中文摘要

Sirt3 调控 TIGAR 影响非小细胞肺癌对 Cisplatin 敏感性的机制研究

研究背景：

针对不同时期的非小细胞肺癌（Non small cell lung cancer，NSCLC）的临床治疗方案各有不同，长期以来I、Ⅱ期癌症患者的首选为外科手术和化学疗法，但是对于Ⅲ、Ⅳ期具有转移性病变的患者这种方法预后较差。如今针对上皮生长因子受体（EGFR）和间质上皮转化因子（MET）突变的分子检测以及间变性淋巴瘤激酶（ALK）和原癌基因 ROS1 易位的分析已成为诊断 NSCLC 的新兴方法，因此靶向治疗能够延长 NSCLC 患者总体生存期。但上述的治疗方案大多数需要抗癌药物顺铂（Cisplatin）的辅助治疗，而随着患者服药时间的增加，大部分患者对 Cisplatin 的敏感性逐渐下降甚至达到耐药的程度，导致药物治疗进入平台期，最后疗效不佳，目前人们对这种现象仍然缺乏深入研究。大量研究报道癌细胞即使在氧气充足的环境下生存也主要依靠无氧糖酵解的糖代谢方式为自身获取能量，即 Warburg 效应，达到 Cisplatin 耐药时期的 NSCLC 就是依靠这种代谢方式为自身快速提供能量，故推测代谢方式的改变可能是影响非小细胞肺癌细胞对 Cisplatin 药物敏感性的重要原因之一。本课题组前期研究发现与正常的肺组织切片比较，非小细胞肺癌患者的免疫组织化学染色切片中的 NAD 依赖的线粒体去乙酰化酶 Sirtuin3（Sirt3）表达更高。Sirt3 可以通过发挥其去乙酰化酶的功能使多种线粒体内的蛋白去乙酰化，从而参与肿瘤细胞的代谢过程。TIGAR (Tp53-induced glycolysis and apoptosis regulator) 作为 p53 诱导的糖酵解和凋亡调节因子，与糖酵解中磷酸果糖激酶 2/果糖-2,6-二磷酸酶(PFK2/FBPase-2)具有相似性，通过降低 F-2,6-BP 的水平抑制 PFK-1 的活性，减少该点下游的糖酵解通量，影响细胞代谢。最近有研究表明，在某些人源性肿瘤细胞系中发现了非依赖于 p53 的 TIGAR 表达，这些 TIGAR 仍具有糖酵解酶的作用。因此探讨 Sirt3、TIGAR、有氧糖酵解在非小细胞肺癌细胞之间的关系，可能为改变非小细胞肺癌对抗癌药物 Cisplatin 的敏感性提供一种新思路。

本研究基于脂质体转染方法改变两种人源非小细胞肺癌细胞系（NCI-H1299、A549）的 Sirt3 表达水平，通过研究联合 Cisplatin 作用下 NSCLC 糖代谢变化和药物敏感性的改变，探讨 Sirt3 影响非小细胞肺癌 Cisplatin 敏感性及联合治疗后糖代谢变化的机制，为克服非小细胞肺癌临床治疗上 Cisplatin 耐受提供新的机制探索与潜在的靶标。

实验方法：

1.用梯度浓度的 Cispaltin 作用于两种非小细胞肺癌细胞系 NCI-H1299 和 A549 细胞，利用 MTT 实验检测 Cispaltin 浓度对 NCI-H1299 和 A549 细胞生存能力的影响；Western blot 法检测 Cispaltin 处理后 NCI-H1299 和 A549 细胞 Sirt3 蛋白表达变化。

2. 运用 Hieff Trans 脂质体核酸转染试剂敲减 NCI-H1299 和 A549 细胞中 Sirt3 的表达，联合低浓度 Cispaltin 作用于 NCI-H1299 和 A549 细胞，利用 MTT 实验检测 Sh-Sirt3 联合 Cispaltin 对 NCI-H1299 和 A549 细胞生存能力的影响；通过 AnnexinV-FITC/PI 双染法和 Western blot 法检测 Sh-Sirt3 联合 Cispaltin 对 NCI-H1299 和 A549 细胞凋亡率的影响。

3. 运用 Hieff Trans 脂质体核酸转染试剂敲减 NCI-H1299 和 A549 细胞中 Sirt3 的表达，联合 Cispaltin 后，利用葡萄糖试剂盒和乳酸试剂盒检测 Sh-Sirt3 及联合 Cispaltin 对细胞葡萄糖摄取量和乳酸生成量影响；通过 Western blot 法检测 Sh-Sirt3 及联合 Cispaltin 对糖代谢相关酶的蛋白表达的影响。

4. 运用 Hieff Trans 脂质体核酸转染试剂敲减 NCI-H1299 和 A549 细胞中 Sirt3 的表达，联合 Cispaltin 后，运用免疫荧光技术以及 Western blot 法检测 Sirt3 和 TIGAR 在细胞中具体定位。

5. 运用 Hieff Trans 脂质体核酸转染试剂敲减 NCI-H1299 和 A549 细胞中 Sirt3 的表达，联合 Cispaltin 后，利用免疫共沉淀技术检测 Sirt3 去乙酰化酶的功能对 TIGAR 表达的影响。

6. 运用 Hieff Trans 脂质体核酸转染试剂在 NCI-H1299 和 A549 细胞中过表达 TIGAR，运用 Western blot 法反向验证 TIGAR 表达变化对 Sirt3 的影响。

7.基于裸鼠移植瘤模型实验对 Sh-Sirt3 增强 NSCLC 对 Cispaltin 敏感性的影响及机制进行验证。

实验结果：

1. Cispaltin 抑制 NCI-H1299 和 A549 细胞的生存，使 Sirt3 蛋白水平增高。

2.与 Control 组相比，Sh-Sirt3 后，NCI-H1299 和 A549 细胞细胞生存率降低，与 Cispaltin 相比，Sh-Sirt3 联合 Cispaltin 后，细胞生存率明显降低、细胞凋亡率明显升高，Sh-Sirt3 增强细胞对 Cispaltin 的敏感性。

3. NCI-H1299 和 A549 细胞 Sh-Sirt3 联合 Cispaltin 后，细胞中葡萄糖摄取和乳酸生成全部有所下降。

4. NCI-H1299 和 A549 细胞 Sh-Sirt3 联合 Cispaltin 后，与 Conrol 组相比，糖酵解相关酶 TIGAR、PFK-1 表达降低，FBPase1 表达增加，NCI-H1299 细胞中 G-6-PD 表达无明显变化，而 A549 细胞中 G-6-PD 表达降低。

5. Sirt3 和 TIGAR 在 NCI-H1299 和 A549 细胞的细胞浆中表达并结合。

6. NCI-H1299 和 A549 细胞 Sh-Sirt3 后，细胞中总乙酰化蛋白表达增加，其中乙酰化 TIGAR 的水平增加，Sirt3 发挥去乙酰化酶功能调控 TIGAR。

7.与 Control 组相比，Sh-Sirt3 联合 Cispaltin 明显抑制了 NCI-H1299 非小细胞肺癌细胞裸鼠模型中肿瘤的生长。

结论：

1. Sh-Sirt3 增强非小细胞肺癌细胞对 Cisplatin 的敏感性，诱导细胞凋亡。

2. Sh-Sirt3 调控 TIGAR 抑制 NSCLC 糖酵解增强细胞对 Cisplatin 的敏感性。具体机制是 Sh-Sirt3 能够增强 p53 和 TIGAR 的乙酰化，致使 TIGAR 表达水平增加，进一步抑制糖酵解关键酶 PFK-1 的活性，逆转细胞糖酵解过程，从而增强对 Cisplatin 的敏感性。

关键词：

非小细胞肺癌，糖酵解，Sirt3，TIGAR

Abstract

the mechanism of Sirt3 regulating TIGAR affecting the sensitivity of non-small cell lung cancer to Cisplatin

Background:

Clinically, there are different treatment schemes for non-small cell lung cancer (NSCLC) in different stages. For a long time, the first choice for patients with stage I、II cancer is surgery and chemotherapy, but for patients with stage III and IV metastatic lesions, this method has a poor prognosis. Nowadays, molecular detection of mutations in epithelial growth factor receptor (EGFR) and mesenchymal epithelial transforming factor (MET), as well as analysis of anaplastic lymphoma kinase (ALK) and proto-oncogene ROS1 translocation have become new methods for diagnosis of NSCLC, so targeted therapy can prolong the overall survival of NSCLC patients. However, most of the above treatment schemes require the adjuvant treatment of cisplatin, an anticancer drug. With the increase of the time taken by patients, the sensitivity of most patients to cisplatin gradually decreases or even reaches the level of drug resistance, leading to the drug treatment entering the platform stage, and the final treatment effect is poor. At present, people still lack of in-depth research on this phenomenon. A large number of studies have reported that cancer cells, even in an environment with sufficient oxygen, mainly rely on anaerobic glycolysis to obtain energy for themselves, that is, Warburg effect. NSCLC, which has reached the stage of cisplatin resistance, relies on this metabolic mode to provide energy for itself quickly. Therefore, we speculate that the change of metabolic mode may be one of the important reasons that affect the sensitivity of non-small cell lung cancer cells to cisplatin. Our previous study found that the expression of NAD-dependent mitochondrial deacetylase Sirtuin3 (Sirt3) was higher in the immunohistochemical staining sections of patients with non-small cell lung cancer compared with normal

lung tissue sections. Sirt3 can deacetylate various mitochondrial proteins by performing its deacetylase function, thus participating in the metabolic process of tumor cells. TIGAR (Tp53-induced glycolysis and apoptosis regulator), as a regulator of p53 induced glycolysis and apoptosis, is similar to the phosphofructose kinase 2/fructose-2,6-diphosphatase (PFK2/FBPase-2) in glycolysis. It inhibits the activity of PFK-1 by reducing the level of F-2,6-BP, reduces the glycolytic flux downstream of this point, and affects cell metabolism. Recent studies have shown that p53 independent TIGAR expression has been found in some human cancer cell lines, and these TIGAR still have the function of glycolytic enzyme. Therefore, exploring the relationship between Sirt3, TIGAR and aerobic glycolysis may provide a new way to change the sensitivity of non-small cell lung cancer to anticancer drug cisplatin.

Objective:

By studying the changes of glucose metabolism and drμg sensitivity of NSCLC under the action of Cisplatin, we explore the effect of Sirt3 on the Cisplatin sensitivity of NSCLC and the changes of glucose metabolism after the combined treatment, so as to provide new mechanism exploration and potential target for overcoming Cisplatin tolerance in clinical treatment of non-small cell lung cancer.

Methods:

1. Cispaltin with gradient concentration was applied to two kinds of non-small cell lung cancer cell lines NCI-H1299 and A549, and the effect of Cispaltin concentration on the viability was detected by MTT assay; Western blot was used to detect the expression of Sirt3 after Cisplatin treatment.
2. Knocked down the expression of Sirt3 in NCI-H1299 and A549 cells, and combinated with low concentration of Cisplatin. The effect of Sh-Sirt3 and Cisplatin on the viability was detected by MTT assay; The effect of Sh-Sirt3 combined with Cisplatin on the apoptosis rate was detected by Annexin V-FITC/PI double staining and Western blot.
3. Knocked down the expression of Sirt3 in NCI-H1299 and A549 cells and then combining with Cisplatin, the glucose uptake and lactate production of

cells were detected with glucose and lactate kits; Western blot was used to detect the protein expression of glucose metabolism-related enzymes.

4. Knocked down the expression of Sirt3 in NCI-H1299 and A549 cells,and then combining with Cisplatin, the specific location of Sirt3 and TIGAR in cells was checked by immunofluorescence assay and Western blot.
5. Knocked down the expression of Sirt3 in NCI-H1299 and A549 cells,and then combining with Cisplatin, the effect of Sirt3 deacetylase function on the expression of TIGAR was detected by immunoprecipitation technique.
6. Over-expressed TIGAR in NCI-H1299 and A549 cells. Western blot was used to reverse verify the effect of TIGAR expression change on Sirt3.
7. The mechanism of Sh-Sirt3 enhancing the sensitivity of NSCLC to Cisplatin was verified based on the nude mice transplanted tumor model experiment.

Results:

1. Cisplatin inhibits the survival of NCI-H1299 and A549 cells and increases the level of Sirt3 protein .
2. Compared with the control group, the survival rate of NCI-H1299 and A549 cells decreased after Sh-Sirt3. Compared with Cisplatin, the survival rate significantly decreased and the apoptosis rate significantly increased after Sh-Sirt3 combined with Cisplatin. Sh-Sirt3 enhanced the sensitivity of cells to Cisplatin.
3. Sh-Sirt3 combined with Cisplatin in NCI-H1299 and A549 cells, the glucose uptake and lactic acid production in cells decreased.
4. After NCI-H1299 and A549 cells Sh-Sirt3 combined with Cisplatin, compared with Conrol group, the expression of glycolytic enzymes TIGAR and PFK-1 decreased, and the expression of FBPase1 increased. The expression of G-6-PD in NCI-H1299 cells did not change significantly, while the expression of G-6-PD in A549 cells decreased.
5. Sirt3 and TIGAR are expressed and bound in the cytoplasm of NCI-H1299 and A549 cells.

6. After Sh-Sirt3, the expression of acetylation protein and TIGAR acetylation increased in the cells, and Sirt3 played the role of deacetylase to regulate TIGAR.
7. Compared with the control group, Sh-Sirt3 combined with Cisplatin significantly inhibited the growth of tumor in the nude mice model of NCI-H1299 non-small cell lung cancer cells.

Conclusion:

1. Sh-Sirt3 enhances the sensitivity of non-small cell lung cancer cells to Cisplatin and induces cell apoptosis.
2. Sh-Sirt3 regulates TIGAR to inhibit NSCLC glycolysis and enhance cell sensitivity to Cisplatin. The mechanism is that Sh-Sirt3 can directly or indirectly promote TIGAR acetylation, increasing TIGAR expression level, further inhibiting the activity of enzyme PFK-1, reversing the cellular glycolytic process, and thereby enhancing sensitivity to Cisplatin.

Key words:

non-small cell lung cancer, glycolysis, Sirt3, TIGAR

目　录

英文缩写词表

英文缩写	英文全称	中文全称
ATP	Adenosine Triphosphate	三磷酸腺苷
DMSO	Dimethyl SμL phoxide	二甲基亚砜
FBS	Fetal bovine serum	胎牛血清
FBPase1	phosphofructose-1,6-bisphosphase	果糖-1,6-二磷酸酶
F-6-P	glucose-6-phosphate	果糖-6-磷酸
G-6-PD	Glucose-6-phosphate dehydrogenase	葡萄糖-6-磷酸脱氢酶
HK	hexokinase	己糖激酶
kDa	kilo Dalton	千道尔顿
MTT	Methylthiazolyl Tetrazolium	甲基噻唑基四唑
NSCLC	non-small cell lung cancer	非小细胞肺癌
PDH	pyruvate dehydrogenasecomplex	丙酮酸脱氢酶复合体
PFK-1	phosphofructokinase-1	磷酸果糖激酶 1
PVDF	Polyvinylidene Fluoride	聚偏二氟乙烯
PMSF	Phenylmethanesulfonyl fluoride	苯甲基磺酰氟
RIPA	Radio Immunoprecipitation Assay	放射免疫沉淀法
SDS	sodium dodecyl sulfate	十二烷基磺酸钠

前 言

目前，肺癌是全国癌症死亡率极高的恶性肿瘤之一，其中非小细胞肺癌（NSCLC）占据 85%。因前期症状较轻，在发现时大多患者已处于晚期阶段，尽管出现了手术切除病灶、靶向治疗的治疗方法，但由于患者对辅助化疗药物 Cisplatin 的敏感性下降直至产生耐药，最后癌症复发导致的死亡率依旧很高，所以寻找新的生物分子并提高非小细胞肺癌细胞对 Cisplatin 的敏感性对未来非小细胞肺癌的治疗具有很大的意义。大量研究报道，与正常细胞相比，即使在氧气充足的环境下癌细胞主要依靠无氧糖酵解的糖代谢方式为自身获取能量，即 Warburg 效应，而达到 Cisplatin 耐药时期的非小细胞肺癌就是依靠这种代谢方式为自身快速提供能量。总而言之，肿瘤细胞对自身进行了代谢重编程，这是肿瘤细胞目前最大的特征，因此改变这种代谢方式为增加非小细胞肺癌细胞对药物敏感性和抑制肿瘤细胞增殖转移寻找到新的治疗思路。

目前大量研究表明，Sirt3 通过去乙酰化酶的活性影响线粒体代谢，TIGAR 作为 Tp53 诱导的糖酵解和凋亡调节因子，不仅与肿瘤细胞的生存息息相关，还能通过调节糖酵解途径改变细胞的代谢变化。前期研究发现，与正常的肺组织相比，非小细胞肺癌细胞的免疫组织化学染色结果中 Sirt3 呈高度表达。本研究基于非小细胞肺癌细胞系（NCI-H1299、A549），首先验证低浓度 Cisplatin 能够刺激 NCI-H1299、A549 细胞 Sirt3 的表达，Sh-Sirt3 能够促进 NCI-H1299、A549 细胞对 Cisplatin 的敏感性，然后基于细胞糖代谢水平探讨在非小细胞肺癌细胞中 Sirt3 通过调控 TIGAR 影响非小细胞肺癌细胞糖酵解，进一步促进非小细胞肺癌细胞对 Cisplatin 敏感性的作用及其机制。本研究通过探究 Sirt3 调控糖酵解和凋亡调节因子 TIGAR 影响 NSCLC 糖酵解水平的机制进而改变 NSCLC 对 Cispaltin 敏感性的影响，为临床提高非小细胞肺癌对 Cisplatin 敏感性的方法提供新思路。