Application of DNA hybridization methods in clinical cytogenetics

PhD thesis

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1. Introduction

1.1. The frequency of chromosomal aberrations in the general population

Detectable chromosomal aberrations are present in 0,6% of liveborns. This means that 600 newborns with chromosomal deficiency are born every year in Hungary. Such defects involve serious physical and mental consequences. Since it is not possible to cure the consequences of chromosomal abnormalities, the precise identification of the endangered and the prevention based on prenatal diagnosis can only help now efficiently.

The opinion that the frequency of chromosomal aberrations in the general population is higher than the 0,6% incidence is hardly disputable. Furthermore, it is very important to take into consideration that the detection of chromosomal abnormalities depends on the method used. Since the above mentioned 0,6% incidence is based on examinations using traditional methods without competence to detect niceties, every methodological flare can be useful to identify previously undetectable aberrations.

The molecular technologies significantly flared the arrange of possibilities to detect aberrations of the genome. It is expectable with good reason that the examination methods based on DNA hybridization – among them the fluorescence in situ hybridization (FISH) and the microsatellite marker analysis – contribute in force to the accurate identification of chromosomal aberrations as much as possible. The etiology of the majority of congenital somatic and mental disorders is namely unknown, and the chromosomal failures which remains undetectable using traditional methods surely show a large proportion among them.

1.2. Possibilities to add to the efficiency of diagnostic procedures

1.2.1. Examination of aberrations not detectable using light microscope

Traditional banding technics are appropriate to reveal numerical and larger (> 10 Mb) structural chromosomal aberrations. Nevertheless, DNA hybridization and the use of fluorescent dies allows to detect fragments of the order of kilobases specifically, hereby resulting in a resolution far exceeding the earlier light microscopic technics.

Using this method we are able to visualise quite small chromosomal regions in the genome revealing the translocation, deletion or duplication of them. The possibility to detect deletions of 3-5 Mb size resulted in recognition of a distinct group of the so-called microdeletion syndromes (best-known are the Prader-Willi, Angelman, DiGeorge, Williams, Miller-Dieker, Wolf-Hirschhorn, Cri du Chat, Kallmann and Smith-Magenis syndromes). This abnormalities with characteristic complex phenotype occur mostly sporadic but familiar cases are also known, in the background of them usually lies a cryptic balanced translocation of one of the parents. A few genes are affected in the microdeletion syndromes which causes a complex phenotype. The „contiguous gene syndrome" apellation is therefore more apposite, referring to collective deletion of functionally unattached genes which are located side by side along the chromosomes.

Moreover, the specificity of FISH permits of labelling of the probes for individual chromosomes with different fluorochromes, in this way we are able to distinguish the chromosomes which have similar appearence in light microscope. This fact is of great significance in the examination of translocations.

An additional advantage of FISH is the ability to detect certain chromosomal regions using appropriate probes in interphase cells. This method can give information about numerical aberration of particular chromosomes allowing the analysis of a number of cells without preparation of metaphases, therefore permits the study of chromosomal mosaicism in different tissues.

The parental origin of certain chromosomes or chromosomal regions can be determined using microsatellite markers which is remarkable in the clinical diagnostic. Namely the euploide state of the cells characterised with the presence of 46 chromosomes not allways comes into existence by fusion of the complete haploid set of either of the parents, and the so-called uniparental disomy can cause abnormal development.

1.2.2. Examination of mosaicism

It can grease the skids to recognize the chromosomal aberrations being present in the general population, if we secede from the traditional supposition that the biological sample being examined represents the whole organism completely when performing diagnostic procedures. Though we are aware for a long while of *mosaicism*, namely of combined presence of cells with different genotype, our knowledge is restricted because of difficulties of the methodology. It is possible to deduce from numerous data that the extant frequency of genetic mosaicism is in fact substantially more frequent than the number of cases recognized.

The somatic mutations of the cells arising in the course of life can cause tumors that in essence corresponds to mosaicism. Though the mutations of chromosomes or genes leading to the formation of tumors can be identified in more and more cases, at the same time other mutations which are existing doubtless do not cause tumors but go hand in hand with functional trouble of the cells can not or hardly demonstrate temporarily.

The function of mutations occuring in the course of intrauterine life can be examined with better chance. Namely, postzygotic mutations evolving in the developing embryo cause congenital malformations, and the abnormal phenotype accordingly can attract attention to the presence of cells with different genotype. Though in our days mosaicism can be demonstrated even on the level of genes in the speckled, circumscribed aberrations of organs or tissues, by the reason of methodological limits talking about mosaicism means to this day mosaic aneuploidy which is the presence of two or more cell-lines in the body containing different chromosomal set.

The extent of retardation or malformation caused by somatic mutations coming into existence in the developing embryo depends on the proportion and distribution of normal and abnormal cells in different tissues. Since in mosaic cases the clinical features are usually slighter compared to persons having exclusively abnormal cells, mosaicism can explain the varied manifestation of certain inherited abnormalities (genetic heterogeneity), segmental and asymmetric developmental deficiencies (problems of skin pigmentation, hemihypertrophy or unilateral hypoplasia in one part or side of the body), intrauterine growth deficiency but normal karyotype („confined placental aneuploidy") and localized congenital malformations.

It can cause difficulties in the practice of genetic counselling when a chromosomal abnormality recurs in a family appearing as „new mutation". Parental

mosaicism – possibly alone in the gonads existing germinal mosaicism can lie behind this problem. The latter can not be examined technically but also the mosaicism existing in the blood lymphocytes can be ruled out only through suitable approach, namely the chance of recognition of an abnormal cell-line with a lower proportion than 15 % is very small because of the few number of cells analysed.

The fact, that the presence of an aneuploid cell-line in other tissues can not be ruled out by the reason of a normal karyotype obtained by the examination of blood lymphocytes makes the detection of mosaicism difficult. It can not be left out of account that the proportion of the abnormal cells in certain tissues can differ from that in the lymphocytes examined routinely, to which in the analysis of genotype-phenotype correlation could pay less attention up to the present because of difficulties of the methods used. The so-called *tissue-mosaicism* could be detected by the analysis of numerous mitoses originating from different tissues which is hard to carry out in the clinical practice at the same time.

The FISH method applied on interphase cells provides better opportunity as earlier also for the examination of mosaicism, which is considerable in respect of understanding the developmental mechanism of the abnormalities or forming an opinion of the prognosis. Using this method relatively numerous cells can be examined on different tissue samples without cell culturing. Hereby arises the opportunity to get data about numerical aberrations of certain chromosomes in other samples than the blood relative quickly without demand on invasive sampling and on analysis of metaphases.

We performed interphase FISH analysis on samples taken from different tissues (lymphocytes, buccal mucous membrane, cultured fibroblast cells, occasionally muscle tissue cells, epithelial cells from the urine and cells of the hair-root) to examine wide-spread the numerical chromosome abnormalities.

2. Aims of the thesis

The colleagues of our department have gotten a lot of experiences for decades in the field of traditional cytogenetic analysis (G-, Q-, C- and R-banding and staining methods) on both pre- and postnatal samples. However the development of molecular cytogenetic technics have made it possible to perform numerous examinations emerging in the clinical practice which cause unsolvable problem for traditional methods. My primary task in the clinical genetics research group was the establishment of some DNA hybridization methods (FISH, mikrosatellite marker analysis) in the most important areas from the point of view of medical attendance, in the interest of expansion of the examination methods in our laboratory which can be used is diagnostic procedures. The scientific aims of this dissertation are attached to the problems which have arisen during diagnostic work. I wanted to analyse the application possibilities of the above mentioned methods, their advantages and limits, respectively, on the basis of the experiences coming from diagnostic examinations and on the ground of the following questions:

- To what extent can be increased the proportion of detection of the constitutional chromosomal aberrations?
- How far contribute the FISH to the phenotype-genotype correlation analysis?
- What importance can be assigned to the chromosomal mosaicism in the human pathology?

- Which advantages has the interphase FISH analysis in the screening of the fetal aneuploidies?
- How can be improved the efficiency of the genetic diagnostic by the segregation analysis of the microsatellite markers?

3. Materials

3.1. According to the clinical indication: first of all samples originating from the patients who appeared in the genetic counselling and suffer from body or mental disorders were treated (apart from tumor samples). In addition to this also fetal cells were examined which arrived at our department within the frameworks of Down screening program.
3.2. According to the source of the cells or of the DNA: lymphocytes, culture of fibroblast and amniotic fluid cells, cells of chorionic villus samples and buccal mucous membrane, epithelial cells from the urine, cells of the hair-root and occasionally the cells of other tissues were examined.

4. Methods used

4.1. Sampling and preparation

For metaphase FISH analysis sterile venous blood was taken. *In order to culture fibroblast cells* skin biopsy was taken following a disinfection by alcohol. *For amniotic cell culture* 20 ml amniotic fluid was centrifugated in sterile tubes (2000 rpm, 10 minutes), the sediment was resuspended and distributed in sterile flasks. The culturing of the cells and the preparation of chromosomes was performed according to standard protocols. *Preparation of hair-root cells:* the hair was pulled out, the root of them was soaked half an hour in 0,9 % NaCl solution. The cells of the hair-root were spread on cleaned glass slides. The slides were immersed in methanol to fix and then dried at room temperature. *Preparation of epithelial cells from the urine:* 20-50 ml urine were centrifugated (5000 rpm, 10 minutes), the supernatant were discarded, the sediment was resuspended in methanol and dropped on cleaned glass slide. The slides were dried at room temperature. *Preparation of buccal smear:* the sampling of the buccal mucus membrane was made using a sterile, flat piece of wood, the cells were spread on a cleaned glass slide, fixed in methanol and dried at room temperature. *Preparation of tissue samples:* the tissue samples taken during surgical intervention were paraffin embedded (as a microscopic section) fixed on glass slide and transported to our laboratory. The paraffin was removed through immersion of the slides for 3 x 10 minutes in xylene at room temperature. The cells were hydrated using 100-90-70-50 % ethanol solutions, then washed in 2 x SSC solution at 75 ºC for 10 minutes.
Until beginning of the FISH procedure the slides were stored at -20 ºC.

4.2. FISH analysis performed on metaphase chromosomes (lymphocytes, fibroblast and amniotic cell cultures): before beginning of the FISH procedure the frozen slides were thawed at room temperature. The analysis was made according to standard protocols.

4.3. Interphase FISH analysis (on buccal smear, epithelial cells from the urine, cells of the hair-root and nuclei of non-dividing cells of other tissues or prenatal samples, respectively): the analysis was performed according to the metaphase FISH protocol, the difference consisted in the posthybridization washing: using certain probes (for example the probe specific to the centromeric region of X chromosome which usually gives cross-hybridization reaction) 45 ºC temperature or 60 % formamide were applied.

Evaluation: the specimens were evaluated in fluorescence microscope using the appropriate filters for the various fluorescent dyes.

4.4. Microsatellite marker analysis

The extraction of the DNA was performed by salting-out procedure (Miller et al., 1988). The primer sequences of the applied microsatellite markers were chosen from the Genome Database. Samples were heated to 94 ºC for 3 minutes, followed by 38 cycles of DNA denaturation (94 ºC for 45 seconds), annealing (55-57 ºC for 30 seconds), and polymerization (72 ºC for 30 seconds). The final 72 ºC incubation was extended to 3 minutes. The amplification was controlled using 2 % agarose gel electrophoresis (staining with ethidium-bromide). The rest of the PCR products was electrophoresed on 8 % polyacrylamide gels and detected by silver staining.

Evaluation: the alleles of the examined person (child) were compared to the alleles of the parents, so the pattern of the alleles were evaluated.

5. Results

During 7,5 years from 1998 to May 2005 cytogenetic examination of 3331 cases altogether occured in our department (1876 karyotype were made from peripheral blood, 1270 amniotic fluid, 83 chorionic villi and 102 fibroblast samples were analysed and evaluated). In this period 889 metaphase and interphase FISH examination and since May 2002 interphase FISH analysis of 890 fetal samples (cells of amniotic fluid and chorionic villi) were performed, the number of the examinations increases year by year continuously.

Among the mentioned 3331 cases 387 were abnormal (11,7 %). In 208 samples (6,25 %) the abnormality could be detected and exactly identified using traditional cytogenetic methods. In 120 cases (3,60 %) the aberration could be recognized by traditional methods but to arrive to the correct result was possible only using FISH method. There were 59 cases (1,78 %) in which the abnormality could not be realized without FISH. On the basis of the above mentioned data it can be said that the application of FISH method gave considerable assistance to the diagnostic work. The results of the individual fields are described below.

5.1. The application of the FISH method on interphase nuclei

5.1.1. Tissue mosaicism

In one of the patient groups – within fall persons in which cases the mosaicism could be detected either by examination of blood lymphocytes or by observation of unusually mild features compared to the characteristic clinical signs – interphase FISH examination was performed on samples taken from different tissues (buccal mucous membrane, fibroblast culture cells from skin biopsy, epithelial cells from the urine, cells of hair-root, occasionally muscle tissue cells) to examine wide-spread the numerical chromosome abnormalities.

In 16 cases – following the traditional cytogenetic examination – interphase FISH analysis occured on the cells of the above mentioned tissues by which means the proportion of normal and abnormal cells with numerical aberration of the chromosomes 9, 13, 18, 21, X and Y was examined (see tables 1 and 2).

Clinical problem:	**Routine cytogenetic examination**	**Peripheral blood: interph. FISH**	**Buccal smear: interph. FISH**	**Epithelial cells from the urine: interph. FISH**	**Fibroblast cells: interphase FISH**	**Other tissues: interph. FISH**
1. case: Turner sy., mosaic marker X chrom.	45,X (80%)/ 46,X, mar(X) (20%)	-	46,X, mar(X) (100%)	45,X (30%)/ 46,X, mar(X) (70%)	-	-
2. case: Turner sy.; short stature, sex. infant., mild features	45, X (100%)	-	45,X (100%)	45,X (100%)	45,X (100%)	Hair-root cells 45,X (100%)
3. case: Turner sy., short stature, minor anomalies	45,X (22%)/ 46,XX (78%)	-	45,X (16%)/ 46,XX (84%)	46,XX (100 %)	-	-
4. case: mental retardation	46,XY(72%)/ 46,XX (28%)	-	46,XY(80%)/ 46,XX (20%)	46,XY(78%)/ 46,XX (22%)	46,XY(95%)/ 46,XX (5%)	Testis biopsy 46,XY (100%)
5. case: Down sy.	46,XX (12%)/ 47,XX,+21 (88%)	-	46,XX (50%)/ 47,XX,+21 (50%)	46,XX (67%)/ 47,XX,+21 (33%)	-	-
6. case: Down sy., mild features	46,XY(10%)/ 47,XY,+21 (90%)	-	46,XY (4%)/ 47,XY,+21 (96%)	46,XY (12%)/ 47,XY,+21 (88%)	-	-
7. case: Down sy., mild features	47,XY,+21 (100%)	-	-	46,XY (88%)/ 47,XY,+21 (12%)	-	-
8. case: mosaic 9 trisomy, multiplex dev. abnorm.	46,XX (83%)/ 47,XX,+9 (17%)	46,XX (86%)/ 47,XX,+9 (14%)	46,XX (84%)/ 47,XX,+9 (16%)	-	46,XX (100%)	Muscle tissue cells: 46,XX (100%)

Table 1: the data of mosaic cases 1-8.

Clinical problem:	Routine cytogenetic examination	Peripheral blood: interph. FISH	Buccal smear: interph. FISH	Epithelial cells from the urine: interph. FISH	Fibroblast cells: interphase FISH	Other tissues: interph. FISH
9. case: mosaic 9 trisomy (unbalanced translocation., multipl. dev. abnorm.)	46,XX,del(7p) (74%)/ 47,XX, del(7p)+9 (26%)	46,XX,del(7p) (74%)/ 47,XX, del(7p)+9 (26%)	46,XX,del(7p) (86%)/ 47,XX, del(7p)+9 (14%)	46,XX,del(7p) (90%)/ 47,XX, del(7p)+9 (10%)	-	-
10. case: mosaic 13 trisomy, mild features	47,XX,+13 (100%)	47,XX,+13 (92%)/ 46,XX (8%)	47,XX,+13 (100%)	47,XX,+13 (100%)	47,XX,+13 (76%)/ 46,XX (24%)	-
11. case: mosaic 18 trisomy, mild features	47,XX,+18 (100%)	47,XX,+18 (75%)/ 46,XX (25%)	47,XX,+18 (94%)/ 46,XX (6%)	-	47,XX,+18 (93%)/ 46,XX (7%)	-
12. case: primer amenorrhaea	45,X0 (30%)/ 46,XY (70%)	45,X0 (34%)/ 46,XY (66%)	45,X0 (34%)/ 46,XY (66%)	45,X0 (40%)/ 46,XY (60%)	-	-
13. case: Edwards or DiGeorge sy.	47,XX,+18 (100%)	47,XX,+18 (90%)/ 46,XX (10%)	-	-	-	-
14. case: Klinefelter sy.	45,X (100%)	45,X (100%)	45,X (100%)	-	-	Testis biopsy 45,X (100%)
15. case: minor anomalies	unsuccesful	47,XX,+18 (10%)/ 46,XX (90%)	-	-	-	-
16. case: Edwards sy.	47,XY,+18 (100%)	47,XY,+18 (64%)/ 46,XY (36%)	47,XY,+18 (64%)/ 46,XY (36%)	-	-	-

Table 2: the data of mosaic cases 9-16.

In the cases of the examined mosaic aneuploidies dissimilar proportion of the abnormal cells taken from different tissues were found in the samples of 14 patients. The remained two cases (2 and 14) fall in the group where the idea of chromosomal mosaicism was raised on the basis of unusual mild clinical features, however, the result of the traditional cytogenetic analysis showed exclusively the presence of abnormal cells. In this two cases neither examination of cells taken from different tissues could detect the presence of normal cells. (Since in the 14th case the results were hard to explain taken into consideration the child's phenotype, further examinations were performed. The findings of them will be described at the 29th case in chapter 5.2.1.)

Further five cases rank in the same group (case 7, 10, 11, 13 and 16) at which the routine cytogenetic analysis showed abnormal cells alone but further examinations could detect the presence of normal cells in other tissues of the body.

It is remarkable that in the cases 2 and 14, in which the slighter clinical features could not be explained by the presence of normal cells in the organism – leastwise in the samples examined -, abnormality of the sex chromosomes was observed, in the remained five cases at the same time the trisomy of chromosomes 13 (case 10), 18 (case 11, 13 and 16) and 21 (case 7). Trisomy 13 and 18 are regarded as incompetible with life in the long run (4 cases among the mentioned 5 rank in this group). Consequently, longer survival or unusually mild features, respectively, give in this group more chance for examination of chromosomal mosaicism and for exact specification of the indication of such analyses.

In 8 cases (case 1, 3, 4, 5, 6, 8, 9 and 12) mosaicism was detected even by the routine cytogenetic examination. The analysis of the cells from different tissues was destined to establish the proportion of normal and abnormal cells, which proved diverse in every cases. In the cases 13, 15 and 16 further examinations of mosaicism were not made because of the death of the newborns.

The results accounted for a few phenomena: variable expressivity (case 8 and 9), not expected survival in lethal disorders (case 10 and 11), Turner (case 1 and 3) and Down syndrome with mild features (case 5, 6 and 7).

Our findings show that mosaicism can be detected with various proportion of abnormal cells in different tissues in the most examined aneuploidies. So in similar cases the analysis of various tissue samples taken with as less invasive method as possible can be required to make exact diagnosis. Additionally, the results confirm the assumption that mosaicism in the human body – including also the different proportion of cells with abnormal genotype among tissues – plays a more significant role in the formation of phenotype and the determination of prognosis as guessed now on the basis of the present knowledge.

5.1.2. Prenatal screening program

Applying the method which was developed in the course of the examination of mosaic cases I had the opportunity to optimize a method for screening of the most frequent aneuploidies on uncultured amniocytes. Within the confines of the screening program with the intent to examine of numerical chromosome abnormalities have been analysed more than 890 samples until this day. Routine chromosome analysis of metaphases and also interphase FISH was performed on every sample to detect numerical abnormality of chromosomes 13, 18, 21, X and Y.

It can be highlighted that the results of the interphase FISH and the side by side performed metaphase analysis differed in neither of the cases. In 30 abnormal cases (3,37 %) numerical aberration of the above mentioned chromosomes were detected. In further 10 cases abnormalities of other chromosomes were found: in 3 samples supernumerary marker chromosomes, in 7 cases in turn several translocations (5 balanced and 2 unbalanced) were revealed in course of the metaphase chromosome analysis. At one of the fetuses having unbalanced translocation was either of the parents a known carrier, therefore the aim of the sampling was first of all the metaphase chromosome analysis. Consequently, among the 890 samples examined up to the present remained altogether two anomalies leading to abnormal phenotype undetected applying interphase FISH method (0,23 %).

The advantage of interphase FISH in contrast to metaphase analysis is that it requires substantial less work (it is true enough, that it demands more financial expenditure), and that the result is available after two days following the sampling. Therefore in our opinion based on our experiences this fast and reliable test as a screening method can replace the metaphase analysis requiring two weeks in cases of certain indications, first of all in advanced maternal age, as already suggested also in the literature.

5.2. The application of FISH on metaphase chromosomes

5.2.1. Structural chromosome aberrations causing multiplex developmental abnormalities

The application of FISH method as a supplement for the traditional cytogenetics technics is an important advance in the exact detection of the most variable chromosomal abnormalities (numerical, structural, mosaic) which is essential to determine the correct diagnosis.

The FISH analyses made in order to identify the structural chromosome abnormalities have been performed using probes specific to whole chromosomes („painting probes”), or to the alpha satellite, centromeric or telomeric region of the individual chromosomes, respectively, and single copy probes, among which were two or more probes hybridized to the specimen target sometimes simultaneously. Additionally, a screening method using specialized probes have been set to detect subtelomeric rearrangements, which can not be revealed with light microscope and the occurrence of which can be expected in idiopathic mental retardation cases.

Hereafter I will describe the accurate identification of altogether 30 cases with structural chromosomal aberrations (tables 3 and 4), among which there are two cases carrying complex rearrangements. In the main part of the demonstrated cases the intention of the FISH examination was the more accurate analysis of the rearrangement or abnormality raised by the result of G-banding. In some cases (5, 25 and 30) further examinations were performed because of special signs in the phenotype of the patient which referred to a certain chromosomal disorder. In the case number 14 the task of FISH analysis was to detect a possible carrier of an unbalanced translocation, since the translocation of the chromosomal region in question can not be revealed using G-banding alone.

Case	Indication of examination	Result of G-banding	FISH result
1.	Turner syndrome	45,X (30%)/46,X,+mar(70%)	45,X/46,X,idic(Y)(q11)
2.	craniodysplasia, muscle-hypotonia, minor anomalies	46,X,del(Yq)	del(Y)(q12)
3.	mental retardation	46,XY/47,XY,+8/47,XY,+mar	Identification of the marker: r(8)
4.	Edwards syndrome	46,XY,t(6;14)	46,XY,t(4;6;14)(q26;q16;p11),del(4)(qter)
5.	hypospadiasis, multiplex dev. abnorm.: del(18q)?	46,XY(?)	46,XY,del(18)(qter)
6.	hypophosphatasia	46,X,del(Xp)	46, X,del(X)(p22.3)
7.	short stature	46,X,del(Xp)	46, X,del(X)(p22.3)
8.	minor anomalies, somatic retardation	46,XY,del(2q)	46,XY,del(2)(q33.3)
9.	short stature	46,XX,t(1;20)	46,XX,t(1;20)(p11.1;q11.1)
10.	hypotonia, developmental delay	46,XX,13q+	46,XX,t(7;13),del(13)(qter)
11.	Down syndrome	46,XX,9p+,10q+,22q-	46,XX,t(10;22)(q26;q12),dup(9p),del(9)(pter)
12.	minor anomalies, delayed psychomot. development	46,XX,18p+	46,XX,der(18),t(18;20)(p11.1;p11.1)pat
13.	short stature, mental retardation	46,XX,13q+	46,XX,dup(13)(q12→q14)
14.	t(5;18) carrier (?)	46,XX(?)	46,XX,t(5;18)(qter;qter)
15.	prenatal screening (Down?)	46,XY (70%)/46,XY,+F (30%)	46,XY (70%)/46,XY,+20 (30%)

3. table: more accurate FISH analysis of structural abnormalities raised by the result of G-banding or by the phenotype of the patient I.

Case	Indication of examination	Result of G-banding	FISH result
16.	trigonocephaly, minor anomalies	46,XY,del(2p)	46,XY,der(2)t(2;17)(p25;q24)pat
17.	minor anomalies	46,XY,t(12;15)(p11;p11)	46,XY,t(12;15)(q13;q15)
18.	minor anomalies, mental retardation	46,XY,t(2;14)	46,XY,t(2;14)(q11;q24)
19.	hyperpigmentation of the skin, café au lait spots	46,XX,r(15)	determination of the breakpoints (see: Morava et al., 2003(a))
20.	minor anomalies	47,XX,+der(15)	47,XX,+der(15)(pter→q14)
21.	minor anomalies	47,XY,+der(9),t(9;21)	47,XY,+der(9),t(9;21)(q13;p13)mat
22.	minor anomalies	46,XX/46,XX,r(18)/45,XX,-r(18)	46,XX,del(18p)/46,XX,r(18)/45,XX,-r(18)
23.	Turner syndrome	45,X	45,X (95%)/46,X,i(Yp) (5%)
24.	prenatal screening (Down?)	46,XY,-B,+C	46,XY,t(2;5)(q32;q14)
25.	gynecomasty (intersexuality?)	46,XX(?)	46,XX,t(Xp;Yp)
26.	minor anomalies	46,XX,14p+	46,XX,der(14),t(14;X)(p12;p11)
27.	prenatal screening (Down?)	46,XX,t(2q;6q)	46,XX,t(2q;6q)(6q;11q)(11q;3q)(3q;2q)
28.	amenorrhaea	46,XY	detection of the SRY region
29.	Klinefelter syndrome	45,X	45,X,t(16;Y)(p13.3;p11.2)
30.	minor anomalies, mental retardation *	46,XX	46,XX,del(9p)

4. table: more accurate FISH analysis of structural abnormalities raised by the result of G-banding or by the phenotype of the patient II. (=screening of subtelomeric rearrangement)*

The extra information provided by FISH have proved essential for solving of a lot of cases in the practice of the last years, among others at one girl who was referred to our department with suspicion of Turner syndrome (case 1), and the routine cytogenetic examination showed two cell-lines (45,X/46,X,+mar), having a marker chromosome in 70 % of the cells. The presence of Y chromosomal fragments is not a rare symptom among Turner syndrome patients, therefore we used a probe specific to α-satellite region of chromosome Y, which identified the marker as an isodicentric Y chromosome. On the basis of the marker's size one could have assumed that the most part of the long arm of chromosome Y is absent, that was confirmed with a probe specific to Yq12 region (Morava et al., 2000c).

The identification of marker chromosomes is not possible using traditional chromosome analysis methods, the FISH can provide help in this cases. In the last years the karyotype mos 46,XY[60]/47,XY,+8[13]/47,XY,+mar[27] was determined in course of the examination of mosaic trisomy 8 syndrome which was detected following toxoplasma infection of the mother in one of our patients (case 3); the marker chromosome proved itself to be ring chromosome 8 on the basis of the analysis performed using a painting probe specific to chromosome 8 (Morava et al., 2002b).

During FISH analysis of an other case (case 19) having ring chromosome 15 was of significant importance the more accurate determination of the breakpoints or the absent regions, respectively, which developed in the course of the ring's formation, apart from the identification of the ring itself, since the phenotype of the child was considerably different compared to similar cases described in the literature earlier (Morava et al., 2003a). The result of the applied single copy and band-specific probes did not refer to deletion of encoding region of significant size (a part of the examinations were made in the Institute of Clinical Genetics, Medical Faculty, Dresden University of Technology in Germany).

In a 7 years old child with short stature and mental retardation (case 13) the cytogenetic analysis showed the presence of a plus segment in the subtelomeric region of chromosome 13. FISH examination of the chromosomal segment in question identificated the fragment as originating from chromosome 13 (painting probe specific to chr. 13), namely as a plus band produced by duplication of 13q14 region (using 13q14 region probe). This result was of great importance because of both the determination of the diagnosis and the further clinical following of the child but the duplicated region can indicate increased osteosarcoma and retinoblastoma risk (Hermann et al., 2000).

The anomaly of the distal part of the long arm of chromosome 2 came up on the basis of the karyotype in a 18-month-old boy (case 8), at whom several minor anomalies and severe growth retardation were found by the clinical examination. The aberration was an interstitial deletion of 2q33.3 band, the accurate definition of that and the determination of the breakpoints required the application of YAC clone probes of the region in question for FISH analysis (I have made the examinations in the Department of Medical Genetics, at the University of Zürich) (Riegel et al., 2001).

The G-banding can be an appropriate method for both recognition and characterization of structural chromosome aberrations, especially translocations, affecting a few bands. However in the cases, in which the aberration affects quite small chromosomal regions sometimes with similar banding pattern, the application of

fluorescent probes is essential. It is exceedingly important, because in the cases of familiar chromosomal rearrangements in the full knowledge of the aberration it is possible to carry out prenatal chromosome examination in a coming pregnancy. Such an examination postulates the application of the FISH method because of both the short time being available and the sometimes poor quality of the metaphases obtained by culturing of amniotic fluid cells. The examination of an already known abnormality on the chromosomes of the fetus can be performed much more expenditious and accurate using specific probes.

The result of G-banding in a child examined because of psychomotor retardation and a pattern of minor anomalies (case 12) referred to the slight enlargement of the short arms of chromosomes 18 and 20, respectively. Since the anomaly of 18p was detectable also on the father's chromosomes in course of the chromosome analysis of the parents, and FISH was at the time of this finding still not available in our department, the interpretation termed this result a possible polymorphism. After initiation of FISH the examination performed using the painting probes specific to the chromosomes mentioned above revealed a reciprocal translocation between the short arms of the chromosomes 18 and 20, respectively, in the father (whole arm translocation), which was present in unbalanced form in the child (monosomy of 18p and trisomy of 20p) (Czakó et al., 2002). Two years ago occured also the examination of the elder sister in the family in order to exclude her carrier state, as well as determination of the genotype of the fetus in a further pregnancy (the above mentioned probes were used for FISH).

Similar to the foregoing, in a child with severe muscle hypotonia, hydrocephalus and joint contractures (case 10) partial trisomy of 7q was detected, which can be explained by the unbalanced inheritance of the balanced translocation t(7;13)(q34;q34) of the mother (Morava et al., 2003b). In the next pregnancy the examination of the fetus was performed using the appropriate painting probes.

A paternal balanced t(2;17)(p25;q24) translocation (than its unbalanced segregation into his child) was revealed applying the probes specific to the telomeric regions of the chromosomes 2 and 17, respectively (case 16) (Czakó et al., 2004).

In a boy with suspicion of Klinefelter syndrome (case 29) an unbalanced translocation was recognized between chromosomes 16 and Y (Kellermayer et al., 2005) in which case the routine analysis showed 45,X karyotype. This surprising result could not be explained without the availability of FISH method. Nevertheless, it came to light in this way that the special region of the Y chromosome was needed for the development of male genitalia is notwithstanding present in his cells, in the background of the mental retardation of the boy stands, however, the tiny deletion of the short arm of chromosome 16.

A child with minor anomalies and mental retardation had normal karyotype (case 30) by reason of G-banding. Since the clinical features referred unambiguously to chromosomal abnormality, a study of the subtelomeric regions were performed revealing a deletion in the subtelomeric region of one of the chromosomes 9. The balanced translocation of this segment could not be detected in the parents (de novo aberration in the child).

Complex chromosomal abnormality have been found altogether in two cases till now. The first patient with complex rearrangement was a 3 years old boy (case 4), who was born at term with low birth weight. He had multiple minor anomalies

(brachycephaly, low set ears, swollen periorbital areas, strabismus, depressed nasal bridge, upturned nose, high arched palate and micrognathia) and overlapping fingers characteristic for trisomy 18, however, congenital heart defect could not be observed. Among the features were also severe growth retardation, muscle hypotonia and psychomotor retardation.

His cytogenetic results referred to a balanced translocation between chromosomes 6 and 14, and to a possible deletion of the short arm of chromosome 4, additionally. Applying the probes specific to 4p16.3 and to chromosomes 4, 6 and 14, respectively, in forms of double hybridizations a complex rearrangement was detected: the distal part of 6q (breakpoint: q16) was translocated to 4q (breakpoint: q26). The segment of the long arm of chromosome 4 (which is found distal to the breakpoint) was translocated to 14p, the probe specific to telomeric 4q showed a deletion on the translocated 4q. By reason of the above mentioned findings it can be assumed that a fourth breakpoint existed on the distal part of 4q, and that the terminal region of 4q participated in the rearrangement. Consequently, the outcome of this complex process is terminal deletion of 4q, which result fits into the phenotype of the child: „4q-syndrome".

The second case showing complex rearrangement was recognized in the course of a prenatal cytogenetic analysis (case 27). Consequently, there was a short time being available for examinations, three rearrangement were detectable during that time, however, on the grounds of that findings the possible role of a fourth chromosome was assumed in this process. Since the mother decided to carry on the pregnancy also in the knowledge of the partial results, further studies were possible only after birth of the child, which demonstrated that it is a complex rearrangement with 4 chromosomes involved and 4 breakpoints affected. The karyotype is: 46,XX,t(2q;6q)(6q;11q)(11q;3q)(3q;2q).

5.2.2. Microdeletion syndromes

Using probes specific to certain chromosomal regions I have studied de novo rearrangements, deletions and duplications, furthermore, a rare familiar cryptic translocation was revealed.

During the period 1998-2005 FISH studies were performed in altogether 412 cases with suspicion of one of the known microdeletion syndromes in patients who have some clinical features characteristic to this type of structural abnormality. The FISH findings were positiv in 59 cases that means that the microdeletion of the critical chromosomal region was detectable (see table 5).

From the patients with Prader-Willi syndrome the examination of a 4-month-old infant in whom at the time of the clinical examination not more than some non-specific features could be observed beyond generalised muscle hypotonia is highlighted. Cytogenetic analysis performed at the age of two month showed a normal karyotype. Further two month later it came to FISH investigation of the region 15q11-13 because meanwhile the recent features have been developed (psychomotor retardation, small hands and feet). The microdeletion of 15q11-13 was confirmed.

In possession of the molecular cytogenetic finding Prader-Willi syndrome could be diagnosed for sure, superseding in this way other examinations which are demanded by the differential diagnostic protocol of the muscle hypotonia in newborns and infants. In the publication written on the score of this case (Erhardt et al., 2000)

we attracted attention to molecular cytogenetic examination of newborns or infants with muscle hypotonia.

Clinical diagnosis	Number of cases	Confirmed microdeletion	Critical region
Prader-Willi syndrome	85	12	15q11-13
Angelman syndrome	89	3	15q11-13
DiGeorge syndrome /VCFS	96	9	22q11.2
Williams syndrome	34	13	7q11.23
Wolf-Hirschhorn syndrome	5	1	4p16.3
Cri du Chat syndrome	6	2	5p15.2
Kallmann syndrome	2	1	Xp22.3
Charcot-Marie-Tooth syndrome	21	1 (duplication)	17p11.2
X-linked ichthyosis	20	8	Xp22.3
Retinoblastoma	11	2	13q14
Smith-Magenis syndrome	22	2	17p11.2
Miller-Dieker syndrome	21	5	17p13.3
In total	**412**	**59**	

Table 5: results of FISH studies performed to detect microdeletions

Within the frameworks of a screening program aimed to study microdeletions of the 22q11.2 chromosomal region examinations due to DiGeorge-, velo-cardio-facial syndrome and congenital heart defects occured (Morava et al., 2000a). This study tried to correlate the phenotype of the affected persons and to determine the incidence of microdeletion in patients with DiGeorge-, velo-cardio-facial syndrome and isolated cardiac anomaly. The intention of the study was to draw recommendations concerning the screening of the deletion 22q11.2 in the above mentioned disorders but the early recognition of the chromosomal aberration is of capital importance in respects of rehabilitation, the estimation of prognosis and family planning (Morava et al., 2000b).

Microdeletion of the same region (22q11.2) was determined in a newborn with partial coronal craniosynostosis and congenital heart malformations. The sequence analysis of the genes frequently studied in craniosynostosis (*FGFR1, FGFR2, FGFR3* és *TWIST*) showed no mutation. Congenital cardiac malformation is a characteristic feature of the 22q11.2 deletion spectrum but craniosynostosis can occur at the same time. Based on the presence of the heart malformation and some of the dysmorphic features including arachnodactyly, FISH analysis was performed with 22q11.2 region specific probe revealing the microdeletion. Since almost 1 % of the patients with 22q11.2 microdeletion were found to have craniosynostosis, which is more frequent

compared to 0,05 % incidence in the general population, we proposed in the article presenting this case (Kárteszi et al., 2004) that patients with coronal craniosynostosis in the presence of congenital heart defect should be screened for the 22q11.2 microdeletion.

We reported on the first Hungarian patient with Smith-Magenis syndrome diagnosed by FISH method in a publication (Kárteszi et al., 2001) in which we recommended to perform FISH analysis of the critical region 17p11.2 in children with mental retardation, speech delay and aggressive behavior.

In two first cousines in a family examined because of Miller-Dieker syndrome the clinical diagnosis was confirmed by FISH analysis of the critical chromosomal region 17p13.3 and the microdeletion was detected in both of the children. The possibility of chromosomal translocation was raised because of the presence of two affected family members, therefore the samples of the parents have been examined. The results showed the 17p13.3 region translocated to the short arm of a group C chromosome in the two fathers (they are brothers).

The translocated chromosome was identified as chromosome 11 with further FISH analyses: both fathers without any clinical features carried the balanced submicroscopic translocation t(11p;17p). There are two carriers among the siblings of the affected cousines, one brother of them has a normal karyotype. We offered the possibility of prenatal diagnosis to the families, to which one of the couples has resorted two times. The metaphase FISH study following amniocentesis showed unbalanced translocation in both of the fetuses and in consequence of that the microdeletion of p13.3 region on the affected chromosome 17.

5.3. Segregation of microsatellite markers

5.3.1. Detection of UPD in somatic and mental retardation of unknown origin

It came to analysis of DNA polymorphism (microsatellite markers) first of all in order to exclude uniparental disomy (UPD).

Because of clinical features denoting to Silver-Russel syndrome (intrauterine and postnatal growth retardation, congenital hemihypertrophy, body asymmetry or characteristic facial features, respectively) the examination of uniparental disomy of the chromosome 7 was performed using microsatellite markers in 12 family, however, UPD was found in neither of them. Taking notice of data published earlier, according to which maternal UPD of chromosome 7 could be detected only in 7-10 % of Silver-Russel syndrome patients, it is not surprising that neither of the families examined showed the abnormality in question.

Paternal UPD of chromosome 11 were studied in 21 families with a child having Beckwith-Wiedemann syndrome (the enlarged tongue, omphalocele, visceromegaly, hemihypertrophy, increased risk of developing specific tumors are characteristic) based on the similar principle. Nevertheless, that the incidence of UPD among Beckwith-Wiedemann syndrome patients is 20 %, positive cases were not found in this group.

UPD of chromosome 15 can cause the features of two characteristic syndromes depending on the parental origin of chromosomes 15 in the cells. The signs of Prader-Willi syndrome are a diminished fetal activity, feeding difficulties after birth, obesity

later, muscular hypotonia, short stature, mental retardation, hypogonadotropic hypogonadism, small hands and feet or typical facial features, respectively. The maternal UPD of chromosome 15 is known in 20-25 % of the cases. Paternal UPD affecting the same chromosome pair causes Angelman syndrome (a condition with features of severe mental and motor retardation, ataxia, hypotonia, epilepsy, absence of speech and unusual facies). Paternal UPD 15 can be established altogether in 1-2 % of the cases.

Microsatellite marker analyses were performed in 60 families aimed to detect maternal or paternal UPD 15 in Prader-Willi and Angelman syndromes, by which maternal UPD 15 were detected in two children with Prader-Willi syndrome. The parents of the child examined earlier asked for prenatal examination (in a subsequent pregnancy of the mother), therefore the analysis was made using the DNA of the fetus obtained by chorion biopsy sampling. The results showed normal biparental segregation according to the expectation.

5.3.2. Examination of the parental origin of supernumerary chromosome 21 in Down syndrome patients

The segregation analyses of chromosome 21 were performed in 14 cases in order to determine whether the non-disjunction leading to trisomy 21 occured during meiosis or mitosis and to establish the parental origin of the supernumerary chromosome 21. The pattern of the markers was not informative in 3 families, showed mitotic (or meiosis II.) non-disjunction in 5 families and meiosis I. segregation failure in 6 cases (in two of them the extra 21 was of paternal, in 3 cases of maternal origin, however, in one of the families the parental origin of the third chromosome 21 could not been defined because of the allele-pairs of the parents which were of the same size). So in almost one third of the cases analysed the non-disjunction was of mitotic/meiosis II.-stage origin, which is according to the data described among cases of free trisomy 21. The two cases in turn in which the extra 21 is of paternal origin are fairly rare.

5.3.3. Identification of the chromosome carrying mutation in monogenic disorders

Microsatellite marker analyses have been performed also in families which are affected by a monogenic syndrome but mutation analysis of the gene in question is not available (e.g. in families whose affected member is not alive anymore and – in absence of sample – the detection of the specific pathogenic mutation is not possible, the other family members, however, would like to minimize the recurrence risk of the disease in their offsprings being born) or the size of the gene not allows of the routine mutation analysis like in neurofibromatosis type I.

In point of view of the genetic counselling the question of carrying Duchenne muscular dystrophy has a significant importance. We tried to establish in 14 families by segregation analysis of the X chromosomes of the affected son, the possibly carrier sister, the parents and grandparents whether the affected (at the time of the examination usually dead) son and his sister shared the same maternal X chromosome. In two families it was possible to exclude safely the sister's carrier state, in other two families the pattern of the markers not allowed of answering the question.

Applying this method it could not be excluded in 9 families that the sister of the affected son is a carrier of the mutation, however, prenatal examinations are possible on the basis of findings, the advantage of this was taken by 4 families up to the present. The results of the examinations showed a presumably carrier female fetus in one pregnancy and male fetuses in 4 pregnancies, each of them had an X chromosome found in the mother's family.

In a family the diagnosis of Duchenne muscular dystrophy was supported by mutation analysis of the dystrophin (DMD) gene performed in two brothers showing deletion of exons 45-50. Since deletions of identical exons were detected in both of the samples, the mother was regarded as obligatory carrier. We performed the analysis of the microsatellite markers of the X chromosome in the family, first of all that of the markers in the region of the DMD gene in order to identify the maternal X chromosome carrying the mutation. The markers in and around the DMD gene showed the two brothers carrying identical X chromosome of the mother. Nevertheless, other markers on the distal short and long arm of the X chromosome referred to inheritance of different maternal X chromosomes. Surprisingly, in the sample of the mother we detected the products of the markers being in the region of the deletion (two alleles of different size), however none of these alleles is present in the children.

On the basis of the results it can be assumed that the mutant DMD gene (the DMD gene carrying the deletion) could be transferred from the original X chromosome to the other member of the homolog pair during a recombination event between the maternal chromosomes. When a chromosomal abnormality recurs in a family appearing as „new mutation", it can cause difficulties in the practice of genetic counselling. Parental mosaicism – possibly alone in the gonads existing germinal mosaicism can lie behind this problem in some cases. In possession of the findings it is also presumable that in the case of the mother of the examined family the question is gonadal mosaicism. (In the Department of Pediatrics, at the University of Szeged further examinations of other female family members were performed in order to get information about their possible carrier state. The results showed no increased risk in neither of them.)

The segregation analysis of chromosome 17 was performed in 16 families with neurofibromatosis type I in order to identify the chromosome 17 which presumably carries the mutation. We managed to do that safely in one family where the inheritance of different chromosomes 17 of the affected parent was detectable in two brothers, which result can be brought into agreement with the different phenotype of the brothers.

6. Assessment of the results

On the basis of the above described results the question put in the chapter „Aims of the thesis" can be answered as follows:

1. On the grounds of our findings it can be drawn that the DNA hybridization technics applicated in the appropriete field ***are able to improve significantly the efficiency of genetic diagnostic***. However, only traditional cytogenetic methods were used in more than half of the 387 abnormal cases (in 208 cases: 53,8 %) analysed since 1998 in the cytogenetic laboratory of our department but in diagnostic procedures of the remainder 46,2 % the FISH method provided valuable information. In 120 cases (31 % of all abnormal cases) the chromosomal aberration was recognized using

traditional methods, the specification and verification of the findings, however, occured by application of FISH. Beside this the FISH was needed not only for the recognition of the abnormality in 59 patients (15,2 %) but also for the correct diagnosis.

Both in Prader-Willi and in Smith-Magenis syndromes – as far as I know – we detected microdeletion in diagnostic procedures first in Hungary and the examination of subtelomeric chromosomal rearrangements was also available at first in our department.

2. The findings obtained by analysis of the individual chromosomal aberrations ***contribute to the understanding of the relationship between phenotype and genotype***. The data presented earlier in the literature were confirmed in several publications (Morava et al., 2000(a), 2000(b) and 2000(c)), in others proposals were put for FISH examination of concrete chromosomal regions in the presence of certain clinical signs (Erhardt et al., 2000; Kárteszi et al., 2001; Kárteszi et al., 2004). We reported data presenting the detailed phenotype in cases of certain affected chromosomal regions, which can be important regarding the prognosis expected in similar cases and the mapping of the individual chromosomal region attached to certain phenotypic abnormalities (Hermann et al., 2000; Riegel et al., 2001; Czakó et al., 2002; Morava et al., 2002(a), Morava et al., 2003(a) and 2003(b); Kellermayer et al., 2005).

3. I have not found reference in the literature for application of interphase FISH method in mosaic cases examined by a protocoll planned in advance. Our results confirm the supposition which was drawn earlier in several case presentations that ***in cases of chromosomal mosaicism the proportion of normal and abnormal cells can vary among different tissues***.

Furthermore, it can be established that in studies detecting mosaicism it is not enough to consider the routine cytogenetic findings based on analysis of metaphases concerning the peripheral blood lymphocytes, because that is not able to detect (or exclude the presence of) the cell-line which is present only in very small proportion in certain cases. Interphase FISH investigation of numerous peripheral blood lymphocytes is reasonable in such cases.

Examinations of cells from different tissues for detection of chromosomal mosaicism is required a.) in disorders with variable expression which can be attached to chromosomal abnormality; b.) in disorders regarded as lethal with unexpected long survival; c.) in cases with suspicion of a chromosomal syndrome where the karyotype of the peripheral blood lymphocytes is normal.

Our results confirm the assumption – which was presented in chapter 1.2.2. – that the mosaicism and the various proportion of cells with abnormal genotype in different tissues of the human body has bigger influence on the phenotype and the prognosis than supposed nowadays on the grounds of the present knowledge.

4. Screening of the fetal aneuploidies is the other important field where interphase FISH is used. The most advantage of the method in this program is the ***fast and reliable result even with expenditure of relative less work***. Furthermore, it is possible to analyse more cells than in the case of metaphase examination which enables it to recognize even mosaic cases showing low proportion of abnormal cells.

5. ***The application of microsatellite markers*** is a unique tool in diagnostic procedures for examination of ***uniparental disomy*** described in several known disorders. In the practice of our department in the last years it could provide the most

facilities in the ***segregation analyses*** performed in the families of patients having Duchenne muscular dystrophy. In addition, using microsatellite marker analysis we obtained data about the pathomechanism of trisomy 21.

7. Conclusions

The application of the DNA hybridization methods in the clinical practice of our department opened ***the door to diagnostic investigations*** in 1.) microdeletion syndromes, 2.) subtelomeric rearrangements and abnormalities, respectively, 3.) cases which can not be revealed using traditional methods (subtle chromosomal abnormalities, rearrangement of chromosomal regions having similar banding pattern), 4.) not dividing cells (detection of mosaicism, screening of common aneuploidies in fetal samples), 5.) cases of uniparental disomy and 6.) monogenic disorders with identification of the chromosome carrying the mutant gene.

In the above mentioned application fields of metaphase FISH method the ***prenatal examinations*** of familiar structural chromosome aberrations are of great significance with which the well known abnormality can be detected reliably within a short time using the appropriate fluorescent probes.

The DNA hybridization methods expand considerably the possibilities of diagnostic investigations provided by the traditional cytogenetic technics, however, it is of great importance to establish that these methods must be used ***as a complement of the banding technics and not instead of them***.

On the grounds of our results also statements can be formulated which are worthy to note ***regarding professional politics***. Suitable financial background is required to introduce this method. However, the outgoings of the microsatellite marker analysis are similar to that of other molecular genetic tests but the FISH analysis is a great charge to a diagnostic laboratory in point of both the costs of probes or other reagents (5-10 000 Ft/test) and that of the acquiring and running of the instruments required by the method (at least 15 M Ft).

A fair number of experiences and relatively long time spent in the practice is needed to the interpretation of the results. The persons who make and evaluate the examination have to possess both molecular genetic and cytogenetic knowledge, furthermore, the perfection in the use of fluorescence microscopy is also essential requirement. It is important to make both examinations in the same laboratory because of the proper interpretation of the findings.

The successful exploration of the individual chromosomal abnormalities depends substantially on the application of the appropriate probes. It is possible to answer accurately the questions raised by the clinical problem, only if the person who makes the analysis and the interpretation of the findings also plans the actions of the diagnostic procedure on the grounds of the available informations. It depends on the team-work of the physicians and the laboratory staff on the one part, and the accurate knowledge of the probes used on the other part.

Therefore, the above delineated examination methods are suggested to be performed only in major laboratories and centres where all conditions (financial background, instruments and well-trained staff) are available for the application of them in diagnostic procedures (especially in prenatal investigations).

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