HBV基因组A1846T变异对病毒复制力及核心启动子活性的上调作用研究

江玲，许智慧，刘妍，李晓东，姚伟明，李韦杰，戴久增，幸绍杰，徐东平

目的 评价乙肝病毒(HBV)基因组核苷酸(nt)A1846T变异对病毒体外复制力及核心启动子(CP)转录活性的影响。方法 385例研究对象包括116例轻中度慢性乙肝(CHB-M)患者，123例重度慢性乙肝(CHB-S)患者和146例慢加急性肝衰竭(AH-F)患者。从患者血清中提取HBV DNA，PCR扩增HBV全长基因组，统计A1846T变异的发生率。挑选代表性A1846T变异株HBV全长序列克隆至pGEM-Teasy载体中，并通过定点突变获得野生型对照。BspQ I/Scal双酶切HBV基因组，转染HepG2细胞，检测病毒复制力和HBsAg表达水平；用PCR分别扩增含nt1846变异型和野生型的HBV CP区片段，构建pG3-CP荧光素酶报告表达载体，转染HepG2细胞，分析检测A1846T变异对荧光素酶表达的影响。结果 HBV A1846T变异发生率为慢性程度加重依次增高，CHB-M、CHB-S和ACLF患者的变异检出率分别为31.03%、42.27%和55.48%(P<0.01)。A1846T变异株的复制力、分泌的HBsAg水平和核心启动子活性较野生株分别提高了320%、28%和85%，常见的CP区A1762T/G1764A双联变异株分别较野生株提高了67%、9%和72%。A1846T变异对提高病毒复制力的影响更弱。结论 A1846T变异显著增加HBV复制力，提高HBsAg表达水平，增强启动子活性，顺式激活下游基因转录表达，提示该变异可能与慢性乙肝重症化发生机制相关。

关键词 肝炎病毒，乙型；肝功能衰竭；突变；启动区；DNA复制

Up-regulation effect of hepatitis B virus genome A1846T mutation on viral replication and core promoter activity

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Objective To evaluate the influence of hepatitis B virus (HBV) genome nucleotide A1846T mutation on the viral replication capacity and the transcription activity of HBV core promoter (CP) in vitro. Methods A total of 385 patients with hepatitis B admitted to the 302 Hospital of PLA were enrolled in the study, including 116 with moderate chronic hepatitis B (CHB-M), 123 with severe chronic hepatitis B (CHB-S), and 146 with acute-on-chronic liver failure (ACLF). Serum HBV DNA was isolated and full-length HBV genome was amplified. The incidence of A1846T was analyzed. Full-length HBV genomes containing 1846T mutation were cloned into pGEM-T easy vector, and the counterpart wild-type 1846A plasmids were obtained by site-directed mutagenesis. The full-length HBV genome was released from recombinant plasmid by BspQ I/Scal digestion, and then transfected into HepG2 cells. Secreted HBsAg level and intracellular HBV core particles were measured 72 hours post-transfection to amplify the replication capacity (a 1.0-fold HBV genome model). 1846 mutant and wild-type full-length HBV genomes were extracted to amplify the fragment of HBV CP region, and the dual luciferase reporter of the pG3-CP was constructed. The luciferase activity was detected 48 hours post-transfection. Results The incidence of A1846T mutation gradually increased with the severity of hepatitis B, reaching 31.03%, 42.27%, and 55.48% in CHB-M, CHB-S and ACLF patients respectively (P<0.01).

Abstract Objective To evaluate the influence of hepatitis B virus (HBV) genome nucleotide A1846T mutation on the viral replication capacity and the transcription activity of HBV core promoter (CP) in vitro. Methods A total of 385 patients with hepatitis B admitted to the 302 Hospital of PLA were enrolled in the study, including 116 with moderate chronic hepatitis B (CHB-M), 123 with severe chronic hepatitis B (CHB-S), and 146 with acute-on-chronic liver failure (ACLF). Serum HBV DNA was isolated and full-length HBV genome was amplified. The incidence of A1846T was analyzed. Full-length HBV genomes containing 1846T mutation were cloned into pGEM-T easy vector, and the counterpart wild-type 1846A plasmids were obtained by site-directed mutagenesis. The full-length HBV genome was released from recombinant plasmid by BspQ I/Scal digestion, and then transfected into HepG2 cells. Secreted HBsAg level and intracellular HBV core particles were measured 72 hours post-transfection to amplify the replication capacity (a 1.0-fold HBV genome model). 1846 mutant and wild-type full-length HBV genomes were extracted to amplify the fragment of HBV CP region, and the dual luciferase reporter of the pG3-CP was constructed. The luciferase activity was detected 48 hours post-transfection. Results The incidence of A1846T mutation gradually increased with the severity of hepatitis B, reaching 31.03%, 42.27%, and 55.48% in CHB-M, CHB-S and ACLF patients respectively (P<0.01).
The replication capacity of 1846T mutants, level of secreted HBsAg, and transcriptional activity of CP promoter were increased by 320%, 28% and 85% respectively, compared with 1846A wild-type strains. While the more common double mutation A1762T/G1764A in CP region was increased by 67%, 9% and 72% respectively, compared with its counterpart wild-type strains. A1846T had a greater influence on viral replication capacity in vitro. Conclusions A1846T mutation could significantly increase the replication capacity of hepatitis B virus, secretion of HBsAg and transcriptional activity of CP promoter, and cis-activate the downstream gene transcription. The finding indicates that HBV genome A1846T mutation might play a role in liver disease progression.

[Key words] Hepatitis B virus; liver failure; mutation; promoter regions; DNA replication

HBV DNA replication is caused by the integration of the HBV genome into the liver cell genome and the expression of viral proteins. The finding indicates that HBV genome A1846T mutation might play a role in liver disease progression.

1 Materials and Methods

1.1 Clinical Material Sample material was obtained from 385 patients with chronic carriers of HBV (CHB-M) who were diagnosed at the Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an, China. The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Xi’an Jiaotong University.

1.2 Primer Design and Synthesis The primer set used for PCR amplification is shown in Table 1.

Tab. 1 Primer sequences for site-directed mutagenesis and PCR amplification of CP region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Note</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>5'-GGGATCCGTCGACTTGTTCTAGGACTCC-3'</td>
<td>Used for site-directed mutagenesis</td>
</tr>
<tr>
<td>P2</td>
<td>5'-GGGATCCGTCGACTTGTTCTAGGACTCC-3'</td>
<td>Used for site-directed mutagenesis</td>
</tr>
<tr>
<td>P3</td>
<td>5'-GGGGGTTACCCACACACAGTTGCTTCCC+3'(Kpn I)</td>
<td>Sense primer for PCR amplification of CP region</td>
</tr>
<tr>
<td>P4</td>
<td>5'-GGGATCCGTCGACTTGTTCTAGGACTCC-3'</td>
<td>Antisense primer for PCR amplification of CP region</td>
</tr>
</tbody>
</table>

HBV DNA replication is caused by the integration of the HBV genome into the liver cell genome and the expression of viral proteins. The finding indicates that HBV genome A1846T mutation might play a role in liver disease progression.

1.3 Methods

1.3.1 HBV DNA full-length clone was used to generate the wild-type and two mutant strains, A1846C and A1846T. The two mutant strains were used to infect HepG2 cells. The results showed that the A1846T mutant had a significantly increased replication capacity compared with the wild-type and A1846C strains.

1.3.2 The effect of the A1846T mutant on the replication of HBV DNA was further investigated. The results showed that the A1846T mutant had a significantly increased replication capacity compared with the wild-type and A1846C strains.
丙醇沉淀，乙醇漂洗，Elution Buffer重溶获得HBV复制中间体核心颗粒DNA。采用Real-time荧光定量法对HBV定量，反映10倍HBV体外复制力。

1.3.5 HBV C区扩增及pGL3-CP荧光素酶表达载体构建 取含有nt1846位点变异型与野生型的T-easy质粒，BspQ I / Sca I双酶切，胶回收3.2kb左右的DNA条带，加T，DNA连接酶后自连。采用普通PCR扩增CPI区，引物为P3、P4(表1)，反应条件：94℃ 3min；94℃ 15s，54℃ 15s，72℃ 30s，35个循环；72℃ 10min。CPI扩增产物胶回收，与pGL3-Basic载体分别进行Kpn I / Bgl II双酶切，胶回收后在T，DNA连接酶作用下将两者定向连接，转化JM109感受态后筛选菌落PCR阳性克隆，提取质粒。行Kpn I / Bgl II双酶切鉴定，酶切鉴定正确的载体按质检测序。

1.3.6 载体质粒瞬时转染及荧光素酶报告基因检测 QiaGen试剂盒提取转基因pGL3-CP荧光素酶表达载体质粒，转染方法同前，以pRL-TK载体作为内参照进行共转染，每孔0.1ug，设置pGL3-Basic为阴性对照，pGL3-Control为阳性对照，48h后裂解细胞，参照荧光素酶检测试剂盒说明书使用单管型多功能检测仪仅对荧光素酶活性进行检测，并用内参对其进行校正。

1.4 统计学处理 应用SPSS 16.0和SAS 9.0软件分析。正态分布的计量资料以±s表示，方差齐采用单因素方差分析，方差不齐采用Kruskal-Wallis秩和检验。计数资料采用X²检验，P<0.05为差异有统计学意义。

2 结 果

2.1 患者临床资料分析 对各组患者进行统计分析，A1846T变异检出率在CHB-M、CHB-S和ACLF三组患者中呈升高趋势，分别为31.03%、42.27%和55.48%(P<0.01)。与ACLF比较，CHB-M和CHB-S的A1846T变异检出率明显降低(P<0.05)，而CHB-M和CHB-S之间差异无统计学意义(P=0.072)。结线性趋势检验，P<0.0001，Z=-3.9805，表明随着病情加重变异率增加。临床资料显示，CHB-M、CHB-S和ACLF患者HBeAg阳性率和HBV DNA载量依次降低(P<0.01)，而谷丙转氨酶(ALT)和总胆红素(TBIL)水平则依次升高(P<0.01，表2)。

表2 患者临床资料分析

<table>
<thead>
<tr>
<th>Tab.2 Clinical data of the patients</th>
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<tbody>
<tr>
<td>Clinical data</td>
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<tr>
<td>Age(year)</td>
</tr>
<tr>
<td>Male/Female</td>
</tr>
<tr>
<td>HBeAg (+/−)</td>
</tr>
<tr>
<td>ALT(U/L)</td>
</tr>
<tr>
<td>TBIL(mmol/L)</td>
</tr>
<tr>
<td>HBV DNA(log10 copy/ml)</td>
</tr>
<tr>
<td>A1846T mutation rate (%)</td>
</tr>
</tbody>
</table>

(1)P<0.05, (2)P<0.01 compared with CHB-M patients; (3)P<0.05, (4)P<0.01 compared with CHB-S patients

2.2 定点突变检测结果 测序证实，经典的双联变异型A1762T/G1764A回复突变为野生型A1762/ G1764，变异型1846T回复突变为野生型A1846。图1显示定点突变鉴定成功。

2.3 1.0倍全长HBV DNA复制力评价 克隆至pGEM-Teasy载体中的全基因组HBV DNA经BspQ I / Sca I双酶切后，胶回收获得1.0倍全长HBV DNA(图2)。变异型的A1762T/G1764A和A1846T复制力较相应野生株分别提高了67%和320%，HBsAg分别提高了9%和28%，表明A1846T变异可显著增加HBV DNA载量，提高病毒复制力及HBsAg的表达。

2.4 pGL3-CP荧光素酶表达载体的构建 基于成功的pGL3-CP荧光素酶载体的构建，成功构建的PGL3-CP荧光素酶报告载体粒经Kpn I / Bgl II双酶切，5.0kb碱基质粒转染为4.8kb的载体片段和243bp的CPI探针片段(图3)，酶切鉴定准确后送DNA测序，结果证实插入的CPI片段方向与载体核苷酸序列相同，除1846位点外其他碱基未发生变化，双荧光素酶报告载体构建成功。

图1 定点突变测序结果

Fig-1 Result of site-directed mutagenesis detected by DNA sequencing

C9. 1762/1764 double-mutation type; C9-W. Wild type; C76. 1846 mutation type; C76-W. Wild type
2.5 启动子活性检测 经双荧光报告系统检测，变异型A1762T/G1764A和相应野生型的启动子活性分别为0.609和0.353，变异型A1846T和相应野生型启动子活性分别为0.795和0.430。变异型分别较相应野生株提高了72%(与文献[7]报道基本一致)和85%，表明A1846T变异型能加强启动子活性，顺式激活下游基因转录，从而增加病毒的产生，与复制力评价结果一致。

3 讨 论

HBV感染后发生重型肝炎的病理机制是由病毒和机体双方决定的。其病变的进展影响因素较多，HBV前C/CP区基因变异可能是其中的重要原因之一。基因变异可能是由多因素决定的，但目前更倾向于是在人类免疫压力的作用下发生的。HBV基因变异可通过增强病毒复制能力、免疫原性和致病性影响机体的免疫应答反应，导致HBV感染持续并加重对肝细胞的损害，因此HBV基因变异可能参与了重型肝炎的发生和发展[6]。前C/CP是HBV复制的关键性调控因子，调控HBV前C基因组mRNA和前基因组mRNA的转录，HBV前C区变异还可使免疫耐受因子HBeAg的合成和分泌减少，进而影响CTL介导的肝细胞杀伤，CP区的变异还可能导致病毒的成熟及分泌、感染性及肝外趋向性等发生改变[9]。CP区的A1762T/G1764A双联变异可使3′端端环结构发生改变，稳定性增强，促进病毒的转录和复制[10]，与肝炎活动性、移植后严重肝病和肝细胞癌密切相关的[11]。新近有研究表明CP区的A1846T变异在ACLF患者中检出率较高，但未分析该变异对病毒生物活性的影响[12]。

本研究发现A1846T变异率随疾病严重程度递增，且变异可导致病毒活力增强，推测加重机体过强的炎性反应是该变异与乙肝重症化相关的内在机制。以经典的A1762T/G1764A作为对照，结果表明，在不同诊断的乙肝患者中，随病情加重，A1846T位点的变异率增加，HBeAg阳性率和HBV DNA载量降低(P<0.01)，而ALT和TBIL水平则依次升高(P<0.01)，提示A1846T变异与乙肝重症化相关。在统计学分析基础上，构建1.0倍HBV复制模型进行复制力评价，排除外源启动子对HBV复制的影响，结果表明A1846T位点变异后，病毒复制力显著升高(变异型较野生株提高320%)，HbsAg检测结果与复制力一致(变异型较野生株提高28%)。进一步进行前C/CP区启动子活性研究，结果表明A1846T变异可增强前C/CP启动子活性(变异型较野生株提高85%)，顺式激活下游基因转录。

以上结果提示，A1846T变异可能与乙肝重症化相关。本研究进一步证实了HBV前C/CP变异与重型肝炎发生之间的关系。为重型肝炎的发病机制研究提供了科学依据。

【参考文献】


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