SARS 病毒 S 和 N 蛋白抗原表位的基因合成、融合表达及纯化鉴定

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摘 要 通过计算机分析 SARS 病毒 N 蛋白和 S 蛋白的氨基酸序列,初步确定含强抗原表位的 N 蛋白片段和 S 蛋白片段,共 560 个氨基酸。选择真核和原核生物均偏爱的密码子,化学合成全新的 SARS 病毒 N 蛋白片段和 S 蛋白片段的基因序列,利用基因工程技术将两个基因片段串联,克隆至质粒 pET28a(+)内的 *Nco* Ⅰ/*Eco*R Ⅰ位点,表达 S 蛋白片段和 N 蛋白片段的融合蛋白。将重组质粒转化大肠杆菌 BL21(DE3),筛选获得了高效表达 SARS 病毒 S 蛋白片段和 N 蛋白片段融合蛋白的工程菌,表达的 SARS 病毒的融合蛋白约占菌体蛋白总量的 30%左右,部分以可溶性形式存在。经离子交换柱和反相高压液相纯化获得了表达的融合蛋白,经初步鉴定,显示该融合蛋白有较好的抗原性和特异性。

关键词 SARS 病毒,抗原表位,基因表达,纯化,抗原性

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严重急性呼吸综合症(Severe Acute Respiratory Syndrome,简写 SARS),是一种急性病毒性传染病。SARS 病毒感染后,潜伏期为 10~14d,发病后的 1 周内重症者可发生呼吸窘迫综合症,死亡率可达 15%左右[1-3]。SARS 病毒是一种新型的冠状病毒,由于这种病毒以前未曾感染过人类,所以人群对该病毒普遍易感。SARS 病毒是一种单股正链 RNA 病毒,其全基因序列已经测定[4-6],GenBank 内已公布了病毒全长基因序列,为利用基因工程技术研究诊断试剂、疫苗及筛选抗病毒药物奠定了基础。

SARS 病毒可用 Vero-E6 细胞体外培养,并且已利用培养的 SARS 病毒建立了检测 SARS 病毒抗体的免疫荧光方法[7]和酶联免疫检测法,但是用培养的 SARS 病毒作抗原检测抗体,会产生假阳性,研制特异的基因重组抗原是建立 SARS 病毒特异抗体检测试剂的发展方向。SARS 感染人体后会刺激机体产生多种抗体,所以在检测血清内的 SARS 病毒抗体时可用多种抗原,以提高抗体检出率。根据对已有病毒抗体的检测经验,一般病毒的壳蛋白均有较强的抗原性,在研制检测抗体的试剂时多数选择该蛋白。SARS 病毒的壳蛋白 N 蛋白具有较高的保守性,在各株 SARS 病毒之间变异较小,所以是用作抗原研制 SARS 病毒抗体检测试剂的理想蛋白。SARS 病毒最外层的蛋白决定了 SARS 病毒的型别特异性,所以在选择特异性抗原以及选择用于疫苗的蛋白时均应包含该蛋白。

预防 SARS 感染的最理想途径是注射疫苗,国内外均在积极开展疫苗的研制,尤其对灭活疫苗的研究进展较快,基因工程疫苗是最终的发展方向。SARS 的 S 蛋白,是介导病毒和宿主细胞结合的主要蛋白,封闭该蛋白的结合位点可阻止病毒感染细胞,所以 SARS 病毒的 S 蛋白是研制疫苗的首选蛋白。

本研究通过计算机分析 SARS 病毒 S 蛋白和 N 蛋白的氨基酸序列,初步确定含强抗原表位的 SARS 病毒的 S 蛋白片段和 N 蛋白片段,选择真核和原核生物均偏爱的密码子,化学合成全新的 S 蛋白片段和 N 蛋白片段的基因序列,将两个基因片段串联,利用基因工程技术表达 S 蛋白片段和 N 蛋白片段的融合蛋白,表达的融合蛋白可用于 SARS 病毒抗体或抗原的检测等研究。

1 材料与方法

1.1 材料

1.1.1 菌种与质粒

宿主菌 BL21(DE3)及表达载体 pET28a(+)为美国 Novagen 公司产品。

1.1.2 分子生物学试剂

限制酶 *Nco* Ⅰ、*Bam*H Ⅰ、*Eco*R Ⅰ及 T4 DNA 连

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接酶为TaKaRa公司产品。质粒纯化试剂盒及从琼脂糖凝胶内回收DNA片段的试剂盒为TaKaRa公司产品。S-Sepharose FF阳离子凝胶为Pharmacia公司产品,POROS 10R1凝胶为ABI公司产品,DTT及IPTG为TaKaRa公司产品。其它试剂为进口或国产分析纯试剂。

1.1.3 抗SARS病毒抗体阳性血清及正常人血清:GBI公司生产的抗SARS病毒抗ELISA试剂盒内的阳性对照,及16份灭活的SARS病毒抗体阳性血清。正常人血清由本实验室保存。

1.1.4 酶联反应材料:酶联板为深圳产96孔板,辣根过氧化物酶(HRP)标记的鼠抗人μ链单克隆抗体购自Sigma公司。其它材料为酶联反应的常规材料。

1.2 方法

1.2.1 基因片段的合成:由大连TaKaRa公司帮助合成。

1.2.2 基因克隆方法:DNA的酶切、连接、电泳;质粒的提取、转化;蛋白的SDS-PAGE分析等一般分子克隆方法按常规方法[8]进行。其它试剂盒按说明书进行操作。

1.2.3 DNA序列分析:用TaKaRa公司质粒纯化试剂盒纯化质粒,用DNA全自动测序仪测序。

1.2.4 表达SARS病毒S蛋白和N蛋白的融合蛋白工程菌的诱导表达及超声裂解。

从接种有5号工程菌的LB平板上,用牙签挑单菌落,接种到含200mL LB液体培养基的三角烧瓶内,加卡那霉素至终浓度60μg/mL,置37℃摇床内培养过夜。次日,将菌液接种到4个分别含200mL LB液体培养基的三角烧瓶,每瓶接种菌液50mL,置37℃摇床内振荡培养1h,然后加IPTG至终浓度0.1mmol/L,30℃诱导表达2h。

将诱导表达融合蛋白的1000mL工程菌离心(8000r/min、10 min、4℃)收集菌体重悬于100mL的细菌裂解液(20 mmol/L PB pH7.4、10 mmol/L EDTA、1 mmol/L DTT、5% 甘油)内,冰浴超声破菌10 min,离心(12000 r/min、30 min、4℃)收集上清和沉淀。

1.2.5 表达SARS病毒S蛋白和N蛋白的融合蛋白的硫酸胺沉淀及包含体溶解:向上清内加入适量饱和硫酸铵溶液,边加边搅拌,使硫酸铵的终浓度为50%,置冰浴内过夜。次日离心(12000 r/min、20 min、4℃)收集沉淀。用500mL平衡液(20 mmol/L PB pH7.4、0.5 mmol/L EDTA、0.2 mmol/L DTT)悬浮沉淀,装入透析袋内,对平衡液透析过夜。离心(12000 r/min、20 min、4℃)收集上清,直接上S-Sepearse FF阳离子柱纯化。包含体用0.5%的Triton X-100溶液超声洗涤2遍,用8mmol/L尿素溶液悬浮,室温溶解1h,离心收集上清,过滤后上S-Sepearse FF阳离子柱纯化。

1.2.6 S-Sepearse FF阳离子柱纯化:用平衡液(20 mmol/L PB、pH7.4、8 mol/L 尿素、0.5 mmol/L EDTA、0.2 mmol/L DTT)冲洗平衡S-Sepharose FF阳离子柱,上样流速1.5 mL/min。上样结束后用平衡液充分洗柱,再依次用含梯度浓度NaCl的平衡液洗脱蛋白,收集各洗脱峰蛋白,用12% SDS-PAGE电泳检测各峰蛋白,确定哪个洗脱峰含表达的SARS病毒S蛋白和N蛋白的融合蛋白。

1.2.7 反相高压液相纯化:先对S-Sepearse FF阳离子柱纯化的S蛋白和N蛋白的融合蛋白透析,透析液为0.5%乙酸,透析后冷冻干燥。此时,从细菌裂解上清及从包涵体内纯化的融合蛋白合并在一起。用0.5%乙酸溶解适量冻干的S蛋白和N蛋白的融合蛋白,上反相高压柱(4.6 × 100mm ,POROS 10R1凝胶,ABI公司产品)进行纯化,上样后用20%乙腈、0.1%三氟乙酸溶液洗柱,流速为3.0mL/min,然后用20%~80%的乙腈溶液梯度洗脱,收集洗脱主峰,冻干保存-20℃。

1.2.8 ELISA试验:采用间接ELISA,用纯化的表达SARS病毒S蛋白和N蛋白的融合蛋白做抗原,检测血清中的抗SARS病毒抗体(IgG或IgM)。用50mmol/L碳酸盐溶液(pH9.6)稀释SARS病毒的S蛋白和N蛋白的融合蛋白,包被酶联板,每孔100μL,4℃过夜。检测步骤参见文献[9]。

2 结果

2.1 SARS病毒S蛋白和N蛋白抗原表位的筛选及其基因片段的化学合成

利用ANTHEWIN等软件,通过计算机分析SARS病毒S蛋白和N蛋白的氨基酸序列(GenBank, No.AY278488),发现N蛋白的N端第1个氨基酸至第258个氨基酸含有较强的抗原决定簇,S蛋白接近N端的第162个氨基酸至第460个氨基酸含有较强的抗原决定簇。选择真核和原核生物均偏爱的密码子,分别化学合成包含上述抗原决定簇的S蛋白和N蛋白的基因序列。在合成的N蛋白基因片段的5′端增加了*Nco* Ⅰ酶切位点(下画线部分)及8个保护碱基(GCGAGTCACC),并在起始密码子ATG之后插入了一个密码子GGA,在3′端增加了*Bam*HⅠ酶切位点(下画线部分)及3个保护碱基(GCG)。在

合成的S蛋白基因片段的5′端增加了 *Bam*HⅠ酶切位点(下画线部分)及两个保护碱基(GT),在3′端增加了终止密码子(TAA)和 *Eco*RⅠ酶切位点(下画线部分)及两个保护碱基(AC)。使合成的两个基因片段易于串联克隆至质粒 pET28a(+)内的 *Nco*Ⅰ和 *Eco*RⅠ酶切位点内。

化学合成的含SARS病毒N蛋白抗原表位基因的DNA序列(796 bp):

GCG AGT CAC CAT GGG ATC TGA TAA CGG TCC GCA GTC AAA CCA ACG TAG TGC CCC CCG CAT TAC ATT TGG TGG ACC CAC AGA TTC AAC TGA CAA TAA CCA GAA TGG AGG ACG CAA TGG GGC AAG GCC AAA ACA GCG CCG ACC CCA AGG TTT ACC CAA TAA TAC TGC GTC TTG GTT CAC AGC TCT CAC TCA GCA TGG CAA GGA GGA ACT TAG ATT CCC TCG AGG CCA GGG CGT TCC AAT CAA CAC CAA TAG TGG TCC AGA TGA CCA AAT TGG CTA CTA CCG AAG AGC TAC CCG ACG AGT TCG TGG TGG TGA CGG CAA AAT GAA AGA GCT CAG CCC CAG ATG GTA CTT CTA TTA CCT AGG AAC TGG CCC AGA AGC TTC ACT TCC CTA CGG CGC TAA CAA AGA AGG CAT CGT ATG GGT TGC AAC TGA GGG AGC CTT GAA TAC ACC CAA AGA CCA CAT TGG CAC CCG CAA TCC TAA TAA CAA TGC TGC CAC CGT GCT ACA ACT TCC TCA AGG AAC AAC ATT GCC AAA AGG CTT CTA CGC AGA GGG AAG CAG AGG CGG CAG TCA AGC CTC TTC TCG CTC CTC ATC ACG TAG TCG CGG TAA TTC AAG AAA TTC AAC TCC TGG CAG CAG TAG GGG AAA TTC TCC TGC TCG AAT GGC TAG CGG AGG TGG TGA AAC TGC CCT CGC GCT ATT GCT GCT AGA CAG ATT GAA CCA GCT TGA GAG CAA AGT TTC TGG TAA AGG CCA ACA ACA ACA AGG CCA AAC TGT CAC TAA GAA ATC TGC TGC TGA GGC ATC TAA AAA GGG ATC CGC G

化学合成的含SARS病毒S蛋白抗原表位基因的DNA序列(916 bp):

GTGGATCC GAA TAC ATC TCT GAC GCA TTC TCT CTG GAC GTT TCC GAA AAG TCT GGT AAC TTC AAA CAC CTG CGC GAG TTC GTG TTT AAA AAC AAA GAC GGT TTC CTG TAC GTT TAC AAG GGC TAC CAG CCG ATC GAC GTA GTT CGT GAC CTG CCG TCT GGT TTT AAC ACT CTG AAA CCG ATC TTC AAG CTG CCG CTG GGT ATT AAC ATC ACT AAC TTC CGC GCT ATC CTG ACT GCT TTC TCT CCG GCT CAG GAC ACT TGG GGC ACT TCT GCT GCA GCC TAC TTC GTT GGC TAC CTG AAG CCA ACT ACC TTT ATG CTG AAG TAC GAC GAA AAC GGT ACT ATC ACT GAT GCT GTT GAC TGC TCT CAG AAC CCA CTG GCT GAA CTG AAA TGC TCT GTT AAG AGC TTT GAG ATC GAC AAA GGT ATT TAC CAG ACC TCT AAC TTC CGT GTT GTT CCG TCT GGT GAC GTT GTG CGT TTC CCT AAC ATC ACT AAC CTG TGC CCG TTT GGT GAA GTT TTC AAC GCT ACT AAA TTC CCT TCT GTC TAC GCA TGG GAG CGT AAA AAA ATT TCT AAC TGC GTT GCT GAT TAC TCT GTG CTG TAC AAC TCT ACC TTT TTC TCT ACC TTC AAG TGC TAC GGC GTT TCT GCT ACT AAG CTG AAC GAC CTG TGC TTC TCC AAC GTT TAC GCA GAT TCT TTC GTA GTT AAG GGT GAT GAC GTA CGT CAG ATC GCT CCA GGT CAG ACT GGT GTT ATC GCT GAC TAC AAC TAT AAA CTG CCG GAC GAT TTC ATG GGT TGC GTT CTG GCT TGG AAC ACT CGT AAC ATT GAC GCT ACT TCT ACT GGT AAC TAC AAC TAC AAA TAT CGT TAC CTG CGT CAC GGC AAA CTC CGT CCG TTC GAA CGT GAC ATC TCT AAC GTG CCG TTC *TAA* GAATTCAC

2.2 表达SARS病毒S蛋白片段和N蛋白片段的融合蛋白重组质粒的构建

提取质粒 pET28a(+),用 *Nco*Ⅰ和 *Eco*RⅠ双酶切,电泳后回收酶切的质粒大片段,溶于去离子水内。用 *Nco*Ⅰ和 *Bam*HⅠ双酶切化学合成的SARS病毒N蛋白基因片段,用 *Bam*HⅠ和 *Eco*RⅠ双酶切化学合成的SARS病毒S蛋白基因片段,分别电泳回收后,溶于去离子水内。

取等摩尔浓度的上述3种酶切后DNA片段,在同一离心管内用T4 DNA连接酶连接,使SARS病毒N蛋白基因片段与S蛋白基因片段经 *Bam*HⅠ酶连接后,插入到载体 pET28a(+)内的 *Nco*Ⅰ和 *Eco*RⅠ位点之间,表达一个SARS病毒S蛋白片段与N蛋白片段的融合蛋白。构建流程见图1。

2.3 重组质粒的筛选与鉴定

将上步连接的重组质粒转化到大肠杆菌BL21(DE3),将转化产物涂布含卡那霉素(60μg/mL)的固体LB培养基上,置37℃培养过夜。次日随机挑选8个转化子菌落(分别标记为1~8号),分别接种到含4 mL液体LB培养基(含卡那霉素 60μg/mL)的试管内,置37℃振荡培养6h,取菌液1mL,离心收菌。分别用50μL去离子水悬浮菌体,沸水煮5min,离心(4℃,12 000r/min)5min,取上清(内有质粒)1μL用作PCR模板,用N蛋白基因片段5′端的引物(5′-GCGAGTCACCATGGGATCTGATAACGGTCCGCAGTCA-AACCAACG-3′)和S蛋白基因片段3′端的引物(5′-GTGAATTCTTAGAAAGGCACATTAGATATGT-3′)组成引物对,PCR扩增连接在一起的SARS病毒N蛋白基因片段与S蛋白基因片段,含串联两个基因片段的阳性重组质粒,应扩增出长约1701bp的串联基因片段。PCR反应浓度为:质粒模板1μL、上下游引物各1μL、10×Buffer 5.0μL、2.5 mmol/L dNTP 4.0μL、

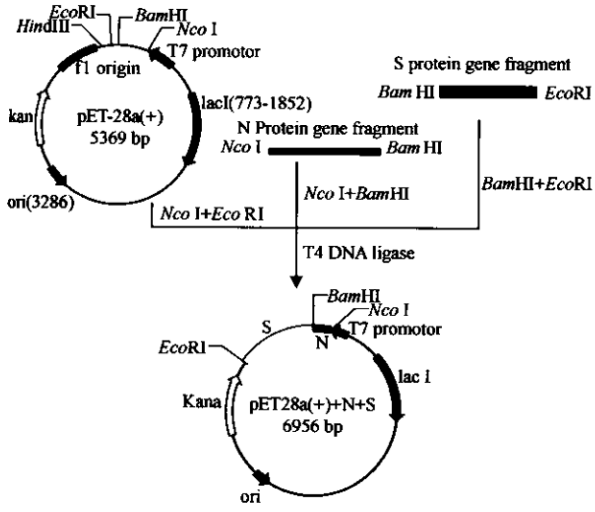

图1　表达SARS病毒的S蛋白片段和N蛋白片段的融合蛋白的重组质粒构建流程图

Fig. 1　Construction of the recombinant plasmid expressing the chimeric protein of SARS virus S and N protein

Taq DNA 聚合酶 0.5μL (2.5 u)、去离子水 37.5μL,总体积 50μL。扩增条件为:94℃ 30s、60℃ 30s、72℃ 60s,30 个循环;最后 72℃延伸 7min。取 PCR 扩增产物 5μL,用 1.2% 的 Agarose 凝胶检测,结果,8 个转化子中,第 2、3、4、5、6 号 5 个转化子扩增出 1701bp 的目的基因片段(见图 2),而第 1、7、8 号 3 个转化子没有扩增出该基因片段。初步证实,有 5 个转化子含有 SARS 病毒 N 蛋白基因片段与 S 蛋白基因片段的串联基因。

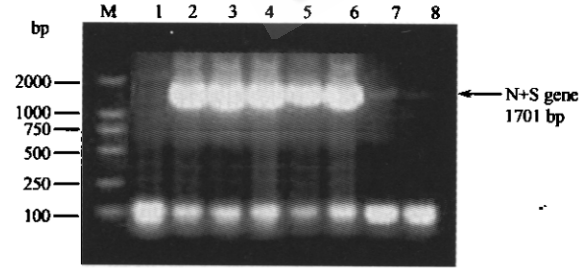

图2　用1.2%的Agarose凝胶检测8个转化子的PCR扩增物

Fig. 2　Analysis of the PCR products from the 8 transformants on 1.2% agarose gel

M: DNA markers (DL2000, TaKaRa); 2, 3, 4, 5, 6: PCR products from No. 2, 3, 4, 5, 6 transformants respectively; 1, 7, 8: The PCR products from No. 1, 7, 8 transformants respectively

提取 5 号重组子的质粒,测定质粒内的 SARS 病毒 N 蛋白基因片段与 S 蛋白基因片段的串联基因序列,DNA 序列分析证实,重组质粒含有串联的 SARS 病毒 S 蛋白基因片段和 N 蛋白基因片段,序列完全正确。

构建的重组质粒表达 SARS 的病毒 S 蛋白片段和 N 蛋白片段的融合蛋白,全长 560 个氨基酸,在其 N 端为 N 蛋白片段,长 259 个氨基酸,C 端为 S 蛋白片段,长 299 个氨基酸,两者之间由甘氨酸和丝氨酸两个氨基酸连接。

2.4　表达融合蛋白工程菌的筛选鉴定

将上述 8 个转化子接种至含 3mL LB 培养基(含卡那毒素 60μg/mL)的试管内,37℃振荡培养 3h,加 IPTG 至终浓度 0.5mmol/L,继续振荡培养诱导 6h,离心收集菌体进行 SDS-PAGE 检测,第 2、4、5、6 号 4 个转化子表达相对分子量约为 60 kD 的 SARS 病毒 S 蛋白和 N 蛋白的融合蛋白,而第 1、3、7、8 号 4 个转化子无此蛋白带(图 3)。

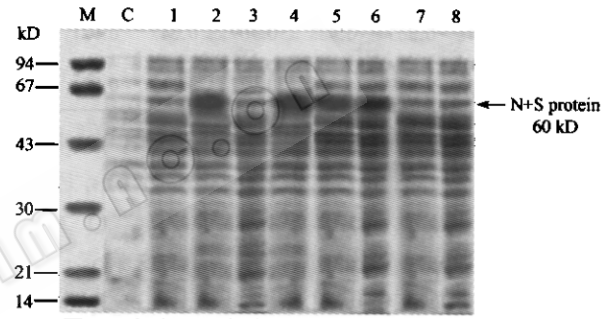

图3　表达S蛋白片段和N蛋白片段融合蛋白的SDS-PAGE分析结果

Fig. 3　SDS-PAGE analysis of the expressed chimeric protein of S and N protein

M: protein markers (Pharmacia); C: the control *E. coli* BL21(DE3); 2, 4, 5, 6: No. 2, 4, 5, 6 transformants expressing the chimeric protein (60kD, indicated with arrow); 1, 3, 7, 8: No. 1, 3, 7, 8 transformants do not express the chimeric protein

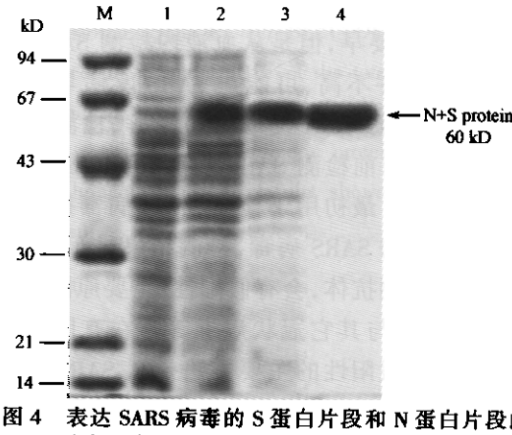

图4　表达SARS病毒的S蛋白片段和N蛋白片段的融合蛋白纯化前后的SDS-PAGE分析结果

Fig. 4　SDS-PAGE analysis of the expressed chimeric protein of SARS virus S and N protein before and after purification

M: protein markers (Pharmacia); 1: control *E. coli* BL21 (DE3); 2: engineered *E. coli* expressing chimeric protein of SARS virus S and N protein; 3: lysate supernatant of the engineered *E. coli* expressing the chimeric protein; 4: purified chimeric protein of SARS virus S and N protein with S Sepharose FF cation exchange column and RP-HPLC

2.5 表达 SARS 病毒 S 蛋白和 N 蛋白的融合蛋白的纯化

将经 S-Sepharose FF 阳离子柱和反相高压液相纯化的融合蛋白进行 SDS-PAGE 分析，结果显示，纯化的 SARS 病毒 S 蛋白和 N 蛋白的融合蛋白显色一条很浓的约 60 kD 蛋白带(见图 4)，无其它蛋白带。纯化的 SARS 病毒 S 蛋白和 N 蛋白的融合蛋白与已知不同浓度的牛血清白蛋白(第五部分)共同电泳、显色后比较，确定纯化的 SARS 病毒的 S 蛋白和 N 蛋白的融合蛋白浓度约为 0.5 mg/mL。

2.6 SARS 病毒 S 蛋白和 N 蛋白的融合蛋白的抗原性鉴定

用 50 mmol/L 碳酸盐溶液(pH9.6)1:500 稀释纯化的 SARS 病毒的 S 蛋白和 N 蛋白的融合蛋白，包被酶联板，检测已知 16 份抗 SARS 病毒 IgG 抗体阳性血清和 1 份抗 SARS 病毒 IgM 抗体阳性血清。结果(表 1)显示，16 份抗 SARS 病毒抗体阳性血清均出现显色反应，1 份抗 SARS 病毒抗体 IgM 阳性血清也出现显色反应。同时检测了 180 份正常人血清，它们的 A_{450} 值均小于 0.05。说明 SARS 病毒的 S 蛋白和 N 蛋白的融合蛋白具有较好的抗原性和特异性。

表 1 用纯化融合蛋白做抗原 ELISA 检测 16 份 SARS 病毒 IgG 抗体阳性血清结果(A_{450})

Table 1 ELISA detection of 16 anti- SARS virus IgG positive sera using the purified protein (A_{450} value)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0.240	0.364	0.503	0.488	0.709	0.547	0.921	0.812	1.024	0.520	1.147	0.234	0.709	1.209	0.334	0.529
0.246	0.320	0.489	0.440	0.737	0.513	0.894	0.851	0.981	0.542	1.234	0.222	0.688	1.114	0.386	0.498

3 讨论

从出现 SARS 到确定其病原为 SARS 病毒、并测定其全基因序列只用了几个月的时间，这主要依赖于现代分子生物学技术。在确定病原微生物之后，主要任务之一就是建立准确的检测方法，国内外相继建立了检测 SARS 病毒抗体的免疫荧光法[7]、酶联免疫法、蛋白抗原芯片，以及检测 SARS 病毒核酸 RNA 的 RT-PCR、基因芯片[10]等。一般情况下，从病人体内检测到 SARS 病毒核酸 RNA 的时间要比检测 SARS 病毒抗体要早，但是从血液内检测 SARS 病毒 RNA 的阳性率并不高，可能与 SARS 发病早期不出现病毒血症、病毒 RNA 提取时易降解、检测敏感度不高等有关。目前检测 SARS 病毒抗体仍然是最主要的检测方法。最初用于检测 SARS 病毒抗体的蛋白抗原是培养的 SARS 病毒，检测结果显示，用这种全病毒抗原检测抗体，会有假阳性，主要原因可能是 SARS 病毒蛋白与其它冠状病毒等存在有同源序列等。克服这种假阳性的主要方法是从 SARS 病毒蛋白中筛选出抗原性强、特异性好的蛋白片段，利用基因工程进行制备。

本研究表达的融合蛋白有较好的抗原性和特异性，为研制抗体检测试剂奠定了基础。用该融合蛋白抗原检测抗体有较多优点：比细胞培养的病毒抗原制备安全、成本低，并且与其它冠状病毒产生交叉反应的可能性小；比分别使用两个单独的蛋白抗原更方便、经济；用这两个蛋白作抗原检测抗 SARS 病毒抗体时，不再需要分别制备这两种蛋白，也不需要调整两个抗原的使用比例，因此应用方便；用捕获法或夹心法检测抗 SARS 病毒抗体时，只需酶标记这一种融合蛋白，不再需要分别标记两个蛋白。

表达的 SARS 病毒的 S 蛋白片段和 N 蛋白片段的融合蛋白部分以包含体和可溶性两种形式存在，可溶性部分易于纯化。包含体经用 8mol/L 尿素溶液溶解后纯化，亦可获得较纯的目的蛋白。制备的融合蛋白纯度会影响检测抗体是的特异性，用阳离子柱纯化的融合蛋白，不用反相高压液相纯化，检测正常人血清时未出现非特异性反应，但在检测发热病人时出现了非特异性反应，可能是发热病人血清内存在细菌蛋白抗体，抗原不纯导致非特异反应。经过反相高压液相纯化后，消除了发热病人血清的非特异性反应。

另外，用我们表达的 S 蛋白片段免疫兔子，制备了高效价的抗血清。并进行了抗血清体外阻断 SARS 病毒感染 VERO-E6 细胞试验，结果显示，该抗血清不能阻断 SARS 病毒感染细胞，提示该 S 蛋白片段不是保护性抗原，病毒和细胞结合时可能与该片段无关。

致 谢 感谢军事医学科学院微生物流行病学研究所周育森博士，帮助进行了抗血清体外阻断 SARS 病毒感染 VERO-E6 细胞试验。

REFERENCES(参考文献)

[1] Ding Y, Wang H, Shen H *et al*. The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. *J Patho*, 2003, **200**(3):282 - 289

[2] Cooke FJ, Shapiro DS. Global outbreak of severe acute respiratory syndrome (SARS). *Int J Infect Dis*, 2003, **7**(2):80 - 85

[3] Peiris J, Lai S, Poon L *et al*. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet*, 2003, **361**:1319 - 1325

[4] Marra MA, Jones SJ, Astell CR *et al*. The genome sequence of the SARS - associated coronavirus. *Science*, 2003, **300**(5624):1399 - 1404

[5] Rota PA, Oberste MS, Monroe SS *et al*. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science*, 2003, **300**(5624):1394 - 1399

[6] Qin E' de, Zhu QY, Yu M *et al*. A complete sequence and comparative analysis of a SARS-associated virus (Isolate BJ01). *Chinese Science Bulletin*, 2003, **48**(10):941 - 948

[7] Fang LQ, Zhang PH, Yang BA *et al*. The application of indirect immuno-fluorescence assay in the diagnosis of severe acute respiratory syndrome. *Chinese Journal of Epidemiology*, 2003, **24**(6):484 - 486

[8] Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd. New York, USA: Cold Spring Harbor Laboratory Press, 1989

[9] LI YX (李越希), WANG XT (汪兴太), TAO KH (陶开华) *et al*. Cloning and expression of human cytomegalovirus gp52 gene in *E. coli*. *Chin J Biologicals* (中国生物制品学杂志), 2000, **13**(1): 9 - 12

[10] Frankish H. SARS genome chip available to scientists: the NIAID hopes that widespread access to the SARS genome chip will catalyse research into effective treatments for the virus. *Lancet*, 2003, **361**(9376):2212

Screening, Expression, Purification and Identification of Antigenic Determinants of SARS Virus S and N Proteins

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Abstract The strong antigenic determinants of the S protein (162 - 460 aa) and N protein (1 - 258 aa) of SARS virus were selected by analyzing the two proteins' amino acid sequences using software ANTHEWIN. The two cDNA sequences of the strong antigenic determinants were synthesized chemically by incorporating the genetic codes that are most suitable for prokaryotic and eukaryotic cells fused together and cloned into plasmid pET28a(+) at *Nco* I / *Eco*R I sites, with the N protein cDNA fragment is at the 5' terminus of the fused genes. The recombinant plasmid was transformed into *E. coli* BL21(DE3). The expressed chimeric protein existed both as soluble protein and inclusion body, which accounted about 30% of the total cellular proteins. The inclusion bodies were washed with 0.5% Triton X-100 solution twice, then dissolved in 8 mol/L urea solution. The soluble protein was purified by S Sepharose FF cation exchange column and RP-HPLC. The purified chimeric protein was identified by ELISA using the anti-SARS IgG or IgM positive human sera and normal human sera, the results showed that it has good antigenicity and specificity, and could be used for developing diagnosis reagent.

Key words SARS virus, antigenic determinants, gene expression, purification, antigenicity

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Fusion Protein of Interleukin 4 and *Pseudomonas* Exotoxin with High Cytotoxicity to Cancer Cells

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Abstract Receptors of human interleukin 4 (hIL4R) have been found to be present on many types of cancers, so they could be good targets for cancer therapy. Previously, a fusion protein (DT4H) was constructed with human interleukin 4 (hIL4) and diphtherial toxin (DT). Although it is high cytotoxic to several cancer cell lines, human immune response to it would be expected in future clinical trials. Here another fusion toxin was constructed by fusing hIL4 to DNA sequence encoding the 253-608 amino acids of *Pseudomonas* exotoxin (PE). The fusion gene was expressed in Escherichia coli which results in a large amount of the fusion toxin (H404K). Purified H404K was very cytotoxic to cancer cell line U251 and moderate cytotoxic to HepG2 and MCF-7, which is similar to DT4H. Furthermore, for the first time we show that its cytotoxicity can not be inhibited by rabbit anti-DT polyclonal antibody. These results suggest that H404K would be expected to have good therapeutic value as an alternative therapy of DT4H in the treatment of some malignancies.

Key words fusion toxin, *pseudomonas* exotoxin, cancer therapy, interleukin 4, IL-4 receptor

Chemotherapy in current use for the systemic treatment of cancer has brought about cures in many patients and decreases in tumor burden. But chemotherapy fails in those patients whose malignant cells are not sufficiently different from normal cells in their growth and metabolism. However, these tumor cells often express surface receptors or other molecules which distinguish them from their surrounding normal tissues. The therapeutic goal of targeted toxin is to target a cytotoxic agent to cell surface molecules which will internalize the cytotoxic agent and result in cell death. Targeted toxins consist of a targeting polypeptide covalently linked to a peptide toxin. The targeting protein or ligand directs the molecule to a cell surface receptor or determinant; the toxin moiety then enters the cell and induces apoptosis by, in most cases, inactivating protein synthesis[1].

Previously, a fusion toxin of IL-4 with diphtherial toxin 1-388 aa (DT4H) has been constructed in our lab, since receptors for IL-4 have been found to be present on many types of cancers including breast cancers, lung cancers, melanomas, renal cell cancers, brain cancers and pancreatic cancers *etc*.[1~7]. And its cytotoxicity to cancer cells has been testified *in vitro*. But, the development of an antibody response to this toxin moiety-diphtherial toxin (DT) will be expected *in vivo* because it is a strong immunogen. This will damage the efficacy of DT4H. A solution to this problem is to use a fusion protein with different kind of toxin moiety. Here, *Pseudomonas* exotoxin (PE) was chosen for this purpose because it is a potent toxin and it has been successfully used in many targeted toxins. Intact PE contains three domains. The cell-binding domain (domain Ⅰ) is commonly replaced with other ligands. The resulting recombinant toxins is a fusion toxin of IL-4 with domain Ⅱ and Ⅲ (PE40), which would be expected to have good therapeutic value as an alternative therapy with DT4H.

Since the carboxyl terminus of native hIL-4 participates in the interaction with its receptor, former fusion toxin IL-4 and *Pseudomonas* exotoxin A showed low affinity. A successful alteration was the application of circularly permuted IL-4[8], in which the native termini of IL-4 were fused each other and ASN^{38} and Lys^{37} were created as its N and C terminus. This kind of reconstruction is based on its three-dimensional structure and analysis of functional glycosylated sites[9,10]. This strategy provides a new option for improving the function of fusion proteins. Here, a circularly permuted form of IL-4 was employed in this construction to improve the binding to its receptor.

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1 Materials and Methods

1.1 Materials

Rneasy minikit was from Qiagen; ThermoScript RT-PCR systerm was provided by Invitrogen; DNA marker, restriction endonucleases, T4 DNA ligase were purchased from TaKaRa; the cloning and expression vectors, *E. coli* host strains were stored in our laboratory.

1.2 Cell lines and cell culture

U251, derived from human glioma, was gained from Institute of Biochemistry and Cell Biology and maintained in RPMI-1640 medium (GIBCO) supplemented with 10% FBS (GIBCO). HepG2 and MCF-7, originating from human liver and breast cancer respectively, were stocked in our lab, and maintained in DMEM medium (GIBCO) with 10% FBS. All the cells were maintained at 37 ℃ in a humidified incubator of 5% CO_2.

1.3 Molecular cloning of mature human IL-4 and *Pseudomonas* exotoxin (PE)

Coding sequence of IL-4 was obtained by RT-PCR from human white blood cells. About 1 μg of total RNA was reverse transcribed to cDNA using RT-PCR kit, 2 μL of cDNA product were then subjected to PCR with the following primers to obtain the coding region of human IL-4. Sense primer: 5′-aaa tcg aca cct att aat ggg tct cac-3′, and antisense primer: 5′-tca gct cga aca ctt tga ata ttt ctc t-3′, which encodes a full-length IL-4 without signal peptide. Coding sequence of *Pseudomonas* exotoxin (PE) lacking of signal peptide came from PCR amplification of genome DNA of *Pseudomonas aeruginosa* (ATCC 27853). Extraction of genome DNA was operated according to Molecular Cloning (the 2nd edition). PE was amplified by PCR with 5′-atg cac ctg ata ccc cat tg-3′ as the forward and 5′-gca gtt act tca ggt cct cgc-3′ as the backward primers. Both of purified PCR products were ligated into pMD-18T (TaKaRa) vector, creating pIL4-T and pPE-T.

1.4 Constructions of the plasmid for expressing fusion protein

The sequences of oligonucleotide primers used in these constructions are as follows: (a) 5′ **ggc ggt aac ggt ggc** cac aag tgc gat atc acc tta ca (GGNGG codons in bold); (b) 5′ **gca agc ttc** ttg gag gca aag atg tc (*Hin*d Ⅲ site in bold); (c) 5′ gct **gga tcc** atg aac aca act gag aag gaa acc t (*Bam*H Ⅰ site in bold); (d) 5′ **gcc acc gtt acc gcc** gct cga aca ctt tga ata ttt ctc (GGNGG codons in bold); (e) 5′ gca **agc ttg** gcg gtc ccg agg gcg gca gcc tg (*Hin*d Ⅲ site in bold); (f) 5′ gcg **cgg ccg** cat tac agt tcg tct ttc ggc ggc tgg ccg (*Not* Ⅰ site in bold).

pHCPIL4 encodes a 6×His fusion protein with cpIL4 (circularly permuted human IL-4). cpIL4 is a protein consisting of two parts of native IL-4, 38-129 aa and 1-37 aa of native IL-4 connected by linker GGNGG. DNA encoding cpIL4 was constructed by the method of gene splicing and by overlap extension (SOE), as described[11], and the product was ligated into pT7473 (derived from pET-21a). Briefly, the first part (1 – 37) and the second part (38 – 129) of mature IL-4 was amplified separately using primers a, b, and c, d, so that the 5′ end of the first fragment and the 3′ end of the second fragment are identical and encode the GGNGG linker. The two fragments therefore annealed in reverse order to form a template for the final PCR step using primers b and c. Primer c and b create *Bam*H Ⅰ and *Hin*d Ⅲ restriction sites and this fragment was ligated to the *Bam*H Ⅰ - *Hin*d Ⅲ site of pT7473.

pH404K encodes fusion protein of cpIL4 and PE40KDEL, a truncated PE. The toxin PE40KDEL (abbreviated PE40K) contains amino acid 253 – 608 of PE followed by KDEL carboxyl terminus, and was generated by PCR amplification of pPE-T with e as the sense and f as the antisense primer. Then PE40K fragment digested with restriction enzymes *Hin*d Ⅲ and *Not* Ⅰ was ligated into the *Hin*d Ⅲ and *Not* Ⅰ sites in the pHCPIL4 plasmid.

1.5 Protein expression and purification

E. coli BL21 (DE3) was transformed with pH404K and inoculated with LB. When OD_{650} reached 0.5, IPTG was added to 1 mmol/L and cells were cultured for an additional 90 min. After sonication of cells, inclusion body was precipitated and solubilized by 8 mol/L urea to a protein concentration of 10 mg/mL. Dithioerythritol was added to a final concentration of 65 mmol/L, and after overnight incubation at 22 ℃ the reduced denatured protein was diluted 100-fold with 100 mmol/L Tris, pH8.0/0.5 mol/L L-arginine/0.9 mmol/L oxidized glutathione/2 mmol/L EDTA. After incubation at 10 ℃ for 36 ~ 48 h, the refolded protein was dialyzed with 20 mmol/L Tris/100 mmol/L urea (pH7.4), then loaded on DEAE-Sepharose Fast Flow and eluted by 0.3 mol/L NaCl. After dialyzed again, the protein solution was loaded onto an equilibrated 4 mL Ni^{2+}/NTA affinity matrix. The column was washed with 20 mL of binding buffer containing 0.075 mol/L imidazole. The recombinant proteins were eluted with 0.25 mol/L imidazole. The purified product was dialyzed against PBS.

1.6 Generation and purification of antibodies to H404K and diphtherial toxin

The antibodies to H404K and DT were generated according to the protocol of reference[12]. Purified H404K and DT were used as immunogens. The mature form of DT was expressed in BL21 (DE3) as inclusion bodies, then were renaturated and purified by DEAE-Sepharose as H404K. Rabbits' serum was collected and purified by ammonium sulphate method.

1.7 Western blot

2 μg of H404K and DT4H was separated by 12% SDS-PAGE. The blot was developed according to the manufacturer's protocol. In this case, anti-DT serum (1:20 000) and AP-conjugated second antibody (1:1000) were used.

1.8 Cytotoxity assay

After exposure to drugs, the viability of several cell lines (U251, HepG2, MCF-7) was determined by the MTT assay. After plating the cells, various concentrations of H404K and human IL-4 (R&D) was added. In some cases, 200 μg antibodies of anti-H404K

and anti-DT were included for inhibition experiments. Cells were cultured for another 36 h, then 0.5 mg/mL MTT was added and absorbance in each well was detected in a microplate reader at 450 nm. The result was expressed as a percent of the control without any toxin.

1.9 Clonogenic assay

The *in vitro* cytotoxic activities of IL-4 cytotoxin on U251 cell were also determined by colony-forming assay. The cells were plated in triplicate in 100mm Petri dishes and exposed to different concentrations of IL-4 cytotoxin (0 ~ 100 nmol/L) at 37 ℃ for 9 days, followed by washing, fixing and staining with 0.25% crystal violet. Colonies consisting of > 50 cells were scored. The percentage of survival colony was determined from the number of colonies remaining in the treated groups divided by the colony number in control group.

1.10 Indirect immunofluorescence staining of IL-4 receptor for H404K

U251 cells were cultured on coverslips for 24 h, then incubated with H404K at 4 ℃ for 4 h. After washing with cold PBS, they were fixed with methanol: acetone (1:1) at room temperature for 10 min. Fixed cells were washed thrice with PBS, incubated at room temperature for 2 h with primary polyclonal rabbit anti-H404K, and diluted 1:200 with 2% BSA/PBS. Coverslips were washed five times and incubated at room temperature for 1 h with secondary FITC-goat anti rabbit IgG (diluted 1:100 with 2% BSA/PBS). Coverslips were washed again and then mounted using 50% glycerol. As to control experiment, only the procedure of incubation with H404K was omitted. The fixed cells were incubated with primary and secondary antibody directly. The cells were observed and digitally photographed using a Nikon fluorescent microscope with Spot software (Diagnostic Instruments).

2 Results

2.1 Cloning of Human IL-4 and PE and Construction of Fusion Toxin

IL-4 was obtained by RT-PCR from human white blood cells, and PE from PCR amplification of genomic DNA of *Pseudomonas aeruginosa*. Their sequences were confirmed by sequencing. In order to construct a fusion toxin for efficiently killing cancer cells, the truncated form of PE (PE40), which is missing the native cell-binding domain, was employed. And it was linked to a circularly permuted IL-4 by a spacer sequence KLGGPE. A 6 × His tag was fused to N-terminus for further convenient purification. So, H404K contains Met, 6 × His, GS for *Bam*H I site, native IL-4 38-129 aa, GGNGG for linker, native IL-4 1-37 aa, KLGGPE for spacer and PE 253 - 608 followed by KDEL.

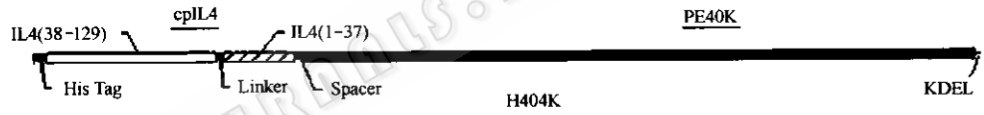

Fig. 1 Schematic drawing of H404K

H404K was constructed by fusing cpIL4 to PE40 with a spacer sequence. cpIL4 consists of 38 - 129 aa and 1 - 37 aa of native IL - 4 connected by a linker. A 6 His tag was retained at the N-terminus. KDEL was followed at the C-terminus. Spacer: KLGGPE; Linker: GGNGG.

2.2 Protein expression and purification

The protein encoded by plasmid pH404K was expressed in *E. coli* BL21 (DE3). The fusion protein represented the main band on SDS-PAGE analysis of the total cell extract and was exclusively present in the form of inclusion bodies. The highly purified form of inclusion bodies, were subjected to dissolution, renaturation and purification as described in Methods. Traditionally, PE fusion toxin is purified by 2 steps of ion exchange and 1 step of gel filter. HPLC is commonly used in this process. This limits the volume of loaded protein greatly. In order to facilitate the purification process, we fused a 6 × His tag to the N-terminus of this protein. Ni^{2+}-NTA column was employed for affinity chromatogram. Through simple two steps, H404K appeared as a single entity and eluted in fractions expected for a protein with a molecular mass of 60 kD, demonstrating 90% purity of the final product.

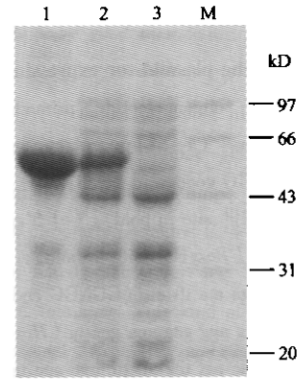

Fig. 2 SDS-PAGE analysis of expression of H404K

M: marker; 1: inclusion bodies; 2 and 3: lysed *E. coli* after and prior to IPTG induction, respectively

2.3 Western Blot Analysis with Anti-DT Antibody

In order to investigate whether there exists a cross reaction between DT and H404K, a western blot analysis was used. Anti-DT polyclonal antibody was raised in rabbits with purified diphtherial toxin. 2μg of H404K and DT4H were blotted and hybridized with 1:20000 anti-DT antibody. The result was shown in Fig.4. A 60 kD band was detected in lane of DT4H and no band was recognized in lane of H404K.

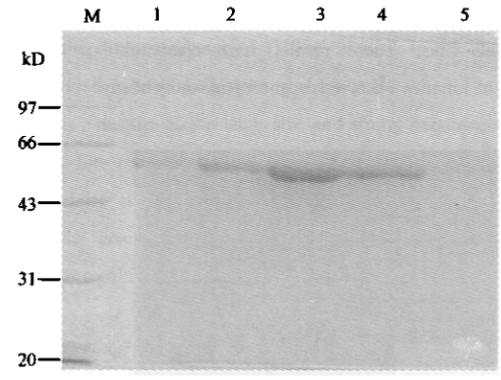

Fig. 3 Analysis of H404K purification with SDS-PAGE

M: marker; 1 ~ 5: designate sequential fractions eluted from Ni/NTA column by 0.25mol/L imidazole

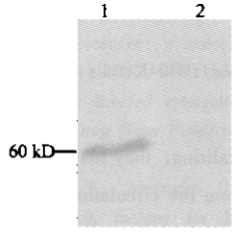

Fig. 4 Western blot analysis of H404K and DT4H

2μg of sample was blotted on PVDF membrane and hybridized with anti-DT and AP-labeled secondary antibody. 1: DT4H; 2: H404K

2.4 Cytotoxicity of H404K on several human cancer cell lines

Several cell lines were tested to determine if H404K is cytotoxic to them. We examined cancers derived from brain, breast, stomach and liver. A glioma cell line, U251, was very responsive to H404K with an IC50 of 0.7 nmol/L. MCF-7 breast cancer and liver cancer HepG2 cell lines had poorer responses with IC_{50} of 30 nmol/L and 25 nmol/L, respectively.

The specificity of H404K cytotoxic action was tested with inhibition experiment of excess recombinant human IL-4 (rhIL4). rhIL4 showed the inhibition of cytotoxic action of H404K at a concentration of 4.25 nmol/L.

In previous research, a fusion protein, DT4H, consisting of truncted DT and IL-4 has shown similar cytotoxicity as H404K and the activity can be inhibitted by the polyclonal anti-DT antibody (data not shown). Since we expect H404K can be served as an alternative agent when anti-DT4H antibody is raised in the treatment of patients. Functional effect on H404K of this antibody was tested on U251 cells. As shown in Fig.6, no significiant inhibition was found at all.

2.5 Inhibition of colony formation of U251 cell lines by IL-4 cytotoxin

We performed a colony-formation assay using the U251 cell line. Two hundred cells were plated in each dish and incubated with various concentrations of IL-4 cytotoxin. After culturing of 9 ~ 14 days, the

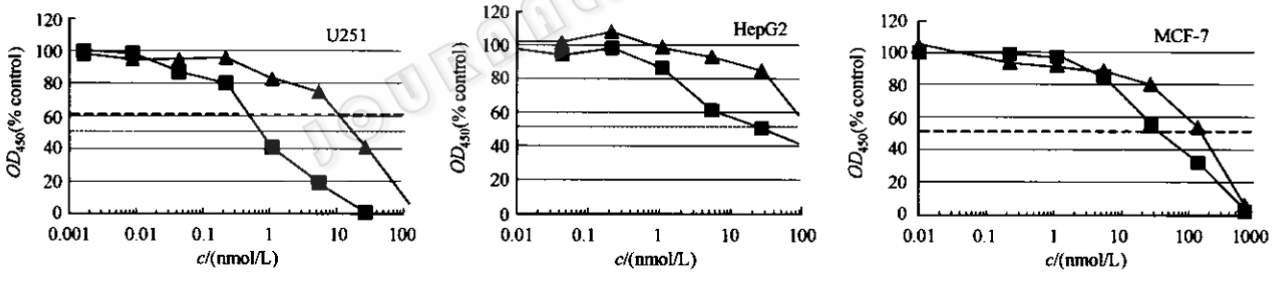

Fig. 5 Cytotoxic activity of H404K on several cancer cell lines and an inhibition of this cytotoxicity by hIL4

The result was expressed as a percent of the control without any toxin. hIL4 was added at a concentration of 4.25nmol/L. The dashed line shows 50% of viability

■H404K; ▲H404K + hIL4

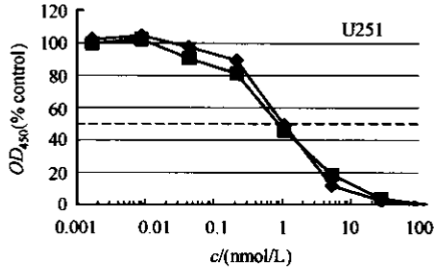

Fig. 6 Antibody Inhibition Assay of H404K

200μg of purified anti-DT and diluted H404K were added in each well of inhibition group

■H404K; ◆H404K + anti-DT

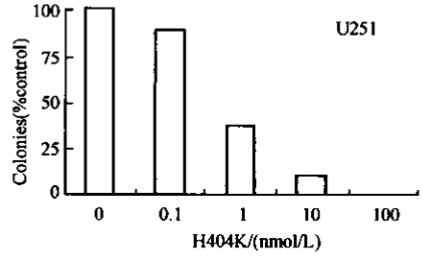

Fig. 7 Inhibition of colony formation by IL-4 cytotoxin

Two hundred U251 cells were allowed to adhere in Petri dishes and cultured with various concentrations(0 ~ 100nmol/L) of H404K for 9 ~ 14 days, the percentages of colonies formed in control and IL-4 cytotoxin-incubated groups were compared.

percentages of colonies formed in control and IL-4 cytotoxin-incubated groups were compared. IL-4 cytotoxin inhibited colony formation in U251 cell line in a concentration dependent manner. The number of colonies in untreated groups regarded as the 100% control value. Less than 1 nmol/L of IL-4 cytotoxin inhibited colony formation by 50% in U251 cell line. These results were comparable with the dose-dependent kinetics observed in the cytotoxicity assay.

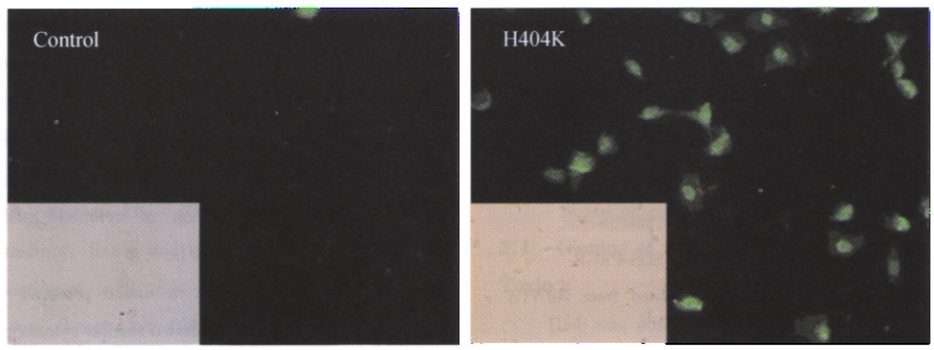

Fig. 8 Indirect immunofluorescence analysis of IL-4 receptor on U251 cell line

Control: cells were cultured on coverslips the incubated with anti-H404K and FITC labeled secondary antibody; H404K: cells on coverslip were incubated with H404K first, then stained with antibodies.

2.6 Immunofluorescence analysis of IL-4 receptor on U251 cell line

To show direct evidence of H404K binding to U251 cells, these cells were first incubated with H404K, indirect immunofluorescence (IF) staining with anti-H404K antibody revealed definitive presence of hybrid protein on the cells. In control experiment, no positive staining was observed when U251 cells were directly incubated with primary and secondary antibodies, which indicated the selectivity of binding.

3 Discussion

It is known that normal resting B cells, T cells, monocytes, and resting or activated bone marrow precursor cells which express low level of IL-4 receptors are not sensitive to cpIL4-PE[13,14]. However, when T cells are activated, IL-4 toxin becomes cytotoxic to these cells because activation of T cells up-regulates IL-4R[15]. However, in the context of the clinical situation, it is not expected that a large number of T cells will be activated *in vivo*. In addition, even if these cells are killed by H404K, it will not cause too much deleterious effects in cancer patients. These results suggest that there exists a therapeutic chance by which one can target IL-4 toxin to cancer cells but cause little collateral damage to immune and non-immune tissues.

There has been a long history of constructing targeted toxins for human cancer treatments. The toxins most commonly modified for the construction of targeted molecules that have been clinically evaluated include DT and PE from bacteria and ricin, Gel and PAP isolated from plants[1]. Since all fusion toxins are immunogenic, treatment ultimately results in an anti-toxin response, especially in patient with solid tumor[16]. Clearly, the development of neutralizing antibodies is detrimental to targeted toxin antitumor efficacy. In many trials, retreatments have been limited to a few cycles because of the development of neutralizing antibodies. Even when the antibodies generated are nonneutralizing, they may form immune complexes and accelerate clearance from the circulation. Therefore, patients treated with the biological agent need to be carefully monitored for the development of these antibodies.

Previously, a fusion toxin of IL-4 with diphtherial toxin 1 ~ 388 aa (DT4H) has been constructed in our lab and humoral immune responses has been expected *in vivo*. In order to continue targeted treatment after antibody appearance, we replaced the diphtherial toxin with *Pseudomonas* exotoxin moiety to construct H404K. But both of enzymatic domains of DT and PE have similar activity, which ADP ribosylates elongation factor 2 (EF-2). In addition, they have similar molecular weight. So, the antibody raised from DT may response to PE, and vice verse. Luckily, blast analysis of these two moieties do not show significant similarity (data not shown). This gives an opportunity that when antibody against one was raised, another could be available. In addition, some patients may have antibody against some toxin prior to treatment, then more options give them more chance to receive targeted treatments. In this research, we mimiced human immune response to DT4H in rabbits. Since a human version of IL-4 was used in the construction, rabbit anti-human IL-4 antibody might be raised if DT4H was directly used in immunization. So, the toxin portion of DT4H was used for injection. The results from western blot and antibody inhibition assay did not show that anti-DT polyclonal antibody could interact with H404K.

In activity assay, H404K showed selectively cytotoxic to several cancer-derived cell lines. Similar to DT4H, U251 was very sensitive. The reason may lie in the high expression level of IL-4 receptor in the cell surface. Since clonogenicity *in vitro* often correlates with *in vivo* malignant phenotype in xenografts, H404K was tested in inhibition of colony formation of U251. The result was consistent with that of cytotoxicity assay. These findings predict that the antitumor activity of

IL-4 toxin will be improved *in vivo* in animal models of human cancer. In addition, the direct evidence of H404K binding to the target cells was also provided in immunofluorescence analysis.

Generally, despite of the large size and strong immunogenicity of fusion toxins, like DT and PE series, their targeted characteristic and potent cytotoxicity still give them a good value in cancer therapy. H404K, maybe combined with DT4H will be a potential agent in addition to the traditional anti-cancer therapeutic.

REFERENCES

[1] Frankel AE, Kreitman RJ, and Sausville EA. Targeted toxins. *Clin Cancer Res*, 2000, **6**(2):326 – 334

[2] Kawakami K, Kawakami M, Husain SR *et al*. Targeting interleukin-4 receptors for effective pancreatic cancer therapy. *Cancer Res*, 2002, **62**(13):3575 – 3580

[3] Kawakami M, Kawakami K, Stepensky VA *et al*. Interleukin 4 receptor on human lung cancer: a molecular target for cytotoxin therapy. *Clin Cancer Res*, 2002, **8**(11):3503 – 3511

[4] Puri RK. IL-4 receptor-directed cytotoxin for therapy of AIDS-associated KS tumors. *Drug News Perspect*, 2000, **13**(7):395 – 402

[5] Strome SE, Kawakami K, Alejandro D *et al*. Interleukin 4 receptor-directed cytotoxin therapy for human head and neck squamous cell carcinoma in animal models. *Clin Cancer Res*, 2002, **8**(1):281 – 286

[6] Joshi BH, Leland P, Asher A *et al*. In situ expression of interleukin-4 (IL-4) receptors in human brain tumors and cytotoxicity of a recombinant IL-4 cytotoxin in primary glioblastoma cell cultures. *Cancer Res*, 2001, **61**(22):8058 – 8061

[7] Puri RK and Siegel JP. Interleukin-4 and cancer therapy. *Cancer Invest*, 1993, **11**(4):473 – 486

[8] Kreitman RJ, Puri RK, and Pastan I. A Circularly Permuted Recombinant Interleukin-4 Toxin with Increased Activity. *Proceedings of the National Academy of Sciences of the United States of America*, 1994, **91**(15):6889 – 6893

[9] Powers R, Garrett DS, March CJ *et al*. The high-resolution, three-dimensional solution structure of human interleukin-4 determined by multidimensional heteronuclear magnetic resonance spectroscopy. *Biochemistry*, 1993, **32**(26):6744 – 6762

[10] Carr C, Aykent S, Kimack NM *et al*. Disulfide assignments in recombinant mouse and human interleukin 4. *Biochemistry*, 1991, **30**(6):1515 – 1523

[11] Horton RM, Hunt HD, Ho SN *et al*. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*, 1989, **77**(1):61 – 68

[12] Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd ed, New York: Cold Spring Harbor Laboratory Press, 1989

[13] Husain SR, Gill P, Kreitman RJ *et al*. Interleukin-4 receptor expression on AIDS-associated Kaposi's sarcoma cells and their targeting by a chimeric protein comprised of circularly permuted interleukin-4 and Pseudomonas exotoxin. *Mol Med*, 1997, **3**(5):327 – 338

[14] Puri RK, Leland P, Kreitman RJ *et al*. Human neurological cancer cells express interleukin-4 (IL-4) receptors which are targets for the toxic effects of IL4-Pseudomonas exotoxin chimeric protein. *Int J Cancer*, 1994, **58**(4):574 – 581

[15] Puri RK, Mehrotra PT, Leland P *et al*. A chimeric protein comprised of IL-4 and Pseudomonas exotoxin is cytotoxic for activated human lymphocytes. *J Immunol*, 1994, **152**(7):3693 – 3700

[16] Pai LH, Wittes R, Setser A *et al*. Treatment of advanced solid tumors with immunotoxin LMB-1: An antibody linked to Pseudomonas exotoxin. *Nature Medicine*, 1996, **2**(3):350 – 353