

学校编码: 10384
学号: 22620081151543

分类号 _____ 密级 _____
UDC _____

廈門大學

硕士学位论文

肺癌细胞 A549 中 Cx43 蛋白与 AKAP95 蛋白
相互作用的研究

The study about interactions between Cx43 and AKAP95 in
lung cancer cells A549

王苏

指导教师姓名: 张永兴 教授
专业名称: 环境科学
论文提交日期: 2011 年 5 月
论文答辩时间: 2011 年 6 月

2011 年 5 月

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果,均在文中以适当方式明确标明,并符合法律规范和《厦门大学研究生学术活动规范(试行)》。

另外,该学位论文为()课题(组)的研究成果,获得()课题(组)经费或实验室的资助,在()实验室完成。(请在以上括号内填写课题或课题组负责人或实验室名称,未有此项声明内容的,可以不作特别声明。)

声明人(签名):

年 月 日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文(包括纸质版和电子版)，允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

()1.经厦门大学保密委员会审查核定的保密学位论文，于
年 月 日解密，解密后适用上述授权。

()2.不保密，适用上述授权。

(请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。)

声明人(签名)：

年 月 日

目 录

摘 要.....	I
ABSTRACT.....	III
缩略词表.....	V
第 1 章 前言.....	1
1.1 细胞间隙连接蛋白家族.....	1
1.2 细胞间隙连接通讯.....	2
1.3 CONNEXIN43 蛋白及磷酸化.....	4
1.3.1 Cx43 蛋白在稳态中的生命周期.....	4
1.3.2 Cx43 蛋白的磷酸化.....	4
1.4 Cx43 蛋白磷酸化相关激酶及对间隙连接的影响.....	5
1.4.1 蛋白激酶 A(cAMP-dependent protein kinase, PKA).....	5
1.4.2 蛋白激酶 C(protein kinase C, PKC).....	6
1.4.3 蛋白激酶 G(protein kinase G, PKG).....	6
1.4.4 丝裂原活化蛋白激酶(mitogen activated protein kinase, MAPK).....	6
1.4.5 酪蛋白激酶 1(casein kinase, CK1).....	7
1.4.6 细胞分裂周期蛋白激酶 2 (Cell Division Cycle protein2, Cdc2).....	7
1.4.7 非受体蛋白酪氨酸激酶.....	7
1.5 Cx43 蛋白去磷酸化与间隙连接.....	8
1.5.1 丝氨酸/苏氨酸磷酸酶(serine/ threonine protein phosphatase, S/ TPP).....	8
1.5.2 蛋白酪氨酸磷酸酶(protein tyrosine phosphatase, PTP).....	8
1.6 Cx43 蛋白病理生理意义.....	9
1.6.1 Cx43 蛋白与肿瘤.....	9
1.6.2 Cx43 蛋白与神经系统疾病.....	9
1.6.3 Cx43 蛋白与心血管疾病.....	10
1.6.4 Cx43 蛋白与生殖系统疾病.....	10
1.6.5 Cx43 蛋白与其他生理、病理过程.....	11
1.7 AKAP 家族及 AKAP95 蛋白.....	11

1.8 本论文的目的、研究内容和意义	13
第 2 章 构建 A549-CX43 稳定细胞株	14
2.1 材料.....	14
2.1.1 实验细胞株及菌种.....	14
2.1.2 质粒.....	14
2.1.3 主要试剂及耗材.....	14
2.1.4 主要仪器及设备.....	15
2.1.5 主要溶液的配制.....	15
2.2 方法	16
2.2.1 质粒的转化和扩增.....	16
2.2.2 质粒的提取.....	16
2.2.3 质粒浓度的测定.....	17
2.2.4 转染细胞.....	17
2.2.5 细胞筛选抗生素浓度的确定.....	18
2.2.6 单克隆筛选.....	18
2.3 结果	18
2.3.1 A549-Cx43 细胞株的鉴定.....	18
2.4 结论	19
第 3 章 正常细胞中与 CX43 蛋白存在相互作用的蛋白质鉴定	20
3.1 材料.....	20
3.1.1 实验细胞株.....	20
3.1.2 主要试剂及耗材.....	20
3.1.3 主要仪器及设备.....	21
3.1.4 主要溶液的配制.....	22
3.2 方法	23
3.2.1 人支气管上皮细胞 16HBE 培养.....	23
3.2.2 人支气管上皮细胞 16HBE 总蛋白的提取.....	23
3.2.3 蛋白浓度测定.....	23
3.2.4 免疫共沉淀(Co-Immunoprecipitation, Co-IP)步骤.....	24

3.2.5 银染.....	25
3.2.6 Micro LC-ESI-LTQ 质谱鉴定.....	25
3.3 结果	26
3.3.1 Co-IP 银染结果	26
3.3.2 Co-IP 条带蛋白鉴定	27
3.4 讨论	29
3.5 结论	30
第 4 章 CX43 蛋白与 AKAP95 蛋白的相互作用.....	31
4.1 材料	31
4.1.1 实验细胞株.....	31
4.1.2 主要试剂及耗材.....	31
4.1.3 主要仪器及设备.....	33
4.1.4 主要溶液的配制.....	33
4.2 方法	35
4.2.1 细胞浆蛋白和胞核蛋白的提取.....	35
4.2.2 测定蛋白浓度.....	35
4.2.3 Co-IP	35
4.2.4 Western blot	36
4.2.5 细胞免疫荧光:	36
4.2.6 PKA 抑制剂 H-89 抑制 PKA 活性	37
4.3 结果	37
4.3.1 核浆蛋白分离效果分析.....	37
4.3.2 Co-IP 验证 Cx43 蛋白和 AKAP95 蛋白相互结合	38
4.3.3 细胞免疫荧光检测 Cx43 蛋白与 AKAP95 蛋白在细胞中的定位.....	40
4.3.4 PKA 活性对 Cx43 蛋白与 AKAP95 蛋白相互作用的影响.....	44
4.4 讨论	46
4.5 结论	48
第 5 章 AKAP95-miRNA 的设计及效果评价	49
5.1 材料.....	49

5.1.1 实验细胞株及菌种.....	49
5.1.2 载体质粒.....	49
5.1.3 主要试剂及耗材.....	49
5.1.4 主要仪器及设备.....	49
5.2 方法	50
5.2.1 miRNA 短序列的设计及合成.....	50
5.2.2 构建 pcDNA6.2-GW/EmGFP-miR-AKAP95 基因质粒.....	51
5.2.3 miRNA 沉默效果评价.....	53
5.3 结果	53
5.3.1 Western blot 检验 AKAP95 miR 四个片段的沉默效果.....	53
5.4 讨论	54
参考文献.....	55
致谢.....	61

Contents

Chinese abstract	I
English abstract	III
Abbreviations	V
1 Introduction	1
1.1 Intercellular connection protein family	1
1.2 Gap Junctional Intercellular Communication	2
1.3 Cx43 and phosphorylation	4
1.3.1 Life cycle of Cx43 in homeostasis.....	4
1.3.2 Phosphorylation of Cx43	4
1.4 Cx43 phosphorylation kinase and effect on GJIC	5
1.4.1 PKA.....	5
1.4.2 PKC.....	6
1.4.3 PKG.....	6
1.4.4 MAPK.....	6
1.4.5 CK1.....	7
1.4.6 Cdc2.....	7
1.4.7 Src.....	7
1.5 Dephosphorylation of Cx43 and GJIC	8
1.5.1 S/ TPP	8
1.5.2 PTP.....	8
1.6 Pathophysiological significance of Cx43	9
1.6.1 Cx43 and tumor.....	9
1.6.2 Cx43 and nervous system disease.....	9
1.6.3 Cx43 and angiocardopathy	10
1.6.4 Cx43 and reproductive system disease	10

1.6.5 Cx43 and other physiological pathology process	11
1.7 AKAP and AKAP95.....	11
1.8 Aim、 research contents and significance.....	13
2 Constructing stable transfection A549-Cx43 cell.....	14
2.1 Materials.....	14
2.1.1 Cells and strains	14
2.1.2 Plasmid.....	14
2.1.3 Reagents	14
2.1.4 Main instruments	15
2.1.5 Primary solutions and buffers	15
2.2 Methods	16
2.2.1 Transformation and amplification of plasmid.....	16
2.2.2 Extraction of plasmid.....	16
2.2.3 Concentration determination of plasmids	16
2.2.4 Transfection of cells.....	17
2.2.5 Determination of antibiotic concentrations for cell screening.....	17
2.2.6 Monoclonal screening.....	18
2.3 Results.....	18
2.3.1 A549-Cx43 cell appraisal.....	18
2.4 Conclusion.....	19
3 Identification of proteins interacting with Cx43 in normal cells.....	20
3.1 Materials.....	20
3.1.1 Cell.....	20
3.1.2 Reagents.....	20
3.1.3 Main instruments	21
3.1.4 Primary solutions and buffers	22
3.2 Methods	23
3.2.1 Cell culture.....	23

3.2.2 Extraction of protein	23
3.2.3 Measuring the protein concentration	23
3.2.4 Co-Immunoprecipitation.....	24
3.2.5 Silver staining	25
3.2.6 Micro LC-ESI-LTQ mass spectrometry.....	25
3.3 Results.....	26
3.3.1 Result of co-IP and silver staining	26
3.3.2 Result of mass spectrometry	26
3.4 Discussion	29
3.5 Conclusion.....	30
4 Interaction of Cx43 and AKAP95.....	31
4.1 Materials.....	31
4.1.1 Cells	31
4.1.2 Reagents.....	31
4.1.3 Main instruments	33
4.1.4 Primary solutions and buffers	33
4.2 Methods	35
4.2.1 Extraction of nuclear and cytoplasmic protein	35
4.2.2 Measuring the protein concentration	35
4.2.3 Co-IP.....	35
4.2.4. Western blot	36
4.2.5 Cellular immune fluorescence	36
4.2.6 Using PKA inhibitor H - 89 inhibit PKA activity	37
4.3 Results.....	37
4.3.1 Analysis of nuclear and cytoplasmic protein separation results	37
4.3.2 Cx43 interaction with AKAP95	38
4.3.3 Results of cellular immune fluorescence	40
4.3.4 Effect of PKA activity on interaction between Cx43 and AKAP95	40
4.4 Discussion	46

4.5 Conclusion	48
5 Design and effect assessment of AKAP95-miRNA	49
5.1 Materials	49
5.1.1 Cells and strains	49
5.1.2 Plasmid.....	49
5.1.3 Reagents.....	49
5.1.4 Main instruments	49
5.2 Methods	50
5.2.1 Design and synthesis of miRNA	50
5.2.2 Establishing of pcDNA6.2-GW/EmGFP-miR-AKAP95	51
5.2.3 Evaluation effect miRNA gene silencing.....	53
5.3 Results	53
5.3.1 Result of Evaluation effect miRNA gene silencing.....	53
5.4 Discussion	54
References	55
Acknowledgments	61

摘要

细胞间隙连接蛋白(Connexin, Cx)是跨细胞膜的蛋白质,是构成细胞间隙连接(gap junction)的重要蛋白。Cx 蛋白表达下降引起细胞间隙连接通讯功能下降或消失,使相邻细胞之间不能沟通信息,细胞之间失去了整体的组织协调功能,丧失了对细胞的正常生长和增殖调控能力,导致正常细胞恶变、肿瘤发生。因此认为细胞间隙连接通讯对于调控细胞生长和增殖以及控制细胞的恶性转化有重要作用。近年来也有研究表明 Cx 蛋白除在细胞膜上形成细胞间隙连接的功能外,尚可直接在细胞内参与细胞周期调节而发挥其对细胞生长和增殖的调控作用。

为了深入研究 Cx43 蛋白非依赖细胞间隙连接通讯功能对细胞生长调节的作用及其可能作用靶点,我们通过免疫共沉淀,串联质谱鉴定出在正常上皮细胞中与 Cx43 蛋白相互结合的 72 种蛋白质。这 72 种蛋白质的功能涉及细胞结构、细胞代谢等诸多生物学过程,如,与细胞结构完整、细胞运动及胞质分裂相关的蛋白质;参与细胞代谢的蛋白质;细胞内蛋白的折叠和组装及蛋白降解相关的蛋白质;参与细胞周期、凋亡调控的蛋白质等。经过对这些蛋白质进行生物学功能归类与分析,我们选定了其中的 AKAP95 蛋白作为 Cx43 蛋白的作用靶点进行深入的实验研究。

利用免疫共沉淀、Western blot 等技术,通过细胞浆核分离方法,证明了在 A549 及 A549-Cx43 细胞中, Cx43 蛋白不仅在细胞浆内表达,同时在细胞核内也有表达; AKAP95 蛋白主要在细胞核内表达,细胞浆没有检测到 AKAP95 蛋白; Cx43 蛋白与 AKAP95 蛋白在细胞核内存在相互作用关系。细胞免疫荧光激光共聚焦显微镜结果显示, Cx43 蛋白主要表达在细胞浆内,少量表达在细胞核内; AKAP95 蛋白主要表达在细胞核内,细胞浆内有少量表达,细胞核内的部分 Cx43 蛋白和 AKAP95 蛋白存在共定位现象。根据上述实验结果,我们认为 Cx43 蛋白调控细胞周期的作用可能存在细胞核内, AKAP95 蛋白可能是 Cx43 蛋白进入细胞核的载体蛋白。

为了研究 PKA 活性是否影响细胞核内 Cx43 蛋白和 AKAP95 蛋白的相互结合,我们利用 PKA 抑制剂 H-89 抑制了 PKA 的活性后,检测了 A549 及 A549-Cx43 细胞中 Cx43 蛋白与 AKAP95 蛋白的相互作用关系,实验结果显示经过 H-89 处

理后, A549 及 A549-Cx43 细胞核内仍然存在 Cx43 蛋白与 AKAP95 蛋白的相互作用关系, 由此推测, PKA 活性可能并不影响 Cx43 蛋白与 AKAP95 蛋白的相互结合关系。但值得指出的是, 用 H-89 处理细胞前, A549 及 A549-Cx43 细胞浆中 Western blot 方法未检测到 AKAP95 蛋白, 但 H-89 处理后细胞浆中检测到一定量的 AKAP95 蛋白, 因此, AKAP95 蛋白在细胞浆内有少量表达, H-89 处理后其表达量有所增加。

实验前期, 进行转染了 Cx43 基因的 A549-Cx43 细胞的构建, 与 A549 细胞相比, A549-Cx43 细胞 Cx43 蛋白表达量增多。后期设计完成 AKAP95 蛋白的 miRNA 基因质粒的构建。设计 4 个分别针对 AKAP95 mRNA 的 1009-1030、1587-1608、1619-1640 和 1895-1916 序列位点进行沉默的 miRNA 短序列。短序列连接到载体质粒后, 瞬时转染 A549-Cx43 细胞, 利用 Western blot 检测 AKAP95 蛋白的表达, 结果显示, 针对 1587-1608 位点沉默 AKAP95 蛋白表达的效果最好。

关键词: Cx43; AKAP95; 肺癌细胞 A549; 免疫共沉淀

Abstract

Connexin (Cx) is important transmembrane protein that constitutes cytomembrane channel structure-gap junction. The expression of Cx reduced causes intercellular gap connection communication (GJIC) decline or disappearance, that makes adjacent cells communicating information disappearance and losing overall coordination function between cells. Cells lost normal regulation ability of cell growth and proliferation lead to normal cells malignant transformation and cancerous tumors. So GJIC is thought having an important role on regulation cell growth, cell proliferation and controlling the malignant transformation of cell, while Cx exerts its inhibiting cellular canceration through formed GJIC. Recent years researches has shown that in the cell membrane Cx except the function of forming intercellular connected directly, it also involves in cell cycle regulation and plays regulation function on the cell growth and proliferation.

In order to research function of Cx43 on cell growth adjustment that not relying on GJIC and its possible targets, we identified 72 proteins interacting with Cx43 in normal epithelial cell 16HBE by co-immune precipitation and tandem mass spectrometry(ms). The functions of these 72 proteins identified in 16HBE cell involved in the cellular structure and the cellular metabolism, and many other biological processes. For example, proteins related cellular structure integrity, movements and cytoplasmic division of cells; Some proteins related with cell metabolism; With protein folding and assembly or degradation; Participating in the cell cycle and apoptosis .Through classification and analysis the biological functions of these proteins, we selected AKAP95 as the targets protein of Cx43 and did in-depth experimental studies.

Through method of cell plasma protein and nucleoprotein extraction respectively and using co-immune precipitation and Western blot, we proved that expression of Cx43 not only in cell plasma protein, but also in A549 and A549-Cx43; The expression of AKAP95 mainly in the nuclei, in cell plasma protein not detected; Cx43

and AKAP95 have an interaction in nucleoprotein. Results of cellular immune fluorescent laser confocal microscope show that expression of Cx43 mainly in cytoplasm, a small amount of expression in the nuclei; AKAP95 mainly expression in the nucleus, a few expression in cell plasma , part of AKAP95 and Cx43 exist co-localization in nuclei. According to above experimental results, we consider function of Cx43 on cell cycle regulation may exist in nuclei, AKAP95 may be as a carrier protein for Cx43 into nuclei.

In order to study whether PKA activity have an effect on interaction of Cx43 and AKAP95 in nuclei, we examined interaction relationship between Cx43 and AKAP95 in A549 and A549-Cx43 that dealing with PKA inhibitors H-89, results show that interaction relationship between Cx43 and AKAP95 still and AKAP95 still exists in dealing with H-89 cells. Thus we speculate that PKA activity have no effect on the interaction between Cx43 and AKAP95. But worth noticing that before using H-89 dealing with cells, AKAP95 wasn't detected in cytoplasm using Western blot in A549 and A549-Cx43 cells; but in plasma of dealing with H-89 cells a certain amount of AKAP95 can be detected. Therefore, expression quantity of AKAP95 increased in cytoplasm in cells that dealing with H-89.

In preliminary experiments, we constructed Cx43 gene carrying A549-Cx43 cells. Compared with A549 cells, expression of Cx43 in A549-Cx43 cells is increased. In advanced stage, we designed and completed AKAP95 miRNA gene plasmid construction. We designed four short sequences that acting respectively on 1009-1030, 1587-1608, 1619-371-380 and 1895-1916 sequence sites of AKAP95 mRNA. After connected short sequences to the carrier plasmid and transient transfecting A549-Cx43 cells, using Western blot tested AKAP95 expression. The results showed that short sequences acting on 1587-1608 sites silencing effect of expression of AKAP95 is best.

Key word: Cx43; AKAP95; Lung cancer cells A549; Co-immunoprecipitation

缩略词表

Cx43	Connexin 43 间隙连接蛋白 43
GJIC	gap junction intercellular communication 细胞间隙连接通讯
PKA	cAMP-dependent protein kinase cAMP 依赖性蛋白激酶 A
PKC	protein kinase C 蛋白激酶 C
PKG	protein kinase G 蛋白激酶 G
MAPK	mitogen activated protein kinase 丝裂原活化蛋白激酶
ERK	extracellular regulated protein kinase 细胞外调节蛋白激酶
BMK-1	Big MAP kinase-1 活化蛋白激酶
JNK	c-Jun N terminal kinase c-Jun 氨基末端激酶
CK1	casein kinase 酪蛋白激酶 1
Cdc2	Cell Division Cycle protein2 细胞分裂周期蛋白激酶 2
PP	protein phosphatase 蛋白磷酸酶
S/ TPP	serine/ threonine protein phosphatase 丝氨酸/苏氨酸磷酸酶
PTP	protein tyrosine phosphatase 蛋白酪氨酸磷酸酶
SHP-1	The Src homology-2-containing protein-tyrosine phosphatase 1 包含 2 个 Src 同源蛋白的酪氨酸磷酸酶
RPTP μ	The receptor protein tyrosine phosphatase 受体蛋白酪氨酸磷酸酶 μ
CRH	Corticotropin releasing hormone 促肾上腺皮质激素释放激素
mRNA	Messenger RNA 信使 RNA
AKAP95	A-kinase anchoring protein 蛋白激酶 A 锚定蛋白 95
biNLS	bipartite nuclear localization signal 二分型核定位信号
NMTS	the nuclear matrix targeting site 核基质靶位点
PCD	premature chromosome decondensafion 染色体超前解集缩
CDK	cyclin-dependent kinase 周期蛋白依赖性蛋白激酶
DAPI	4',6-diamidino-2-phenylindole 4',6-二脒基-2-苯基吲哚
AP	Ammonium Persulfate 过硫酸铵

Degree papers are in the "[Xiamen University Electronic Theses and Dissertations Database](#)". Full texts are available in the following ways:

1. If your library is a CALIS member libraries, please log on <http://etd.calis.edu.cn/> and submit requests online, or consult the interlibrary loan department in your library.
2. For users of non-CALIS member libraries, please mail to etd@xmu.edu.cn for delivery details.

厦门大学博硕士论文摘要库