours. Culture medium BrDU (Sigma Aldrich Company LTD, Irvine, UK) 200 µg/ml was added and incubated again at 37°C for 4 hours and 30 minutes. Finally,
bribidization mixture was added: 20 μl for blocking reagent and 15 μl for genome DNA mixture respectively. Some microlitres of mRNA were added additionally in DNA mixture, 20 μl from blocking reagent were placed on slide, covered by coverslip and hybridized in humid chamber at 37°C. One hour later the coverslip was removed, 15 μl from the probe were placed on the slide and hybridized for 48-72 hours in a humid chamber saturated with 20% formamide and 2xSSC. Washing comprised 4 steps: 1 minute in solution 1xPBS/0.5 Tween 20 at room temperature, 10 minutes in fresh solution 1xPBS/0.05 Tween 20 at room temperature, 30 minutes in solution 50% formamide/2xSSC at 42°C and 10 minutes in solution 1xPBS/0.05 Tween 20 at room temperature. The slides were spin dried for 1 minute at 1200 rpm. Scanning at 532 nm and 635 nm was performed using GenePix 4000B scanner (Molecular Devices). All further data analysis was simultaneously performed with Excel (Microsoft Inc.) and Looptool (ESAT-SISTA, University of Leuven, Leuven, Belgium).

Quantitative-PCR: Primers were selected using PrimerExpress 2.0.0 ABI Prism software (Applied Biosystems, Lennik, Belgium). A penalty score less than 130 was used to further analyze the selected oligonucleotides. The primers and the amplicons were separately checked to exclude any repetitive sequences by using the BLAST program from the NCBI (www.ncbi.nlm.nih.gov/BLAST) and University of California, Santa Cruz (UCSC) in silico PCR (http://genome.cse.ucsc.edu). Q-PCR was carried out using the qPCR mastermix Plus for SYBR Green I without UNG (Eurogentec, Liege, Belgium) according to the manufacturer’s instructions. The final volume of 15 ml contained 0.75 mM of both forward and reverse primers, 7.5 ml of reaction buffer, and 50 ng of DNA per reaction. PCR was carried out in duplicate. An 81-base pair DNA fragment within the p53 gene (forward: 5'-CCCAAGCAATGGATGATTTGA-3' and reverse: 5'-GAGCCTCAGTGGACCTTGGGT-3') was used as a control amplicon. Q-PCR was done with the locus-specific oligonucleotides of interest on an ABI PRISM 7000 Sequence Detection System (SDS) according to the manufacturer's instruction manual (Applied Biosystems, Lennik, Belgium). The amplification results and the melting curve were analyzed with the ABI Prism 7000 SDS software version 1.1 (Applied Biosystems). DNA levels were normalized to the gene p53 and relative differences were calculated according to the relative quantitation method.

DNA extraction, leukocyte suspension preparation and part of FISH with locus specific probes were carried out in the Department of Medical Genetics, University Hospital, Plovdiv, Bulgaria; subtelomeric FISH in the Human Genetic Institute, University Hospital, Hamburg, Germany; MLPA in the Department of Clinical Genetics, Rigshospitalet, University of Copenhagen, Denmark, and array-CGH in the Center for Human Genetics, University Hospital Gasthuisberg, Leuven, Belgium.

RESULTS
76 patient were investigated as follows: 19 by subtelomeric FISH, 34 by MLPA, 13 consecutively by MLPA and array-CGH, and 10 by array-CGH. Abnormal results were found in 15 patients (19.7%): 3 (15.8%) detected by subtelomeric FISH, 5 (10.6%) by MLPA and 7 (30.4%) by array-CGH. The 15 abnormalities were 7 deletions, 4 duplications and 4 complex aberrations (Table 1).

DISCUSSION
This study proposes diagnostic guidelines for the screening of individuals with MR/MCM, describes the incidence of cryptic chromosome aberrations as a cause of MR/MCM and analyzes genotype-phenotype correlations in patients with chromosome imbalance.

We used three techniques (subtelomeric FISH, MLPA, array-CGH) to screen 76 MR/MCM patients aged between 0 and 18 years with apparently normal karyotype. In all tested individuals at least three from the following criteria were present: MR of unknown etiology, dysmorphic features, congenital malformation, positive family history, normal routine karyotype. Cryptic abnormalities were found in 15 patients (19.7%): 3/19 (15.8%) were detected by FISH, 5/49 (10.6%) by MLPA, 7/23 (30.4%) by array-CGH. These data confirm the higher detection rate of array-CGH. This technique is suitable to screen for cryptic chromosome imbalances in the whole human genome. Similar results were published by other authors. Novelli et al. described the incidence of subtelomeric aberrations detected by FISH as 16.3%, Rooms et al. and Koolen et al. 5.2% and 6.7% subtelomeric rearrangements depicted respectively by MLPA.26,12,16 Using array-CGH Menten et al. found 20% submicroscopic abnormalities among MR/MCM patients.3 The slightly higher incidence of aberrations detected
<table>
<thead>
<tr>
<th>N</th>
<th>Chromosome aberration by ISCN(^1)</th>
<th>Detection method</th>
<th>Confirmed by</th>
<th>Inherited/de novo</th>
<th>Parents analyzed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>46,XX.ish del(17p13.3)de novo</td>
<td>sMLPA(^2)</td>
<td>LS-FISH(^4)</td>
<td>de novo</td>
<td>LS-FISH</td>
</tr>
<tr>
<td>2.</td>
<td>46,XY.ish del(4p16.3)de novo</td>
<td>sFISH(^3)</td>
<td>LS-FISH</td>
<td>de novo</td>
<td>LS-FISH</td>
</tr>
<tr>
<td>3.</td>
<td>46,XX.ish del(13p13)de novo</td>
<td>sFISH</td>
<td>LS-FISH</td>
<td>de novo</td>
<td>LS-FISH</td>
</tr>
<tr>
<td>4.</td>
<td>46,XX.ish del(2q37.3)mat, polymorphic variant</td>
<td>sFISH</td>
<td>LS-FISH</td>
<td>Maternal karyotype: 46,XX.ish del(2q37.3)</td>
<td>LS-FISH</td>
</tr>
<tr>
<td>5.</td>
<td>46,XX, arr cgh 1q44(RP11-399B15)x1 de novo</td>
<td>array-CGH</td>
<td>LS-FISH</td>
<td>de novo</td>
<td>LS-FISH</td>
</tr>
<tr>
<td>6.</td>
<td>46,XY, arr cgh 22q11(XX-91c)x1</td>
<td>array-CGH</td>
<td>LS-FISH</td>
<td>Not determined</td>
<td>Not available</td>
</tr>
<tr>
<td>7.</td>
<td>46,XX, arr cgh 19p12(RP11-359H18)x1de novo</td>
<td>array-CGH</td>
<td>LS-FISH</td>
<td>de novo</td>
<td>LS-FISH</td>
</tr>
<tr>
<td>8.</td>
<td>46,XY, arr cgh 17q12(RP5-906A24)x3</td>
<td>array-CGH</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not available</td>
</tr>
<tr>
<td>9.</td>
<td>46,XX, arr cgh 11p15(RP11-11A9)x3</td>
<td>array-CGH</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not available</td>
</tr>
<tr>
<td>10.</td>
<td>46,XY, arr cgh 8p11.1(CTD 2115H11)x3</td>
<td>array-CGH</td>
<td>Q-PCR</td>
<td>Not determined</td>
<td>Not available</td>
</tr>
<tr>
<td>11.</td>
<td>46,XY, arr cgh 22q11(83H3-&gt;CHKAD-26-1)x3</td>
<td>array-CGH</td>
<td>Q-PCR</td>
<td>Not determined</td>
<td>Not available</td>
</tr>
<tr>
<td>12.</td>
<td>46,XY ish der(4)(4;8)(p16;q24.3)pat</td>
<td>sMLPA</td>
<td>LS-FISH</td>
<td>Not determined</td>
<td>Not available</td>
</tr>
<tr>
<td>13.</td>
<td>46,XX,del 11qter, dupl 4pter</td>
<td>sMLPA</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not available</td>
</tr>
<tr>
<td>14.</td>
<td>46,XX, ish der(8)(t(8;20)(p23.3;p13)de novo</td>
<td>sMLPA</td>
<td>LS-FISH</td>
<td>de novo</td>
<td>LS-FISH</td>
</tr>
<tr>
<td>15.</td>
<td>46,XX ish der(1)t(1;11)(p36.3;q25)mat</td>
<td>sMLPA</td>
<td>LS-FISH</td>
<td>Maternal karyotype: 46,XX ish t(1;11)(p36.3;q25)</td>
<td>LS-FISH</td>
</tr>
</tbody>
</table>

\(^1\) ISCN – International System for Human Cytogenetic Nomenclature 1995
\(^2\) sMLPA – subtelomeric MLPA
\(^3\) sFISH – subtelomeric FISH
\(^4\) LS-FISH – locus specific FISH
by array-CGH may be probably explained by the high selectivity of our cases. Thirteen individuals were subsequently analyzed with MLPA and array-CGH. MLPA did not detect subtelomeric rearrangements, but array-CGH revealed 4 additional alterations. From our data and the data of previously published reports we suggest array-CGH to be the most reliable and feasible technique to screen individuals with MR/MCM. This technique promises to become an important tool in the clinical diagnostic setting and will, at least partially, replace standard karyotype.

Taking into account these data it is clear that some of them were not very rare. For most of the cases a genotype-phenotype correlation was present: 2 cases of 4p deletion syndrome (Wolf-Hirschhorn syndrome), one case of 22q11 deletion syndrome, one case of 22q11 duplication syndrome, one case of 1p36 deletion syndrome, one case of 13q deletion (11 Mb in size), one case of 11q deletion syndrome or Jacobsen syndrome, one case of 1q44 deletion syndrome. However, in addition some rare variants were found (Table 1). Rare variants could be subdivided in two groups. The first group included previously not described or rarely described rearrangements: 17q12 duplication (113 kb in size); 19p12 deletion (177 kb in size); 8p11 duplication (143 kb in size). The second group contained alterations located in well known chromosome regions, but showing a different phenotype probably due to the smaller aberration size. This imbalance was a 120 kb large duplication in 11p15. Rare variants will be published separately.

Out of all 15 aberrant cases 6 were of de novo origin, 2 were inherited from a parent carrier of a balanced translocation, 1 case was a polymorphic variant inherited from a healthy mother. Unfortunately, in 6 cases parents could not be tested.

CONCLUSIONS

The imbalances described above can contribute to the delineation of new microdeletion syndromes and prove that array-CGH is a fast, reproducible and sensitive method to screen for submicroscopic aberrations among individuals with MR/MCM.

REFERENCES


Материал и методы: Настоящая работа описывает результаты и сравнивает три метода с помощью которых обнаруживаются цитогенетически неустановленные хромосомные аберрации у 76 детей с УО и/или ВММ в нормальных кариотипах при рутинном Г-ленточном анализе.

Результаты: Субмикроскопические хромосомные аномалии обнаружены у 15 пациентов (19,7%) как следуют методом флюоресцентной in situ гибридизации (FISH) со специфическими ДНК зондами для субтеломерных участков у 3 от 19 исследованных (15,8%); методом зависимой от локирующих зонд множественной амплификации (MLPA) со специфическими зондами для субтеломерных участков – у 5 от 47 (10,6%) и методом векторной сравнительной геномной гибридизации (array-cohortaive genome hybridization, array-CGH) – у 7 от 23 (30,4%) обследованных. Аноморфные находки составили...
из 7 делеций, 4 дупликаций и 4 комбинированных aberrаций. Из 15 aberrаций 6 — это aberrации de novo и 2 — унаследованы от родителей, носителей балансированных транслокаций.

Дискуссия: По сравнению с имеющимися в литературе данными наблюдаемая авторами частота слежка повышена. Среди диагностированных дефектов встречаются и хорошо известные клинические синдромы, и некоторые редкие варианты.

Выводы: Настоящее исследование утверждает высокую диагностическую эффективность молекулярно-генетического скрининга, что касается скрытых хромосомных aberrаций в случаях идентификации УО и дисморфизма. Техника афта-CGH, по мнению авторов настоящей работы, самая подходящая и имеет высокую диагностическую стоимость.