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THE RISK OF EARLY LIVER ALLOGRAFT DYSFUNCTION IS ASSOCIATED WITH THE TLR-4 GENE GENOTYPE IN THE RS913930 SEQUENCE AND IS IMPLEMENTED VIA HMGB1 NUCLEAR PROTEIN, KUPFFER CELLS AND IL-23 ACTIVATION

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Aim. To evaluate the associations of genotypes of clinically relevant nucleotides rs11536865, rs913930 and rs5030717 of the TLR-4 gene with the risk of development and severity of early allograft dysfunction after liver transplantation. **Materials and methods.** A case-control study enrolling 71 patients was organized. Inclusion criteria: DBD liver transplantation. Exclusion criteria: living related liver transplantation, reduced graft transplantation, recipient's age fewer than 18. **Results.** Within rs5030717 there were identified three genotypes: AA (81.6%) and two genotypes with the minor G-allele: AG (12.6%) and GG (5.6%). Within rs913930 there identified three genotypes: TT (59.1%) and two genotypes with the minor C-allele: C/T (29.5%) and CC (11.2%). The rs11536865 studying revealed no polymorphism (GG genotype). The early allograft liver dysfunction (EAD) developed in 19.7% of patients, the severe EAD in 11.2% of patients, septic complications in 14%, acute cellular rejection in 23.9% of cases. The C/T genotype of the TLR-4 gene in the SNP rs913930 sequence was closely associated with the EAD development (OR 4.8 to 1; p = 0.047; 95% CI 1–23.4). Patients with the donor's liver C/T genotype had a reliably higher proportion (%) of the HMGB1 positive hepatocytes in the donor's bioptate, 21 (17–29%) vs the CC+TT genotypes, 16 (10–19%) (Mann–Whitney test, p = 0.01). The CD68 expression in the liver bioptate at the donor's stage was reliably higher in the carriers of heterozygotes in the SNP rs913930 (C/T genotype) and in the SNP rs5030717 (AG genotype), (Mann–Whitney test, p = 0.03). Significant positive correlation between the CD68 expression in the donor's liver bioptates and the IL-23 level in the hepatic vein has been determined in an hour after the portal reperfusion (ρ = 0.62; p = 0.04) as well as between the HMGB1 expression in the donor's liver bioptates and the AST level in 24 hours after the reperfusion (r = 0.4; p = 0.02). The HMGB1 staining in the donor's liver bioptates was higher in the EAD patients, 21 (20; 29) cells/mm^2 in comparison with the patients without EAD, 16 (12; 18) (Mann–Whitney test, p = 0.0036). **Conclusion.** The early allograft liver dysfunction is associated with the genetic predisposition caused by the TLR-4 gene polymorphism and is implemented via the HMGB1, Kupffer cells and IL-23 activation.

Key words: liver transplantation, allograft dysfunction, gene polymorphism, TLR-4, Kupffer cells.

INTRODUCTION

Using both standard criteria and extended criteria donors is associated with the risk for the EAD, caused by the ischemia reperfusion injury (IRI) [1]. Nowadays, there is no reliable objective technique available to assess the donor's liver graft allowing estimating the risk of the graft dysfunction occurrence, to evaluate the development of other complications and possible lethal outcome after liver transplantation.

A number of phenotypic and technical factors of the graft dysfunction attributed to the donor (age, serum sodium level, degree of liver steatosis, arterial hypotension, longtime of cold and warm ischemia, donor on ALV longer than 7 days, DCD donor, split or reduced graft) and those attributed to the recipient (MELD score and urgent transplantation) are known [1, 2]. However, the clinical observation shows that EAD severity and outcome are widely variable under equal phenotypic

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and technical risk factors. It might be attributed to the fact that IRI is determined genetically to some extent, and a definite genetic profile characteristic for both cold preservation and reperfusion injury corresponds to it [3].

IRI caused liver damage is a typical example of DAMP effect (damage-associated molecular patterns, DAMPs). Currently known pattern of the DAMP-associated damage includes the HMGB1 nuclear protein interaction (as a result of the hepatocyte necrosis) with the TLR-2, TLR-4, TLR-9 receptors and the Kupffer cells activation leading to IL-23 and other cytokines secretion by the activated macrophages and endothelial cells, γδ-T cells initiation for generating IL-17 and neutrophil chemotaxis in the liver [4].

According to the current understanding, the TLR-4 receptor plays a key role in the systemic inflammatory response and ischemia-reperfusion injury development and acceleration [5]. Experimental studies showed that blocking of TLR-4 receptors leads to reduction of early inflammatory response and IRI in liver transplanted mice [6]. In addition, TLR-4 gives rise to the endothelial cells hyperactivation under IRI even when Kupffer cells are absent and serves as mediator in the steatotic liver graft dysfunction [7, 8].

The results of certain clinical study showed that the minor alleles of rs11536865, rs913930 and rs5030717 sequences of TLR-4 gene were associated with the liver graft failure in the North America population [9]. Based on these data we have made a hypothesis that in addition to the phenotypic and technical aspects (age, serum sodium level, liver steatosis degree, arterial hypotension, etc.), EAD is caused by genetic predisposition associated with the TLR-4 gene polymorphism brought into effect due to the specific pathological processes typical for transplantation, i. e. the deceased donor's systemic inflammatory response and the recipient's ischemia reperfusion injury.

Therefore, evaluation of the genotypes association of the clinically valid rs11536865, rs913930 and rs5030717 nucleotides sequences of the TLR-4 gene with the risk of development and evidence of the early allograft liver dysfunction became the aim of the study.

MATERIALS AND METHODS

In order to determine the clinical associations with the TLR-4 gene polymorphism, we carried out a case-control study for determining the EAD association with various genotypes of the TLR gene in the rs11536865, rs913930 and rs5030717 sequences. The study included 71 patients and was performed from November 2013 till April 2015. Inclusion criteria: deceased donor liver transplantation corresponding to the standard criteria (AST and ALT levels below 200 IU/l, estimated liver steatosis below 40%, serum Na level < 165 μmol/l, age < 60 years old, vasopressor administration was allowed). Exclusion criteria: living donor liver transplantation, reduced graft transplantation, recipient's age < 18 years old. The patients' characteristics are summarized in Table 1.

Table 1

Summary of patients' characteristics

#	Index	Median (25%; 75%)
1	Patient's age, years	50 (40; 56)
2	MELD	17 (13; 24)
3	Donor's age, years	40 (29; 49)
4	Total ischemia time, min	480 (400; 530)
5	Warm ischemia time, min	45 (40; 55)
6	Liver steatosis, %	10 (3; 20)

The blood samples were taken from the left hepatic vein by puncturing in an hour after the portal reperfusion in order to determine the IL-6, 8, 17, 23, TNF-a, MIP-1a and P-selectin levels. The liver biopsy was performed in 2 hours after the portal reperfusion for the immunohistochemical staining for CD68 and HMGB1. Special oligonucleotide primers were selected for amplification of the TLR-4 gene three locus containing the

Table 2

Sequence of oligonucleotides for the gene TLR-4 polymorphisms determination

SNPs*	OBSERVED*	MAF*	Location**	Primers
rs11536865	C/G	C = 0.0427	5' (–728)	F:CCTCAAAGCCATGAGTCACC R:TCTTTCAAGGCTCTCTCTCCA Size: 247 bp***
rs5030717	A/G	G = 0.0958	intron 2 (–833)	F:TGGTTGGTAAACCTCTGCCTA R:AGGAGGTGAAGTGAACAGCAA Size: 174 bp
rs913930	C/T	C = 0.1769	3' UTR (–7083)	F:TGTGGGTGGTTATTCTCCATT R:CAAATGCTTGGCTTAAGAATCA Size: 227 bp

* – http://www.ncbi.nlm.nih.gov/snp.

** – Donor Polymorphisms of Toll-Like Receptor 4 Associated with Graft Failure in Liver Transplant Recipients / William S. Oetting et al. // Liver transplantation. 18: 1399–1405, 2012.

*** – bp (base pair).

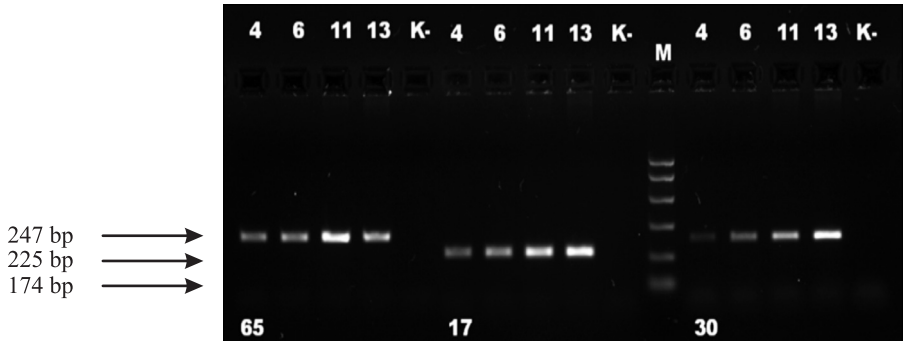

Fig. 1. PCR-specific products of three TLR-4 gene loci containing SNP: rs11536865 (65) – 247 bp; rs5030717 (17) – 174 bp; rs913930 (30) – 227 bp; K- – the negative reference sample; M – marker

polymorphisms (SNP), rs11536865 (G/C), rs5030717 (A/G), rs913930 (T/C) (Table 2) [9, 10].

In order to get PCR-specific products of three studied TLR-4 gene loci (Fig. 1) PCRs were performed in 20 μL of the reactive mixture containing 1 × PCR buffer, 0.2 mM dNTPs, 0.2 μL of Phusion Green Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Finland), 0.25 μM of each – of the direct and of the reverse primer (Primetech, Belarus), and 5 μL of the genome DNA.

In order to perform PCR the genome DNA was isolated from the donors' blood samples using the kit for DNA isolation Nucleosorb (Primetech, Belarus). BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) was used for carrying out the sequencing PCR. Sequencing was performed at 3500 Genetic Analyzer (Life Technologies, USA). For analysis of sequencing results Sequencing Analysis software (Life Technologies, USA) was used.

Early allograft liver dysfunction was determined by AST, ALT, bilirubin, and INR levels and in accordance with the criteria proposed by Olthoff K.M. et al. during the first 7 days after the operation [11]. Severe EAD was determined by Salvalaggio P.R. et al. criteria [12].

The numerical values distribution was recognized abnormal. Therefore, the average values were presented as median with 25% and 75% quartiles. The findings were analyzed statistically using STATISTICA 8 software for Windows. Literature sources were searched for in the electronic databases Medscape and Pubmed (NLM) using Endnote®Web software for Macintosh.

RESULTS

Three genotypes within the rs5030717 borders were identified: AA (homozygote) and two genotypes with minor G alleles – AG (heterozygote) and GG (homozygote, Fig. 2).

Three genotypes within the rs913930 borders were identified: TT (homozygote) and two genotypes with minor C alleles – C/T (heterozygote) and CC (homozygote, Fig. 3).

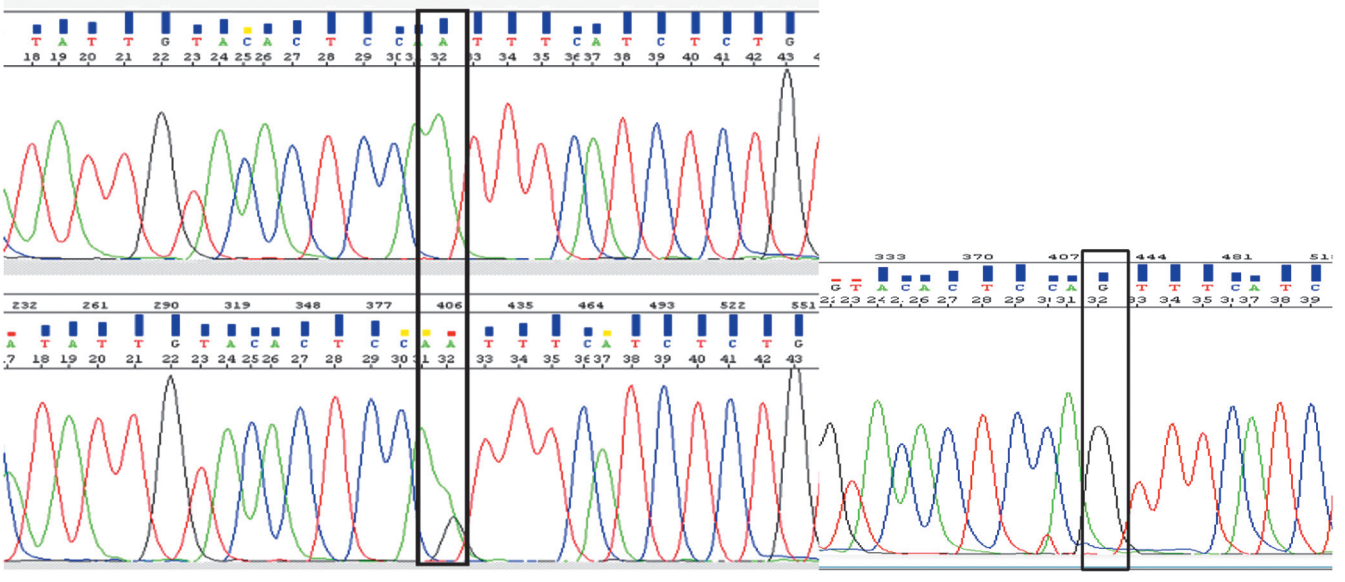

Fig. 2. SNP rs5030717, localized in intron 2 (–833) of TLR-4, A>G. AA and AG genotypes (on the left downwards) and GG genotype (on the right) were identified

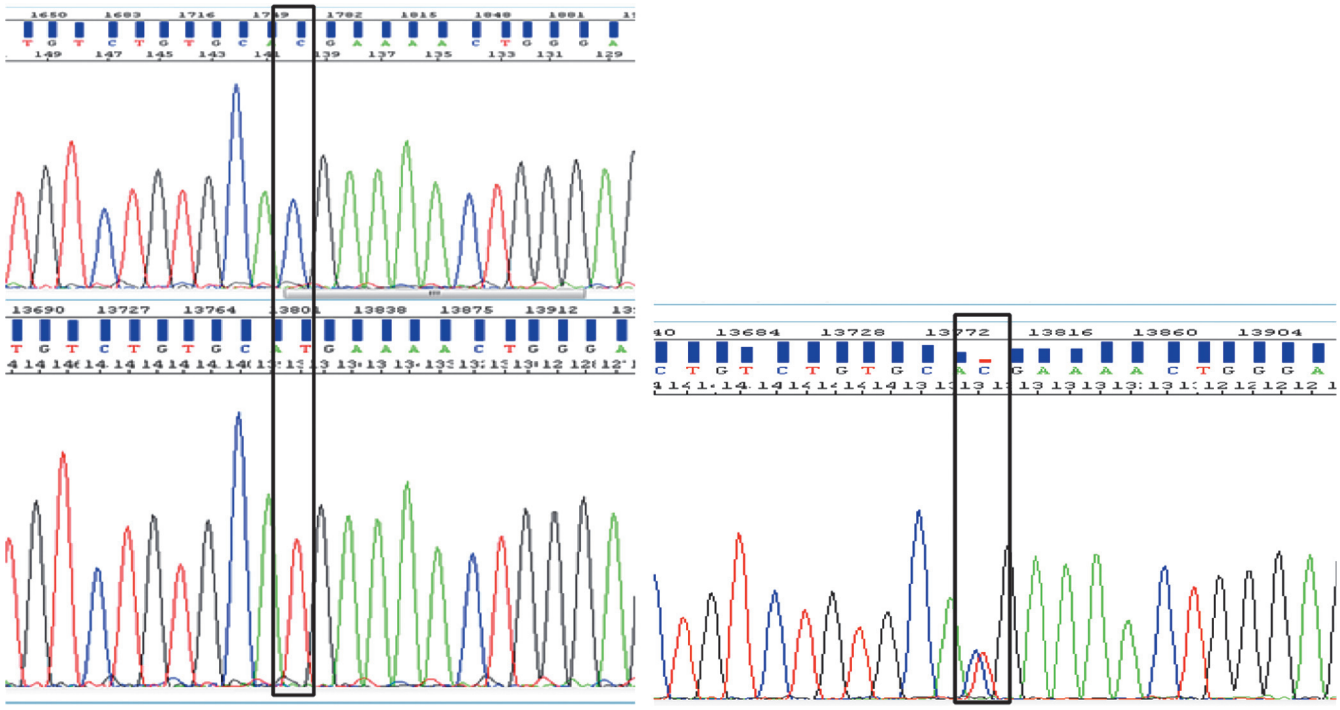

Fig. 3. SNP rs913930, localized in –7083 after 3' UTR of TLR-4 gene, T>C. TT genotype and CC genotype (on the left) and C/T genotype (on the right) were identified

Table 3

Clinical associations of TLR-4 gene polymorphisms (C/T vs. CC+TT) in rs913930 sequence

№		C/T genotype, n = 21	CC+TT genotypes, n = 50	p
1	EAD, n (%)	6 (28.5)	8 (16)	0.3
2	Severe EAD*, n (%)	**5 (23.8)**	**3 (6)**	0.04
3	Septic complications after transplantation, n (%)	3 (14.2)	7 (14)	0.7

EAD – early allograft dysfunction; * – Salvalaggio's criteria.

No polymorphism was found while studying rs11536865 (each sample had the GG genotype). Clinically valid polymorphism variant – C allele (minor allele) was not identified in the studied samples.

The genotypes frequency in SNP rs913930 was 59.1% (42/71) for TT, 29.5% (21/71) – for TC and 11.2% (8/71) – for CC. 81.6% of donors (58/71) had AA genotype in SNP rs5030717, 12.6% of them (9/71) were AG heterozygotes with the A minor allele, and 5.6% (4/71) were homozygotes with the GG minor allele.

The early liver graft dysfunction occurred in 14 of 71 patients (19.7%), severe EAD – in 8 patients (11.2%), septic complications developed in 10 recipients (14%), acute cell rejections were observed in 17 recipients (23.9%). While analyzing the C/T genotype influence on the risk of EAD development through a comparison with that of the CC+TT genotypes it was found that the frequency of moderate and severe EAD was significantly and considerably higher (23.8%) in transplanted patients with donor's liver C/T genotype of rs913930 than in case of CC+TT homozygous genotypes (6%; Fisher exact test, p = 0.04). The EAD frequency determined by Olthof's criteria and the number of septic complications did not differ reliably in the TC and CC+TT genotypes groups (Table 3).

The regression analysis showed that C/T genotype in the SNP rs913930 sequence of TLR-4 gene is strongly associated with early allograft dysfunction development (Odds ratio 4.8 to 1; p = 0.047; 95% CI 1–23.4).

The analysis of polymorphism associations of the TLR-4 gene in the SNP rs913930 sequence with immunohistochemical (CD68, HMGB1) and serological (VEGF, AST, ALT, procalcitonin) markers showed that transplanted patients with donor's liver of C/T genotype had a reliably higher proportion of HMGB1 (%) positive hepatocytes in the donor's bioptate, 21% (17–29%) than in case of the CC+TT genotypes, 16% (10–19%) (Mann–Whitney test, p = 0.01; Fig. 4).

When analyzing the influence of the CC+C/T genotypes on the risk of EAD development by comparing with that of the TT genotype it was found that the frequency of moderate and severe EAD was significantly and considerably higher (20.6%) in transplanted pa-

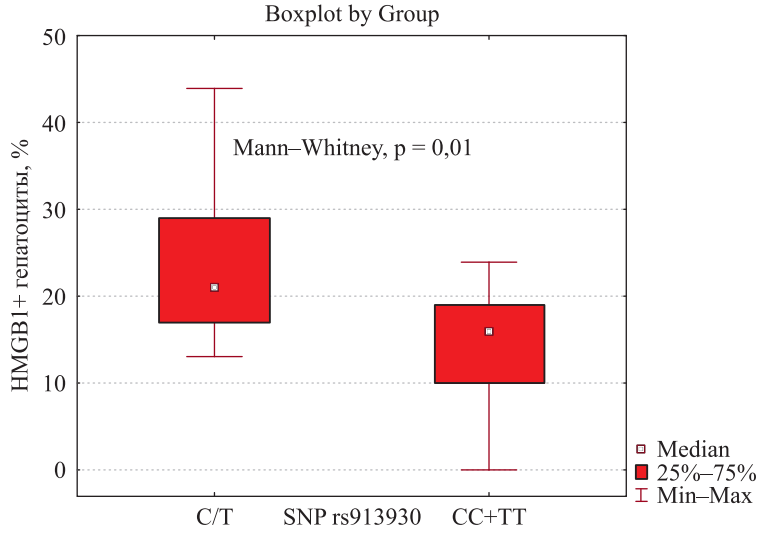

Fig. 4. Difference of HMGB1+ hepatocytes in the donor's liver bioptates with C/T and CC+TT genotypes

Table 4

Clinical associations of TLR-4 gene polymorphisms (CC+C/T vs. TT) in rs913930 sequence

№		CC+C/T genotypes, n = 29	TT genotype, n = 42	p
1	EAD, n (%)	7 (24.1)	7 (16.6)	0.6
2	Severe EAD*, n (%)	**6 (20.6)**	**2 (4.7)**	0.04
3	Septic complications after transplantation, n (%)	5 (17.2)	5 (11.9)	0.7

EAD – early allograft liver dysfunction; * – Salvalaggio's criteria.

tients with donor's liver the CC+C/T genotype in the rs913930 sequence than in case of the TT homozygote genotypes (4.7%; Fisher exact test, p = 0.04). The EAD frequency determined by Olthof's criteria and the number of septic complications did not differ reliably in the CC+C/T and the TT genotypes groups (Table 4).

The regression analysis showed that the TLR-4 gene allele C in the SNP rs913930 sequence (CC+C/T genotypes) is associated, though the confidence value is marginally significant, with the EAD development (odds ratio 5.2 to 1; p = 0.05; 95% CI 0.9–28).

The analysis of the TLR-4 gene polymorphism associations in the SNP rs913930 sequence with the immunohistochemical (CD68, HMGB1) and serological (VEGF, AST, ALT, procalcitonin) markers showed that the patients transplanted with donor's liver with CC+C/T genotypes had a reliably higher proportion of HMGB1 (%) positive hepatocytes in the donor's bioptate, 19.5% (17–29%) than in case of the TT genotype, 16% (10–19%) and a tendency to a higher proportion of HMGB1 (%) positive hepatocytes in the post-operative bioptates (Mann–Whitney test, p = 0.06; Fig. 5).

When analyzing the CD68 cells expression (cell/mm^2) in the liver bioptates at the sampling stage and after the reperfusion it is was found that the numbers of CD68 positive cells in the bioptates of the donor-carriers of heterozygotes for SNP rs913930 (C/T genotype) and of the donors-carriers of heterozygotes for SNP rs5030717 (AG genotype) (at the sampling stage) differed reliably (Mann–Whitney test, p = 0.03; Fig. 6).

The analysis of the TLR-4 gene polymorphism associations in the SNP rs5030717 sequence influence on the risk of EAD development showed that EAD, severe EAD occurrence, septic complications and the cell rejection frequencies (patients ratio, %) did not differ reliably in the recipients with the A/G+GG genotypes and with the AA and A/G genotypes grafts (p = 0.7; 0.8; 0.6; 0.9, respectively) comparing with the AA+GG genotypes (p = 0.8; 0.3; 0.3; 0.9, respectively).

The analysis of the CD68 and HMGB1 expressions demonstrated a reliable correlation between the CD68 expression in the donor's liver bioptates and the IL-23 level in the graft's hepatic vein in an hour after the reperfusion (ρ = 0.62; p = 0.04) and between the HMGB1 expression in the donor's liver bioptates and the AST level in 24 hours after the reperfusion (r = 0.4; p = 0.02) (Fig. 7).

A further analysis of the association of the IHC test markers of the hepatocytes and Kupffer's cells activation showed that the HMGB1 expression in the donor's liver bioptates differed reliably (Mann–Whitney test, p = 0.0036) in the groups with and without development of EAD after the liver transplantation being higher in the EAD patients, 21 (20; 29) cells/mm^2 than in patients without EAD, 16 (12; 18) cells/mm^2.

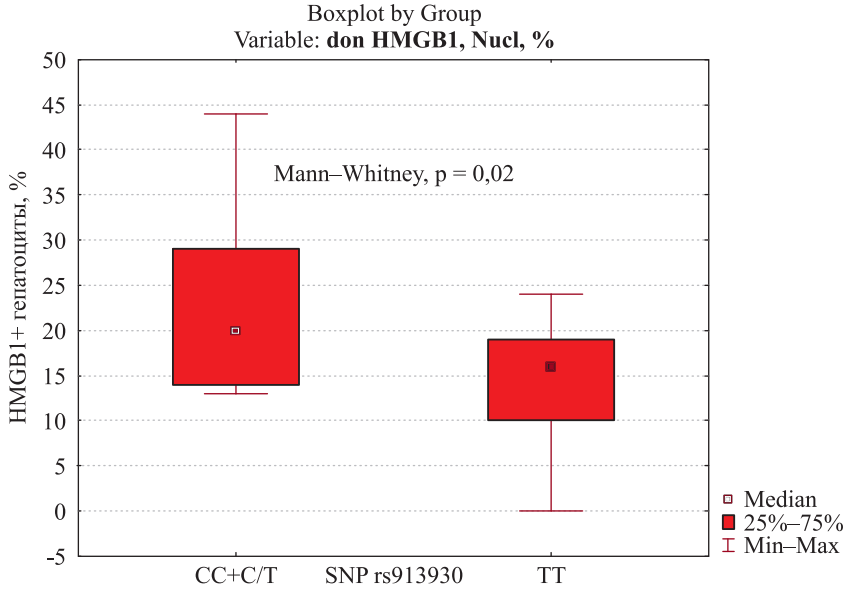

Fig. 5. Dissimilarity of HMGB1+ hepatocytes in the donor's liver bioptates with CC+C/T and TT genotypes

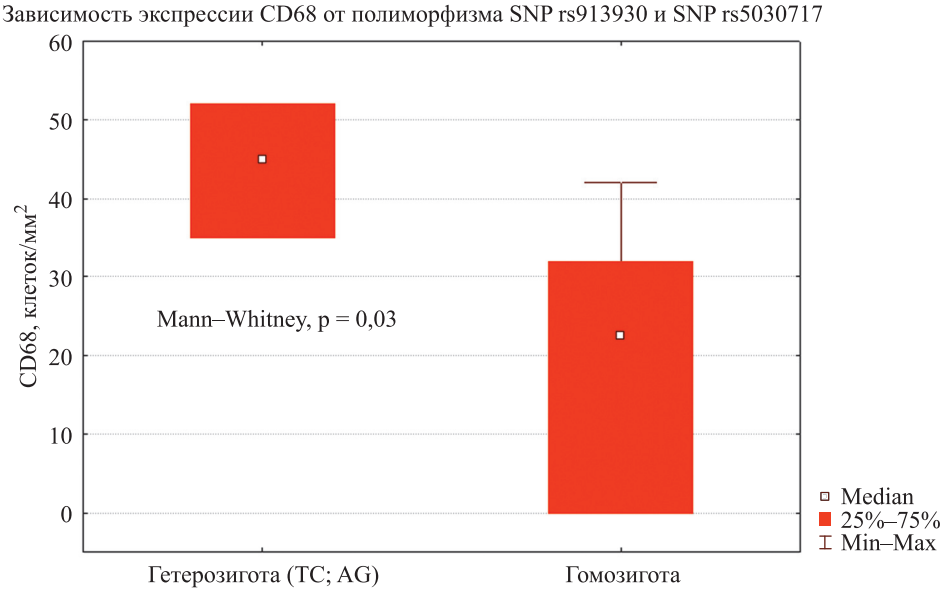

Fig. 6. CD68 expression in the livers of the donors-carriers of TC and AG heterozygotes in comparison with those with homozygotes

The microphotographs of the donor's liver bioptates with different HMGB1 expression, transplantations of which were associated either with a non-complicated course or with EAD development are shown on Fig. 8 and Fig. 9.

DISCUSSION AND CONCLUSIONS

The study demonstrated clinical significance of the TLR-4 gene polymorphisms for the EAD development in patients from Eastern Europe. From three sequences studied, the rs913930 nucleotide sequence is associated with the EAD.

Based on the data obtained, it can be stated that the genetic factors are brought into effect only in case of severe EAD development when the AST and the ALT levels exceed 3000 IU during the first 24 hours after the reperfusion. The TLR-4 gene both genotypes in the rs913930 sequence containing the minor allele C are associated with the risk of severe EAD development.

We have established that the genetic determinancy of EAD risk development includes the HMGB1 nuclear protein activation, Kupffer's cells (CD68) expression in the donor's liver and the IL-23 intrahepatic secretion within 1 hour after the portal reperfusion. The HMGB1 activation, CD68 expression in the donor's liver and the IL-23 precede the clinical picture of severe EAD in particular.

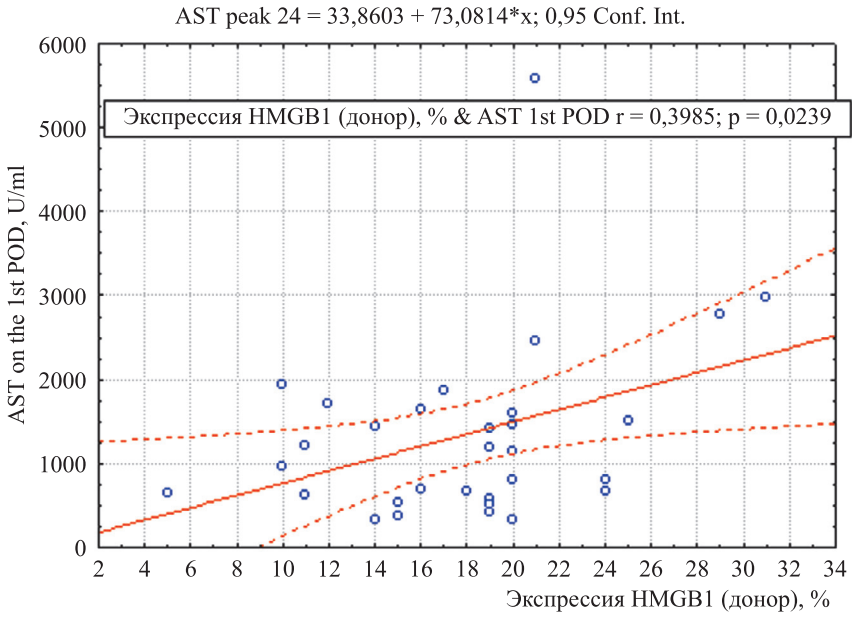

Fig. 7. The AST level correlation with the HMGB-1 expression in the donor's liver during the first post-operative 24 hours

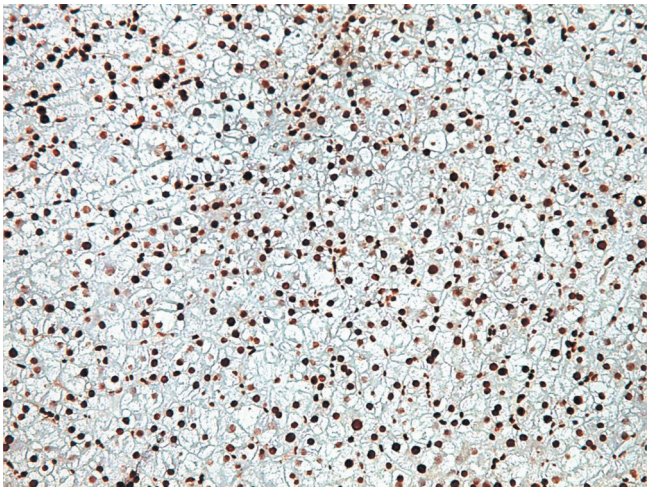

Fig. 8. HMGB1 expression in the donor's liver bioptates, the transplantation of which wasn't associated with EAD development

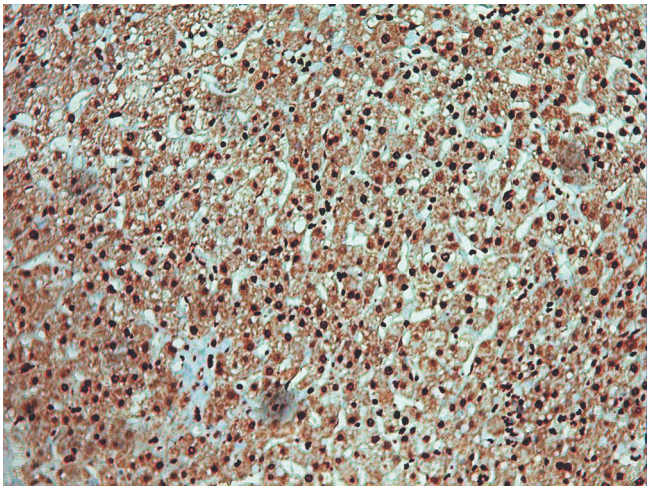

Fig. 9. HMGB1 expression in the donor's liver bioptates, the transplantation of which was associated with EAD development

The data obtained confirm the scientific hypothesis that EAD can be caused by the liver injury resulted from the systemic inflammatory response to the brain death and the bacterial translocation in the deceased donor, and EAD can be caused by the genetic factors brought into effect as a result of the specific pathological processes accompanying transplantation in addition to phenotypic and technical factors.

The main limitation of the study is a number of observations. However, the choice of the suitable study design and compliance with the relevant requirements (Fisher exact test followed by the regression analysis) allow us making the clinically substantiated conclusions that the TLR-4 gene genotype in the rs913930 SNP is associated with the EAD and is implemented via the HMGB1 nuclear protein, Kupffer's cells, and IL-23 activation.

The findings of the study may be taken as a basis for objective assessing of DCD liver graft, identification of the graft dysfunction risks, development of other complications and death after the liver transplantation and, therefore, may contribute to the reduction of the severity and probability of complications through early start of specific therapy.

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