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66034T/C polymorphism of the human pregnane X receptor (hPXR) as potential risk factor for drug resistance in epilepsy – Preliminary study

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ABSTRACT

Introduction and objectives: Recent research has suggested that genetic factors may play an important role in the development of drug resistance in epilepsy. It is not clear which gene loci are responsible for the drug-resistant phenotype. Studying certain nuclear receptors may be helpful in predicting drug response, as they regulate drug transporting proteins and enzymes involved in their metabolism. This study focuses on one of these receptors, the human pregnane X receptor (hPXR).

The objective was to examine the link between selected single nucleotide polymorphisms (SNPs) 69789A/G rs 7643645 and 66034T/C rs 13059232 hPXR and the lack of response to epilepsy treatment.

Materials and methods: 73 patients diagnosed with drug-resistant epilepsy were included in the study. The diagnoses were made according to the criteria published by The International League Against Epilepsy (ILAE) in 2010. The control group was comprised of a group of 122 healthy volunteers. Genetic material isolated from the peripheral blood of the participants was analyzed with TagMan Genotyping Assays in search of the selected hPXR polymorphisms.

Results: The distribution of genotypes of the 66034T/C rs 13059232 hPXR polymorphism was significantly different in the group with drug-resistant epilepsy and the control group.

In the drug-resistant group the CC genotype was significantly more common compared to the control group (50.7% vs 35.2%) $p = 0.0339$.

The distribution of 69789 A/G rs 7643645 hPXR genotypes was comparable in both groups.

Conclusions: There is potential association between hPXR and drug resistance but its relevance for the development of drug-resistant phenotype remains to be studied.

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1. Introduction

Approximately one third of epileptic patients suffer from seizures, despite correct antiepileptic treatment. This is what is understood by “drug-resistant”, “intractable” or “refractory” epilepsy. Several risk factors for this form of the disease had been identified thus far, such as the onset of symptoms in the first year of life, high frequency of seizures before starting treatment, the presence of several types of seizures in one patient, a family history of epilepsy, structural changes of the brain, malformations of cortical development, hippocampal sclerosis, brain tumors, tuberous sclerosis and postinflammatory injuries, among others [1].

Nevertheless it is often difficult to pinpoint the cause of drastically different responses to treatment with antiepileptic drugs (AED) in patients with the same diagnosis and type of epileptic seizures [2,3]. Genetic factors modifying the pharmacokinetic and pharmacodynamic properties of said drugs may be a cause of the phenomenon.

Examples of such factors include genetic polymorphisms of certain microsomal enzymes (CYP2C9, CYP2C19) [4–6], P-glycoprotein (also known as the multidrug resistance-associated protein) [7–11], the dysfunction of GABAA receptors [12–14] and potassium channels [15] modifying their pharmacodynamic properties.

The genetic polymorphism of the Pregnane X Receptor gene (*hPXR*) is a potential risk factor for resistance to antiepileptic drugs.

The first reports of the *hPXR* receptor appeared in 1998 when the pregnanes were identified [16]. Pregnanes are the natural ligands of the *PXR* receptor, after which the receptor was named.

PXR is a nuclear receptor involved in the induction of cytochrome P450 enzymes (CYP2B6, CYP2B9, CYP2C8, CYP2C9, CYP3A4) and protein transporters such as P-glycoprotein [17–20].

These two mechanisms were considered to be plausible sources of the drug resistance phenomenon in epilepsy, and have since then been subject to much research.

It was later discovered that *hPXR* regulates the transcription of over 150 genes, of which many are determinant of drugs' pharmacokinetics and their effects on the body.

CYP3A, MDR1, OATP2 [21], MRP1, MRP2, MRP3 [22–24] and UGT1A1 [25] genes are among the best documented ones.

It seems reasonable to assume that the genetic variations within *hPXR* may indirectly influence many other genes that collectively determine the individual's ability to metabolize certain drugs and their response to treatment.

The promoter and intron 1 of the *hPXR* gene seem to be particularly involved in influencing the activity of the P450 CYP3A4 – the isozyme responsible for metabolizing antiepileptic drugs, among others. Out of 89 known single nucleotide polymorphisms (SNP) of the promoter and intron 1, several modify the activity of CYP3A4: SNP 44477T>C, SNP 63396C>T, SNP 56348C>A, SNP 69789A>G and SNP 66034T>C [26].

Based on that data, we designed this study to try to assess the link between two SNPs, 69789A/G rs7643645 and 66034T/C rs13059232 *hPXR*, and drug-resistant epilepsy.

2. Material and methods

2.1. Patients

73 patients (36 women and 37 men, with an average age of 41.9 ± 16.1 years old) with epilepsy not responding to treatment took part in the study. All patients were followed by the Department of Neurology and Stroke at the Central Veterans' Hospital in Lodz between 2013 and 2014. The formal criterion for the inclusion was a diagnosis of drug-resistant epilepsy according to the 2010 consensus definition by the International League Against Epilepsy (ILAE). This definition describes the failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom. Patients who presented other risk factors predisposing them to negative outcomes, i.e. disease onset before 1 year of age, family history of epilepsy and structural changes of the brain, were not included in the study. All patients had undergone an imaging study of the brain, an EEG and a thorough neurological examination. The control group was comprised of 122 healthy volunteers (65 women and 57 men), between ages 25 and 65, with an average age of 37.3 ± 11.2 years old. Genetic material was isolated from peripheral blood samples. All participants signed a written notice of informed consent after first receiving all relevant information. The study was approved by the Bioethical Committee attached to the Medical University of Lodz, approval no. RNN/263/13/KB.

2.2. Genotyping analysis

Genomic DNA was isolated from 200 µL of blood using the Blood DNA Purification Kit (EURex) according to the manufacturer's protocol. DNA was quantified using a PicoDrop spectrophotometer (PicoDrop Limited) and either immediately used for PCR reaction or stored at –20 °C. The *hPXR* gene single-nucleotide polymorphisms were analyzed using commercially available pre-made Taq Man SNP Genotyping Assays (Thermo Fisher): polymorphism C/T (rs13059232) – assay ID: C_30747427_20 and polymorphism A/G (rs7643645) – assay ID: C_1834250_10.

The PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems) in 25 µL reaction volume containing 10 ng DNA, 12.5 µL TaqMan® Universal PCR Master Mix and 1.25 µL (40×) of the appropriate TagMan® SNP Genotyping Assay. The PCR thermal cycling was as follows: initial denaturation at 95 °C for 10 min; 40 cycles of 15 s at 92 °C and 1 min at 60 °C. Each 96-well plate contained tested samples and 3 reaction mixtures without DNA template (no-template control). End-point fluorescent intensities of each probe were monitored using 7900HT Fast Real-Time PCR System (Applied Biosystems). The genotypes were determined automatically and verified visually using Sequence Detection System 2.3 Software.

2.3. Statistical analysis

Both descriptive and inferential statistical methods were used to analyze the data. Proportions, or fractions, were calculated

to describe the groups. In cases of entire groups and larger subgroups, these proportions were presented as percentages. Because grouping the participants by their genotypes yielded many small subgroups, these results were left as fractions.

Pearson's Chi-squared test was used to compare how often each of the possible genotypes was found in the studied group and the control group. The Chi-squared test was also used to search for additional possible links between specific genotypes, frequency of seizures and the number of used drugs.

Reference charts were used to determine whether the results of various comparisons were statistically significant.

The distribution of genotypes in both studied and control groups was checked against the Hardy-Weinberg principle. Calculations were carried out using the Chi-squared test.

The cutoff point for the Hardy-Weinberg principle was $p < 0.05$.

3. Results

The distribution of genotypes of both studied polymorphisms in the studied and the control group complied with the Hardy-Weinberg equilibrium ($p > 0.05$).

An analysis using the Pearson's Chi-squared test revealed a significant difference in the distribution of the various genotypes of the 66034T/C rs 13059232 hPXR SNP in the studied and the control group. The CC genotype was statistically more common ($p = 0.0339$) in the studied group (50.7%) than in the control group (35.2%). The results for the remaining two genotypes were not statistically significant (Table 1).

The distribution of genotypes of the other studied SNP, the 69789 A/G rs 7643645 hPXR, did not reveal significant differences between the two groups. These results are presented in Table 2.

The analysis of the coincidence of both hPXR polymorphisms (Table 3) revealed that certain combinations of

Table 1 – Genotypic distributions of the hPXR 66034T/C polymorphism in relation to AED response.

Genotype	Studied group		Control group		p
	N	%	N	%	
CT	29	39.7%	61	50.0%	0.1637
CC	37	50.7%	43	35.2%	0.0339
TT	7	9.6%	18	14.8%	0.2964
Total	73	100.0%	122	100.0%	

Bold indicates significant value at $p < 0.05$.

Table 2 – Genotypic distributions of the hPXR 69789 A/G polymorphism in relation to AED response.

Genotype	Studied group		Control group		p
	N	%	N	%	
AG	35	47.9%	57	46.7%	0.8684
AA	30	41.1%	43	35.2%	0.4140
GG	8	11.0%	22	18.0%	0.1852
Total	73	100.0%	122	100.0%	

Table 3 – Genotypic distributions of the hPXR polymorphisms combination.

Combination of polymorphisms	Studied group		Control group		p
	N	%	N	%	
CT/AG	22	30.1%	39	32.0%	0.7896
CT/GG	5	6.8%	7	5.7%	0.7546
CC/AA	27	37.0%	27	22.1%	0.0249
CC/AG	10	13.7%	14	11.5%	0.6474
TT/AG	3	4.1%	4	3.3%	0.9236
CT/AA	1	1.4%	15	12.3%	0.0155
TT/AA	1	1.4%	1	0.8%	0.7149
TT/GG	3	4.1%	13	10.7%	0.1795
CC/GG	1	1.4%	2	1.6%	0.6504
Total	73	100.0%	122	100.0%	

Bold indicates significant value at $p < 0.05$.

polymorphisms occur significantly more commonly in either the studied or the controlled group.

The Chi-squared test confirmed ($p = 0.0249$) that the CC/AA combination was significantly more common in the studied group (37.0% vs 22.1% in the control group).

In the control group it was the CT/AA genotype association that proved to be non-random ($p = 0.0155$), with 1.4% prevalence in the studied group but 12.3% in the control group.

The correlation between patient genotype and the frequency of epileptic seizures was statistically significant ($p < 0.001$). The CC/AA genotype was the most strongly correlated with the frequency of seizures. Half of the patients with this genotype suffered from frequent seizures (0.50 fraction). They were also common in the CC/AG genotype (0.20 fraction) and the CT/GG genotype (0.33 fraction). None of the patients with the TT/GG genotype suffered from frequent or very frequent seizures (Table 4).

All patients in the studied group were treated with 2 or 3 antiepileptic drugs. No statistically significant correlation was found between the number of drugs used and patients' genotypes ($p > 0.05$) (Table 5).

4. Discussion

The hPXR receptor regulates the drug metabolism by means of expression of certain enzymes, drug transport through cell membranes, and their excretion. The genetic variety within the hPXR gene is therefore a likely cause of differences in individuals' responses to antiepileptic drugs and consequently the efficacy thereof.

Few mentions of the significance of the hPXR variance in epilepsy have been published so far.

A Malaysian study tried to establish whether the G7635A hPXR polymorphism changed outcomes in monotherapy with either of the two most commonly used antiepileptic drugs: valproic acid and carbamazepine. No significant differences were found for the group of 685 Malaysian patients [27].

An analogous study focusing on 11193T>C and 11156A>C hPXR polymorphisms found no significant differences in responses to antiepileptic drugs in the Chinese population [28].

A Japanese study from march 2014 looked for correlation between the polymorphisms of (PXR)*1B gene, hepatocyte

Table 4 – Relationship between genotype and frequency of seizures.

Seizure frequency	CC/AA		CT/AG		CC/AG		CT/GG		TT/GG	
	N	Fraction	N	Fraction	N	Fraction	N	Fraction	N	Fraction
Very frequent	2	0.08	–	–	1	0.10	–	–	–	–
Frequent	11	0.42	2	0.10	1	0.10	2	0.33	–	–
Rare	13	0.50	18	0.90	8	0.80	4	0.67	3	1.00
Total	26	1.00	20	1.00	10	1.00	6	1.00	3	1.00

Very frequent: >10 seizures a month; frequent: 5 > x > 10 seizures a month; Rare: <5 seizures a month.

Table 5 – Relationship between the genotype and the number of drugs used.

Number of drugs used	CC/AA		CT/AG		CC/AG		CT/GG		TT/GG	
	N	Fraction	N	Fraction	N	Fraction	N	Fraction	N	Fraction
2	20	0.77	11	0.55	9	0.90	6	1.00	2	0.67
3	6	0.23	9	0.45	1	0.10	–	–	1	0.33
Total	26	1.00	20	1.00	10	1.00	6	1.00	3	1.00

growth factor 4 α (*HNF4 α*) rs2071197 (c.115+308G>A), cytochrome P450 3A5*3 and the response to treatment with carbamazepine in 168 patients. Even though neither of these genetic factors significantly changed the response to treatment in isolation, the combination of the *PXR*1B* and *HNF4 α* rs2071197 polymorphisms had an influence on the metabolism of carbamazepine [29].

We have not found studies pointing to a specific polymorphism of *hPXR* increasing the risk of drug resistance in the current body of literature.

While it is acknowledged that epileptic patients responding well to treatment might have formed a more effective control group, we saw issues with this approach.

In our opinion, selection of patients with epilepsy who demonstrate a “good response” to an antiepileptic drug maintained for 10–20 years (for sake of statistical comparisons) is a difficult task, because a drug is usually gradually discontinued after 3 years of effective treatment. The treatment is re-instated if seizures appear again after discontinuation of the drug.

Evolution of epilepsy into drug-resistant disease is another possible scenario. According to our observations, majority of patients with drug-resistant epilepsy are being treated for many years (usually more than 10) – and often at the beginning of the treatment their seizures were drug-responsive. At that time it is not possible to predict if the drug-resistance will develop in the future. Probably there is a group of patients who demonstrate long-term response to an appropriately chosen monotherapy, but the size of that group is low, and that kind of group could not be assembled within the limited time of material collection. Analysing results of the study a question may be also asked if there is an association between the 66034T/C polymorphism of the *hPXR* gene and epilepsy itself. Authors have presented a causal relationship between *hPXR* and a possible effect on pharmacokinetics of anti-epileptics. There have been no reports from the GWAS study indicating association of that locus with epilepsy itself. Of course we cannot exclude the possibility of such association.

The study was designed as a screening aimed at determination if there really was a potential association (deduced on a

purely theoretical basis) between *hPXR* and drug-resistance. Based on the presented results it may be stated that such association exists, although other interpretations are also plausible. To confirm it finally the study group should be increased in size and some additional control groups should be created, e.g. patients responding to anti-epileptic therapy or drug-resistant schizophrenia etc.

Despite the limitations mentioned above, this study is the first of its kind to demonstrate a potential link between 66034T/C polymorphism of the *hPXR* gene and resistance to antiepileptic treatment.

We have also observed a correlation between certain genotypes predisposed to drug-resistant epilepsy and a higher frequency of epileptic seizures.

We believe that the preliminary results are compelling and warrant further research in the area, as well as a validation of our findings in other populations.

5. Conclusion

The analysis of our results suggests a possible association between the genotype 66034CC in terms of the 66034T/C polymorphism of *hPXR* gene and the absence of a satisfactory response to anti-epileptic treatment, although other interpretations are not completely excluded. It was observed that the 66044CC genotype may predispose to increased frequency of epileptic seizures, and therefore be indirectly correlated with severity of the condition. Considering the fact that the control group was composed of healthy individuals, at the current stage of research a potential correlation between the 66034T/C polymorphism and epilepsy as such is possible. A correlation of that kind has not been mentioned in available literature. Our results constitute a starting point for further studies using larger and ethnically different populations, including responders to treatment.

Conflict of interest

None declared.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals.

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