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复制缺陷型HCV C基因腺病毒表达载体的构建包装及鉴定

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Construction, package and identification of replication-deficient recombinant adenovirus expression vector of HCV C

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Abstract

AIM:To construct a replication-deficient recombinant adenovirus expression vector of HCV C.

METHODS:The HCV core gene was cloned at the downstream of CMV promoter of the adenoviral shuttle plasmid pAd.CMV-link.1, and the resultant recombinant plasmid pAd. HCV-C was cotransfected into 293 cell together with plasmid pJM17 containing adenoviral genome, then the adenovirus expression vector was obtained, and identified by infecting test,electronic microscope observation and PCR co-amplification. The plasmid pAd.HCV-C was identified by endonuclease, PCR and sequencing.The expressive activity of adenovirus vector was identified by immunofluorescence and Western blot.

RESULTS:HCV core gene in the inserted DNA of pAd.HCV-C was confirmed by endonuclease, PCR and sequencing. Results of infecting test, electronic microscopic observation and PCR co-amplification showed that the adenovirus vector had been constructed successfully. Expression of HCV core antigen was proved in the HepG2 cells by immunofluorescence and Western blot.

CONCLUSION:The replication-deficient recombinant adenovirus vector can express HCV core antigen in HepG2 cells.This study established a foundation for further study on HCV vaccines and gene therapy for hepatitis C.

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摘要

目的:构建能表达HCV C基因的复制缺陷型腺病毒表达载体.

方法:将HCV H株C区基因定向插入到腺病毒穿梭质粒pAd.CMV-Link.1中,获得重组质粒pAd.HCV-C,再与pJM17共转染293细胞,包装腺病毒表达载体.通过酶切、PCR及测序对穿梭质粒进行了鉴定.对腺病毒载体进行了感染性鉴定、电镜鉴定及双引物PCR鉴定.利用间接免疫荧光法和Western blot检测了腺病毒载体在人肝癌细胞$HepG_2$中的表达.

结果:酶切、PCR及测序鉴定证实，穿梭质粒插入片段为HCV C区基因.包装的腺病毒载体具有良好的感染性，可以在293细胞中形成病毒颗粒，腺病毒载体内携带HCV C区基因，并可以在$HepG_2$细胞中表达HCV C抗原.

结论:包装成功的复制缺陷型腺病毒载体可以在$HepG_2$细胞中表达HCV C抗原，为丙型肝炎的基因治疗及疫苗的进一步研究奠定了基础.

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0 引言

腺病毒具有宿主范围广、感染率高、包装容量大、繁殖滴度高、不发生整合及安全性好、性质稳定、载体制备较容易等特点[1-5], 成为继逆转录病毒载体后被广泛应用的载体系统.丙型肝炎病毒(HCV)感染是当前危害人类健康的重要传染病[6], 至今尚无特异性的预防和治疗措施[7,8].基因治疗作为一种控制HCV感染的新途径, 正在受到人们的重视[9-13].因而，构建并包装携带HCV C基因的腺病毒表达载体，在丙型肝炎的预防和基因治疗中具有重要意义.

1 材料和方法

1.1 材料 pBRTM/HCV1-3011质粒含HCV-H株除5'CR外的全部HCV序列, 由美国华盛顿大学Rice教授惠

赠[14];E_1区缺失的复制缺陷型腺病毒穿梭质粒 pAd.C M V-Link.1,腺病毒重组质粒 pJM17 和转化有腺病毒E_1区基因的包装细胞 293 细胞均为李谨革博士惠赠;大肠杆菌 E.coli JM 109 及 HepG2 细胞为本室保存. Hind Ⅲ, Bgl Ⅱ,T_4DNA 连接酶, Taq DNA 聚合酶,dNTP, PCR 产物回收试剂盒及 Plasmid Purification Kit 均为 Promega 公司产品; 脂质体 Lipofectamine 2000、 DMEM 及胎牛血清均为Gibco公司产品; 小鼠抗HCV C 单克隆抗体购自中国预防科学院.PCR引物设计参考已发表的HCV H株C区序列设计1对引物, 在每条引物的5 ’端引入相应的酶切位点及保护碱基, P_1(上游引物, 5 ’ 端含有 Bgl Ⅱ位点及起始码):5 ’GAAGATCTATGAGCAC GAATCCTA AACCT3 ’;P_2(下游引物,5 ’ 端含有 Hind Ⅲ位点及终止码): 5 ’ CCAAGCTTTTAGGCTGAAGCGGGCACAGT 3 ’插入的 HCV C 区序列两端腺病毒载体特异性引物设计为：P_3(上游引物): 5 ’ ATAGAAGACACCGGGACCGAT CCAGCCTCC 3 ’ P_4(下游引物): 5 ’ TCTAGAGTCGACGGA TCCTACGTAGGTACC 3 ’,均由大连宝生物公司合成.

1.2 方法

1.2.1 HCV C基因腺病毒载体的包装　将构建好的携带HCV C区基因的腺病毒穿梭质粒[15] pAd.HCV-C与腺病毒重组质粒 pJM17 用 Lipofectamine 2 000 共转染 293 细胞, 培养 24 h 后,加第 1 层空斑琼脂糖凝胶复合物(配方为:2 × MEM 10 mL; FBS 2 mL; 16 g/L Agar 6.8 mL; 30 g/L 谷胺酰胺 0.8 mL; 双抗 0.2 mL; Hepes 0.4 mL; 75 g/L $NaHCO_3$ 0.8 mL; NEAA 0.2 mL), 每孔 3 mL, 37 °C培养 96 h, 加第 2 层空斑琼脂糖凝胶复合物(配方同第 1 层), 每孔 3 mL, 37 °C培养 96 h, 加第 3 层空斑琼脂糖凝胶复合物(配方在第 1 层的基础上加 14 g/L 中性红 0.6 mL), 每孔 3 mL, 37 °C培养 24 h, 可出现空斑.挑取分化良好的单个空斑,再感染 293 细胞,出现 CPE(cytopathic effect)时收获细胞并制成细胞冻融液(含包装好的腺病毒载体),-70 °C冻存备用.

1.2.2 HCV C 基因腺病毒载体的鉴定　感染性鉴定用冻存的腺病毒载体感染 70-80 % 融合的 293 细胞,37 °C培养 2-3 d，观察 293 有无 CPE 出现.电镜鉴定用冻存的腺病毒载体感染 293 细胞,37 °C培养 2-3 d 后,293 细胞出现CPE.将293细胞收获,制作电镜标本,电镜下观测细胞内有无病毒样颗粒形成. 双引物PCR鉴定用冻存的腺病毒载体再感染293细胞,待出现CPE时吸取1 mL培养液,提取病毒DNA,采用插入的HCV C区基因特有引物P_1 P_2和腺病毒载体特有引物 P_3 P_4,进行双引物 PCR 鉴定.腺病毒载体表达的免疫荧光鉴定用重组腺病毒感染HepG2细胞, 以抗HCV C单抗为一抗做间接免疫荧光实验, 检测重组腺病毒载体在 HepG2 细胞中表达 HCV C 抗原的情况.腺病毒载体表达的 Western Blot 鉴定,把腺病毒感染的 HepG2 细胞裂解后进行 SDS-PAGE 凝胶电泳, 然后将蛋白条带电转移到硝酸纤维素膜上，以抗 HCV C 单克隆抗体为一抗(1：10),以 HRP- 羊抗鼠 IgG 为二抗(1：100)进行 Western Blot 实验，检测重组腺病毒载体在 HepG2 细胞中表达 HCV　C 抗原的情况.

2　结果

2.1 感染性鉴 用腺病毒载体感染 293 细胞,37 °C培养 2-3 d, 发现 293 细胞圆缩、脱落,出现 CPE.表明包装成的腺病毒载体感染性良好,可以在 293 细胞中 E1 基因产物的反式作用下大量增生,并阻断宿主细胞 DNA 和蛋白质生成,最终导致细胞营养耗竭而死亡.

2.2 电镜鉴定　用腺病毒载体感染 293 细胞 2-3 d 后,293 细胞出现CPE.将293细胞收获,制作电镜标本,在电镜下观测,发现有病毒样颗粒形成(图 1).未感染腺病毒的 293 细胞未发现有病毒样颗粒形成.

2.3 双引物 PCR 鉴定　腺病毒感染的 293 细胞出现 CPE 时提取病毒DNA,采用插入的HCV C区基因特有引物P_1 P_2和腺病毒载体特有引物 P_3 P_4,进行双引物 PCR 鉴定, 结果出现 573 和 857 两个条带(图 2), 未感染腺病毒的 293 细胞提取的 DNA 用相同的引物进行 PCR，未见条带，表明包装的腺病毒载体内携带 HCV C 基因.

2.4 腺病毒载体表达的免疫荧光鉴定　用重组腺病毒感染 HepG2 细胞 72 h 后,以抗 HCV C 单抗为一抗做间接免疫荧光实验, 获得阳性结果(图 3),证明重组腺病毒载体可以在 HepG2 细胞中表达 HCV C 抗原.

2.5 腺病毒载体表达的 Western Blot 鉴定　Western Blot 检测表明, 腺病毒载体可以在HepG2细胞中表达HCV C 抗原, 该抗原可与抗 HCV C 单克隆抗体特异性结合,其分子质量为 22 ku (图 4).

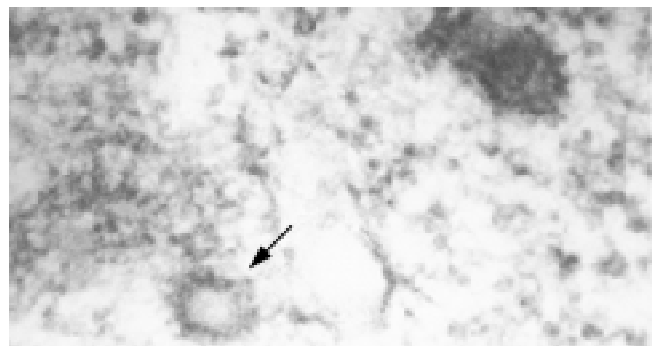
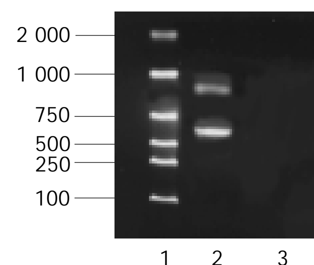


图 1　293 细胞中病毒样颗粒的形成(× 100k)



1:Marker (DL-2000); 2:重组腺病毒载体; 3:阴性对照

图 2　重组腺病毒载体双引物 PCR 鉴定.

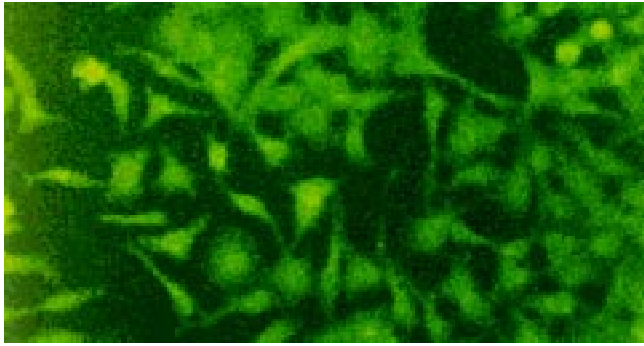
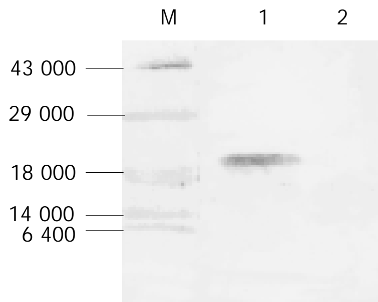


图3 间接免疫荧光法检测HCV C抗原在Hep G2细胞中表达.



M:蛋白质marker;
1:腺病毒感染的HepG2细胞;
2:未感染腺病毒的HepG2细胞
图4 Western Blot法检测HCV C抗原在HepG2细胞中表达.

3 讨论

利用腺病毒具有与细胞表面受体结合并将其基因导入宿主细胞的特性,将控制病毒复制的基因及部分病毒复制非必需基因去除后可制备成腺病毒载体.因腺病毒载体具有宿主广泛、可感染静止及分裂期细胞、包装容量大及不整合等优点[1],成为继逆转录病毒之后被广泛应用的病毒载体.我们构建的pAd.HCV-C是E1区缺失的复制缺陷型腺病毒.由于E1区基因为腺病毒复制所必需,因此需要由转化了E1区基因的包装细胞即293细胞提供反式补偿才能复制和扩增.这种复制缺陷的重组腺病毒仍具有感染靶细胞的能力但不能复制,因而不会直接造成靶细胞的损害.由于腺病毒基因组较大(36 kb),不便直接进行分子克隆,因此,在构建腺病毒载体时,将腺病毒基因组左端制备成含E1区缺失的穿梭质粒(pAd.CMV-Link.1),将目的基因HCV C插入其多克隆位点后(通过酶切、PCR及测序三重鉴定，证实目的基因HCV C已正确插入穿梭质粒pAd.CMV-Link.1中)[14]再与含腺病毒基因组的质粒(pJM17)共转染至293细胞内进行同源重组.由于在pJM17中包装信号(Ψ)缺失不能被包装成病毒[15], 必须与含有包装信号的pAd.CMV-Link.1重组后才能形成感染性病毒,因此,有重组病毒形成后形成的空斑大多为含有目的基因的阳性克隆.本实验中,感染性试验证明了腺病毒载体具有良好的感染活性;电镜鉴定从形态学上展示了腺病毒载体的包装过程;采用插入的HCV C区基因特有引物和腺病毒载体特有引物,进行双引物PCR鉴定后,证明包装的腺病毒载体内携带HCV C基因;用重组腺病毒感染Hep G2细胞后,通过间接免疫荧光实验发现腺病毒载体可以在7721细胞中表达HCV C抗原,证实了其表达功能的完整性.

近年由于血液, 血制品和经血治疗方法的广泛应用及吸毒现象的日益严重, HCV的感染越来越引起人们的重视[16-21].HCV感染常导致慢性肝炎、 肝硬变、甚至肝癌及其他肝外病变[22-33]. HCV基因序列的高度变异性,主要表现为病毒各种基因型, 亚型及准种变异的存在和免疫逃避现象, 使病毒得以逃避宿主免疫系统的作用而造成病毒的持续感染. 由于其极易变异,型别众多,在体内呈准种分布,因而难以治疗及预防[34-36].HCV感染力弱,没有理想的细胞模型,除黑猩猩外也没有方便的动物模型,这使HCV致病机制及疫苗的研究遇到了极大的困难[37].

要获得有效的防治HCV感染的疫苗,必须克服HCV多型、 易变带来的困难. HCV中和抗体位点所在膜区(E区)呈高度变异性[38-45], 机体感染HCV后易发生免疫耐受, 因此, 采用传统方法难以获得对不同HCV株型同时具有防治功效的疫苗. 要克服这种免疫耐受现象, 就必须避开高变区, 选择相对保守区进行实验, 同时诱导机体产生体液免疫及对预防和清除不同株型HCV感染具有重要作用的细胞免疫应答.因此, 本文选用在我国常见的HCV Ⅱ型H株的高度保守区C区基因作为目的基因, 插入到腺病毒表达载体中, 构建了能表达HCV C基因的腺病毒载体，为以后进一步探讨抗HCV重组腺病毒疫苗及T细胞疫苗奠定了基础[46-48].

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