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Research on Genotyping of Hepatitis B Virus and its Response Influence of Genovariation on Cellular Immunology

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The purpose of this paper is understanding the feature of HBV genovariation from HB patients in Qingdao and discussing the influence of HBV YMDD variation and genotyping on cellular immunity level. The research method is to detect peripheral blood T lymphocyte subsets by flow cytometry (FCM), using enzyme-linked immunosorbent assay (ELISA) to detect serum cytokine interferon (IFN-γ), tumor necrosis factor (TNF-α) and interleukin 2 (IL - 2)] level, applying real-time fluorescent polymerase chain reaction (PCR) technique in serum detection of gene mutation, HBV DNA quantitative monitoring and detection of genotyping of 820 cases of hepatitis b patients and making a direct sequencing of PCR product .The result is that 154 cases of YMDD variation in 820 patients with HBV infection was detected and the variation rate is18.78%; the YMDD variation difference between different genotyping was statistically significant, P"ÿ0ÿ05 and HBeAg positive people are more likely to variate; the difference of YMDD variation between different genotyping of HBV infected person clinical genotyping was statistically significant(P"ÿ0ÿ05), among which LC(36/113)0CHB(97/368) is primary; difference of gender,age and DNA capacity from different group was not statistically significant(Pÿ0ÿ05), then select 91 cases of chronic hepatitis b patients, 19 cases was Leu60Val mutation and variation rate was 20.9% , fulminant hepatitis grouphighest was the highest with the mutation rates increase gradually; leval of Val60 IFN -γ and TNF -α level mutant group was obviously increasing(t is 2.584; 4.766,P<0.01), ratio of CD4+/CD81 went up(t=2.275, P<0.05). Conclusion is that: Va16O variants may increase affinity with human I leucocyte antigen (HLA - I) affinity or raise the expression of HLA- I molecules - or to activate a lot of cytotoxic T releasing lymphocyte cytokine and at the same time exerting immunological effect in part of liver which improve the lethality to the host. Monitoring of HBV YMDD mutation has important clinical significance to treatment, prognosis and vest of HB patient.

Key words: YMDD variation; genotyping: HBV; cell immune response.

Hepatitis B virus short for HBV is double strands of DNA of annular part and composed of 3200 base pairs that can be classified into long minus strand and short plus strand. L strand has four open code reading area, S,C,P, and X and its variation appear in any area. The outstanding feature of HBV in replication is that mispairing of one or more nucleotide will lead to variation because RNA polymerase and reverse transcriptase lack of correcting feature after reverse replication by RNA. HBV variation may be natural variation or happen in the induction of immune stress and antiviral drug therapy[1]. There are 120 million HBV infectors in China. Antiviral drug therapy is mainly adopted in clinic in order to prevent chronic hepatitis B developing to liver

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cirrhosis and cancer. However, time of antiviral drug therapy is positive related to YMDD variation rate. The variation of change can cause the biological characteristics of hepatitis b virus, for example, the drug resistance will lead to the recurrence of hepatitis. We studied correlation between YMDD variation, HBVDNA capacity, HBV genotype , HBeAg and clinical typing of 820 cases of serum hepatitis B from NB infectors in recent two years, selected 91 cases of chronic HB infectors, measured peripheral blood T cell subsets (CD4 +, CD81) and serum levels of cytokines (interferon γ, INF -α), tumor necrosis factor (TNF -α) and interleukin 2 (interleukin 2, IL - 2)], and discussing the relationship of HBV core Val60 variant and cellular immune level.

MATERIALS AND METHOD

Sample and source

Serum of 820 cases of HBV infectors are coming from outpatients and inpatients in Qingdao infectious diseases hospital from March,2012 to December,2012, including 642 cases of male from 14 to 77, 178 cases of female from 12 to 71. Among them, 126 cases are ASC, 105 cases are AH, 368 cases are CHB, 113 cases are LC, 91 cases are SH and 17 cases of PHC. Clinical diagnosis is conforming to the standard of Viral hepatitis prevention plan that is revised in the tenth national viral hepatitis and liver disease academic conference in 2000[2].

Detection reagent

HBV parting detection reagent was provided by Shanghai Zhijiang science and technology company. Ration and variation detection reagent of HBVDNA was provided by Kaijie biology engineering limited company in Shenzhen. Detection reagent of HBV mark was provided by Shanghai Kehua biology engineering limited liability company.

Experimental method

HBV Variation detection

Extract HBVDNA by routine using HBVYMDD mutant nucleic acid detection kit. Then add reaction liquid of mutation HBV polymerase chain reaction(PCR) to augment. The condition of augment is 37°C 3min, 92°C 1min, (93°C 2s, 42°C 20s, 72°C 20s) 40 circulation, 37°C 3min. After PCR augment, analyze the result according to positive control and the instrumentation is Roche LIGHTCYCLE.

HBV genotyping detection

We adopted genotyping detection knit according to instruction book. The condition of augment is 37°C 2min, 94°C 2min, (94°C 10s, 62°C 40s) 40 circulation. After PCR augment, analyze the result according to positive control.

HBVDNA ration detection

Adopt real time fluorogenetic quantitative PCR method according to instruction book. Knit sensibility is 103IU/mL, and instrumentation is Mx3000.

HBv-M detection

Adopt ELISA method according to the instruction book.

Serum cytokines detection

IFN-γ TNF- α and IL-2 knit was purchased from America Gibco company. Adopt double antibody sandwich enzyme-linked immunosorbent method to detect. Primary antibody is biochemistry antibodies and second antibody is streptavidin which is marked by horse radish peroxidase. Draw curve by standard substance and calculate concentration of the sample when absorbancy was 429 nm.

Detection of peripheral blood T cell subsets

Adopt flow cytometry and select disposable reagent (TriTESTTM CD4 FITC+ CD8PE + CD3PerCP) that is marked by trichromatic fluorescent to stain. Fluorescence labeling reagents, red blood cell cracking liquid and the same type contrast is all from American BD Company. Take 1 mL anticoagulated blood and adjust the amount of hemameba to(4.0~10)x109/L and add 20 µl of CD3/CD4/CD8 three color fluorescent antibody and 100µl of mixing anticoagulated blood. Mix it and keep it in dark place under indoor temperature for 30 min. Add 1ml red blood cell cracking liquid into test tube and mix. Put in the dark place under indoor temperature for 15 min and do computer test.

1~4 adopted SPSS17.0 software and apply chi-square to collect. $P<0.05$ meaned the difference is statistically significant. Comparison of rate in 5~6 was tested by . Comparison of mean was tested by t. Variance analysis was tested by F.

RESULTS

HBV variation detection result

Material

Among 820 HBV infectors, 154 cases were YDDM variation and variation rate is 18.78%. 95 cases are YIDD variation and account for 61.69%. 51 cases were YVDD variation and account for 33.12%. 8 cases were YIDD/YVDD variation and account for 5.19%.

Relationship between HBV genovariation and gender and age

Difference of male and female infectors was not statistically significant (P<0.05). There are four age groups. And the difference between age was not statistically significant as showed in Table 1.

Statistical method

27 cases were HBV genotype B. 729 cases were type C. 3 cases are mixed type BC. Most of these cases were type C.

Correlation between HBV variation and HBeAg

Difference of positive HBeAg of patient and YMDD variation is statistically significant (P<0.05), HBeAg(+) is more likely to variate compared with HBeAg(-).

Correlation between HBV variation and HBV DNA

Difference of HBV capacity and HBV variation is not statistically significant (P<0.05) as showed in Table 4.

Correlation between HBV genovariationand HBV clinical classification

Patients with chronic hepatitis were divided into aa60 variant plant group and aa60 wild plant group. Comparing the changes of cytokine levels and distribution of T cell subgroup, we found that IFN -γ, TNF-and IL - α 2 levels of aa60 variant group of were higher than that of wild group. The difference is outstanding comparing IFN-γ and TNF-α level of variant plant group with wild group respectively. CD4ASrelative abundance of variant

Table 1. Correlation between HBV genovariation and gender and age

Types of variation	Cases	Gender		Age			
		Male	Female	20	21~40	41~60	>60
YMDD(-)	666	527	139	10	255	351	50
YMDD(+)	154	115	39	1	47	93	13

Table 2. Correlation between HBV genovariation and HBV genotype

Variation types	Cases	Genotype			
		Type B	Type C	Mixed type BC	Unclassified type
YMDD(-)	666	24	579	2	61
YMDD(+)	154	3	150	1	

Table 3. Correlation between HBV variation and HBeAg

Variation types	Cases	HBeAg(+)	HBeAg(-)
YMDD(-)	666	313	352
YMDD(+)	154	127	27

Table 4. Correlation between HBV variation and HBV DNA

Variation types	Cases	HBV DNA capacity					
		103~104	104~105	105~106	106~107	107~108	≥ 108
YMDD(-)	666	93	135	148	137	99	40
YMDD(+)	154	17	27	33	31	27	9

group and ratio of CD4+/CD8ASis higher than wild plant group and CD8+ relative abundance is obviously lower than wild plant group, among which difference of CD4+/CD81 h ratio was outstanding (Table 6 and 7).

IFN-γ:interferon γ, TNF-α tumor necrosis factor α IL-2: interleukin-2; compared with wild group; $^{*}t'_{TNF-\alpha} = 4.766, P < 0.05$,.

**t_{IFN} -λ =2.584, P<0.01

**t_{IL} -2 =0.533, P<0.01

Table 5. Correlation between HBV variation and HBV clinical classification

Variation types	Cases	Clinical classification					
		ASC	AH	CHB	LC	SH	PHC
YMDD(-)	666	125	101	271	77	77	15
YMDD(+)	154	1	4	97	36	14	2

Table 6. Comparison of serum cytokines level of aa 60 variant plant group and wild plant group ($\bar{x} \pm s$ pg/ml)

Groups	Cases	IFN-γ	TNF- α	IL-2
Variant group	19	53.3±14.8	50.1±10.3	31.6±13.3
Wild group	72	41.1±19.0	35.6±16.1	29.5±15.2

Table 7. Comparison of distribution of peripheral blood T cell subsets in aaa60 variant plant group and wild plant group

Groups	Cases	CD4+	CD8+	CD4+/CD81
Variant group	19	47.1±11.1	29.8±10.0	1.8±0.5
Wild group	72	45.9±14.2	35.0±16.2	1.4±0.7

$t_{CD4^+} = 0.341,\ t'_{CD8^+} = 1.731, t_{CD4^+/CD8^+} = 2.275, P < 0.05$

DISCUSSION

HBV is a highly variating virus in the form of quasi kind existing in the body. Variant plant that with strong adaptively was augment to take the place of former wild plant that is superiority plant in specific environment. Wild plant can also recover to be superiority plant. Now antiviral therapy is adopted in order to prevent hepatitis being liver cirrhosis and liver. The longer time of using lamivudine, the higher rate of variation[3]. It lead to virus drug resistance changing into the main obstacle influencing therapeutic effect. The research showed that age and gender is not statistically significant with DNA capacity and YMDD variation (P < 0.05), which conform to the result of Xiangtian[4]. The research found that after DNA capacity of patients with chronic hepatitis B to achieve stable quantity of low copy, if genovariation appear then there is risk of clinical drug resistance, which leads to recurrence of hepatitis and deterioration of illness. Thus in the process of therapy, it is necessary to detect the YMDD variation in clinical therapy. According to the standard of heterogeneity of whole genome sequences of HBVed8%, HBV can be classified into A ^ÿ H genotype. The distribution of HBV genotype is featured by race and region. The main genotype in mainland is A,B,C and D· Among groups of asymptomatic carriers, CHB, liver cirrhosis and cancer, type C become more and type become less[6]. Some researches hold that genotype C and cirrhosis liver were independent risk factors of liver cancer[7]. This experiment showed HBV

genotype of hepatitis B infectors in Qingdao were mainly type B and type C and type C was more. Difference of YMDD variation and genotype was statistically significant (P>0.05), that is, genotype Care more likely to occur YMDD variation to induce drug resistance and increase the difficulty of clinical therapy in process of HB therapy. Therefore, detecting YMDD genotype and mutation at the same time can provide a basis for doctors predicting the development of the disease so that they can set out a personal therapeutic schedule. Clinic and virology research showed that front area of C/C and BCP variation was closely connected with the development of chronic liver disease. This research showed that YMDD variation and HBV infectors clinical classification difference is statistically significant, which was mainly distributed in Group LC and CHB. Rate of positive is 31. 86% and 26<36% while positive rate of other group is all lower than 15%. It illustrated that result of HBV variation will significantly enhance HBV replication, deteriorate inflammation of liver cells, necrosis and fibrosis and worse clinical outcomes[8]. HBV itself does not directly injury liver cells. immune response and immune pathological reaction that is induced by virus is the key to hepatitis b.C T L is the main executor of cellular immune response and the critical factor of removing HBV[9,10]. CTL can develop immunological effect by secreting cytokines including T N F-αand IFN-γ[11,12]that possess antiviral effect. IFN- have the function of activating CD4+0CD 8+ and NK cell have the function of killing liver cell, which is an important mechanism. And T N F- is closely related to the necrosis of liver cell. Thus, CTL after activation can induce the injury of liver cell and serious hepatic failure in antiviral effect.

All in all, HBV variation has a big influence on therapy, prognosis and vest. Serology can be diagnosed to be negative after HBV variation and DNA can be diagnosed to be positive, which lead to clinical leak detection, wrongly using of drug and therapy delay. Thus, detection of HBV provides a strong basis for clinical treatment and a reliable basis for doctors to adopt solution or develop new therapy solution.

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