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IMPLEMENTATION OF FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) AS A METHOD FOR DETECTING MICRODELETION SYNDROMES – OUR FIRST EXPERIENCES

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A b s t r a c t: Fluorescent *in situ* hybridisation (FISH) is a complementary cytogenetic method which has an important role in discovering unsolved cases of mental retardation and multiple anomalies. The ability of this method to detect complex and cryptic chromosomal rearrangements exceeds the resolution of the usual cytogenetic banding techniques; therefore it has a wide implementation in modern cytogenetic laboratories – in routine work, as well as for research purposes.

We analysed 19 patients with microdeletion syndromes – 9 patients with Williams syndrome, 4 patients with Prader-Willi syndrome, and 6 patients with DiGeorge syndrome. On the basis of evaluation of facial dysmorphism and the presence of specific major anomalies, all the patients met the criteria for the diagnosis of the syndrome. FISH studies were performed, confirming the suspected syndrome in patients.

Key words: fluorescent *in situ* hybridization, microdeletion syndromes, dysmorphology.

Introduction

Chromosomal imbalances are responsible for a number of cases with mental retardation. Many dysmorphic syndromes are explained by changes in the number and structure of chromosomes, even more precisely with high-

resolution banding techniques. However, some of the well-known syndromes remain undiscovered after a conventional chromosome study. Despite many different banding techniques and analysis through cytogenetic imaging systems, conventional karyotyping has certain disadvantages: despite a constant improvement in chromosomal culture and banding techniques, subtle structural rearrangements of chromosomes which are less than one band (1-10MB) remain invisible (as in microdeletion, microduplication syndromes and cryptic translocations). Moreover nonmitotic cells cannot be evaluated for specific chromosomal changes (i.e. in Killian-Pallister syndrome, where analysis of a buccal smear is needed). Often the origin of some marker chromosomes cannot be easily recognized.

The *in situ* hybridization method was employed for the first time in 1969 by Gall and Pardue, and was modified in 1981 by Langer, who used a non-radioactive *in situ* hybridization procedure. The technique of fluorescence *in situ* hybridization has been developed as a complementary method for rapid gene mapping within the Human Genome Project [1].

FISH provides a link between two techniques: conventional cytogenetics and molecular genetics [2, 3]. The process of hybridization is highly specific and occurs only between the probe and analysed specimen within a specific target locus of a chromosome. The sensitivity of FISH can be very high – up to 10 Kb, which is beyond the resolution of conventional cytogenetics [4, 5]. The principle of the method is based on the hybridization of complementary, single-stranded nucleic acid previously labelled with a fluorescent tag to a complementary sequence on a fixed chromosome spread.

The method has a wide range of applications in cytogenetic laboratories for routine analysis in prenatal (preimplantational) diagnostics, detection of microdeletions, microduplications, marker chromosomes and other cryptic and complex chromosomal rearrangements. This technique has been widely used for research purposes such as gene mapping, analysis of nuclear organization during the lifetime of a cell, to follow the dynamics of DNA reparation, philogenetic studies, etc. [6, 7].

Fluorescence *in situ* hybridization can detect numerical and structural rearrangements of chromosomes utilizing band-specific [8], centromere-specific [9] and chromosome-specific [10] probes.

In this context many dysmorphic conditions accompanied with mental retardation have been shown to be associated with microdeletion and microduplication of specific chromosomes. These conditions, known as microdeletion and microduplication syndromes, are clinically well defined (Table 1).

In most of the microdeletion syndromes, the deleted region usually involves many neighbouring genes. The phenotypic diversity between patients with the same syndrome originates from the different size of the deleted region,

covering a variable number of deleted genes. Therefore, microdeletion syndromes are also known as contiguous gene syndromes.

Table 1 – Табела 1

Most common microdeletion syndromes [11,12]
Најчести микроделециони синдроми [11,12]

Syndrome	Clinical features	Probe/ Chormosome Locus	% Cases with deletion
Angelman syndrome	Ataxia Inappropriate laughter Mental Retardation	SNRPN/ 15q11.2	70%
Cri du chat syndrome	High-pitched cry Microcephaly Mental retardation	D5S23/ 5p15	>99%
DiGeorge syndrome	Dysmorphic facies Hypoplasia or aplasia of Parathyroid/Thymus	TUPLE1/ 22q11.2	>95%
Velocardiofacial syndrome	Conotruncal Cardiac Defect Hypernasal Speech	TUPLE1/ 22q11.2	>70%
Kallmann syndrome	Dysmorphic elfin facies Cardiac defect Mental retardation	KAL/ Xp22.3	
Miller-Dieker syndrome	Dysmorphic facies Seizures	D17S379/ 17p13.3	>90%
Prader-Willi syndrome	Neonatal hypotonia Obseity Hypogonadism Mental retardation	SNRPN/ 15q11.2	70%
Smith-Magenis syndrome	Dysmorphic facies Mental retardation Self-destructive behaviour Craniofacial changes Sleep disturbances	D17S29/ 17p11.2	99%
Steroid Sulfatase Deficiency	Ichthyosis, possible cryptorchidism, corneal opacities	STS / Xp22.3	85%
Williams syndrome	Elfin facies Cardiac defect Mental retardation	ELN/ 7q11.23	>95%
Wolf-Hirschhorn syndrome	Dysmorphic facies Mental retardation	D4S96/ 4p16.3	>99%

The probes used can be specific for the gene that is defective (as in Miller-Dieker syndrome) or is located to or near the critical region and contain several genes usually with a length of 110–150 Kb (as in Williams syndrome). These probes are highly sensitive, yet in a small number of cases, due to the different genetic mechanisms (very small deletions, point mutations, imprinting) the results can be a false negative although a particular syndrome is clinically present.

Some of the probes (e.g. Prader-Willi/Angelman; DG/VCFS) are the same for two different syndromes, but the gene responsible for their appearance is different [13]. For example, the candidate gene for Prader-Willi syndrome is SNRPN, while the gene for Angelman syndrome is UBE3A although both of them are located on 15q11–13.

There are some other methods for the detection of the microdeletions, such as MLPA (multiple ligation dependent probe ligation) and STR (short tandem repeats), which can be used if additional data are needed, such as estimation of the length of the deleted region, or concerning the parental origin.

Material and methods

Patients with the three most common microdeletion syndromes were included in this study – 9 patients with Williams syndrome, 4 patients with Prader-Willi syndrome and 6 patients with DiGeorge syndrome. A dysmorphic profile was established in all of them, including major and minor anomalies that had been described for each syndrome.

In our investigation standard FISH protocol was used. Metaphase spreads from peripheral blood leukocytes were used for the analysis. Pretreatment of the slides was done with pepsin, PBS, followed by dehydration with graded ethanol series. Highly sensitive probes were used for detecting Williams syndrome (7q11.23, Cytocell Technologies, Cambridgeshire, UK, Aquarius, Cat.No. LPU 011), Prader-Willi syndrome (15q11, Cytocell Technologies, Cambridgeshire, UK, Aquarius Cat.No. LPU 005) and DiGeorge syndrome (22q11.2, Cytocell Technologies, Cambridgeshire, UK, Aquarius Cat.No. LPU 004). The probe and the metaphase preparation on slide were denatured in a 70% formamid/2xSSC solution at 72^{o}C for 3 minutes, followed by fast cooling on ice. The denatured probe was placed onto the slide and hybridization was carried out over 18 hours at 37^{o}C. The slide was counterstained with a solution of DAPI/mounting medium. Fluorescent labels attached to the probes allowed direct visualization of the targeted region with a fluorescent microscope (Olympus BX51) equipped with a set of filters; DAPI for counterstaining, FITC for visualization of green and TRITC for that of red. The software used for image analysis was obtained from Video Test-FISH (St. Petersburg, Russia).

Results

A clinical evaluation of the patients was performed including all minor and major anomalies for the particular syndrome, according to the literature and to the data obtained by a specific software programme for dysmorphology caled

London Dysmorphology Database (Oxford University Press, London, UK). The most apparent features in all the patients analysed are presented in Table 2. The proportion of the observed clinical signs is determined in the three analysed groups separately – nine patients with Williams syndrome, four patients with Prader-Willi syndrome and six patients with DiGeorge syndrome (Table 2).

Table 2 – Табела 2

Percentage of dysmorphic features present in analysed patients
Процентуална застапеност на присутните дисморфични стигмати кај анализираните пациенти

Williams syndrome n = 9		**Prader-Willi syndrome n = 4**		**DiGeorge syndrome n = 6**	
clinical feature	**%**	**clinical feature**	**%**	**clinical feature**	**%**
Short stature	78%	Postnatal roblems	100%	Microcephaly	83%
Elfin (long) facies	56%	Obesity	100%	Cleft palate	50%
Big ears	100%	Hypotonia	100%	Mental retardation	100%
Protruding philtrum	100%	Short stature	100%	Microretrognathia	100%
Prominent lips	100%	Small hands/feet	100%	Prominent nose	100%
Depressed nose	100%	Temporal narrowing	100%	Malar flatness	100%
Epicanthus	67%	Almond-shaped palpebrae	100%	Thickened helix	100%
Malar flatness	100%	Up-slanted eyes	100%	Low-set ears	100%
Clinodactyly	100%	Epicanthus	100%	Wide nasal root	100%
Hypoplastic nails	56%	Strabismus	100%	Narrow palpebral fissures	100%
Hallux valgus	44%	Thin nose	100%	Microstomia	100%
Mental retardation	100%	Hypogonadisms	50%	Hexodactily	17%
Characteristic behaviour	56%	Blond hair	50%	Slender hands/fingers	100%
Hypercalcaemia	44%	Obsessive eating	100%	Hypotonia	100%
Cardial anomaly	78%	Seizures	25%	Swallowing difficulties	50%
Disturbed dentition	78%	Short stature	100%	Cardiac anomaly	83%

All the analysed cases met the major criteria for the specific syndrome (Figure 1). All of the cases with Williams syndrome had an elfin face and characteristic "cocktail party person" behaviour which were the main clinical features of the syndrome. Specific cardiac anomaly of the aorta or pulmonary artery was found in 77% of analysed patients, while hypercalcaemia was noted in 44% of them. The auxologic characteristics of patients with Prader-Willi syndrome were studied: the weight of all Prader-Willi patients was above the 97‰ curve while the height was between 3 and 10‰ curve for the age. Hypogonadism –

small testes and penis – was noted in the two male patients. Small hands, as well as the specific facial features – almond-shaped eyes and temporal narrowing – were seen in all the patients. The main feature in most of the DiGeorge patients was severe cardiac anomaly, i.e. tetralogy of Fallot and truncus arteriosus, while in two patients a ventricular septal defect was noted. One patient had no cardiac defect. Other main features of the syndrome: specific facial appearance – micrognathia and a prominent nose – were present in all the patients. A palatal cleft was seen in half of the analysed patients with DiGeorge syndrome.

All the patients had moderate developmental delay and mental retardation.

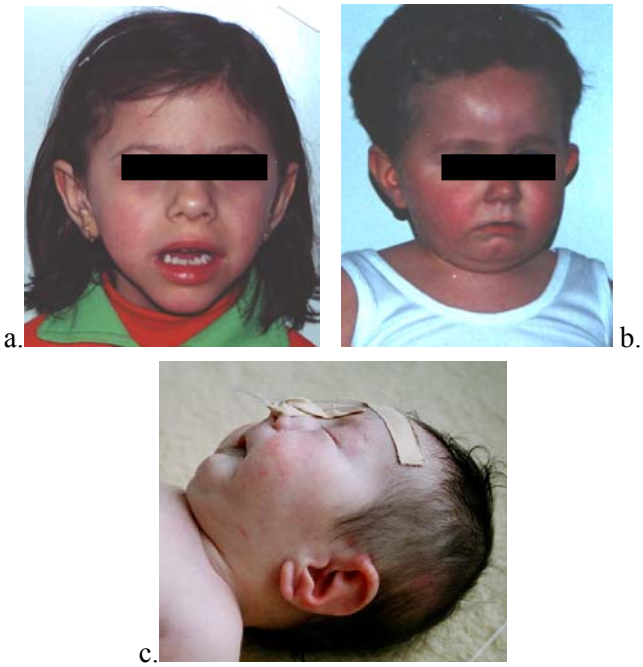

Figure 1 – Facial appearance of children with a) Williams, b) Prader-Willi and c) DiGeorge syndrome
Слика 1 – Лицеви карактеристики кај три синдроми: а)Williams; b) Prader-Willi; v) DiGeorge

The conventional karyotype analysis in most of the patients was normal, except for one patient with Prader-Willi syndrome with translocation 15/15. In FISH preparations, the deleted signal from one of the analysed chromosomes (7q11 for Williams syndrome, 15q11 for Prader-Willi syndrome and 22q11 for DiGeorge syndrome) was found in all the metaphase spreads, as well as in the interphase nuclei (figure 2). No mosaic line with nondeleted chromosomes was detected in any of the patients.

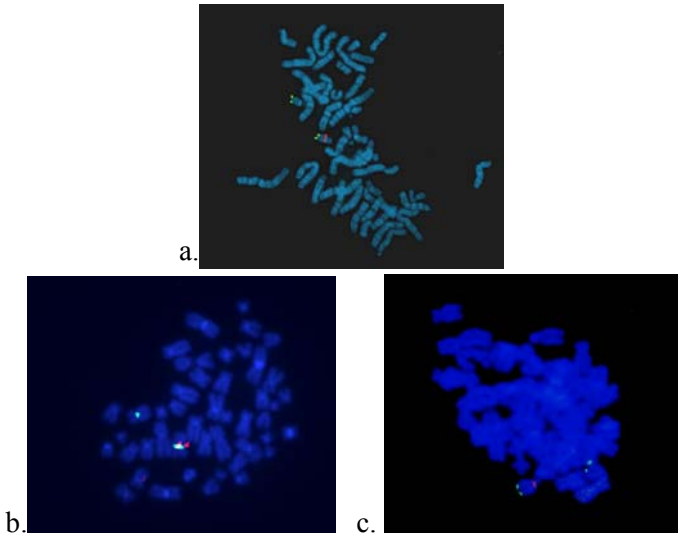

Figure 2 – FISH analysis in some of our patients: a) 22q11.2 including TUPLE1 and neighbouring genes; b) 7q11.2 encompassing ELN gene; c) 15q11.2 with SNRPN gene. In all photos the green signal corresponds to the centromeric probe, and the red fluorophore is a targeted locus specific probe. A deleted signal in one of the analysed chromosomes confirms the diagnosis of the syndrome

Слика 2 – FISH анализа кај некои од нашите пациенти: а) 22q кој го содржи генот TUPLE1, како и соседните гени; б) 7q11.2 со вклучен ELN ген; в) 15q11.2 со SNRPN ген. На сите фотографии зелениот сигнал кореспондира со центромерната проба, додека црвениот сигнал е баранатa локус специфична проба. Сигналот што недостасува на еден од испитуваните хромозоми е доказ за постоечкиот синдром

Discussion

Microdeletion syndromes are genetic syndromes that are associated with small chromosome deletions that are beyond the resolution power of conventional banding karyotyping analysis. Recognition of the phenotype almost always relies on detecting and combining minor dysmorphic stigmata characteristic of the syndrome.

The spectrum of clinical features usually depends on the number and type of the deleted genes. Therefore, deletion of 7q11.2 chromosome in Williams syndrome can be variable in its size and starting point. In this respect, the ELN gene (responsible for disorganized elastin fibres and an anomal aortic arch) is always deleted, which contributes to recognizing typical features inherent in the syndrome [15]. According to the specific dysmorphism, all of our patients had deletion of the ELN gene, which was confirmed by FISH.

One of the syndromes with a very complex genetic background, Prader-Willi (PW) syndrome, is caused by inactivation of the father's genes within the PW region on chromosome 15q11.2. In 70% of the cases the syndrome is a result of the microdeletion of paternal chromosome 15, in 28% of the cases there is a uniparental isodisomy (either chromosomes originated from the mother, or there is an apparent translocation 15/15). In less than 2% of the cases, there is a mutation within the responsible gene – SNRPN (small nuclear ribonucleoprotein) [16]. In our small series of four patients with PW syndrome, the patients had most of the specific signs of the syndrome. In three of them, there was a deletion of 15q11.2 in all analysed mitoses, and in one child a balanced translocation of chromosomes 15/15 was found. However, some of the children who have PW due to the imprinting mutation could not be identified by FISH. Therefore methylation of the SNRPN gene should be evaluated if suspicion of the syndrome still exists.

The group of syndromes associated with deletion of the 22q is characterized by substantial clinical variability. DiGeorge syndrome has the most difficult clinical appearance; therefore it relies upon the deletion of the complete critical region. The complete clinical spectrum of the deletion of 22q11 is called CATCH22, which includes: velocardiofacial syndrome, conotruncal anomaly face syndrome, CHARGE syndrome, asymmetric crying face syndrome, isolated cardiopathy, etc. In general, larger deletion leads to the most severe clinical picture, although there are data in the literature showing the opposite. Interestingly, there are family studies showing the specific deletion in a child with the full-blown clinical spectrum of DiGeorge syndrome, but the presence of only a few noncardial features of the syndrome in the parent [17]. In this study six patients with DiGeorge deletion were evaluated, and all of them had a complex cardiac anomaly, as well as deletion of the 22q11.2 region confirmed by FISH.

The number of clinically recognizable microdeletion syndromes is constantly increasing. Some of them exhibit a newly delineated complex of dysmorphic signs (such as Mowat-Wilson syndrome, del 1p36) [18]. Some of them have been recognized by medical geneticists for a long time, and now are considered as microdeletion syndromes. For example, Williams syndrome was described in 1961, but its etiology was elucidated only in 1995, on the basis of elastine gene deletion located on 7q11.23.

Careful selection of patients with a specific set of dysmorphic signs is a prerequisite for a successful application of some of the commercial probes [12, 19]. Since some of the microdeletion syndromes give a vide variety and overlapping of the phenotypic signs, clinical diagnosis can be difficult and application of FISH probes is a prerequisite for confirmation. If a deletion is found, parental karyotype analysis is also needed in order to assess recurrence risk and provide proper genetic counselling.

Conclusion

Chromosomal rearrangements are identified as a substantial cause of mental retardation and multimalformative syndromes. Conventional karyotyping has an indisputable role in discovering major chromosomal changes, yet many conditions with small chromosomal aberrations remain unsolved. Primarily used as a research tool, at present FISH is a sensitive molecular cytogenetic method which points to a specific chromosomal aberration. Various applications of FISH represent a powerful tool in the diagnosis of many well-known syndromes. The method allows an increase in the number of solved cases of mental retardation and malformative syndromes. Implementation of this method makes genetic counselling of the affected families possible.

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Резиме

ВОВЕДУВАЊЕ НА ФЛУОРЕСЦЕНТНАТА *IN SITU* ХИБРИДИЗАЦИЈА (FISH) КАКО МЕТОД ЗА ДЕТЕКТИРАЊЕ НА МИКРОДЕЛЕЦИОНИТЕ СИНДРОМИ – НАШИ ПРВИ ИСКУСТВА

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Техниката на флуоресцентна *in situ* хибридизација (FISH) е комплементарна цитогенетска метода која има важна улога во откривањето на причината за ментална ретардација и мултипно-малформативните синдроми. Можноста на оваа техника да ги детектира комплексните и малите хромозомски реаранжмани ја надминува резолуцијата на вообичаените цитогенетски техники, заради што има широка примена во модерните цитогенетски лаборатории – во рутинската работа, како и во истражувачки цели.

Анализирани се 19 пациенти со микроделециони синдроми – 9 пациенти со синдромот на Williams, 4 пациенти со Prader-Willi и 6 пациенти со син-

дромот на DiGeorge. Направена е евалуација на лицевиот дисморфизам, како и присуството на специфични мајорни аномалии. Сите пациенти поседуваа најголем број знаци специфични за синдромот. Со помош на техниката на флуоресцентна *in situ* хибридизација кај сите деца беше докажан соодветниот синдром.

Клучни зборови: флуоресцентна *in situ* хибридизација, микроделециони синдроми, дисморфологија.

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