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厦门大学

硕　士　学　位　论　文

抗人 DcR3 多克隆抗体、单克隆抗体的研制及酶联免疫分析方法的建立

Preparation of Polyclonal Antibody and Monoclonal Antibody against Human DcR3 and Establishment of Enzyme-linked Immunosorbent Assay

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摘要

诱骗受体 3 (decoy receptor 3，DcR3，又称 TR6 或 M68) 是 1998 年 Pitti RM 等发现的一种可溶性肿瘤坏死因子受体 (TNFR) 超家族成员，其互补脱氧核糖核苷酸（Completementary Deoxyribonucleic Acid , cDNA）开放阅读框共编码 300 个氨基酸，N 端前 29 个氨基酸为信号肽序列，随后是四个半胱氨酸富集区（CRD）,其分子量约 35KD，有一个 N 糖基化位点。DcR3 能与 TNF 家族成员：Fas 配体 (Fas ligand, FasL) 、LIGHT[(herpes virus entry mediator(HVEM)-L] 和 TL1A 竞争性地结合，并抑制其介导的细胞凋亡，从而导致机体对肿瘤细胞和活化的自身免疫细胞的耐受，与多种恶性肿瘤、自身免疫性疾病、移植排斥等方面密切相关。研究表明，DcR3 在胚胎肺、脑、肝和成人的脾脏、结肠、气管、胃、脊髓及淋巴结中低表达，而在肺癌、胃肠道肿瘤、脑恶性胶质瘤、胰腺癌和肝癌等恶性肿瘤组织中及自身免疫性疾如系统性红斑狼疮 (SLE)、急性肠内皮细胞炎、矽肺患者的外周血单个核细胞 (PBMC) 中都有不同程度的高表达且在肿瘤患者血清及组织中浓度的高低与其恶性度呈正相关，因此 DcR3 在肿瘤早期排查、手术切除效果评估等方面具有重要的临床意义。但是, 目前对于 DcR3 在肿瘤的发生发展中具体的作用模式以及 DcR3 基因的表达调控机制的研究尚不清楚，为进一步研究 DcR3 生物学特性， 为肿瘤的预防、诊断及治疗提供新的研究策略。本文在成功构建原核表达载体 pET-22b(＋)/ DcR3 并转化到大肠杆菌 *Rosetta-gami* 基础上，经过高效表达和纯化，获得了较高纯度的 DcR3 蛋白。应用自制的 DcR3 蛋白分别免疫新西兰大白兔和 Balb/c 小鼠，经过一系列筛选和检测，制备了兔抗人 DcR3 多克隆抗体（pAb）和鼠抗人 DcR3 单克隆抗体 (mAb) 并初步应用于 DcR3 的检测。为了检测体液中的可溶性的 DcR3 蛋白，我们以 pAb 为包被抗体，mAb 为酶标抗体，建立了双抗夹心 ELISA 方法并初步进行了效果评价，以研究探讨 DcR3 在肿瘤发生、发展中的作用和机制。

我们首先扩大培养转化的大肠杆菌 *Rosetta-gami*，在最适温度和最佳的 IPTG 浓度条件下，实现目的蛋白在大肠杆菌 *Rosetta-gami* 中的高效表达，超声破碎目的蛋白并行可溶性分析。用 Ni^{2+}- 树脂亲和层析柱纯化目的蛋白，

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SDS-PAGE、间接 ELISA 及 Western blot 分析等鉴定蛋白产物。结果显示：表达目的蛋白主要以包涵体的形式存在，且表达量占菌体总蛋白的 30% 以上，SDS-PAGE 分析显示相对分子质量 (Mr) 为 33 000，经 Ni^{2+}- 树脂亲和层析纯化后，表达蛋白的纯度达 95%，间接 ELISA 及 Western blot 显示纯化蛋白与抗人 DcR3 单克隆抗体发生特异结合。因此，我们成功表达、纯化和复性到高纯度 DcR3 融合蛋白，为进一步研究其功能及应用奠定了基础。

将纯化复性后的 sDcR3 融合蛋白采用背部多点注射法免疫 2 只雄性新西兰大白兔，末次加强免疫一周后，颈动脉放血收集血清并对其进行纯化及特性鉴定。纯化复性后的 sDcR3 融合蛋白采用背部多点注射法免疫 6 只雄性 Balb/c 小鼠，待小鼠抗体效价达 1×10^{-5} 以上时取其脾细胞在 PEG3000 作用下与骨髓瘤细胞株 Sp2/0 进行融合（5：1），HAT 培养基选择培养、间接 ELISA 方法筛选阳性杂交瘤细胞、有限稀释法克隆化培养。选择抗体阳性强、细胞生长好的克隆，进行扩大培养，建立分泌特异性抗体的杂交瘤细胞株、保存。将杂交瘤细胞接种于同系小鼠腹腔，诱导并收集腹水，辛酸-饱和硫酸铵分步沉淀法及 Protein G 亲和层析法纯化单克隆抗体并对其进行鉴定。结果表明纯化后多克隆抗体效价达 2×10^{-8}，抗体蛋白浓度为 12.3 g/L，纯化后的抗人 DcR3 pAb 能够识别 DcR3 融合蛋白、SW480 细胞裂解液。HAT 选择培养 4 天后可见融合的细胞克隆，融合率为 95.0% (285/300)，阳性克隆率为 90.0% (270/300)，有限稀释法对阳性克隆进行 3 次亚克隆，共获得 4 株分泌特异性抗体的杂交瘤细胞株，分别命名为 4 株可分泌 DcR3 mAb 的杂交瘤细胞系 ZZ-393 、ZZ-394、ZZ-151 和 ZZ-268。其中 DcR3 mAb ZZ-268 的 Ig 亚类为 IgG1（κ型）；ZZ-393 、ZZ-394、ZZ-151 的 Ig 亚型为 IgM，其腹水抗体效价为 1×10^{-4}～1×10^{-6}，Western blot 显示 ZZ-268 细胞株分泌的单抗均可特异性地识别 DcR3 蛋白。所建立的杂交瘤细胞株经反复冻存、复苏、体外传代培养半年以上仍能稳定分泌高效价单克隆抗体。兔抗人 DcR3 pAb 和鼠抗人 DcR3 mAb 的成功制备，为深入研究 DcR3 蛋白的生物学特性和恶性肿瘤的临床诊断、治疗和监测预后提供了一个可靠手段。

我们经过反复的多抗与单抗和单抗与单抗的配对、分析、筛选，最后建立了以 pAb 为包被抗体，ZZ-268-HRP mAb 为检测抗体的双抗夹心 ELISA 方法。在建立测定 DcR3 双抗夹心 ELISA 方法中，由于我们采用了标记一抗的方法（直

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接标记 mAb），与间接 ELISA 相比可以大大提高抗原检测的含量，检测抗原的灵敏度可达 124 pg/mL，具有良好线性关系的检测区间为 0.5～20 ng/mL，标准曲线的线性回归方程的相关系数可达 0.99 以上。加入法测定回收率在 95.3～110.5%之间，组内及组间平均变异系数均小于 10%，表明该检测方法具有良好的灵敏度和较高的准确性。

总之，本文在经过目的蛋白的高效表达、纯化和复性后，成功制备了高纯度的 DcR3 融合蛋白。将纯化复性后的 DcR3 融合蛋白分别免疫新西兰大白兔和 Balb/c 小鼠，最终获得了效价高、特异性强的抗人 DcR3 pAb 和 4 株单克隆抗体。以抗人 DcR3 pAb 为包被抗体，以直接标记辣根过氧化物酶的抗人 DcR3 mAb 为检测抗体，建立了灵敏度高、稳定性好的双抗体夹心 ELISA 方法。我们建立的双抗夹心 ELISA 方法灵敏、特异性、准确性较好，为临床快速、简便的定量检测微量 DcR3 提供了有力的手段。

关键词：诱骗受体 3；蛋白表达；多克隆抗体；单克隆抗体；酶联免疫吸附测定

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Abstract

Decoy receptor 3, DcR3 (also known as TR6 and M68), a soluble protein, belongs to the tumor necrosis factor receptor (TNFR) superfamily and was identified by Pitti RM in 1998. The DcR3 mRNA contains one open reading frame which encodes 300 amino acids those of the first 29 constitutes the signal peptidesequence that is followed by four tandem cysteine-rich domains (CRDs). Its protein relative molecular mass is 35000 approximately. DcR3 protein can compete for binding to the TNF family ligands such as Fas ligand (FasL), LIGHT, and TL1A with the ligand's bona fide receptors, Fas, HVEM, and DR3, respectively. Because of lacking a transmembr-ane region in its cDNA sequence, DcR3 can block Fas ligand (FasL), LIGHT, and TL1A to mediate the apoptosis which is the most common physiological form of cell death and occurs during embryonic development, tissue remodeling, immune regulati-on, and tumor regression. DcR3 mRNA was detected weakly in a wide array of nomal human tissues such as embry on lung, brain, liver, spleen, colon, trachea, stomach, spinal cord and lymph node, while at high levels in some malignant tumors tissue such as lung cancer, colon adenocarcinoma, esophageal carcinoma, gastric carcinoma, hepatoma, pancreatic carcinoma and some autoallergic diseases such as SLE, acute intestinal epithelial inflammatory cells, peripheral blood mononuclear cells (PBMC), silicosis patients by Northern blot、Western blot and so on. In addition, the DcR3 protein level is positively correlated with the tumor grade of the patients. Thereforce, dectecting the DcR3 level has an important clinical significance in early investigation of tumors and assessment of surgical resection results. But, the mechanism of this soluble protein in the cancer occurance and development is not well understood now. In order to go futher into its biological function and find new strategies for cancer prevention, diagnosis and treatment, we undertook below studys. Based on the success of prokaryotic expression vector pET-22b(+)/DcR3, we transformed it into *E.coli Rosetta-gami* and obtained a high purity of DcR3 protein through the efficient

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expression and purification. Two New Zealand white rabbits and six Balb/c mice were immunized with self-made DcR3 protein. Then rabbit anti-human DcR3 polyclonal antibody (pAb) and mouse anti-human DcR3 monoclonal antibody (mAb) were produced after a series of screening and detection. At last, sandwish ELISA which coated with pAb, detected with mAb ZZ-268-HRP has been established to detect soluble DcR3 and assessed its detective effects in order to investigate roles and mechanisms of DcR3 in cancer development.

In order to achieve the target protein high expression in *E.coli Rosetta-gami*, we cultured the *E.coli Rosetta-gami* at the optimum temperature and optimum IPTG concentration. The harvested *E.coli Rosetta-gami* was disintergrated with ultrasonication. The targent protein was purified by Ni-NTA affinity column and the product was analyzed by SDS-PAGE, identified by indirect ELISA and Western blot. We found that the targent protein was mainly present in inclusion bodies and reached 30% of the total protein. SDS-PAGE showed a new protein band in a relative molecular mass of 33000. The targent protein purity reached 95% after cleaning, denaturation lysis and purification. The purified protein showed good antigenicity with anti-human DcR3 monoclonal antibody by indirect ELISA and Western blot. So, DcR3 fusion protein was successfully expressed, purified and renatured, which laid a foundation for the further study of its function and application.

Two male New Zealand white rabbits were immunized with the purified fusion protein on the back by the multi-point injection. The serum were collected and purified, a week after the last booster immunization, the pAb protein's characteristics were identified. Similarly, six Balb/c mice were immunized with purified fusion DcR3 protein, spleen cells which were collected when the titer reach above 1×10^{-5} and myeloma cells Sp2/0 were fused by PEG3000. Hybridoma cells which excreted specific antibodies were obtained by selective culturing with HAT medium, screening by indirect ELISA, cloning by limiting dilution assay. In order to prevent loss, the hybridoma cell strains which excreted specific antibodies were collected and storaged. Hybridoma cell strains were inoculated into abdominal cavity of homologous series mice to induce ascites. The mAb protein was purified by caprylic acid-saturated

ammonium sulfate fractional precipitation and Protein G affinity chromatography. The pAb and mAb specificity, subtype, titers were identified by ELISA and Western –blot. The result showed that the value of purified pAb against DcR3 reached 2×10^{-8} and the concentration of that was 12.3 g/L. The purified pAb could specific recognize DcR3 fusion protein and lysate of SW480 cells. The cloned cells were found after cell fusion and HAT selective culture for 4 days, and the fusional rate was 95.0% (285/300)，the positive cloning efficiency was 90%(270/300). Four hybridoma cell strains which secreted specific mAb were obtained. We named them ZZ-393, ZZ-394, ZZ-151 and ZZ-268. ZZ-268 mAb was determined as IgG1 subtype and ZZ-393, ZZ-394 and ZZ-151 mAbs were IgM subtype. These mAbs ascites titer reached 1×10^{-4}～1×10^{-6} by limiting dilution assay. ZZ-269 mAb could specific recogonized DcR3 protein by Western-blot. These hybridoma cell strains still kept excreting high-titer mAbs via freezing, reanimating repeatedly and serial subcultivation in vitro for six months. The successful preparation of rabbit anti-human DcR3 pAb and mouse anti-human DcR3 mAb set up the basement of the research for further to study the biological function and provided a reliable tool for malignant tumor diagnosis, treatment and monitoring.

We finally established the Sandwish ELISA which coated with pAb and detected with mAb ZZ-268-HRP to detect soluble DcR3 in the human biological fluids after a series of matching, analysis, and screening. The double-antibody sandwich ELISA can greatly improve the detecting content of DcR3 comparing with the indirect ELISA because of using the HRP to direct labeling mAb. The assay had a detection of 124 pg/mL with a dynamic range of 0.5～20 ng/mL on the standard curve. The correlation coefficients of the standard curve was more than 0.99. The recovery range was 96.2～110.5%. Intra-assay and inter-assay average CVs were less than 10%, which indirates a good sensitivity and high accuracy of this detection method.

In conclusions, the confusion protein DcR3 was successfully prepared after a series of targeting protein high expression, purification and renaturation. Two New Zealand white rabbits and Balb/c mice were immunized with self-made DcR3 protein. anti-DcR3 pAb and mAbs with high titer, better specificity were acquired. Sandwich

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ELISA which coated with pAb and detected with HRP labed mAb was sensitive and precise to quantify DcR3. The established of sandwich ELISA which has high sensitive, specifity, and accuracy will base a substantial foundation for clinical to detect DcR3 conveniently and quickly.

Key words: Decoy receptor 3; protein expression; polyclonal antibody; monoclonal antibody; enzyme linked immunosorbent assay

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