Association between response to lamivudine treatment and tumor necrosis factor-α (TNF-α) promoter polymorphism in chronic hepatitis B

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The Master's Thesis
submitted to the Department of Medical Science
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medical Science

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December 2006

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December 2006

ACKNOWLEDGEMENTS

초등학교 시절에 읽었던 과학상자라는 책에서 뿌리에는 감자가 잎에서는 토마토가 열린 식물을 보았습니다. 생명과학이라는 개념을 몰랐던 그 시절에 '나도 언젠간 저걸 만들 거야!' 라는 막연한 꿈이 있었습니다.
시간이 지나면서 잊혀져간 꿈을 찾아가기 위해 남들과는 다르게 많이 돌아서 온 것 같습니다. 하지만 지나간 시간에 대한 후회보다는 현재의 상황에서 열심히 노력하여 오늘과 같은 결과를 얻게 된 것 같습니다.

지난 2년간, 생명과학을 공부하는 학생으로의 자세와 태도뿐만 아니라, 인생을 살아가면서 가져야할 가치관에 대해 많은 가르침을 주신 한광협 교수님께 온 마음을 다해 감사드립니다. 실험실 생활동안 항상 친형과 같은 따뜻한 웃음으로 학문적·실험적 도움을 주신 안상훈 선생님, 부족한 논문에 대해 아낌없는 조언을 해주신 박전한 교수님께도 감사드립니다.
환자들의 진료로 바쁘시지만 부족한 저의 논문을 위해 많은 정보를 제공해 주신 김도영 선생님, 늘 변함없는 모습으로 열심히 생활하시는 김자경 선생님, 오랫동안 생활은 못했지만 많은 도움을 주신 이현웅, 김화숙, 윤영준, 박준용 그리고 이진형 선생님께도 감사드립니다. 학자로서의 모범을 보여주시며, 앞으로 제게 많은 가르침을 주실 김균환 선생님께도 감사드립니다. 아무것도 모르는 처음의 저를 가르치느라 고생하신 장혜영 선생님, 멀리 포항에서 열심히 연구하고 있는 정말 고마운 친구 용욱이, 동기로서 많이 챙겨주고 도움을 주지 못해 미안한 명은이, 4층에서 동물실험으로 고생하는 숙인이, 잠깐 생활했지만 편하게 대해주는 효정이 에게도 감사드립니다.
실험실 생활을 시작하면서 알게 되었지만, 학자로서의 자세와 많은 실험적 조언을 해주신 경민형, 차분한 성격과 모범적인 생활로 저에게 자극이 되어주신 용섭형, 말수는 적지만 따뜻한 웃음으로 늘 반겨주신 재형형에게

도 감사의 말씀을 전합니다. 많은 시간을 함께 보내진 못했지만, 만날 때마다 조언과 격려를 해주신 멀리 인천에 계신 진환형, 항상 열심히 하는 모습으로 나를 반성하게 해준 거흔, 지은, 성녕 그리고 진수에게도 감사합니다.

끝으로, 사고뭉치로 말썽만 피우는 막내아들을 항상 응원해주시고, 걱정해주시는 어머니, 표현은 안하시지만 늘 아들생각으로 마음 쓰시며 기도해주신 아버지께 항상 사랑하고 감사드리며, 더욱더 열심히 공부하는 모습을 보여드릴 것을 약속드립니다. 멀리 부산에서 많은 도움을 주신 큰누님, 매형 그리고 귀여운 우리 윤서에게도 감사합니다. 서울에서 같이 사는 동안 많이 싸우기도 했지만 항상 못난 동생 걱정을 하는 작은 누님에게 이 지면을 통해 고마운 마음을 전합니다.

2006년 11월 박 용 광 드림

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Abstract

Association between response to lamivudine treatment and tumor necrosis factor-α (TNF-α) promoter polymorphism in chronic hepatitis B

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(Directed by Professor Kwang-Hyub Han)

Lamivudine (LMV) is a potent nucleoside analog to inhibit hepatitis B virus (HBV) replication. Recently, it was reported that LMV treatment can restore HBV-specific T-cell mediated immune response in patients with chronic hepatitis B. HBV-specific cytotoxic T-lymphocytes (CTLs) suppress expression and replication of HBV gene through the induction of tumor necrosis factor-alpha (TNF-α). It is well known that TNF-α plays a critical role in HBV immunopathogenesis. In chronic HBV infection, it was reported that TNF-α was activated in the liver and the lack of TNF-α induced impaired proliferation of HBV-specific CTLs in mice. TNF-α secretion is regulated at transcriptional and post-transcriptional level and TNF-α promoter polymorphism influence TNF-α expression. TNF-α promoter polymorphism at -238 and -308 positions is

reported to affect the production of TNF-α. Thus, we investigated whether TNF-α promoter polymorphism at -308 and -238 positions affect the response to LMV treatment in patients with chronic hepatitis B (CHB).

In this study, we examined 225 patients with CHB. All patients were treated with LMV at least for 12 months and followed up for 6 months after the end of therapy. These patients were classified into two groups according to the response to LMV. Responders were patients who showed normalization of serum ALT level, loss of HBeAg and sustained HBV-DNA negative after therapy, while non-responders had the detection of HBeAg and viral breakthrough during LMV treatment. Genomic DNA was extracted from whole blood and PCR for TNF-α promoter region was performed. The PCR products were purified and analyzed by direct sequencing.

Two hundred twenty five patients with CHB were classified into 103 patients of responders and 122 patients of non-responders. TNF-α promoter A allelic polymorphism at position -238 was present in 19 of 122 non-responders and 5 of 103 responders. There was an association between TNF-α promoter A allelic polymorphism at position -238 and response to LMV treatment in patients with CHB (responders vs. non-responders, p=0.009). TNF-α promoter A allelic polymorphism at position -308 was present in 6 of 122 non-responders and 7 of 103 responders. There was no relationship between TNF-α promoter A allelic polymorphism at position -308 and response to LMV treatment in patients with CHB.

These results suggest that the non-response to LMV treatment in patients with CHB may be related to TNF-α promoter -238 A allelic polymorphism.

Key words : Hepatitis B virus, lamivudine, tumor necrosis factor alpha, polymorphism

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I. INTRODUCTION

Hepatitis B virus (HBV) is a noncytopathic enveloped double-stranded DNA virus that causes acute and chronic liver diseases. HBV infection in adult is usually acute and completely recovered. However, 5-10% of acutely infected adults become persistently infected status and develop chronic hepatitis. HBV infection in neonatal period and childhood is rarely cleared and over 90% of such children become chronically infected.[1] These persistent infection increases the risk of developing cirrhosis and hepatocellular carcinoma (HCC). The risk of progression to chronic hepatitis is related to interaction between HBV and host

immune response.[1]

Because viral hepatitis is a liver disease caused by the destruction of HBV-infected hepatocytes, the clearance of HBV without killing the infected cells is required for noncytolytic intracellular viral inactivation by cytotoxic T lymphocytes (CTLs)-derived cytokines induced by virus-activated antigen presenting cells macrophage, dendritic cell and B-cell. Human leukocyte antigen (HLA) class I molecules, complexed with HBV antigenic peptides derived from intracellular cytosolic processing, provide information of intracellular invasion to CD8+T lymphocytes. HBV-specific CTLs abolish intracellular HBV gene expression and replication by inflammatory cytokines in HBV transgenic mice.[2] As the significance of host immune response to HBV increases, many research groups studied alleles of HLA and cytokines from host. Among various potential factors in HBV infection, tumor necrosis factor-alpha (TNF-α) has been widely studied.

TNF-α is a potent pro-inflammatory cytokine, mediating an anti-neoplastic, and anti-viral action and immunomodulatory activity, mainly secreted by macrophage and T-cell.[3] In chronic HBV infection, the secretion of TNF-α is activated in the liver,[4, 5] and TNF-α can inhibit the expression of HBV antigen in HBV transgenic mice.[6] Moreover, the lack of TNF-α induces impaired proliferation of HBV-specific CTLs.[7] Thus, the production of TNF-α plays an important role in HBV infection and is regulated by transcriptional and post-transcriptional process. The polymorphic variations in TNF-α promoter region is reported to affect the production of TNF-α.[8, 9, 10]

Among the various single nucleotide polymorphisms (SNPs) in TNF-α promoter region, -238 and -308 G to A polymorphisms were vigorously examined to reveal the association of HBV infection. TNF-α promoter -238 G

to A polymorphism is known to associated with chronic hepatitis B[11] and progression of HBV infection.[12] TNF-α promoter region -308 G to A polymorphism is significantly associated with HBV clearance and antibody production.[13]

Lamivudine (LMV) is the negative enantiomer of the deoxycytidine analogue 2'-deoxy-3'-thiacytidine [Fig. 1]. LMV is metabolized within HBV-infected hepatocytes to the active 5'-triphosphate form by addition of phosphate groups.[14] The active LMV 5'-triphosphate mimics deoxycytidine triphosphate and is incorporated into newly synthesised HBV-DNA to cause chain termination by competitive inhibition of reverse transcriptase activity.[15] Recently, LMV is reported to restore HBV-specific T-cell immune response in patients with chronic hepatitis B (CHB)[16] and HBsAg-specific cytotoxic T lymphocytes can mediate the suppression of HBV gene expression and replication through the induction of TNF-α.[17]

The immunomodulatory effect of LMV and the polymorphism of TNF-α promoter region may affect treatment response in patients with CHB. Thus, we examined whether TNF-α promoter polymorphism at -308 and -238 positions affect the response to LMV treatment in patients with CHB.

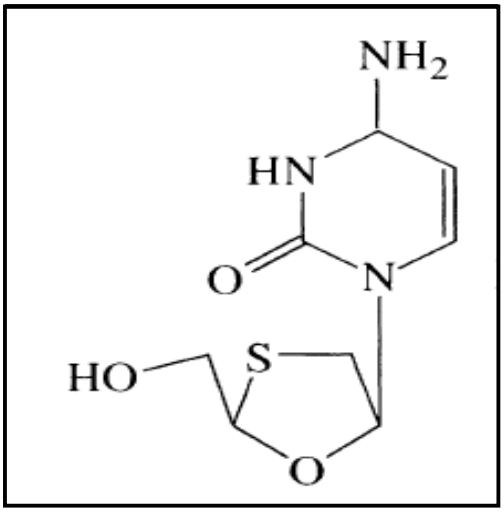


Figure 1. Chemical structure of Lamivudine.

The drug contains a sulphur atom instead of carbon at the 3′ position of the sugar ring, which does not allow chain elongation by phosphodiester bond formation, in the absence of the normal 3′ hydroxyl group. (adapted from Karayiannis P, 2003)[14]

II. MATERIALS AND METHODS

1. Study subjects

A total of 225 Korean patients with CHB were treated with LMV for more than 12 months and followed up for 6 months after the end of therapy. The 89 healthy control subjects were negative for all HBV markers suggesting no exposure to HBV before the test. All patients were enrolled from Severance Hospital of Yonsei University College of Medicine between January 2001 and September 2006.

These patients were classified into two groups according to the LMV response ; "Responders" had normalization of alanine aminotransferase (ALT) level, loss of HBeAg and sustained HBV-DNA negativity after therapy, while "Non-responder" had HBeAg and positive HBV-DNA even during LMV treatment. All patients had no serological evidence for hepatitis C virus, hepatitis D virus and human immunodeficiency virus co-infection.

Genomic DNA was isolated from peripheral venous blood using a NucleoGen Genomic DNA isolation Kit (NucleoGen, Seoul, Korea) according to the manufacturer's instructions and subsequently stored at 4℃ until analysis.

2. Analysis of TNF-α promoter polymorphisms

A 328-base pair fragment containing the -308 and -238 locus of the TNF-α gene promoter was amplified with forward primer TNF-396 (5' TTCCTGCATCCTGTCTGGAA 3') and reverse primer TNF-69 (5' CAGCGGA AAACTTCCTTGGT 3')[8] in a final volume of 25㎕ containing 50~100 ng of genomic DNA, 20 pmol of each primer, 0.2 mM dNTPs, 2 U of *Taq*

polymerase (iNtRON biotechnology, Seoul, Korea) and manufacturer's standard polymerase chain reaction (PCR) buffer. Amplification was performed in a GeneAmp PCR system 9700 (Perkin Elmer Corp., Norwalk, CT, USA). PCR conditions were 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 sec, 5 5℃ for 30 sec and 72℃ for 50 sec, with final extension at 72℃ for 7 min. PCR products were identified by electrophoresis, then PCR products were purified with PCR purification kit (iNtRON biotechnology, Seoul, Korea) and analyzed by direct sequencing (bionex Co. Ltd, Seoul, Korea).

3. Statistical analysis

The frequencies of TNF-α promoter polymorphism were compared among healthy controls, responders and non-responders by chi-square test. Chi-square test was used for comparison between genotype and allelic gene frequency comparisons for small numbers. All statistical tests were performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA). P value less than 0.05 was regarded as significant.

III. RESULTS

1. Clinical characteristics of responders and non-responders

Two hundred twenty five patients with CHB were grouped into 103 patients of responders and 122 patients of non-responders according to the LMV response. In responders group, 80 were males and 23 were females and the mean age of subjects was 40.3 years (range 18 ~ 76 years). HBV-DNA was negative (< 0.5 pg/ml) and the mean level of ALT is within normal range as 21.9 IU/ml. In non-responders group, 102 were males and 20 were females and the mean age of subjects was 43.6 years (range 22 ~ 66 years). The mean level of HBV-DNA and ALT were 518.4 pg/ml and 156.9 IU/ml, respectively (Table 1).

Table 1. The clinical characteristics of subjects

Characteristics	Responders (n = 103)	Non-responders (n = 122)
Sex (Male : Female)	80:23	102:20
Mean age (range)	40.3 (18~76)	43.6 (22~66)
HBV DNA (pg/ml)	< 0.5*	518.4*
ALT (IU/ml, mean)	21.9*	156.9*

Note. n, Number of investigated patients; *, mean value

2. Direct sequencing analysis of TNF-α promoter at -308 and -238 position

To investigate TNF-α promoter polymorphisms performed dye terminator sequencing. A 328 base pair fragment containing the -308 and -238 locus of the TNF-α gene promoter was analyzed. TNF-α promoter G allele and G to A transition at -308 and -238 positions were observed [Fig. 2, 3].

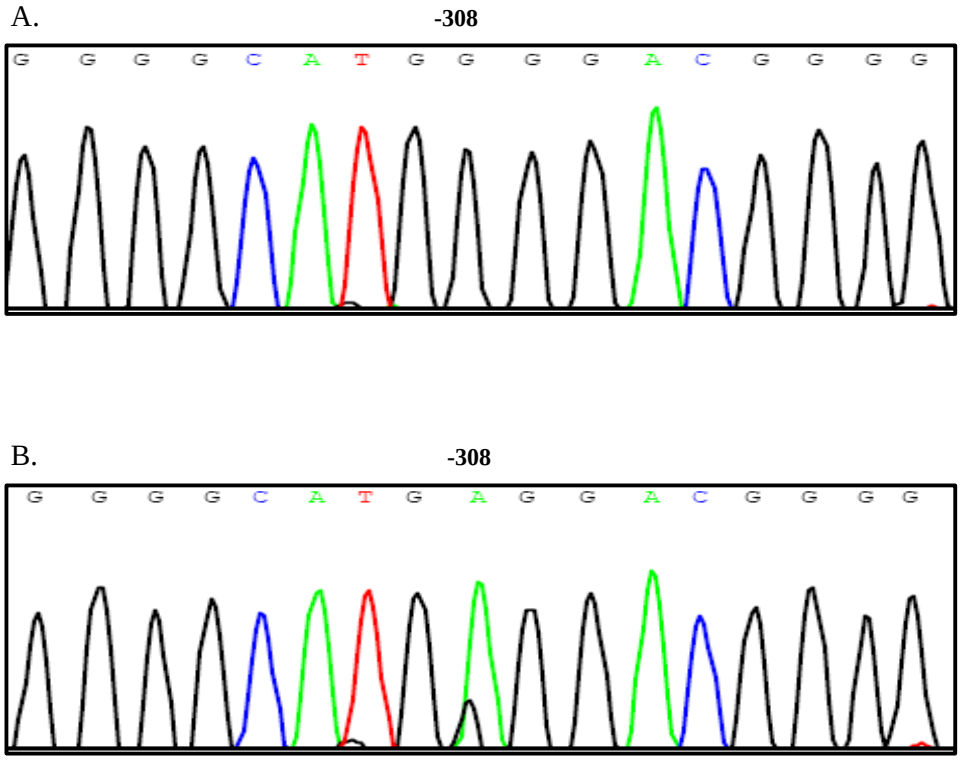
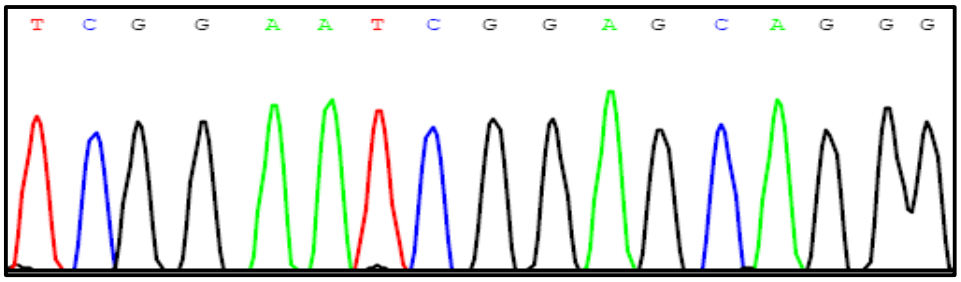


Figure 2. The chromatogram of TNF-α promoter at -316 to -300 region.
A. The wild type (G/G genotype) sequence of TNF-α promoter at -316 to -300.
B. TNF-α promoter G to A transition at -308 position.

A. **-238**



B. **-238**

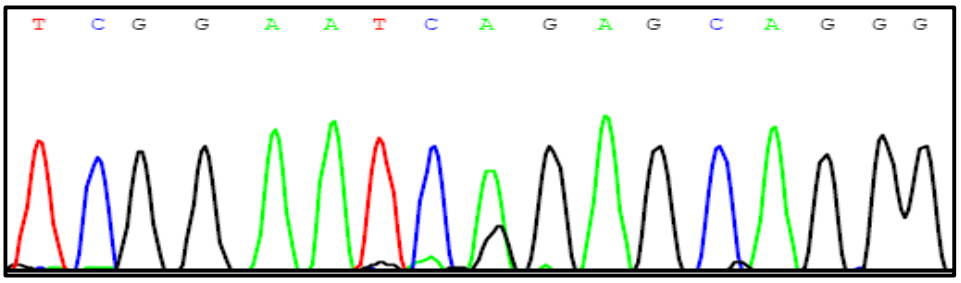


Figure 3. The chromatogram of TNF-α promoter at -246 to -230 region.
A. The wild type (G/G genotype) sequence of TNF-α promoter at -246 to -230.
B. TNF-α promoter G to A transition at -238 position.

3. Distribution of TNF-α promoter genotypes and alleles at -308 and -238 positions

The genotypic frequencies of TNF-α promoter at -308 and -238 positions are shown in Table 2. TNF-α promoter A/A genotype at -308 position was presented in 2 of 89 control groups and 2 of 225 CHB patients, while no one showed TNF-α promoter A/A genotype at -238 position in all investigated groups.

The allelic frequencies of TNF-α promoter at -308 and -238 positions are shown in Table 3. TNF-α promoter A allelic polymorphism at -308 position observed in 7.9% of control group and 3.3% of CHB patients with statistically significant difference (p = 0.015). TNF-α promoter A allelic polymorphism at -238 position observed in 2.4% of responders and 7.8% of non-responders. There was an association of TNF-α promoter A allelic polymorphism at -238 position and response to LMV treatment (p = 0.012). TNF-α promoter G allelic polymorphism at -308 position observed in 96.1% of responders and 97.1% of non-responders.

Table 2. Frequency of TNF-α promoter genotypes in control, responders and non-responders

Genotype	Control n=89 (%)	CHB		
		Responder n=103 (%)	Non-responder n=122 (%)	Total n=225 (%)
TNF-α -308				
G/G	77 (86.5)	96 (93.2)	116 (95.1)	212 (94.2)
G/A	10 (11.2)	6 (5.8)	5 (4.0)	11 (4.9)
A/A	2 (2.3)	1 (1.0)	1 (0.9)	2 (0.9)
TNF-α -238				
G/G	85 (95.5)	98 (95.1)	103 (84.4)	201 (89.3)
G/A	4 (4.5)	5 (4.9)	19 (15.6)	24 (10.7)
A/A	—	—	—	—

note. n, Number of investigated patients; CHB, chronic hepatitis B

Table 3. Frequency of TNF-α promoter allelic polymorphism at -308 and -238 region

TNF-α promoter Polymorphism	Control	CHB		
		Responder	Non-responder	Total
-308 region				
G allele	164 (92.1)	198 (96.1)	237 (97.1)	435 (96.7)
A allele	14 (7.9)†	8 (3.9)	7 (2.9)	15 (3.3)
-238 region				
G allele	174 (97.8)	201 (97.6)	225 (92.2)	426 (94.7)
A allele	4 (2.2)	5 (2.4)	19 (7.8)*	24 (5.3)

note. Number in parentheses represent percentage values.

†P = 0.015, Control vs. CHB; *P = 0.012, non-responders vs. responders

IV. DISCUSSION

To investigate whether TNF-α promoter polymorphism at position -308 and -238 affect the response to LMV treatment in patients with CHB, a group of 225 CHB patients with LMV therapy were selected and grouped according to the LMV response. The present study is a first evidence that the non-response to LMV treatment in patients with CHB associated with TNF-α promoter A allelic polymorphism at -238 position (Table 3, p=0.012).

Patients with chronic HBV infection are generally hyporesponsive to HBV protein, and the level of T cell reactivity at this stage of infection is significantly weaker than in acute self-limited hepatitis B.[1] Boni et al.[16, 22] reported LMV effect on HLA class I and II restricted T cells activity restore HBV-specific T cells responsiveness in patients with CHB. Thus, this immuno-modulatory effect of LMV may enhance recognition to the clearance of HBV infection in patients with CHB.

In HBV immunopathogenesis, HBV-specific CTLs abolish intracellular HBV gene expression and replication by inflammatory cytokines (i.e. TNF-α and INF-ɣ),[2] and TNF-α can especially inhibit the expression of HBV antigen in HBV transgenic mice.[6] Moreover, the lack of TNF-α induces impaired proliferation of HBV-specific CTLs.[7] Thus, the level of TNF-α plays a critical role in clearance of HBV. The production of TNF-α was regulated by transcriptional level which was determined by the binding capabilities of allelic-specific binding elements with *trans* factors. It was reported that TNF-α promoter region bound regulatory DNA-binding proteins, and the variation in this site might affect the activities of the promoters.[8] TNF-α promoter G to A polymorphism at -308 position has been shown to be associated with elevated

TNF-α transcriptional activity.[9, 19] On the other hand, polymorphisms at position -863 C to A and -238 G to A transition have been reported to be associated with reduced TNF-α promoter activity.[20, 21] Some research groups studied to association between the production of TNF-α and other TNF-α promoter polymorphisms (i.e. -1031, -857, -376, -244),[10, 13, 18] however the function of these polymorphic variation is precisely unknown whether the production of TNF-α is affected. TNF-α promoter region -238 G to A polymorphism is known to associated with CHB[11] and progression of HBV infection.[12] TNF-α promoter region -308 G to A polymorphism is reported to be significantly associated with HBV clearance and antibody production.[13]

Antigen presenting cells (APCs) deliver viral antigen presentation to T lymphocytes through the MHC class I, II. The variations of MHC gene deliver inappropriate viral antigen presentation to T lymphocytes[23], so it may affect HBV infection. Ahn et al.[24] reported that HLA-DRB*13 associate with HBV clearance in Korean. Other groups also reported association of HLA-DRB*13 and HBV clearance in Caucasian[25] and African[26]. The TNF-α gene is located between major histocompatibility complex (MHC) class I and MHC class II. To investigate the linked polymorphism between TNF-α and HLA on the outcome of HBV infection, some research groups studied association of TNF-α promoter polymorphism and HLA types in patients with HBV infection.[13, 27, 28] In point of this, the linked polymorphism between TNF-α promoter and HLA types may profitable to the present study and needed more investigation.

Although our results demonstrated TNF-α promoter polymorphism at -238 position was associated with LMV response, we did not include the influence of the TNF-α production by TNF-α promoter polymorphism at -238 position in CHB patients with LMV treatment. Moreover our data had a small investigated population. So, the present study needed for the functional study and large of

population.

In the present study, we showed that TNF-α promoter -238 A allelic polymorphism is one of the host factor associated with the LMV treatment response. Thus, TNF-α promoter polymorphism may be a helpful information to the clinical research of LMV response in patients with CHB.

V. CONCLUSION

This study demonstrated that TNF-α promoter A allelic polymorphism at -238 position associated with the response to LMV treatment in patients with CHB. These results suggest that TNF-α promoter -238 A allelic polymorphism may be one of the factors related the non-response to LMV treatment in patients with CHB.

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Abstract (In Korean)

만성 B형 간염에서 라미부딘 치료 반응과 tumor necrosis factor-α 유전자 다형성과의 관계

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박 용 광

만성 B형 간염 치료제로 B형 간염 바이러스(HBV)의 역전사 효소를 억제하는 라미부딘이 가장 많이 사용되고 있다. 최근 연구에서 만성 B형 간염 환자의 cytotoxic T lymphocytes (CTLs)의 기능을 회복시키는 라미부딘의 면역조절 기능이 보고 되었다. HBV 특이적인 CTLs은 tumor necrosis factor-α (TNF-α)의 분비를 통해 HBV의 복제와 유전자 발현을 억제하며, 이러한 면역반응은 효과적으로 HBV를 제거하도록 유도한다. TNF-α는 만성 B형 간염 환자의 간에서 분비량이 증가하며, promoter부위의 유전적 다형성에 따라서 전사 단계와 전사 후 단계에서 TNF-α 분비량이 조절된다. TNF-α promoter -308 부위의 유전적 다형성은 B형 간염의 자연치유와, -238 부위의 유전적 다형성은 B형 간염의 진행성과 관련이 있다고 보고 된 바 있기에, 본 연구는 TNF-α promoter 유전적 다형성에 따른 만성 B형 간염에서 기존의 항바이러스 치료제인 라미부딘 치료반응과의 연관성을 규명하고자 하였다.

만성 B형 간염환자 중 라미부딘 장기간 투약 치료(12개월 이상)를 받은 환자 225명을 대상으로, 치료 전과 치료 후 ALT 수치와 HBV-DNA 수치 검사, HBeAg의 임상적 결과에 따라 반응 군(responders, 103명)과 비반응 군(non-responders, 122명)으로 분류하였고, 정상 성인 대조군 89명과 비교하였다.

TNF-α promoter -308 부위의 G allele은 라미부딘 치료 반응 군에서 96.1%, 비반응 군에서는 97..1%였고, A allele type은 반응 군에서 3.9%, 비반응 군 2.9%로 두 군 간의 통계적 유의성은 관찰되지 않았다. 반면 TNF-α promoter -238 부위의 경우 G allele은 치료 반응 군에서 97.6%, 비반응 군에서 92.2%였다. A allele은 치료 반응 군에서 2.4%, 비반응 군에서는 7.8%로 관찰되어 TNF-α promoter -238 부위의 A allele과 라미부딘 비반응 군 간의 통계적 유의성이 관찰되었다.

만성 B형 간염환자에서 라미부딘에 의해 HBV의 항원 인지능력이 증가하여 CTLs 매개 면역 반응이 회복될 수 있지만, TNF-α promoter -238 부위의 유전적 다형성에 의해 TNF-α의 분비량이 감소하게 되면 라미부딘에 대한 치료반응이 어려워질 수도 있다. 따라서 본 연구의 결과는 라미부딘의 치료반응에 TNF-α promoter -238 부위의 유전적 다형성이 영향을 줄 수도 있다는 것을 암시한다. 하지만, 만성 B형 간염 환자의 검체수가 적어 더 많은 환자에 대한 분석과 대상 환자를 통한 TNF-α promoter에 대한 추가적인 연구가 필요하겠다.

핵심되는 말 : 만성 B형 간염, 라미부딘, Tumor necrosis factor-α, 유전자 다형성