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厦门大学

硕 士 学 位 论 文

构建 TNFAIP8 干扰及原核表达载体并制备 TNFAIP8 抗体

Construct the TNFAIP8 interference and prokaryotic expression vector and prepare TNFAIP8 antibody

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摘 要

恶性肿瘤的发病率在世界范围内逐渐增高，而肿瘤的发生与一些肿瘤相关基因的改变相关，即癌基因与抑癌基因之间的平衡被打破造成的，非正常的生活方式、各种污染、激素变化、辐射、药物及基因等因素也是其诱因。

肿瘤坏死因子 α 诱导蛋白 8 (tumor necrosis factor α inducedprotein-8, TNFAIP8)是转录因子 NF-κB 可诱导的抗凋亡，致癌分子，分子量为 21kD 的胞浆蛋白。TNFAIP8 在肾癌及乳腺癌中高表达，过表达的 TNFAIP8 可导致乳腺癌、肺癌、肾细胞癌和食管鳞状细胞癌的进展，因其在细胞凋亡信号转导、肿瘤发生、侵袭及转移过程中具有重要调控作用而逐渐成为研究的热点。

为了进一步探索 TNFAIP8 在肿瘤发生和发展中的作用，我们拟构建其原核表达载体 TNFAIP8-PET-22b(+)，并表达纯化 TNFAIP8 蛋白。同时应用 TNFAIP8 蛋白免疫新西兰兔制备其多抗；使用 TNFAIP8 蛋白免疫 BALB/c 鼠融合并筛选杂交瘤细胞株、制备其单克隆抗体并鉴定抗体活性。

为了深入的探究干扰表达 TNFAIP8 后对肿瘤细胞的影响及其在肿瘤发生发展中的作用机制，我们设计并合成针对人 TNFAIP8 基因的 RNA 干扰序列，将其连接到 pSIREN-RetroQ 载体，构建 TNFAIP8 基因的 shRNA 重组质粒。经脂质体法转染 TNFAIP8-shRNA-pSIREN-RetroQ 干扰质粒、载体 pSIREN-RetroQ 质粒和 GFP-pSIREN-RetroQ 质粒至人肺腺癌细胞系 A549 细胞，并检测其干扰 TNFAIP8 表达的效果。荧光显微镜检测带有 GFP 基因质粒的表达情况以确认转染效率，应用 RT-PCR 以及 Western-Blot 方法检测并筛选在 mRNA 和蛋白水平干扰效率最佳的干扰片段。通过干扰 A549 细胞 TNFAIP8 蛋白的表达，确定 TNFAIP8 对肿瘤细胞生长的影响。

研究结果表明，我们成功构建 TNFAIP8-PET-22b（+）表达载体，筛选到 TNFAIP8 高表达菌株，确定最适诱导条件为 0.6 mM IPTG 诱导 10h，纯化复性后的 TNFAIP8 蛋白纯度在 90％以上。Western-Blot 结果表明所纯化的蛋白可与抗 TNFAIP8 抗体发生特异结合。TNFAIP8 免疫新西兰兔获得 2 株多克隆抗体，其效价分别为 10^4 和 10^5。检测得出制备的多克隆抗体不可以应用于 Western-Blot

和免疫组化。TNFAIP8 免疫 BALB/c 鼠后融合筛选出 8 株杂交瘤细胞株，其分泌的抗体效价分别是 10^4、10^6、10^4、10^5、10^6、10^4、10^4 和 10^6，经鉴定 1 号至 5 号可应用于 Western-Blot。

针对 TNFAIP8 基因的不同片段，我们成功设计和构建了三条干扰质粒 TNFAIP8-shRNA-pSIREN-RetroQ。GFP-pSIREN-RetroQ 对照质粒转染 A549 细胞 48h 后，荧光显微镜下可观察到大量绿色荧光蛋白的表达，其转染效率为 80%，经 RT-PCR 和 Western-Blot 证实 TNFAIP8-shRNA1-pSIREN -RetroQ 能有效干扰并显著抑制细胞内 TNFAIP8 基因的表达。应用终浓度 100μg/mL 的 aDR_5ScFv 分别作用转染 TNFAIP8-shRNA1 干扰质粒和空载质粒的 A549 细胞 16h，流式结果显示前者早、晚期凋亡率均有增加，前者凋亡率为 36.3%高于后者 11.53%，说明 TNFAIP8 具有抑制肿瘤细胞凋亡的作用。

本实验成功获得 TNFAIP8-PET-22b（+）原核表达载体，纯化到 TNFAIP8 蛋白；获得 2 株 TNFAIP8 多克隆抗体；筛选的 5 株单克隆抗体可用于 Western-Blot 检测；成功获得 TNFAIP8-shRNA-pSIREN-RetroQ 干扰质粒；通过流式检测发现降低 TNFAIP8 表达可以提高细胞对 aDR_5ScFv 诱导凋亡的敏感性。

关键词：原核构建表达　TNFAIP8　RNA 干扰　凋亡

Abstract

Irregular lifestyles, pollution, radiation, hormone changes, drugs, genes and other factors are causing the incidencement of growing malignant tumors all over the world, while cancers are due to the changes of tumor-related genes, that is breaking the balance between oncogenes and tumor suppressor genes.

Tumor necrosis factor alpha induced protein 8 (tumor necrosis factor- α inducedprotein-8, TNFAIP8, TNFAIP8) was a 21kDa cytoplasmic protein who can induce the apoptosis and carcinogenic, also can be induced by transcription factor NF-κB. TNFAIP8 was highly expressed in renal cell carcinoma and breast carcinoma, overexpression of TNFAIP8 can lead to breast cancer, lung cancer, renal cell carcinoma and squamous cell carcinoma of the esophagus.TNFAIP8 become the hot topic of research because it has an important regulating role in the process of its signal transduction in cell apoptosis, carcinogenesis, invasion and metastasis in tumors.

In order to further explore the role of TNFAIP8 in the tumorigenesis and development of tumers, we plan to construct prokaryotic expression vector TNFAIP8-PET-22b (+) ,express and purify TNFAIP8 protein. Immune the New Zealand rabbits and with purified protein TNFAIP8 to preparate the corresponding titer polyclonal antibody and detect its activity. Preparate monoclonal antibody by using TNFAIP8 protein immune BALB/c mice screen mice fusion hybridoma cell lines and identify the activity of it.

According to the characteristics of shRNA and in order to further explore the effects on tumor cells after interference the expressing of TNFAIP8, we build shRNA recombinant plasmid targeting human TNFAIP8 gene by designing and synthesizing RNA interference on TNFAIP8 gene sequence and connect it to the pSIREN-RetroQ carrier. Transfection TNFAIP8-shRNA-pSIREN RetroQ interference plasmid,carrier PSIREN RetroQ plasmid and GFP-pSIREN-RetroQ plasmid to human lung adenocarcinoma cells A549 cells by the method of liposome

and detect the interference on TNFAIP8 expression. Detect the expression of GFP gene to confirm the transfection efficiency by fluorescence microscope and filter out the highest interference efficiency plasmid by detecting the mRNA and protein levels using RT-PCR and Western-Blot methods. Determine TNFAIP8 effects on tumor cell growth by interfering TNFAIP8 protein expression in A549 cells.

Sequencing results showing that we successfully built TNFAIP8-PET-22b (+) expression vector and selected the high expression bacterial by inducing strain. Conform the optimum condition was 0.6 mM IPTG induct for 10 h and puritied TNFAIP8 protein was over 90%. Western-Blot results showed that the purified protein can be specificly combined with TNFAIP8 antibody. Immune the New Zealand rabbit and obtained 2 polyclonal antibodies whose strains titer were respectively 10^4 and 10^5. The preparated polyclonal antibodies cannot be applied to Western-Blot and Immunehistochemistry. Using TNFAIP8 protein immune BALB/c mouse to integrate and filter out 8 hybridoma cell strains,the titers of their secretory antibodies were respectively 10^4, 10^6,10^4, 10^6, 10^6, 10^4, 10^6 and 10^4. The No. 1 to No. 5 monoclonal antibodies could be applied to Western-Blot. Successfully built three interference TNFAIP8 gene plasmid TNFAIP8-shRNA-pSIREN-RetroQ which were consistent with the expected sequences. The green fluorescent protein expression were detected by fluorescence microscope 48 h after GFP-pSIREN-RetroQ plasmid was transfected to A549 cells and the transfection efficiency was 80%. RT-PCR and Western-Blot results confirmed TNF-AIP8-shRNA1-pSIREN-RetroQ could effectively interfer- ence and significantly inhibit the expression of TNFAIP8. Flow cytometry result showed that with 100μg/mL aDR5ScFv acted 16h on the A549 cells separately 24h after transfected with TNFAIP8-shRNA1 interference plasmid and no-load plasmid, the late and early apoptosis rates of the former one was rising, the former apoptosis was 36.3% higher than the latter 11.53%,explaining that TNFAIP8 has effects on inhibiting tumor cell apoptosis.

This experiment successfully acquired the high expression TNFAIP8-PET -22b(+) prokaryotic expression vector and purified high purity TNFAIP8 protein. Obtained high titer polyclonal antibody and the screened 5 monoclonal antibody

could be applied to Western-Blot. Successfully built TNFAIP8-shRNA-pSIREN -RetroQ interference plasmid, which could significantly reduce the TNFAIP8 expression both in gene and protein levels. Flow cytometry testing found reduced the expression of TNFAIP8 could improve the sensitivity of cells toward apoptosis induced by aDR5ScFv.

Keywords: prokaryotic construct and expression;TNFAIP8;RNA interference; apoptosis.

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