

人脂联素重组克隆载体构建*

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摘要 目的:构建含有人脂联素(adiponectin, ad)基因的重组克隆载体 pEGFP-N1-AD,为进一步研究 AD 与脂质代谢及代谢综合征关系奠定基础。方法:根据 Gene-Bank 中已经公布的 AD 设计引物,从含有目的基因的质粒克隆模板中,利用 PCR 方法钓取目的基因。将 AD 基因克隆到真核表达载体 pEGFP-N1 中。酶切、PCR、及测序鉴定。结果:PCR、酶切及测序鉴定证实目的基因正确克隆至真核表达载体 pEGFP-N1 中,测序结果与 Gene-Bank 报道一致。结论:成功构建了 AD 重组克隆真核表达载体。

关键词 脂联素;慢病毒载体;构建;表达

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Reconstruction of Eukaryotic Expression Vector Containing Human Adiponectin Gene*

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ABSTRACT Objective: To construct the vector (pEGFP-N1-AD) containing human adiponectin (AD) gene so as to investigate the effects of adiponectin in the lipid metabolism and the syndrome of metabolism. **Methods:** AD gene was amplified from the plasma by polymerase chain reaction (PCR) technique whose primer was designed according to the announced sequences of AD gene in gene bank, then cut by the endonucleases and directionally cloned into the pEGFP-N1 vector and analyzed by endonuclease cutting, PCR and gene sequencing analysis. **Results:** Through PCR, endonuclease cutting and gene sequencing, the target gene was verified to be correctly cloned to vector pEGFP-N1. **Conclusion:** Recombinant eukaryotic expression vector containing human adiponectin protein was successfully constructed.

Key words: Adiponectin; Vector; Construction

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随着对肥胖研究的不断深入,脂肪组织作为内分泌器官已得到大家的共识。由脂肪细胞分泌的脂源性细胞因子如抵抗素、脂联素等在基础及临床的研究中越来越受到重视。脂联素(Adiponectin / APb0)是 1995 年 Scherer 在小鼠的 3T3-L1 脂肪细胞系中首次分离克隆的,命名为脂肪细胞补体相关蛋白 30, 亦称 apM1 或 adipQ 等。它是至今为止发现的唯一一种当脂肪组织容积变大时而其血浆浓度下降的脂肪细胞因子。临床和实验研究显示脂联素的浓度与肥胖相关的疾病有关,包括心血管疾病、胰岛素抵抗和炎症等^[1-2]。

本研究采用真核载体系统,成功构建了含 AD 基因的真核细胞表达载体,为进一步在体内外研究 AD 的功能奠定了基础。

1 材料和方法

1.1 材料

1.1.1 仪器 Western Blotting 专用垂直电泳槽,转膜仪, DYY-11

电泳仪,凝胶成像仪,紫外分光光度计,高速冷冻离心机,倒置荧光显微镜,PCR 仪,细菌摇床及培养箱等。

1.1.2 基本材料 TRIZOL 购自 Invitrogen 公司,RT-PCR 试剂盒,购自 Takara 公司,In-Fusio kit 购自 BD 公司,pEGFP-N1 载体,Xho I/BamH I 酶切,购自上海吉凯基因化学技术有限公司;大肠杆菌 Top10 为本实验室保存,DNA Marker 购自 Fermentas 公司;蛋白质 Marker 购自晶美生物有限公司,Plasmid 抽提 Kit 购自 QIAGEN 公司,引物合成和基因测序由上海吉凯基因化学技术有限公司完成。Lipofectamine2000 购自 Invitrogen 公司。

1.2 方法

1.2.1 获取目的基因 从含有目的基因的质粒克隆模板中,利用 PCR 方法钓取目的基因。PCR 反应条件:94℃预变性 30 s,94℃变性 30 s,55℃退火 30 s,72℃延伸 2 min,30 cycles,72℃最终延伸 10 min。1%琼脂糖凝胶电泳鉴定。引物设计如下:F: 5'-CCGCTCGAGATGCTGTTGCTGGGAGCTG-3';R: 5'-CGGGAT-

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CCCGGTTGGTGTTCATGGTAGAGAAG -3'(下划线标出的是保护碱基,粗体部分是 Xho I 和 BamH I 酶切位点,斜体部分是目的基因 5' 端和 3' 端部分序列,用于 PCR 扩增目的基因序列)。

1.2.2 重组克隆构建 pEGFP-N1 载体经 Xho I/BamH I 酶切 (50 μ l 酶切体系:纯化 DNA 质粒 1 μ g/ μ l 2 μ l,10Xbuffer 5 μ l, 100XBSA 0.5 μ l, Xho I 酶 10 μ g/ μ l :1 μ l, BamH I 酶 10 μ g/ μ l :1 μ l ; H₂O:40.5 μ l 37 $^{\circ}$ C :1h)。目的基因酶切 PCR 产物纯化后交换连接入酶切真核表达载体(10 μ l 连接体系:酶切回收的载体 DNA 100 ng/ μ l :1 μ l, 酶切回收的 PCR 产物 100 ng/ μ l :1 μ l, 10 $\times$ T4 噬菌体 DNA 连接酶缓冲液 :1 μ l, T4 噬菌体 DNA 连接酶 :1 μ l, dd H₂O :7 μ l 4 $^{\circ}$ C :12 h)。同时设载体和酶切 PCR 产物对照。制备克隆连接液,准备转化。连接产物 10 μ l 转化至感受态大肠杆菌 Top10, AMP 抗性平板筛选,挑取最小的阳性单克隆, LB 培养基 37 $^{\circ}$ C 200 r/min 摇床过夜培养,然后小抽质粒。进行 PCR 鉴定,其中 PCR 引物:F 5'-CGCAAATGGGCGGTAG-GCGTG-3', R:5'-CGGGATCCCGTTGGTGT CATGGTAGA-GAAG -3', PCR 反应条件:94 $^{\circ}$ C 预变性 3s, 94 $^{\circ}$ C 变性 30 s, 60 $^{\circ}$ C 退火 30 s, 72 $^{\circ}$ C 延伸 30 s, 30 cycles; 72 $^{\circ}$ C 最终延伸 6 min, 1% 琼脂糖凝胶电泳鉴定;再对重组慢病毒载体进行 Xho I/BamH I 酶切酶切(条件同前)。初步鉴定正确后送测序。

2 结果

2.1 获取目的基因

通过 PCR 获取的目的基因及双酶切片段 1% 琼脂糖凝胶电泳鉴定可见 799bp 和 788bp 的条带见图 1 和图 2。DNA Marker 片段为 5 kb、3 kb、2 kb、1.5 kb、1kb、750 bp、500 bp、250 bp 和 100 bp。

2.2 重组克隆构建鉴定

重组克隆 PCR 鉴定结果见图 3。测得目的基因序列与 GENE BANK 报道一致,说明重组克隆构建成功。

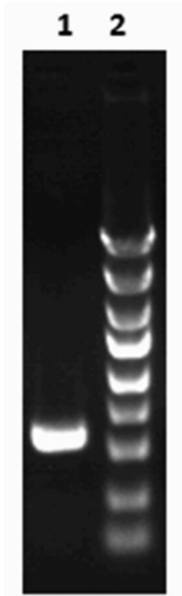


图 1 PCR 获取 AD 基因片段
Fig.1 AD gene fragment obtained by PCR
注:1 AD 基因片段 2 DNA 标准

Note:1 AD gene digested fragment; 2 DNA Marker

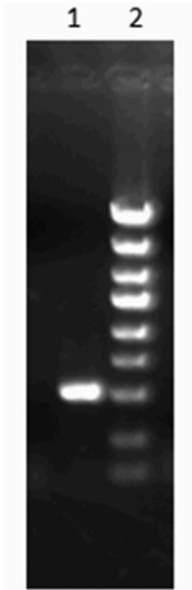


图 2 AD PCR 产物双酶切片段
Fig.2 AD PCR products digested with Xho I/BamH
注:1 酶切 AD 基因片段 2 DNA 标准
Note:1 AD gene fragment; 2 DNA Marker

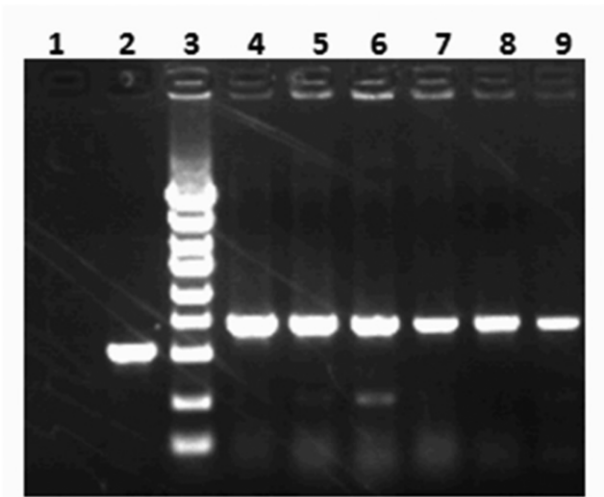


图 3 pEGFP-N1-AD 重组克隆酶切鉴定结果
Fig.3 Identification of recombinant pEGFP-N1-AD with Xho I/BamH
注:1 阴性对照 2 阳性对照(有确定片段插入载体) 3 DNA 标准 4-6 pEGFP-N1-AD 克隆
Note: 1 Negative Control; 2 Positive Control(with identified fragment); 3 DNA Marker; 4-6 recombinant pEGFP-N1-AD

3 讨论

脂联素是一种脂源性胶原样蛋白,属于胶原蛋白超家族,具有 244 个氨基酸多肽,相对分子量为 30Kd^[1]。人类编码脂联素的基因 apM1 位于 3 号染色体 q27 处,全长 16 kb^[1]。

脂联素作为一种脂肪细胞因子在脂质代谢及其相关疾病中起重要作用。首先,脂联素与血脂代谢密切相关。对 293 例健康人群的研究发现,血浆脂联素水平与 apo B 和甘油三酯及 HOMA 指数负相关,与 apo A1、HDL 胆固醇和 LDL 颗粒大小正相关。多元回归分析发现血浆甘油三酯水平影响脂联素与 LDL 颗粒之间的关系。血浆脂联素与 HDL2b 和 HDL2a 正相

关,与 HDL3a、HDL3b 和 HDL3c 负相关。提示血浆脂联素水平与致粥样硬化脂蛋白谱下降有关^[3]。在绝经后妇女中,脂联素与 VLDL-甘油三酯分泌速率负相关,与 VLDL-甘油三酯分解速率正相关。脂联素增加有利于 VLDL-甘油三酯的代谢^[4]。对青少年血清脂联素和瘦素的测定发现,肥胖者的血清脂联素水平低于正常者,脂联素与 12-13 岁儿童肥胖比率呈负相关^[5]。对多囊卵巢综合症患者的研究发现脂联素与腰臀比、胰岛素和 HOMA 指数负相关,与 HDL 胆固醇正相关。脂联素与胰岛素抵抗、HDL 胆固醇和腹部脂肪的关系不受二甲双胍治疗的影响^[6]。脂联素与甘油三酯负相关不受肥胖影响^[7]。脂联素与男性 VLDL-apoB 浓度及代谢密切相关^[8]。脂联素与 VLDL 颗粒的代谢有关。老年妇女血浆脂联素水平随年龄增加升高。与 HDL 胆固醇正常组相比, HDL 胆固醇低的个体脂联素水平较低。绝经妇女脂联素水平与 HDL 胆固醇密切相关,提示脂联素可能抵抗粥样硬化作用通过增加绝经妇女 HDL 胆固醇^[9]。

其次脂联素是一种认为改善胰岛素分泌和敏感性/增加脂肪氧化分解的脂肪细胞因子并具有抗炎症作用和防止粥样硬化作用^[10]。脂联素有抗糖尿病特性^[10]增加胰岛素信号^[11]和抑制肝脏糖原生成酶抑制肝脏细胞葡萄糖生成^[12]。升高的循环脂联素浓度与低代谢综合症的发病率相关^[13]。事实上,低浓度的血浆脂联素与代谢综合症的每个组分有关^[7]。肥胖、胰岛素抵抗、高血压和血脂异常。血清脂联素水平与肥胖和 2 型糖尿病患者全身胰岛素的敏感性直接相关^[14]对 117 例日本不同程度的葡萄糖耐量异常患者的血清脂联素测定发现 HOMA-AD(空腹葡萄糖水平乘空腹胰岛素水平再除以血清脂联素水平)较 HOMA-IR 与作为胰岛素抵抗的金标准指标更为显著负相关,并能弥补 HOMA-IR 在高血糖症中应用的缺陷, HOMA-AD 可作为一个简单而又确实的确定胰岛素抵抗的新指标,甚至在高血糖症患者^[15]。对 714 例男性和 364 例女性的颈动脉内膜厚度(carotid intima-media thickness,IMT)和脉搏速率(pulse wave velocity,PWV)及血清脂联素的研究发现白介素-6 水平与 IMT 和 PWV 正相关,脂联素在男性与 IMT 负相关,白介素-6 或超敏 C 反应蛋白或低脂联素与代谢综合征个体的 IMT 增加相关。白介素-6 和脂联素是男性早期动脉改变的重要的危险因素^[16]。

脂联素在调节胰岛素功能和能量平衡中起重要作用^[17]。脂联素通过激活肝脏和肌肉的 AMP-激活的蛋白激酶调节胰岛素的作用^[18]脂联素在不同肥胖/糖尿病动物模型中有减低葡萄糖水平作用和这种降糖作用与刺激胰岛素分泌无关,但增加胰岛素的敏感性^[19]。尽管脂联素在血糖代谢中的作用机理不太清楚,实验证据表明脂联素减少肝脏葡萄糖的生成,肌肉组织糖利用增加^[17]。

除对糖代谢发生作用,脂联素对血管和心脏行使重要功能,特别是保护内皮不受缺血/再灌注的伤害。其行使抗炎症、抗凋亡和抗肥大作用^[13]。脂联素通过控制单核细胞黏附到血管内皮,对血管保护^[20]。在人类,低脂联素水平与内皮功能紊乱的严重程度密切相关^[21]。低脂联素血症与内皮依赖的血管舒张降低有关^[22]。

脂联素通过调节信号传递机制减低不同刺激引起的炎症

反应,而且脂联素的这种抗炎症特性可解释其对心血管和代谢失调的有利作用,包括粥样硬化和胰岛素抵抗^[23]。脂联素负调脂肪组织 CRP 和肿瘤坏死因子的表达。通过 cAMP-PKA 依赖途径脂联素负调内皮细胞白介素 8、血管细胞黏附分子-1 和反应性氧的生成,通过 AMPK 激活激活内皮一氧化氮合成酶。在心肌细胞,脂联素通过激活环加氧酶 2-前列腺素 E2 信号减低肿瘤坏死因子的生成。在巨噬细胞,脂联素通过激活核因子- κ B 抑制肿瘤坏死因子和白介素 6 的生成。在人巨噬细胞中,脂联素还具有增加白介素 10 的表达增加金属蛋白酶组织抑制剂-1 的生成^[24]。通过抑制 A 类清道夫受体的表达阻止巨噬细胞向泡沫细胞转变^[25]。脂联素除作为胰岛素敏感因子外,许多证据提示脂联素是保持血管动态平衡的重要因子。脂联素缺乏是内皮功能紊乱、高血压、冠状动脉心脏病、心肌梗死和其他心血管并发症的独立危险因素。在动物模型中发现,脂联素水平升高能减轻血管功能紊乱。脂联素通过对血管系统的直接作用行使其血管保护作用,如增加内皮一氧化氮的生成、抑制内皮细胞的激活和内皮与白细胞相互作用、增加吞噬作用和抑制巨噬细胞激活、巨噬细胞形成泡沫细胞和血小板聚集^[26]。

本研究成功构建了含脂联素基因的真核细胞表达载体,为进一步开发脂联素测定试剂和研究脂联素的功能奠定了基础。

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