Effect of met-enkephalin on chromosomal aberrations in the lymphocytes of the peripheral blood of patients with multiple sclerosis

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Abstract

Endogenious opiod met-enkephalin throughout previous research manifested cytoprotective and anti-inflammatory effects. Previous research suggests that met-enkephalin has cytogenetic effects. Reducement in the frequency of structural chromosome aberrations as well as a suppressive effect on lymphocyte cell cycle is found. It also reduces apoptosis in the blood samples of the patients with immune-mediated diseases. Met-enkephalin exerts immunomodulatory properties and induces stabilization of the clinical condition in patients with multiple Sclerosis (MS). The goal of the present research was to evaluate met-enkephalin in vitro effects on the number and type of chromosome aberrations in the peripheral blood lymphocytes of patients with MS. Our research detected disappearance of ring chromosomes and chromosome fragmentations in the cultures of the peripheral blood lymphocytes treated with met-enkephalin (1.2 μg/mL). However, this research did not detect any significant effects of met-enkephalin on the reduction of structural chromosome aberrations and disappearance of dicentric chromosomes. Chromosomes with the greatest percent of inclusion in chromosome aberrations were noted as: chromosome 1, chromosome 2 and chromosome 9. Additionally, we confirmed chromosome 14 as the most frequently included in translocations. Furthermore, met-enkephalin effects on the increase of the numerical aberrations in both concentrations applied were detected. Those findings should be interpreted cautiously and more research in this field should be conducted.

KEY WORDS: met-enkephalin, chromosomal aberrations, multiple sclerosis, peripheral blood lymphocytes.

INTRODUCTION

Met-enkephalin is one of the simplest endogenous opioid peptides within the enkephaline family. Endogenious opioid peptides share amino sequence of tyrosine-glycine-glycine-phenylalanine (aka Opioid motif), and contain one or more copies of met-enkephalin (Tyr-Gly-Gly-Phe-Met) and leu-enkephalin (Tyr-Gly-Gly-Phe-Leu). Opioid receptors (OP) are detected in human phagocytic leukocytes, with a direct binding of naloxone in lymphocytes and thrombocytes [1]. Met-enkephalin binds with high affinity to OP1 (δ) receptors, and with low affinity to OP3 (μ) receptors. Additionally, it specifically binds to receptors on T lymphocytes which are not morphin receptors [1, 2]. As a potential receptor on human lymphocytes a complementary transcript of met-enkephalin is isolated, with a single sequence that matches cytokine receptor γ chain [3]. Multiple sclerosis (MS) is a progressive disease followed by development of the neurological deterioration. Relapsing/remitting form of the disease is highly sensible to immunosuppressive therapy. However, with the extended duration, the response-rate to the treatment tends to decrease as well. As a result of an assumption which claims the existence of the inflammation and of the neurodegenerative phase, patients with MS are recommended for early immunomodulatory treatment [4, 5]. Due to immunomodulatory properties met-enkephalin was applied in clinical studies and it effect on the stabilization of the clinical conditions of MS was documented [6, 7]. It also manifests in vivo citoprotective effects [8]. Met-enkephalin mostly induces immunostimulation when applied in low doses, and immunosuppresion when applied in higher doses. Higher doses of met-enkephalin exerted suppressive effect in ex-

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perimental treatment of allergic encephalomyelitis [2, 9]. A role of the released cytokines and of Th1 cells differentiation disorder are implied in an immuno mediated demyelization [4]. The research of the cerebrospinal fluid in MS patients reveals an increase in the levels of immunoglobulin (lg) and mononuclear pleocytosis. Furthermore the same studies showed the numerous somatic gene mutations in the variable region of the lg Heavy Chain in the cerebrospinal fluid B cells in MS patients [10,11]. Research by Štambuk et al. [3] detected a significant reduction in the frequencies of the structural chromosome aberration in human lymphocytes of the peripheral blood of MS patients.

MATERIALS AND METHODS

The research was conducted at the Neurological Clinic of the Clinical and University Center of Sarajevo, and the Center for Human Genetics of the Faculty of Medicine, University of Sarajevo.

Samples

Blood samples were obtained from seven female patients in relapse in a test tube containing heparin. The eligibility criteria were MS diagnosis as per McDonald Diagnostic Criteria, depicted on existence of objective proofs of at least two lesions (MRI or evoked potentials), or at least two clinically diagnosed symptomatic disease episodes. Patients included in the study were never treated with interferon, and had not received pulse corticosteroid treatment over the past six months.

Tested substance

Met-enkephalin (Biotechnology Laboratories Richmond, USA) was dissolved in distilled water and kept at a temperature of -18°C. Prior to adding to culture, it was kept at a room temperature (18-23°C) up to five minutes. The met-enkephalin concentration per culture 2 (C2) was 1.2 μg/ml and per culture 3 (C3) 120 μg/ml. Control culture (C1) was not incubated with the tested substances.

Research design

Blood samples were cultivated following method described by Moorhead et al. [12], with the incubation of cultures during 72 hours and the application of Colcemid stock solution 25 mcg/ml (0.2 ml) two hours before completion of incubation period. After microscopic analysis of chromosome preparations by standard procedure (Giemsa staining), the identification of rearranged chromosomes was conducted by destaining and applying the G-band technique. The total number of chromosomes included in structural aberrations was determined in the following manner: numerical analysis did not include chromosomes with gaps; its number was separately analyzed. It is deemed that the single chromosome was included when the following structural aberration existed: chromosome/chromatide break, acentric fragment, ring fragment, minute, acentric ring, ring chromosome, marker chromosome. Two chromosomes are deemed included when the following structural aberration existed: dicentric chromosome and translocation.

Statistical analysis

The collected data was statistically processed by computer software SPSS v.11 (Statistical Package for Social Sciences©, March 2004). For the purpose of the hypothesis testing, we used a non-parametric testing for correlated samples, Wilcoxon Signed Ranks Test. Findings from control culture (C1) and cultures incubated with various concentrations of tested substances (C2, C3) were compared.

RESULTS

Female patients included were 34 to 60 years old (41.89±9.17), while the total number of hospitalizations due to MS was from one up to 6 hospitalizations (2.67±1.80). The recorded values of fibrinogen ranged between 9.10-15.90 μmol/L (12.44±2.52).

Our research reviewed 200 mitosis per each tested culture. The total number of chromosomes included in structural aberrations are presented in Table 1. and detected aberrations in Figure 1.

TABLE 1. Descriptive statistics for structural chromosomes aberrations

Culture	N	$\overline{X}$ ± SD	Median	Xmin	Xmax
C1	7	6.14 ± 2.67	5	4	12
C2	7	5.29 ± 4.68	4	0	13
C3	7	4.29 ± 1.98	4	1	7

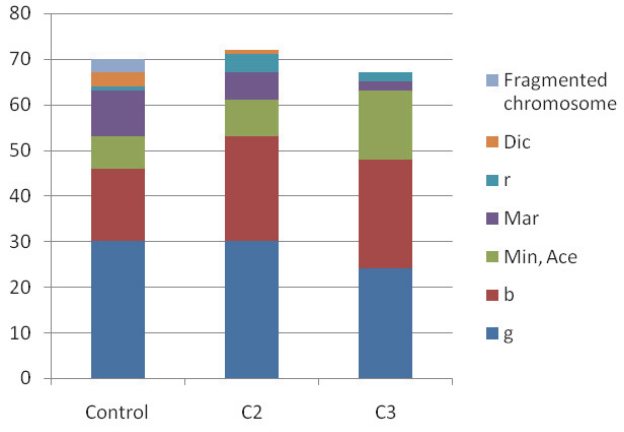

FIGURE 1. Number of the total detected structural aberrations

TABLE 2. The frequency of associated chromosomes in structural aberrations, expressed in percentages

Chromosome	C1(%)	C2(%)	C3(%)
1	17.24	19.35	8.00
2	6.90	11.29	14.00
3	6.90	4.84	8.00
4	1.72	1.61	3.00
5	5.17	3.23	6.00
6	3.45	-	2.00
7	1.72	6.45	3.00
8	8.62	1.61	12.00
9	13.79	17.74	14.00
10	1.72	3.23	2.00
11	5.17	3.23	2.00
12	3.45	9.68	-
13	3.45	-	3.00
14	5.17	3.23	-
15	-	-	-
16	-	-	-
17	-	3.23	-
18	3.45	-	-
19	-	-	3.00
20	-	-	2.00
21	-	-	-
22	-	-	-
X	3.45	4.84	3.00
Unidentified chromosomes	n=5	n=4	n=5

Aberration not detected in a certain chromosome

TABLE 3. Descriptive statistics for numerical chromosome aberrations

Culture Code	N	$\overline{X} \pm SD$	Median	Xmin	Xmax
C1	7	3.29 ± 2.69	2	1	9
C2	7	7.00 ± 2.83	7	3	12
C3	7	5.14 ± 4.91	4	2	16

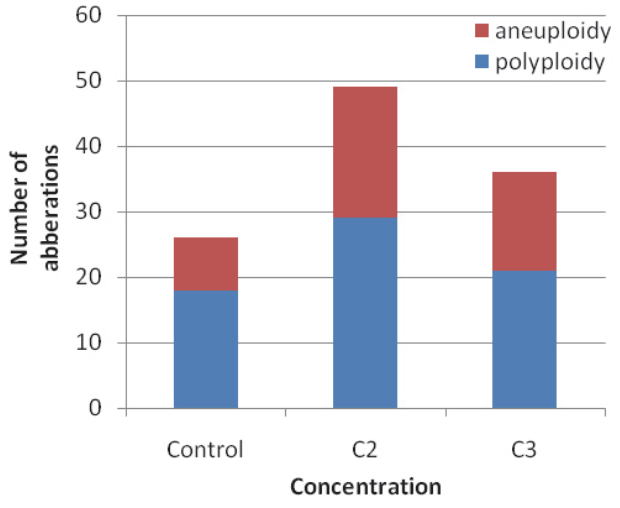

FIGURE 2. Number of detected numerical aberrations

Among detected structural aberrations the highest presence of gaps, breaks and marker chromosomes was documented. The ring chromosomes and the chromosome fragmentation were present within the C1 only; while dicentric chromosomes were detected in C1 and C2. When control cultures were compared to incubated ones, no statistically significant differences were recorded either in the number of chromosomes included in structural aberrations (C1 vs C2, p=0.527; C1 vs C3: p=0.089) or in the number of mitosis with aberrations (C1 vs C2, p=1.000; C1 vs C3, p=0.581). The identified chromosomes included in structural chromosome aberrations are displayed in Appendix 1. The frequency of engagement of certain chromosomes in aberrations, expressed in percentages, is shown in Table 2. For the calculation of frequency of associated aberrations with a familiar origin, the following were included: gap, break, translocation, chromosome fragmentation, dicentric and ring chromosomes. The majority of translocated marker chromosomes was detected in C1, while chromosome 14 was mostly included in translocations. Basic descriptive statistics for the numerical aberrations is displayed in Table 3. The number of all detected numerical aberrations is listed in Figure 2. When control cultures were compared to incubated ones, a statistically significant increase in number of numerical aberrations was detected in the incubated cultures (C1 vs C2, p=0.027; C1 vs C3, p=0.039). When observing polyploidy, the most fequent was the presence of endoreduplication, while triploidy and tetraploidy were somewhat rarer. Additionally, hyperdiploidy was detected. Furthermore, after G-banding the most frequent engagement in polysomy was of the X chromosome. Among the detected aneuploidy, the trisomy and tetrasomy of the X chromosome were dominant (Figure 3). When control cultures were compared to incubated ones, a statistically significant increase in the number of poliploidy was detected in the culture treated with a lower concentration of met-enkephalin (C1 vs C2, p=0.034), while no significant difference was documented when controls were compared to cultures treated with higher concentration of met-enkephalin (C1 vs C3, p=0.131). No statistically significant difference existed in the number of endoreduplications (C1 vs C2, p=0.157; C1 vs C3, p=0.334). A statistically significant increase in the number of aneuploidy existed in cultures incubated with lower met-enkephalin concentration compared to control cultures (C1 vs C2, p=0.026). No statistically significant difference was revealed when a culture incubated with a higher concentration was compared to control ones (C1 vs C3, p=0.236). Mitotic index was determined as a percent of lymphocytes in mitosis (M1+M2), counted on 300 lymphocytes. No statistically significant difference in mitotic index existed between

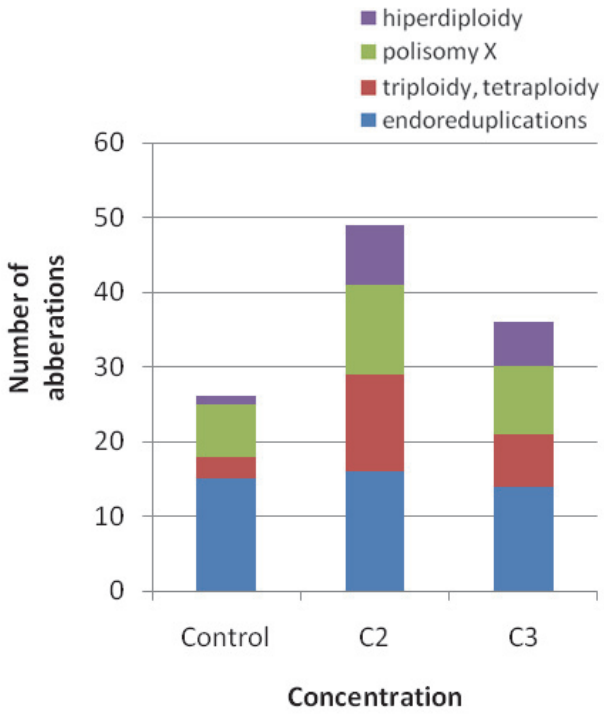

FIGURE 3. Structure of detected numerical aberrations

the control culture and the cultures incubated with various concentrates of substance used for testing (C1 vs C2, p=0.674; C1 vs C3, p=0.753).

DISCUSSION

Our research detected disappearance of ring chromosomes and chromosome fragmentations in the cultures treated with met-enkephalin. Similar to our results, the study by Štambuk et al. [3] showed disappearance of ring chromosomes and chromosome fragmentation after in vitro treatment of cultures with met-enkephalin (1.2 μg/mL) and incubation period of 48 hours. However, in contrast to their results, our research did not reveal significant effect of met-enkephalin on the reduction of the number of structural aberrations and on disappearance of dicentrical chromosomes. Furthermore, within the five-days cell culturing with incorporated 3H-thymidine, Štambuk et al. [3] documented significant reduction in the number of cells reaching the third stage of mitosis and a significant increase in a number of first metaphase. Chromosomal fragile sites expressed through an increased frequency in gaps and breaks are identified, as well as a presence of conservation of fragile sites throughout evolution [13, 14]. Re et al. [14] suggested that fragile sites via modulated gene expression can participate in the regulation of the cell responsivity rate to oncogenic stress and DNA damage. Ilyinskikh et al. [15] calculated the expected frequency of chromatic aberrations which are induced by radiation (8.44 up to 2.04 from the first up to 22nd chromosome), while the number of breaks increases with an increase in absolute chromosome length. The longest chromosomes in our research were also most frequently included in aberrations (chromosome 1 in C1 and C2, and chromosome 3 in C3). The frequency of chromosome 9 in structural aberrations was the most prominent. It is difficult to interprete the noticed impact of the met-enkephalin on the number of aberrations in treated cultures. The relationship between the ploidy disorders in malignant cells and an increase in the cell growth potential was suggested [16]. A hypothesis on aneuploidy as chromatic base of cancerogenesis was established [17]. Our research detected dominant engagement of X chromosome in hyperploidy and polysomy. According to data obtained from the Mittleman Data Base on chromatic aberrations in malignant diseases [18], the trisomy of X chromosome is most frequently related to acute lymphoblastic leukemia and lymphoblastic lymphoma. Previous research using met-enkephalin do not point out cancerogenous potential of this peptide; rather it shows quite the opposite [19-22]. When applied with paclitaxel, met-enkephalin enhances the inhibition of tumor growth of squamous cells head and neck carcinoma [19, 20]. Furthermore, throughout the experiment, aneugenic potential of opioids morphine and noskapine was detected [21, 22]. Genotoxic in vitro effects of noskapine were not confirmed in vivo, probably due to fast metabolism and low systemic bioavailability of the medication [21]. Experimental study by Cheng et al. [23] also suggests that met-enkephalin inhibits cell proliferation of various human and animal cells – probably by inducing an expression of inhibitors of cyclin-dependant kinases.

CONCLUSION

In conclusion, although the application of met-enkephalin in the culture of peripheral blood lymphocytes of MS patients did not manifest statistically significant protective effects, it influenced the disappearing of serious structural aberrations such as ring chromosomes and fragmentation of chromosomes. Met-enkephalin showed an impact on the number of numerical aberrations in both treated cultures, which certainly demands further in vivo evaluation.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

[1] Plotnikoff NP, Faith RE, Murgo AJ, Herberman RB, Good RA. Methionine enkefalin: A new cytokine--human studies. Clin Immunol Immunopathol 1997; 82(2): 99-101.

[2] Janković BD. Enkephalins and immune inflammatory reactions. Acta Neurol (Napoli) 1991; Oct; 13 (5):433-41.

[3] Štambuk N, Kopjar N, Šentija K, Garaj-Vrhovac V, Vikić-Topić D, Marušić-Della Marina B. et al. Cytogenetic Effects of Met-enkephalin (Peptid M) on human lymphocytes. Croat Chem Acta 1998; 71(3): 591-605.

[4] Hauser LS, Goodin SD. Multiple sclerosis and other demyelinating diseases. In: Harrison's Principles of Internal Medicine, 16th Edition. Edited by D.L. Kasper, E. Braunwald, A.S. Fauci, S.L. Hauser, D.L. Longo, J.L. Jameson, McGraw-Hill Medical Publishing Division, 2005, pp. 2461-2471.

[5] Rieckmann P, Traboulsee A, Devonshire V, Oger J. Escalating immunotherapy of multiple sclerosis. Therapeutic Advances in neurological Disorders 2008;1:181-192.

[6] Štambuk N, Brinar V, Štambuk V, Svoboda-Beusan I, Rabatić S, Mazuran R. et al. Peptide M (Lupex(R)) immunotherapy in multiple sclerosis, optic neuritis and uveitis. Int. J. Thymology, 5 (1997) 448–464.

[7] Štambuk N, Brinar V, Štambuk, V, Svoboda-Beusan, I, Mažuran, R, Rabatić, S, Marušić-Della Marina, B. et al. Peptid-M (LUPEX®) Effects on the Immune Responseand Clinical Status in Uveitis, Optic Neuritis and Multiple Sclerosis, in: S.Ohno, K. Aoki, M. Usui, and E. Uchio (Eds.), Uveitis Today, Excerpta Medica ICS1158, Elsevier, Amsterdam, 1998, pp. 319–322.

[8] Konjevoda P, Stambuk N, Vikic-Topic D, Boban-Blagaic A, Vikic-Topic S, Mrljak V, Pavan J, Ramadan P, Biđin Z. Protective Effects of Met-enkephalin
on Alcohol Induced Gastric Lesions. Croat Chem Acta 2000; 73 (4): 1111-1121.

[9] Zagon IS, Rahn KA, Bonneau RH, Turel AP, McLaughin PJ. Opioid growth factor suppresses expression of experimental autoimmune encephalomyelitis. Brain Research, 2010; 1310:154-161.

[10] Qin Y, Duquette P, Zhang Y, Talbot P, Poole R, Antel J. Clonal Expansion and Somatic Hypermutation of VH genes of B Cells from Cerebrospinal Fluid in Multiple Sclerosis. J Clin Invest 1998; 102(5):1045-1050.

[11] Corcione A, Casazza S, Ferretti E, Giunti D, Zappia E, Pistorio A. et al. Recapitulation of B cell differentiation in the central nervous system of patient with multiple sclerosis. Proc. Natl. Acad. Sci. USA 2004; 101(30):11064-11069.

[12] Moorhead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA. Chromosome preparations of leukocytes cultured from human peripheral blood. Exp Cell Res 1960; Sep;20: 613-616.

[13] Debacker K, Kooy F. Fragile sites and human disease. Human Molecular Genetics, 2007; 16(2):150-158.

[14] Re A, Cora D, Puliti AM, Caselle M, Sbrana I. Correlated fragile site expression allows the identification of candidate fragile genes involved in immunity and associated with carcinogenesis. BMC Bioinformatics, 2006; 7:413

[15] Ilyinskikh NN, Ilyinskikh IN, Ilyinskikh EN. Chromosome breakage at sites of oncogenes in a population accidentally exposed to radioactive chemical polution. Mutagenesis 1999; 14(1):83-86.

[16] Šulić S, Panić L, Đikić I, Volarević S. Deregulation of cell growth and malignant transformation. Croat. Med. J. 2005; 46(4):622-638.

[17] Duesberg P, Li R, Sachs R, Fabarius A, Upender BM, and Hehlmann R. Cancer drug resistance: The central role of the karyotype, Drug Resist. Updat. (2007), doi:10.1016/j.drup.2007.02.003

[18] Mitelman Database of Chromosome Abberations in Cancer Mitelman F, Johanson B, and Martens F (Eds.), 2009; Avaliable at http://cgap.nci.nih.gov/Chromosomes/Mitelman [accessed: November 2009]

[19] McLaughlin PJ, Jaglowski JR, Verderame MF, Stack BC, Leure-Dupree AE, Zagon IS. Enhenced growth inhibition of squamous cell carcinoma of the head and neck by combination therapy of paclitaxel and opioid growth factor. Int J Oncol, 2005; 26(3): 809-816.

[20] Jaglowski JR, Zagon IS, Stack BC Jr, Verderame MF, Leure-Dupree AE, Manning JD. et al. Opioid growth factor enhances tumor growth inhibition and increases the survival of paclitaxel-treated mice with scuamous cell carcinoma of the head and neck. Cancer Chemother Pharmacol, 2005; 56(1): 97-104.

[21] Lakshman Kumar P. Genotoxic evaluation of morphine, buprenorphine, pentazocine and noscapine by micronucleus and Comet assay in albino mice. Indian Journal of Pharmacology 2007; 39(6): 265-268.

[22] Schuler M, Muehlbauer P, Guzzie P, Eastmond DA. Noscapine hydrochloride disrupts the mitotic spindle in mammalian cells and induces aneuploidy as well as polyploidy in cultured human lymphocytes. Mutagenesis, 1999; 14(1):51-56.

[23] Cheng F, Mclaughlin PJ, Verderame MF and Zagon IS The OGF-OGFr Axis Utilizes the p16INK4a and p21WAF1/CIP1pathway to restrict normal cell proloferation. Molecular biology of the cell, 2009;20:319-327.

APPENDIX 1. Identified chromosomes for cultures not treated with met-enkephalin

	P1	P2	P3	P4	P5	P6	P7
1	1r (p;q), Break 1p, Gap 1q and 1q		Gap 1q	Break 1q	Gap 1q, 1q i 1q		Gap 1q
2	Gap 2q	t(2q;14q)		t(2p;14q), Gap 2q			
3	Gap 3q			Gap 3q		Break 3p	Gap 3q
4				Gap 4q			
5		Break 5p	Gap 5q			Break 5p	
6	Gap 6q			Break 6p			
7	Gap 7q						
8	Gap 8q	Frag 8, Gap 8q				Gap 8q	Break 8q
9	Break 9q and 9q, Gap 9q and 9q			Gap 9q i 9q			Break 9q, Gap 9q
10		Gap 10q					
11	Break 11p			Dic(Xq;11q), Gap 11p			
12		Frag 12				Dic(12q;18q)	
13					Break 13q		Break 13q
14		t(2q;14q)		t(2p;14q)			Break 14q
18		Gap 18q				Dic(12q;18q)	
X				Dic(Xq;11q)			X(q24)

Index: t- translocation, p- upper row, q- lower row, c- centromere area, frag- fragmentation
Identified chromosomes in cultures treated with met-enkephalin

	P1	P2	P3	P4	P5	P6	P7
Culture 2							
1	Dic(1p;12q) Break 1q	Gap 1q, 1q and 1q	Break 1q	t(1q;2q)	Gap 1q		Break 1q and 1q, Gap 1q and 1(c)
2	Gap 2q	Gap 2p		t(1q;2q), Mar 2p	Gap 2(c)	Gap 2(c)	Break 2p
3	Break 3q and 3q					Gap 3(c)	
4					Break 4q		
5				Break 5q			Break 5q
7	t(7q;14q) Break 7p		Gap 7q		t(7q36;14)		
8							Break 8p
9	Gap 9q		Break 9p	Break 9q	Break 9p, Gap 9q	Gap 9q and 9q	Break 9q and 9q, Gap 9q and 9q
10			Gap 10p				Gap 10q
11				Gap 11q and 11q			
12	Dic(1p;12q)		Break 12q and 12q, Gap 12p		Gap 12q		Break 12p
14	t(7q;14q)				t(7;14q12)		
17	Break 17q			Gap 17q			
X					Break Xq, Gap Xp		Break Xp
Culture 3							
1			Break 1p	Gap 1(c) and 1q			Gap 1q
2	Break 2p		Break 2q	Break 2p	Gap 2p and		
2(c)	Break 2q	Gap 2q					
3				Break 3q	t(3p;4q), Break 3p, Gap 3p		
4	Break 4q				t(3p;4q)		
5	Break 5p		Break 5p	Break 5q			
6		Gap 6q					
7	Gap 7q			Gap 7p			
8	Break 8q and Gap 8p				Gap 8q		Break 8p and 8q, Gap 8p
9	Gap 9q and 9q		Gap 9q, 9q and 9q		Break 9q, Gap 9q		
10				Gap 10q			
11				Break 11q			
13	Gap 13q				Gap 13q		
19	Break 19p					Gap 19q	
20							Mar 20
X	Break Xp						Gap Xq

Index: t- translocation, p- upper row, q- lower row, c- centromere area, frag- fragmentation