Cytogenetic Investigations in Immunological and Gastrointestinal Diseases and Malformations in Children

PhD Thesis

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Abbreviations

CD   Coeliac Disease
CSI   Centromere Separation Index.
EMA anti-endomysium antibody
ID   Immunodeficiency
IgA   Immunoglobulin A
PCD Premature Centromere Division
SCE Sister Chromatid Exchange

Introduction

Mitosis is the process of eukaryotic cell division that leads to two identical daughter cells. Chromosomes are replicated during the S phase and condense into compact structures during prophase of cell division. Each of them consists of two identical sister chromatids held together by the centromere. In prometaphase and metaphase chromosomes are aligned along the metaphase plate of the spindle apparatus at the equatorial plane in the midline of the cell. Spindle fibers are bound to the kinetochores (structures associated with the centromere of each chromosome) and during anaphase the centromeres divide and the separated sister chromatids are pulled to the opposite poles by spindle fibers. By the end of cell division two daughter cells are produced with the same genetic component as the parent cell.

To prepare a standard karyotype any population of dividing cells providing metaphase cells is required. Most of the time blood is the sampled tissue as lymphocytes can easily be induced to proliferate, but other sources of tissue e.g. skin (cultured fibroblasts), bone marrow, amniotic fluid, chorionic villi, etc. also can be used when appropriate.

Routine technique for karyotype preparation – brief summary

1. A sample of blood is drawn and coagulation is prevented by addition of heparin.

2. Mononuclear cells are purified from the blood by centrifugation through a dense medium that allows red cells and granulocytes to pellet, but retards the mononuclear cells (lymphocytes and monocytes).

3. The mononuclear cells are cultured for 3-4 days in the presence of a mitogen like phytohemagglutinin, which stimulates the lymphocytes to proliferate madly.

4. Mitosis is arrested in metaphase by a solution of colchicine. At the end of the culture period, when there is a large population of dividing cells, the culture is treated with colchicine, which

disrupts mitotic spindles and prevents completion of mitosis. This greatly enriches the population of metaphase cells.

5. The lymphocytes are harvested and treated briefly with a hypotonic solution. This makes the nuclei swell osmotically and greatly aids in getting preparations in which the chromosomes do not lie on top of one another.

6. The swollen cells are fixed, dropped onto a microscope slide (forcing the chromosomes into a single plane) and dried.

7. Slides are stained after treatment to induce a banding pattern. When they are stained, the mitotic chromosomes have a banded structure that unambiguously identifies each chromosome of a karyotype. Without banding it is impossible to distinguish between among chromosomes.

8. Once stained slides are prepared, they are scanned to identify "good" chromosome spreads (i.e. the chromosomes are neither too long nor too compact and are not overlapping), which are photographed.

9. The photomicrograph is cut apart and the individual chromosomes are arranged into a definitive karyogram. Alternatively, a digital image of the chromosomes can be cut and pasted using a computer. If standard staining was used, the orderly arrangement is limited to grouping like-sized chromosomes together in pairs, whereas if the chromosomes were banded, they can be unambiguously paired and numbered.

Based on the size and shape of the chromosomes of a somatic cell human chromosomes are divided into 7 groups and sex chromosomes.

Centromere separation

Existence of a sequence

It is a crucial point of cell division when sister chromatids forming chromosomes are separated at the centromere and they move to daughter cells. Aisenberg was the first to report in 1935 that this separation is a non-random process. During the mid 70's Méhes and Vig independently published the sequence of centromere separation in human leukocytes. It was striking that chromosomes 2, 17 and 18 separated earlier and the acrocentric chromosomes did not show any signs of division when all others had already separated. Later on other teams joined this research, and centromere separation was investigated not only in different human cells but in other species, as well. Subsequent reports confirmed the original observations. As a result of this research the normal sequence of centromere separation was determined in humans.

Assessment of Centromere Separation

For the investigation of centromere separation a meticulous analysis of several hundreds or thousands of mitoses is required. Centromere separation can be assessed best on traditionally stained, non-banded chromosomes on the one hand, meanwhile on the other hand G-banding is necessary for the individual chromosomes to be clearly identified. This is a difficult and time consuming process to find late metaphases with clear separation of chromosomes which can be still identified by their G-banding patterns. Beyond the previously mentioned difficulties subjectivity in the assessment of centromere division also contributes to complexity when determining the separation order.

There are 2 methods used for the assessment of centromere separation:

1. **Premature Centromere Division** (PCD) introduced by Méhes is used to determine the susceptibility to too early (premature) separation. During analysis only mitoses showing not more than 3 complete separations are taken into account. Complete separation is considered to be present when no connection between the sister chromatids can be seen on microscopic examination. The number of separations for an individual chromosome in a large series is divided by the value calculated based on a "random" separation order. The result reflects the tendency for premature separation. ("Calculated value" is the total number of separations observed in 100 mitoses divided by 23.)

2. Vig introduced a scoring system called the **Centromere Separation Index** (CSI).

   By this method the centromeres that had not divided at all were given a score of 0, those that had just begun to separate scored as 1, and the ones showing clear separation, i.e. no connection between the sister chromatids, were scored as 2.

   The scores obtained were pooled for individual pairs of chromosomes. These totals were then divided by the highest value in the series, providing relative values (CSI) against a given value of 1 for the earliest separating pair. The higher the CSI for a chromosome, the earlier was its position in the centromere separation sequence.

Based on repeated observations in different laboratories and despite all the difficulties in the assessment of centromere separation it is now proven and accepted that during metaphase in human mitotic cells chromosomes do not separate randomly.

> Chromosomes 2 and 18 are the first to separate, followed by chromosomes 4, 5, X, 12, 3 and 17. Chromosomes 1, 7, 8, 9, 11, and 16 separate late and the large acrocentrics (13-15) are the last ones in this order.

The more marginal the position of a certain chromosome in the separation sequence the more accurately can its position be determined. On the other hand, when assessing chromosomes in the middle of the separation order uncertainty due to subjectivity has to be taken into account.

Consistency of Separation Sequence

When investigating separation sequence in different living beings sequences were found to be species-specific. Based on findings published so far the following factors have <u>NO INFLUENCE</u> on the separation order:

> length of chromosome, position of centromere, size of heterochromatic part, time or temperature of incubation, type of medium used, colchicine treatment, type of hypotonic solution or process, drugs, radiation or gender.

The role of aging has not been unravelled yet. Based on species specificity and consistency it is highly likely that the sequence of centromere separation is a genetically determined, species-specific phenomenon unalterable by exogenous factors known to date.

Separation Sequence and Disorders – brief summary

It is well known that factors affecting cell division may cause chromosome injury and may disrupt chromosome pairing or migration. Chromosome aberration arising from this may serve as a basis for a latent or manifest disease which may be inherited in certain cases.

1) **Aneuploidy:** Alteration of centromere separation sequence may lead to non-disjunction causing aneuploidy. When investigating trisomic children and their parents a higher frequency of too early, too late or absent separation of that specific chromosome were reported.

2) **Structural abnormalities:** Too early or too late separation of chromosomes may occur with structural chromosomal abnormalities. Not only out-of-phase separation in subjects with chromosome deletion (13q14) and translocation (3p;19q) were reported but late separating fused D-group chromosomes (D/D fusions) as well.

3) **Carcinogenesis:** Chromosome aberrations play a crucial role in carcinogenesis. According to previously published papers altered centromere separation sequence may be an indicator for mutagenic effect therefore may play a role in carcinogenesis. Attention was called to the relationship between PCD, aneuploidy, mutagenic agents, chromosome breakage, immunological changes and malignancy. Altered centromere separation sequence was shown in several types of malignancy.

Aims of this thesis

1. To investigate whether "normal" centromere separation sequence can be influenced or altered by an extrinsic factor such as vanadate which is well-known to affect cell division;
2. to introduce an objective method for analyzing centromere separation;
3. to investigate chromosomal abnormalities and premature centromere separation in children with congenital immunodeficiencies;
4. to investigate chromosome fragility and premature centromere division in children with coeliac disease;
5. and to investigate chromosomal abnormalities and associated congenital abnormalities in newborns with gastrointestinal malformations.

STATISTICAL ANALYSIS

All statistical analysis was performed using Excel or the Statistical Package for the Social Sciences (SPSS) for Windows 7.5, 8.0 and 11.5 softwares. Statistical significance of the differences between groups was evaluated using the Fisher's exact or Chi-square or Student's *t*-test when appropriate. Differences were considered to be statistically significant when probability values were smaller than 5% (i.e. $P<0.05$).

Investigations

The Effect of Vanadate on the Centromere Separation Sequence

INTRODUCTION

The existence of a "normal" sequence of centromere separation in human mitoses has repeatedly been described. Too early or too late separation may lead to trisomy or monosomy of the given chromosome, therefore alteration of the centromere separation sequence may lead to aneuploidy and may be an indicator of chromosome instability. This raises the question of whether this phenomenon can be influenced by mutagenic factors. To our knowledge only a few studies have dealt with this problem so far. In this study we made an attempt to analyze the centromere separation sequence in human lymphocytes exposed to vanadium, a well-known cytotoxic agent.

MATERIALS AND METHODS

Routine chromosome preparations of peripheral blood lymphocyte cultures were prepared. To

examine the effect of vanadate, 5% NaVO$_3$ was given to the cultures in various amounts and for various times in the following combinations: 0.51, 1.02 and 2.56 μmol/ml, respectively, each for 2.5 hours, 2.56 μmol/ml for 3 and 4 hours, 5.12 μmol/ml for 2.5 and 4 hours, and 2.56 μmol/ml for 6 hours. Since vanadate concentrations above 2.56 μmol/ml and exposures of more than 4 hours proved to be so toxic that the preparations contained no evaluable mitoses, only the lowest and highest exposures allowing normal mitoses were further investigated. Simultaneous cultures of the same blood sample not treated with vanadate served as controls. Mitotic indices were determined by counting at least 2,000 cells from 5 different fields and expressed as number of mitoses at any stage per 1,000 cells.

In order to see the separation more clearly, the chromosomes were not banded. This meant that only the main groups and the most characteristic individual chromosomes could be identified. The sequence of centromere separation was characterized by the centromere separation index (CSI) discussed previously.

Table 1. **Mitotic index and centromere separation index (CSI) values in control lymphocyte cultures and in those of slight and intensive vanadate exposure**

	Control	Vanadate-treated cultures	
		0.51 μmol/ml 2.5 hours	2.56 μmol/ml 4 hours
Mitotic index (1/1000)	49.5	26.0[A]	24.5[A]
CSI			
Number of mitoses	95	91	93
Chromosome or group			
1	0.11	0.15	0.08
2	1.00	0.99	1.00
3	0.47	0.51	0.47
4-5	0.61	0.60	0.58
6-X-12	0.50	0.56	0.52
13-15	0.00	0.01	0.01
16	0.02	0.08	0.03
17-18	0.97	1.00	0.93
19-20	0.45	0.50	0.44
21-22-Y	0.00	0.01	0.02

[A] $p < 0.001$ against control.

The differences in CSI values of the three groups are statistically not significant.

RESULTS

The findings in the three groups, i.e. low and high vanadate exposures and control, are summarized in **Table 1.** As shown by the figures, the mitotic indices were significantly lower in the vanadate-treated cultures than in the untreated controls. Apart from 2 gaps, no breaks and other structural aberrations were found in any of the slides examined. The relative CSI values for both the slight and heavy exposures are almost identical with those of the control group. Since individual chromosomes could not be identified, only the mean values of chromosome groups are given, which demonstrate very similar tendencies of separation. As in the controls, in the cells treated with vanadate chromosomes 2, 17 and 18 also showed very early division, whereas chromosomes 1 and 16 and the acrocentrics were the last to separate. This sequence corresponds to the findings of several earlier studies from different laboratories.

Cytogenetic Investigations in Children with Congenital Immunodeficiencies

INTRODUCTION

In previous studies it has been reported that congenital immunodeficiencies (ID) are associated with a higher frequency of structural chromosome aberrations. In addition, a few syndromes combine ID with fragility of chromosomes - ataxia telangiectasia, Bloom's syndrome and Nijmegen breakage syndrome. Beyond centromeric instability of certain chromosomes somatic recombination of the arms of these chromosomes and a marked tendency to formation of multibranched configurations were also registered in the recently reported ICF (Immunodeficiency, Centromeric heterochromatin instability and Facial anomalies) syndrome.

The aims of this study were:

- To investigate numerical and structural abnormalities of chromosomes and premature centromere division in children with various type of ID.

- To determine if there is any correlation between classic features of chromosome instability (i.e. break, gap, sister chromatid exchange, dicentric, ring formation, etc.) and previously uninvestigated "out of phase" centromere separation.

PATIENTS AND METHODS

"Routine" lymphocyte cultures of 12 patients with different types of ID - hypogammaglobulinaemia 5, variable ID and IgA deficiency 3-3 each and septic granulomatosis 1, respectively - (age: 7

months-11 years, male:female ratio: 9:3) were analyzed and compared to healthy controls (n=6). These lymphocyte cultures were prepared according to the method described previously.

The following abnormalities/features were recorded in at least 60 mitoses of each individual:

Hypo-, Hyperdiploidy, Gap, Breaks, Structural abnormalities (dicentric, ring formation, translocation), Sister Chromatid Exchange (SCE) rate and Premature Centromere Division (PCD)

Beyond the classic features of chromosome instability, like structural abnormalities and SCE, attention was focused on PCD. When analyzing data all efforts were made to be as objective as possible, therefore a simplification was made in PCD analysis. The centromere separation sequence was not determined in each mitosis, only the percentages of those metaphases are given in which 3 or more completely separated centromeres could be seen. Based upon literature and our previous data, fewer than 4% of routine lymphocyte cultures contain such metaphases.

Table 2. **Chromosome breaks, sister chromatid exchange (SCE) and premature centromere division (PCD) in patients with immunodeficiency (ID) and in controls**

Patients	Analyzed mitoses	Chromatid break/cell	Chromosome break/cell	SCE	Mitoses ≥ 3 PCD (%)	PCDs per mitosis of chr. 13-15.
Controls (n=6)	86	0,009	0,004	4,1 (2,1-6,0)	2,2 (1-4)	0,004
1.	70	0,014	0,014	4,6	4,2	0
2.	85	0,012	0	3,7	2,3	0
3.	76	0	0	-	2,6	0
4.	100	0,02	0,01	6,1	5,0	0,01
5.	70	0	0	4,4	2,8	0
6.	60	0,017	0	3,9	6,7	0
7.	66	0,061*	0,03	9,7	13,7*	0,182*
	100	0,1*	0,04*	11,6*	19,0*	0,210*
8.	100	0,01	0	-	2,0	0,10
9.	80	0,025	0,037*	8,5	22,5*	0,137*
	100	0,06*	0,02	7,9	17,0*	0,06*
10.	64	0	0,016	3,6	1,6	0
11.	75	0,013	0,013	4,5	4,0	0,013
12.	60	0,017	0	5,8	3,4	0,017

Mitoses ≥3 PCD (%): Percentages of metaphases with 3 or more complete PCD *p< 0,05
PCDs per mitosis of chr. 13-15.: Number of PCDs of chromosomes 13-15 per mitoses

RESULTS

As **Table 2.** shows, the frequencies of chromosome breaks, SCE and PCD were similar in most of the children with ID compared to healthy controls. However, in patients 7 and 9 (with hypogammaglobulinaemia and variable ID, respectively) significantly higher numbers of structural aberrations, SCE and PCD were found. To rule out the possible effect of a recent viral infection, drug effect or technical artefact during lymphocyte preparation these investigations were repeated after 4-6 months, and the findings were similar again. In these 2 children the classical features of chromosome instability and PCD were parallel. In this study neither ICF nor any other specific syndrome could be identified in any of the 12 children with ID.

It is well-known that subjectivity can not be eliminated when analyzing centromere separation. However, in these 2 patients not only PCD was noted. Frequent separation of the large acrocentric chromosomes, which are known to be among the last ones in centromere division, also was observed. Separation of these D-Group chromosomes is not seen normally.

Objective Analysis of Centromere Separation

INTRODUCTION

The existence of a genetically determined sequence of centromere separation in human mitotic chromosomes seems to be established. Disturbance of this "normal" pattern may lead to nondisjunction, causing aneuploidy, malignancies and various conditions related to chromosomal instability. In spite of the increasing number of such observations, the pathogenetic role of altered centromere separation and PCD has failed to achieve a widespread acceptance, mainly because of the criticism that visual evaluation of the separation grade of a centromere (i.e. the sister chromatids) under the light microscope cannot be accurate and objective. Here we describe a simple computer-based method for the precise measurement of centromere distances within chromosomes by means of image analysis and public software that makes an objective and exact staging of centromere division possible.

MATERIALS AND METHODS

Well-spread metaphases of routine Giemsa-stained chromosome preparations of blood lymphocyte cultures were selected in a NIKON microscope using 400x primary magnification. The microscope was completed with a charge-coupled device camera. Appropriate fields were captured and transferred to a computer installed with the public NIH Image Version 1.55 program. After identification of individual chromosomes on the grey scale digital image, the density slice option

was selected. Within this, a line perpendicular to the longitudinal axis of the chromosome, exactly across both sister centromeres, was drawn on the monitor image and the density histogram (plot) of this line was created in each case by the software. Centromere separation distance could be obtained directly by measuring the length between the 2 density peaks representing the 2 centromeres. The distance between the separating centromeres could be given in pixels or in micrometers after setting the pixel/ micrometer ratio. Images were also analyzed in the binary (black and white) option. By using binary transformation of the image, the separation of the sister chromatids became even clearer, although the fine density differences corresponding to the centromere structure stained with Giemsa and the background grey level were lost. Thus, both grey scale and binary functions of the software gave interesting information concerning the stage of centromere division.

RESULTS

Some 15-20 mitoses of 5 healthy subjects (3 males) with normal karyotypes were analyzed. In each mitosis, the centromere separation distances of the individual chromosomes were compared with each other and aligned according to their separation grade. The measurement was quick and objective and resulted in a "normal" centromere separation sequence that was similar to that observed in earlier studies, i.e. chromosomes 2, 18, 17, 4, 5 and X divided early, whereas chromosome 16 and the acrocentrics were the last to separate.

The advantages of this method are obvious in the determination of PCDs in the mitoses of a patient with Fanconi anaemia. In this case, in addition to completely intact and partially divided chromosomes, PCD of the large acrocentrics is the most conspicuous finding. A single density peak can be found over the centromere of the intact chromosome; two separate peaks with an obvious connection are seen in the case of partial centromere separation, whereas a complete PCD is characterized by a density curve declining to the background level between the two peaks.

Chromosome Fragility and Premature Centromere Division in Coeliac Disease

INTRODUCTION

The frequent association of coeliac disease (CD) with type 1 diabetes mellitus and other autoimmune diseases is well known. This and the increased risk of malignancy in CD raised the question of the possible role of genetic instability as an underlying condition. The results of previous studies regarding chromosome instability in treated and untreated coeliac patients are still controversial. Premature centromere division (PCD) was found to be a possible marker for

chromosome instability. The aim of our study was to investigate chromosome fragility and PCD in patients with CD.

PATIENTS AND METHODS

Spontaneous and bleomycin-induced chromosome fragility and PCD were determined in 48- and 72-hour peripheral blood lymphocyte cultures of 22 patients with CD. In all cases diagnosis of CD was established by ESPGHAN criteria. The male/female ratio was 12/10, ages were 3-19 yrs (mean: 12.5), and duration of disease was 0-13 yrs (mean: 5.9) in this cohort. Similarly prepared cultures of 18 healthy individuals (M/F ratio=9/9) served as controls. Simultaneously anti-endomysium antibody (EMA) was determined and a questionnaire regarding the diet, febrile illness, vaccination and exposure to conditions causing chromosome fragility was obtained. According to this questionnaire no febrile illness, vaccination or exposure to conditions causing chromosome fragility was registered within 6 weeks prior the investigation in any of the subjects. To estimate the effect of gluten-free diet, newly diagnosed patients (n=2) and non-compliant patients (n=5) were also enrolled.

Routine peripheral blood lymphocyte cultures with an incubation time of 48 and 72 hours were prepared. Bleomycin-treated cultures were prepared as routine cultures, with addition of 30 μg/ml bleomycin for the last 5 hours of incubation. A G-banded slide (resolution of approximately 400 bands) was made in each subject for routine karyotyping. The remaining slides were Giemsa stained only and the following abnormalities were registered: chromatid and chromosome breaks, rearrangements, and dicentric and ring chromosomes.

On final analysis only the percentages of mitoses with aberrations were registered irrespective of their nature or number per mitoses. This part of the study was in accordance with the bleomycin test described previously. Cell divisions demonstrating more than 3 PCD (PCD>3%) were also registered. For the assessment of these abnormalities 50-100 mitoses were evaluated in all subjects at 48 and 72 hours of incubation time in routine and bleomycin-treated cultures, respectively. Frequency of chromosome aberration and PCD was compared in coeliac patients and controls.

RESULTS

All the karyotypes were normal, i.e. no chromatid or chromosome breaks, rearrangements, or dicentric or ring chromosomes were identified. The number of spontaneous chromosome aberrations in routine cultures was found to be very low (between 0 to 3% in each patient) and no differences were seen between coeliac patients and controls. Though frequencies of mitoses with chromosome aberrations were significantly higher in bleomycin-treated cultures compared to

routine cultures in both patient groups, there was no significant difference between coeliac and control patients in spontaneous and bleomycin-induced chromosome fragility (**Table 3.**).

Table 3. **Percentages of mitoses with chromosome aberrations in routine and bleomycin-treated cultures (%, mean ± SD)**

	Routine		Bleomycin treated	
	48 hours	**72 hours**	**48 hours**	**72 hours**
Coeliac (n=22)	0.38±0.74 [A]	0.1±0.29 [C]	42.2±11.68 [A]	34.7±16.56 [C]
Control (n=18)	0.25±0.46 [B]	1.2±1.23 [D]	40.6±9.02 [B]	35.3±9.97 [D]

A,B,C,D = p<0.05

The frequency of PCD was similarly between 0-5%, in both the coeliac and control patients, although with a high rate of 8-23% in 4 coeliac patients.

Analysis of Gastrointestinal Malformations, Associated Congenital Abnormalities, and Intrauterine Growth in Neonates

INTRODUCTION

In contrast with other malformations, congenital anomalies of the gastrointestinal tract have been scarcely investigated. The prevalence of gastrointestinal malformations with special reference to congenital abnormalities and their relation to intrauterine growth in neonates were analyzed.

PATIENTS AND METHODS

Among the 4,241 newborn infants treated during the 14-year period between 1987 and 2000 in the Neonatal Intensive Care Unit of the Department of Paediatrics, Faculty of Medicine, University of Pécs, 278 (6.55 %) had gastrointestinal malformations. We recorded associated congenital abnormalities, chromosomal abnormalities verified by 150-to 400-band karyotyping, malformation syndromes and associations, gestational age, and birth weight. As defined in previous studies, newborns with birth weights below the gestational age-specific 10th percentile were classified as small for gestational age.

RESULTS

In 241 neonates, excluding patients with Hirschsprung disease and pyloric stenosis, 304 gastrointestinal malformations were found. Gastrointestinal malformation alone was found in 108 patients, whereas in 133 cases it was observed as one of multiple anomalies. Of these 133 patients, a specific syndrome or association could be identified in 27 and 9 cases, respectively. Using cytogenetic investigations and evaluation of major and minor congenital anomalies, no classified syndromes or associations could be diagnosed in 97 patients. Skeletal disorders were the most frequently associated anomalies in these patients. Compared with the normal population, the frequency of intrauterine growth retardation was significantly increased ($P < 0.001$). The proportions of patients with intrauterine growth retardation were 38.9% and 30.8% in the isolated and multiple malformation groups, respectively. Because ours is a tertiary centre, no epidemiologic data could be calculated from the 4,241 newborns treated in the neonatal intensive care of our department.

Discussion and Practical Consequences of the Studies

1. The effect of vanadate on the centromere separation sequence

Vanadate is regarded as an inhibitor of cell development and division, especially of chromosomal movement. The decreasing mitotic index values in the present study also referred to such an inhibition. At the same time, vanadate did not alter the sequence of centromere separation.

Practical consequences

A. Different vanadium compounds may have different effects on cell metabolism and division. The present data show that Na-vanadate also is toxic in that it slows the division of cultured lymphocytes and lowers their mitotic rate.

B. It has been shown in previous studies that various factors of cell culture and preparation, such as temperature, medium, culture time, colchicine, hypotonic shock, and calcium do not alter the sequence of centromere separation. The fact that even toxic levels of vanadate were ineffective in this respect provides further evidence for the suggestion that the centromere separation sequence is hardly at all influenced by environmental factors but rather is a species-specific, genetically determined phenomenon. On-going analysis of possible exogenous and endogenous factors influencing the separation sequence seems to be required.

2. Cytogenetic investigations in children with congenital immunodeficiencies

Two out of 12 children with ID showed signs of chromosome instability. This could be demonstrated not only by the classic methods but the high percentages of metaphases showing PCD, as well.

Practical consequences

A. Based on the present findings it is important to stress that PCD – ignored as "artefact" previously – can be recognized effortlessly in routine chromosome preparations. This observation has a significant impact on future research.

B. These data and our previous findings in Fanconi anaemia and ataxia teleangiectasia suggest that well-marked aberrant separation sequence and too early separation can be considered as a sign of chromosome instability. Therefore analysis of centromere separation should be integrated when investigating chromosome instability.

3. Objective analysis of centromere separation

Introduction of a simple digitalized image analysis system – by combining light microscopy and a PC based image analyzer software – is described that makes an objective and exact staging of centromere division possible.

Practical consequences

The method is simple, quick and relatively inexpensive, provided that an image analysis system is available. It offers an accurate and objective way to determine centromere division. It may also be utilized in retrospective analyses of old slides, e.g. in family investigations, in which centromere anomalies are sought as possible signs of chromosome instability.

4. Chromosome fragility and premature centromere division in coeliac disease

Irrespective of the method applied, no differences in the rate of chromosome breakage were found between coeliac patients and controls. Fragility was not correlated with diet, duration of gluten enteropathy, or age or sex of the patients. The fragility tests showed no chromosome instability in CD. The significance of unusually high frequencies of PCD (8-23%) observed in a few patients is not clear, but the phenomenon deserves further studies from the aspect of the relation of gluten enteropathy to malignancies.

Practical consequences

Our results support the findings of *Kolacek et al.* but did not show any correlation between the duration of diet and fragility. The same correlation can not be excluded between PCD and diet

although our findings are not consistent. It is possible that these different features of instability may manifest themselves differently in the same condition as suggested by Fundia earlier.

Despite discrepancies it is highly likely that classic gluten enteropathy is not necessarily associated with chromosome instability. Two years later our suggestion was confirmed by *Kolacek et al*. They found that frequency of chromosome aberrations in peripheral blood lymphocytes in CD patients decreased significantly on a gluten-free diet. According to their opinion genomic instability is a secondary phenomenon, possibly caused by chronic intestinal inflammation.

Continuing our investigation in children with CD we found 2 girls with 45,X/47,XXX karyotype (Méhes et al. 2007). Coeliac disease associated with sex chromosome abnormalities – especially with Ullrich-Turner syndrome – has been reported before, but this type of mosaicism in CD has not been published so far.

These findings have at least two implications:

A. Screening for CD in patients with Turner syndrome should be performed and small bowel biopsy is recommended in positive cases. Undiagnosed and untreated CD has a much higher risk of malignant lymphoma and other neoplasms compared to that in CD patients following a strict gluten-free diet; in addition, untreated CD may impair growth hormone therapy in the affected patients.

B. Cytogenetic investigation is warranted in CD patients if there is any clinical suspicion, especially in girls with short stature.

5. Analysis of gastrointestinal malformations, associated congenital abnormalities and intrauterine growth in neonates

The distribution of gastrointestinal malformations in this study was similar to that reported in the literature. The frequency of intrauterine growth retardation was significantly increased in patients with gastrointestinal malformations compared with the normal population. Analysis of associated anomalies found that an unexpectedly large number of skeletal disorders exceeded numbers of heart and urogenital malformations (45.4%, 41.2% and 31.9%, respectively).

Practical consequences

Gastrointestinal malformations are often associated with intrauterine growth retardation and mostly complicated by skeletal anomalies. The association among these disorders – not published before – requires further investigation. However, from a practical point of view, skeletal anomalies – beyond cardiac and urogenital disorders – should be sought in newborns with gastrointestinal malformations.

Summary

According to previous observations chromosome aberration, immunodeficiency, malformation, malignancy, reproductive loss, altered growth and development do not exist on their own only. Instead various degrees of overlap exists among them in certain cases.

Most likely non-specific genetic instability may serve as a basis for these conditions resulting in different clinical manifestations. This hypothesis is supported not only by several references cited above but by the findings and results of this thesis, as well.

Contribution was made to the following areas:

A. Overlap between chromosome aberrations and immunodeficiency,

B. Overlap between chromosome aberrations and coeliac disease (autoimmune disease),

C. Overlap between malformations and intrauterine growth retardation.

With respect to the overlap of malignancy and malformation, in a systematic analysis that we undertook (not discussed in this thesis), when prospectively screening children with malformations and their relatives for malignancies we found significantly more malignancies in grandparents of malformed children compared to controls, raising the possibility of increased risk for malignancy at a higher age (Hadzsiev et al. 2006).

Despite widespread investigations analysing the role of centromeres in cell division there is little knowledge about regulation of centromere separation. The processes controlling separation order are almost unknown. Further studies on this topic would enable us to understand the mechanisms of this process.

On a final note it is important to stress that experimental and clinical investigations presented in my thesis show that even difficult and exciting problems can be investigated by simple methods providing data for further research.

Publications

ARTICLES RELATED TO THE THESIS

1) **Tárnok A,** Méhes K, Kosztolányi Gy. No effect of vanadate on the centromere separation sequence.
    Acta Biol Hung 1993;44(2-3):297-301.   **IF:0,203**

2) Méhes G, **Tárnok A**, Pajor L, Méhes K. Objective analysis of centromere separation.
    Hum Genet 1996;97:365-366.   **IF: 2,445**

3) **Tárnok A.** Analysis of gastrointestinal malformations, associated anomalies and intrauterine growth in neonates.
    Gyermekgyógyászat 2000;1:39-44. *(In Hungarian with English abstract)*

4) **Tárnok A,** Méhes K. Gastrointestinal malformations, associated congenital abnormalities and intrauterine growth.
    J Pediatr Gastroenterol Nutr 2002;34:406-409.   **IF: 2,078**

5) Bajnóczky K, **Tárnok A,** Horváth M, Ruszinkó V, Kosztolányi Gy, Méhes K. Coeliac disease is not associated with chromosome fragility.
    Gyermekgyógyászat 2002;6:645-651. *(In Hungarian with English abstract)*

6) Hadzsiev K, **Tárnok A**, Kosztolányi Gy, Méhes K. Excess of malignancies in grandparents of children with malformations?
    Acta Biol Hung 2006;57:137-140.   **IF: 0,688**

7) Méhes K, Horváth M, **Tárnok A.** Celiac disease is relatively frequently associated with sex chromosome aberrations. *(In Hungarian)*
    Orv Hetil 2007;148(4):187.

PUBLISHED ABSTRACTS RELATED TO THE THESIS

1) Méhes K, Gyurkovits K, Kosztolányi Gy, **Tárnok A**. Frühzeitige Zentromertrennung bei Kindern mit Immundefizienz.
    Monatsschr Kinderheilk 1993;141(Suppl 1):S55 #P9. *(In German)*   **IF: 0,194**

2) **Tárnok A.** Analysis of gastrointestinal malformations in neonates.
    Z Gastroenterol 1999;37:458 #217.   **IF: 0,857**

3) **Tárnok A,** Bajnóczky K, Ruszinkó V, Kosztolányi Gy, Méhes K, Horváth M. Chromosome fragility and premature centromere division in celiac disease.
    J Pediatr Gastroenterol Nutr 2004;39(Suppl 1):S229 #P455   **IF: 1,764**

OTHER ARTICLES AND PUBLISHED ABSTRACTS

A. Original papers

1) Illés T, Than P, Kajtár P, **Tárnok A**, Bellyei Á, Kardos M. Changes in the treatment of osteosarcoma in our department.
Magy Traumatol 1997;5:375-383. *(In Hungarian)*

2) Méhes K, **Tárnok A**, Morava É, Sándor Gy, Antal I, Vermes É, et al. Investigation of health status of children living near Garé.
Népegészségügy 1998;4:3-7. *(In Hungarian)*

3) Adamovich K, **Tárnok A,** Szauer E. Successful treatment of right atrial thrombus secondary to central venous catheter.
Orv Hetilap 1999;140:1467-1470. *(In Hungarian with English abstract)*

4) Tárnok A, Vincze Á. Endoscopic ligation of oesophageal varices in childhood
Gyermekgyógyászat 2004;4:479-483. *(In Hungarian with English abstract)*

5) Veres G and participants of the Hungarian IBD registry. First year (2007) analysis of the Hungarian pediatric inflammatory bowel disease registry (HUPIR).
Gyermekgyógyászat 2008;5:282-287. *(In Hungarian with English abstract)*

6) Müller KE, participants of the Hungarian IBD registry and Veres G: Prevalence of extraintestinal manifestation in inflammatory bowel disease according to the Hungarian Pediatric IBD registry in 2008.
Gyermekgyógyászat 2010;1:15-21. *(In Hungarian with English abstract)*

B. Letter(s) to the Editor

1) Tárnok A, Koós Z, Domán I. Blount disease (tibia vara) in Bardet-Biedl syndrome.
Am J Med Genet 2003,123A:306–308.   **IF: 2,334**

C. Reviews and Editorials

1) Tárnok A. Epidemiology, diagnosis and treatment of H. pylori infection in childhood.
Gyermekorvos Továbbképzés 2003;1:48-54. *(In Hungarian)*

2) Tárnok A. Constipation in childhood.
Gyermekorvos Továbbképzés 2006;3:188-193. *(In Hungarian)*

3) Tárnok A. Therapeutic endoscopy in children.
Gyermekgyógyászat 2007;5:42-46. *(In Hungarian with English abstract)*

4) Tárnok A. Role of oligosaccharides in infant nutrition.
Gyermekorvos Továbbképzés 2007;4:227-229. *(In Hungarian)*

D. **Published Abstracts**

1) Horváth M, Weisenbach J, **Tárnok A**. Gallbladder disease in infants and children.
   Z Gastroenterol 2000;38:408 #44. **IF: 0,887**

2) **Tárnok A,** Kabács Á. Prevalence of Helicobacter pylori infection in child communities of Pécs.
   Z Gastroenterol 2001;39:424 #180. **IF: 0,803**

3) **Tárnok A**, Pintér A, Farkas A, Weisenbach J, Kustos Gy, Horváth L, Battyány I, Kalmár K. Haemobilia – cause of severe life-threatening gastrointestinal bleeding.
   Z Gastroenterol 2002;40:360 #135. **IF: 0,837**

4) **Tárnok A,** Horváth M, Papp K, Szabó É, Jakobik V, Decsi T. Low contribution of eicosapentaenoic and dihomo-gamma-linolenic acids to plasma lipids in children with celiac disease.
   J Pediatr Gastroenterol Nutr 2006;42(5):E18 **IF: 2,067**

5) **Tárnok A,** Knisely AS, Taylor RM, Dhawan A. Perivenular cell drop-out in paediatric liver transplant recipients: Outcome and prognostic implications.
   J Pediatr Gastroenterol Nutr 2006;42(5):E72 **IF: 2,067**

6) **Tárnok A**, Mosdósi B, Berki T, Rózsai B, Stankovics J. Interleukin-2 receptor alpha-subunit deficiency and spontaneous perforation secondary to cytomegalovirus colitis.
   Z Gastroenterol 2006;44:439 #144. **IF: 1,293**

7) **Tárnok A,** Talián GC, Jakobik V**,** Decsi T, Melegh B: Plasma carnitine concentrations and carnitine ester profiles in children with celiac disease..
   Z Gastroenterol 2008;45:516 #114. **IF: 0,880**

8) Veres G, Papp M, Lakatos P**,** et al. First 2-year analysis of prospectively diagnosed patients with inflammatory bowel disease (IBD) in the Hungarian Pediatric IBD Registry (HUPIR)
   J Pediatr Gastroenterol Nutr 2009;48(Suppl 3):E42-43 PG2-22 **IF: 2,132**

9) **Tárnok A**, Kasza G. Successful outcome of multiple arterial embolisation in a 16-year-old girl with ulcerative colitis.
   Z Gastroenterol 2009;47:485 #104. **IF: 0,880**

10) Marosvölgyi T**, Tárnok A**, Szabó É, Decsi T. Fatty acid composition of plasma phospholipids in children with celiac disease and type-1 diabetes mellitus.
    Acta Paediatrica 2009;98(Suppl 460):184 #474. **IF: 1,517**

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