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MSRV *POL* SEQUENCE COPY NUMBER AS A POTENTIAL MARKER OF MULTIPLE SCLEROSIS

Mariola Zawada[1], Izabela Liweń[1], Monika Pernak[3], Danuta Januszkiewicz-Lewandowska[1,2,3], Karina Nowicka-Kujawska[1], Jolanta Rembowska[1], Krzysztof Lewandowski[3], Hanna Hertmanowska[4], Mieczysław Wender[5], Jerzy Nowak[1,#]

[1]Institute of Human Genetics, Polish Academy of Sciences, Strzeszyńska 32, PL 60-479 Poznań, Poland; [2]University of Medical Sciences, Szpitalna 27/33, PL 60-572 Poznań, Poland; [3]Department of Medical Diagnostics, Dobra 38, PL 60-595 Poznań, Poland; [4]State Hospital, Department of Neurology, Juraszów 17/19, PL 60-479 Poznań, Poland; [5]Neuroimmunological Unit, Medical Research Center, Polish Academy of Sciences, Przybyszewskiego 49, PL 60-355 Poznań, Poland

MSRV pol sequence copy number as a potential marker of multiple sclerosis. M. ZAWADA, I. LIWEŃ, M. PERNAK, D. JANUSZKIEWICZ-LEWANDOWSKA, K. NOWICKA-KUJAWSKA, J. REMBOWSKA, K. LEWANDOWSKI, H. HERTMANOWSKA, M. WENDER, J. NOWAK. Pol. J. Pharmacol., 2003, 55, 869–875.

Multiple sclerosis (MS) is a neurological disease in which demyelination in the brain and spinal cord is observed. The causal influence of bacterial/viral infections and genetic/immune factors in the etiology of multiple sclerosis is suggested. Multiple sclerosis-related retrovirus (MSRV) is one of the potential agents, which can lead to development of the disease.

The aim of cytogenetic studies was assessment of MSRV *pol* sequence copy number in patients with MS compared to normal individuals.

Cytogenetic slides with interphase nuclei and extended chromatin fibers were prepared from peripheral blood of 16 patients with MS and 10 healthy individuals.

Fluorescence *in situ* hybridization (FISH) with biotinylated product of polymerase chain reaction was used in order to analyze MSRV *pol* sequence copy number in the examined material. Detection of MSRV *pol* probe was carried out by immunological reaction with avidin-fluorescein and biotinylated anti-avidin.

MSRV *pol* sequence copy number was significantly greater in MS patients than in normal individuals. Using FISH technique to extended chromatin fibers, it was observed that MSRV *pol* exists as tandem repeats on various chromosomes. The increased number of MSRV *pol* sequence has been found on chromatin fibers of MS patients as compared to healthy controls.

Key words: *multiple sclerosis (MS), MSRV, pol sequence, FISH, interphase nuclei, chromatin fibers*

[#] *correspondence*; e-mail: nowakjs@rose.man.poznan.pl

Abbreviations: ECFs – *extended chromatin fibers,* FISH – *fluorescence in situ hybridization,* FITC – *fluorescein,* MS – *multiple sclerosis,* MSRV – *multiple sclerosis-associated retrovirus,* PCR – *polymerase chain reaction,* SCID – *severe combined immunodeficiency,* SSC – *sodium chloride + sodium citrate,* SSCP – *sodium chloride + sodium citrate + sodium phosphate*

INTRODUCTION

Multiple sclerosis (MS) is the disease of the central nervous system, in which affected individuals have disseminated foci of demyelination in the brain and spinal cord. The prevalence of MS disease ranges from 30 to 100 cases per 100 000 individuals.

The etiology of MS is still unknown and may involve autoimmune and genetic components. The important pathogenic role can be also played by bacteria (*Chlamydia pneumoniae*, *Helicobacter pylori*) and viruses (i.e. HTLV-1, HHV6, HERV-Tar, HERV-7q, Herpes simplex type B).

In early work by Perron et al. [17], an extracellular activity of reverse transcriptase in leptomeninges cell culture from cerebrospinal fluid of patients with MS was reported. They also observed the presence of viral particles in culture of monocytes from MS patients and lack of them in normal individuals [19]. Next year Perron et al. [18] successfully completed virus transfer into non-infected cells *in vitro*. In 1997 a retrovirus was isolated from cells of leptomeninges and also from immortalized B lymphocytes of MS patients, and finally was called multiple sclerosis-associated retrovirus (MSRV) [16]. Virions related to MSRV-RNA were also detected and isolated from plasma of MS patients [7].

Menard et al. [11, 12] also described studies concentrated on MSRV and its relationships to MS. An activity of reverse transcriptase MSRV-RNA and cytotoxin were detected in both supernatant of monocyte/macrophage cultures and cerebrospinal fluid of patients with MS. Furthermore, cytotoxin induced death of primary mouse cortical glial cells, immortalized mouse astrocytes and oligodendrocytes *in vitro*. Importantly, the toxicity of this agent was observed only in MS cases.

MSRV virus is suggested to be one of the potential factors, which can be related to pathogenesis of MS. MSRV virus is taxonomically connected with the large and diversified group of human endogenous retroviruses (HERV). HERV sequences can comprise about 1% of human genome and MSRV is related to HERV-W group. Family HERV-W sequences are widely distributed on 7q21-q22, 14q11-q12, 21q22.3 and Xq22 chromosomes [1–4, 20].

Recently, MSRV retroviral particles or the envelope recombinant protein were shown to display superantigen activity *in vitro*. The pathogenicity of MSRV retroviral particles was evaluated in a hybrid animal model: severe combined immunodeficiency (SCID) mice grafted with human lymphocytes and injected intraperitoneally with MSRV virion. MSRV-injected mice developed acute neurological symptoms and died few days after injection. Necropsy revealed disseminated and major brain hemorrhages. Moreover, in the group of infected animals reverse transcriptase-polymerase chain reaction (RT-PCR) analyses showed circulating MSRV-RNA in serum, whereas overexpression of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ was evidenced in spleen RNA [6].

MSRV-RNA *pol* sequence together with *gag* and *env* comprise the retroviral genome, including oncogene viruses. Homologies of these sequences exist in many genomes of *Aves* and *Mammalia*. Analysis of MSRV *pol* sequence is usually carried out by RT-PCR and sequencing techniques [6, 8, 14]. Recently, Nowak et al. [14] proposed an introduction of fluorescence *in situ* hybridization (FISH) technique to analysis of MSRV *pol* sequence.

MATERIALS and METHODS

MSRV *pol* sequence was detected in peripheral blood cells of 16 patients with clinically definite MS (in the relapsing/remitting stage of MS), aged 20–57 years (median 37.9 years). The median duration of the disease was 34 months (ranging from 2 to 172 months). All patients were examined during the period of clinical activity of the disease. The control group consisted of 10 healthy individuals, aged 20–56 years.

Preparation of cytogenetic slides: interphase nuclei and extended chromatin fibers

Heparinized peripheral blood (about 2 ml from each person) was treated with hypotonic solution (0.4% KCl), and was incubated at 37°C for 20 min. Subsequently, cell nuclei were treated thrice with

cold fixative (methanol:acetic acid, 3:1) and during fixations the material was incubated at –20°C for 20 min. Interphase nuclei pellet was spread on cold microscope slides and dried at room temperature.

The fresh cells from peripheral blood were used for preparation of extended chromatin fibers (ECFs) according to Haaf and Ward [9] with modifications. Heparinized peripheral blood (about 5 ml from each person) was washed with sterile PBS buffer several times. The cells were overspread on microscopic slides in Cytospine (Shandon Southern) at 600 rpm. Then the slides were treated with concentrated salt solution (15 mM NaCl, 2 M Tris, 0.5 M EDTA, 1 mM H_2PO_4 pH 8.0 + 90% glycerol) at room temperature for 30 min. Chromatin fibers were fixed by incubation of slides in absolute ethanol for 30 min.

DNA probe preparation

In experiments, the probe consisted of PCR – MSRV *pol* sequence product (435 bp length) labeled with biotin in the nick translation reaction (Boehringer Mannheim, Germany). In order to increase the amount of biotin particles in the probe, two different, labeled nucleotides were used (Boehringer Mannheim, Germany; ENZO, USA). Hybridization mixture was composed as follows: formamide, SSCP, H_2O, dextran sulfate. Complete hybridization mixture with probe was stored at –20°C.

Fluorescence *in situ* hybridization (FISH)

All cytogenetic slides were treated with RNase (1:100 from 10 mg/ml stock) for 1 h at 37°C. Slides were washed in 2 x SSC, dehydrated in 70%, 85%, 96% ethanol and dried at room temperature. Denaturation of each slide was carried out for 2-min in 70% formamide/2xSSC solution. The fixation of denatured DNA and its dehydration was performed in a series of increasing ethanol concentrations. Slides were dried at room temperature. The time of 12 min and the temperature of 80°C were sufficient for denaturation of the probe. The edges of microscope cover glasses were sealed with Fixogum. Then, the probes were hybridized with nuclei or chromatin fibers in moist chamber at 37°C for 48 h.

Post-hybridization washes were conducted in formamide/2xSSC solution at 40°C. Detection of MSRV *pol* probe was carried out during immunological reaction: avidin-fluorescein (FITC) and biotinylated anti-avidin (Vector Laboratories, USA). The slides, stained by propidium iodide were analyzed in fluorescence microscope Axiophot (Zeiss, Germany), and the *isis* software (MetaSystems GmbH, Germany) for digital analysis of microscopic images was used.

RESULTS

The presence of MSRV *pol* sequence in each analyzed interphase nuclei from both MS patients and healthy individuals was detected with the use of FISH technique. To assess the number of MSRV *pol* sequence copies we counted fluorescent signals in the interphase nuclei.

Significant differences in number of MSRV *pol* sequence copies between patients with MS and healthy individuals were observed. The number of *pol* sequence copies per interphase nucleus in MS patients came up to 50, and in two cases it was higher than 50 (Tab. 1). In healthy individuals, this parameter came up only to 20 (Tab. 3). The average value of MSRV *pol* copy number/interphase nucleus was also different in both studied groups. In MS patients this value ranged from 6.18 to 24.02 (Tab. 2), while in control group it was lower, rang-

Table 1. MSRV *pol* sequence copy number in interphase nuclei of patients with MS

MSRV *pol* copy number								
Patients	1–5	6–10	11–15	16–20	21–30	31–50	> 50	% of interphase nuclei
MS1	20	40	17	10	10	3		
MS2	20	39	16	13	12			
MS3	4	20	26	34	14	2		
MS4	2	20	10	14	36	18		
MS5	4	40	26	12	16	–	2	
MS6	16	36	16	22	8	2		
MS7	16	30	24	10	14	6		
MS8	30	32	28	4	4	2		
MS9	48	36	14	2				
MS10	24	52	12	2	4	6		
MS11	6	20	26	38	10			
MS12	16	30	32	16	6			
MS13	12	38	30	10	8	2		
MS14	2	30	44	14	8	2		
MS15	2	4	16	22	28	26	2	
MS16	18	18	28	22	12	2		

Table 2. Analysis of MSRV *pol* sequence in patients with MS

Patients	Minimum number of MSRV *pol* copies in interphase nucleus	Maximum number of MSRV *pol* copies in interphase nucleus	Average of MSRV *pol* copies / interphase nucleus	Number of analyzed interphase nuclei
MS1	2	42	11.57	30
MS2	2	29	11.1	100
MS3	4	34	15.42	50
MS4	5	45	21.14	50
MS5	2	56	13.86	50
MS6	3	46	12.5	50
MS7	2	42	13.8	50
MS8	1	44	9.36	50
MS9	2	17	6.18	50
MS10	2	34	9.78	50
MS11	3	26	14.44	50
MS12	2	21	11.18	50
MS13	2	34	11.58	50
MS14	5	48	13.68	50
MS15	4	54	24.02	50
MS16	2	38	13.08	50

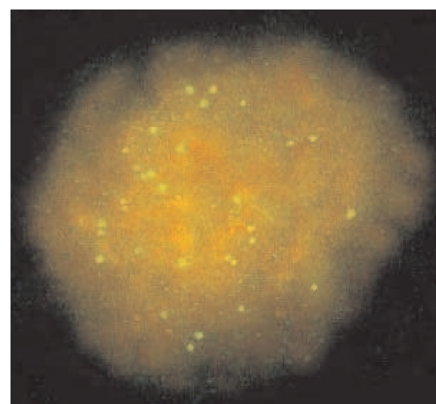

Fig. 1. FISH with biotinylated probe to interphase nucleus of MS patient

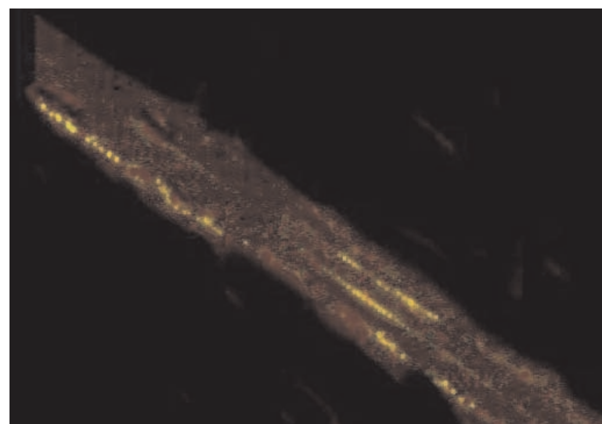

Fig. 2. FISH with biotinylated probe to chromatin fibers of MS patient

ing between 3.22 and 6.48 (Tab. 4). Possibility of nonspecific binding of the probe was eliminated by restriction conditions of fluorescence *in situ* hybridization, and especially by post-hybridization washes and proper temperature.

Thus, we can suggest that MSRV *pol* sequence is present in number of copies in healthy individuals as well as in patients with MS.

MSRV *pol* sequence occurs in number of copies in MS cases

The cytogenetic analysis of interphase nuclei and chromatin fibers of MS patients revealed many copies of MSRV *pol* sequence in the genome (Fig. 1, 2). Moreover, the tandemly dispersed *pol* repeats were observed.

In two cases with MS, the MSRV *pol* sequence copy number was higher than 50 but the most of all MS patients (ten persons) had from 31 to 50 copies of MSRV *pol*. In three patients, number of *pol* copies ranged from 21 to 30, and only in one case the value was in the range between 16 and 20. Among all MS patients, none displayed less than 16 copies of MSRV *pol* (Tab. 1).

Analysis of MSRV *pol* sequence in patients with MS showed that the minimum amount of copies ranged from 1 to 5, while maximum ranged from 21 to 56. The average value of MSRV *pol* copies/interphase nucleus was estimated to be 6.18 to 24.02. The number of analyzed interphase nuclei was 100 in 1 case, 50 in 14 cases and 30 in 1 case (Tab. 2).

MSRV *pol* sequence in healthy individuals

The presence of many copies of MSRV *pol* sequence in both interphase nuclei and chromatin fibers of healthy individuals was noted (Fig. 3, 4).

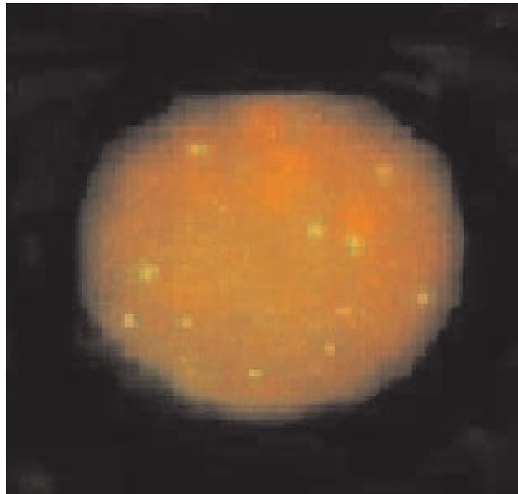

Fig. 3. FISH with biotinylated probe to interphase nucleus of healthy individual

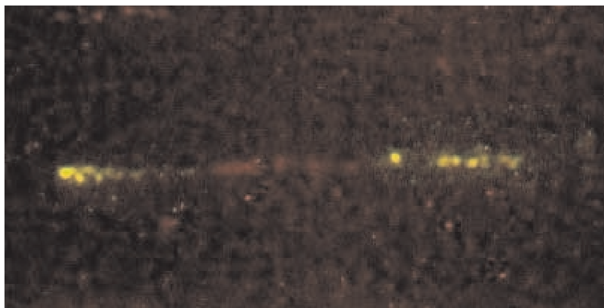

Fig. 4. FISH with biotinylated probe to chromatin fibers of healthy individual

Moreover, MSRV *pol* was found as a tandemly repeated sequence on chromatin fibers.

In five healthy individuals, MSRV *pol* sequence copy number ranged from 16 to 20, in three cases this value was estimated as 11 to 15 and in two cases as 6 to 10 (Tab. 3).

Analysis of MSRV *pol* sequence in healthy individuals showed that the minimum number of copies was 1 to 2, while the maximum was 8 up to 20. The average of MSRV *pol* copies/interphase nucleus ranged from 3.22 to 6.48. The number of analyzed interphase nuclei was 100 in 1 case, 50 in 8 cases and 15 in 1 case (Tab. 3).

Table 3. MSRV *pol* sequence copy number in interphase nuclei of healthy individuals

MSRV *pol* copy number								
Healthy individuals	1–5	6–10	11–15	16–20	21–30	31–50	> 50	% of interphase nuclei
C1	40	52	6	2				
C2	78	20	2					
C3	82	18						
C4	92	8						
C5	46	38	10	6				
C6	48	50	2					
C7	44	48	6	2				
C8	71	24	4	1				
C9	47	47	6					
C10	64	30	4	2				

Table 4. Analysis of MSRV *pol* sequence in healthy individuals

Healthy individuals	Minimum number of MSRV *pol* copies in interphase nucleus	Maximum number of MSRV *pol* copies in interphase nucleus	Average of MSRV *pol* copies / interphase nucleus	Number of analyzed interphase nuclei
C1	2	18	6.4	50
C2	1	12	4.12	50
C3	2	8	3.54	50
C4	1	9	3.22	50
C5	2	20	6.48	50
C6	2	12	5.56	50
C7	2	16	6.18	50
C8	2	19	5.01	100
C9	2	11	5.7	15
C10	2	18	5.1	50

DISCUSSION

Molecular biology methods are fundamental tools in analysis of MSRV in patients with MS and healthy individuals. The potential utility of cytogenetic techniques in the studies of MSRV *pol* sequence practically was not described in the references before. Introduction of FISH into MS studies creates novel experimental and diagnostic possibilities because of the higher resolution of this method in comparison with classic cytogenetic techniques.

Nowak et al. [14] early described integration of MSRV *pol* sequence into human genomic DNA of healthy individuals using FISH. MSRV *pol* was

found to be repeated and tandemly dispersed sequence on chromatin fibers. In the present work conducted on chromatin fibers, we showed not only integration of MSRV into genome of MS patients but we also observed a repeated chains of *pol* sequence on various fibers (chromosomes), which were longer than in healthy individuals (Fig. 2).

Now, we cannot explain the relationship between increased number of MSRV *pol* sequence and MS. From our and other studies, it appears that MSRV *pol* sequence can resemble other HERV sequences, which are widespread in human genome [10, 16, 22]. At the same time, extracellular viral expression was observed. Virus particles are detected in plasma, cerebrospinal fluid, supernatant of peripheral blood cells and in some cell cultures of MS patients [7, 13, 15]. Virus particles are detected relatively rarely in blood of healthy individuals [5, 8, 21].

The cytotoxin has been detected in cerebrospinal fluid of patients with MS [11, 12, 13]. It is possible that the mutation in gene or genes regulating the MSRV *pol* sequence amplification could occur. Thus, cytotoxin production can be a consequence of this mutation. This toxic factor called gliotoxin is present only in active cases of MS and is a stable glycosylated protein of 17 kDa, which is highly toxic for glial cells. It should be taken into consideration that gliotoxin may represent an initial pathogenic factor leading to neuropathological features of MS, like demyelination and blood brain barrier disruption [12, 13].

It should be noted that, independently of the relationship between MSRV and MS, the analysis of *pol* sequence provided valuable data about the differences between the MS patients and healthy individuals. This distinction can be observed and analyzed at the cytogenetic level. It is notable that the FISH technique is very useful in the studies of MS.

We suggest that MSRV *pol* sequence can be a diagnostic marker of MS, and probably in the future can play important prognostic and monitoring role. Similarly, other authors confirmed usefulness of MSRV analysis in MS, but they carried out molecular studies of RNA-MSRV in extracellular fluids [16]. Continuation of cytogenetic analysis with the application of FISH technique in studies of larger group of MS patients and healthy individuals, including patients with other autoimmune diseases is indicated.

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