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鞭毛蛋白 FliC 突变体 /HPV 18 L2N 融合蛋白的表达与纯化 *

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摘要 目的:人乳头瘤病毒(HPV)的持续性感染导致女性宫颈癌的发生。HPV 的次要衣壳蛋白 L2 可以诱发交叉中和多种型别 HPV 的中和抗体,但是单独免疫 L2 诱发的抗体滴度较低。鼠伤寒沙门氏菌鞭毛蛋白 FliC 是一种有效的佐剂。删除 FliC 超变区域的突变体可与外源抗原融合表达并且显著增强外源抗原特异性抗体的产生。本研究旨在构建鞭毛蛋白 FliC 超变区删除突变体与 HPV 18 L2N(aa.13-154)的融合基因,通过大肠杆菌原核表达系统表达 FliC 突变体与 HPV 18 L2N 的融合蛋白并纯化,为研究鞭毛蛋白的佐剂活性及新型 HPV 18L2 疫苗奠定基础。**方法:**以鼠伤寒沙门氏菌鞭毛蛋白编码基因 fliC 为模板,通过重叠 PCR 法构建删除 fliC D3 区域(fliC△D3)、D3+CD2a 区域(fliC△D3CD2a)、D3+D2 区域(fliC△D2D3)的突变体,同时将 HPV 18 L2N 基因插入置换突变体的超变区删除区域。含有重组基因的表达载体在大肠杆菌中诱导表达, 经 SDS-PAGE 及 Western blot 鉴定分析。表达的融合蛋白经 Ni-Sepharose 亲和层析纯化及 Q-Sepharose 离子交换层析去除内毒素。纯化后的融合蛋白经 Native-PAGE 鉴定分析,通过鲎试剂凝胶法测量蛋白溶液中的内毒素含量。**结果:**构建了 pET22b-fliC△D3/18 L2N、pET22b-fliC△D3CD2a/18 L2N、pET22b-fliC△D2D3/18 L2N 重组载体。重组载体在大肠杆菌以包涵体形式高效表达,且主要以单体形式存在。**结论:**通过原核表达及层析法纯化,成功获得了无热源、高纯度的鞭毛蛋白 FliC 突变体与 HPV 18 L2N 的融合蛋白,为增强 HPV L2 免疫原性提供了一种新的途径,为进一步研制 HPV 18 L2 疫苗奠定了基础。

关键词:鞭毛蛋白;人乳头瘤病毒 18; L2;突变体

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Expression and Purification of FliC Mutant/ HPV 18 L2N Fusion Proteins*

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ABSTRACT Objective: The persistent infection of human papillomavirus causes cervical cancer in women. The minor capsid protein L2 can induce neutralizing antibody that cross-neutralize different HPV genotypes, but L2 alone can only induce low-titer neutralizing antibody. The FliC of Salmonella typhimurium is a potent adjuvant. FliC mutant whose hypervariable region was deleted can express as fusion protein with foreign antigen and enhance production of foreign antigen-specific antibody. This study is to construct fliC hypervariable region deleted mutants and fliC mutant/18 L2N recombinant genes, express fusion proteins of fliC mutants with HPV 18 L2N in E.coil, further to purify them. It established a foundation for studying the adjuvant activity of FliC and a novel HPV 18 L2 vaccine. **Methods:** FliC△D3 (D3 region deleted), fliC△D3CD2a (D3 and CD2a region deleted) and fliC△D2D3 (D2 and D3 region deleted) genes were constructed by overlap PCR based on Salmonella typhimurium fliC gene. HPV 18 L2N gene was fused to replace the internal deleted region of fliC△D3, fliC△D3CD2a, fliC△D2D3. The expression vectors containing recombinant genes were expressed in E.coil and analyzed by SDS-PAGE and Western blot. Fusion proteins were purified by Ni-Sepharose affinity chromatography and endotoxin was removed by Q-Sepharose ion-exchange chromatography. The purified proteins were analyzed by Native-PAGE and residual endotoxin was quantified by LAL test. **Results:** pET22b-fliC△D3/18 L2N, pET22b-fliC△D3CD2a/18 L2N, pET22b-fliC△D2D3/18 L2N recombinant genes were constructed and highly expressed in E.coil as inclusion body. Fusion proteins were validated to be monomeric by Native-PAGE. **Conclusion:** Through prokaryotic expression and purification by chromatography, we successfully obtained apyrogenic FliC mutant/18 L2N monomeric fusion proteins with high purity. This implied a novel way to improve immunogenicity of L2 and laid a basis for its application in the research of a novel HPV 18 L2 vaccine.

Key words: Flagellin; Human papillomavirus 18; L2; Mutant

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前言

宫颈癌是女性全球发病率第二的恶性肿瘤，年发病率约49.3万，死亡27.4万[1]。宫颈癌的发生与高危型人乳头瘤病毒(human papillomavirus，HPV)的持续性感染有关[2]。HPV 18在宫颈癌的中检出率仅次于HPV 16，且HPV 18相关的肿瘤患者的相对死亡率是HPV 16相关肿瘤患者的2.4倍[3]。HPV次要衣壳蛋白L2多肽可诱发产生交叉中和多种HPV的中和抗体[4,5]，但中和抗体滴度较低，因此增强L2的免疫原性是L2疫苗的研究重点。

鞭毛蛋白是细菌鞭毛的主要结构蛋白，可以通过TLR5通路诱导炎症反应和树突状细胞(Dendritic cell，DC)的成熟[6]。鞭毛蛋白可分为D0、D1、D2、D3区域，D0、D1区序列高度保守，D2和D3区域编码序列变异程度较高，为高变区。D1区域含TLR5的识别区域[7]。现有研究表明，抗原序列插入置换鞭毛蛋白高变区形成的融合蛋白可以有效增强抗原的免疫原性[8-10]，但同时也会引发人体强烈的炎症反应[11-13]。

本实验通过overlap PCR法构建三种鼠伤寒沙门氏菌鞭毛蛋白FliC删除不同区域的突变体，将HPV 18 L2N插入置换鞭毛蛋白删除区域，通过大肠杆菌表达并经层析法纯化了三种融合蛋白，为研制HPV 18 L2疫苗及研究鞭毛蛋白的佐剂活性奠定了基础。

1 材料与方法

1.1 材料

含有鼠伤寒沙门氏菌（Salmonella enterica subsp. enterica serovar Typhimurium）鞭毛蛋白fliC基因的pGEX-fliC质粒由北京生命科学研究所邵峰实验室惠赠；HPV 18 L2N原核优化基因由本室构建保存；限制性内切酶NdeI、NheI、AflII、HindIII均购自美国NEB公司；2× Es Taq MasterMix购自中国北京康为世纪生物技术公司；Tryptone及Yeast Extract均购自美国OXID公司；鲎试剂购自中国湛江安度斯生物公司；Ni-Sepharose及Q-Sepharose填料介质购自美国GE公司；质粒小量提取试剂盒及小量琼脂糖凝胶DNA回收试剂盒购自中国北京庄盟国际生物基因科技有限公司；其他试剂均为国产分析纯。

1.2 FliC 突变体 /HPV 18 L2N 融合基因的构建及表达鉴定

1.2.1 FliC 突变体 /HPV 18 L2N 融合基因的构建 通过overlap PCR法构建fliC△D3、fliC△D3CD2a、fliC△D2D3的三种突变体。fliC△D3为fliC删除基因D3编码区域(aa.190-283)突变体；fliC△D3CD2a为fliC删除基因D3+CD2a编码区域(aa.190-344)的突变体；fliC△D2D3为fliC删除基因D2D3编码区域（aa.177-401）的突变体。fliC△D3、fliC△D3CD2a、fliC△D2D3构建方法相似，所用引物列于Table 1。以fliC△D3突变体构建为例，以pGEX-fliC为模板，以fliC△D3(1-189 AA)上下游PCR扩增A片段(fliC aa.1-189)，以fliC△D3(284-494 AA)上下游引物PCR扩增B片段(fliC aa.284-494)。得到的B片段的5'端有21 bp碱基对与A片段是重叠的，利用overlap PCR法，以A片段及B片段PCR产物为模板(各约50 ng)，用fliC△D3(1-189 AA)上游引物和fliC△D3(284-494 AA)下游引物进行overlap PCR反应，扩增fliC△D3基因。三种fliC突变体基因5'均带有NdeI酶切位点，3'端带有HindIII酶切位点，且删除区域加入了NheI、AflII酶切位点，其中fliC△D2D3删除区域加入了两个连续拷贝的柔性肽GGGGS编码基因。将以上fliC突变体通过NdeI及HindIII定向克隆至pET22b载体。18 L2N(aa.13-154)原核优化基因通过NheI及AflII定向克隆至FliC突变体删除区域。构建后的pET22b-fliC△D3/18 L2N、pET22b-fliC △D3CD2a/18 L2N、pET22b-fliC △D2D3/18 L2N经上海生工生物工程公司测序验证正确。

1.2.2 FliC 突变体 /18 L2N 融合基因的表达鉴定 将测序正确的pET22b-fliC△D3/18 L2N、pET22b-fliC△D3CD2a/18 L2 N、pET22b-fliC△D2D3/18 L2N重组质粒分别转化BL21(DE3)感受态细胞，经氨苄青霉素抗性筛选后，挑取阳性单克隆37℃培养过夜。取30 μL培养过夜的工程菌接种至3 mL Amp+ LB液体培养基中，培养至OD600约为1时，加入异丙基1-1硫代-β呋喃半乳糖苷(IPTG)至终浓度为0.5 mM，30℃诱导表达4 h。收集菌体，部分处理后进行SDS-PAGE分析，部分超声破碎后分离上清与沉淀分别进行SDS-PAGE分析，以判断蛋白的可

表1 PCR 扩增 FliC 突变体所用引物列表

Table 1 Oligonucleotide primers designed for PCR amplification for generation of internal deletion FliC mutants.

Forward (5'-3')	Reverse (5'-3')
FliC△D3 (1-189 AA) GGAATTCCATATGGCACAAGTCATTAATAC	AGCATTTGCCTTAAGGCTAGCTCCTGTAACAGTTGCAGC
FliC△D3 (284-494 AA) GCTAGCCTTAAGGCAAATGCTGATTTGACA	CCCAAGCTTACGCAGTAAAGAGAGGAC
FliC△190-344 (1-189 AA) GGAATTCCATATGGCACAAGTCATTAATAC	CGTAGTATTCTTAAGGCTAGCTCCTGTAACAGTTGCAGC
FliC△190-344 (345-494 AA) GCTAGCCTTAAGAATACTACGAAATACACT	CCCAAGCTTACGCAGTAAAGAGAGGAC
FliC△D2D3 (1-176 AA) GGAATTCCATATGGCACAAGTCATTAATAC	CACCCTTAAGGCTAGCCGACCCACCGC- CACCTTGTTGCACATTCAGCGT
FliC△D2D3 (402-494 AA) TCG G CTAGCCTTAAGGGTGGGGGTGGGAGCACAACCA- CCGAAAACCCG	CCCAAGCTTACGCAGT AAAGAGAGGAC

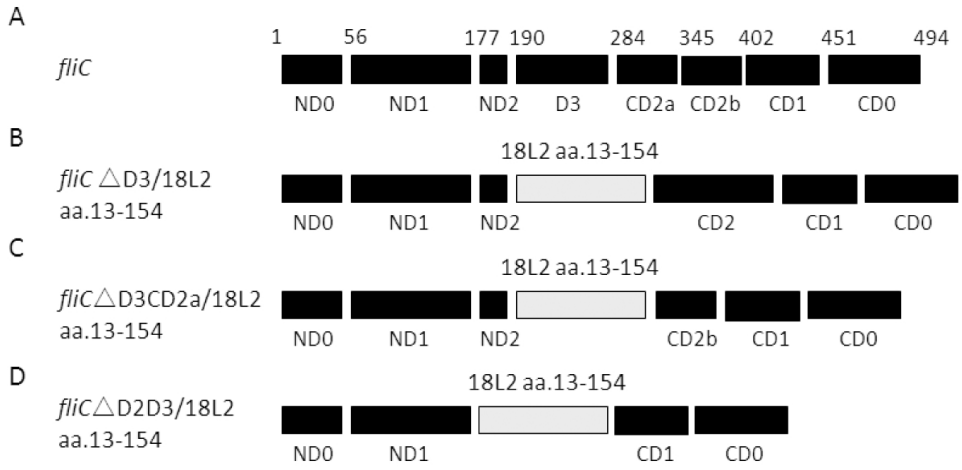

图 1 fliC 及 fliC 突变体 -18L2 N 融合基因示意图

Fig.1 Linear schematic representation of fliC and fliC mutant-18L2N fusion genes

(A) Full-length flagellin coding gene (fliC), beginning amino acid numbers of each domain are given. (B) HPV 18 L2N gene was genetically fused to the the hypervariable region to replace domain 3 of fliC (fliC△D3/18L2N) or D3+CD2a domains of fliC (fliC△D3CD2a/18L2N, C) or D2+ D3 domains of fliC (fliC△D2D3/18 L2N, D)

溶性。SDS-PAGE 电泳后，转印到 PVDF 膜上，用含 5% BSA 的 PBST 室温封闭 1 h 后用小鼠抗 Histag 单克隆抗体（1:5000 稀释）4℃ 孵育过夜。PBST 洗涤 3 次，HRP- 山羊抗小鼠 IgG（1:5000 稀释）室温孵育 1 h，PBST 充分洗涤 3 次后，western blot 试剂盒显色。

1.3 FliC 突变体 /18 L2N 融合蛋白的纯化

由于三种融合蛋白均以包涵体的形式表达，且 C 末端均带有 6× Histag，因此纯化路线相同。取 300 mL 工程菌，经 0.5 mmol/L 的 IPTG，30℃ 诱导 4 h。离心后 PBS 重悬细胞，超声破碎，收集沉淀作为包涵体的粗提物。包涵体粗提物经 20 mL 包

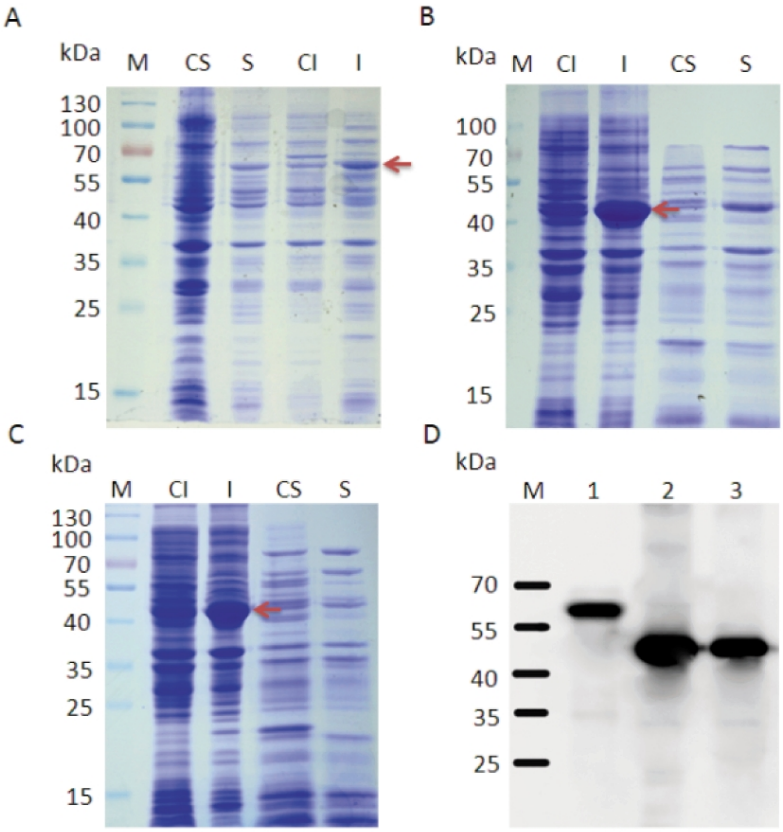

图 2 SDS-PAGE 分析 FliC 突变体 -18 L2N 融合蛋白的表达

Fig.2 SDS-PAGE analyses of the expression of FliC mutant-18 L2N fusion proteins

M: protein marker; C: control; S: fusion protein supernatant fraction; I: fusion protein insoluble fraction; CS: control supernatant fraction; CI: control insoluble fraction. (A) FliC△D3/18 L2 N (B) FliC△D3CD2a/18 L2N (C) FliC△D2D3/18 L2N (D) Western blot analyses of FliC mutant-18 L2N fusion proteins, lane 1: FliC△D3/18 L2N I; lane 2: FliC△D3CD2a/18 L2 N I; 3: FliC△D2D3/18 L2N I

涵体洗涤液（50 mmol/L Tris，100 mmol/L NaCl，10 mmol/L EDTA，0.5% Triton X-100，pH 8.0）洗涤二，加入 20 mL Ni-Sepharose binding buffer（20 mmol/L Na_3PO_4，8 mol/L 尿素，20 mmol/L 咪唑，pH 7.4），变性溶解后按 Ni-Sepharose 操作手册纯化融合蛋白。纯化后的各个组分进行 SDS-PAGE 分析。

1.4 FliC 突变体 /18 L2N 融合蛋白去除内毒素及内毒素检测

由于融合蛋白不结合 Q-Sepharose，主要在流穿中，而内毒素带负电可以结合 Q-Sepharose，因此收集流穿部分就可去除融合蛋白中的内毒素。收集的蛋白溶液采用凝胶法按鲎试剂操作手册测定其中的内毒素含量。鲎试剂灵敏度为 0.125 EU/mL。

1.5 Native-PAGE 检测 FliC 突变体 -18 L2N 融合蛋白

为了检测融合蛋白是否以单体形式存在，对融合蛋白分别进行了三种处理：1、70℃ 加热处理 15 min；2、加入 50 mM DTT，70℃ 加热处理 15 min；3、不加热也不加 DTT。三种融合蛋白均采用以上三种处理方式进行处理，其中 70℃ 加热 15 min 可以解离鞭毛蛋白因疏水作用结合的寡聚体，而加入 DTT 并且加热处理用以确保鞭毛蛋白融合蛋白充分解离，以进行 Native-PAGE 检测。

2 结果

2.1 FliC 突变体 /HPV 18 L2N 融合基因的表达

由于鞭毛蛋白的主要抗原表位存在于鞭毛蛋白超变区（D2、D3 区），因此，为了减弱鞭毛蛋白自身抗原性，我们删除了鼠伤寒沙门氏菌鞭毛蛋白 FliC 部分或全部超变区，构建了三种 fliC 突变体（fliC△D3、fliC△D3CD2a、fliC△D2D3）。同时将 HPV 18 L2N 原核基因插入置换超变区删除区域，构建的三个融合基因如图 1 所示。三种融合基因均能在 BL21（DE3）中高效表达，并且主要以包涵体形式存在（图 2）。

2.2 FliC 突变体 /HPV 18 L2N 融合蛋白的纯化

由于三种融合蛋白 C 末端均带有 6× Histag，因此均可以通过 Ni-Sepharose 亲和层析纯化。以包涵体的形式存在的融合蛋白，通过高速离心及包涵体洗涤液多次洗涤后，其纯度可达 90% 以上，通过 Ni-Sepharose 亲和层析进一步精纯。经 SDS-PAGE 分析发现纯化后的产物只有一条特异性条带（图 3A-C）。融合蛋白经透析后 100%复性可溶（图 3D）。

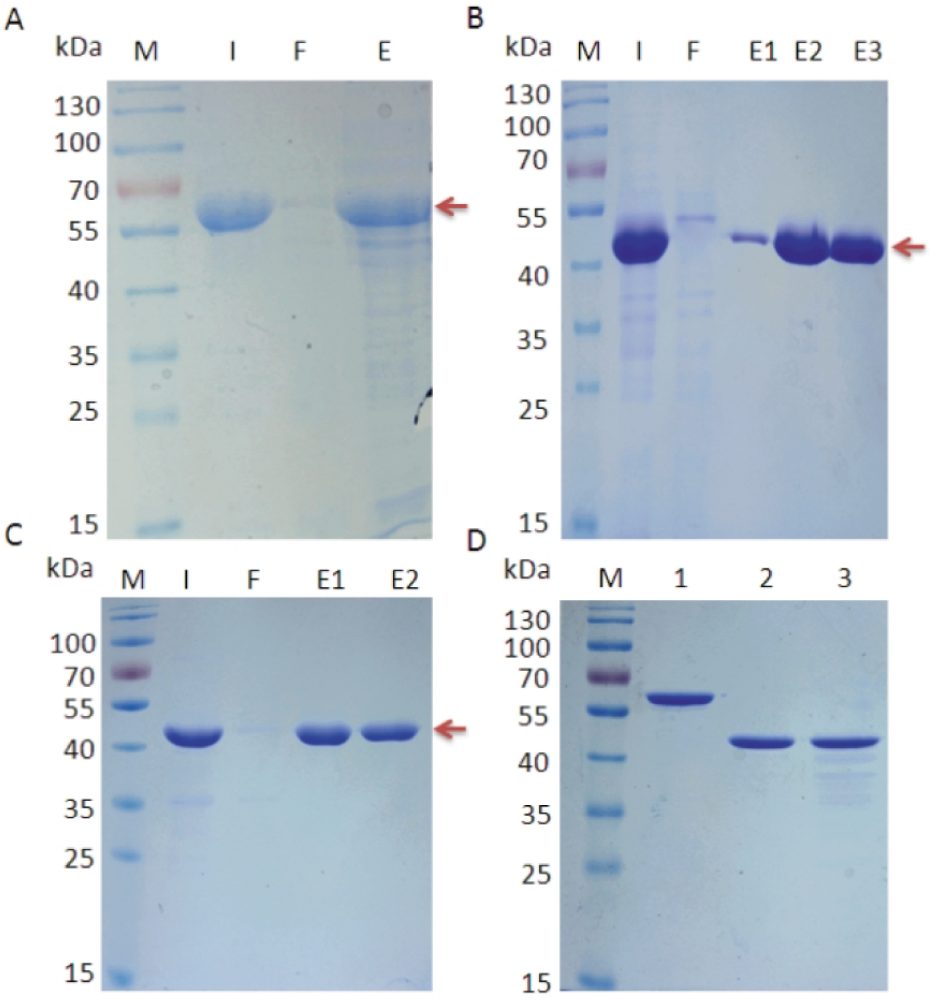

图 3 SDS-PAGE 分析经 Ni-Sepharose 亲和层析纯化后的 FliC 突变体 /18 L2N 融合蛋白

Fig.3 SDS-PAGE analyses of FliC mutant/18 L2N fusion proteins purified by Ni-Sepharose affinity chromatography

M: protein marker; I: fusion protein inclusion body; F: flow through; E: Eluted fraction. (A) FliC△D3/18 L2 N (B) FliC△D3CD2a/18 L2N (C) FliC△D2D3/18 L2N. (D) renatured FliC mutant/18 L2N fusion proteins, M: protein marker; lane 1: FliC△D3/18 L2N; lane 2: FliC△D3CD2a/18 L2N; lane 3: FliC△D2D3/18 L2N

2.3 FliC 突变体 /18 L2N 融合蛋白去内毒素及内毒素检测

用 Q-Sepharose 阴离子交换层析去除内毒素样品经由鲎试剂凝胶法测定分析，结果显示 FliC △D3/18 L2N、FliC △D3CD2a/18 L2 N、FliC△ D2D3/18 L2N 内毒素含量均小于 50 EU/mg。

2.4 Native-PAGE 检测 FliC 突变体 /18 L2N 融合蛋白

Native-PAGE 检测发现 FliC△ D3/18 L2N、FliC△ D3CD2a/18 L2N、FliC△ D2D3/18 L2N 融合蛋白非变性组条带单一，且与相应的加热组及 DTT+ 加热组相比较条带大小一致。说明三种融合蛋白均以单体形式存在，未见寡聚化(图 4)。

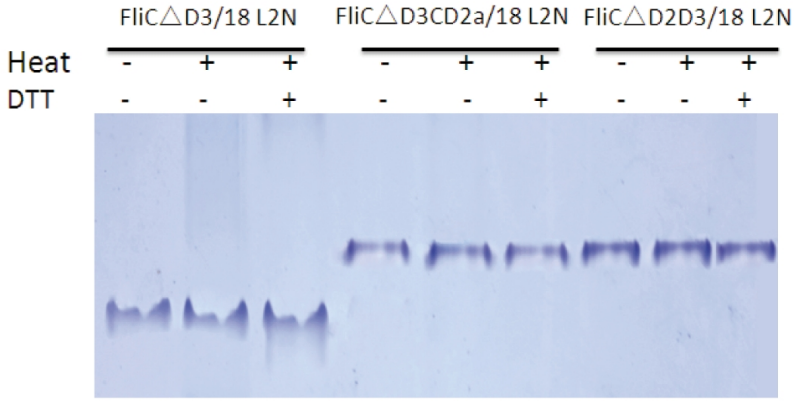

图 4 Native-PAGE 检测 FliC 突变体 /18 L2N 融合蛋白

Fig.4 Native-PAGE analyses of FliC mutant/18 L2N fusion proteins

Heat: heated at 70℃ for 15 min; DTT: reduced with 50 mmol/L DTT

3 讨论

鞭毛蛋白能够有效激活 TLR5，具有良好的佐剂效应。Smith[14]等发现，删除鼠伤寒沙门氏菌鞭毛蛋白 FliC aa.185-306 (D3、D2 部分区域)不会影响 TLR5 识别鞭毛蛋白。Liu G[15]等发现鞭毛蛋白特异性的抗体会阻碍鞭毛蛋白的 TLR5 信号传导，而删除鞭毛蛋白 FliC aa.174-400(D3、D2、ND1 部分区域)可以最大限度降低 FliC 特异性抗体，同时保留 FliC 的佐剂活性。由此可见，FliC 超变区并不是 FliC 的佐剂活性区域。然而，现有研究没有比较删除部分或者全部 FliC 超变区的突变体的佐剂活性，因此我们设计了删除 FliC D3、D3+CD2a、D3+D2 不同区域的三种突变体，为研究新型鞭毛蛋白佐剂奠定了基础。

全长鞭毛蛋白与外源抗原的融合蛋白以可溶性蛋白形式表达，删除超变区的鞭毛蛋白突变体与外源抗原的融合蛋白则主要以包涵体的形式表达[8, 16, 17]。这可能是因为该区域的删除对其鞭毛蛋白的空间折叠产生了影响，造成表达时包涵体的形成。现有研究中鞭毛蛋白与外源抗原的融合蛋白主要通过 Ni-Sepharose 亲和层析及 Q-Sepharose 阴离子交换层析或者凝胶层析纯化[10, 18, 19]。本实验构建的三种融合蛋白均主要以包涵体形式表达，表达量可达菌体总蛋白的 20%~30%，且可以通过简单的洗涤及 Ni-Sepharose 亲和层析纯化。三种融合蛋白均可通过直接透析复性，不需要繁琐的梯度复性，且蛋白 100%复性可溶。经纯化及复性后的蛋白纯度较高，且内毒素含量较低。研究表明鞭毛蛋白经聚合作用形成的纤维状鞭毛蛋白聚合体，其 TLR5 的刺激活性与鞭毛蛋白单体相比降低了 95%[14]。为了鉴定本实验所制备的三种融合蛋白的主要存在形式(单体或者多聚体)，我们对三种融合蛋白分别做了三种处理，经 Native-PAGE 分析发现，三种融合蛋白均主要以单体形式存在。因此本实验构建的三种融合蛋白可以用于后续研究。

综上，本实验成功表达并纯化了 FliC△ D3/18 L2N、FliC△ D3CD2a/18 L2N、FliC△ D2D3/18 L2N 融合蛋白。计划进一步研究三种融合蛋白在小鼠模型中诱发交叉中和抗体的能力，并且检测免疫血清中鞭毛蛋白自身抗体的差异，从而选择一种诱发高滴度交叉中和抗体且低滴度鞭毛蛋白自身抗体的融合蛋白作为新型广谱 HPV 疫苗的候选。同时也可以以此研究删除鞭毛蛋白不同区域对于鞭毛蛋白佐剂活性及自身免疫原性的影响，为今后利用鞭毛蛋白作为佐剂的研究提供理论基础。

参考文献(References)

[1] Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002 [J]. CA Cancer J Clin, 2005, 55(2): 74-108

[2] Bosch FX, Lorincz A, Munoz N, et al. The causal relation between human papillomavirus and cervical cancer [J]. J Clin Pathol, 2002, 55 (4): 244-265

[3] Lombard I, Vincent-Salomon A, Validire P, et al. Human papillomavirus genotype as a major determinant of the course of cervical cancer[J]. J Clin Oncol, 1998, 16(8): 2613-2619

[4] Roden RB, Yutzy WHt, Fallon R, et al. Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes[J]. Virology, 2000, 270(2): 254-257

[5] Pastrana DV, Gambhira R, Buck CB, et al. Cross-neutralization of cutaneous and mucosal Papillomavirus types with anti-sera to the amino terminus of L2[J]. Virology, 2005, 337(2): 365-372

[6] Ramos HC, Rumbo M, Sirard JC. Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa[J]. Trends Microbiol, 2004, 12(11): 509-517

[7] Samatey FA, Imada K, Nagashima S, et al. Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling [J]. Nature, 2001, 410(6826): 331-337

[8] Liu G, Tarbet B, Song L, et al. Immunogenicity and efficacy of flagellin-fused vaccine candidates targeting 2009 pandemic H1N1 influenza in mice[J]. PLoS One, 2011, 6(6): e20928

[9] Mizel SB, Graff AH, Sriranganathan N, et al. Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates[J]. Clin Vaccine Immunol, 2009, 16(1): 21-28

[10] Bargieri DY, Rosa DS, Braga CJ, et al. New malaria vaccine candidates based on the Plasmodium vivax Merozoite Surface Protein-1 and the TLR-5 agonist Salmonella Typhimurium FliC flagellin[J]. Vaccine, 2008, 26(48): 6132-6142

[11] Treanor JJ, Taylor DN, Tussey L, et al. Safety and immunogenicity of a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125) in healthy young adults[J]. Vaccine, 2010, 28(52): 8268-8274

[12] Taylor DN, Treanor JJ, Sheldon EA, et al. Development of VAX128, a recombinant hemagglutinin (HA) influenza-flagellin fusion vaccine with improved safety and immune response [J]. Vaccine, 2012, 30 (39): 5761-5769

[13] Turley CB, Rupp RE, Johnson C, et al. Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults[J]. Vaccine, 2008, 29(32): 5145-5152

[14] Smith KD, Andersen-Nissen E, Hayashi F, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility[J]. Nat Immunol, 2003, 4(12): 1247-1253

[15] Nempont C, Cayet D, Rumbo M, et al. Deletion of flagellin's hypervariable region abrogates antibody-mediated neutralization and systemic activation of TLR5-dependent immunity [J]. J Immunol, 2008, 181(3): 2036-2043

[16] Song L, Zhang Y, Yun NE, et al. Superior efficacy of a recombinant flagellin:H5N1 HA globular head vaccine is determined by the placement of the globular head within flagellin [J]. Vaccine, 2009, 27(42): 5875-5884

[17] Huleatt JW, Jacobs AR, Tang J, et al. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity[J]. Vaccine, 2007, 25(4): 763-775

[18] Huleatt JW, Nakaar V, Desai P, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin[J]. Vaccine, 2008, 26(2): 201-214

[19] Hong SH, Byun YH, Nguyen CT, et al. Intranasal administration of a flagellin-adjuvanted inactivated influenza vaccine enhances mucosal immune responses to protect mice against lethal infection[J]. Vaccine, 2012, 30(2): 466-474

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[4] VEGF Inhibition Study in Ocular Neovascularization (V.I.S.I.O.N.) Clinical Trial Group. Year 2 efficacy Results of 2 randomized controlled clinical trials of pegaptanib for neovascular Age-Related macular degeneration[J]. Ophthalmology, 2006, 113: 1508

[5] 李婧,邢怡桥,贺涛, 等. 姜黄素对氧诱导的视网膜新生血管形成的影响[J]. 中华眼底病杂志, 2010, 26(3): 227-230
Li Jing, Xing Yi-qiao, He Tao, et a1. Effects of carcumin on the oxygen-induced retinal neovasularization[J]. Chin J Ocul Fundus Dis, 2010, 26(3): 227-230

[6] Kim JS, Choi JS, Chung SK. The effect of curcumin on corneal neovascularization in rabbit eyes[J]. Curr Eye Res, 2010, 35(4): 274-280

[7] Takehana Y, Kurokawa T, Kitamura T, et al. Suppression of laser-induced choroidal neovascularization by oral tranilast in the rat [J]. Invest Ophthalmol Vis Sci, 1999, 40: 459-466

[8] Grisanti S, Tatar O. The role of vascular endothelial growth factor and other endogenous interplayers in age-related macular degeneration[J]. Prog Retin Eye Res, 2008, 27: 372-390

[9] Miller JW, Le Couter J, Strauss EC, et al. Vascular endothelial growth factor a in intraocular vascular disease [J]. Ophthalmology, 2013,120 (1): 106-114

[10] Spilsbury K, Garrett KL, Shen WY, et al. Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization [J]. Am J Pathol, 2000, 157(1) :135-144

[11] Ciulla TA, Rosenfeld PJ. Antivascular endothelial growth factor therapy for neovascular age-related macular degeneration[J]. CurrOpin Ophthalmol, 2009, 20: 158-165

[12] Zhou H, Beevers CS, Huang S. The targets of curcumin [J]. Curr Drug Targets, 2011, 12: 332-347

[13] Motterlini R, Foresti R, Bassi R, et al. Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress[J]. Free Radic Biol Med, 2000, 28(8): 1303-1312

[14] Mrudula T, Suryanarayana P, Srinivas PN, et al. Effect of curcumin on hyperglycemia-induced vascular endothelial growth factor expression in streptozotocin-induced diabetic rat retina[J]. Biochem Biophys Res Commun, 2007, 361: 528-532

[15] Premanand C, Rema M, Sameer MZ, et al. Effect of curcumin on proliferation of human retinal endothelial cells under in vitro conditions[J]. Invest Ophthalmol Vis Sci, 2006, 47: 2179-2184

[16] Kulkarni SK, Bhutani MK, Bishnoi M. Antidepressant activity of curcumin: involvement of serotonin and dopamine system[J]. Psychopharmacology, 2008, 201(3): 435-442

[17] Yu WG, Xu G, Ren GJ, et al. Preventive action of curcumin in experimental acute pancreatitis in mouse [J]. Indian J Med Res, 2011, 11 (5): 717-724

[18] Allegri P, Mastromarino A, Neri P. Management of chronic anterior uveitis relapses: efficacy of oral phospholipidic curcumin treatment. Long-term follow-up[J]. Clin Ophthalmol, 2010, 21: 1201-1206

[19] Lal B, Kapoor AK, Agrawal PK, et al. Role of curcumin in idiopathic inflammatory orbital pseudotumours[J]. Phytother Res, 2000, 14: 443 -447

[20] Nita Chainani-WU, D.M.D., M.P. H. Safety and Anti-Inflammatory Activity of Curcumin: A Component of Tumeric [J]. The Journal of Alternative and Complementary Medicine, 2003, 9(1): 191-198