应用核酸适配子检测细胞因子的新方法-ELONA 法

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摘 要 以人肿瘤坏死因子(Human tumor necrosis factor, hTNF-α)特异性的核酸适配子为检测分子建立了酶联寡聚核苷酸吸附试验(Enzyme-linked Oligonucleotide assay, ELONA)方法,用于 hTNF-α 的检测。通过 SELEX(Systematic Evolution of Ligands by Exponential Enrichment)方法从随机 RNA 库中筛选到与 hTNF-α 特异结合的 RNA 适配子。根据其序列,用体外转录方法合成生物素标记的 RNA 适配子,并对其进行了氨基修饰以增加其稳定性。以 hTNF-α 的单克隆抗体为捕获分子,生物素标记的 hTNF-α 特异性 RNA 适配子为检测分子建立了 ELONA 方法,并对这种检测方法的灵敏度、精密度和准确度等进行了分析。同时用 ELONA 和 ELISA 方法检测了正常人血清中的 hTNF-α 水平,并对检测结果进行比较。结果显示,ELONA 方法的灵敏度为 100 pg/mL,具有较好的精密度和准确度。ELONA 法的检测结果与 ELISA 法检测结果基本一致。该方法适用于血清、细胞培养上清等多种生物标本中各种细胞因子及其它蛋白的检测。

关键词 SELEX, hTNF-α, Aptamer, ELONA, ELISA
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指数富集式配基系统进化(Systematic Evolution of Ligands by Exponential Enrichment, SELEX)技术是通过多轮的选择和扩增过程(PCR 或 RT-PCR),从随机寡聚核苷酸库中筛选特异性适配子(Aptamer)的组合化学技术,具有靶分子范围广,筛选出的适配子亲和力和特异性高等特点。Tuerk 和 Gold 于 1990 年创立了基本的 SELEX 方法[1],此后,科研工作者们在各个领域采用此技术进行了广泛的探索和运用,目前,已经建立了大规模自动化筛选技术,可以在短期内筛选到大量的适配子。在 SELEX 技术建立十几年的期间,已经筛选出大量的适配子并开展了应用于临床诊断的研究[2]。与抗体相比,寡聚核苷酸适配子作用的靶分子范围更广,结合能力更强,其解离常数(kd)多在 pmol/L ~ nmol/L 之间,甚至强于天然配基;与靶分子结合的特异性更强;筛选周期更短,并且其筛选过程可以自动化。核酸适配子比抗体的分子小,方便体内影像诊断和治疗;如接上硫代磷酸,可用于细胞内诊断和治疗。在体外化学合成,可以保证时间、质量和数量;特异性和亲和力不受组织或样品中非靶蛋白的干扰;适配子变性与复性可逆且速度快,可反复使用、长期保存和常温运输[3]。因此,凡是涉及抗体的诊断领域,几乎都可以用寡聚核苷酸适配子代替。

hTNF-α 是巨噬细胞/单核细胞活化产生的一种多功能细胞因子,具有广泛的生物学活性,同时 hTNF-α 也是一种重要的致病因子,与许多自身免疫性疾病如:成人类风湿性关节炎(RA)、儿童多发性风湿性关节炎(JRA)、Crohn's 病等密切相关,因此,hTNF-α 水平的测定对这些疾病的诊断和治疗具有重大的指导意义[4]。目前,主要应用双抗体夹心法来检测血液、尿、唾液和其他体液中 hTNF-α 的水平。它以捕捉抗体与固相载体结合,然后加入待检样品,以另一个抗体为检测抗体与待测抗原结合,然后加入酶标记的抗体孵育,最后加入底物进行检测。

本研究利用 SELEX 技术,从随机寡聚核苷酸库中筛选到与 hTNF-α 高特异性结合的 RNA 适配子。以 hTNF-α 单克隆抗体为捕捉抗体,以 2'-氨基修饰的 RNA 适配子为检测物质,建立了检测 hTNF-α 的新方法——ELONA (Enzyme-linked oligonucleotide assay)法。

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1 材料和方法

1.1 材料

DNA模板由大连 TaKaRa 公司合成，氨基修饰的嘧啶核苷酸购于美国 Ambion 公司，生物素标记的尿嘧啶核苷酸购于美国罗氏公司，T7 体外转录试剂盒购于 Promega 公司，寡核苷酸的纯化试剂盒购于 Qiagen 公司，hTNF-α 的 ELISA 检测试剂盒购于 DIAClone 公司，HRP 标记的链亲和素购于中山公司，与 hTNF-α 特异结合的 RNA 适配子 T3.11 由我们实验室通过 SELEX 方法从随机 RNA 库中筛选得到。

1.2 方法

1.2.1 生物素标记的 RNA 适配子的制备：我们通过 SELEX 方法从随机 RNA 库中筛选到与 hTNF-α 特异结合的 RNA 适配子 T3.11，相应的 DNA 模板序列为 5′-TAATACGACTCACTATAGTTCGGCTCCCCCT CATAGACTACTGTTAAGCCTCGAGGT-3′。以带有 T7 启动子的双链 DNA 为模板，加入嘌呤核苷酸和氨基标记的嘧啶核苷酸（其中尿嘧啶核苷酸与生物素标记的尿嘧啶核苷酸的比例为 3∶1），T7 RNA 多聚酶，缓冲液和 RNA 酶抑制剂，37℃孵育 1 h，然后加入 RQ1 DNA 酶 1 μL，37℃孵育 15 min 消化 DNA 模板[5]。转录得到的适配子经聚丙烯酰胺凝胶电泳鉴定后，用 Qiagen 公司的小片段回收试剂盒纯化。

1.2.2 检测 hTNF-α 的 ELONA 法：用包被液（0.05 mol/L，pH9.6 碳酸盐缓冲液）稀释 hTNF-α 单克隆抗体至 1 μg/mL，100 μL/孔包被酶标板，4℃过夜；次日，用洗液（含 0.05% Tween-20 的磷酸盐缓冲液）洗板 3 次，2% 牛血清白蛋白（BSA）于 37℃封闭 1 h，弃封闭液，洗板 3 次[6]。

以磷酸盐缓冲液对 hTNF-α 标准品做 2 倍连续稀释后加入上述包被好的酶标板，100 μL/孔，37℃孵育 1 h；洗板 3 次，每孔加入洗液稀释的生物素标记的适配子（1 μg/mL）于 37℃孵育 1 h；洗板 3 次，每孔加 100 μL 辣根过氧化物酶标记的链亲和素（1∶400 稀释），37℃温育 1 h；洗板 3 次，每孔加 100 μL TMB 和 H_2O_2 底物溶液，室温避光显色 5 min；每孔加 50 μL 2 mol/L 硫酸终止反应，用酶标仪测定 450 nm 时的吸光度值，绘制标准曲线。

分析 ELONA 方法的精密度和准确度时，在包被好的酶标板中加入相应浓度的 hTNF-α 标准品，检测方法同上。根据测得的吸光度值计算相应的 hTNF-α 浓度。检测正常人血清样品的方法同上。每次检测都设立内参照孔和阴性对照孔。

1.2.3 ELISA 法检测 hTNF-α：用包被液稀释 hTNF-α 单克隆抗体包被多孔酶标板，包被方法同上，检测方法按照试剂盒说明书进行操作，用酶标仪测定 450 nm 时的吸光度值。

1.2.4 数据的统计和分析：采用 SPSS 统计软件中方差分析法进行各组数据分析和组间差异的比较。

2 结果

2.1 生物素标记的 RNA 适配子的制备

以合成带有 T7 启动子的 57 个碱基对的双链 DNA 为模板，通过体外转录方法获得生物素标记的 40 个碱基的 RNA 适配子。转录产物的聚丙烯酰胺凝胶电泳鉴定见图 1。结果表明我们通过体外转录方法获得了氨基修饰的生物素标记的适配子。

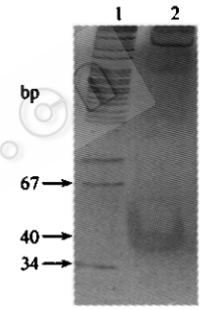

图 1 PAGE 鉴定 RNA 适配子

Fig.1 PAGE of RNA aptamer

1:57 base dsDNA;

2:40 base ssRNA(Product of in vitro transcription)

2.2 ELONA 方法的灵敏度、精密度和准确度分析

用磷酸盐缓冲液对已知浓度的 hTNF-α 标准品进行稀释，每个浓度设置 6 个复孔，使 hTNF-α 的终浓度为 4000 pg/mL、2000 pg/mL、1000 pg/mL、500 pg/mL、250 pg/mL、125 pg/mL、62.5 pg/mL。根据检测结果绘制标准曲线，见图 2。图上显示了每一点误差的均值，变异系数为 4.8%，线性相关系数 r^2 = 0.99。对已知数据进行最佳直线拟合，直线回归方

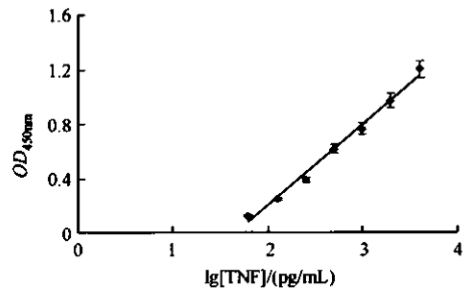

图 2 ELONA 方法检测 hTNF-α 水平的标准曲线

Fig.2 Standard curve of hTNF-α by ELONA

程为 $Y = 0.6882X - 1.0475$。将 hTNF-α 从 4000 pg/mL起倍比稀释,吸光度值大于孔的吸光度值为 N,标准差为 SD,理论灵敏度的吸光度值为 N + 2.5SD。经过计算,它的灵敏度为 100 pg/mL。

对低、中、高三个已知浓度的 hTNF-α 样品进行检测,同样的检测分别在不同的天重复 5 次,见表 1。低浓度组的理论值为 125 pg/mL,均值为 142.8 pg/mL,组内变异和组间变异分别为 3.1%和 6.3%;中浓度组的理论值为 500 pg/mL,均值为 537.2 pg/mL,组内变异和组间变异分别为 3.3%和 4.2%;高浓度组的理论值为 2000 pg/mL,均值为 1902.2 pg/mL,组内变异和组间变异分别为 2.7%和 2.9%。可见三组的变异系数都小于 5%。

我们把已知浓度的 hTNF-α 标准品用磷酸盐缓冲液进行稀释,每个浓度设置 3 个复孔,通过 ELONA 结果计算测得的 hTNF-α 的水平,实测值与理论值的比率为回收率。hTNF-α 的理论浓度从 62.5 pg/mL 到 4000 pg/mL,回收率为 94.97% ~ 121.60%,平均回收率为 106.77%,CV 在 4.20% ~ 6.01%见表 2。结果显示,实测值与理论值很接近,其中 hTNF-α 浓度越高,实测值越接近理论值。hTNF-α 浓度较高时,实测值低于理论值,hTNF-α 浓度较低时,实测值高于理论值。

表 1　hTNF-α ELONA 精度分析

Table 1　hTNF-α ELONA Precision analysis

Reference	Theoretical value/(pg/mL)	Mean/(pg/mL)	Standard deviation	Intra-assay CV	Inter-assay CV
high	2000	1902.2	55.42	2.7%	2.9%
medium	500	537.2	22.68	3.3%	4.2%
low	125	142.8	8.96	3.1%	6.3%

表 2　hTNF-α 标准品回收率分析

Table 2　Parallelism of hTNF-α standard

Theoretical value /(pg/mL)	Assay value /(pg/mL)	Standard deviation	CV/%	Recovery/%
4000	3852.67	161.98	4.20	96.32
2000	1907.67	85.87	4.50	95.38
1000	949.67	43.68	4.60	94.97
500	537.00	29.46	5.49	107.40
250	289.33	12.50	4.32	115.73
125	145.00	8.72	6.01	116.00
62.5	76.00	3.61	4.74	121.60

2.3　人血清样品的检测

分别用 ELISA 和 ELONA 方法检测了 10 个正常人的血清样品中 hTNF-α 水平,结果见表 3。ELISA 方法测得 hTNF-α 水平为 91 ~ 181 ng/mL($n = 10$),ELONA 方法测得 hTNF-α 水平为 107 ~ 202 ng/mL($n = 10$)。ELONA 方法检测结果高于 ELISA 方法。

表 3　ELONA 和 ELISA 方法检测正常人血清中 hTNFα 含量

Table 3　ELONA and ELISA values of hTNFα in normal human sera(pg/mL)

Sample	1	2	3	4	5	6	7	8	9	10
ELONA	148	107	136	199	142	129	168	137	145	202
ELISA	127	91	124	181	129	107	143	117	123	179

3　讨论

在核酸研究早期,DNA 和 RNA 的信息贮存和传递功能备受关注,而对它们的高级结构所发挥的生物功能却鲜有问津,直到核酶和寡聚核苷酸适配子的发现才把研究者的视线转移至后者。近来的研究表明,单链的 DNA 和 RNA 在溶液中能折叠成一定的三维构象,且具有一定的刚性,是许多靶分子理想的高亲和力配基[7]。由于 RNA 分子易形成发卡、口袋、假节、G 四聚体等二级结构,能产生比 DNA 分子更复杂的三维构象,所以筛选随机 RNA 库更易获得与靶分子具有高亲和力的适配子。

本实验以 hTNF-α 的单克隆抗体为捕捉分子,用 SELEX 筛选得到的特异性 RNA 适配子代替抗体做为检测分子,建立了 ELONA 方法。实验结果显示,这种检测方法的准确度、精密度、可重复性很好,该方法的灵敏度为 100 pg/mL。我们用 ELONA 方法和经典的 ELISA 方法检测正常人血清中的 hTNF-α 水平,ELONA 方法检测值偏高,这可能是因为血清中含有一定浓度的 hTNF-α 或者与 hTNF-α 结构类似的蛋白所致。结果提示,我们在筛选适配子的过程中可以以血清为靶物质进行反向筛选,去除与血清特异性结合的适配子,进一步提高检测的特异性。

适配子与单克隆抗体一样,可以作为捕捉分子或检测分子,但同一个适配子不能同时发挥捕捉和检测两方面的作用,因为它们竞争结合配体(抗原)的相同部位。要解决这个问题,可以通过改变筛选

条件和方法或核酸库的类型(RNA/DNA),或通过进一步筛选配基-靶复合物来得到第二个不同的适配子[8]。

寡核苷酸配基作为诊断试剂,单独或与抗体组合应用于多种诊断模式已显示出其独特的优越性,特别是可以弥补抗体在诊断领域中应用的不足[9]。随着SELEX过程的自动化,会有大量的高特异性适配子问世,将在实验诊断中发挥巨大作用。但适配子不可能完全代替已经成熟的抗体技术,两者结合发展会更好的解决诊断实验中的难题。

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Novel Methods to Detect Cytokines by Enzyme-linked Oligonucleotide Assay

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Abstract The development of the systematic evolution of ligands by exponential enrichment (SELEX) process has made it possible to isolate oligonucleotide sequences with the capacity of recognizing virtually any class of target molecules with high affinity and specificity. These oligonucleotide sequences, referred to as "aptamers", are useful as a class of molecules that rival antibodies in diagnostic applications. Aptamers are different from antibodies, yet they mimic properties of antibodies in a variety of diagnostic formats. To meet the shortcomings of antibodies, aptamers have the following advantages. Aptamer does not depend on animals, cells, or even *in vivo* conditions and produced by chemical synthesis with extreme accuracy and reproducibility. Once denatured, functional aptamers could be regenerated easily within minutes. They are stable to long-term storage and can be transported at ambient temperature. We describe here an enzyme -linked oligonucleotide assay that use a SELEX-derived RNA aptamer to detect hTNFα. In order to protect from nuclease attack, the RNA aptamer was modified by replacement of 2'-NH2 for 2'-OH at all ribo-purines. In a sandwich micro-platc assay, hTNFα monoclonal antibody was coated on the surface of the plate, biotin-labeled RNA aptamer was used as a detect molecle. HTNFα was diluted by pooled human serum as standard, and streptavidin-horseradish peroxidase-substrate system was added for detection. Accuracy, precision, sensitivity, specificity of ELONA method were analyzed. The levels of hTNF-α in normal human serum samples were assayed by the ELONA and the ELISA processes. The resultes demonstrate that a sandwich assay using a SELEX-derived RNA aptamer has parameters for accuracy, precision, sensitivity, specificity well within the limits expected of a typical enzyme-linked assay. There is no significant difference between the results of ELONA and ELISA. The minimum detection level was 100 pg/mL. This method will be useful for detection of almost all the cytokines and other protein molecules.

Key words SELEX, hTNF-α, aptamer, ELONA, ELISA

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Humoral Immune Response Elicited by Plasmid DNA Containing HGV E2 Gene Fragment

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Abstract In order to study the feasibility of E2 gene fragment of hepatitis virus G(HGV) as a component of DNA vaccine against the hepatitis virus G infection, a 559bp DNA fragment encoding HGV E2 was cloned into plasmid pCMV-S from pThioHis-E2 in the same reading frame with HBsAg gene to form a recombinant plasmid named pCMV-S-E2. BALB/c mice of Kunming strain were immunized with purified plasmid DNA of pCMV-S-E2 by intra-muscularly inoculation. The immunizations were boosted twice at an interval of 14 days. The whole blood was collected from mice orbit on the day-8 after the last boost. Mice sera were screened by ELISA to determine the humoral immune response using E2-GST fusion protein as the immobilized antigen and the sera from mice immunized with pCMV-S as control. The result indicated that the immunization with plasmid DNA of pCMV-S-E2 could induce quite strong humoral immune response.

Key words DNA vaccine, E2, hepatitis G virus

GBV-C/hepatitis *G* virus (*HGV*) identified in 1995 - 1996 by *Linnen et al*. and *Simons et al*. as a transfusion-transmissible virus is becoming worldwide spread infectious pathogen. The infection causes acute and chronic hepatitis, cirrhosis and liver cancer[1, 2, 3].

The virus is transmitted via the same routes as HCV. The principal transmission routes are blood transfusion, injection of blood derivates and other parenteral ways[1, 2]. The high risk groups are injection drug abusers, patients of hemodialysis, blood donors and recipients of blood derivates and also patients with hemophilia. HGV infection rate presented in blood donors is about 1.5% ~ 1.7%. HGV RNA positive patients were about 3% in the population of hemodialysis patients. Recent research of *Sentjens et al* found that HGV/GB virus could be transmitted by blood in patients undergoing open-heart surgery[4]. HGV transmission between mother and children occurs obviously[5]. Sexual conduct is another possible transmission way[1]. So protecting people from HGV infection becomes an important health issue.

HGV is a positive, single-stranded *RNA* virus. Its genome containing approximately 9400 nucleotides shares about 25% ~ 40% homology to *HCV*, and has a genomic organization resembling that of *flaviviridae*. In analogy with other *flaviviruses*, the viral envelope protein, *E2*, probably locating on the surface of the virus is presumed to play an important role in binding the virus to target cells.

It would be interesting to know whether E2 could induce immune response against HGV infection. The previous study found that the humoral immune response to E2 was associated with losing detectable HGV viraemia[6]. Appearance of E2 specific antibodies might serve as a useful marker for diagnosing recovery from HGV infection[6, 7]. Thus if E2 DNA fragment is introduced into animal body mediated by eukaryotic expression vector, e.g. pCMV-S (known also as pRc/CMV-HBs), the expressed E2 encoded by E2 gene fragment would elicit immune responses which could prevent the immunized animal from HGV infection.

In this work, HGV E2 gene fragment was inserted into plasmid pCMV-S yielding the plasmid pCMV-S-E2, and the immune response induced with pCMV-S-E2 plasmid DNA was characterized. The result showed that the DNA immunization could induce a quite strong humoral immune response.

1 Material and methods

1.1 Material

1.1.1 Strains, plasmids and animal: *Escherichia coli* strain DH5α [supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96

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thi-1 relA1] and *Escherichia coli* BL21 (DE3) strain [hsdSgal (λcIts857 indl sam7 nin51acUV-T7 gene 1)] were preserved in the lab.

Plasmid pThioHis -E2 containing HGV E2 was a gift from Chinese Drug and Biological Product Examination Institute[8]. Plasmid pGEX5X1-E2 containing HGV E2 fragment was constructed by Wang *et al*[9]. Plasmid pCMV-S containing HBsAg gene fragment was a gift given by Victoria Knudson of Aldevron Corp.

Six weeks aged BALB/c mice of Kunming strain were provided by Experimental Animals Centre at Wuhan University.

1.1.2 Reagents: Restriction DNA endonucleases, PCR Kit, T4 phage DNA Ligase and RNase A were purchased from MBI Fermentas and Sino-American Biotechnology Company, respectively.

Reagents for ELISA, purifying GST-E2 fusion protein, and for recovering DNA from agarose gel were prepared according to the methods described previously[10, 11, 12] Polyclonal antibodies against GST and E2 protein were prepared with the way mentioned previously[10, 13, 14, 15].

PCR primers were synthesized by Shanghai Sangon Biotechnology Company.

1.1.3 Instrument: ELISA photometer was purchased from Universal Microplate Reader. Bio-Tek instruments, Inc (type EL × 800). Sonicator for destructing *E. coli* was purchased from Sonics. Vibra cell™. UV-VIS Recording spectrophotometer (Model Ultrospec 3100 pro) was purchased from Amersham Pharmacia Biotech. Refrigerated centrifuge (Model 5810R) was purchased from Eppendorf.

1.1.4 Software for data processing and DNA/protein sequence analysis: Vector NTI 9.0 advanced (InforMax®) purchased from InorMax, Inc. was employed for DNA/protein data processing and analyzing.

1.2 Methods

1.2.1 Construction of recombinant plasmid *pCMV-S-E2*L:

(1) Amplifying E2 DNA fragment from plasmid *pThioHis-E2* by PCR: PCR primers for amplifying E2 fragment from pThio-His-E2 were designed as follows:

Sense primer:

5′-CGA TGA CAA GGT ACC ATG GAT GAG CTC GAG ATC TTC-3′

Kpn I

Anti-sense primer:

5′-CTG GGC CCC CCG TGA GAC CCG CAC C-3′

Apa I

PCR was conducted according to the standard procedure described by (*Sambrook et al*)[16].

(2) Insertion of E2 DNA fragment into *pCMV-S*: A 559bp E2 gene fragment amplified with PCR was digested with *Apa* I and *Kpn* I, recovered from agarose gel[12] and ligated with plasmid pCMV-S linearized with *Apa* I and *Kpn* I[16].

The ligation product was introduced into E. coli DH5α competent cells. Plasmids isolated from several transformants were analyzed with restriction mapping by both *Kpn* I and *Apa* I. Recombinant plasmids containing a 559 bp E2 gene fragment were named as pCMV-S-E2.

1.2.2 Expression and purification of fusion protein GST-E2: The recombinant *E. coli* BL21 (DE3)/pGEX5-X1-E2 was cultured in LB medium, and expression of fusion protein GST-E2 was induced with IPTG. The cells were harvested and sonicated according to the previously described methods[9, 17, 18]; GST-E2 was purified as described in the GST Gene Fusion System manual from Pharmacia Biotech[11].

1.2.3 Immunizing mice with plasmid DNA of pCMV-S-E2:

(1) Preparation of plasmid pCMV-S-E2 DNA for immunization: A large scale of recombinant plasmid pCMV-S-E2 was prepared from a bulk culture of *E. coli* DH5α pCMV-S-E2 in LB medium containing 100μg/mL of ampicillin according to the previously described methods[16, 19, 20]. RNA was removed by treatment with RNase A and minor contaminant proteins were eliminated with Phenol: Chloroform extraction and then mixed with 1/10 volume of 3mol/L sodium acetate and two volume ethanol and stored overnight at − 20 °C. Plasmid DNA was recovered by centrifugation at 10000 × g for 15min. The plasmid DNA pellet was washed with 70% of ethanol then dissolved in physiological salt solution [0.9% of NaCl]. The quantity and purity of isolated plasmid DNA was assessed spectrophotometrically. Purified plasmid DNA was diluted with physiological salt solution to obtain a final concentration of 1 mg/mL.

(2) Immunization of mice with plasmid DNA: Fifteen of six-week old female BALB/c mice of Kunming strain were randomly divided into three groups (five mice each) named as group-A, -B and -C, respectively. In group A, mice were injected with physiological salt solution. The five mice in group B were injected with 50μg of pCMV-S plasmid DNA and mice of group C were injected with 50μg of pCMV-S -E2 plasmid DNA. To improve DNA absorption, 100μL of 2% Neocaine solution was injected into quadriceps of right hind limb of the mice three days before each injection. The immunizations were boosted twice at an interval of 14 days. The whole blood was collected from mice orbit on the day-8 after the last boost.

1.2.4 Determination of the titer of antibodies against protein E2 in the mice sera: Antibody titers of the mice sera were determined by ELISA[10] by using GST-E2 fusion protein purified with Glutathione affinity column chromatography as coated antigen and the sera collected from mice immunized with plasmid DNA of pCMV-S as control. The immuno-reaction between the antibodies and GST-E2 fusion protein was detected with horseradish peroxidase labeled goat-anti-mouse IgG secondary antibodies[14, 19, 21, 22].

2 Results

2.1 Construction of recombinant plasmid *pCMV-S-E2*

In order to create a DNA fragment containing HCV E2 coding sequences for inserting it into *pCMV-S* in the same reading frame with HBsAg, E2 DNA fragment on plasmid pThioHis-E2 was modified with

the way of oligonucleotide mediated mutagenesis by using the designed primer pair. The modified 559bp long *E2* gene fragment digested with *Kpn* Ⅰ and *Apa* Ⅰ was inserted into pCMV-S at the site of *Kpn* Ⅰ and *Apa* Ⅰ (See Figure 1). As shown in Figure 2 plasmid pCMV-S-E2 was successfully constructed and identified with restriction mapping and PCR amplifying using the designed primer pair.

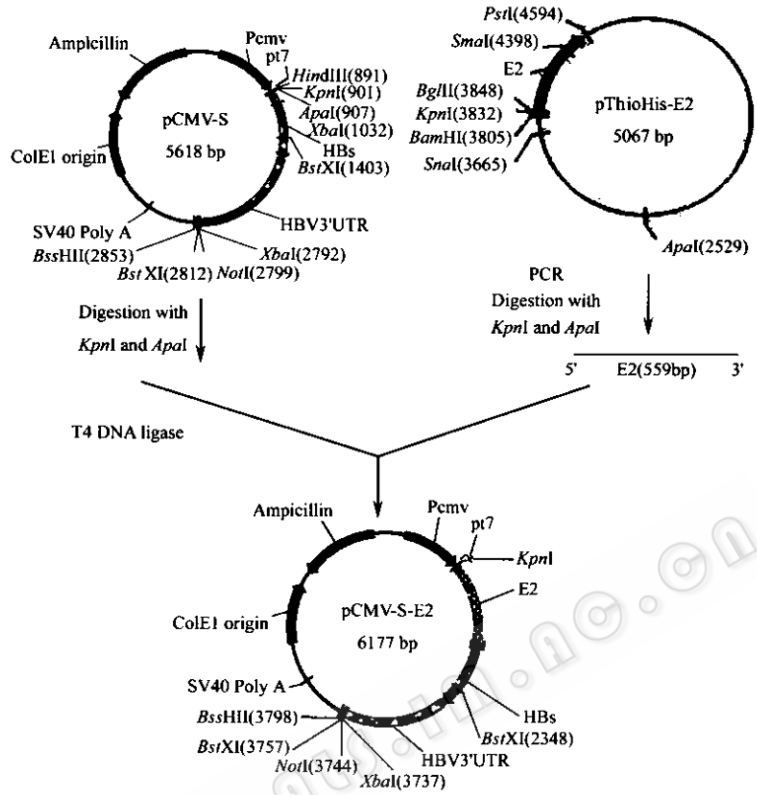

Fig.1 Construction of the recombinant plasmid pCMV-S-E2

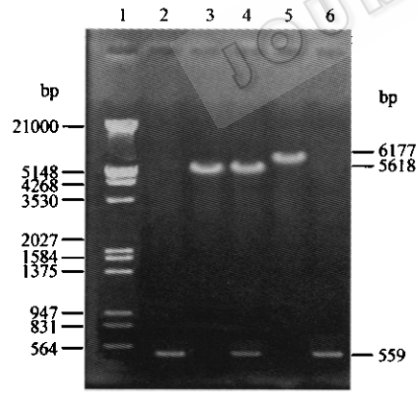

Fig.2 Restriction mapping of the recombinant plasmid of pCMV-S-E2

1: λDNA/*Eco*R Ⅰ + *Hin*d Ⅲ molecular marker;

2: E2 DNA fragment amplified from plasmid DNA of pThioHis-E2 by PCR;

3: plasmid pCMV-S DNA digested with both *Apa* Ⅰ and *Kpn* Ⅰ;

4: plasmid pCMV-S-E2 DNA digested with both *Apa* Ⅰ and *Kpn* Ⅰ;

5: plasmid pCMV-S-E2 DNA digested with *Apa* Ⅰ;

6: E2 DNA fragment amplified from plasmid DNA of pCMV-S-E2 by PCR

To further confirm the correctness of the insertion, plasmid DNA of pCMV-S-E2 was sent to Shanghai Dingan Biology Science Co. LTD for sequencing analysis using the same primer pair. The sequencing verified that HGV E2 gene fragment was correctly inserted into pCMV-S.

2.2 Expression of fusion protein GST-E2 in *E. coli* BL21 (DE3)/ pGEX5X1-E2

The preliminary experiments indicated that the expression level of E2 protein in the prokaryotic system of *E. coli* TOP 10 pThioHis was too low to provide enough amount of purified E2 protein as antigen for detecting antibodies elicited by plasmid DNA of pCMV-S-E2. To overcome this difficulty, plasmid pGEX-5X-1-E2 was employed for expression E2 protein in a form of GST-E2 fusion protein. The purified GST-E2 fusion protein could be detected as a single protein band with appearance molecular weight of 49kD on the SDS-PAGE gel as shown in Figure 3. The molecular weight (MW) of this fusion protein corresponds to the deduced MW of GST-E2 fusion protein and this is also in somehow in conformity with the sum of MW of GST and E2.

To further identify the purified fusion protein and confirm its immunological reactivity, two Western blot experiments were performed using anti-GST and anti-E2 antibodies as probe, respectively. As shown in Figure 4, the anti-GST antibodies could specifically recognize the protein bands located at 49kD either in the lane of affinity chromatography purified sample or in the lane of the

cell lysate of *E. coli* BL21 (DE3)/ pGEX-5X-1-E2 induced with IPTG. Figure 5 indicated that anti-E2 protein antibodies could also specifically recognize the band of the 49 kD GST-E2 fusion protein.

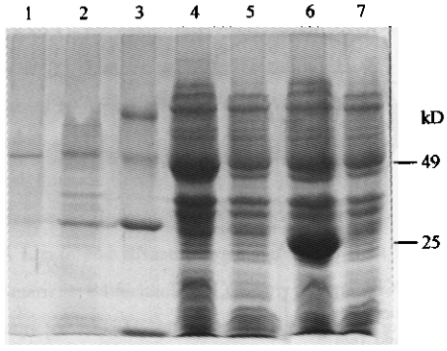

Fig.3 Expression and purification of fusion protein GST-E2

1: GST-E2 fusion protein purified by affinity chromatography

2: cell lysate supernatant of *E. coli* BL21 (DE3)/pGEX-5X-1-E2 induced by IPTG

3: protein MW marker

4: cell lysate of *E. coli* BL21 (DE3)/pGEX-5X-1-E2 induced by IPTG

5: uninduced *E. coli* BL21 (DE3)/pGEX-5X-1-E2 cell lystate

6: cell lysate of *E. coli* BL21 (DE3)/pGEX-5X-1 induced by IPTG

7: uninduced *E. coli* BL21 (DE3)/pGEX-5X-1 cell lystate

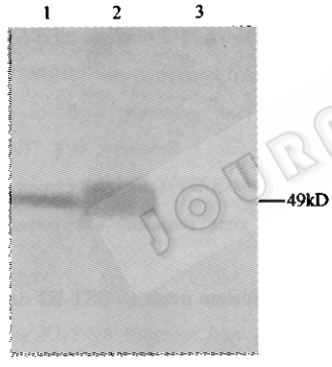

Fig.4 Western Blot probed with anti-GST antibodies to verify authenticity of GST-E2 fusion protein

1: Fusion protein GST-E2 Purified with affinity column chromatography

2: BL21 (DE3)/pGEX-5X-1 E2 cell lysate induced by IPTG.

3: Uninduced *E. coli* BL21 (DE3)/pGEX-5X-1-E2 cell lysate

2.3 Plasmid DNA of pCMV-S-E2 could induce humoral immune response in mice

To verify the immunogenesity of plasmid DNA of pCMV-S-E2, *BALB/c* mice were injected intra-muscularly with plasmid DNA of pCMV-S-E2 and pCMV-S (as control). To determine the purity of the plasmid DNA, ratio of OD_{260} to OD_{280} (as an index of DNA purity) was taken. The ratio was large than 1.8, which indicated that the purity of plasmid DNA was suitable for DNA immunization[19, 23, 24, 25].

Mice sera were collected at the 6th week after first injection and screened for antibody *IgG* class with *ELISA* using the GST-E2 fusion protein as immobilized antigen. As indicated in Figure 6, the level of antibodies against E2 protein in mice inoculated with *pCMV-S-E2* DNA increased obviously comparing with those in mice inoculated with plasmid DNA *pCMV-S*, in which there was no detectable immuno-reaction. To ensure the existence of *anti-E2* antibodies in the mice sera recognized only the portion of E2 in the GST-E2 fusion protein, *GST* protein purified with Glutathione affinity column chromatography as an antigen was also put into the assay. The result indicated that no antibodies against GST were detected see Figure 6.

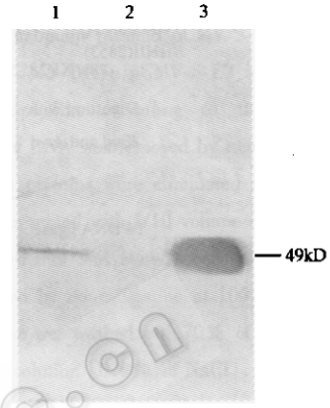

Fig.5 Western Blot probed with rabbit anti -E2 polyclonal antibody to verify authenticity of GST-E2 fusion protein

1: GST-E2 fusion protein purified with affinity column chromatography

2: uninduced *E. coli* BL21 (DE3)/pGEX-5X-1 E2 cell lysate

3: cell lysate of *E. coli* BL21 (DE3)/pGEX-5X-1 E2 induced by IPTG

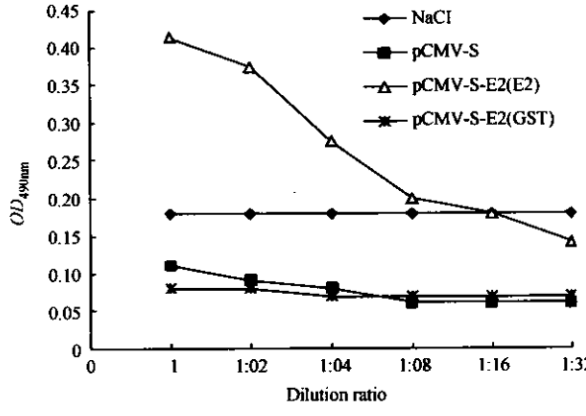

Fig.6 Humoral Immune Response in Immunized mice with either Recombinant Plasmid DNA of pCMV-S or pCMV-S-E2. ELISA was done by using E2-GST Fusion Protein as coated antigen, except that indicated

(Cut-off Value was determined as the average of negative controls = 0.18)

3 Discussion

One of the first advantages of the genetic immunization is that the side effects are almost negligeable, and as far as the data indicate,

have a superior efficacy[30, 31]. DNA vaccine have the added advantages, which are their ease of construction, the low expense of mass production, their high temperature stability, and their ability to induce a full spectrum of exceptionally long-lasting immune responses including cytolytic T cells, they are also immunogenic after oral application[32].

Recently developed DNA vaccine technology has shown to be of inducing specific antibody responses in mice against virus proteins, including influenza virus A, HIV-1 and hepatitis B virus[15]. It has also been reported that this method may have both protective and therapeutic values[28] and may provide an approach to cure persistent viral infections.

In this respect, gene fragment of HGV could be a good candidate for developing DNA vaccine and for its use as an adjunct to antiviral therapy.

In this work, we constructed a recombinant plasmid, pCMV-S-E2, by inserting the HGV envelope protein E2 gene fragment into eukaryotic expression vector pCMV-S under the control of cytomegalovirus (CMV) promoter. Using directly intra-muscularly inoculation in the mice model, humoral immune response was characterized and verified for its value and potential as anti-HGV vaccine. As shown in Figure 6 the level of antibodies against HGV *E2* protein in the mice sera inoculated with plasmid DNA of *pCMV-S-E2* was quite high ($P > 0.18$), which demonstrated that DNA based immunization with plasmid DNA of pCMV-S-E2 induced a satisfying humoral immune response is consistent with the results of previously reported works where plasmid DNA was used as a vaccine[26, 27, 28, 29].

Based on other relevant researches, investigators believe that a strong cellular and humoral immune response would be required to prevent human from infection of HGV and a high efficient vaccine could be formulated by including both plasmid pCMV-S-E2 and E2 protein as the components of a Hepatitis G vaccine. Supported by relevant research achievements, pCMV-S vector in this work seems to be a successful and promising vector for DNA immunization.

This study should be undertaken in nonhuman primate before a vaccine development program settled. Experimentally infected chimpanzees seem to be the most susceptible animal model. Thus, any value of DNA vaccine against HGV infection could be evaluated and demonstrated in nonhuman primate model, like chimpanzee, before vaccine development.

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