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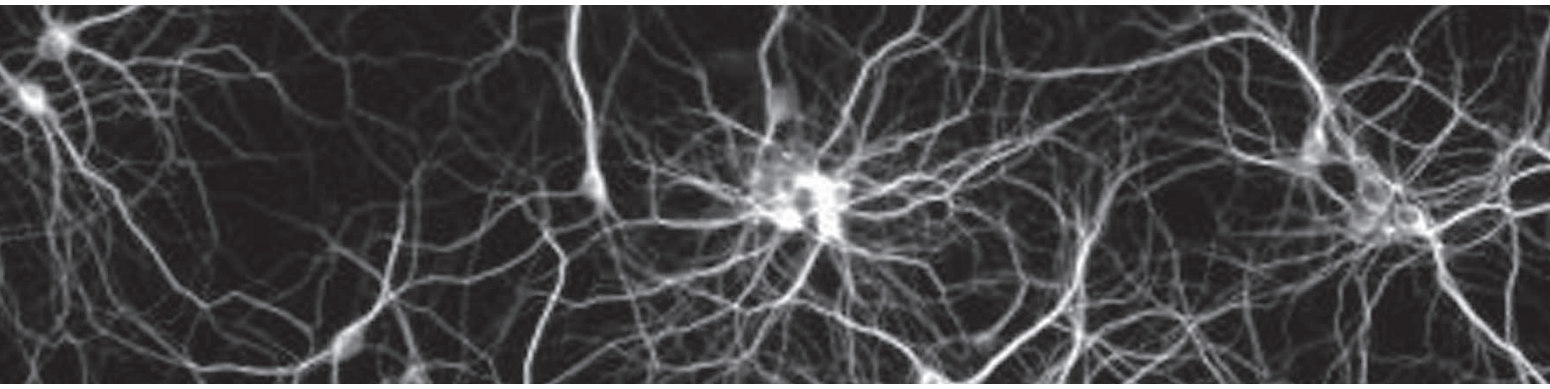

ABCB1 gene variants as predictors of multidrug-resistant epilepsy in Croatian population

D. Sporiš, S. Bašić, N. Božina[1], T. Babić[2], S. Hajnšek[3], J. Sertić[1], I. Šušak, I. Marković

ABSTRACT - P-glycoprotein (Pgp) is a drug efflux transporter and is the encoded product of the human multidrug resistance gene ABCB1 (MDR1). Pgp is expressed in the blood-brain barrier (BBB) and blood-cerebrospinal fluid (CSF) barrier, and reduces the brain penetration of antiepileptic drugs (AEDs). Functional polymorphism or overexpression of Pgp in the BBB of patients with epilepsy may play a role in pharmacoresistance. The aim of this study was to investigate the possible association of ABCB1 gene polymorphisms C1236T in exon 12, G2677T in exon 21, and C3435T in exon 26 with the development of resistance to antiepileptic therapy. All patients enrolled in the study had an established diagnosis of partial complex epilepsy with or without secondary generalisation and have been suffering from it for more than two years. Patients were devided into two groups. The first group comprised 57 patients refractory to the current therapy, while group 2 consisted of 48 patients with well-controled seizures. Results of our study showed statistically significant difference in the allele and genotype frequency of ABCB1 G2677T between resistant and nonresistant patients. Haplotype G2677/C3435/C1236 was overrepresented among resistant patients. According to our results, ABCB1 variants C1236T, G2677T and C3435T might be associated with therapeutic response to AED in patients with partial epilepsy with or without secondary generalization and represent a possible predictive factor for pharmacoresistance.

Key words: ABCB1 gene, epilepsy, multi-drug resistance, polymorphism

INTRODUCTION

Epilepsy is characterized by recurrent spontaneous seizures and is one of the most common neurological disorders (1). Despite advances in antiepileptic drug (AED) therapy, about one-third of patients with epilepsy are resistant to drug treatment. Most

Dubrava University Hospital, Department of Neurology, Zagreb, Croatia

[1] Zagreb University Hospital Center, Center for Functional Genomics and Clinical Institute of Laboratory Diagnosis, Zagreb, Croatia

[2] Research, CNS Scientific Affairs, Maidenhead, UK

[3] Zagreb University Hospital Center, University Department of Neurology, Zagreb, Croatia

patients with refractory epilepsy are resistant to several, if not all, AEDs despite the fact that these drugs act by different mechanisms (2). Epilepsy was the first central nervous system (CNS) disorder for which drug resistance was associated with enhanced expression of multidrug transporters in the brain (3). In addition to intrinsic and acquired overexpression of multidrug transporters in the blood brain barrier (BBB) of patients with epilepsy, functional polymorphisms of these transporters might contribute to pharmacoresistance (4).

P-glycoprotein (Pgp), the encoded product of the human multidrug-resistance gene ABCB1, is a drug efflux transporter of particular clinical relevance because many drugs are substrates and/or inhibitors, among which there are several major AEDs, including phenytoin, phenobarbital, carbamazepine, lamotrigine, topiramate and gabapentin (5–10).

P-glycoprotein is widely localized in normal tissues including the apical membrane of the gastrointestinal tract, blood cells, the biliary canalicular membrane of hepatocytes, and the luminal membranes of proximal tubular epithelial cells in the kidney, and thus limits the cellular uptake of xenobiotics by excreting these compounds into the bile, urine and intestinal lumen (11–13). Pgp is expressed in the BBB or blood-cerebrospinal fluid (CSF) barrier, combining them to reduce the brain penetration of AEDs.

More than 50 single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms in the large ABCB1 gene have been reported (14), and mutations at positions 2677 and 3435 have been associated with alteration of Pgp expression and/or function (14–16). A silent C to T transition in exon 26 of ABCB1 (3435C>*T*) has been associated with differences in Pgp levels and activity (17). The polymorphism has also been associated with Pgp function, and with clinical conditions such as drug-resistant epilepsy (18). However, available data suggest that this polymorphism may not directly cause altered Pgp transport activity but may be associated with one or more causal variants in the stretch of linkage disequilibrium surrounding it (19). In a study from Austria (20), in which functional variants in ABCB1 were genotyped in patients with temporal lobe epilepsy, a common ABCB1 gene haplotype CGC/CGC was identified, which was significantly associated with the risk of pharmacoresistance. A few other studies confirmed the association of polymorphism, linkage disequilibrium and specific haplotype combinations with pharmacoresistance in patients with epilepsy (21–24). However, other studies failed to replicate an association of polymorphism in ABCB1 gene with multidrug-resistant epilepsy (25–32).

The aim of this study was to investigate the possible association of ABCB1 gene polymorphisms in exon 21 G2677T, exon 26 C3435T and exon 12 C1236T with the development of antiepileptic drug resistance in Croatian population.

PATIENTS AND METHODS

Patients

This cross-sectional study is part of the ongoing pharmacogenomic study of epilepsy in Croatian population and has been approved by the Ethics Committee of the Zagreb University Hospital Center. Patients were consecutively recruited through the Referral Center for Epilepsy, University Department of Neurology, and laboratory methods were done at the Center for Functional Genomics and Clinical Institute of Laboratory Diagnosis. All patients enrolled in the study had an established diagnosis of partial complex epilepsy with or without secondary generalization and had been suffering from it for more than two years. They were divided into two groups. Group 1 consisted of 57 patients refractory to current therapy, while group 2 included 48 patients with well-controlled seizures. Refractory epilepsy was defined as one or more seizures *per* month during the past year, while on therapy with two or more established AEDs at the maximally tolerated doses. The patients with well-controlled seizures were free from seizures in the same period. Compliance was determined by measuring serum concentration of anticonvulsants (33,34). Seizure frequency was recorded from the patient medical records, seizure diaries and patient interview. Patients in-between these two groups were excluded from the study. Patients with a history of CNS infection, head trauma, brain tumor, cerebrovascular disease, neurodegenerative and psychiatric diseases, or pseudoattacks were excluded from the study. All patients signed an informed consent to participate in the study. In all patients, the allele, genotype and haplotype frequencies were determined as well as the number of AEDs and MDR1 substrates, age at onset, duration of epilepsy and interictal electroencephalogram (EEG). Association with ABCB1 gene polymorphisms was compared between the two groups.

The mean patient age was 42.64 years (SD 13.91). Non-responders took a significantly higher mean number of medications (χ^2=55.322; ss=4; *P*<0.001), as compared with responders (2.9 *vs*. 1.9 antiepileptics). Furthermore, the mean number of AEDs, substrates of MDR1 transporter, was statistically significantly higher in the group of non-responders as compared with the group of responders (2.6 *vs*. 1.6). In the non-responder group, most patients were taking three (49%) or two (47%) AEDs, MDR1 substrates. In the responder group, 48% were taking one AED, MDR1 substrate, and 46% were taking two AEDs, MDR1 substrates. Carbamazepine was the most frequently administered AED (n=95, 88%), either as monotherapy or in combination with other drugs. Lamotrigine was used by 54 (50.5%), phenobarbital by 46 (43%), valproate by 23 (21%) and topiramate by 18 (16.8%) patients. Gabapentin and phenytoin were used by 7/6 patients.

Genotyping procedures

Genotyping of 1236C/T, 2677 G/T/A and 3435C/T variants of the ABCB1 gene was performed by the PCR-RFLP and real-time PCR methods according to the previously described procedures (35–40).

Statistical analysis

A test for Hardy-Weinberg equilibrium using Markov chain method (41) and the linkage-disequilibrium likelihood-ratio test between loci whose gametic phase is unknown (42) were performed, as implemented in Arlequin ver. 3.01 (43). Haplotype frequencies were estimated using Expectation-Maximization algorithm implemented in the same program, leading to maximum likelihood estimates of haplotype frequency. The χ^2-test was used for pair-wise comparisons of allele frequencies between the groups. Log likelihood ratio tests were performed to compare distributions of the estimated haplotypes between the groups, as well as comparisons of genotype frequencies. *P* values less than 0.05 were considered statistically significant. The χ^2-test or t-test was performed for comparison of age at onset, duration of epilepsy, interictal electroencephalogram, antiepileptic drugs and MDR1 substrates between genotypes and in resistant *vs*. non-resistant patients. All statistical analyses were carried out using the SPSS 11.5 (SSPS Inc., Chicago, IL, USA) statistical software package. The pattern was calculated with Altman's algorithm and power of 80%.

RESULTS

All samples were successfully genotyped. We found no case of 2677A variant. Genotype frequencies of the ABCB1 2677GG, 2677GT and 2677TT in the sample were 35, 48 and 22; of the 3435CC, 3435CT and 3435TT 26, 44, 37; and of the 1236CC, 1236CT and 1236TT 33, 41 and 25, respectively.

No significant deviations from the expected Hardy-Weinberg proportions were observed in the total sample and at the loci (ABCB1 G2677T: *P*=0.43; C3435T: *P*=0.08; C1236T: *P*=0.516). Test result for linkage disequilibrium (LD) between the loci was significant among all loci. LD between exon 21 G2677T and exon 26 C3435T: χ^2=94.97, *P*<0.001, ss=1; LD between exon 21 G2677T and exon 12 C1236T: χ^2=58.72, *P*<0.001, ss=2; and LD between exon 26 C3435T and exon 12 C1236T: χ^2=40.49, *P*<0.001, ss=2.

Pair-wise comparisons of the allele frequency between resistant and non-resistant patients revealed a statistically significant difference for exon 21 G2677T (*P*=0.041, χ^2=4.19, ss=1), while in exons 26 and 12 there was no statistically significant difference (Table 1). Patients with allele G of G2677T had 85% odds ratio (31% risk ratio) for resistance recorded in patients with T allele. Analysis of various C3435T alleles in exon 26 did not yield statistical difference (p=0.124) according to antiepileptic therapeutic efficacy. Analysis of different C1236T alleles in exon 12 yielded no statistical difference (*P*=0.821) according to antiepileptic therapeutic efficacy (Table 1).

A statistically significant difference was found in genotype based analysis of exon 21 G2677T (*P*=0.016, likelihood ratio G= 8.214, ss=2), but not in exon 26 C3435T and exon 12 C1236T (Table 1). Patients with G/T allele had a statistically significantly lower chance for pharmacoresistance as compared with patients with G/G allele. Patients with T/T allele did not show statistical difference according to resistance. C3435T polymorphism in exon 26 did not show significant correlation with pharmacoresistance, although patients with C/T allele showed a statistically significantly lower chance for resistance as compared with patients with C/C allele. C1236T polymorphism in exon 12 did not show statistical difference according to antiepileptic therapeutic efficacy (Table 2).

Likewise, statistically significant differences were found in the distribution of the estimated haplotypes between the groups. Haplotype G2677/C3435/C1236 was overrepresented among resist-

Table 1. *Distribution of allele and genotype frequencies of ABCB1 exon 21 G2677T, exon 26 C3435T and exon 12 C1236T between subjects who are resistant and non resistant to treatment*

Locus			Resistant	Non resistant	Total	OR (95%C.I.)
Exon 21 G2677T	[a]Allele	G	73	45	118	1.85 (1.02-1.69)
		T	43	49	92	1
	[b]Genotype	GG	26	9	35	1
		GT	21	27	48	0.27 (0.10-0.70)
		TT	11	11	22	0.35 (0.11-1.07)
Exon 26 C3435T	[a]Allele	C	59	37	96	1.59 (0.95-1.70)
		T	59	59	118	1
	[b]Genotype	CC	19	7	26	1
		CT	21	23	44	0.34 (0.12-0.96)
		TT	19	18	37	0.39 (0.13-1.15)
Exon 12 C1236T	[a]Allele	C	67	40	107	0.91 (0.71-1.20)
		T	45	46	91	1
	[b]Genotype	CC	23	10	33	1
		CT	21	20	41	0.46 (0.17-1.20)
		TT	12	13	25	0.40 (0.14-1.18)

[a] exon 21 G2677T , p=0.041, x^2 = 4.19, ss=1; exon 26 C3435T, p=0.124, x^2=2.37, ss=1; exon 12 C1236T p=0.821, x^2=0.05, ss=1
[b] exon 21 G2677T, p=0.016, likelihood ratio G= 8.214, ss=2; exon 26 C3435T, p=0.103, likelihood ratio G=4.739, ss=2; exon 12 C1236T p=0.176, likelihood ratio G= 3.615, ss=2

Table 2. *Distribution of genotype combination frequencies of ABCB1 exon 21 G2677T, exon 26 C3435T and exon 12 C1236T between subjects who are resistant and non resistant to treatment.*

Genotype combination	Resistant n=56	Non-resistant N=43	OR (95%C.I.)	p
GG-CC-CC	17	3	1	
GT-CT-CT	11	11	0.18 (0.4-0.78)	0.022
TT-TT-TT	7	5	0.25 (0.05-1.33)	0.103
Others	21	24	0.15 (0.4-0.60)	0.007

Likelihood ratio G=9,641; ss= 3; p= 0,023

ant patients (likelihood ratio G=9.641; ss= 3; *P*=0.023) (Table 2); in the analysis of genotype combination frequencies of ABCB1 exon 21, 26 and 12, genotype combination GG/CC/CC was statistically significantly overrepresented among resistant patients (likelihood ratio G=9.641; ss=3; *P*=0.023) (Table 3).

There were significant differences between resistant and non-resistant patients in the age at disease onset (mean age ± SD: 11.8 ± SD 6.37 *vs.* 23.4 ± SD 11.57, t=8.448, ss=105, *P*<0.001) and duration of illness (mean value ± SD: 30.4 ± SD 11.97 *vs.* 19.9 ± SD 11.59, t=4.495, ss=105, *P*<0.001). Furthermore, in the resistant group of patients we found significantly higher focal altered EEG findings (χ^2=11.545, ss=1, *P*<0.001).

DISCUSSION

As indicated by the data presented, ABCB1 variants seem to be associated with AED response in patients with partial complex epilepsy.

Many studies aimed to highlight the role of intestinal Pgp in drug interactions, of P-glycoprotein expressed in the BBB for drug penetration into the CNS, the role of pharmacological inhibition of Pgp function to increase drug concentrations in sanc-

Table 3. *Distribution of haplotype frequencies of of ABCB1 exon 21 G2677T, exon 26 C3435T and exon 12 C1236T between subjects who are resistant and non resistant to treatment.*

Haplotype	G2677T	C3435T	C1236T	Resistant n=51	Non resistant n=44	OR (95%C.I.)
H1	G	C	C	50,47	22,41	1
H2	G	C	T	6,54	8,30	0,35 (0,11-1,14)
H3	T	C	T	12,26	6,78	0,80 (0,26-2,52)
H4	G	T	T	2,74	4,51	0,27 (0,06-1,22)
H5	T	C	C	0	2,89	x
H6	T	T	C	4,27	8,52	0,22 (0,06-0,80)
H7	T	T	T	33,73	33,19	0,48 (0,24-0,96)

Likehood ratio G=12,99; ss=3; p=0,043.

tuary sites (e.g., for the HI virus), and for the potential role of MDR1 polymorphisms for Pgp expression, drug disposition, adverse drug reactions and disease risk (44). Active drug transport is now considered an important additional mechanism limiting drug accumulation in multiple tissues, including the CNS. Despite the magnitude of extensive research, many conflicting data still need to be clarified.

AED resistance could be either the result of each single polymorphic variant of ABCB1 gene, or of many different polymorphic variants in the wide spectrum of linkage disequilibrium.

An important characteristic of refractory epilepsy is that most patients are resistant to most, and often to all AEDs (2). This argues against epilepsy-induced alterations in specific drug targets as a major cause of pharmacoresistant epilepsy. It points out other mechanisms such as decreased drug uptake into the brain by seizure-induced overexpression of multidrug transporters in the BBB (45). Our results are in accordance with the findings reported by Hung *et al.*, Zimprich *et al.* and Kwan *et al.* (20,22,46), confirming the possible role of ABCB1 gene variants in pharmacoresistance. The results of the presented study confirmed the existence of linkage disequilibrium of 12, 21 and 26 exon of ABCB1 gene that could be responsible for altered function of drug transporter and as a consequence for refractory epilepsy. Association between combinations of GG/CC/CC genotypes and pharmacoresistance in patients with partial complex epilepsy has been reported. Furthermore, the analysis showed that carriers of GG2677 genotype had more often diagnosis of refractory epilepsy. Single GCC haplotypes in the resistant group showed a statistically significantly difference in comparison with single TTT and TTC haplotypes in the nonresistant group. Siddiqui *et al.* (18) were the first to report on the association between single nucleotide polymorphisms in the ABCB1 gene and refractory epilepsy. That study led to a hypothesis that drug resistance in epilepsy might be genetically determined. Patients with AED-resistant epilepsy were more likely to carry 3435CC genotype, which is associated with increased Pgp expression in comparison to 3435TT genotype.

Soranzo *et al.* (19) suggest that C3435T polymorphism might not be the only cause of the altered Pgp activity. They identified three intron polymorphisms that are associated with 3435C>T polymorphism. Their study showed the possible importance of linkage disequilibrium for the Pgp function. Our results are in part consistent with the data reported by Hung *et al.* (22). They demonstrated association between C3435T, C1236T and G2677T polymorphisms with AED resistance. They emphasize that the CGC, TGC and TTT haplotypes and haplotype combinations CGC/CGC, CGC/TGC, CGC/TTT and TGC/TTT were found more often in the resistant group in comparison with nonresistant group. Zimprich *et al.* (20) performed genotyping for functional ABCB1variants in patients with temporal lobe epilepsy and found the 2677GG/3435CC genotype to be significantly associated with resistance. Kwan *et al.* found that in 464 Chinese epilepsy patients, the ABCB1 intronic polymorphism rs3789243 and the coding polymorphism 2677, and haplotypes containing them might be associated with drug resistance (46). Contrary to our results and the studies mentioned above, many other studies did not confirm positive association between ABCB1 polymorphisms and multidrug resistance in epilepsy patients (25–32).

Some clinical features could be associated with drug resistance, in particular early onset of seizures, high seizure frequency prior to treatment initiation, a history of febrile seizures, type of seizures (especially partial seizures) or epilepsy, structural brain lesions, and various malformations of cortical development (47). The present study revealed differences in main characteristics between the resistant and nonresistant group. Earlier onset and longer duration of the disease were recorded in the former, and earlier epilepsy progression from partial seizure to secondary generalized form of seizure in the latter. Contrary to the nonresistant group, patients in the resistant group had significantly higher focally altered EEG findings. Aikia *et al*. (48) report that focal epileptic EEG activity could be one of the predictive factors for pharmacoresistance, which is confirmed by our results.

An important fact is the compelling evidence that candidate gene strategies are dependent on ethnic stratification, and divergent results could be due to ethnic and racial differences in frequency distribution of polymorphic alleles. Significant interethnic variability was documented for frequencies of MDR1 variants. The frequency of exon 26, C3435 allele is 43%-54% in Caucasians, 46%-61% in Asians and 73%-90% in Africans (17,39,40,49). Documented frequency of exon 21, G2677 allele is 57% in Caucasians, 43% in Japanese and 34% in Indian population (50). Some studies point out that the high frequency of C3435 allele in Africans could be connected to the high frequency of more aggressive and drug resistant tumors, like breast carcinoma in subjects of African origin (51,52).

In conclusion, according to our results, ABCB1 variants C1236T, G2677T and C3435T might be associated with therapeutic response to AED in patients with partial epilepsy with or without secondary generalization and represent the possible predictive factor for pharmacoresistance.

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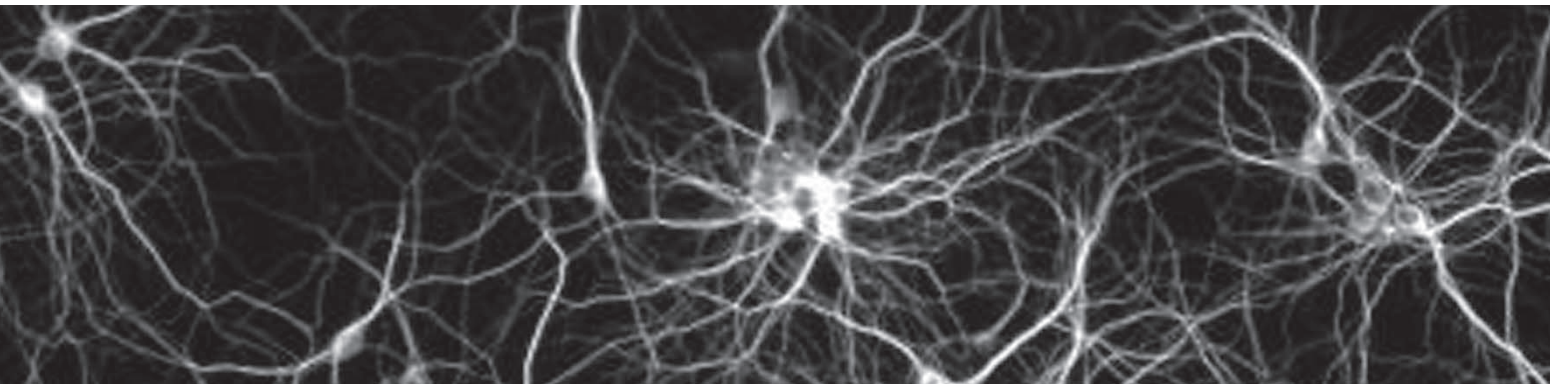
Address for Correspondence: Davor Sporiš, MD, Department of Neurology, Dubrava University Hospital. Av. Gojka Šuška 6, 10000 Zagreb, Croatia; E-mail: davor.sporis@kbd.hr

Varijante gena ABCB1 kao prediktora epilepsije višestruko rezistentne na lijekove u hrvatskoj populaciji

SAŽETAK - P-glikoprotein (Pgp) je transmembranski transporter koji je kodirani ABCB1 genom višestruke rezistencije na lijekove (MDR1). Pgp je izražen na krvno-moždanoj i krvno-likvorskoj barijeri, a smanjuje transport antiepileptika u mozak. Funkcionalni polimorfizam ili pojačana ekspresija Pgp-a na krvno-moždanoj barijeri mogu imati ulogu u farmakorezistenciji pacijenata s epilepsijom. Cilj ovog istraživanja bio je ispitati moguću povezanost polimorfizama gena ABCB1 C1236T egzona 12, G2677T egzona 21 i C3435T egzona 26 s razvojem rezistencije na antiepileptičku terapiju. Svi bolesnici koji su uključeni u istraživanje imali su postavljenu dijagnozu parcijalne kompleksne epilepsije sa sekundarnom generalizacijom ili bez nje, a bolovali su duže od dvije godine. Bolesnici su bili podijeljeni u dvije skupine. Prvu je skupinu činilo 57 bolesnika koji su bili refraktorni na trenutnu terapiju, dok je drugu skupinu činilo 48 bolesnika sa zadovoljavajuće kontroliranim napadajima. Rezultati našeg istaživanja pokazuju statistički značajnu razliku alela i genotipske frekvencije ABCB1 G2677T između rezistentnih i nerezistentnih pacijenata. U rezistentnih je pacijenata bila prisutna pojačana ekspresija haplotipa G2677/C3435/C1236. Prema dobivenim rezultatima, ABCB1 varijante C1236T, G2677T i C3435T bi mogle biti povezane s terapijskim odgovorom na antiepileptike u pacijenata s parcijalnom epilepsijom sa sekundarnom generalizacijom ili bez nje te bi mogli biti mogući prediktivni faktor farmakorezistencije.

Ključne riječi: ABCB1 gen, epilepsija, višestruka rezistencija na lijekove, polimorfizam



Concentrations of GH, IGF-1 and insulin in CSF of healthy people

Erv. Bilić, Ern. Bilić[1], V. Kušec, K. Zibar[1], D. Čerimagić[2*], P. Konjevoda, B. Tripković[3], D. Delimar[3], M. Žagar

ABSTRACT – Growth hormone (GH), insulin-like growth factor-1 (IGF-1) and insulin are involved in brain development, and also act as neuroprotective factors. The values of these hormones in healthy population have not been published so far. We measured GH, IGF-1 and insulin concentrations in cerebrospinal fluid (CSF) of 57 healthy people (35 male and 22 female) undergoing lumbar puncture for spinal anesthesia before surgery of the knee joint. In men, the mean (SD; min-max) CSF concentrations of GH, IGF-1 and insulin were 0.67 (0.11; 0.50-0.90) μU/mL; 7.49 (0.92; 6.00-9.00) μg/L and 0.71 (0.13; 0.40-0.90) μU/mL, respectively. In women, the mean CSF concentrations of GH, IGF-1 and insulin were 0.69 (0.10; 0.50-0.90) μU/mL; 7.32 (0.95; 6.00-9.00) μg/L and 0.68 (0.13; 0.40-0.90) μU/mL, respectively. None of the study hormones showed a statistically significant sex difference. There was no significant bivariate correlation between GH, IGF-1 and insulin. The hormones did not correlate with age either. Determination of the normal range of GH, IGF-1 and insulin in CSF could help identify deviations in CSF hormone status in particular neurologic diseases. These values could prove important in the screening, diagnosis and management of various diseases involving the central nervous system.

Key words: cerebrospinal fluid, growth hormone, healthy people, insulin, insulin-like growth factor 1

Medical School University of Zagreb, University Department of Neurology, Department of Neuromuscular Disorders and Clinical Electromyoneurography, Zagreb University Hospital Center, Zagreb, Croatia

[1] Medical School University of Zagreb, University Department of Pediatrics, Department of Oncology and Hematology, Zagreb University Hospital Center, Zagreb, Croatia

[2*] Dubrovnik General Hospital, Department of Neurology, Dubrovnik, Croatia

[3] Zagreb University Hospital Center, University Department of Orthopedic Surgery, Zagreb, Croatia

INTRODUCTION

Growth hormone (GH), insulin-like growth factor-1 (IGF-1) and insulin are involved in brain growth and development, and also act as neuroprotective factors (1-3). To the best of our knowledge, the values of these hormones in healthy population have not been published so far. Heinze *et al*. (4) have reported concentrations of GH, IGF-1, insulin-like growth factor binding protein-3 (IGFBP-3) and insulin-like growth factor binding protein-2 (IGFBP-2) in cerebrospinal fluid (CSF) of patients suffering from viral infection, leukemia, Hodgkin's disease or multiple sclerosis (MS). The authors only took samples without pathologic alterations in routine CSF analysis findings. However, their study subjects suffered from systemic diseases and could not be considered as healthy people. We measured GH, IGF-1 and insulin concentrations in CSF from 57 patients (35 male and 22 female) aged 11-71 (mean 45.56; SD 17.548) years undergoing lumbar puncture for spinal anesthesia before knee joint surgery. They had no recent history data on any other disease besides knee trauma.

In our previous study, we measured the concentrations of GH, IGF-1 and insulin in CSF and serum of healthy people serving as a control group for comparison with the values recorded in patients suffering from amyotrophic lateral sclerosis (ALS). We found ALS patients to have significantly lower CSF levels of GH, IGF-1 and insulin than healthy people (5).

The stimulatory action of GH on the proliferation of cerebrocortical brain cells could be mediated by an antiapoptotic action of GH that promotes cell survival (6). It is unclear whether GH is synthesized within the nervous tissue or it is solely taken up from the circulation (4). The high density of GH receptors in the choroid plexus (CP) suggests a possible receptor-mediated transcytosis transport (7-9). IGF-1 acts as a neuroprotective survival factor under pathologic conditions such as stroke, brain trauma, MS, or Alzheimer's disease (AD) (10-13). It is also effective in slowing the progression of motor neuron degeneration in wobbler mice (14,15). IGF-1 may reduce myelin breakdown and promote myelin regeneration in demyelinating diseases (16,17). The inhibitory effect on apoptosis is associated with the prevention of bcl-2 reduction by a mechanism resulting in the phosphorylation of the apoptotic BAD protein (18). Insulin in brain does not stimulate glucose metabolism in neurons; it stimulates glucose uptake in rat brain glial cells and human glioblastoma and enhances glycogen accumulation in astroglia-rich primary cultures from neonatal rat brain (19-22).

Since examinations of these substances in CSF have only recently been introduced and as yet experimentally used in clinical evaluation, relevant data on their normal values are still lacking. The aim of this study was to measure the concentrations of these factors in healthy people in order to enable recognition of abnormal values in particular neurologic diseases, having in mind how important role they play in physiologic neuroprotective processes.

PATIENTS AND METHODS

The study included 57 patients that underwent lumbar puncture for spinal anesthesia before knee joint surgery. They had no recent history data on any other disease except for knee trauma. The group consisted of 35 males and 22 females (mean age 45.56, SD 17.548 years). The patients were examined at University Department of Neurology, Zagreb University Hospital Center in Zagreb, Croatia. The approval for this study was granted by the Ethics Committee of the School of Medicine, University of Zagreb and Zagreb University Hospital Center, Zagreb, Croatia. Providing anonymity and informed consent obtained, the approval permitted CSF sampling when it could be safely obtained during lumbar puncture performed for anesthesia in knee joint surgery. An informed consent was obtained from each study participant.

CSF samples were obtained by lumbar puncture between 8.00 AM and 10.00 AM considering the well-known circadian rhythm of GH and IGF-1 concentrations in serum. All samples were immediately centrifuged, frozen in small aliquots and stored at -80 °C until analysis. To exclude the possible CSF contamination with blood constituents, only samples with less than 15 erythrocytes/μL were analyzed. The CSF leukocyte content was within the normal limits (<5 cells/μL). The CSF GH level was determined using the polyclonal immunoradiometric assay (IRMA) kit (BioSource hGH-IRMA, KIP1081, Nivelles, Belgium) according to the manufacturer's instructions (23). The detection level was 0.2 μU/mL. The CSF IGF-1 level was determined using the two-site immunoenzymometric assay (IEMA) according to the manufacturer's instructions (AC-27PL-GB; OCTEIA IDS Inc., Fountain Hills, AZ, USA), with sensitivity defined as the concentration corresponding to the

mean plus 2 SD of 20 replicates of the zero Calibrator (1.9 μg/L). The CSF insulin level was measured by using the microparticle enzyme immunoassay (MEIA) following the manufacturer's instructions (Abbott IMX system, 2A10, Abbott Laboratories, Wiesbaden, Germany); the sensitivity was 1.0 μU/mL.

The software package SPSS 17.0 (SPSS inc, Chicago, IL, USA) was used throughout data analysis. As all data showed normal distribution, the results were presented as mean ± SD. Due to the descriptive aim of the study, the minimal and maximal values were reported, irrespective of the normality of data distribution. Differences were tested using Student's t-test and correlations using Pearson's correlation test. $P<0.05$ was considered statistically significant.

RESULTS

The mean (SD; min-max) CSF concentration of GH was 0.68 (0.10, 0.50-0.90) μU/mL for the study group as a whole, 0.67 (0.11, 0.50-0.90) μU/mL for men, and 0.69 (0.10, 0.50-0.90) μU/mL for women (Fig. 1a, Table 1). The values showed normal distribution (skewness=0.222). The mean CSF concentration of IGF-1 was 7.42 (0.93, 6.00-9.00) μg/L for the study group, 7.49 (0.92, 6.00-9.00) μg/L for men, and 7.32 (0.95, 6.00-9.00) μg/L for women (Fig. 1b, Table 1). IGF-1 concentration also showed normal distribution (skewness=0.029). The mean CSF concentration of insulin was 0.70 (0.13, 0.40-0.90) μU/mL for the study group, 0.71 (0.13, 0.40-0.90) μU/mL for men, and 0.68 (0.13, 0.40-0.90) μU/mL for women (Fig. 1c, Table 1). Insulin concentration also showed normal distribution (skewness=-0.356). CSF concentrations of GH, IGF-1 and insulin are presented in Fig. 1a-c, respectively. Results according to sex subgroups are shown in Table 1. There were no sex differences in CSF concentrations of GH (t=-0.625; P=0.534), IGF-1 (t=0.662; P=0.510) or insulin (t=0.744; P=0.460). CSF concentration of GH did not correlate significantly either with IGF-1 (r=0.054; P=0.691) or with insulin (r=0.140; P=0.298) (Fig. 2a,b). There was no significant correlation between CSF concentrations of IGF-1 and insulin either (r=0.036; P=0.793)

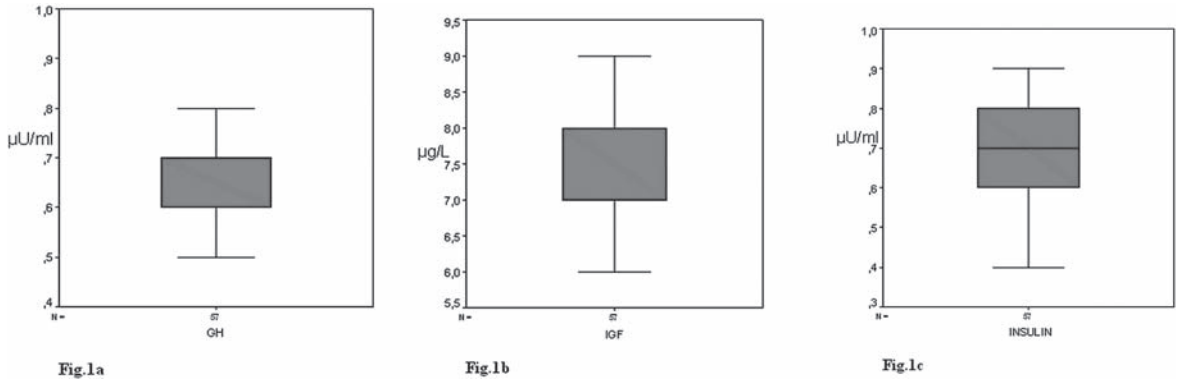


Fig. 1. *The mean CSF concentration of GH, IGF-1 and insulin in healthy people (N=57): (a) GH (μU/mL); (b) IGF-11 (μg/L); (c) insulin (μU/mL).*

Table 1. *Gender differences in CSF concentrations of GH, IGF-1 and insulin in healthy people.*

	Gender	n	Mean	SD	Min	Max	t	*P*
GH μU/mL	m	35	0.67	0.11	0.50	0.90	-0.625	0.534
	f	22	0.69	0.10	0.50	0.90		
IGF-1 μg/L	m	35	7.49	0.92	6.00	9.00	0.662	0.510
	f	22	7.32	0.95	6.00	9.00		
Insulin μU/mL	m	35	0.71	0.13	0.40	0.90	0.744	0.460
	f	22	0.68	0.13	0.40	0.90		

SD = standard deviation; GH = growth hormone; IGF-1 = insulin-like growth factor-1; m = male; f = female; Min = minimum; Max = maximum; n = number

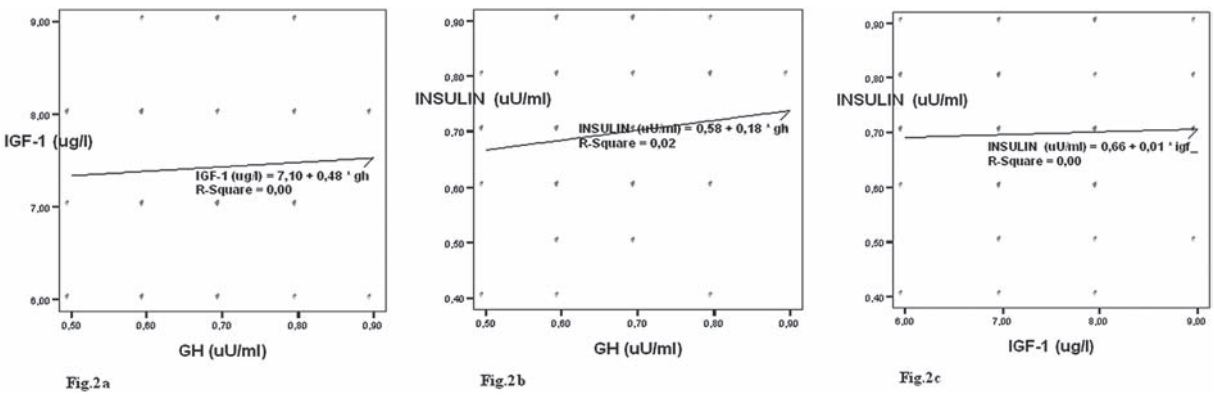


Fig. 2. *Relation between GH, IGF-1 and insulin in CSF of healthy people (n=57), linear regression: (a) GH (μU/mL) vs. IGF-1 (μg/L); (b) insulin (μU/mL) vs. GH (μU/mL); (c) insulin (μU/mL) vs. IGF-1 (μg/L).*

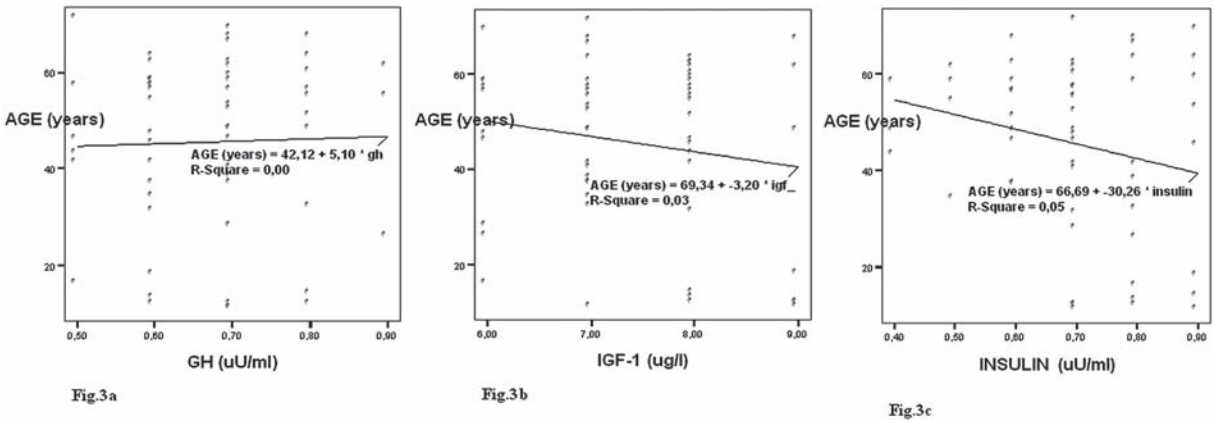


Fig. 3. *Relation between GH, IGF-1, insulin and age (years) in CSF of healthy people (N=57), linear regression: (a) GH (μU/mL) and age (years); (b) IGF-1 (μg/L) and age (years); (c) insulin (μU/mL) and age (years).*

(Fig. 2c). There was no significant correlation between age and CSF concentrations of GH (r=0.030; *P*=0.824), IGF-1 (r=-0.169; *P*=0.209) and insulin (r=-0.277; *P*=0.090) (Fig. 3a-c).

DISCUSSION

This is a report on the concentrations of GH, IGF-1 and insulin in CSF of healthy people. The mean CSF GH was 0.68 (0.10) μU/mL, IGF-1 7.42 (0.93) μg/L and insulin 0.70 (0.13) μU/mL. The values of all three study parameters showed normal distribution. None of the study hormones showed any statistically significant sex difference. There was no significant bivariate correlation between GH, IGF-1 and insulin. The hormones did not correlate with age either.

Early studies of the brain glucose metabolism established the axiom that insulin is not required for glucose utilization by the central nervous system (CNS). A corollary to this concept was the belief that circulating insulin is incapable of crossing the blood-brain barrier (BBB). While the first of these tenets remains unchallenged, the second has been subjected to detailed scrutiny, following the identification of both insulin and its receptor in the adult mammalian brain (24-26). Some brain areas like olfactory bulb and hypothalamus have immunoreactive insulin concentrations that are two- to three-fold those of other brain regions (27-29). Insulin receptors appear to be concentrated on the CSF-facing surface of choroidal epithelium (24,30), and it is possible that insulin receptors may contribute to CSF insulin removal (24,30,31). In contrast to its action in peripheral tissues, insulin does not stimulate glucose metabolism in neurons. Glucose transport, glucose oxidation, and glycogen synthesis in cultured fetal chick neurons are not altered by insulin (20,24). Evidence suggests a regionally specific effect of insulin on brain glucose metabolism (32). Insulin does not seem to influence basal cerebral glucose metabolism or transport of glucose into the brain (32,33). *In vitro* studies showed that insulin regulated glucose uptake by glial cells, but did not influence neuronal glucose uptake (32,34). Insulin receptor mRNA concentrations in

the rat CNS are maximal at birth and decline to minimal levels in adult brain (35). Studies in human and animal models have shown that an increase in brain insulin has a cognition-enhancing effect (34). Cognitive dysfunction and dementia have recently been proven to be common and underrecognized complications of diabetes mellitus (DM) (36). Patients suffering from AD and ALS were found to have lower than normal CSF levels of insulin (5,36). Too much insulin in the brain may be associated with reduced amyloid-β clearance due to competition for their common and main depurative mechanism, the Insulin Degrading Enzyme (36). On the other hand, hyperglycemia by producing hyperinsulinemia may lead to an increased production of Reactive Oxygen Species (ROS), protein glycation and oxidative stress, some of the processes important even for physiologic brain aging. Neurons have been shown to share more similarities with the insulin-producing pancreatic islet cells than with any other cell type. The root of this similarity is uncertain, but it may lie in the islets' evolution from an ancestral insulin-producing neuron (37). Not more than 10 years ago, the brain was described in medical textbooks as "an insulin insensitive organ". Evidence for the presence of insulin and its receptors in the CNS has challenged this notion in recent years (38-40).

The insulin-like growth factors (IGFs) first appeared early in phylogeny about 600 million years ago and have increased in number through gene duplication (41). In mammalians, IGFs are expressed in all tissues and are found in many biological fluids (42,43). IGF-1 promotes differentiation, proliferation and prevents apoptosis of brain-derived cells, and helps in myelinization, dendrite growth and cytoskeleton protection (44). The mechanism by which IGF-1 prevents cells from entering a death program has not been completely defined, but the phosphatidylinositol kinase pathway is implicated and it seems that neuroprotective action of IGF-1 is linked to the Bcl family (45,46). Furthermore, IGF-1 is thought to have a neuromodulatory function affecting glutamic acid decarboxylase and choline acetyltransferase activities as well as dopamine uptake (47,48). The levels of IGF-1 and its binding proteins may be altered in CSF in various neurologic disorders like autism, ALS, AD and MS (5,42,49,50). In rats, IGF is secreted by the choroid plexus (51). During embryonic development, IGF-1 mRNA is detectable in many brain regions, its expression being particularly high in the midbrain and cerebral cortex (42,52). IGF-1 is also expressed in the leptomeninges and choroid plexus, enabling growth factors to diffuse to their sites of activity (42,53). In most neurons, IGF-1 transcription decreases significantly postnatally, correlating with the degree of cell maturation and reaching low levels in adults (54). Systemic IGF-1 is not readily transported through BBB, and therefore the local production of IGF-1 is the main and primary source of this neural growth factor for the brain (42). IGF in the CSF can pass through the ependymal layer and reach the brain parenchyma (55). Choroid plexus-derived IGF might be important for cellular survival and recovery shortly after injury, before the neural tissue starts to produce its own IGF. Neurotrophic factor is therefore secreted into the brain ventricles and conveyed by CSF bulk flow to various regions of the brain and spinal chord, bringing many neurons in contact with this valuable molecule necessary for physiologic functioning of neural tissue (56). For example, the exercise in physiologic conditions causes increase in circulating IGF-1 levels, which is proven to be neuroprotective in the cases of various brain injuries, and when IGF-1 uptake into the CSF is blocked, the neuroprotective effect is lost (57).

GH was isolated in 1944 and used for therapeutic purposes in the 1960s (58). The DNA encoding GH was cloned in 1979 and recombinant GH was approved for clinical use in 1985 (59). Favorable effects of GH substitution on the metabolism, cardiovascular system and body composition have been described, but during the past two decades, the effects of somatotropic axis on the CNS have come into the focus of interest. In fact, the stimulatory action of GH on the proliferation of cerebrocortical brain cells could perhaps be mediated by an antiapoptotic action of GH that promotes cell survival. GH is present in the brain of human embryos during the 8th week of development, prior to its appearance in the pituitary gland at the end of the first trimester (60). It remains unclear whether these effects are mediated directly by GH or by its mediator, IGF-1 (61). In human brain, GH receptor can be found in many brain areas. They are detected in highest concentrations in choroid plexus, like those for IGF-1 and insulin, but also in the hippocampus, basal ganglia and hypothalamus. A reduction in GH binding sites in the brain is seen with increasing age (62). The somatotropic axis plays a central role in the development and growth of the CNS. Distinction between GH and IGF-1 mediated effects is often difficult, but transgenic mouse models have shown that overproduction of GH induces an increase in body size and motoneuron size, whereas overproduction of IGF-1 induces increase in body size only (6,61).

The possible roles of insulin, GH and IGF-1 in the pathogenesis of neurodegenerative diseases (Parkinson's disease, AD, ALS and Huntington's disease) have already been investigated (63,65). It is also necessary to clarify the role of these neuroprotective and neurotrophic factors in neurologic diseases and mechanisms involved in cell death (apoptotic or other).

Considering the pain, side effects and risks associated with the procedure of lumbar puncture, it may be difficult to compose an adequate control group for investigations of GH, IGF-1 and insulin in humans. We decided to measure CSF concentrations of insulin, GH and IGF-1 in healthy subjects to help in future investigations focused on their concentrations and roles in various neurologic diseases and to try to establish their standard values. Determination of these neural growth factors in CSF of healthy people could be important for distinction of their pathologic values in certain neurologic and other diseases (66,67). These parameters could possibly serve as markers of disease activity, or as a screening method, which is impossible without the knowledge of their normal levels.

All the neural growth factors measured, GH, IGF-1 and insulin, are involved in neural development and they are probably an important component in the physiologic neuroprotective processes as well as in brain aging. It is surprising that despite all their known important roles in brain development, aging and possible rejuvenation, they have shown modest therapeutic effects in the treatment of neurodegenerative diseases. It seems likely that GH, insulin and IGF-1 do not cross BBB efficiently enough for therapeutic goals and therefore should be administered intrathecally in various therapeutic strategies.

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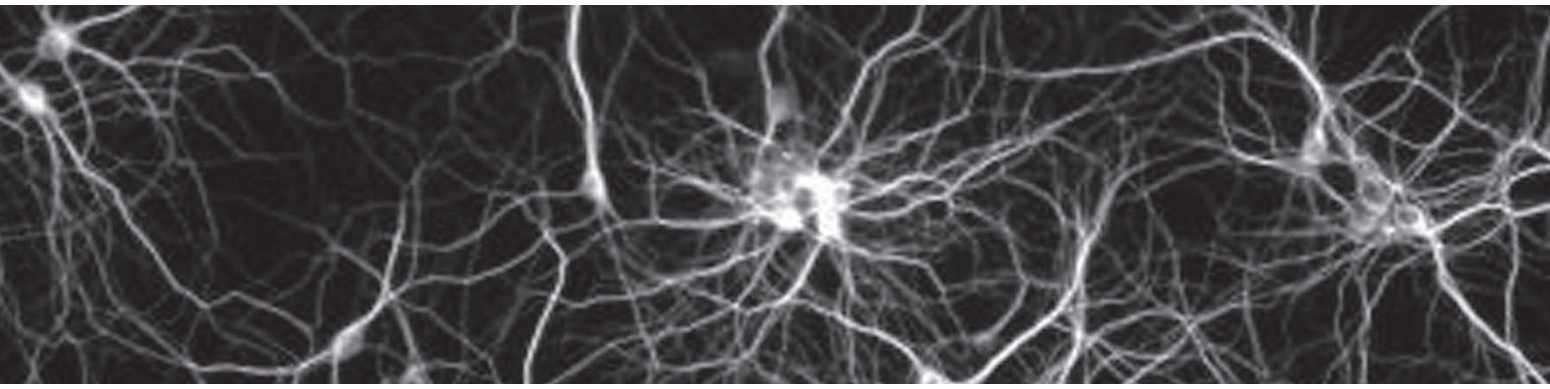
Address for Correspondence: Ervina Bilić, MD, PhD.; e-mail: ervina.bilic@mef.hr

Koncentracije hormona rasta, inzulinu sličnog čimbenika rasta 1 i inzulina u likvoru zdravih osoba

SAŽETAK – Hormon rasta (engl. *Growth Hormone* - GH), inzulinu sličan čimbenik rasta-1 (engl. *Insulin-like Growth Factor*-1 - IGF-1) i inzulin imaju značajnu ulogu u razvoju središnjega živčanog sustava i u cjeloživotnom procesu neuroprotekcije. Koncentracije ovih hormona u likvoru zdravih osoba dosada nisu poznate. Mjerene su koncentracije GH, IGF-1 i inzulina u likvoru 57 neurološki zdravih osoba (35 muškaraca i 22 žene) podvrgnutih operaciji koljena u spinalnoj anesteziji. U likvorima muških ispitanika koncentracije (SD; min-maks) GH, IGF-1 i inzulina su bile 0,67 (0,11; 0,50-0,90) μU/mL; 7,49 (0,92; 6,00-9,00) μg/L i 0,71 (0,13; 0,40-0,90) μU/mL. U likvorima ispitanika ženskog spola koncentracije GH, IGF-1 i inzulina su bile 0,69 (0,10; 0,50-0,90) μU/mL; 7,32 (0,95; 6,00-9,00) μg/L i 0,68 (0,13; 0,40-0,90) μU/mL. Nisu nađene statistički značajne razlike koncentracije navedenih hormona u likvoru između ispitanika muškog i ženskog spola. Nije dokazana korelacija između vrijednosti koncentracija ispitivanih parametara i dobi ispitanika niti značajna bivarijatna korelacija koncentracija GH, IGF-1 i inzulina. Određivanje normalnog raspona vrijednosti koncentracija hormona GH, IGF-1 i inzulina u likvoru može biti polazna točka za daljnje istraživanje promjena njihovih koncentracija u specifičnim neurološkim bolestima. Mjerenje ovih parametara u neurološkim bolestima moglo bi poslužiti kao laboratorijska metoda probira, dijagnostički korak ili jedan od načina praćenja učinkovitosti pojedinih terapijskih postupaka kod različitih neuroloških bolesti.

Ključne riječi: likvor, hormon rasta, zdrave osobe, inzulin, inzulinu sličan čimbenik rasta 1



Immunoreactivity and characterization of oligosaccharide determinants in glycoproteins isolated from peripheral nerve and *Campylobacter jejuni* O:19

A. Poceva-Panovska, K. Brezovska, A. Grozdanova[1], S. Apostolski[2], Lj. Suturkova

ABSTRACT − The biology of glycoconjugates and immunopathological responses in reference to the diseases of the nervous system is an area of intensive research. In the last decade, several carbohydrate structures that are target determinants in peripheral nerve diseases have been isolated and partially characterized. Experimental evidence indicate structural similarity between oligosaccharide determinants present in peripheral nerve glycoconjugates and bacterial carbohydrate structures, suggesting that molecular mimicry between bacterial and neural oligosaccharides may have a potential role in the development of autoimmune postinfectious neuropathies. In this study, galactosyl *N*-acetylgalactosamine binding glycoproteins were isolated from human peripheral nerve and bacteria *Campylobacter jejuni* O:19, using peanut agglutinin (PNA) lectin affinity chromatography. Isolated glycoproteins were detected with immunoblot analysis using periodate oxidation and biotinylated peanut agglutinin. Sera from patients with Guillain-Barré syndrome were tested on immunoblot for reactivity to the previously isolated glycoproteins. We detected immunoreactive glycoproteins with similar electrophoretic mobility present in the isolates from peripheral nerve and *Campylobacter jejuni*. N-linked oligosaccharides were released from these immunoreactive glycoproteins, fluorescently labeled and enzymatically sequenced with highly specific exoglycosidases. Further analysis with fluorophore-assisted carbohydrate electrophoresis demonstrated the presence of similar oligosaccharide structures in glycoprotein isolates. Structural similarity and immunoreactivity between human peripheral nerve

Faculty of Pharmacy, Institute for Applied Chemistry and Pharmaceutical Analysis, University Ss. Cyril and Methodius, Skopje, Macedonia

[1] Faculty of Pharmacy, Institute of Pharmaceutical Chemistry, University Ss. Cyril and Methodius, Skopje, Macedonia

[2] Institute of Neurology, Clinical Centre of Serbia, Belgrade, Serbia

and *Campylobacter jejuni* glycoproteins was detected. This finding indicates the possible role of the isolated bacterial glycoprotein as an antigenic determinant involved in the pathogenesis of Guillain-Barré syndrome.

Key words: *Campylobacter jejuni,* Guillain-Barré syndrome, immunoreactivity, glycoproteins, peripheral nerve

INTRODUCTION

Antibodies involved in the pathogenesis of autoimmune neuropathies usually are directed against carbohydrate epitopes of glycoconjugates. Several carbohydrate-containing structures that are target determinants in peripheral nerve diseases have been identified and characterized. These antigenic structures include molecules of glycoproteins, glycolipids and glycosphingolipids (1).

In Guillain-Barré syndrome (GBS), a prototype of postinfectious autoimmune disease, the gram-negative bacterium *Campylobacter jejuni* (*C. jejuni*) is a frequent antecedent pathogen with overall prevalence of 32% (2). Numerous *C. jejuni* serotypes have been reported in association with GBS. A strong association between *C. jejuni* serotype and GBS has been reported by Kuroki *et al.* (3), with a predominance of serotype O:19, present in 81% of GBS population (4).

Analysis of *C. jejuni* lipopolysaccharide (LPS) showed that the terminal structure (Gal(β1-3)GalNAc β1-4 [NeuAc α1-3]Galβ) is identical to the terminal tetrasaccharide of the GM1 ganglioside, a glycosphingolipid that is mostly abundant in the human peripheral nervous system. These were the first findings that demonstrated the existence of molecular mimicry between neural tissue gangliosides and the infectious agent isolated from patients with GBS (5,6). Further animal studies supported the hypothesis that expression of ganglioside-mimicking structures of *C. jejuni* LPS is a triggering factor for the induction of antiganglioside antibodies and development of GBS (7-10).

Several studies have reported reactivity of GM1 positive GBS patient sera to Gal(β1-3)GalNAc glycoproteins from human peripheral nerve (11) and glycoproteins isolated from *C. jejuni* (12,13). Flagellar glycoprotein was isolated and purified from the reference strain of *C. jejuni* O:19 and the results obtained support the hypothesis that in GBS patients, antiflagellar antibodies are induced during *C. jejuni* infection (14). In addition, the glycosyl modifications that are surface exposed in the flagellar filament appear to be highly immunogenic (15). These findings suggest that the presence of non LPS antigens may also be involved in the development of the disease. The association of glycosylated flagellin with the development of GBS remains speculative, but the possibility of molecular mimicry between flagellar glycoproteins and eukaryotic glycoproteins exists (16).

Since most of the research in this field is oriented toward the lipopolysaccharide antigens in bacteria and ganglioside structures in the nervous system, we investigated other nonlipopolysaccharide antigens in bacteria and glycoproteins in human peripheral nerve. In this study, we analyzed the carbohydrate composition of two N-linked glycoproteins isolated for human peripheral nerve and *C. jejuni*, which were recognized by GBS patient sera, using a technically simple biochemical method of fluorophore-assisted carbohydrate electrophoresis (FACE).

MATERIAL AND METHODS

Isolation of Gal(β1-3)GalNAc binding glycoproteins from peripheral nerve and C. jejuni serotype O:19

Human sciatic nerve was obtained postmortem from a 45-year-old male patient who died from non-neurological disease (Institute of Forensic Medicine, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Macedonia) and kept frozen at -70 °C until use. It was delipidated in chloroform:methanol (1:2) and solubilized in 0.5% Triton X-100, 0.4% SDS with protease inhibitor cocktail.

C. jejuni serotype O:19 (ATCC 700 297) was cultured on Campylosel® (bioMerieux, La Balme, France) in microaerophilic conditions using Campy Gen® (Oxoid, Basingstoke, UK) at 37 °C for 48 hours. The identity of *C. jejuni* was confirmed with microscopic examination, staining according to Gram and biochemical tests, at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje. Hot phenol-water method, described by

Westphal and Jann (17) was adapted for extraction of proteins from the phenol phase. Proteins from the peripheral nerve and *C. jejuni* were further purified with affinity chromatography using 2-mL column with agarose bound peanut agglutinin (Sigma-Aldrich, St. Louis, USA) (11).

Identification of isolated glycoproteins

Isolated glycoproteins from the peripheral nerve and *C. jejuni* were separated with SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) on 7.5% gel and analyzed on Western blot.

Carbohydrate structures were detected using periodate oxidation as prescribed in Bio-Rad immunoblot kit for glycoprotein detection. Detection of PNA-binding glycoproteins was done using lectin from *Arachis hypogaea* (peanut) conjugated with biotin (Sigma-Aldrich, St. Louis, USA). For visualization, avidin-peroxidase in dilution 1:1000 (Sigma-Aldrich, St. Louis, USA) and chromogen diaminobenzidine (SIGMA FAST™, Sigma-Aldrich, St. Louis, USA) were used.

Immunoreactivity of Gal-β(1-3)-GalNAc binding glycoproteins from peripheral nerve and C. jejuni

Sera from eight male and two female patients (aged 40-62 years) originating from Serbia, diagnosed with GBS, were provided by the Institute of Neurology, Clinical Center of Serbia in Belgrade. Sera from one female and two male blood donors were used as negative controls. Antibody titer to gangliosides GM1 and GA1 in these patients was determined by ELISA using intra-laboratory protocol standardized according to the method suggested by INCAT (European Inflammatory Neuropathy Cause and Treatment) group (18,19). Sera (1:200) were also tested for reactivity to the isolated glycoproteins on Western blot, following separation of the glycoproteins on 7.5% polyacrylamide gel. Peroxidase conjugated anti-human IgM and IgG (Sigma-Aldrich, St Louis, USA) were used as secondary antibodies (1:500). Visualization was done with diaminobenzidine (SIGMA FAST™, Sigma-Aldrich, St. Louis, USA).

Analysis of oligosaccharides in immunoreactive glycoprotein

The glycoproteins from peripheral nerve and *C. jejuni* that gave signal when incubated with patient sera (~60 kDa) were isolated using preparative SDS PAGE (7.5% gel) and lyophilized. Isolated glycoproteins were characterized by oligosaccharide profiling and sequencing, using the method of fluorophore-assisted carbohydrate electrophoresis (FACE) (20-22).

Enzymatic release of N-linked oligosaccharides

Enzymatic release of asparagine linked (*N*-linked) oligosaccharides from immunoreactive glycoproteins from peripheral nerve and *C. jejuni* was done using Peptide *N*-glycosidase F (PNGase-F) (Glyko® ProZyme Inc., San Leandro, Ca, USA) by a protocol prescribed by Glyko®. After overnight incubation at 37 °C with PNGase F, the *N*-linked oligosaccharides released were labeled with fluorophore (8-aminonaphthalene-1,3,6-trisulfonate, ANTS), followed by the addition of reducing agent (sodium cyanoborhydride, $NaCNBH_3$). The labeled oligosaccharides were separated on 21% polyacrylamide gel using commercial *N*-linked oligosaccharide buffer (Bio-Rad®). Determination of the relative migration of the oligosaccharides was done using a mixture of glucose oligomers ranging from $Glucose_1$ to larger than $Glucose_{20}$ (glucose ladder). Separated oligosaccharides were visualized in UV chamber (long UV) and analyzed using TotalLab® (Nonlinear Dynamic, UK) image software.

Enzymatic sequencing of N-linked oligosaccharides

Oligosaccharide band from the profiling gel was cut out and glycans were extracted and subjected to sequential enzymatic digestion and electrophoresis. Following enzymes purchased from Glyco® were used: neuraminidase (NANase III) specific for all α2-3,6,8,9 linked *N*-acetylneuraminic acid; β-galactosidase (GALase III) specific for β1-4 linked galactose; βN-acetylhexoaminidase (HEXase III) specific for β1-2,3,4,6 linked *N*-acetylglucosamine and α-mannosidase (MANase II) specific for α1-2,3,6 linked mannose. Oligosaccharide sequencing was performed with series of enzyme digests by sequencing protocol obtained from Glyko® *N*-linked oligosaccharide sequencing kit.

The structure of the glycan was determined by comparing the electrophoretic migration patterns of the digestion products with the glucose ladder. Relative mobility shift after releasing of each monosaccharide unit was given in the protocol. No changes in the migration indicated that sugar was not present or not in the correct linkage. The se-

quencing gels were analyzed using TotalLab® image software.

RESULTS

Identification of the isolated glycoproteins

Following the electrophoretic separation and immunoblotting, the isolated glycoproteins from peripheral nerve and *C. jejuni* were visualized with periodate oxidation and with biotin labeled lectin from *Arachis hypogaea* (peanut agglutinin, PNA) that specifically binds to the Gal-β(1-3)-GalNAc determinant. It was shown that isolates from both peripheral nerve and *C. jejuni* contained few PNA-binding glycoproteins with molecular masses of approximately 200 kDa, 120 kDa, 70 kDa and 60 kDa (Fig. 1).

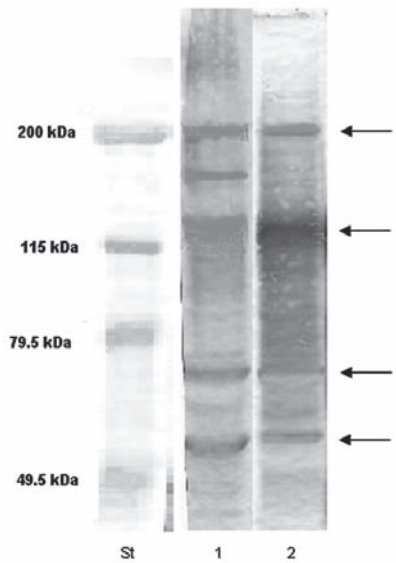


Fig. 1. *Immunoblot detection of (1) peripheral nerve and (2) C. jejuni glycoproteins with biotinylated PNA.*

Immunoreactivity of Gal-β(1-3)-GalNAc binding glycoproteins from peripheral nerve and C. jejuni

In order to test immunoreactivity of the isolated Gal-β(1-3)-GalNAc binding glycoproteins, we used sera from patients with GBS. First, antibody titers of patient sera to GM1 and AG1, the gangliosides that are frequently associated with the syndrome, were determined by indirect ELISA method. Elevated titers of antiganglioside antibodies were found in approximately 70% of tested sera. When immunoreactivity of isolated glycoproteins to GBS patient sera was tested, 50% of the sera were found to have a similar reactivity pattern to glycoproteins with a molecular weight of 60-70 kDa, present in peripheral nerve and *C. jejuni* (Fig. 2). Negative controls showed no reactivity to peripheral nerve and *C. jejuni* glycoproteins. There was no correlation between the anti-ganglioside antibody titer and reactivity to the isolated glycoproteins.

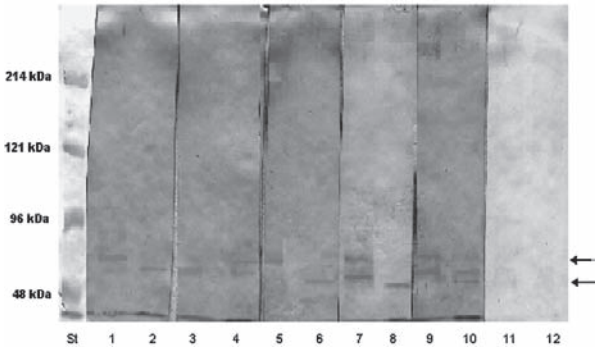


Fig. 2. *Immunoreactivity glycoproteins isolated from peripheral nerve (lanes 2, 3, 6, 8 and 10) and C. jejuni (lanes 1, 4, 5, 7 and 9) with five GBS patient sera. Negative control sera (PN, lane 11; C. jejuni, lane 12).*

Enzymatic release of N-linked oligosaccharides (oligosaccharide profiling)

Oligosaccharide profiles of peripheral nerve and *C. jejuni* N-linked oligosaccharides in immunoreactive glycoproteins, obtained after enzymatic release, labeling and electrophoretic separation are shown in Fig. 3. The migration position is reported in terms of degree of polymerization (DP), which corresponds to the sample migration in relation to the migration of the glucose ladder standards (lane 1).

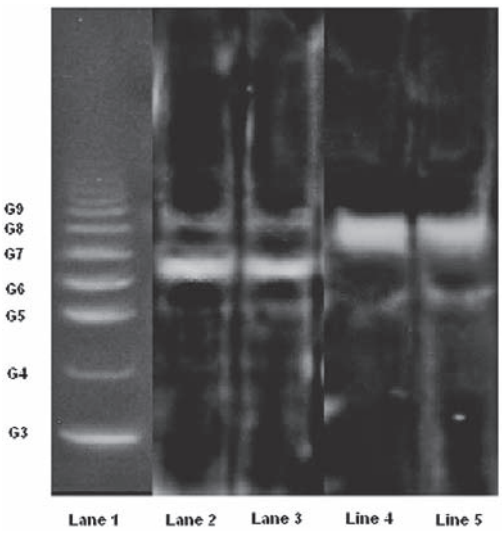


Fig. 3. *Oligosaccharide profiles of N-linked oligosaccharides isolated from peripheral nerve (lanes 2 and 3) and C. jejuni (lanes 4 and 5) immunoreactive glycoprotein; lane 1: glucose ladder (St).*

According to the software analysis, profiles of oligosaccharide released from immunoreactive glycoprotein from peripheral nerve and from *C. jejuni* contained one major band with 6.58 DP and 7.6 DP, respectively. In comparison with the values for the mobility of ANTS labeled *N*-linked oligosaccharides, peripheral nerve oligosaccharide is a monosialylated bi-antennary complex type, while *C. jejuni* has the asialo bi-antennary complex type of *N*-linked oligosaccharide.

Enzymatic sequencing of N-linked oligosaccharides

The sequencing gel for peripheral nerve oligosaccharides is shown in Fig. 4. Lane 1 represents the oligosaccharide (OS) profile and lane 2 OS with no enzymatic digestion. The probe in lane 3 contains NANase III, which cleaves terminal sialic acid residues. In Fig. 4, the upwards shift in 1.1 DP relative to the glucose ladder indicates that one sialic acid residue was removed. Digestion with NANase II and GALase III cleaves the glycan further (lane 4) and band shifting of 2.06 DP indicates that two galactose residues were removed. Digestion with HEXase produces band shift of 1.63 DP, which corresponds to removal of two *N*-acetylglucosamine residues (0.75 DP units/*N*-acetylglucosamine removed) (lane 5). Treatment with MANase II gives mobility shift of 1.36 DP units indicating the cleavage of two mannose residues. Digestions with HEXase and MANase determined the number of antennae in the glycan. Since two *N*-acetylgalactosamine residues were linked to trimannosyl core,

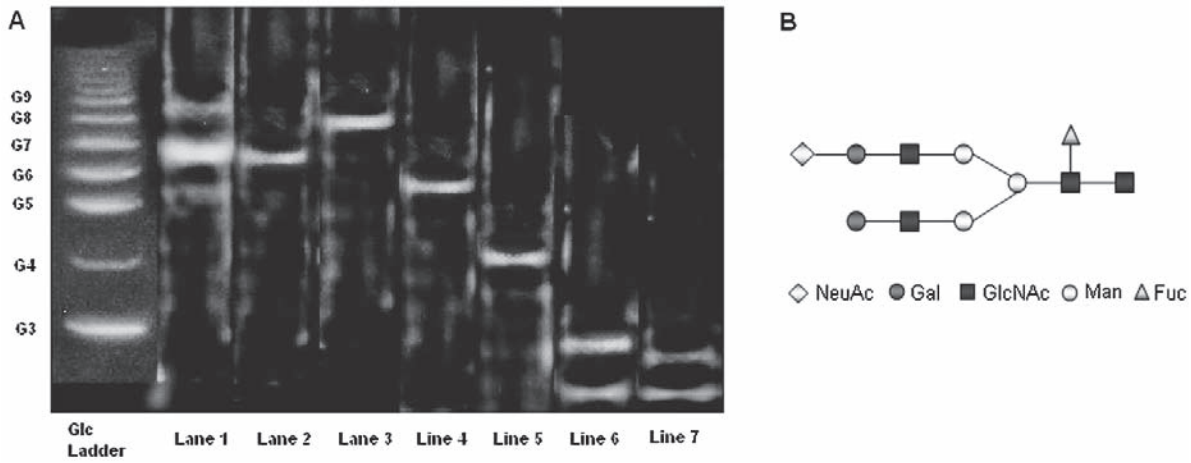


Fig. 4. *(A) Sequenced analysis of N-linked oligosaccharides from peripheral nerve. Lane Glc Ladder: glucose ladder; lane 1: oligosaccharide (OS) profile; lane 2: OS without enzyme; lane 3: OS with NANase III; lane 4: OS with NANase III + GALase III; lane 5: OS with NANase III + GALase III + HEXase III; lane 6: OS with NANase III + GALase III + HEXase III + MANase II; lane 7: core standard; (B) proposed structure based on the analysis.*

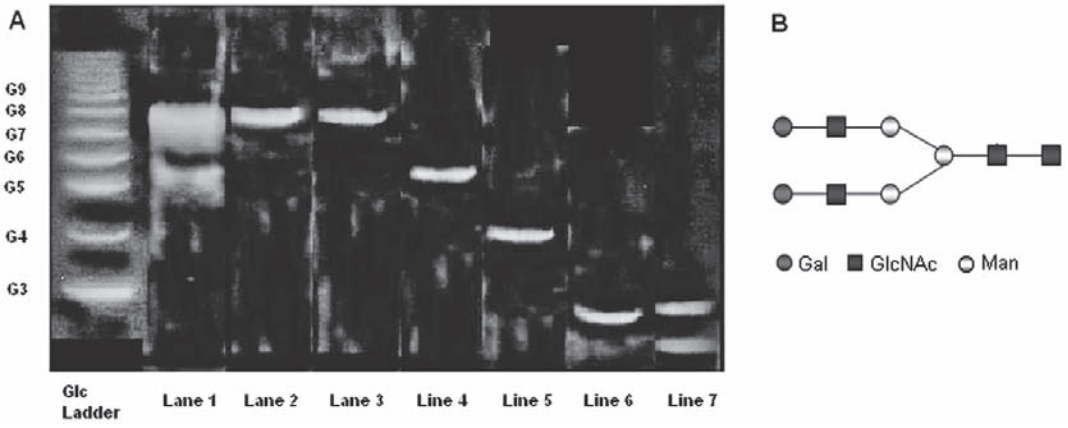


Fig. 5. *(A) Sequence analysis of N-linked oligosaccharides from Campylobacter jejuni: lane Glc Ladder: glucose ladder; lane 1: oligosaccharide (OS) profile; lane 2: OS without enzyme; lane 3:OS with NANase III; lane 4: OS with NANase III + GALase III; Lane 5: OS with NANase III + GALase III + HEXase III; lane 6: OS with NANase III + GALase III + HEXase III + MANase II; lane 7: core standard; and (B) proposed structure based on the analysis.*

the glycan was determined to be of a bi-antennary structure.

To determine the presence or extent of core fucosylation, a mixture of ANTS labeled trimannosyl core oligosaccharides with and without α1-6 fucose was separated in lane 7. The core residue in line 6 co-migrated with fucosylated core standard (upper line). From this analysis, the identity of the glycan derived from peripheral nerve is monosialylated, bi-antennary galactosylated complex type of *N*-linked oligosaccharide with fucosylated core (Fig. 4B).

The sequencing gel for *C. jejuni* oligosaccharides is shown in Fig. 5. In lane 3, there was no shift in mobility, suggesting that sialic acid is not present in oligosaccharide structure. Band shift of 2.15 DP in lane 4 indicates the loss of two galactose residues. *N*-acetylglucosamine (lane 5) produced band shift of 1.64 DP, which corresponds to mobility shifts of two *N*-acetylglucosamine residues. Digestion with MANase II gives band shift in 1.46 DP indicating the presence of 2 mannose residues. The core of the glycan structure is non-fucosylated because it migrates closer to the band of non-fucosylated core in the core standard. The identity of the glycan derived from *C. jejuni* is of asialylated, biantennary galactosylated complex type of *N*-linked oligosaccharide with non-fucosylated core (Fig. 5B).

DISCUSSION

The Guillain-Barré syndrome (GBS) is the most common form of acute neuromuscular paralysis in developed countries (2), but the pathogenesis is still in debate. Research in the field was predominated by the discovery that infections with the gram-negative *Campylobacter jejuni* frequently precede GBS and by the finding of antibodies against various peripheral nerve gangliosides in serum from GBS patients. These anti-ganglioside antibodies recognize the oligosaccharide portion of the gangliosides. This was demonstrated by the cross-reactivity of anti-ganglioside antibodies with gangliosides and other glycoconjugates with homologous oligosaccharide moieties. Anti-GM1 antibodies frequently cross-react with GD1b and asialo-GM1, suggesting that they bind to the Gal(β1-3)GaINAc-structure, which these glycolipids have in common (23). The Gal(β1-3)GaINAc-structure is also widespread in glycoproteins (24), and human monoclonal anti-GM1 antibodies cross-react with glycoproteins in peripheral nerve extracts (25). It is therefore unknown whether anti-ganglioside antibodies bind with gangliosides or other target structures in peripheral nerves. There are studies that report antibodies against the outer membrane proteins to be frequently found in serum from German GBS patients with *C. jejuni* infections (14). The presence of glycosylated proteins in bacteria such as flagellar protein raises the possibility of molecular mimicry to glycosylated moieties on human proteins.

In our study, we demonstrated that isolated bacterial glycoprotein shows similarity in the carbohydrate structure with human peripheral nerve glycoprotein. Enzymatic sequence analysis of the glycans derived from immunoreactive glycoproteins present in peripheral nerve and *C. jejuni* indicated the presence of two galactose, two *N*-acetylgalactosamine and two mannose residues differing only in the presence of one residue of terminal sialic acid and fucosylated core in peripheral nerve oligosaccharide. An important finding in our research was that 50% of GBS patient sera, when tested on Western blot, showed a similar reactivity pattern to glycoprotein isolates from human peripheral nerve and *C. jejuni*. We also observed that there was no correlation between the anti-GM1 and AG1 ganglioside antibody titer and immunoblot reactivity, to the isolated glycoproteins. This finding supports the hypothesis that the pathology of GBS may be mediated by cross-reactive autoantibodies directed against the GalGalNAc epitope of glycoproteins, and their production may be triggered by homologous antigen in *C. jejuni*.

Structural similarity in the oligosaccharide portion and immunoreactivity of these glycoproteins indicates that they are potentially cross-reactive and may contribute to the disease development. Animal model studies and *in vivo* testing of the isolated glycoproteins will further demonstrate their antigenic potential and possible role in the pathogenesis of GBS.

CONCLUSION

Results from this study revealed structural similarity in oligosaccharide portion and immunoreactivity of the glycoproteins isolated from peripheral nerve and *C. jejuni*, indicating that they are potentially cross-reactive determinants and may contribute to the development of GBS associated with antecedent *C. jejuni* infection. Further structural characterization and *in vivo* analysis of their antigenic potential will elucidate their possible involvement in the development and pathogenesis of GBS.

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Address for Correspondence: Ana Poceva-Panovska, Pharm. MSci, Vodnjanska 17, 1000 Skopje, Macedonia; e-mail: anpo@ff.ukim.edu.mk

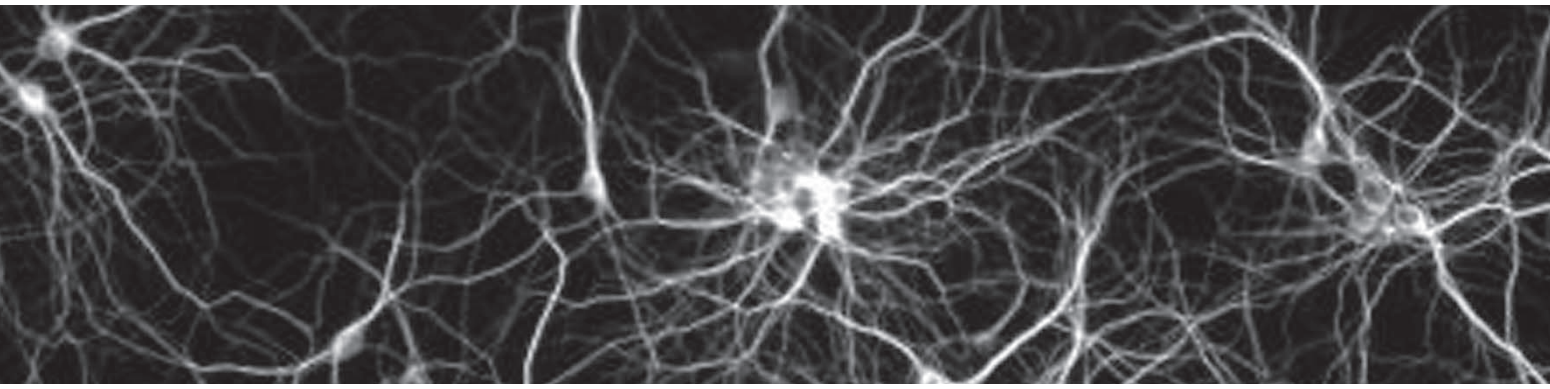
Imunoreaktivnost i karakterizacija oligosaharidnih determinanti u glikoproteinima izoliranima iz perifernog živca i *Campylobacter jejuni* O:19

SAŽETAK - Biologija glikokonjugata i imunološki odgovori u odnosu na bolesti živčanog sustava područje su intenzivnog istraživanja. Posljednjih deset godina izolirano je i djelomično karakterizirano nekoliko ugljikohidratnih struktura koji su ciljne determinante kod bolesti perifernog živca. Eksperimentalni nalazi ukazuju na strukturnu sličnost između oligosaharidnih determinanti prisutnih u glikokonjugatima perifernog živca i bakterijskih ugljikohidratnih struktura ukazujući da molekulska mimikrija između bakterijskih i živčanih oligosaharida može imati ulogu u razvoju autoimunih postinfekcijskih neuropatija.

U ovoj smo studiji izolirali glikoproteine koji vežu galaktozil *N*-acetilgalaktozamin iz ljudskog perifernog živca i bakterije *Campylobacter jejuni* O:19 koristeći lektinsku (*peanut agglutinin* - PNA*)* afinitetnu kromatografiju. Izolirani glikoproteini otkriveni su imunoblot analizom uporabom oksidacije periodata i biotinilirani PNA. Serumi pacijenata s Guillain-Barréovim sindromom testirani su imunoblotom na reaktivnost prethodno izoliranih glikoproteina. Otkrili smo da su imunoreaktivni glikoproteini sa sličnom elektroforetskom pokretljivošću prisutni u izolatima iz perifernog živca i *Campylobacter jejuni*. Iz tih imunoreaktivnih glikoproteina oslobodili smo *N*-povezani oligosaharid, fluorescentno obilježili i enzimski sekvencionirali s visoko specifičnim egzoglikozidazama. Daljnja analiza uz pomoć tehnike fluoroforno potpomognute elektroforeze ugljikohidrata pokazala je prisutnost sličnih oligosaharidnih struktura u glikoproteinskim izolatima.

U ovom je radu otkrivena strukturna sličnost i imunoreaktivnost između glikoproteina izoliranih iz ljudskog perifernog živca i bakterije *Campylobacter jejuni* O:19. Taj nalaz ukazuje na moguću ulogu izoliranog bakterijskog glikoproteina kao antigenske determinante uključene u patogenezu Guillain-Barréova sindroma.

Ključne riječi: *Campylobacter jejuni*, Guillain-Barréov sindrom, imunoreaktivnost, glikoproteini, periferni živac



Cognitive assessment in dementia: initial approach in outpatient clinic

M. Gregorič Kramberger, S. Popović, Z. Pirtošek

ABSTRACT – A diagnosis of dementia should be made only after comprehensive assessment, which inevitably includes history taking, cognitive and mental state examination, physical examination, a review of medication in order to identify and minimize use of drugs that may adversely affect cognitive functioning, and other appropriate investigations. Clinical cognitive assessment in those with suspected dementia should include examination of attention and concentration, orientation, short- and long-term memory, praxis, language and executive function. As part of this assessment, formal cognitive testing should be undertaken using a standardized instrument. Formal neuropsychological testing should form part of the assessment in cases of mild or questionable dementia.

Key words: assessment, cognition, diagnosis, dementia

OVERVIEW

Dementia is a clinical state characterized by the loss of function in at least two cognitive domains. When making a diagnosis of dementia, features to look for include memory impairment and at least one of the following: aphasia, apraxia, agnosia and/or disturbances in executive functioning. To be significant the impairments should be severe enough to cause problems with social and occupational functioning and the decline must have occurred from a previously higher level. It is important to exclude delirium when considering such a diagnosis. When approaching the patient with a possible dementia, taking thorough history is crucial. Clues to the nature and etiology of the disorder are often found following careful consultation with the patient and carer. A focused cognitive and physical examination is useful and the presence of specific features may aid in diagnosis. Certain investigations are mandatory and additional tests are recommended if the history and examination indicate particular etiologies. It is useful when assessing a patient with cognitive impairment in the clinic to consider the following straightforward questions:

- Is the patient demented?
- If so, does the loss of function conform to a characteristic pattern?

Ljubljana University Medical Center, Department of Neurology, Ljubljana, Slovenia

- Does the pattern of dementia conform to a particular pattern?
- What is the likely disease process responsible for the dementia?

Due understanding of cognitive function and its anatomical correlates is necessary in order to ascertain which brain areas are affected. We shall illustrate how the history and examination, including bedside cognitive testing, are used in diagnosis (1).

TAKING HISTORY AND EXAMINATION OF THE COGNITIVELY IMPAIRED PATIENT

It is vital to obtain a history from a relative or close friend in addition to the patient history if they can provide one. It is useful to interview the patient and the accompanying person separately. The absence of a concerned relative or friend at the appointment may lessen the likelihood of dementia in a patient complaining of memory problems. Interviewing the patient separately enables the cooperation and language skills to be assessed without them being masked by interruptions or assistance from a third party. It also allows an assessment into the degree of insight of the affected individual. Conversation with the patient may be as important as any formal cognitive assessment (2).

In brief cognitive assessment, which should be done in all cases, the presence of word finding difficulties, paraphasic errors, and inappropriate behavior should be sought. The assessment must be divided into a number of domains or systems and each of them has to be examined. These are:

1. Alertness and arousal
2. Attention and concentration
3. Orientation
4. Memory
5. Language
6. Visuospatial and constructive functions
7. Frontal lobe and fronto-subcortical functions
8. Other dominant (left) hemisphere functions: calculation, praxis, right-left orientation, finger gnosis
9. Other nondominant (right) hemisphere functions: dressing apraxia, neglect phenomena, agnosias
10. Insight and judgment

Assessment of the level of consciousness and attention processes is crucial as disturbance in these domains can influence the performance on other tests. Familiarity with the common bedside tests for each function is important (Hodges, 1994). For most purposes, a screening battery such as Folstein's Mini-Mental State Examination (MMSE) is a good starting point. It tests orientation, immediate and recent memory, concentration, arithmetic ability, language and praxis (5). It is easy to administer and takes only 5-10 minutes. It has reasonable sensitivity but low specificity, and may be used for serial evaluations. The score is out of 30, and 27 or less is indicative of impairment. A score less than 25 is definitely abnormal. The MMSE may be normal in the presence of subtle impairment, and if this is suspected, detailed evaluation is recommended.

It is useful to combine the MMSE with the Clock Drawing Test in which the patient is asked to draw a clock-face and draw in the hands to indicate 11:10. This tests the patient's constructional abilities and, more importantly, planning and organization or frontal lobe function. Examination of the frontal lobes is central to many neuropsychiatric disorders and the following clinical tests for this are suggested:

1. Observing behavior: impulse control, motivation, affective regulation, relationships.
2. Motor and expressive language.
3. Primitive reflexes: grasp, palmomental, snout, pout, glabellar tab.
4. Verbal fluency: letter-saying as many words-not proper nouns-as possible in one minute beginning with the letter F or A or S), category (naming as many objects from one category as you can in one minute, such as animals).
5. Motor sequencing: Luria's hand sequences (e.g., alternating repeatedly between making a fist and a ring with one hand and then the other-fist-ring test, alternating between a fist, palm and cut movement with one hand and then the other).
6. Reasoning and conceptualization: similarities, differences, proverbs.
7. Planning and organization: clock drawing (ask the patient to draw a clock face and put in numbers and hands to indicate 11:10.

With regard to the tests used, it is important to remember that tests are rarely pure, and usually are influenced by a number of cognitive functions. For example, simple tests like 'serial sevens' may be in-

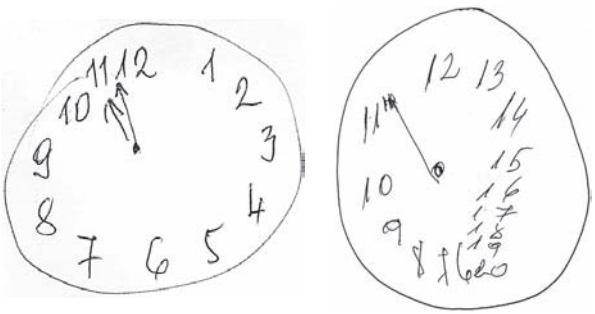


Fig. 1. *Abnormal clock drawing test.*

fluenced by impairment of attention, short-term memory, and calculation ability. A battery of tests is therefore necessary to determine which function is really disturbed. Failure on one test must be followed up with other tests before a dysfunction is established. All cognitive tests are designed to be administered in a particular manner. Significant departure from a standard administration may render the test invalid. Repetition of the same test may lead to an improvement in performance because of what is known as 'practice effect'. For detailed assessment, a referral to a clinical neuropsychologist is necessary. We should also be aware that bedside testing has the potential of confounding the formal assessment of a neuropsychologist if that were to follow. Therefore, one should use only the tests that are meaningful for bedside assessment.

RATING SCALES

The widely used MMSE provides useful information in grading established dementia but does have limitations, particularly in detecting early disease. It contains a crude test of delayed recall, with only three items being employed and not enough time allowed between registration and recall. It lacks a timed test to detect problems with verbal fluency (3). The Addenbrooke's cognitive assessment has been developed to address the deficiencies of the MMSE (7). It also has the advantage of being brief enough to allow the clinician to use it within the time constraints of a new patient appointment. It should be noted that even the Addenbrooke's cognitive assessment is no match for formal neuropsychological assessment. Such services are, however, patchy, and in some services are non-existent, so the clinician must remain competent at assessing cognition.

The focused examination of the patient with dementia is central in cognitive assessment. Aside from the mental state examination and specific tests of cognitive function, it is important to examine the neurological system in any patient with possible cognitive impairment. Neurological examination is, however, often normal in the early stages of many neurodegenerative dementias and specific abnormalities may point to rarer or potentially treatable causes of dementia. It is important to assess the patient at rest for any involuntary movements, including chorea, tremor, dystonia, and myoclonus (which may be spontaneous or stimulus sensitive). The muscles should be observed for fasciculations. The presence or absence of primitive reflexes (frontal release signs) should be determined. Ocular examination should involve careful assessment of visual acuity, papillary responses, eye movements, optic discs, and visual fields. Assessment of speech and swallowing may reveal the presence of bulbar features. Examination for pyramidal or extrapyramidal signs is important and gait should be assessed wherever possible. Ataxia is unusual in Alzheimer's disease, dementia with Lewy bodies, and frontotemporal dementia; its presence should raise the possibility of a different cause. The presence or absence of apraxia should be assessed by asking the patient to perform alternating hand movements or copy gestures (4). Peripheral neuropathy may be present and when cooperation allows signs of this should be sought.

Examination of other systems is also useful in looking for evidence of multisystem disease. In addition to neurological examination, patients should be assessed for signs of immune compromise (predisposing to opportunistic infections such as progressive multifocal leukoencephalopathy, toxoplasmosis or primary cerebral lymphoma possibly indicating HIV/AIDS). Features of systemic disease may indicate an underlying neoplasm, vasculitis, infection, or a metabolic disorder. Uveitis may indicate sarcoidosis, Behcet's disease, or multiple sclerosis. The presence of cardiac disease, hypertension, or a previous transient ischemic attack or stroke may suggest cerebrovascular disease. Armed with the above theoretical knowledge regarding memory and its subdivisions along with how to elicit information from history taking and examination, we can now return to trying to achieve a diagnosis in a patient with possible dementia (6).

CONCLUSION

It is impractical to examine everything in cognitive assessment, and as in most other areas of neurology, the history remains pre-eminent in guiding subsequent examination. The central role of an in-

formant, and the ability to immediately test the hypotheses generated during history taking, distinguish this means of neurological assessment. In some patients, it is not possible to reach a firm diagnosis after a single cognitive assessment, even when combined with a formal neuropsychological report. This is particularly true for the mild stages of neurodegenerative diseases, and reflects the relative insensitivity of both clinical and imaging assessment to early pathology. Longitudinal follow up and repeated assessment in such cases is invaluable, and should not be forgotten.

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Address for Correspondence: Asist. Prof. Milica Gregorič Kramberger, MD, Ljubljana University Medical Center, Department of Neurology, Zaloška 2, 1000 Ljubljana, Slovenia. www.kobz.si/en; e-mail: milica.krambeger@gmail.com

Procjena kognitivnog stanja kod demencije: prvi pristup u ambulanti

SAŽETAK - Dijagnozu demencije trebalo bi postaviti tek nakon sveobuhvatnog pregleda koji neminovno uključuje povijest bolesti (anamnezu), ispitivanje kognitivnog i mentalnog stanja, fizikalni pregled, osvrt na terapiju u cilju identificiranja i smanjena upotrebe lijekova koji bi mogli negativno utjecati na kognitivno funkcioniranje i druge odgovarajuće pretrage. Ako se sumnja na demenciju, klinički kognitivni pregled bi trebao obuhvatiti ispitivanje pažnje i koncentracije, orijentacije, kratkoročnog i dugoročnog pamćenja, praksije, jezičnih i egzekutivnih funkcija. U okviru te procjene trebalo bi poduzeti formalno kognitivno testiranje korištenjem standardiziranih instrumenata. U slučajevima blagih demencija ili kada se demencija dovodi u pitanje u procjenu bi trebalo uvrstiti formalno neuropsihološko testiranje.

Ključne riječi: kognitivno stanje, demencija, dijagnoza, procjena

Upute autorima

NEUROLOGIA CROATICA, službeno glasilo Hrvatskoga neurološkog društva i Hrvatskoga neurokirurškog društva, izdaje Klinika za neurologiju, Klinički bolnički centar Zagreb, četiri puta na godinu. Neurologia Croatica objavljuje radove iz područja kliničke neurologije, temeljnih neuroznanosti i drugih pridruženih područja.

Uza stručne i znanstvene radove, Neurologia Croatica objavljuje kratka priopćenja, prikaze slučajeva, prethodna izvješća i preglede, pisma uredniku te objave stručnih i znanstvenih skupova.

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Baršić B, Lisić M, Himbele J *et al*. Pneumococcal meningitis in the elderly. Neurol Croat 1992;41:131-140.

Knjige

Critchley M. The ventricle of memory. New York: Raven Press, 1990.

Poglavlje u knjizi

Geschwind N. The borderland of neurology and psychiatry: some common misconceptions. In: Bensom DF, Blumer D, eds. Psychiatric aspects of neurologic disease. New York: Grune and Stratton, 1975:1-9.

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Baršić B, Lisić M, Himbele J *et al*. Pneumoccocal meningitis in the elderly. Neurol Croat 1992; 41: 131 - 140.

Books

Critchley M. The ventricle of memory. New York: Raven Press, 1990.

Chapter in a book

Geschwind N. The borderland of neurology and psychiatry: some common misconceptions. In: Bensom DF, Blumer D, eds. Psychiatric aspects of neurologic disease. New York: Grune and Stratton, 1975; 1 - 9.

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