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硕 士 学 位 论 文

Semaphorin3Fc 单克隆抗体的制备鉴定及初步应用

Preparation and Characterization of Semaphorin3Fc Monoclonal Antibody and the Preliminary Application

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摘 要

研究背景和目的

抑癌基因发生异常是癌症发生的机制之一。人类基因的很多区域发生改变时可导致细胞失控性生长。Sema3F 作为肺癌 19 个候选抑癌基因之一，是III型 Semaphorin（即神经导向因子，最初作为 Neuropilins 的配体在神经系统中发挥排斥神经节轴突和调节神经系统发育的作用）家族的一个成员。目前有研究发现，与正常组织相比 Sema3F 蛋白在肿瘤细胞中的表达普遍存在下调的趋势，并在血管形成以及肿瘤增殖、凋亡、粘附和转移等中起着负向调节作用。此外，诸多体内或者体外实验已经证明，Sema3F 的表达水平同样影响到肿瘤的发生发展等过程。以上证据提示 Sema3F 可能是一种潜在的肿瘤分子标记物。然而，由于其多种受体的参与导致的功能多样性和信号通路的复杂性，导致 Sema3F 在肿瘤生物学行为方面的研究还存在着诸多争议。因此，深入研究 Sema3F 在肿瘤生物学行为中的作用和相关信号通路，对于进一步阐明 Sema3F 作用机制、寻找肿瘤新的治疗靶点将具有重要意义。Sema3F 的 C 末端是其发挥作用的重要结构域，该结构经过弗林蛋白酶（furin）的蛋白水解作用被激活，从而与相应的受体相互作用。基于上述背景，本文旨在通过传统的杂交瘤技术制备一株靶向 Sema3F 的 C 末端的单克隆抗体，通过鉴定该抗体的特性并探索该抗体在实际中的应用，从而为开展 Sema3F 的临床诊断、预测预后以及阐明其在肿瘤细胞中的作用机制提供基础和技术支持。

实验方法

利用 Sema3F 的 C 末端融合蛋白（Sema3Fc）作为免疫原，免疫 4-6 周龄雌性 Balb/c 小鼠，通过细胞融合技术构建可以稳定分泌靶向 Sema3F 的单克隆抗体的杂交瘤细胞株。然后扩大培养杂交瘤细胞，注射入 Balb/c 小鼠腹腔中获得含有 Sema3Fc 单克隆抗体（Sema3Fc mAb）的腹水。通过 rProtein A 亲和柱纯化抗体，冻干机冻干抗体；SDS-PAGE 电泳分析纯化后的抗体纯度；间接 ELISA 鉴定腹水中抗体以及纯化冻干后抗体的活性；硫氰酸盐洗脱法测定腹水中抗体的相对亲

和指数。然后，采用 Western blotting、细胞免疫荧光、流式细胞术、免疫细胞化学染色实验以及免疫组织化学染色实验分别检测制备的单抗与肝癌细胞株和肝癌组织中 Sema3F 蛋白的结合情况。利用自制的 Sema3FcmAb 初步建立定量检测 Sema3F 的竞争 ELISA 方法，建立标准曲线，分析方法的灵敏度和最低检测限。此外，免疫组织化学染色方法检测肿瘤组织芯片中 Sema3F 蛋白，分析其在多种肿瘤组织、配对癌旁组织以及不同分期的乳腺癌组织中的表达情况。

实验结果

我们成功获得了 2 株稳定分泌 Sema3Fc mAb 的杂交瘤细胞株，分别命名为 G5 mAb 和 E8 mAb。通过腹腔注射杂交瘤细胞得到的腹水经 rProtein A 亲和柱纯化得到了纯度较高的抗体（抗体纯度约为 90 %，浓度约为 2 mg/mL）；间接 ELISA 结果表明两株杂交瘤细胞株分泌的 Sema3Fc mAb 滴度都在 1×10^{-5} 左右；硫氰酸盐洗脱法表明 E8 株抗体相对亲和力指数达到了 2.5mol/L，具有较高的亲和力。Western blotting 结果显示 E8 mAb 与 Sema3Fc 融合蛋白及两株肝癌细胞株 HepG2、BEL-7402 总蛋白中的 Sema3F 蛋白分别在 10KDa、90KDa 和 120KDa 处有一结合条带，说明抗体与线性的 Sema3F 可以很好的结合，并具有较高的特异性；流式细胞术、细胞免疫荧光和细胞化学染色实验结果表明，单抗与肝癌细胞株中立体结构的 Sema3F 蛋白也可以很好地结合，并具有很高的特异性；免疫组织化学染色结果表明 E8 mAb 可以特异性地结合肝癌组织中的 Sema3F 蛋白。应用自制的抗体通过竞争 ELISA 方法建立了定量检测 Sema3F 的标准曲线，得到的回归方程为 Y=0.0954x+7.1839，相关系数 R^2=0.9948。以抑制率达到 50%时的竞争物（标准样品）浓度为灵敏度和以抑制率达到 10%时的竞争物浓度为最低检测限，分析得到灵敏度和最低检测限分别为 448.806ng/mL 和 29.52ng/mL。组织芯片免疫组化结果显示，Sema3F 在多种肿瘤组织及配对癌旁组织中均有不同程度的表达；在不同分期乳腺癌中，Sema3F 的表达情况存在着差异。

结论

1 本实验成功制备筛选出了 2 株稳定分泌 Sema3Fc mAb 的杂交瘤细胞株并制备纯化了单克隆抗体。

2 纯化后的 G5 和 E8 株单克隆抗体经间接 ELISA 方法检测，抗体效价均能达到 1×10^{-5}，经抗体亚型鉴定，E8 株细胞分泌的单抗亚型为 IgM。

3 经 Western blotting、流式细胞术、细胞免疫荧光、细胞化学染色以及免疫组织化学染色鉴定，E8 mAb 能与肝癌细胞株 BEL-7402、HepG2 以及肝癌组织中 Sema3F 蛋白特异性结合。

4 应用 E8 mAb 初步建立了定量检测 Sema3F 的竞争 ELISA 方法。

5 初步应用 E8 mAb 对组织芯片中多种肿瘤组织中 Sema3F 表达进行定性检测，发现 Sema3F 在某些肿瘤中表达下调，提示可能参与肿瘤的发生发展。

关键词：Semaphorin3F；单克隆抗体；肿瘤；检测；竞争 ELISA

Abstract

Background and Purpose:

That Tumor Supress Gene(TSG) suffered to be mutant or abnormal expression is one of mechanism contributing to the initial of cancer. Many regions of human gene could lead cells to grow out of control when they changes. As one of 19 candidate TSGs of lung cancer, Sema3F is one member of class 3 Semaphorins ,which is also called collapsins and firstly identified as negative mediator of axon guidance during neuronal development. Semaphorins(Semas), also called collapsins, is firstly identified as negative mediator of axon guidance during neuronal development. As a member of Semas, Semaphorin3F(Sema3F) is a functional tumor suppress gene. In the recent research, it is found that Sema3F along with its receptors are extensively expressed in tumor cells and play crucial role in angiogenesis and tumor growth, apoptosis, adhesion and metastasis, besides, sema3F may be invoved in regulation of tumor magligant phenotype and possesses independent signaling pathway . clinical studies found that the lower expression of Sema3F is tightly related to prognosis and metastasis of liver cancer, indicating that Sema3F may be a potential prognosis mark. Further, various experiment in vivo or in vitro demonstrated that the expression of Sema3F similarly has effect on progression of tumor. Further understanding of function of Sema3F in tumor biological behavior and relative signaling pathway will be of great significance to explain the mechanism underlying in these functions and find new target. C-terminu is importan domain, which Sema3F lies on to exert functions, and the domain needs to be activated by furin therefore interacting with corresponding receptors. Basing on the background above, this study aims to produce monoclonal antibody (mAb) against Sema3F by hybridoma technique and identify the characters of this antibody with expectation to provide basic and technique support for clinical diagnosis, and finally apply the antibody to preliminarily explore the relationship between the expression of Sema3F and tumor pathological grades.

Methods:

Sema3Fc was used as antigen to immune 4-6 week old Balb/c mice and establish hybridoma stably secreting Sema3Fc mAb through the hybridoma protocol by Kohler and Milstein. Hybridoma was intraperitoneally injected into Balb/c mice to acquire ascites. The obtained ascites was purified by rProteinA column, freez-dried and suffered to SDS-PAGE; indirect ELISA was used to determine the antibody titer and thiocyanate elution method wad used to determine the relative affinity of mAb. In addition, with mAb, regular experiments in lab such as western blotting , flow cytometry immunofluorescence analysis, immunocytochemical staining and immunohistochemical staining assay were used to detect Sema3F expression in liver cancer cells line HepG2 and BEL-7402 and liver tissues, meanwhile assessing the specificity of E8 mAb. Later, with produced Sema3Fc mAb, we developed competitive ELISA method for quantitative detection of Sema3F, established standard curve and analyzed sensitivity and detection limit of the method. Besides, immunohistochemical staining assay was used to detect the expression of Sema3F in tumor tissue chips.

Results:

We successfully established two hybridoma stably secreting Sema3Fc mAb. Though intraperitoneally injecting hybridoma, we obtain the ascites containing Sema3Fc mAb. After processing by rProteinA column and freeze-drying, the purity of Sema3Fc mAb is as high as 90 % and concentration is 2mg/mL; indirect ELISA showed that Sema3Fc mAb got the titer of 1×10^{-5}; thiocyanate elution method indicated the relative affinity of mAb is 2.5mol/L. In addition, Western blotting showed the bands of 10 KDa, 90KDa and 120 KDa respectively, indicating E8 mAb could bind to both recombinant Sema3Fc and full-length Sema3F in cancer cells; Flow cytometry, immunofluorescence staining, immunocytochemistry and immunohistochemica staining results indicated that Sema3F mAb could specifically combine Sema3F expressed in liver cancer cells line and liver tissue. With produced E8 mAb, we successfully established ELISA method for detection of Sema3F and

obtained standard curve. The regression equation of the curve is Y=0.0954x+7.1839, the relative coefficient R^2=0.9348. We designated the corresponding competitor concentration as sensitivity when the inhibition rate reached 50%, and as detection limit when 10%. Therefore, the sensitivity and detection limit is 448.806ng/mL and 29.52ng/mL respectively.

Conclusions:

1 We successfully establish two hybridoma cell lines stably secreting Sema3Fc mAb and produce the abundant monoclonal antibody.

2 The titer of Sema3Fc mAb two hybridoma secreting is about 1×10^{-5} by indirect ELISA method; according to isotype identification, the mAb belongs to IgM.

3 Identified by western blotting, flow cytometry, immunocytofluorescence, immunocytochemical staining and immunohistochemical staining, the prepared E8 mAb could specifically bind to Sema3F protein expressed in liver cell lines BEL-7402 and HepG2, also in liver tissue.

4 We preliminarily developed a competitive ELISA method for quantitative detection of Sema3F

5 With E8 mAb as detector, we analyzed the expression of Sema3F protein in clinical pathological tissues by immunohistochemical staining, and found the expression of Sema3F is downregulated in some tumor, indicating that Sema3F may involved in progression of tumor.

Key words: Semaphorin3F; Monoclonal antibody; Tumor; Detection; Competitive ELSIA

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