P.O.Box 2345 Beijing 100023, China
Fax: +86-10-85381893
Email: wcjd@wjgnet.com www.wjgnet.com

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HCV-Fc融合基因疫苗真核表达载体的构建及表达

冯志华,王全楚,周永兴,郝春秋,聂青和

冯志华,王全楚,周永兴,郝春秋,聂青和,中国人民解放军第四军医大学唐都医院全军感染病诊疗中心 陕西省西安市 710038
冯志华,女,1964-11-08生,山西省忻州市人,汉族,医学博士,副教授,副主任医师,硕士研究生导师. 主要从事病毒性肝炎治疗方面的研究,发表论文22篇. 参加编写医学专著4部.
国家自然科学基金资助课题, No.30170822
项目负责人:冯志华,710038,陕西省西安市灞桥区新寺路1号,中国人民解放军第四军医大学唐都医院全军感染病诊疗中心. fengzh@fmmu.edu.cn
电话:029-3577742 传真:029-3537377
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Construction and expression of chrimeid plasmid pHCV-IgFc

Zhi-Hua Feng, Quan- Chu Wang, Yong-Xing Zhou,Chun-Qiu Hao, Qing-He Nie

Zhi-Hua Feng,Quan-Chu Wang,Yong-Xing Zhou,Chun-Qiu Hao, Qing-He Nie,The Center of Diagnosis and Treatment of Infection Diseases of PLA, Tangdu Hospital, the Fourth Military Medical University, Xi' an 710038, Shan' xi Province, China
Supported by the National Natural Science Foundation of China, No. 30170822
Correspondence to: Zhi-Hua Feng,The Center of Diagnosis and Treatment of Infection Diseases of PLA,Tangdu Hospital, the Fourth Military Medical University, Xi' an 710038, Shaan' xi Province, China. fengzh@fmmu.edu.cn
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Abstract

AIM:To construct a recombinant cherimal plasmid of HCV-Fc that can express HCV core protein and IgG Fc.

METHODS:The HCV core gene derived from the plasmid pBRTM/HCV1-3011 by using polymerase chain reaction (PCR) was inserted into the backward position of cytomegalovirus (CMV) immediate early promotor element of Fc plasmid (pIgFc), then the recombinant plasmid pHCV-IgFc was obtained.

RESULTS:The insert DNA of pHCV-IgFc was HCV core and Fc gene conformed by endonuclease,PCR and sequencing. HCV core gene and Fc gene expressed transiently with Lipofectamine 2000 coated in human hepatoblastoma 7 721 cells, which was conformed by immunofluorescence.

CONCLUSION:Recombinant cherimal plasmid vector pHCV-IgFc can express HCV core and Fc gene transiently in 7 721 cells. It may be useful in transfection of dendritic cells and development into dendritic cell vaccince.

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摘要

目的:构建能表达HCV C-Fc融合基因的真核表达载体,为进一步修饰和转染树突状细胞,制备能高效表达HCV C和Fc基因的树突状细胞疫苗做准备.

方法:用分别含有*Kpn* Ⅰ和*XhoI* Ⅰ酶切位点的HCV C区基因上、下游引物,以含有HCV H株基因序列的质粒pBRTM/HCV1-3011为模板,通过PCR扩增获得HCV C区基因片段回收后,以*Kpn* Ⅰ和*XhoI* Ⅰ双酶切,定向插入到含IgG1 Fc基因的质粒pIgC3双粘端位点之间,获得重组表达质粒pHCVFc. 通过*Kpn* Ⅰ双位点酶切、PCR及插入片段序列测定对质粒进行了鉴定. 以抗HCV C单克隆抗体为一抗,利用间接免疫荧光法检测了pHCV-IgFc在人肝癌细胞7 721中的瞬时表达.

结果:酶切、PCR及测序鉴定证实,pHCV-IgFc插入片段为HCV C区基因片段,免疫荧光法检测表明其可以在7 721细胞中瞬时表达.

结论:构建的质粒pHCV-IgFc可以在7721细胞中瞬时表达HCV C区基因,为研究HCV C-Fc融合基因修饰的树突状细胞的功能奠定了基础.

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0 引言

丙型肝炎病毒(HCV)感染是当前危害人类健康的重要传染病,至今尚无特异性的预防和治疗措施. 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[1-23]. 树突状细胞具有最强的抗原呈递能力,可广泛用于肿瘤和感染性疾病的免疫治疗,其诱导的强烈的CTL反应在HCV基因治疗中具有很好的应用前景[24-27]. 我们构建了HCV C-Fc融合基因真核表达载体,并证实其可以在7721细胞中瞬时表达HCV C基因,为下一步制备转染和表达HCV C及Fc融合基因的树突状细胞疫苗奠定了基础.

1 材料和方法

1.1 材料 pBRTM/HCV1-3011质粒(HCV-H)含除5'NCR外的全部HCV序列,是由美国华盛顿大学Rice教授惠赠;含Fc基因的质粒载体pIgFc3(Fc上游含*Kpn* Ⅰ,*BamH* Ⅰ单酶切位点)为张新海博士惠赠;大肠杆菌MC1061及人肝癌细胞7 721株均为本室保存. *Kpn* Ⅰ,*BamH* Ⅰ,T4DNA连接酶,Taq DNA聚合酶,dNTP,

PCR产物回收试剂盒及P1asmid Purification Kit均为Promega公司产品. 脂质体Lipofectamie 2000，1 640培养基及胎牛血清均为Gibco公司产品；小鼠抗人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株核心区序列设计l对引物. 根据克隆所需，在每条引物的5端引入相应的限制性内切酶识别序列及保护碱基，采用DNA *SIS*和*OLIGO*核酸分析软件在微机上进行分析. 引物由宝生物(大连)有限公司合成，序列如下：P1 (HCV C基因上游引物，5’端含有*Kpn* Ⅰ位点及起始码) 5’TTG GTACCATGAGC ACGAATCCTAAACCT3’；P2 (HCV C下游引物，5’端含有*BamH* Ⅰ位点及插入碱基以保证下游Fc基因三联密码子顺读)：5’ATTGGATCCTTGGCTGAAGCGGG CCACAGT3’.

1.2 方法

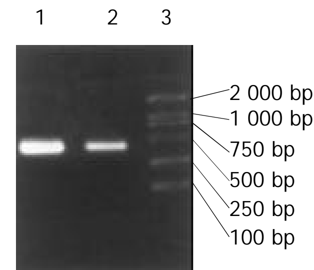
1.2.1 pHCV-IgFc质粒的构建 参照文献. 以pBRTM/HCV1-30ll质粒为模板，用合成的分别含有*Kpn* Ⅰ及*BamH* Ⅰ限制性内切酶识别序列的HCV C区基因上下游引物进行PCR扩增. PCR在100 μL的反应缓冲液中进行(50 mmol/L KCl，10 mmol/L Tris-HCl，pH9.0 25 ℃，1.5 mmol/L $MgCl_2$，1 g/L Triton X-100)，其中含上下游引物各100 pmol，dNTP各25 μL，模板质粒5 μL，*Taq*聚合酶1U，三温循环的程序为：预变性94 ℃ 5 min，变性94 ℃ 45 s，退火55 ℃ 45 s，延伸72 ℃ 1 min，35个循环后，72 ℃再延伸10 min，PCR产物回收(按试剂盒说明书操作)后，用*Kpn* Ⅰ/*BamH* Ⅰ双酶切，获得572 bp的HCV C基因片段，即目的DNA；含Fc基因的载体pIgFc3亦用*Kpn* Ⅰ/*BamH* Ⅰ双酶切，切胶回收获得3 500 bp的载体DNA. 目的DNA与载体DNA在16 ℃下经T4 DNA连接酶连接16 h (连接体系为:目的DNA 0.4 μg，载体DNA 0.1 μg，10×Buffer 2 μL，T4 DNA连接酶16 μL，加无离子水至20 μL)，获得重组质粒. 将重组质粒转化到用氯化钙制备的新鲜感受态大肠杆菌*E.coli* MC1061中，挑取阳性克隆，扩增后提取并纯化质粒，得到含HCVC-Fc融合基因的真核表达质粒pHCV-IgFc.

1.2.2 pHCV-IgFc的鉴定 酶切鉴定:将重组质粒pHCV-IgFc和空载体质粒pIgFc3分别用*Kpn* Ⅰ单酶切. 10 g/L琼质糖凝胶电泳鉴定. PCR鉴定:分别以重组质粒pHCV-IgFc和空载体质粒pIgFc3为模板，用前面合成的HCVC区基因上游及下游引物在相同的反应体系中进行PCR扩增，PCR产物用10 g/L琼质糖凝胶电泳鉴定. 测序鉴定：为确保重组质粒pHCV-IgFc中插入序列及读码框的正确性，对其进行测序鉴定，由宝生物(大连)有限公司的自动测序仪完成.

1.2.3 pHCV-IgFc在人肝癌细胞株772l中的瞬时表达 将重组质粒pAd. HCV-C用lipofectamine 2000转染7 72l细胞，72 h后收集爬片细胞，以小鼠抗HCV-C单克隆抗体为一抗做间接免疫荧光实验，检测重组载体pHCV-IgFc插入的HCV-C基因在人肝癌细胞株7721中的瞬时表达情况.

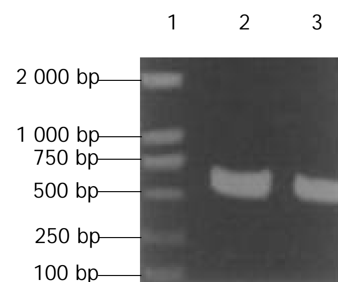
2 结果

2.1 重组质粒pHCV-IgFc的双位点酶切鉴定 插入的HCV-C基因片段含有另一个*Kpn* Ⅰ位点.重组质粒pHCV-IgFc经*Kpn* Ⅰ双位点酶切后，可切下300 bp片段，空载体质粒pIgFc3只用一个*Kpn* Ⅰ酶切位点. 电泳酶切片段，证明插入片段和载体大小均正确(图1):



Line 1,2 重组质粒pHCV-IgFc经Kpn Ⅰ双位点酶切后300 bp片段; Line3 Marker (DL-2000)
图1 重组质粒的酶切鉴定.

2.2 重组质粒pHCV-IgFc的PCR鉴定 利用前面合成的PCR引物，以重组质粒pHCV-IgFc为模板进行PCR[14]，反应产物电泳后发现有572 bp的条带(插入的HCV C基因)存在；对照组用相同的引物. 以空载体质粒pIgFc为模板进行PCR，反应产物电泳后未见条带存在(图2). 证明重组质粒pHCV-IgFc中成功地插入了HCV C基因.



Line l Marker (DL-2000); Line2-3;重组质粒pHCV-IgFc PCR产物572 bp的条带
图2 重组质粒的PCR鉴定.

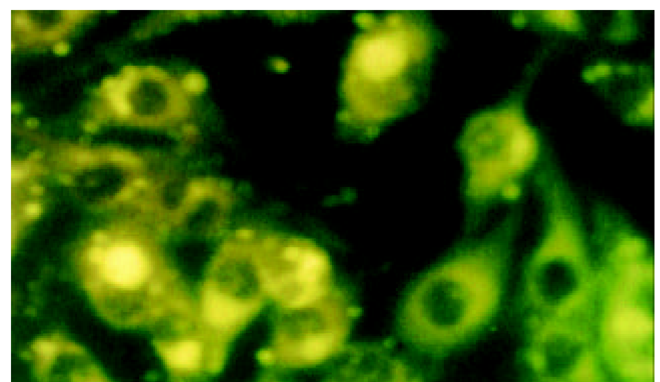


图3 重组质粒pHCV-IgFc在人肝癌细胞株7 721中的瞬时表达.

2.3 重组质粒pAd.HCV-C的测序鉴定 重组质粒pHCV-IgFc由宝生物(大连)有限公司测序，插入片段正是HCVH株C区基因，结果如下:

2.4 重组质粒pHCV-IgFc在人肝癌细胞株7 721中的瞬时表达 转染了重组质粒pHCV-IgFc的人肝癌细胞株7 721细胞，以抗HCV C单克隆抗体为一抗，进行间接免疫荧光检测，获得阳性结果(图3);未转染的7 721细胞进行间接免疫荧光检测，未见阳性结果. 表明pHCV-IgFc在7 721中瞬时表达了HCV C抗原.

3 讨论

丙型肝炎病毒(HCV)感染常导致慢性肝炎、肝硬变、甚至肝癌及肝外病变. HCV基因序列的高度变异性以及机体细胞免疫功能低下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，因而难以治疗及预防. 基因疫苗是将编码抗原的外源基因插入合适的表达载体中， 再以载体免疫机体， 使目的基因在机体内高效表达，达到基因治疗的目的. 丙型肝炎基因疫苗诱导特异性免疫应答产生已被大量的实验室证实[28]. 但普遍存在着抗体滴度不高，CTL杀伤力不强等问题. 其原因可能与HCV感染后，抗原提呈细胞(APC)，尤其是树突状细胞(dendritic cells，DC)数量减少、功能降低有关[29]. 如何绕过或提高DC免疫功能低下, 诱导出更强有力的 、更具广泛性的免疫应答，应当成为基因疫苗和免疫治疗亟待解决的问题之一.

一个理想的抗原呈递策略，应当是全方位、强有力的诱导 CD_8^+CTL、CD_4^+Th及B细胞应答，这样才能更有效的清除病原体及肿瘤细胞. 近年来，以免疫球蛋白(主要是IgG1)的Fc段为基础构建的新型免疫分子进展很快，有望用于新型免疫复合外物制剂的研制. IgG1的Fab与Fc是功能相对独立的 片段，Fab中的可变区负责与抗原结合，而Fc段能与免疫效应细胞的Fc γ受体(Fc γ R)结合，发挥其调节免疫效应、细胞炎症反应、细胞毒效应以及激活吞噬细胞的作用. DC本身表面带有Fc γ R，因此由Fc γ R介导的吞噬细胞内在化的过程可加强免疫应答的 抗原提呈.

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[30-34]. HCV中和抗体位点所在膜区(E区)呈高度变异性，机体感染HCV后易发生免疫耐受. 因此，采用传统方法构建的以产生中和抗体为主的体液免疫型疫苗难以获得对不同HCV株型同时具有防治功效的疫苗. 要克服这种困难，就必须诱导机体产生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[35-39]. Saito *et al* 研究发现，仅用HCV E区基因免疫难以诱导有效的细胞免疫应答. HCV CE1区含有5个CTL表位，以HCV CE1区基因免疫机体，可产生较强的针对不同株型HCV的CTL反应，且杀伤效应至少可持续100 d以上. 因此，我们选用在我国常见的HCV Ⅱ型H株C区基因作为目的基因，插入到含人IgG1 Fc段基因的载体pIGC3中，以此为基础构建了HCV C-Fc的可分泌型真核表达载体pHCV-IgFc. 经双酶切、PCR扩增及测序三重鉴定，证实插入片段为HCV C区基因. 重组质粒pHCV-IgFc在人肝癌细胞7 721中瞬时表达了HCV C抗原，证实了其表达功能的完整性. 以上工作为以后制备能表达HCV C及Fc抗原的树突状细胞疫苗提供了物质保证，为进一步探讨抗HCV基因疫苗及树突状细胞疫苗奠定了基础[40,41].

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