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HCV 核蛋白羧基端信号肽基因缺失突变体的构建、鉴定和真核表达

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Expression of a C-terminal deleted mutant of hepatitis C virus core protein in P815 cell line

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Abstract

AIM: To construct a recombinant eukaryotic expression vector encoding C-terminal deleted mutant of hepatitis C virus (HCV) core protein and express it in P815 cell line.

METHODS: cDNA for HCV core protein was obtained from patients with chronic HCV infection by RT-PCR and employed as template to amplify the gene fragment of truncated core protein (C507). The truncated core cDNA was modified by two restriction enzymes and cloned into pCI-neo, which was named as pCI-C507. By using lipofectamine 2000, the recombinant was transferred into P815 cells. 24 hours after transfection, the cells were collected and examined by confocal microscopy.

RESULTS: The cDNA of HCV core protein was analyzed with phylogenetic analysis and confirmed to be genotype Ib. pCI-C507 was identified by digestion with restriction enzymes and confirmed by DNA sequencing. The expression of pCI-C507 (P169) was observed in the cell plasma under confocal microscopy while only a little was in the nucleus.

CONCLUSION: The recombinant pCI-C507 was correctly constructed and effectively expressed in P815 cells, which can be used for DNA vaccination study.

Key Words: Hepatitis C virus; Core protein; P815 cell line; C-terminal deleted mutant

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摘要

目的: 构建羧基末端缺失核蛋白的真核表达载体并在小鼠肥大细胞瘤细胞株 P815 细胞中表达.

方法: 用 RT-PCR 方法从西安地区丙型肝炎患者血清中扩增 HCV C 区及部分 E1 区基因并进行克隆、测序, 以此为模板扩增羧基末端缺失突变核蛋白基因片段(C507), 将其定向克隆入真核表达载体 pCI-neo 中并转染 P815 细胞, 经间接免疫荧光染色、激光共聚焦显微镜检测细胞中突变核蛋白(P169)的表达.

结果: 从患者血清中成功克隆出 HCV C cDNA, 构建了编码羧基末端缺失核蛋白的重组质粒 pCI-507, 并经酶切鉴定和测序证实;间接免疫荧光染色表明P169主要在 P815 细胞胞质中表达, 少数可见于细胞核内.

结论: 重组体 pCI-507 构建正确, 其表达产物 P169 在 P815 中得到有效表达, 为在此基础上的 DNA 免疫研究提供了实验依据.

关键词: 丙型肝炎病毒; 核蛋白; P815 细胞; 羧基末端缺失

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0 引言

丙型肝炎病毒(HCV)感染人口已经达到全球人口的3%，其中80%的感染者发展为持续性感染，最终将导致慢性肝炎、肝硬化或肝细胞癌，但目前尚无有效的治疗和预防方法[1-2]. 研究表明，HCV感染后人体不能产生足够强和广泛的细胞免疫反应是原因之一. 各国学者试图通过DNA免疫来提高机体对HCV 的免疫反应，以达到治疗慢性丙型肝炎的目的[3-6]. 由于核蛋白的序列高度保守，且具有多个B细胞和T细胞抗原位点，是细胞免疫反应的主要靶抗原，因此，核蛋白特异性免疫的研究一直十分活跃. 大量的研究结果表明，利用完整核蛋白基因构建的DNA疫苗诱导的免疫反应弱，为此，人们尝试用多种方法来提高核蛋白特异性的免疫反应[7-9]. 有研究发现HCV核蛋白的羧基末端抑制核蛋白在原核细胞中的表达，而截去羧基末端的核蛋白表达水平明显提高[10-11]. 我们设计构建了羧基末端基因缺失的核蛋白真核表达重组体，以期提高核蛋白在真核细胞中的表达水平，为增强核蛋白特异性免疫反应探索新的途径.

1 材料和方法

1.1 材料 MMLV逆转录酶、DNA聚合酶、限制性内切酶*Eco*RI，*Sal*I、真核表达载体pCI-neo均系Promega公司产品. 胶回收试剂盒购自Clontech公司. 小量质粒抽提试剂盒购自上海华舜生物技术公司. DNA连接酶购自华美生物技术服务公司. 胰蛋白胨、酵母提取物为Oxford公司产品. Lipofectamine2000™ Reagent购自Invitrogen公司. FITC标记山羊抗人IgG荧光抗体系鼎国生物技术公司进口分装试剂. RPMI1640为GIBCO公司产品. 胎牛血清购自杭州四季青生物制品公司. *E. coli*JM109和小鼠肥大细胞瘤细胞株P815均由本室保存. 抗HCV抗体及HCV RNA双阳性血清采自本科住院的慢性丙型肝炎患者.

1.2 方法 收集抗HCV抗体及HCV RNA双阳性血清，以Trizol Reagent抽提总RNA，MMLV逆转录酶合成第一链;以其为模板，利用PCR方法进行cDNA的扩增，扩增产物包括核蛋白、部分E1蛋白和非编码区基因. 引物为:HCV+1:5’-GTACTGCCTGATAAGGTGCTT-3’;HCV+2:5’-CTGATAGGGTGCTTGCGAGT-3’;HCV-1:5’-TTCA(GT)CATCAT(AG)TCCCA(AGCT)GCCA-3’;HCV-2:5’-CA(GT)CATCAT(AG)TCCCA(AGCT)GCC-3’;将PCR产物克隆入载体质粒PGEM-T Easy，转化大肠杆菌*E. coli*JM109，利用酶切法进行初步筛选，阳性克隆进行序列测定和系统发生树分析. 以上面得到的HCV cDNA为模板，扩增羧基末端22个氨基酸缺失的核蛋白基因片段. 上游引物:5’-GTGCGAATTCATGAGCACGAATCCTAAAC-3’;下游引物:5’-TCGCGTCGACTCAAGGAAGGTTCCCTGTTGC-3’;上游引物设计了*Eco*RI酶切位点，下游引物设计了*Sal*I酶切位点，目的片段预计为507 bp. PCR反应体积为50 μL，引物浓度0.2 μmol/L，扩增参数:94℃预变性5 min，94℃变性1 min，55℃退火1 min，72℃延伸1 min，35个循环后72℃延伸10 min，PCR产物进行10 g/L琼脂糖凝胶电泳分析. PCR产物经*Eco*RI和*Sal*I双酶切，用胶回收方法进行纯化后与真核表达载体pCI-neo中的相应位点连接. 将连接产物转化新鲜制备的感受态*E. coli*JM109，将阳性克隆菌落接种于含氨卞青霉素的LB培养基中过夜培养，按说明书操作进行质粒的小量提取，经*Eco*RI和*Sal*I双酶切鉴定，并交上海生物工程公司双向测序. 测序证实编码正确的重组质粒采用脂质体转染法进行转染，按照说明书操作. P815细胞在37℃、50 mL/L CO_2条件下以100 g/L胎牛血清-RPMI1640培养. 处于对数生长期的细胞以无血清、无抗生素的RPMI1640洗涤后移至24孔板中，调整细胞浓度为8×10^5/孔. 在无菌Ep管中以无血清、无抗生素RPMI1640分别稀释质粒1 μg和Lipofectamine2000 3 μL，终体积均为50 μL. 将质粒和Lipofectamine 2000混合，置室温20 min后，将上述混合物加入24孔板，与细胞轻轻混匀，置37℃、50 mL/LCO_2条件下培养4 h后换液，以100 g/L胎牛血清-RPMI1640继续培养24 h，收获细胞. 转染空白pCI-neo质粒的P815细胞作为阴性对照. 收获的细胞用PBS清洗后制作细胞滴片，风干后以4℃丙酮固定10 min，50 mL/L山羊血清-PBS封闭20 min. 滴加抗HCV阳性混合血清(1∶10稀释)作为第一抗体，37℃湿盒中反应60 min，反应结束后以PBS冲洗细胞3次，每次5 min. 滴加FITC标记山羊抗人IgG作为第二抗体(1∶60稀释)，37℃湿盒中反应60 min，反应结束后以PBS冲洗细胞3次，每次5 min. 风干后以500 mL/L甘油-PBS封片，置荧光显微镜下观察.

2 结果

2.1 HCV核蛋白及部分E1区基因cDNA克隆 5份丙型肝炎患者血清标本中，2例RT-PCR产物为阳性，大小约为1 000 bp，与预期相符. 将PCR产物克隆入PGEM-T Easy载体中，用酶切方法从每份PCR产物的转化产物中各筛选出3个阳性克隆，经序列测定，各有2个克隆正确. 阳性克隆分别命名为HCV-CE1-2-1，HCV-CE1-2-2，HCV-CE1-3-3，HCV-CE1-3-4. 经系统发生树分析，2例患者的HCV基因型均为1 b型，且基因序列有一定差异，证实PCR产物无污染.

2.2 HCV核蛋白羧基末端缺失突变体基因真核表达载体的构建和鉴定 以HCV-CE1-3-3为模板扩增的基因

片段长度为507 bp，命名为C507. PCR产物经*Eco*RI和*Sal*I双酶切后克隆入载体质粒pCI-neo，获得阳性克隆pCI-C507，经*Eco*RI单酶切和*Eco*RI、*Sal*I双酶切鉴定，可见约500 bp的目的基因片段及5 472 bp的载体片段(图1). DNA双向测序证实整个插入片段读码框架正确.

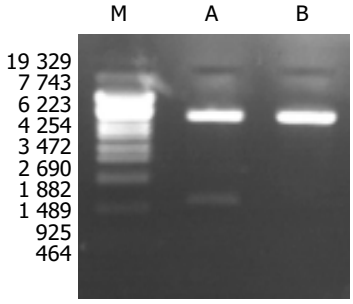

图1 重组质粒pCI-C507酶切鉴定. M: DNA marker λ EcoT14; A: pCI-C507 digested by *Eco*RI and *Sal*I; B: pCI-C507 digested by *Eco*RI.

2.3 HCV核蛋白在真核细胞中的表达 pCI-C507转染的P815细胞经间接免疫荧光染色后进行激光共聚焦分析，结果显示，在pCI-C507转染的P815细胞中，阳性信号大部分定位于细胞质内，分布不均匀，少数细胞的细胞核内呈阳性染色(图2A)，而转染了pCI-neo空载体的P815细胞则无荧光信号(图2B). 表达产物命名为P169. 初步证实目的蛋白P169在真核细胞中正确表达并具有天然抗原性.

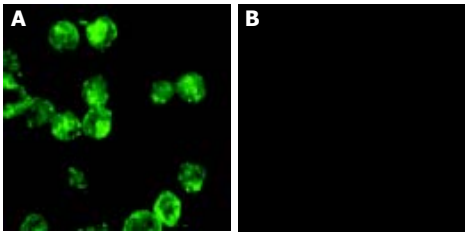

图2 重组质粒pCI-C507转染P815细胞表达P169蛋白(10 × 40). A: 阳性; B: 阴性.

3 讨论

在HCV感染过程中，核蛋白是细胞免疫反应的主要靶抗原之一. 在急性感染早期抗HCV抗体及CTL反应水平高者，最终往往清除了病毒，即使遭到再次感染其病情也相对轻微，而抗HCV抗体及CTL反应水平低者则最终转为慢性持续性感染[12-13]. 因此，有效的核蛋白特异性免疫反应对预防和清除HCV感染具有至关重要的意义. 以往的研究表明，以完整核蛋白基因组构建的重组核酸疫苗虽然能够诱导核蛋白特异性的体液和细胞免疫，但产生的特异性抗体水平低. 采用联合免疫佐剂如HBsAg，GM-SF，IL-12等多种细胞因子的免疫策略使得核蛋白特异性反应有一定程度的提高，但仍未达到理想的效果[14-20]. 核蛋白在体内的低水平表达可能是造成核蛋白特异性免疫水平低下的重要原因之一. 因此，提高核蛋白的表达水平可能为增强核蛋白特异性免疫效应提供一条新的途径. Nishimura *et al*[21]构建的ELI-a启动子调控下表达的核蛋白重组质粒能促进核蛋白在哺乳动物细胞的表达，单次免疫小鼠后所诱导出的核蛋白特异性CTL杀伤率达到50%(效靶比80∶1)，提示通过提高核蛋白的表达水平使核蛋白特异性免疫反应得到了明显提高，为这一途径的开发提供了有利的支持.

完整的核蛋白有191个氨基酸，其羧基末端约20个氨基酸序列是包膜蛋白E1的信号肽[22]，在大肠杆菌内的表达过程中，带有该信号序列的完整核蛋白表达水平极低，而截去该信号序列后核蛋白的表达水平得到显著提高，提示该信号序列在核蛋白的原核表达过程中起到抑制作用[10-11]. 此外，核蛋白的羧基末端还是诱导细胞凋亡的必要条件之一，可能通过诱导感染HCV的淋巴细胞的凋亡来达到延长病毒生存的目的[23]. 有人用截去羧基末端的核蛋白基因重组质粒免疫小鼠，诱导出的核蛋白特异性抗体的滴度远远高于慢性丙型肝炎患者血清核蛋白特异性抗体滴度;同时核蛋白特异性T淋巴细胞增生反应及杀伤细胞活性也显著升高[24-26].

我们针对我国主要流行的HCV1b型，成功构建了羧基末端缺失突变核蛋白基因的真核表达载体pCI-507，经激光共聚焦分析证实，其编码的P169多数定位于细胞质内，少数进入细胞核内，与文献[27-30]报道一致. 初步证实P169能够在哺乳动物细胞中有效表达，为进一步的核蛋白特异性免疫反应研究提供了实验依据.

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