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Associative Prognostic Parallels between the Polymorphism rs231775 and rs5742909 of Ctla-4 Gene and HBV-Liver Cirrhosis Progression in Children

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Abstract The objective. To establish the prognostic significance of ctla-4-gene polymorphisms of rs231775 (+49A>G) and rs5742909 (+318C>T) in the progression of HBV-cirrhosis of the liver (HBV-LC) in children. Methods. We examined 88 sick children with HBV-CP (according to Child-Pugh: class A – 29.5%, class B - 27.8%, class C – 42.7%), aged 6-18 years, among whom there were 75% boys and 25% girls. The duration of HBV-CP was 7.8±0.2 years. The diagnosis was established on the basis of clinical biochemical and instrumental test such as ultrasound with Dopplerography and fibroelastometry using SMI and ASQ technology. Verification was done using HBV - methods of ELISA and PCR. Prognostic criteria of the MELD/PELD scale were used. Genotyping was performed by PCR-RFLP analysis of rs231775 (+49A>G) and rs5742909 (+318C>T) polymorphisms of the ctla-4 gene. The prevalence of alleles and genotypes among cases and controls was compared according to the Hardy-Weinberg equilibrium law using the $\chi 2$ criterion. The control group consisted of 90 practically healthy children. Results. Investigation of genetic polymorphisms of rs231775 (+49A>G) and rs5742909 (+318C>T) of the ctla-4 gene in patients with HBV-CP provided the possibility to establish associative-prognostic links only with the carrier of the positional variant +49A/G, which was manifested by high expression of the gene compared to healthy donor children and indicated a genetic predisposition to the formation of liver cirrhosis in cases of HBV infection. At the same time, the carriage of the G-allele in the homozygous mutant GG position was associated with a high probability of rapid decompensation of the pathological process in liver and development of frequent complications. While the A-allele is in the AA-homozygote position was associated with a compensated progression of the disease. Determined gender differences including the high expression of the G-allele of the ctla-4 gene characteristic for boys, particularly in the homozygous mutant variation of GG, partially substantiates the phenomenon of high susceptibility and prevalence of HBV infection among boys. Conclusion. In contrast to the polymorphism of +318C>T, the existing difference in the prevalence of polymorphism +49A/G alleles of the ctla-4 gene proves the susceptibility to the formation of characteristic progression of HBV-LC in children and justifies the fact that this gene serves to be a partially active mechanism in the development and progression of the pathological process in case of HBV persistence. The GG genotype carriage in the positional variant +49A/G of the ctla-4 gene can be considered to be one of the HOST factors for the dominant susceptibility of boys to the formation of HBV-LC and, in general, for the prediction of the unfavorable progression of the disease in children.

Keywords Liver cirrhosis, HBV infection, Ctla-4 gene, Polymorphisms +49A>G and +318C>T, Clinic, Prognosis, Children

1. Introduction

The problem of liver cirrhosis (LC) is caused not only by the complexity of pathogenesis, the severity of complications, insufficient effectiveness and high cost of antiviral treatment, but also by the infection of the most susceptible to chronic HBV infection groups of the population, particularly children (up to 64.7–95% of cases), the development of early disability and deaths (2.7-5.4% of all cases of childhood mortality) [1,2,3, et al.]. LC can be the final stage of the development of both progressive and latent forms of chronic HBV infection, where the development of hepatocellular carcinoma can reach up to 70-90% of cases, which significantly reduces the survival rate of sick children [4-9]. The course of the disease is mainly associated not only

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with viral factors, but also with the encoded genetic fund of the macro-organism. In particular, the processes of fibrogenesis are regulated by specific genes, among which we consider ctla-4 gene, which triggers such pathological processes as apoptosis, proliferation and differentiation mediated through a cascade of immunopathological reactions [10-13]. At its core, ctla-4 is an inhibitory receptor that restricts T-cell activation. Its gene modifications (-318C/T, 49G/A, 7-30A/T, and others) are associated with various degrees of ctla-4-mediated effect on T-cell activation and response to interferon therapy, as well as with an increased risk of chronic infection [14,15]. In this regard, the role of ctla-4 genetic determinants is being actively studied, which makes it possible to diagnose and predict the course of the disease, the risk of complications and unfavorable outcome with greater accuracy [16,17]. These facts emphasize the relevance of studying many aspects of the problem in order to solve such important issues as increasing the duration and improving the quality of life of the children with HBV-liver cirrhosis.

2. The Objective

To establish the prognostic significance of polymorphisms rs231775 (+49A>G) and rs5742909 (+318C>T) of ctla-4 gene in the progression of HBV- liver cirrhosis (HBV-LC) in children.

3. Materials and Methods

We examined 88 sick children with HBV-LC, aged 6-18 years, among whom there were 75% boys and 25% girls. According to Child-Pugh classification there were Class A 29.5%, class B 37.5%, and class C 33% of children. Prognostic criteria of the PELD (<12 years) and MELD (>12 years), scale were used [18]. The duration of the disease was 7.8 ± 0.2 years. The diagnosis was established on the basis of clinical biochemical and instrumental tests (ultrasound on the PHILIPS HD3 scanner with Dopplerography of the vessels of the liver, spleen and portal system; shift-wave elastometry (SWE, ASQ); MRI, EGDS). We also performed microbiological tests of biological fluids (blood, ascites liquid, urine, sputum, feces). Verification of HBV, HCV and HDV was carried out by ELISA methods using HUMAN kits (Germany) on a MULTISCANFC device and Real Time PCR (HBV-DNA) with hybridization-fluorescence detection on a BIO-RADiQ5 amplifier (USA) using Ampli Cens RHBV-FL kits (Russia). Genotyping was performed by PCR-RFLP analysis of rs231775 (+49A>G) and rs5742909 (+318C>T) polymorphisms of ctla-4 gene. Restriction primers for the determination of polymorphism A49G and C318T of ctla-4 genes were selected using the online programs NCBIPrimersTool and Nebcutter. PCR amplification was performed using the PCRCore kit (Isogen, Russia). Weights of restricted products were as follows: AA – healthy genotype – 162 bp/21bp; AG – heterozygous genotype – 162 bp/91bp/71bp; GG – mutant genotype – 91bp/71bp; CC – healthy genotype – 226bp/21bp; CT –heterozygous genotype – 226bp/130bp/96bp/21bp and TT – mutant the genotype is 130 bp/96 bp/21bp. The prevalence of alleles and genotypes among cases and controls were compared according to the Hardy-Weinberg equilibrium law using the $\chi 2$ criterion. The control group consisted of 90 practically healthy children. The results of clinical and laboratory tests were processed by the method of variation statistics.

4. Results and Discussion

Genotyping of rs5742909 polymorphism (+318C>T) of ctla-4 gene in patients with HBV-LC and practically healthy children showed the absence of associative relationships, i.e. all examined children were carriers of a healthy homozygous genotype +318CC (Table 1). In contrast, the study of rs231775 polymorphism (+49A>G) revealed the presence of associations with ctla-4 gene in sick children with the prevalence of the following genotypes: homozygous AA in 14.8% of cases, heterozygous AG - 31.8% and mutant GG genotype in more than half of the cases – 53.4%. At the same time, statistical significance to healthy donors was obtained in homozygous AA- and GG-carrier variants. In general, healthy donor children had characteristic homozygous AA genotype (66.6%).

Intragroup analysis of the prevalence of allele polymorphism +49A>G of ctla-4 gene in the studied groups revealed an associative link of the G-allele with the development of HBV-LC (Table 2), where its frequency significantly exceeded that of healthy children and the associative link of the A-allele carriage among healthy children ($p<0.001$).

Table 1. Prevalence of ctla-4 genotypes among children with HBV- liver cirrhosis and healthy donors, %

Subject	Polymorphisms of ctla-4 gene					
	A49G (rs231775)			C318T (rs5742909)		
	AA	AG	GG	CC	CT	TT
Children with HBV-liver cirrhosis, n=88	14.8±3.8 [a] *	31.8±4.9 [c]	53.4±5.3 [b] *	100	-	-
Healthy donor children, n=90	66.6±4.9 [a]	30.0±4.8 [c]	3.3±1.8 [b]	100	-	-

Note: * - reliability of differences to the group of healthy donor children;
[a] – between genotypes AA/AG, [b] – AA/GG, [c] – AG/GG ($p<0.05$-0.01).

Table 2. Positional +49A/G prevalence of ctla-4 gene alleles among the examined children

Allele	Children with HBV-liver cirrhosis p=176		Healthy donor children p=182		P
	abs	%	abs	%	
A-allele	54	29.6±3.3	147	81.6±2.8	<0.001
G-allele	128	70.3±3.3*	33	18.3±2.8 *	<0.001

Reliability of differences: P – between the examined groups;
* - between A and G alleles (p<0.001); p – total number of alleles.

Based on the immunogenetic mechanisms of regulation of ctla-4 gene of the same membrane receptor, functioning as one of the checkpoints of the immune response at the level of inhibition of already activated regulatory T-lymphocytes (Treg), responsible for the release of cytokines and coordinated lysis of infected cells [12,14,19], we can make assumption about the consequences of improper induction and function of Treg on the side of ctla-4 regulation, eventually determining the progression of the disease. Relatively high G-expression in conditions of chronic HBV persistence in the examined sick children, on the one hand, indicates excessive involvement of activated T cells in the pathological process. On the other hand, it shows a genetic predisposition to the development of the disease. An explanation for this assumption can also be seen in scientific studies confirming that in cases of mutation G-allele was associated with a decrease in the control of T-cell activation due to a change in the avidity of ctla-4 molecule binding to the co-receptor of antigen-presenting cells B7.2 (CD86), thus contributing to the development of an intense inflammatory reaction of the body, autoimmune processes, intensification of fibrotic and apoptic phenomena in tissues [17,20,21].

This assumption was confirmed in the analysis of the clinical progression of HBV-LC in children dependently on the polymorphism +49A/G of ctla-4 gene (Fig. 1), where in cases of carrying a mutant heterozygous GG genotype, the majority (61.7%) of patients were decompensated (Class "C" according to Child-Pugh) with predominance of syndromes such as cholestatic (93%), hemorrhagic (91.4%) and prolonged sub-febrile fever (48.2%, p<0.05-0.001 compared to other groups). When carrying the heterozygous AG genotype, more than half of the sick children (53.6%) had compensated progression of HBV-LC (Class B), which was also associated with higher basic inflammatory activity with the presence of G-allele, compared to sub-compensated patients (Class "A"), who with the homozygous AA position all had a stable progression of the disease.

At the same time, it was noticeable that the prevalence of complications of HBV-LC, predetermining the prognosis of the disease, in the form of the development of hepatic encephalopathy (PE), bleeding from varicose veins of the esophagus (VEV) with the development of hepatic coma, hepatorenal syndrome (HRS) and the addition of bacterial infection (BI) also depended on the carriage of ctla -4 genotypes (Table 3). Thus, hepatic cell insufficiency with the development of PE occurred in the majority of patients G-allele carriers (GG-78.7%; AG-60.7%, p<0.001 to patients with the AA genotype). Bleeding from VEV also developed most often among the patients carrying G-allele, particularly in cases of homozygous GG-carrier (91.4%, p<0.001). These were mainly patients with decompensated HBV-CP. At the same time, the endoscopic stage of esophageal varicose nodes correlated with the sonographically determined stage of portal hypertension, and the probability of bleeding was high in cases with portal hypertension III stage and the III-IV stages of VEV. In addition to the size of the VEV, the increased (2.9 times) risk of bleeding depended on the presence of red color sings on varicose veins and the severity of the disease. After bleeding, the Child-Pugh score significantly increased (p<0.05). Relapses of bleeding during the year were observed in 46.8% (22 children) of the patients with ctla-4 GG genotype. Among them, prognostic indicators of the MELD|PELD scale were in the zone of high probability of mortality (29.9±0.49 points, p<0.02-0.001 to patients with AA and AG genotypes).

Among the other complications, HRS in the general sample was registered in 24 (27.2%) sick children, and significantly more often in GG-genotype carrier (40.6%). The prevalence of BI (in the form of pneumonia, pleuritis, spontaneous peritonitis, myocarditis, urinary tract infection, etc.) concomitant to HBV-LC in children similarly depended on the carriage of ctla-4 genotypes, where G-allele was also widely represented (AG-85.7% and GG-95.7%). In the range of etiological factors of BI among the carriers of G-allele, in 44% of cases multi-resistant strains such as enterobacteria ESBL (28%) and staphylococcus MRSA (16%) were detected.

Table 3. Incidence of HBV-cirrhosis complications in children dependent on ctla-4 genotypes, %

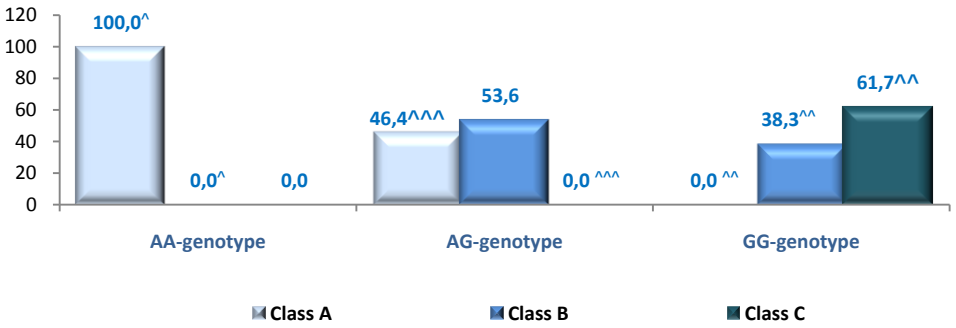
Indicator	Genotypes of polymorphism +49A>G ctla-4			P_{AA-AG}	P_{AA-GG}	P_{AG-GG}
	AA (n=13)	AG (n=28)	GG (n=47)			
Hepatic encephalopathy	30.7± 12.7	60.7±9.2	78.7±5.9	<0.001	<0.001	>0.05
Bleeding from varicose veins of esophagus	46.1±13.8	64.3±9.0	91.4±4.0	<0.05	<0.001	<0.05
Hepatorenal syndrome	7.7±7.3	14.3±6.6	40.6±7.1	<0.05	<0.001	<0.05
Bacterial infection	38.4±13.4	85.7±6.6	95.7±2.9	<0.05	<0.05	>0.05

P – reliability of differences between the examined groups.

Table 4. Prevalence of genotypes of polymorphism +49A>G of ctla-4 gene dependent on the gender of sick children, %

Sick children with HBV-LC					
Boys, n=66			Girls, n=22		
Genotypes of polymorphism +49A>G ctla-4					
AA	AG	GG	AA	AG	GG
4.5±2.5^	33.3±5.8^^^	62.1±5.9^^	45.4±10.6	40.9±6.6	13.6±7.3^^

Reliability of differences between genotypes: ^ - AA и AG; ^^ - AA и GG; ^^^ - AG и GG (p<0.05-0.001).



Reliability of differences between genotypes: ^ – AA/AG; ^^ – AA/GG; ^^^ - AG/GG (p<0.05-0.001).

Figure 1. The prevalence of polymorphism genotypes +49A>G of ctla-4 gene dependent on the severity of HBV-liver cirrhosis in children, %

According to the laws of epigenetic theory of gender differences of the risk of development of diseases predetermined by genetics, which may be based on various violations of the mechanisms of genomic imprinting, changes in nucleic acid molecules, and other faults leading to phenotypic mutations with the consequent development of the disease, the associative links of the carriage of genotypes and alleles of ctla-4 gene with the gender of sick children were analyzed. Comparative differentiation according to the gender (Table 4) revealed a significant prevalence of registration of the mutant GG genotype among the boys (62.5%), while among the girls AA (45.4%) and AG (40.9%) genotypes were detected with almost the same frequency. Allelic differences were characterized by relevance of boys to G-allele (72% vs. 27.9% of A-allele, p<0.001), particularly in the homozygous carrier variant, which may partially prove the phenomenon of high susceptibility and infection rate among the boys with HBV infection, since it is the G-allele that is the main regulator of T-cellular activation of immunity. In contrast, A-allele (60% versus 40% of G-allele, p<0.02) prevailed in girls, which in the homozygous variant represents a healthy genotype and is not dominant in the mechanisms of the receptor apparatus of regulatory T-lymphocytes.

5. Conclusions

Genetic polymorphisms rs231775 (+49A>G) and rs5742909 (+318C>T) of ctla-4 gene in patients with HBV-LC were associated only with the carriers of the positional variant +49A/G, which was manifested by high expression of the gene compared to healthy donor children and indicated a genetic predisposition to the development of liver cirrhosis in cases of HBV infection. At the same time, the carriage of the G-allele homozygous mutant GG position was associated with a high probability of rapid decompensation of the pathological process in liver and the development of frequent complications. While A-allele in the AA-homozygote position was associated with a compensated progression of the disease. The established gender differences such as high expression of G-allele of ctla-4 gene characteristic for boys, especially with homozygous mutant variation of GG partially substantiates the phenomenon of high susceptibility and infection rate among boys with HBV viral infection, since the main regulatory ability of T-cell immunity is associated with the G-allele.

The existing difference in the prevalence of polymorphism alleles +49A/G of ctla-4 gene and the susceptibility to the development of HBV liver cirrhosis in children justifies the fact that this gene serves to be a partially active mechanism in the development and progression of the pathological process in cases of HBV persistence. Consequently, the 49-position carriage of the mutant GG variation of ctla-4 gene can be considered to be one of the HOST factors in the prediction of unfavorable progression of HBV cirrhosis in children, which allows timely definition of risk groups at the stage of primary diagnosis and its preventive measures, respectively.

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