Hsa-miR-99a-5p 在胆囊癌中的功能与机制研究

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Research on the Functions and Mechanisms of Hsa-miR-99a-5p in Gallbladder Cancer

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摘要

研究背景

胆囊癌是发病率较低但恶性程度极高的肿瘤，其发病率在消化道肿瘤中排第六位。目前根治性手术切除是唯一可能治愈胆囊癌的方法，但由于其早期缺乏特异性症状，大部分患者一经发现已至进展期，根治性切除率低，故预后极差，五年生存率不足 5%。miRNA 是一类小分子非编码 RNA。它可以通过与靶基因的 3'UTR 区结合来参与对下游分子与信号通路的调控作用。miRNA 在许多恶性肿瘤的发生发展过程中都扮演了至关重要的角色。

方法

1、 通过前期的高通量 miRNA 芯片检测了胆囊癌组织以及其相应的癌旁正常组织中 miRNA 的表达情况，同时结合 TCGA 数据库的资料，筛选出 miR-99a-5p 作为研究对象。

2、 采取实时荧光定量 PCR 的方法检测了胆囊癌患者癌组织及其相应的癌旁组织中 miR-99a-5p 的表达情况，并分析 miR-99a-5p 与胆囊癌患者的临床病理资料以及预后的相关性。

3、 通过细胞功能学实验和体内动物实验研究 miR-99a-5p 对胆囊癌细胞的增殖和侵袭转移能力是否存在调控作用。

4、 通过生物信息学方法预测 miR-99a-5p 的靶基因，使用双荧光素酶报告基因试验检测 miR-99a-5p 是否与靶基因的 3'UTR 区产生作用，并用 Western blot 实验检测 miR-99a-5p 是否能在蛋白水平抑制靶基因的表达，同时判断 miR-99a-5p 是否对相关信号通路和 EMT 相关指标产生影响。

5、 用细胞功能学实验验证 miR-99a-5p 的候选靶基因 SMARCA5 在胆囊癌中的相关功能，并用回复实验验证 miR-99a-5p 能够通过靶定 SMARCA5 实现抑制胆囊癌细胞侵袭和转移的功能。

结果

1、通过 miRNA 芯片分析结合实时荧光定量 PCR 验证，发现 miR-99a-5p 在胆囊癌患者癌组织中普遍低表达，且 miR-99a-5p 的低表达与胆囊癌患者的不良预后具有显著相关性；

2、体内体外实验证明 miR-99a-5p 的过表达通过调控 PI3K/AKT 通路和 EMT 作用抑制了胆囊癌细胞的侵袭和转移功能；

3、MTOR 和 SMARCA5 确为 miR-99a-5p 的直接下游靶基因；

4、SMARCA5 能够促进胆囊癌细胞的侵袭和转移，且直接介导了 miR-99a-5p 抑制胆囊癌侵袭转移功能的作用。

结论

miR-99a-5p 在胆囊癌患者癌组织中普遍低表达，通过靶基因 MTOR、SMARCA5 以及 PI3K/AKT 通路和 EMT 作用抑制了胆囊癌的侵袭转移功能，miR-99a-5p 可能成为胆囊癌综合治疗的潜在靶点。

关键词： 胆囊癌； miRNA； 靶基因；侵袭转移；SMARCA5

Research on the Functions and Mechanisms of Hsa-miR-99a-5p in Gallbladder Cancer

Abstract

Background

Gallbladder cancer(GBC) is a tumor with low incidence but high degree of malignancy, which is the sixth most common gastrointestinal tumors. At present, radical resection is the only way to cure GBC. However, because of lack of specific early symptoms, most patients have been found to have progress. The radical resection rate is low and the prognosis is poor, with a five-year survival rate less than 5 %. miRNAs, a kind of small molecular noncoding RNA, can be involved in the regulation of downstream molecules and signaling pathways by binding to the 3'UTR of the target genes. miRNAs play a vital role in the development and progression of many malignancies.

Method

1. The expression of miRNAs in GBC tissues and peritumoral tissues was detected by high-throughput miRNAs microarray. MiR-99a-5p was selected as the research object according to the TCGA database.

2. The expression of miR-99a-5p in GBC tissues and peritumoral tissues was identified by qRT-PCR. The correlation between miR-99a-5p and clinicopathological data and prognosis of GBC patients was analyzed.

3. The effect of miR-99a-5p on proliferation and invasion/metastasis of GBC cells was investigated by functional experiment and in vivo animal experiment.

4. The target genes of miR-99a-5p were predicted by bioinformatics method. Whether miR-99a-5p interacted with the 3'UTR of the target genes was detected by dual luciferase reporter assay. Western blot was used to detect the level of target genes which could be inhibited by miR-99a-5p, also to identify whether miR-99a-5p affected the relevant signaling pathways and EMT-related markers.

5. The function of SMARCA5 in GBC was verified by functional experiment. The rescue experiment was processed to show whether miR-99a-5p could inhibit the invasion and metastasis of GBC cells by targeting SMARCA5.

Result

1.MiR-99a-5p expression in tumor tissues was significantly lower in patients with GBC, which was significantly correlated with the poor prognosis.

2. Experiments in vitro and in vivo showed that overexpression of miR-99a-5p inhibited the invasion /metastasis of GBC cells by regulating PI3K / AKT pathway and EMT.

3. MTOR and SMARCA5 were the direct downstream target genes of miR-99a-5p.

4.SMARCA5 could promote the invasion/metastasis of GBC cells and directly mediated miR-99a-5p inhibition of GBC invasion/metastasis.

Conclusion

MiR-99a-5p was commonly low expressed in tumor tissues of GBC. Low expression of miR-99a-5p was involved in the invasion/metastasis of GBC regulating by MTOR, SMARCA5, PI3K / AKT pathway and EMT. miR-99a-5p may be a potential target for the treatment of GBC.

Key words Gallbladder cancer; miRNA; target gene; invasion and metastasis; SMARCA5

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符号及缩略词

英文缩写	英文全称	中文全称
GBC	Gallbladder cancer	胆囊癌
RNA	Ribonucleic Acid	核糖核酸
miRNA	MicroRNA	微小 RNA
mRNA	Messenger RNA	信使 RNA
EMT	Epithelial-mesenchymal transitions	上皮间质转化
TCGA	The Cancer Genome Atlas	癌症和肿瘤基因图谱
PCR	Polymerase Chain Reaction	聚合酶链式反应
qRT-PCR	Quantitative real-time PCR	实时定量荧光聚合酶链式反应
FBS	Fetal bovine serum	胎牛血清
PBS	Phosphate buffer saline	磷酸缓冲盐溶液
MTT	Diphenyl tetrazolium bromide	四甲基偶氮唑盐
HE	Hematoxylin-eosin	苏木素和伊红
SPF	Specific pathogen free	无特定病原体
PVDF	Polyvinylidene Fluoride	聚偏氟乙烯
SDS	Sodium dodecyl sulfate	十二烷基硫酸钠
PAGE	Polyacylamide gel electrophoresis	聚丙烯酰氨凝胶电泳
3'UTR	3'untranslated region	3'末端非编码区
siRNA	Small interference RNA	小干扰 RNA
DEPC	Diethyl Pyrocarbonate	焦碳酸二乙酯

绪论

胆囊癌(gallbladder cancer, GBC)是一种发病率相对较低，但恶性程度极高的消化系统肿瘤，是胆道系统中最常见的恶性肿瘤，发病率在消化道肿瘤中排在第六位。胆囊癌好发于远东地区及智利，世界范围内死亡率居恶性肿瘤的第十位，严重影响人类健康。在中国，胆囊癌的发病率为 3.9/10 万人[1]。如能早期发现，行根治性手术可以达到比较满意的 5 年生存率。但胆囊癌起病较为隐匿，且早期缺乏特异性的症状，一经发现，许多患者已经进展到中晚期，失去了行根治性手术的机会，且目前放化疗等手段不能达到满意的治疗效果，总体 5 年生存率仅为 5%[2]。因此，早期诊断是改善胆囊癌预后的关键。且研究发现，胆囊癌所导致的死亡病例中超过 90%为肿瘤复发转移所致[3]。因此，关于胆囊癌发病以及复发侵袭转移机制的研究有助于寻找新的胆囊癌肿瘤标志物和靶向药物治疗的新靶点，对于改善胆囊癌诊疗水平以及患者预后具有重要意义。

随着分子生物学技术的快速发展，人类基因组计划已经全部完成。研究发现非编码 RNA 的数量要远远超过编码 RNA 的数量，这其中微小 RNA 又是研究最为广泛的一类。微小 RNA（miRNAs）是一类长度约为 18~25 个碱基的小分子非编码 RNA。它可以与靶基因的 3'UTR 区结合来参与调控靶基因的表达，通过对靶基因 mRNA 直接进行降解或者抑制蛋白翻译过程，在转录后水平来实现对靶基因表达量的调控，从而在细胞的增殖、侵袭、黏附、转移、凋亡等功能中扮演重要角色，同时通过影响下游分子及信号通路来发挥作用[4,5]。越来越多研究指出，miRNAs 在组织中的异常表达与肿瘤的发生发展密切相关。例如，有学者发现 miR-26a 在恶性肿瘤的淋巴结转移灶中高表达，通过对 PTEN 的靶向作用激活 PI3K/AKT 通路[6]。MiR-199a-3p 被证实通过直接作用于多个基因，例如 MET、IKK β 和 ERK-2，来调节炎症和 ERK-MAPK 通路，后者与缺血-再灌注损伤具有密切联系[7]。

近年来，随着对肿瘤性质研究的深入，miRNA 在调控肿瘤发生发展中的重要角色不断被证实。虽然各种研究表明，许多 miRNA 在不同肿瘤组织中出现表达量的上调或下调，但其具体的调控机制并未完全明了。胆囊癌的病因至今尚未明确，公认的与胆囊癌预后相关的因素包括肿瘤部位、术前黄疸、肿瘤细胞分化程度、淋巴结转移情况等[8]。目前有数个基于芯片分析技术和生物信息学的研究已发现一些与胆囊癌发生发展密切相关的 miRNA，并且初步阐明了一些相关机制。例如 Chang 等[9]通过高通量 miRNA 筛选从 880 个 miRNA 中发现数个具有较高研究价值的 miRNA，其表达与胆囊癌的分期和预后密切相关，其中 miR-20a

在胆囊癌组织中高表达，通过 Western blot 试验发现，胆囊癌细胞株转染了 miR-20a 后，Smad7 蛋白表达明显减少，细胞的增殖、侵袭以及转移显著性受到抑制，因此认为 miR-20a 可能是胆囊癌的新型肿瘤标志物及治疗靶点。另外，miR-20a 受到 TGF-β 的炎性因子的刺激，从而促进上皮间质转化（epithelial-mesenchymal transitions, EMT）进一步影响胆囊癌的侵袭转移。

Hsa-miR-99a-5p (以下均称 miR-99a-5p) 位于 21 号染色体 q21 位置，已有相关研究证明其与许多肿瘤的发生发展密切相关，例如在肝细胞肝癌、前列腺癌、头颈部鳞癌、膀胱癌、卵巢癌中 miR-99a-5p 都被证明具有异常表达[10-14]，并且研究证实其参与肿瘤细胞的增殖、侵袭、转移、分化、凋亡等过程。其低表达在上述肿瘤中均被认为是预后不良因素。但目前仍未有 miR-99a-5p 在胆囊癌中的相关研究。故 miR-99a-5p 在胆囊癌中的作用及相关机制都亟待探索。

第一部分：胆囊癌组织样本中差异 miRNA 的筛选和验证

1. 前言

胆囊癌是恶性程度极高的消化道恶性肿瘤，也是胆道系统中最常见的恶性肿瘤，发病率约为 3.9/10 万人，在消化道肿瘤中排第 6 位[1]。由于胆囊癌起病较为隐匿，且早期缺乏特异性的症状，一经发现，许多患者已经进展到中晚期，失去了行根治性手术的机会，且目前放化疗等手段不能达到满意的治疗效果，总体 5 年生存率仅为 5%[2]。因此，早期诊断是改善胆囊癌预后的关键。且研究发现，胆囊癌所导致的死亡病例中超过 90%为肿瘤复发转移所致[3]。因此，关于胆囊癌发病以及复发侵袭转移机制的研究有助于寻找新的胆囊癌肿瘤标志物和靶向药物治疗的新靶点，对于改善胆囊癌诊疗水平以及患者预后具有重要意义。

微小 RNA（miRNAs）是一类长度约为 18~25 个碱基的小分子非编码 RNA。它可以通过与靶基因特异性结合来参与调控靶基因的表达，通过对靶基因 mRNA 直接进行降解或者抑制蛋白翻译过程，在转录后水平来实现对靶基因表达量的调控，从而在细胞的增殖、侵袭、黏附、转移、凋亡等功能中扮演重要角色，同时通过影响下游分子及信号通路来发挥作用[4,5]。越来越多研究指出，miRNAs 在组织中的异常表达与肿瘤的发生发展密切相关。所以研究恶性肿瘤中差异表达的 miRNAs 对揭示肿瘤的发生发展，为诊断和治疗恶性肿瘤提供新思路都具有重要意义。

具体到胆囊癌，为了寻找并筛选胆囊癌中是否存在重要的差异表达 miRNAs，本课题组在前期采取了高通量 miRNA 芯片的方法对 4 例胆囊癌癌组织及其相对应的癌旁组织进行了检测[15]。另外，我们结合了癌症和肿瘤基因图谱 (TCGA) 数据库的分析，并采用实时定量荧光 PCR 的方法进行大规模的验证，最后在胆囊癌中特异性靶定了 miR-99a-5p 进行研究，同时对 miR-99a-5p 的表达水平与胆囊癌患者的临床病理资料及预后的关系进行了分析。

2. 材料与方法

2.1 实验材料

2.1.1 人胆囊癌组织样本的收集和保存

全部胆囊癌组织样本收集自 2013 年至 2015 年于上海交大医学院附属新华医院普外科

住院治疗的 41 例因胆囊癌行根治性手术的患者，术前均未接受非手术治疗。所有标本均由组织病理学检查确诊。同时收集这些患者新鲜的胆囊癌组织以及相对应的癌旁组织。手术标本离体后即刻放入液氮中保存，以备提取组织总 RNA、反转录后进行实时荧光定量 PCR 检测。配对的癌旁组织是指至少距离胆囊癌组织边缘 2cm 的，且镜下未发现癌细胞存在的正常胆囊黏膜组织。在这 41 例胆囊癌患者中，共有男 13 例，女 28 例，年龄 49-88 岁，平均年龄为 67 ± 11 岁。其中高或中分化腺癌占 78.0%（32/41），低分化腺癌占 22.0%（9/41）。进展期胆囊癌（III-IV 期）患者占 63.4%（26/41）。有局部淋巴结转移的患者共有 14 例。TNM 分期以及组织分化程度分级均参见第七版美国癌症联合委员会指南。随访频率为术后 2 年每 3 个月 1 次，之后每 6 个月 1 次。随访时间根据手术日期起始，直至末次随访（2016 年 11 月）或死亡时为随访截止日期。术后平均随访时间为 10 个月（1~26 个月）。所有标本的获取均已告知患者及其家属并获得同意，本研究已经过上海交通大学医学院附属新华医院伦理委员会批准开展。

2.1.2 Realtime-PCR 试剂

名称	厂家
Trizol 试剂	Invitrogen 公司
PrimeScript® RT reagent Kit with gDNA Eraser 逆转录试剂盒	Takara 公司
Mir-X™ miRNA First-Strand Synthesis Kit miRNA 逆转录试剂盒	Takara 公司
SYBR *Premix Ex Taq* (Tli RNaseH Plus) 定量 PCR 试剂盒	Takara 公司
SYBR Premix Ex Taq II (Tli RNaseH Plus)定量 PCR 试剂盒	Takara 公司
异丙醇	国药集团化学试剂有限公司
氯仿	上海润捷化学试剂有限公司

2.1.3 主要实验仪器和设备

名称	厂家
PCR 仪	LifePro 公司
Step One Plus 96 孔 Real Time PCR 仪	Applied Biosystems 公司
MDF-382E(CN)医用低温箱	日本三洋公司
台式高速低温离心机 5424R	艾本德公司
超净工作台	上海实验设备有限公司
NanoDrop2000 超微量分光光度计	美国热电公司
200μL/1.5ml EP 管	爱思进生物技术（杭州）有限公司

2.2 实验方法

2.2.1 人胆囊癌及其相对应正常组织样本的总 RNA 提取

取液氮中冻存的胆囊癌及其相对应正常组织样本切割成黄豆大小，置于 1.5ml EP 管内，分别加入 1mL 的 Trizol，再加入研磨钢珠，用组织匀浆破碎仪于 4℃进行匀浆。将获得的匀浆液于高速低温离心机中 4℃、12000rpm 离心 5min 后吸取上清液，以备提取总 RNA 用。

提取总 RNA 的步骤如下：

1. 向处理后的样品中加入 200μL 氯仿，上下颠倒 EP 管剧烈振荡 15s， 将 EP 管于冰上静置 5min。
2. 4°C，12000 rpm，离心 15min。
3. 吸取适量上层透明水相加入新的洁净 1.5ml EP 管中，加入等体积的异丙醇，混合均匀后冰上静置 10min。
4. 4°C，12000 rpm，离心 10min。
5. 弃去上清后加入 1mL 75%的乙醇轻轻洗涤白色 RNA 沉淀。
6. 4°C，7500 rpm，离心 5min。
7. 弃去上清，空气中干燥 15min 后用 20μL 体积的 DEPC 水溶解 RNA 沉淀。
8. 使用 NanoDrop2000 分光光度计测定 A260/A280 以及 RNA 浓度，A260/A280 应在 1.9-2.1 之间。
9. 总 RNA 按照 mRNA 逆转录试剂盒的说明书进行逆转录。反应条件为：

第一步：42℃ 3min（去除基因组 DNA）

第二步：37℃ 15min

85℃ 5s

10. MiRNA 逆转录试剂盒按照说明书进行逆转录。反应条件为：

37℃ 60min

85℃ 5min

11. 剩余 RNA 冻存于-80℃ 下备用。

2.2.2 实时荧光定量 PCR 法验证 miRNA 芯片结果

miR-99a-5p 引物设计如下：

Forward primer：5'-AACCCGTAGATCCGATCTTGTG-3'

由北京天根生化科技有限公司合成。实时荧光定量 PCR 所用 U6 引物为 Mir-X™ miRNA First-Strand Synthesis Kit miRNA 逆转录试剂盒所提供。

按以下组份配制 PCR 反应液：

试剂	使用量	终浓度
SYBR Premix Ex Taq II(2×)	10μl	1x
Forward Primer (10μM)	0.8μl	0.2μM
Uni-miR Reverse Primer (10μM)	0.8μl	0.2μM
ROX Reference Dye (50×)	0.4μl	1x
Template（cDNA 溶液）	2μl	*2
ddH_2O	6μl	
Total	20μl	*4

反应条件：

95℃ 30s

95℃ 5s
60℃ 34s
} 40 个循环

胆囊癌组织与其相对应的癌旁组织中 miR-99a-5p 相对量采用 $2^{-\Delta Ct}$ 法测定。Ct 值为 PCR 扩增产物的荧光信号强度达到所设定的阈值时经历的扩增循环数。ΔCt=组织中 miR-99a-5p 的 Ct 平均值-组织中 U6 的 Ct 平均值。胆囊癌患者组织中的 miR-99a-5p 相对量用 $2^{-\Delta\Delta Ct}$ 法测定，公式如下：ΔΔCt=胆囊癌组织中 ΔCt-癌旁组织中 ΔCt。

2.3 统计分析

使用 SPSS 21.0 软件进行数据分析，miR-99a-5p 在胆囊癌组织及其相对应癌旁组织中比较采用配对 t 检验； miR-99a-5p 的表达与临床病理特征之间的关系采用 $\chi 2$ 检验。生存曲线分析则采用 Kaplan-Meier 法进行，生存曲线显著性检验采用 Log-rank 法；单因素及多因素分析运用 Cox 回归法。计量资料采取(平均数 ± 标准差)的形式表示。以 $P<0.05$ 认为具有统计学意义。

3. 实验结果

3.1 胆囊癌组织中差异 miRNA 的筛选

为了寻找并筛选胆囊癌中是否存在重要的差异表达 miRNAs，本课题组在前期使用了 Aglient 人 miRNA 表达谱芯片(v19.0)的方法对 4 例胆囊癌癌组织及其相对应的癌旁组织进行了检测[15]。该种芯片共有 2588 条已经确认的人成熟 miRNA 的探针，检测时以 U6 作为内参 miRNA。结果显示，人胆囊癌样本中发现了两条在癌组织中显著过表达的 miRNAs 和 11 条在癌组织中显著低表达的 miRNAs (差异倍数>2，P 值<0.05) (图 1-1)。

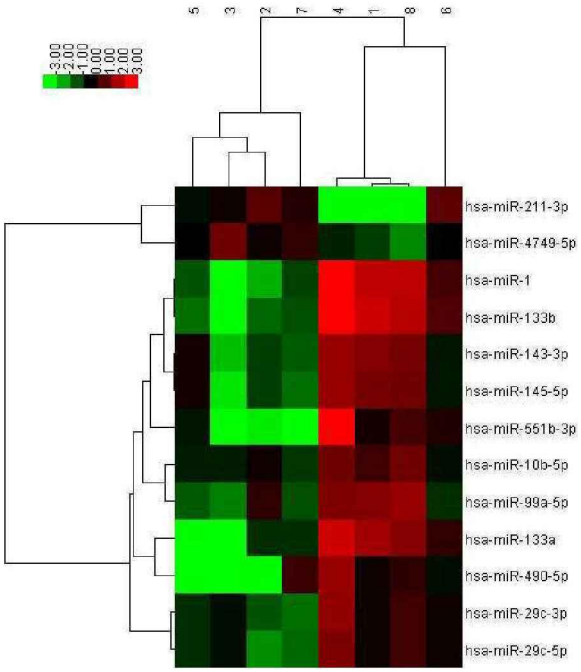


图 1-1：胆囊癌癌组织与癌旁组织样本中存在差异表达的 miRNAs 聚类分析热图[15]。

Figure 1-1：Heat map diagram of differentially expressed miRNAs in GBC tissues by clustering

analysis [15].

热图右边一列为筛选出的 miRNA 名称，上方为胆囊癌样本编号，左边四个样本为胆囊癌组织，右边四个样本为癌旁正常组织。红色区域代表相对高表达，蓝色区域代表相对低表达。

芯片结果提示胆囊癌组织中存在着较多有价值的差异表达 miRNA，这也间接证实了 miRNA 表达的异常对胆囊癌病情的发展可能具有重要意义。根据前期的功能初筛试验，在这 13 条差异表达的 miRNAs 中，我们初步靶定了 miR-99a-5p 作为主要研究对象。

3.2 TCGA 数据库中 miR-99a-5p 表达情况分析

为了进一步验证筛选结果的合理性，我们通过查找 TCGA 数据库的方式来判断 miR-99a-5p 在胆囊癌中是否有研究价值。由于 TCGA 数据库中未收录胆囊癌相关的病例资料，我们选择与胆囊癌来源相近的肝癌及肝内胆管癌作为分析对象。图 1-2 显示，在肝癌和肝内胆管癌的癌旁组织中，miR-99a-5p 均表现为明显高表达，癌组织中表达量则普遍偏低。TCGA 数据库的分析结果间接证明了 miR-99a-5p 在胆囊癌中可能具有潜在的研究价值。

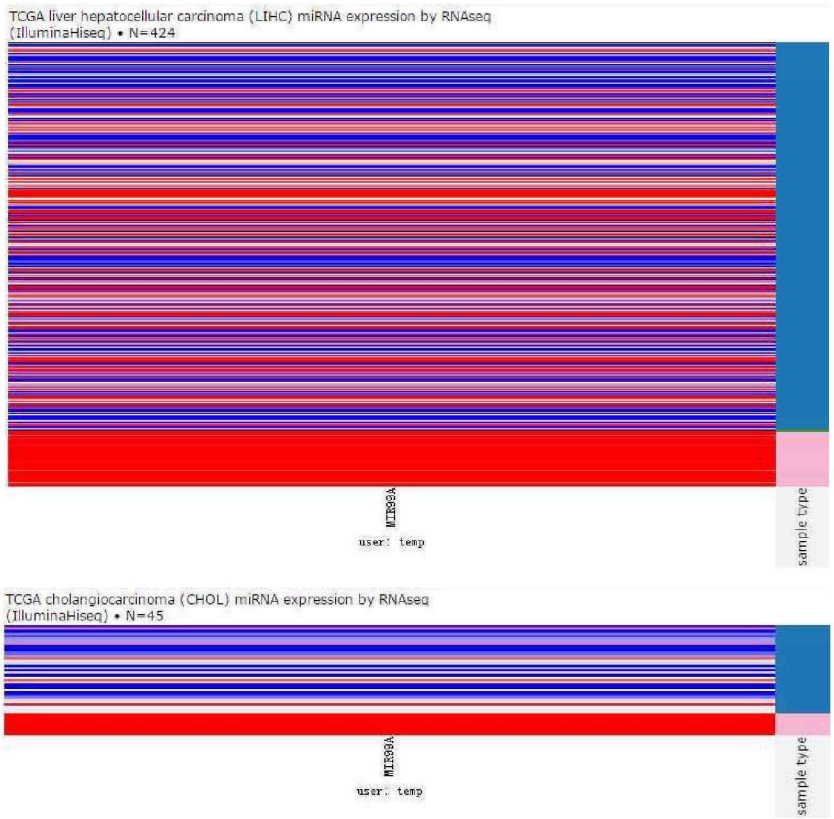


图 1-2：TCGA 数据库中肝癌(上)和肝内胆管癌(下)癌组织与癌旁组织 miR-99a-5p 相

对表达量对比。

Figure 1-2：Comparison of miR-99a-5p relative expression levels in hepatocellular carcinoma (upper) and intrahepatic cholangiocarcinoma (lower) tumor tissues and peritumoral tissues in TCGA database.

（左列：miR-99a-5p 表达量。红色代表高表达，蓝色代表低表达。右列:样本类型。蓝色代表癌组织，粉色代表癌旁组织。）

3.3 胆囊癌组织样本中 miR-99a-5p 的表达量

为了验证上述芯片数据结果，我们选择通过实时荧光定量 PCR 的方法检测 41 例胆囊癌组织及其相对应的癌旁组织中 miR-99a-5p 的表达含量，同时通过瀑布图来表示 miR-99a-5p 在胆囊癌患者中的表达情况。图 1-3 显示 miR-99a-5p 在胆囊癌癌组织中的表达量显著低于对应癌旁组织，癌旁组织 miR-99a-5p 的平均表达量是癌组织中的 2.79 倍，差异具有统计学意义(P<0.0001)。

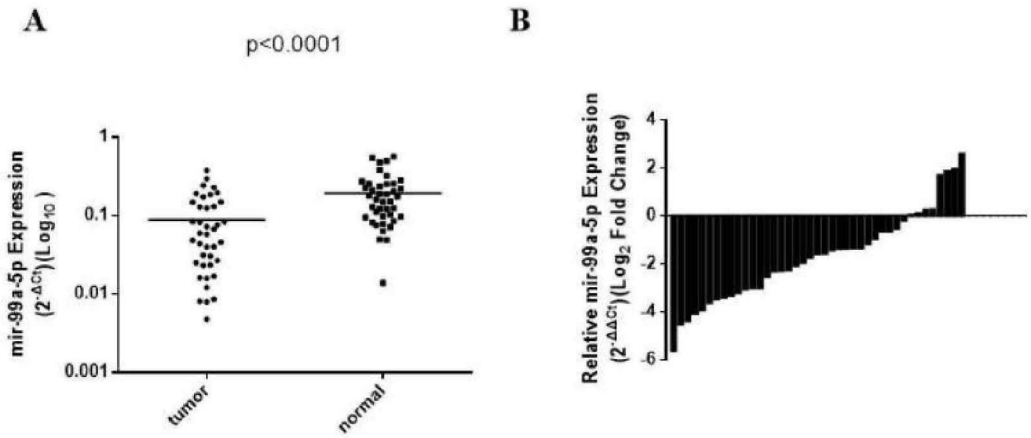


图 1-3：miR-99a-5p 表达量的检测。

Figure 1-3: Evaluation of miR-99a-5p expression levels.

（A）miR-99a-5p 在 41 例胆囊癌癌组织及其对应癌旁组织中的相对表达量。癌旁组织 miR-99a-5p 的平均表达量是癌组织中的 2.79 倍，使用配对 t 检验法进行分析，P<0.0001；

（B）胆囊癌患者癌组织与癌旁组织中 miR-99a-5p 表达量比较。

3.4 miR-99a-5p 表达量与胆囊癌患者的临床病理资料和临床预后的相关性分析

为了研究 miR-99a-5p 表达量与临床病理特征之间是否存在潜在关系，我们通过上述患

者组织样本中 miR-99a-5p 的表达情况将 41 例胆囊癌标本分成两组：miR-99a-5p 低表达组和 miR-99a-5p 高表达组。分组标准为癌组织中 miR-99a-5p 表达量超过平均值者为高表达，反之为低表达。通过采用 χ2 检验和 Fisher 检验分析，结果显示 miR-99a-5p 的表达只与淋巴结转移相关（P=0.009），而与性别、年龄、肿瘤大小、分化程度、浸润深度及 TNM 分期无明显关系（均 *P*>0.05）（表 1-1）。

表 1-1：miR-99a-5p 相对表达量与胆囊癌患者的临床病理特征之间的关系

Table 1-1: Association between miR-99a-5p expression and the clinicopathological characteristics in GBC

临床病理因素	n	miR-99a-5p 相对表达量		χ2	*P*
		低表达 (n=27)	高表达 (n=14)		
性别					
男	13	8 (29.6%)	5(35.7%)	0.158	0.691
女	28	19 (70.4%)	9(64.3%)		
年龄(岁)					
＜60	10	6 (22.2%)	4 (28.6%)	0.158	0.691
≥60	31	21 (77.8%)	10(71.4%)		
肿瘤大小(cm)					
＜3	15	9(33.3%)	6 (42.9%)	0.360	0.548
≥3	26	18(66.7%)	8 (57.1%)		
分化程度					
高、中分化	32	21(77.8%)	11(78.6%)	0.003	0.954
低分化	9	6 (22.2%)	3(21.4%)		
肿瘤浸润深度(AJCC)					
Tis-T_2	16	9 (33.3%)	7(50.0%)	1.076	0.300
T_3-T_4	25	18 (66.7%)	7(50.0%)		
淋巴结转移					
是	**14**	**13 (48.1%)**	**1(7.1%)**	**6.894**	**0.009**
否	**27**	**14 (51.9%)**	**13 (92.9%)**		
TNM 分期(AJCC)					
I-II	15	8 (29.6%)	7 (50.0%)	1.649	0.199
III-IV	26	19 (70.4%)	7 (50.0%)		

接着，通过生存分析我们可以发现，低表达组与高表达组患者生存时间存在明显差异（中位生存期分别为 10.5 个月和 20.0 个月，*P*=0.014）。1 年生存率分别为 48.4%和 76.0%（图 1-4）。

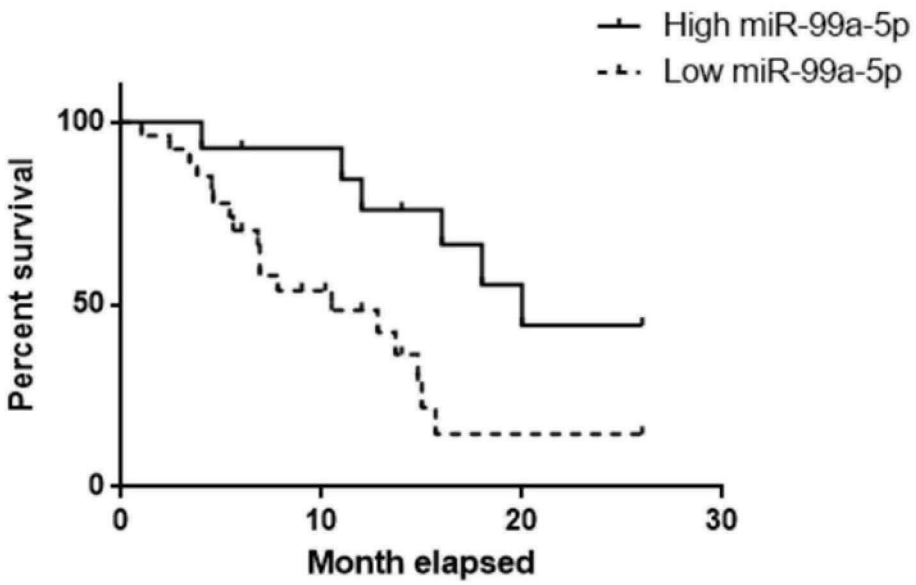


图 1-4：miR-99a-5p 表达量与胆囊癌患者生存时间的生存关系曲线。

Figure 1-4: The relationship between the expression of miR-99a-5p and the survival time of GBC patients

而且，单因素和多因素分析发现，淋巴结转移和 miR-99a-5p 表达量都是胆囊癌患者预后的相关因素（表 1-2）。

表 1-2 胆囊癌临床信息和预后的单因素和多因素分析

Table 1-2 Univariate and multivariate analysis between clinicopathologic characteristics and prognosis in GBC patients

临床病理因素	单因素分析		多因素分析	
	HR (95%CI)	*p*	HR (95%CI)	*p*
年龄 (＜60 *vs.*≥60)	0.680 (0.311-1.591)	0.365	-	-
性别 (男 *vs.*女)	0.383 (0.124-1.049)	0.069	-	-
肿瘤大小 (cm) (≥3 vs.＜3)	0.506 (0.164-1.522)	0.233	-	-
分化程度 (高或中 *vs.*低)	2.314 (0.850-6.262)	0.085	-	-
浸润深度(AJCC) (Tis-T2 *vs.*T3-T4)	0.698 (0.366-1.316)	0.506	-	-
淋巴结转移 (是 vs.否)	**3.278 (1.345-7.969)**	**0.006**	**1.310 (0.418-5.384)**	**0.022**
TNM 分期(AJCC) (I-II *vs.*III-IV)	0.421 (0.087-1.911)	0.245	-	-
手术方式 (根治性 *vs.*姑息性)	1.663 (0.619-4.498)	0.357	-	-
miR-99a-5p 表达量 (低表达 vs.高表达)	**2.469 (1.027-6.167)**	**0.040**	**1.952 (0.165-5.103)**	**0.014**

CI, 置信区间;HR, 相对风险系数

4. 讨论

随着关于恶性肿瘤研究的不断深入，肿瘤中非编码 RNA 领域逐渐成为了科学家们研究的一个热点。许多关于恶性肿瘤疾病的研究发现，非编码 RNA 都对恶性肿瘤的发生发展起到了至关重要的作用[16-20]。miRNA 又是其中的重要组成部分，目前有许多证据表明其在多种肿瘤中具有异常表达，它对恶性肿瘤的功能及其影响机制的研究也日渐增多[21-23]。本部分研究通过高通量 miRNA 芯片的方法对胆囊癌患者组织中差异表达的 miRNA 做了初步筛选，得到了 11 条癌中低表达的 miRNA 和 2 条癌中高表达的 miRNA。针对这些 miRNA，我们课题组在前期根据在胆囊癌中的细胞功能学试验对其进行了初筛，初步判定 miR-99a-5p 在胆囊癌中具有抑癌的功能。然后在 TCGA 数据库上查找与胆囊癌来源相近的肝癌以及肝内胆管癌的数据，发现 miR-99a-5p 在肝胆肿瘤患者癌组织中明显低表达。故我们选定了 miR-99a-5p 作为研究对象。

MiR-99 家族是进化过程中最古老的 miRNA 家族之一，包括 miR-99a、miR-99b 和 miR-100[24]。MiR-99a 位于 21 号染色体 q21 位置，已被证明在多种肿瘤组织中表达受到抑制，且能影响肿瘤的相关功能[25-27]。Lee 等[28]对 221 例胰腺癌患者中 miRNA 与患者术后生存期之间的关系进行了回顾性分析，发现 miR-99a-5p 与患者切缘情况、肿瘤分期、病理分化程度、术后 CA199 水平等密切相关，且在胰腺癌组织中表达量明显降低，是影响患者预后的独立因素。Yu 等[29]发现在非小细胞肺癌中，miR-99a-5p 与晚期终末期和肿瘤转移显著相关，提示不良预后，随后的功能学研究发现过表达 miR-99a-5p 能够在体外抑制非小细胞肺癌细胞的增殖、侵袭转移功能，而且能通过 AKT1 的介导起到肿瘤抑制作用。Feng 等[30] 用 Taqman 探针及茎环法实时荧光定量 PCR 方法检测了 100 对膀胱癌组织和癌旁组织中的 miR-99a-5p 表达水平，同时也分别检测了膀胱癌患者与正常人群血清中 miR-99a-5p 的表达水平，表明了膀胱癌患者癌组织及血清中 miR-99a-5p 通常为低表达，且其低表达与侵袭表型相关，对膀胱癌细胞进行过表达处理能抑制其增殖作用。

在本部分研究中，我们对比分析了 41 例胆囊癌患者癌组织与对应癌旁正常组织的 miR-99a-5p 的表达量，结果发现 miR-99a-5p 在胆囊癌组织中明显低表达，与前述相关研究结果相符。由此推断，miR-99a-5p 在胆囊癌中可能起到抑癌作用，但需进一步体外体内功能学试验来证实。在此基础上，我们着重分析了 miR-99a-5p 的表达量与胆囊癌患者临床病理资料的关系。结果显示 miR-99a-5p 的低表达与淋巴结转移显著相关，这预示着 miR-99a-5p 可能会影响胆囊癌的侵袭转移功能。回顾文献我们可以发现，许多研究证实了 miR-99a-5p 能够

影响肿瘤的侵袭转移功能，而支持 miR-99a-5p 能抑制恶性肿瘤生长的研究也不在少数。所以下一步我们的研究重点将放在 miR-99a-5p 是否能影响胆囊癌的增殖以及侵袭转移功能上。与此同时，通过预后生存分析我们可以发现，miR-99a-5p 的低表达与不良预后密切相关。所以 miR-99a-5p 对于胆囊癌来说可能是个潜在的诊断和治疗靶点。

5. 小结

1. 通过 miRNA 芯片分析结合实时荧光定量 PCR 验证，发现 miR-99a-5p 在胆囊癌患者癌组织中普遍低表达，并可能与胆囊癌的淋巴结转移有关；
2. miR-99a-5p 的低表达量与胆囊癌的不良预后具有显著相关性。

第二部分：miR-99a-5p 在胆囊癌中的功能研究

1. 前言

胆囊癌是恶性程度极高的消化道恶性肿瘤，也是胆道系统中最常见的恶性肿瘤，发病率在消化道肿瘤中排在第六位，严重影响了人类健康。在中国，胆囊癌的发病率为 3.9/10 万人[1]。如能早期发现，行根治性手术可以达到比较满意的 5 年生存率。但胆囊癌起病较为隐匿，且早期缺乏特异性的症状，一经发现，许多患者已经进展到中晚期，失去了行根治性手术的机会，且目前放化疗等手段不能达到满意的治疗效果，总体 5 年生存率仅为 5%[2]。因此，早期诊断是改善胆囊癌预后的关键。且研究发现，胆囊癌所导致的死亡病例中超过 90%为肿瘤复发转移所致[3]。因此，关于胆囊癌发病以及复发侵袭转移机制的研究有助于寻找新的胆囊癌肿瘤标志物和靶向药物治疗的新靶点，对于改善胆囊癌诊疗水平以及患者预后具有重要意义。

目前许多研究证实，miRNA 在恶性肿瘤中通过异常表达来影响其发生发展过程，且 miRNA 可以影响肿瘤相关的多个信号通路，参与肿瘤的增殖、侵袭转移、凋亡、周期阻滞等过程。而在肿瘤组织中低表达的 miRNA 通常表现为抑癌的功能，通过靶向负调节一些促癌基因来实现抑制肿瘤增殖、侵袭转移等功能，如 miR-204、miR-140-5p 等[31-32]。而另一类 miRNA 可以表现出促癌功能，在肿瘤组织中过表达。例如 Liu 等[33]指出，miR-19b 可以通过抑制抑癌基因 PTPRG 的功能来推动乳腺癌的发生发展。

在上一部分实验中，我们通过芯片筛选出了 miR-99a-5p 为在胆囊癌组织中低表达的 miRNA，并通过组织样本的实时定量荧光 PCR 实验得到了验证，初步提示 miR-99a-5p 可能在胆囊癌中发挥抑癌的作用。由于 miR-99a-5p 与胆囊癌的关系还未有相关报道，所以本部分我们将着重研究 miR-99a-5p 对胆囊癌细胞功能的影响，并且进一步确认 miR-99a-5p 是否起着抑癌的作用。

2. 材料与方法

2.1 实验材料

2.1.1 细胞

胆囊癌细胞系 GBC-SD、NOZ、EHGB-1 和 OCUG 均购买自中国科学院上海生命科学研究院细胞资源中心。

2.1.2 动物

体内试验选用 4-6 周龄 SPF 级裸鼠均购自中国科学院上海动物实验中心。试验用裸鼠均根据上海交通大学医学院附属新华医院伦理委员会指导饲养于 SPF 环境内。

2.1.3 主要实验所用试剂

细胞培养所用试剂

名称	厂家
DMEM 培养基	Gibco 公司
William's 培养基	Gibco 公司
胎牛血清	Gibco 公司
胰蛋白酶-EDTA	Gibco 公司
青霉素-链霉素	Gibco 公司

细胞增殖转移实验相关试剂

名称	厂家
CCK8 试剂	上海翊圣生物科技有限公司
Transwell 小室	Corning 公司
基质胶小室	Corning 公司
4%多聚甲醛	国药集团化学试剂有限公司
结晶紫染色液	碧云天公司

细胞转染、实时定量荧光 PCR 实验相关试剂

名称	厂家
Trizol 试剂	Invitrogen 公司
PrimeScript® RT reagent Kit with gDNA Eraser 逆转录试剂盒	Takara 公司
Mir-X™ miRNA First-Strand Synthesis Kit miRNA 逆转录试剂盒	Takara 公司
SYBR *Premix Ex Taq* (Tli RNaseH Plus) 定量 PCR 试剂盒	Takara 公司
SYBR Premix Ex Taq II (Tli RNaseH Plus)定量 PCR 试剂盒	Takara 公司
异丙醇	国药集团化学试剂有限公司
氯仿	上海润捷化学试剂有限公司
Rfect 小核酸转染试剂	常州百代生物科技有限公司

蛋白印迹实验主要试剂

名称	厂家
过硫酸铵	碧云天公司
RIPA 裂解液	碧云天公司
丙烯酰胺	碧云天公司
四甲基乙二胺	碧云天公司
6×上样缓冲液	碧云天公司
三羟甲基氨基甲烷	上海翊圣生物科技有限公司
甘氨酸	上海翊圣生物科技有限公司
十二烷基磺酸钠	上海翊圣生物科技有限公司
脱脂奶粉	上海翊圣生物科技有限公司
20×三乙醇胺缓冲盐水溶液	上海翊圣生物科技有限公司

吐温-20	上海翊圣生物科技有限公司
聚偏氟乙烯膜	伯乐公司
兔抗人 E-cadherin、N-cadherin、Vimentin 单抗	CST 公司
兔抗人 GAPDH 抗体	上海泊湾生物科技有限公司
兔抗人 MTOR 抗体	CST 公司
兔抗人 SMARCA5 抗体	艾博抗公司
辣根过氧化物酶(HRP)偶联山羊抗兔/鼠二抗	碧云天公司
蛋白 Marker	赛默飞世尔科技公司
化学发光底物试剂盒	赛默飞世尔科技公司
蛋白定量 BCA 试剂盒	碧云天公司

2.1.4 仪器与设备

名称	生产厂家
1300 系列 A2 生物安全柜	赛默飞世尔科技公司
501A 型数显超级恒温水浴锅	上海申安医疗器械厂
LDZX-40 型立式自动电热压力蒸汽灭菌锅	上海浦东荣丰科学仪器有限公司
台式高速低温离心机 5417R	艾本德公司
DMI3000B 型倒置荧光显微镜	徕卡公司
温控摇床	上海精密科学仪器有限公司
常温高速离心机	日本日立公司
DK-420BS 电热恒温水温箱	上海贺德实验设备有限公司
FA2004A 型电子分析天平	上海精密科学仪器有限公司
90-1A 磁力搅拌器	上海赛鸽电子科技有限公司
超纯水系统	默克密理博公司
YX280A 手提式不锈钢蒸汽消毒器	上海三申医疗器械有限公司
-20℃/4°C 立式冰箱	中国海尔公司

YDS-10 贮存型液氮生物容器	成都金凤液氮容器有限公司
D1008E 掌上离心机	赛洛捷克公司
MDF-382E(CN)医用低温箱	日本三洋公司
2.5μl 、20μl、200μl 、1 ml 移液器	艾本德公司
AF100 制冰机	斯科茨曼公司
550 型酶标仪	美国伯乐公司
血球计数板	上海医药工业研究院
0.22μm 微孔尼龙滤膜	上海医药工业研究院
HERAcell 150i CO_2 细胞培养箱	赛默飞世尔科技公司
96、24、6 孔细胞培养板	无锡耐思生物科技有限公司
Step One Plus 96 孔 Real Time PCR 仪	Applied Biosystems 公司
稳流稳压电泳仪	上海精密科学仪器有限公司
SDS-PAGE 生物蛋白电泳仪	美国伯乐公司
Mini Trans-Blot 转印槽	美国伯乐公司
湿电转膜仪	美国伯乐公司
自动化学发光凝胶成像分析仪	美国通用电气公司

2.2 实验方法

2.2.1 细胞培养

人胆囊癌细胞株 NOZ 和 EHGB-1 购自中国科学院上海生命科学研究院。NOZ 细胞采用含 10%胎牛血清(FBS)的 william's 培养基，EHGB-1 细胞采用含 15%FBS 的 DMEM 培养基，GBC-SD、OCUG 细胞采用含 10%FBS 的 DMEM 培养基培养，培养基中均含 100U/ml 的青霉素和链霉素。通常将细胞置于 100mm 培养皿中进行培养，培养环境为 37℃及 5%CO_2，在对数生长期时使用含 EDTA 的胰酶于 37℃消化 3min，用含血清的培养基中止消化并收集细胞于 1. 5ml Eppendorf 管中。细胞离心条件为 1500rpm、3min。

2.2.2 miR-99a-5p 过表达与敲减试剂的合成与构建

miR-99a-5p 模拟物与抑制剂由广州市锐博生物科技有限公司合成。miR-99a-5p 过表达慢病毒 PGMLV-CMV-MCS-EF1-ZsGreen1-T2A-Puro-miR99a 由上海吉凯基因化学技术有限公司构建并浓缩。

2.2.3 实时荧光定量 PCR 方法检测细胞内 miRNA 表达量

细胞总 RNA 提取方法，反转录体系及反应条件参见第一部分 2.2.1。实时定量荧光 PCR 体系及反应条件参见第一部分 2.2.2。miR-99a-5p 引物序列参见第一部分 2.2.2。

2.2.4 细胞计数

取洁净盖玻片放置于血球计数板中央，充分混匀细胞悬液后，用 10μL 移液器吸取 10μL 体积的细胞悬液，沿盖玻片的一边缓慢打入细胞悬液，使细胞悬液布满盖玻片与血球计数板之间的空隙，注意不能产生气泡，也不能从盖玻片四周溢出。静置 1min，待细胞沉降于计数板上。在显微镜下观察计数板，计数四个角大方格内细胞的总数。对于压线细胞，只计入压在左边线和上线的细胞，细胞团则按单个细胞计数。按以下公式计算细胞悬液的密度：细胞密度=(4 个大方格细胞总数/4)×10^4 个/ml。

2.2.5 Cell Counting Kit-8(CCK8)试验检测细胞体外增殖能力

CCK8 的主要成分是水溶性四唑盐 (2-(2-甲氧基-4-硝苯基)-3-(4-硝苯基)-5-(2,4-二磺基苯)-2H-四唑单钠盐)，它是一种类似于 MTT 的化合物，在电子耦合试剂的存在下，能被线粒体中一些脱氢酶还原，生成橙黄色的具有水溶性的产物甲瓒。该种试剂被细胞内的脱氢酶生物性还原后生成的甲瓒能被直接溶解在培养基中。活细胞的数目越多，形成产物的颜色越深。对于同一种细胞，产物溶液的吸光度和细胞的总数目呈线性关系。该法于 MTT 法相比无需用 DMSO 溶解甲瓒，灵敏度更高，线性范围更宽，且对细胞毒性小，使用较为安全简便。

具体步骤如下：

1. 根据转染或感染情况对细胞进行分组，每组细胞设置 5 个副孔。在 96 孔细胞培养板上分别标记好 d0~d5，每组细胞拟共铺 30 个孔，检测时间点分别为铺板后 6h、24h、48h、72h、96h、120h。

2. 将处于对数生长期的细胞进行消化，离心，重悬制成细胞悬液后计数。

3. 以 1000 个细胞/孔的密度，每孔 100μL 体积将细胞铺于 96 孔板中，周围各孔铺上 100μL PBS，置于 5%CO_2 培养箱中 37℃ 培养。

4．用完全培养基稀释 CCK8 试剂，比例为 10：1。

5. 每到检测时间点时取出相对应的 96 孔板，每孔加入 100μL 稀释过的 CCK8 试剂，37℃ 继续培养 1-4h。

6. 用酶标仪测定波长为 450nm 处的 OD 值，计算细胞的生长情况并绘制曲线。

2.2.6 克隆形成试验

克隆形成试验通常用来测定单个细胞形成单克隆的能力。单细胞在体外环境持续增殖 6 代以上所组成的细胞群称为一个克隆，每个克隆有超过 50 个细胞组成，用相应方法染色后肉眼即可观察。常用方法有平板和软琼脂克隆形成实验。由于本实验中所使用胆囊癌细胞均为贴壁生长细胞，故我们采取平板克隆形成实验的方法。克隆形成能力是反映细胞的群体依赖性以及增殖能力的重要指标。该实验关键点是细胞悬液接种的密度和细胞克隆分布的均匀程度。若细胞接种密度过大，则相邻克隆容易汇合，若密度过小则难以观察到明显的细胞克隆。其过程是将细胞以一定密度接种在培养板中，定期检查细胞状态，必要时换液。当单个细胞克隆生长至超过 50 个细胞时终止培养，染色后肉眼观察克隆数量，并镜下观察单个克隆生长情况。

具体步骤如下：

1. 取相应实验组和对照组细胞以 PBS 清洗一次后用含 EDTA 的胰酶 37℃消化 3min，用含 FBS 的培养基中止消化后 1500rpm 离心 3min。然后用完全培养基重悬细胞，用细胞计数板进行计数。

2. 在六孔板中加入 1.5ml/孔的相应完全培养基，根据细胞计数结果计算所需细胞悬液体积，以 1000 个细胞/孔的密度接种于六孔板中，每组细胞设置 3 个复孔，用移液器反复轻柔吹打细胞，使单细胞分散均匀。将六孔板置于 5%CO_2 细胞培养箱中，37℃培养。

3. 定期观察接种细胞的状态，必要时给细胞换液。当对照组细胞大部分克隆的细胞数超过 50 个时，吸去各孔培养基，用 PBS 溶液冲洗各孔一次，4%多聚甲醛固定 15min 后用 0.1% 结晶紫溶液染色 10 分钟，肉眼观察大体克隆形成数，并用倒置显微镜观察单个克隆中细胞情况。记录超过 50 个细胞的克隆总数。

2.2.7 细胞划痕愈合试验

细胞划痕愈合实验(Wound healing assay)的基本原理为在单层长满的细胞中制作划痕以产生供细胞愈合的空白区域，然后观察划痕周边细胞向中间迁移的过程，即划痕愈合。相同时间范围内划痕变得越窄，则细胞迁移能力越高。具体步骤如下：

1. 取相应实验组和对照组细胞消化计数，分别接种到6孔板中，每孔30万个细胞，使细胞过夜后长满。

2. 先用marker笔在直尺的辅助下，在6孔板背后均匀地划横线，每隔1cm一道，横穿过孔。每孔至少划3条横线。

3. 用灭菌的200μL移液枪枪头尽量垂直于背后的横线在细胞生长平面划痕，枪头尽量与板底保持垂直，使每条划痕都成一条直线。

4. 划完后用PBS轻轻洗涤板孔3次，以去除脱落细胞。

5. 每孔添加1.5ml的无血清DMEM培养基。放置于5% CO2培养箱内，37°C培养。

6. 分别于划痕形成0、24和48小时后，在镜下观察、拍照，并测量划痕宽度。

2.2.8 Transwell 小室迁移/侵袭试验

Transwell 小室为带一层半透膜的容器，分为有基质胶和无基质胶的两种小室。在下室加入含 FBS 或趋化因子的培养基，将细胞用无血清培养基稀释后加入上室，培养一定时间后细胞通过半透膜穿入下室。通过结晶紫染色，可以在镜下观察穿过半透膜的细胞数，借此分别判断细胞的侵袭或迁移能力。

具体步骤如下：

1. 实验前将带基质胶的Transwell小室置于24孔细胞培养板孔中，上下室分别加入500μL无血清培养基，置于37°C培养箱中水化2小时。进行细胞迁移试验时省略此步骤。

2. 取相应实验组和对照组细胞用胰酶消化，PBS清洗2次，用相对应的无血清培养基重悬后进行计数。

3. 取2万个细胞加入每个Transwell小室的上室，用无血清培养基调整细胞悬液体积至200μL。在每个小室的下室内加入500μL含20% FBS的相对应完全培养基。置于5%CO2 培养箱内，37°C培养24小时。

4. 24h后取出所有Transwell小室，吸弃小室上室内液体，用PBS清洗一次，在每个下室加入700μL 4%多聚甲醛，固定20min后，下室用0.1%结晶紫溶液染色10min。染色结束后，下室浸入PBS中轻轻清洗三次，用棉签轻轻擦去上层未迁移细胞。在400倍显微镜下随机观察五

个不同视野，计数细胞。计数结束后轻轻取下薄膜，将其浸入33%醋酸溶液脱色，用酶标仪测量洗脱液在570nm处的OD值，定量测定反应细胞数。

2.2.9 裸鼠皮下成瘤试验

为了检测 miR-99a-5p 在体内对胆囊癌细胞增殖能力的影响，将相应实验组和对照组 NOZ 细胞系接种于裸鼠的左侧腋窝下，观察肿瘤生长情况，以评价细胞在体内成瘤生长能力。

具体步骤如下：

1. 取相应实验组和对照组 NOZ 细胞系，用胰酶消化，离心。用 PBS 洗涤细胞两次。

2. 用 PBS 制作细胞悬液，密度为 $2*10^7$ 个细胞/ml。

3. 取 4-6 周龄的 BAL B/C 裸鼠进行试验。用 100μL 1%戊巴比妥钠麻醉裸鼠。

4. 以带 6 号针头的 1 ml 胰岛素注射器抽取细胞悬液接种裸鼠，每只裸鼠接种细胞悬液体积为 0.2ml，接种细胞量约为 $4x10^6$ 个，接种部位为裸鼠左侧腋窝皮下，接种前用 75% 酒精消毒皮肤，接种时由裸鼠左侧腰部稍靠上部位进针，确保进针点与接种点的距离小于针头长度，不刺破皮肤或肌肉层。麻醉清醒后裸鼠以 SPF 级别饲养。

5. 定期检测肿瘤生长情况并量取肿瘤的长短径。肿瘤体积按照以下公式计算，体积(mm^3)=(瘤长径 × 瘤短径 ^{2})/2。

6. 4 周后处死裸鼠，取出肿瘤称重、拍照，浸泡于 4%多聚甲醛中，保存于 4℃，用于后续免疫组化染色。

2.2.10 裸鼠脾脏注射肝转移模型建立

为了检测miR-99a-5p在体内对胆囊癌细胞转移能力的影响，将相应实验组和对照组NOZ细胞系注射入裸鼠的脾脏内，然后切除脾脏。45天后，处死裸鼠，分离肝脏组织，观察肝脏转移结节形成情况，以评价细胞在体内转移能力。

具体步骤如下：

1. 取相应实验组和对照组 NOZ 细胞系，用胰酶消化，离心。用 PBS 洗涤细胞两次。

2. 用 PBS 制作细胞悬液，密度为 $2*10^7$ 个细胞/ml。

3. 取 4-6 周龄的 BAL B/C 裸鼠进行试验。用 100μL 1%戊巴比妥钠麻醉裸鼠。

4. 术野用 75% 酒精棉球消毒，取裸鼠左上腹斜行切口约 1.5cm，用组织镊从腹腔中将脾脏轻轻拉出少许，以 1 ml 胰岛素注射器抽取细胞悬液对裸鼠脾脏进行注射，细胞悬液体积为

0.15ml，注射细胞量约为 $3x10^6$ 个，注射时间约 2 分钟，可见脾脏被膜出现肿胀变白现象，注射完毕后用棉签压迫针眼 2-3 分钟止血并防止细胞外渗形成腹腔内种植转移。然后结扎脾脏血管，将脾脏切除后缝合伤口。手术操作过程需要严格遵守无菌原则。麻醉清醒后裸鼠以 SPF 级别饲养。

5. 45 天后处死裸鼠，取出肝脏组织拍照，浸泡于 4%多聚甲醛中，保存于 4℃，用于后续试验。

2.2.11 蛋白印迹(Western Blot)试验

将相应细胞用PBS洗涤后用细胞刮板刮下，收集，用含50 mM Tris-HCl的RIPA裂解液重悬，冰上静置20分钟。4℃ 14000rpm离心10min，吸取上清液，并用BCA蛋白定量试剂盒进行蛋白浓度测定。蛋白悬液与上样缓冲液混合，100℃加热5分钟使蛋白变性，-20℃保存备用。

SDS-PAGE蛋白电泳步骤：

1. 按相应比例配方配制分离胶与浓缩胶，并按照计算好的蛋白体积分别上样。

2. 以60V恒压电泳蛋白质，待溴酚蓝条带到到达浓缩胶与分离胶边界时，调整电压为120V继续电泳。当溴酚蓝条带跑到玻璃板底部时关闭电源，停止电泳。

3. 电泳结束后取出凝胶，裁剪成适当大小的长方形。取转膜板，从黑面到白面依次按照海绵、3层滤纸、胶、PVDF膜、3层滤纸、海绵的顺序组装，确保彼此之间准确对齐并没有产生气泡。夹紧转模板放入转膜槽中，加入配好的转膜液，320mA恒流电泳2h，电泳槽需在冰浴中进行转膜。

4. 转膜结束后，打开转膜板，用镊子轻轻夹出膜，将膜用TBST缓冲液在摇床上漂洗3次，每次5min。漂洗结束后，将膜置于5%脱脂牛奶封闭液中室温封闭1h。

5. 封闭结束后，在室温下将膜用TBST缓冲液漂洗3次，每次5min。然后用1：1000稀释的一抗覆盖住膜的蛋白面，室温孵育1h。

6. 一抗孵育完成后将膜用TBST缓冲液漂洗3次，每次5min。然后用1：1000稀释的二抗覆盖住膜的蛋白面，室温孵育45min。孵育结束后用TBST缓冲液漂洗3次，每次5min。

7. 将洗净的膜上覆盖显影液，用化学发光仪拍摄照片，进行条带分析。

2.2.12 统计方法

所有计量资料均采用(平均数 ± 标准差)的形式表示。应用SPSS 21.0 软件进行数据统计分析。处理组和对照组间比较采用Student's t检验，以$P<0.05$ 认为具有统计学意义。所有实验数据取自三次平行重复实验结果的平均值。

3. 实验结果

3.1 miR-99a-5p 对胆囊癌细胞增殖能力的影响

首先，我们通过实时定量荧光 PCR 的方法检测了四株胆囊癌细胞系中 miR-99a-5p 的表达量，观察 miR-99a-5p 在胆囊癌细胞系中的表达水平。结果显示，miR-99a-5p 在 NOZ、EHGB-1 细胞系中表达量偏低，在 GBC-SD 和 OCUG 细胞系中表达量较高。因此，我们选用 NOZ 和 EHGB-1 细胞系进行过表达处理，而用 GBC-SD 细胞系进行抑制剂处理。

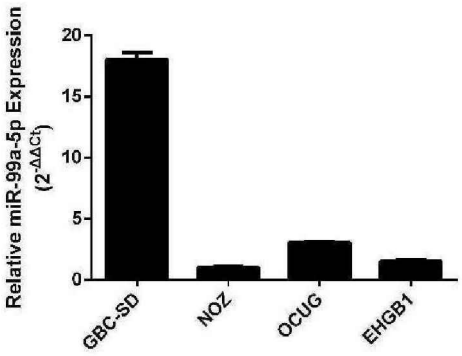


图 2-1：miR-99a-5p 在四株胆囊癌细胞系中的相对表达量。

Figure 2-1：Relative expression levels of miR-99a-5p in 4 GBC cell lines.

以 NOZ 细胞系中 miR-99a-5p 的表达量为 1。

为了验证 miR-99a-5p 的过表达和抑制表达的效果，我们使用了实时定量荧光 PCR 法测定 NOZ 和 EHGB-1 细胞感染了 miR-99a-5p 过表达慢病毒之后 miR-99a-5p 的表达水平。通过图 2-2 可以发现细胞系中 miR-99a-5p 的表达水平得到了显著上调。同样地，化学合成的抑制剂 miR-99a-5p inhibitor 也能够显著抑制 GBC-SD 细胞系中 miR-99a-5p 的表达。

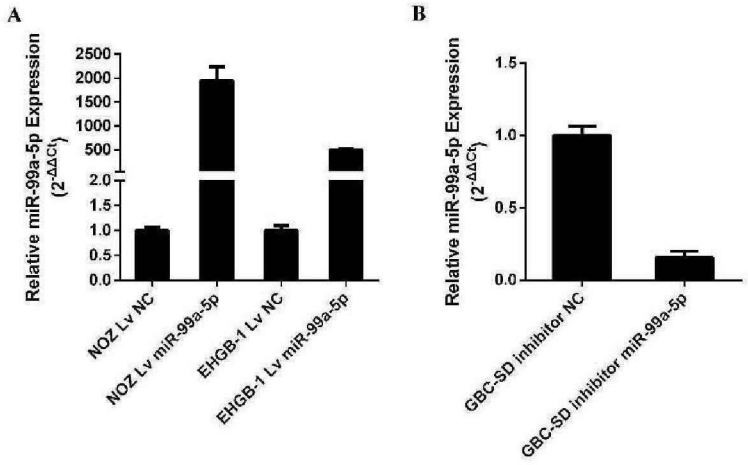


图 2-2：胆囊癌细胞系经处理后 miR-99a-5p 的相对表达量。

Figure 2-2: Relative expression levels of miR-99a-5p after treatment in GBC cell lines.

（A） NOZ 以及 EHGB-1 感染 miRNA 过表达慢病毒和 (B) GBC-SD 转染 miRNA 抑制剂后 miR-99a-5p 的相对表达量。

接着我们进行 miR-99a-5p 影响胆囊癌细胞体外增殖能力的试验。通过 CCK8 试验，我们发现虽然 P 值小于 0.001，但从细胞增长曲线来看，过表达 miR-99a-5p 对 NOZ 和 EHGB-1 细胞系仅有极弱的抑制作用，而抑制 miR-99a-5p 的表达对胆囊癌细胞增殖则没有影响(图 2-3)。

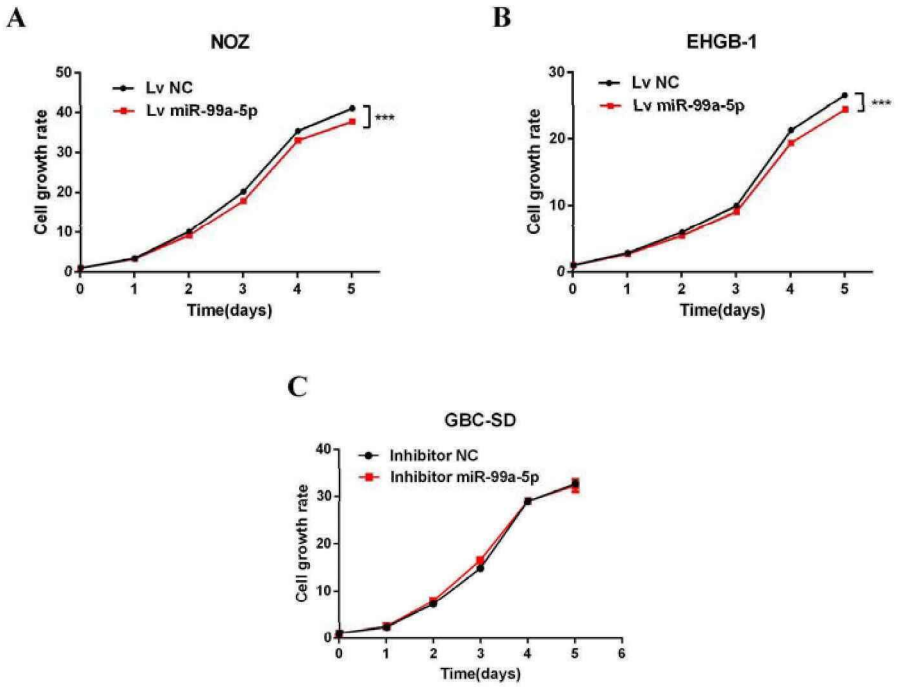


图 2-3：MiR-99a-5p 对胆囊癌细胞系增殖能力的影响。

Figure 2-3: Effect of MiR-99a-5p on proliferation of GBC cell lines.

（A:NOZ 细胞系；B:EHGB-1 细胞系；C:GBC-SD 细胞系。横坐标 0 点代表铺板后 6h。纵坐标以铺板后 6h 的 OD450 值为 1。***：P<0.001）

克隆形成试验能够检测单个细胞增殖形成集落的能力，将经过过表达慢病毒和抑制剂处理的胆囊癌细胞接种于六孔板中，每孔1000个细胞，待对照组细胞单个克隆细胞数大于50个时进行结晶紫染色，肉眼观察形成的克隆总数，然后镜下观察单个克隆内细胞数。结果见图2-4，过表达与抑制表达 miR-99a-5p 均无法改变胆囊癌细胞系的克隆形成能力。

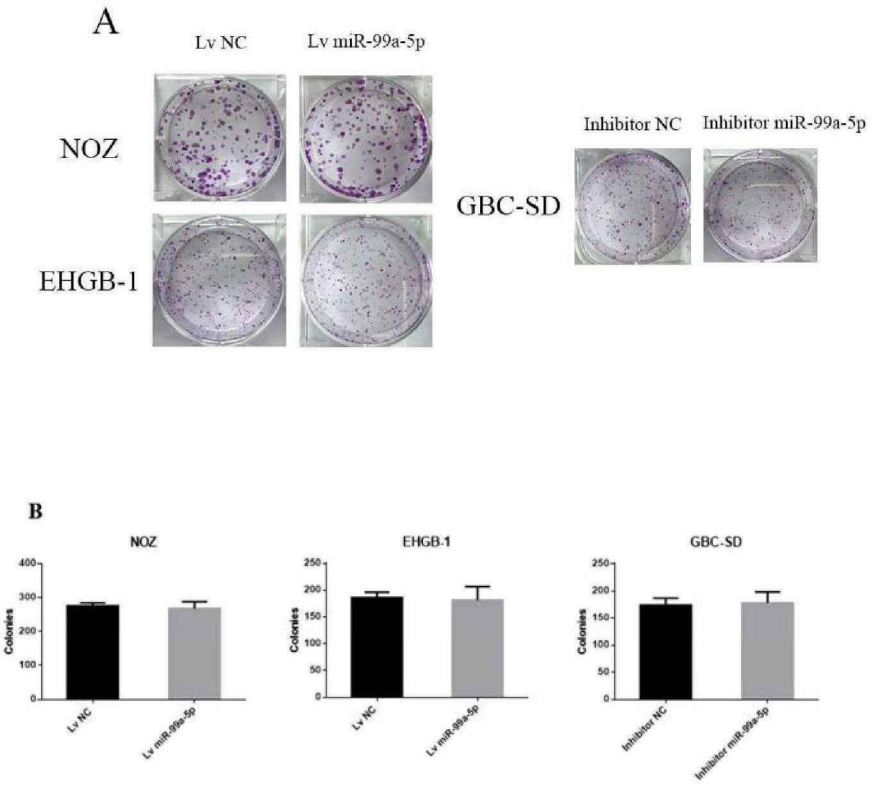


图 2-4：MiR-99a-5p 对胆囊癌单个细胞体外增殖能力的影响。

Figure 2-4: The effect of miR-99a-5p on the proliferation of GBC single cells in vitro.

(A)经抑制剂和过表达慢病毒处理后的胆囊癌细胞克隆形成图片；（B）胆囊癌细胞系克隆数柱状图。结果均无统计学差异。

为了更进一步明确 miR-99a-5p 是否对胆囊癌细胞增殖能力有影响，我们将 NOZ 细胞用慢病毒感染来过表达 miR-99a-5p，然后将细胞悬液注射至裸鼠腋下，四周后将裸鼠处死。如图2-5所示，过表达 miR-99a-5p 后裸鼠皮下瘤生长能力稍有抑制，但并无统计学差异。以上试验共同说明了 miR-99a-5p 不能明显影响胆囊癌细胞的增殖能力。

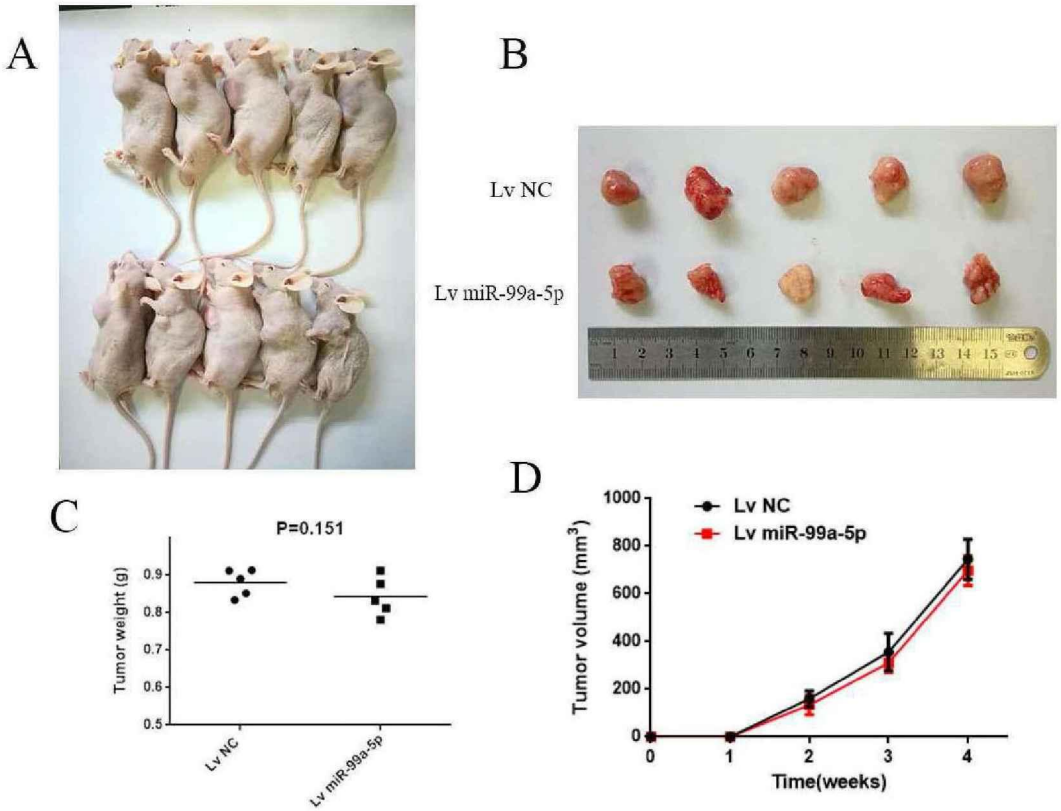


图 2-5：MiR-99a-5p 对 NOZ 细胞系体内增殖的影响。

Figure 2-5: Effect of miR-99a-5p on the growth of NOZ cells in vivo.

(A)裸鼠成瘤大体图；(B)取出的皮下瘤照片；(C)皮下瘤重量散点图；(D)皮下瘤生长曲线。结果均无统计学差异。

3.2 miR-99a-5p 对胆囊癌细胞转移侵袭能力的影响

在以上研究的基础上，为了继续研究 miR-99a-5p 对胆囊癌细胞转移侵袭能力的影响，我们进行了体外的 Transwell 小室试验和细胞划痕愈合实验。通过有基质胶和无基质胶的 Transwell 小室，我们分别能够检测细胞的体外侵袭和转移能力。如图2-6所示，Transwell 小室试验显示过表达 miR-99a-5p 能够显著抑制胆囊癌细胞系 NOZ 和 EHGB-1的侵袭转移能力，而经过 miR-99a-5p Inhibitor 处理后，穿过小室的 GBC-SD 细胞明显增多。

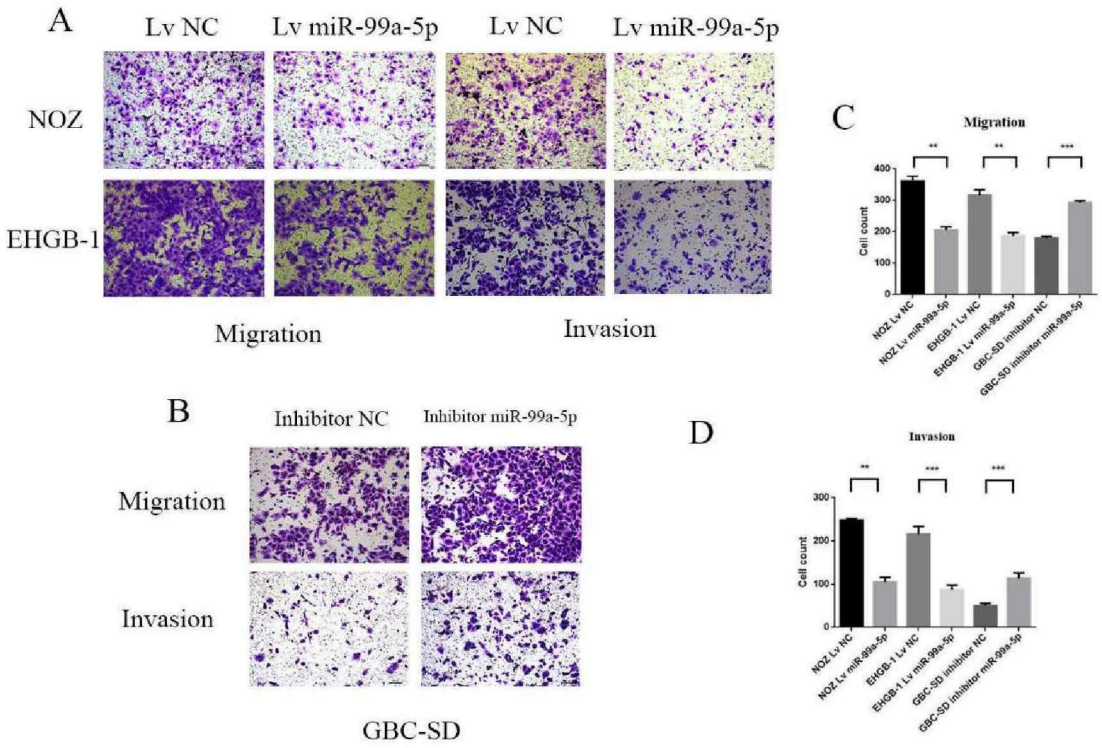


图 2-6：胆囊癌细胞系 Transwell 迁移/侵袭实验。

Figure 2-6: Tranwell chamber invasion/metastasis assay in GBC cell lines.

（A）NOZ、EHGB-1 迁移/侵袭细胞镜下图；（B）GBC-SD 迁移/侵袭细胞镜下图；（C）各细胞系迁移实验细胞数统计图；（D）各细胞系侵袭实验细胞数统计图。**: $P<0.01$，***: $P<0.001$。

同时进行的细胞划痕愈合试验获得了同样的结果。三组细胞中，经过 miR-99a-5p 处理后的细胞48h 后划痕宽度明显较对照组细胞大，提示其划痕愈合速度最慢，细胞迁移能力减弱。相反地，miR-99a-5p Inhibitor 处理后的细胞划痕愈合速度增加，划痕宽度明显减小(图2-7)。以上试验共同表明了 miR-99a-5p 能够明显地抑制胆囊癌细胞在体外的侵袭和转移能力。

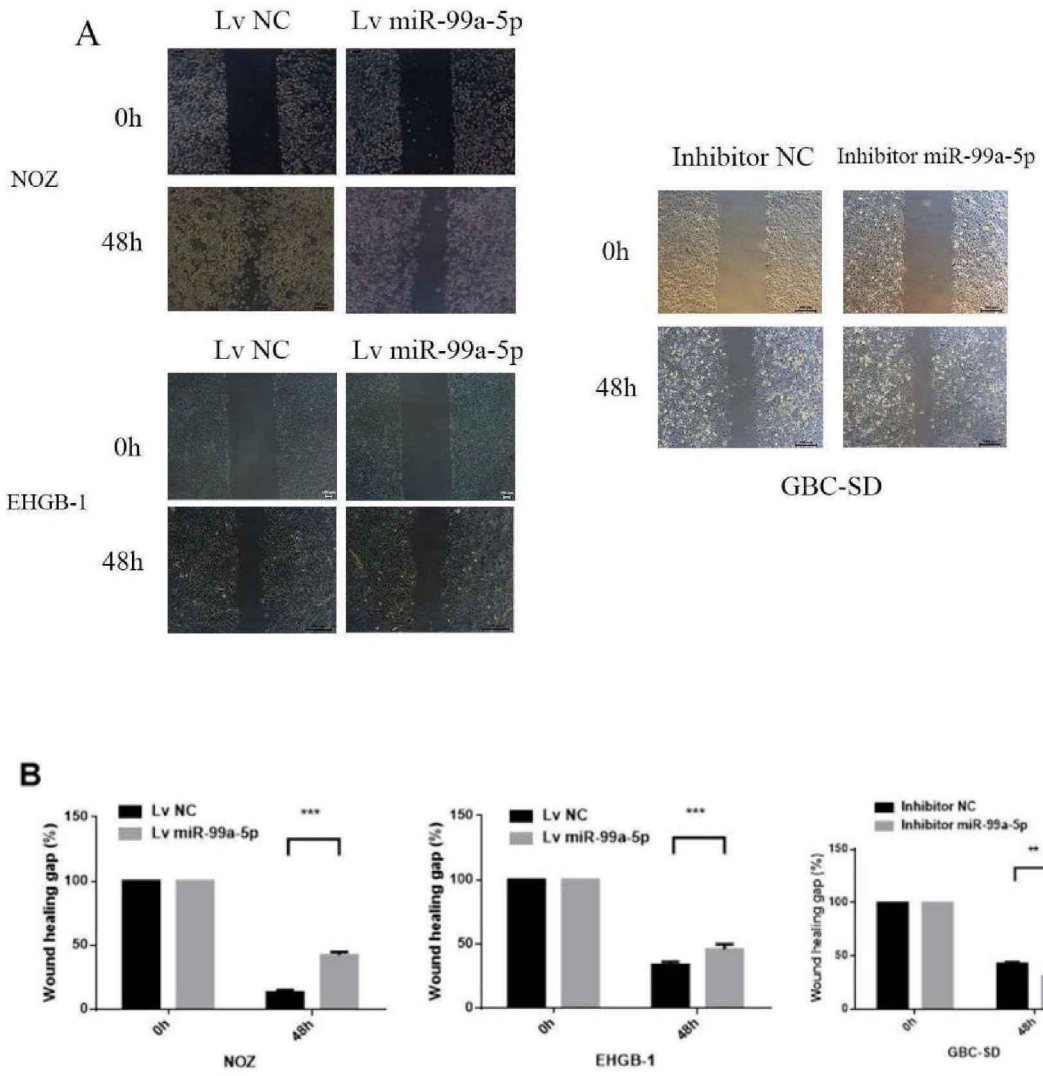


图 2-7：胆囊癌细胞系划痕愈合实验。

Figure 2-7: wound healing assay in GBC cell lines.

(A)镜下观察胆囊癌细胞系 48 小时之内划痕愈合情况；(B)对划痕愈合距离进行定量分析。**：P<0.01；***：P<0.001.

裸鼠脾脏注射肝转移是一种能够反应肿瘤细胞在体内转移能力的动物模型，常用于消化道恶性肿瘤的体内模型构建。我们把感染了 miR-99a-5p 过表达慢病毒的 NOZ 细胞系通过脾脏注射至裸鼠体内来评估 miR-99a-5p 在动物体内对胆囊癌细胞转移能力的影响。我们发现对照组细胞在同样的时间里在裸鼠中有更高的肝转移发生率(3/5 vs 1/5)，肝脏的 HE 染色也同时证实了转移结节的存在(图2-8)。以上结果表明 miR-99a-5p 能够在动物体内起到抑制胆囊癌细胞转移能力的作用。

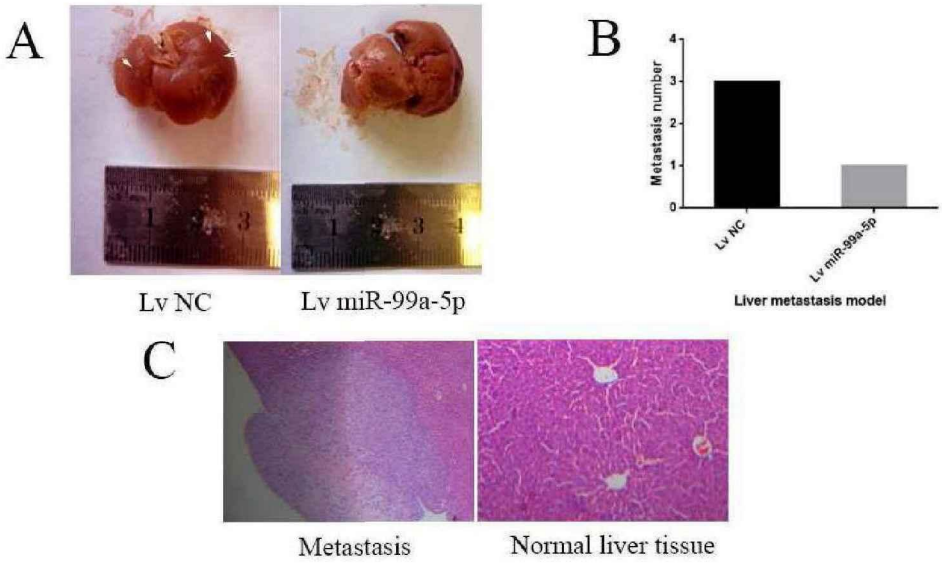


图 2-8：裸鼠脾静脉肝转移模型。

Figure 2-8: Splenic-liver metastasis model of nude mice.

(A)两组裸鼠肝脏典型大体图片，箭头指向转移灶；(B)发生肝转移的个体数；（C）正常肝和转移灶 HE 染色对比图。

此外，由于在许多肿瘤中，侵袭转移的发生于 EMT 作用直接相关，所以我们也检测了 miR-99a-5p 处理后 EMT 相关蛋白的表达水平变化。通过 Western Blot 试验，我们可以发现 miR-99a-5p 的过表达使与上皮细胞相关的蛋白 E-cadherin 的表达水平显著升高，而代表间质细胞水平的标志物 N-cadherin 和 Vimentin 的表达水平则有所下降（图 2-9）。该结果说明了 miR-99a-5p 能够抑制 EMT 过程的发生，从而达到减弱胆囊癌细胞侵袭转移能力的作用。

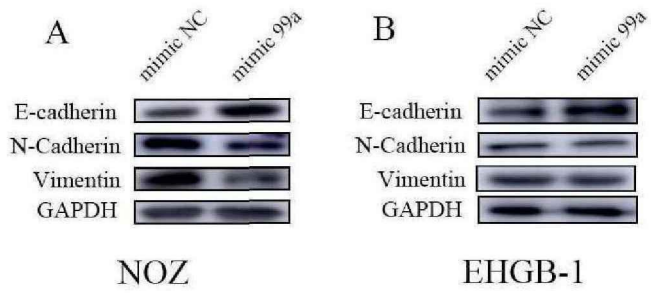


图 2-9：NOZ 和 EHGB-1 细胞系中过表达 miR-99a-5p 后用 WB 检测 EMT 相关蛋白的表达。

Figure 2-9: The expression of EMT-related proteins was detected by WB after overexpression of miR-99a-5p in NOZ and EHGB-1 cell lines.

4. 讨论

近年来，胆囊癌在诊断及治疗方面取得了一些进展，但早期的淋巴结转移和对肝脏的直接侵犯，以及根治性手术后容易局部复发和淋巴结转移仍然是限制胆囊癌患者获得长期无病生存的重要原因[34]。所以研究如何抑制胆囊癌的侵袭转移功能是提高胆囊癌治疗效果的关键。但目前胆囊癌高侵袭转移能力的原因和机制尚未被阐明。

miRNA 是一类单链的小分子非编码 RNA，在进化上相对保守，通过与特异性靶基因的 3'UTR 区结合来直接降解靶基因 mRNA 或者抑制靶基因的 mRNA 的翻译过程来实现对靶基因的调控。与其它恶性肿瘤研究的情况类似，近些年来，miRNA 在胆囊癌领域的研究也不在少数[35-38]。

在本部分研究中，我们通过对胆囊癌细胞系进行过表达与抑制表达 miR-99a-5p 的处理，然后分别通过 CCK8、克隆形成、细胞划痕愈合、Transwell 小室试验以及体内动物试验等方法探究 miR-99a-5p 对胆囊癌增殖及侵袭转移功能的影响。我们发现，miR-99a-5p 表达的改变对胆囊癌细胞的增殖功能影响不大。但用过表达慢病毒处理之后，胆囊癌细胞系 NOZ 和 EHGB-1 穿过有基质胶或无基质胶 Transwell 小室的细胞数目明显减少，细胞划痕愈合时间也明显延长。反之，用 miR-99a-5p 抑制剂处理胆囊癌细胞系之后，胆囊癌细胞的侵袭转移能力得到了增强。在体内试验中也得到了类似的结论。这充分说明了 miR-99a-5p 无论在体外还是在体外都参与了胆囊癌的侵袭转移过程。

肿瘤的侵袭转移过程相当复杂，涉及到许多效应分子、信号通路及肿瘤微环境方面的影响。虽然关于胆囊癌的研究有很多，但究竟是哪种分子或是哪个信号通路主导了胆囊癌的侵袭转移过程，目前仍未有定论。Bao 等[39]发现 miR-101 可以靶向抑制促癌基因 ZFX，实现一部分对胆囊癌发展的功能调节。另外，miR-101 可以对 Smad 通路产生影响，抑制了 TGF-β 介导的 EMT 过程，实现了对胆囊癌的侵袭转移等功能的抑制。而另一项研究发现，miR-29c-5p 通过靶定 CPEB4 基因来负向调节胆囊癌的增殖和转移功能，同时也能直接抑制 MAPK 通路，从而增强 miR-29c-5p 的抑癌效果[15]。我们还需要进一步的深入研究来明确 miR-99a-5p 是如何在胆囊癌中发挥调控侵袭转移功能的作用。

5. 小结

1. miR-99a-5p 无论在体内还是体外对胆囊癌细胞的增殖功能影响均不明显；
2. miR-99a-5p 可以体内体外抑制胆囊癌细胞的侵袭转移；
3. miR-99a-5p 影响了胆囊癌细胞的 EMT 过程，从而导致了对其侵袭转移能力的抑制。

第三部分：miR-99a-5p 通过靶定 MTOR 和 SMARCA5 影响胆囊癌的功能

1. 前言

胆囊癌是恶性程度极高的消化道恶性肿瘤，也是胆道系统中最常见的恶性肿瘤，发病率在消化道肿瘤中排在第六位，严重影响了人类健康。在中国，胆囊癌的发病率为 3.9/10 万人[1]。如能早期发现，行根治性手术可以达到比较满意的 5 年生存率。但胆囊癌起病较为隐匿，且早期缺乏特异性的症状，一经发现，许多患者已经进展到中晚期，失去了行根治性手术的机会，且目前放化疗等手段不能达到满意的治疗效果，总体 5 年生存率仅为 5%[2]。因此，早期诊断是改善胆囊癌预后的关键。且研究发现，胆囊癌所导致的死亡病例中超过 90%为肿瘤复发转移所致[3]。因此，关于胆囊癌发病以及复发侵袭转移机制的研究有助于寻找新的胆囊癌肿瘤标志物和靶向药物治疗的新靶点，对于改善胆囊癌诊疗水平以及患者预后具有重要意义。

截至目前，人类已经发现了上万个 miRNA 分子，他们与下游的目标靶蛋白构成了复杂的作用网络。数据显示有超过 30%的蛋白质在一定程度上收到了 miRNA 的直接或间接调控[40]。与传统基因组相比，miRNA 相关的对肿瘤生物学行为的调控网络更为庞大。miRNA 通过碱基互补配对原则与靶基因的 3'UTR 区进行结合，直接对 mRNA 进行降解或者影响其后的蛋白翻译过程。一个 miRNA 可能针对下游多个靶基因产生作用，而一个蛋白可能也受到多个 miRNA 的调控[41]。对下游靶基因的预测及研究是了解 miRNA 影响肿瘤机制的重要研究手段。

在上一部分中，我们明确了 miR-99a-5p 对胆囊癌细胞侵袭转移能力的影响，起着抑癌的作用。为了了解它的调控机制，我们采取生物信息学的工具来寻找和预测其下游的候选靶基因，并用双荧光素酶报告基因试验和蛋白免疫印迹试验来进行验证。同时，我们也将对可能参与调控 miR-99a-5p 功能的潜在信号通路进行研究，从而更加完善其作用机制。

2. 材料与方法

2.1 实验材料

2.1.1 主要实验试剂

名称	厂家
双荧光素酶报告系统试剂盒	美国普洛麦格公司
质粒小提中量试剂盒(无内毒素)	北京天根公司
Viafect 试剂	美国普洛麦格公司
Lipofectamine2000	Invitrogen 公司
无水乙醇	国药集团化学试剂有限公司
二甲苯	国药集团化学试剂有限公司
醋酸	国药集团化学试剂有限公司

其余试剂请参见本文第二部分“2.材料与方法”。

2.1.2 主要实验仪器

仪器名称	厂家
化学发光仪	美国伯乐公司

2.2 实验方法

2.2.1 通过生物信息学方法预测 miR-99a-5p 的靶基因

对于miR-99a-5p靶基因的生物信息学预测分析，本部分实验采用miRDB、TargetScan、picTar和miRanda预测工具完成。

2.2.2 双荧光素酶报告基因实验

我们采用双荧光素酶报告基因实验验证候选靶基因与miR-99a-5p的直接作用关系。包含

MTOR、SMARCA5的3'UTR区域的双荧光素酶报告基因质粒及阴性对照突变体质粒的合成由上海龙钱生物科技有限公司完成。野生型质粒包含miR-99a-5p的结合位点，而突变型质粒在miR-99a-5p结合位点进行随机序列突变。PCR产物插入pmirGLO载体。MTOR-3'UTR pmirGLO质粒或SMARCA5-3'UTR pmirGLO质粒与相应的miRNA模拟物或抑制剂在293T细胞中共转染。48小时后使用双荧光素酶报告基因实验试剂盒进行萤火虫荧光素酶和海肾素荧光素酶活性的检测。使用海肾素荧光素酶活性进行相对荧光活性的标准化。

共转染具体步骤：

1、转染前一天将 293T 细胞铺到 24 孔板中，每孔铺 10 万细胞，置于 5%CO2 细胞培养箱中 37℃培养，使第二天转染时细胞融合度达到 50%。

2、每孔细胞转染 10ng 质粒和 1μL 的 miRNA 的模拟物或抑制剂，使用 Lipofectamin2000 转染试剂 1μL。

3、在一个 1.5ml EP 管中加入 50μL 的 optiMEM，再加入 10ng 的相应质粒和 1μL 的相应 miRNA 模拟物或抑制剂，轻柔混匀；另一个 1.5ml EP 管中加入 50μL 的 optiMEM，再加入 Lipofectamin2000 转染试剂 1μL，室温下静置 5min 后将两个 EP 管中的试剂混匀，室温下共同孵育 20min。

4、将培养的细胞取出，用 PBS 清洗所有细胞一次后，在每个培养孔中加入 400μL 含 FBS 不含双抗生素的 DMEM 培养基。

5、将孵育好的混合物按加入对应孔中，轻柔混匀，将细胞继续放回培养箱中培养，6h 后更换为完全培养基培养。转染 48h 后进行双荧光素酶报告基因试验。

2.2.3 靶基因过表达质粒的构建与转染

SMARCA5 CDS区(Coding sequence)的全长被由上海龙钱生物科技有限公司插入pCMV质粒的MCS区（图3-1），构建成为pCMVGFPPuro05(-)SMARCA5质粒，并且转染至胆囊癌细胞系。使用嘌呤霉素筛选出稳定表达SMARCA5的克隆。使用实时荧光定量PCR和蛋白印迹试验验证SMARCA5的表达水平。空载体作为空白对照组。

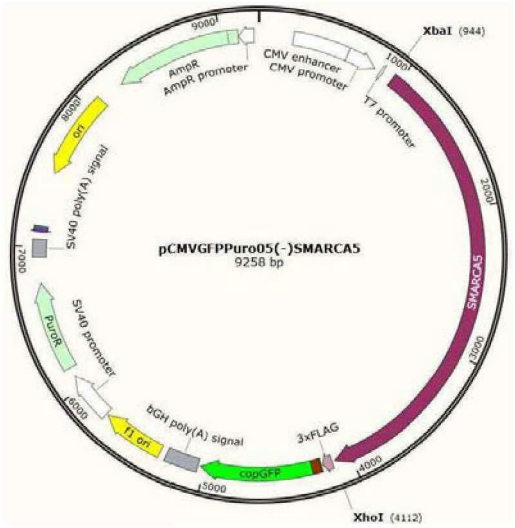


图3-1：pCMVGFPPuro05(-)SMARCA5的质粒图谱。

Figure 3-1：Vector map of pCMVGFPPuro05(-)SMARCA5.

质粒转染具体步骤：

1、转染前一天将细胞铺到六孔板中，置于 5%CO2 细胞培养箱中 37℃培养，使第二天转染时细胞融合度达到 70%。

2、每孔细胞转染 2μg 质粒，使用 viafect 转染试剂 6μL。

3、将相应体积的质粒和 6μL/孔的 viafect 试剂共同加入 300μL/孔的 optiMEM 培养基中，轻柔混匀，室温下共同孵育 15min。

4、将培养的细胞取出，用 PBS 清洗所有细胞一次后，在每个培养孔中加入 1700μL 含 FBS 不含双抗生素的对应培养基。

5、将孵育好的含质粒的 optiMEM 按 300μL/孔体积加入对应孔中，轻柔混匀，将细胞继续放回培养基中培养，6h 后更换为完全培养基培养。

6、转染 24h 后继续后续试验。

2.2.4 小干扰 RNA 合成

针对SMARCA5的小干扰RNA（siRNA）和阴性对照siRNA由上海拓然生物有限公司合成，并用RFect转染试剂转染至胆囊癌细胞中。使用实时荧光定量PCR和蛋白印迹试验验证SMARCA5的表达水平。SMARCA5的小干扰RNA序列：5'-GUCAGAGUGUUCCGCUUUA-3'。

2.2.5 主要实时定量荧光 PCR 引物列表

名称	序列
MTOR-F	5'- AGCCGAAGGAGATGCAGAAG -3'
MTOR-R	5'- TTCGGACCAGCTCGTTAAGG -3'
SMARCA5-F	5'- GCATGAGCTGTGGTCACTTC -3'
SMARCA5-R	5'- GCCTTAATTCGACGAAGGAG -3'
GAPDH-F	5'-AGAAGGCTGGGGCTCATTTG-3'
GAPDH-R	5'-AGGGGCCATCCACAGTCTTC-3'

2.2.6 统计学分析

采用 SPSS 21.0 软件进行统计学分析。组间比较则采用 t 检验方法，计量资料均由(平均数 ± 标准差)表示。以 $P<0.05$ 为有统计学意义。所有实验数据为三次重复实验得出的平均值。

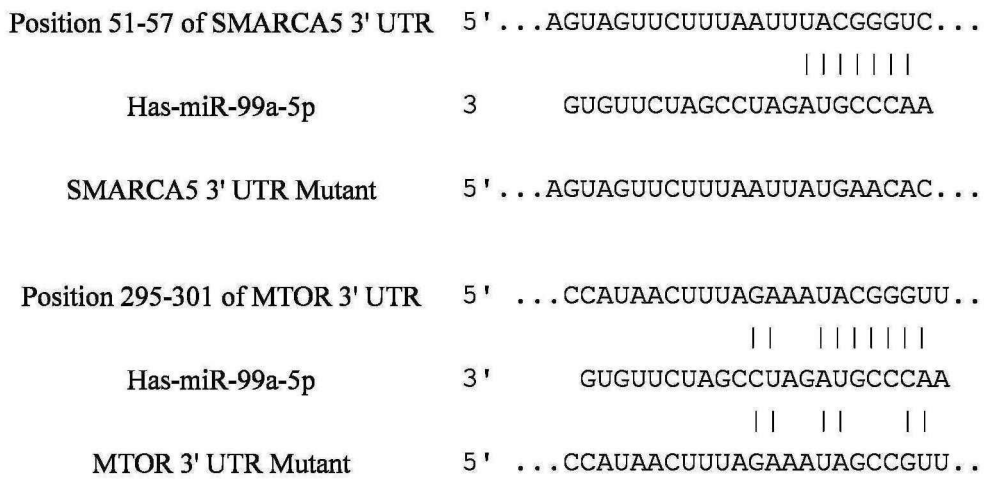
3. 实验结果

3.1 miR-99a-5p 的靶基因预测

通过运用生物信息学技术的分析，我们可以预测与 miRNAs 直接作用的下游靶基因，从而更加深入研究其影响肿瘤细胞生物学行为的机制。在本研究中，我们选择了 miRDB、TargetScan、picTar 和 miRanda 等在线数据库作为预测工具。根据预测结果，我们选择了在这四个数据库中均排名靠前的基因 SMARCA5 和 MTOR 作为候选靶基因的对象。

表 3-1：生物信息学预测 miR-99a-5p 与候选靶基因 SMARCA5 和 MTOR 的结合位点。

Table 3-1: Binding site of miR-99a-5p to candidate target genes SMARCA5 and MTOR predicted by Bioinformatics.



3.2 双荧光素酶报告基因试验验证 miR-99a-5p 的靶基因

为了验证 miR-99a-5p 与候选靶基因是否能够直接作用，我们分别构建了带有 MTOR 和 SMARCA5 基因 3'UTR 区的野生型和突变型双荧光素酶报告基因的质粒，将它们与 miR-99a-5p 模拟物以及抑制剂共转染之后，通过双荧光素酶报告基因试剂盒来检测相应组别荧光素酶的活性。试验结果显示，MTOR 和 SMARCA5 野生型 3'UTR 质粒与 miR-99a-5p 模拟物共转染之后，与突变型质粒组相比，均能明显使相应荧光素酶活性降低(图 3-2 A)。另外，我们还将 miR-99a-5p 模拟物与对照模拟物分别与 MTOR 和 SMARCA5 野生型 3'UTR 质粒共转染。结果发现，miR-99a-5p 模拟物与对照组相比均能明显下调荧光素酶的活性。相反地，miR-99a-5p 抑制物与靶基因的野生型 3'UTR 质粒共转染能够增强荧光素酶的活性(图 3-2 B,C)。

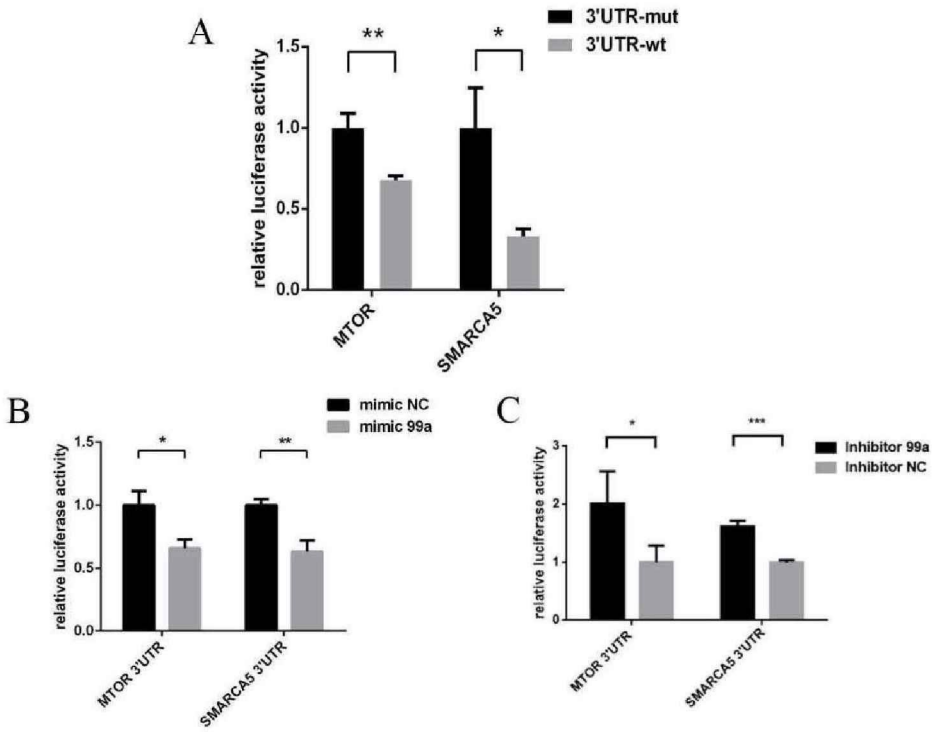


图 3-2：MiR-99a-5p 抑制 MTOR 和 SMARCA5 3'UTR 报告基因活性。

Figure 3-2: Relative activity of luciferase reporters of 3'UTR plasmids of MTOR and SMARCA5 inhibited by miR-99a-5p.

(A) MTOR 和 SMARCA5 野生型 3'UTR 质粒与 miR-99a-5p 模拟物共转染; (B) miR-99a-5p 模拟物与对照模拟物分别与 MTOR 和 SMARCA5 野生型 3'UTR 质粒共转染; (C) miR-99a-5p 抑制物与对照抑制物与靶基因的野生型 3'UTR 质粒共转染。*: P<0.05; **: P<0.01; ***: p<0.001。

3.3 miR-99a-5p 对候选靶基因 MTOR 和 SMARCA5 表达量的影响

为了验证 miR-99a-5p 对候选靶基因 MTOR 和 SMARCA5 在 mRNA 水平和蛋白水平表达量的影响，我们使用了实时荧光定量 PCR 和蛋白印迹试验分别检测了过表达 miR-99a-5p 后胆囊癌细胞内 MTOR 和 SMARCA5 在 mRNA 水平和蛋白水平表达量的变化。如图 3-3 所示，在 NOZ 和 EHGB-1 两株胆囊癌细胞中，过表达 miR-99a-5p 不能造成候选靶基因在 mRNA 水平上表达量的改变，但能显著抑制靶基因的蛋白表达量。

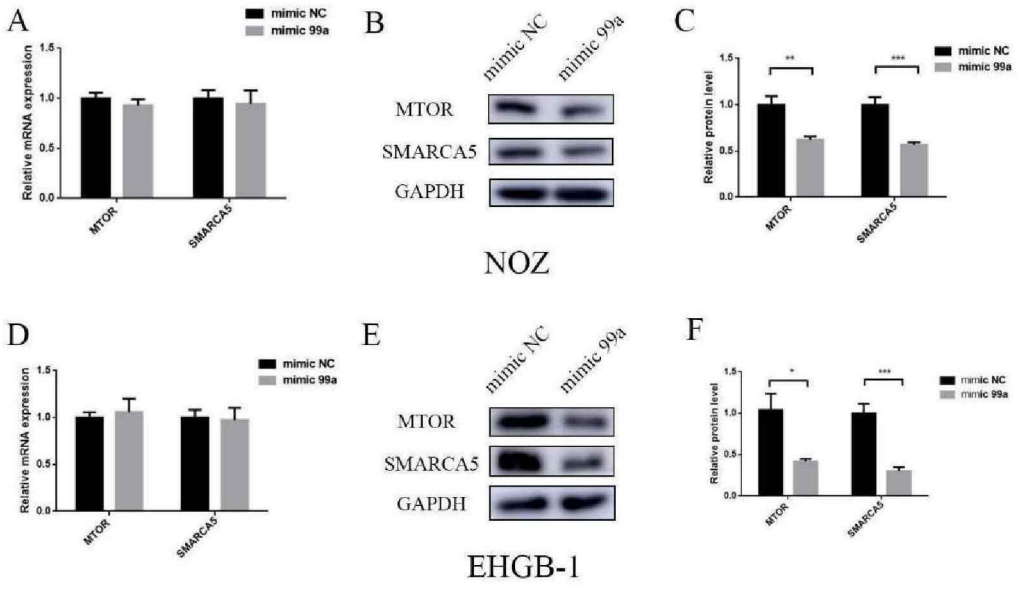


图 3-3：过表达 miR-99a-5p 后 NOZ 和 EHGB-1 细胞中 MTOR、SMARCA5 的 mRNA 和蛋白水平表达量。

Figure 3-3: mRNA and protein levels of MTOR and SMARCA5 in NOZ and EHGB-1 cell lines after overexpressing miR-99a-5p.

(A)过表达 miR-99a-5p 后 NOZ 细胞系中 MTOR 和 SMARCA5 mRNA 表达水平变化；(B) (C) 过表达 miR-99a-5p 后 NOZ 细胞系中 MTOR 和 SMARCA5 蛋白表达水平变化；(D) 过表达 miR-99a-5p 后 EHGB-1 细胞系中 MTOR 和 SMARCA5 mRNA 表达水平变化；(E) (F) 过表达 miR-99a-5p 后 EHGB-1 细胞系中 MTOR 和 SMARCA5 蛋白表达水平变化。 *: P<0.05; **: P<0.01; ***: p<0.001。

综合上述试验结果，我们可以得出结论：MTOR 和 SMARCA5 都是 miR-99a-5p 的直接靶基因。

3.4 SMARCA5 在胆囊癌患者组织中的表达以及与临床病例资料和预后的相关性

为了了解 SMARCA5 在胆囊癌患者组织中的表达情况，我们利用 qRT-PCR 检测了 30 对胆囊癌组织及其对应癌旁组织中 SMARCA5 的表达量。结果证明 SMARCA5 在胆囊癌组织中的表达量显著高于癌旁组织(P<0.001)。

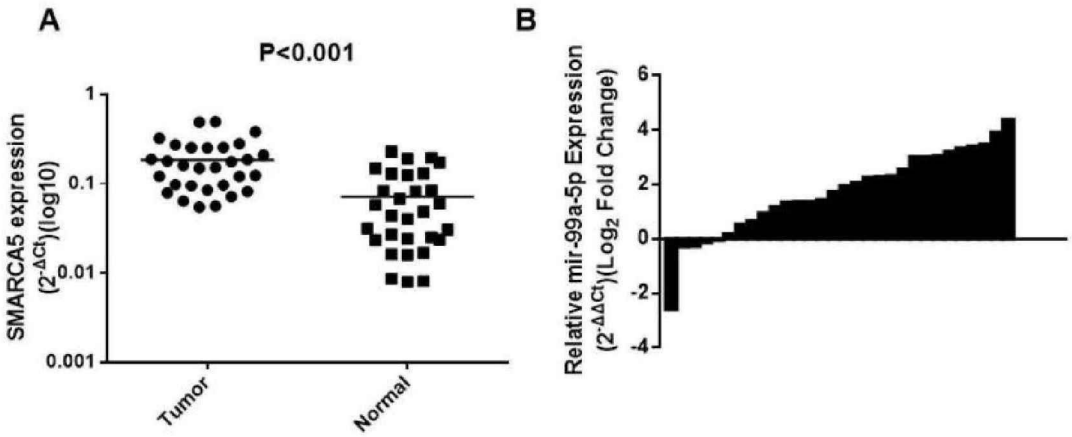


图 3-4: SMARCA5 在胆囊癌组织中的表达量检测。

Figure 3-4: Expression of SMARCA5 in GBC tissues.

（A）SMARCA5 在 30 对胆囊癌组织和其癌旁组织中的表达量；（B）SMARCA5 在胆囊癌组织与癌旁组织中的表达量对比。

为了进一步明确 SMARCA5在胆囊癌中的促癌作用，我们将上述患者以 SMARCA5在胆囊癌组织中的平均表达量为界，分为高表达组合低表达组。通过临床病理资料分析显示SMARCA5表达量与淋巴结转移（*P*=0.009）和 TNM 分期密切相关（P=0.02）（表3-1）。生存分析证实，SMARCA5高表达患者预后明显不良(图3-5)。高表达组和低表达组中位生存时间分别为6.9个月和18个月，1年生存率分别为45.0%和70.7%。并且淋巴结转移和 SMARCA5表达量高低可以作为胆囊癌患者预后的独立生存因素（表3-2）。

表 3-1：SMARCA5 相对表达量与胆囊癌患者的临床病理特征的关系

Table 3-1：Association between SMARCA5 expression and the clinicopathological characteristics in GBC

临床病理特征	例数	SMARCA5 表达		χ^2	p
		高表达(n=11)	低表达(n=19)		
年龄（岁）					
＜60	9	4 (44.4%)	5 (55.6%)	0.335	0.563
≥60	21	7 (33.3%)	14 (66.7%)		
性别					
男	12	6(50.0%)	6 (50.0%)	1.531	0.216
女	18	5 (27.8%)	13 (72.2%)		
组织分化					
高-中分化	23	8 (34.8%)	15 (65.2%)	0.151	0.698
低分化	7	3 (42.9%)	4 (57.1%)		
T 分期					
Tis-T_2	9	4 (44.4%)	5 (55.6%)	0.335	0.563
T_3-T_4	21	7 (33.3%)	14 (66.7%)		

淋巴结转移					
无	22	5 (22.7%)	17 (77.3%)	6.903	0.009
有	8	6 (75.0%)	2 (25.0%)		
TNM 分期					
0-I	11	7 (63.6%)	4 (36.4%)	5.440	0.02
II-IV	19	4 (21.1%)	15 (78.9%)		

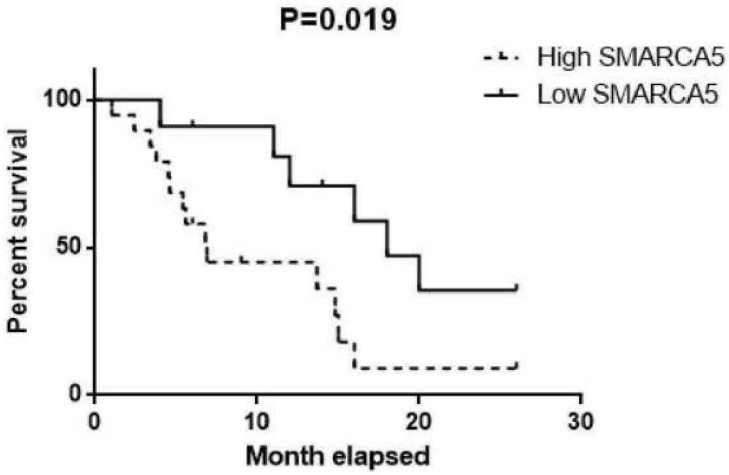


图 3-5：SMARCA5 高表达组和低表达组患者的生存曲线分析。

Figure 3-5：Survival analysis of GBC patients correlated with SMARCA5 expression.

表 3-2 胆囊癌患者临床病理因素和 SMARCA5 表达与预后关系的单因素和多因素分析

Table 3-2 Univariate and multivariate analysis of the relationship between

因素	单因素分析		多因素分析	
	HR (95% CI)	*p*	HR (95% CI)	*p*
年龄（<60 / ≥60）	0.411 (0.213-0.776)	0.226	-	-
性别（男 / 女）	0.522 (0.260-1.071)	0.082	-	-
组织分化（高-中分化 / 低分化）	1.262 (0.816-1.881)	0.321	-	-
T 分期（T3-T4 / Tis-T2）	1.096 (0.595-2.127)	0.808	-	-
淋巴结转移（有 / 无）	**6.252 (2.527-14.661)**	**<0.001**	**8.116 (2.564-23.609)**	**<0.001**
TNM 分期（III-IV / 0-II）	**0.326 (0.161-0.639)**	**0.001**	**0.348 (0.134-0.841)**	**0.012**
SMARCA5 表达量	**6.248 (3.146-12.651)**	**<0.001**	**2.613 (1.150-6.346)**	**0.036**

HR：相对风险系数；CI：置信区间。

3.5 miR-99a-5p 通过 PI3K/AKT 通路影响胆囊癌细胞的侵袭转移能力

为了进一步深入研究 miR-99a-5p 是如何对胆囊癌细胞的侵袭转移功能起到调控作用，我们通过蛋白印迹试验（Western Blot 试验）来检测与 MTOR 相关的 PI3K/AKT 信号通路中相关蛋白水平的改变。如图 3-6 所示，在 NOZ 和 EHGB-1 细胞系中过表达 miR-99a-5p 能使

AKT 蛋白的磷酸化水平下降，从而抑制 PI3K/AKT 信号通路的促癌功能。

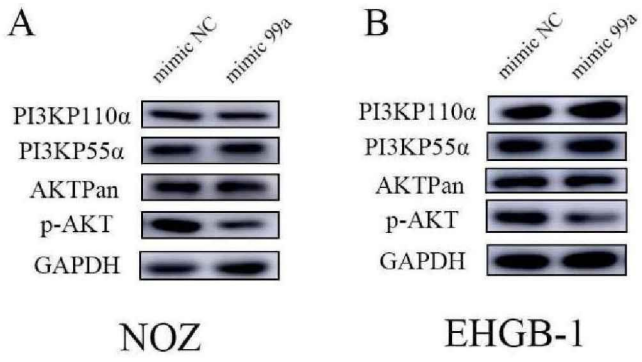


图 3-6：过表达 miR-99a-5p 后 NOZ 与 EHGB-1 细胞系中 PI3K/AKT 通路表达水平变化。

Figure 3-6: Expression levels of proteins involved in PI3K / AKT signaling pathway after overexpression of miR-99a-5p in NOZ and EHGB-1 cell lines.

3.6 SMARCA5 对胆囊癌细胞增殖及侵袭转移能力的影响

SMARCA5 基因在胆囊癌中的作用和功能目前仍然处于未知状态。为了了解 SMARCA5 对胆囊癌细胞的增殖和转移能力是否会产生影响，我们分别用 siRNA 和过表达质粒对胆囊癌细胞进行了敲减和过表达的处理(图 3-7)。通过 CCK8 试验，我们发现无论是对 NOZ 和 EHGB-1 细胞系进行敲减还是过表达，都不会对胆囊癌细胞的增殖能力产生显著影响(图 3-7)。

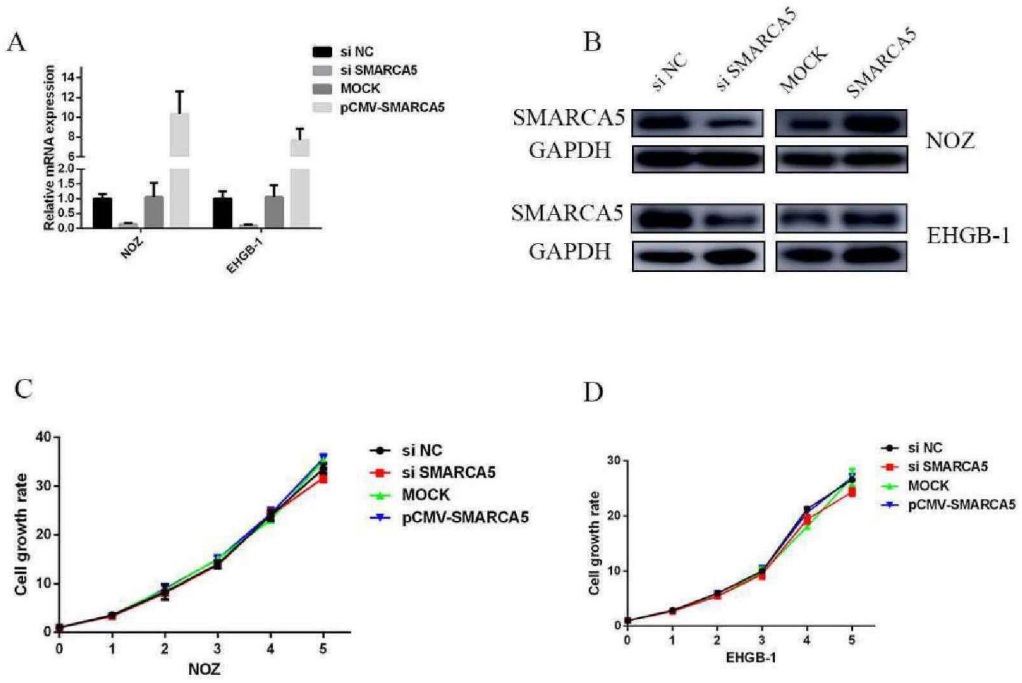


图 3-7：敲减和过表达 SMARCA5 后对胆囊癌细胞增殖功能的影响。

Figure 3-7: Effects on proliferation of GBC cells after knockdown and overexpression of SMARCA5. (A) (B)分别用 qRT-PCR 和 WB 检测敲减和过表达 SMARCA5 的效果；(C) (D)为用 CCK8 试验在 NOZ 和 EHGB-1 中检测 SMARCA5 对增殖功能的影响。

而根据有基质胶和无基质胶的 Transwell 小室试验结果，我们能够发现，敲减了 SMARCA5 之后，胆囊癌细胞的体外侵袭和转移能力明显下降，而过表达 SMARCA5 则有着相反的效果(图 3-8)。以上试验结果与 miR-99a-5p 对胆囊癌细胞增殖及侵袭转移能力的影响恰好相反。这符合了 SMARCA5 是 miR-99a-5p 的一个下游靶基因的假设。

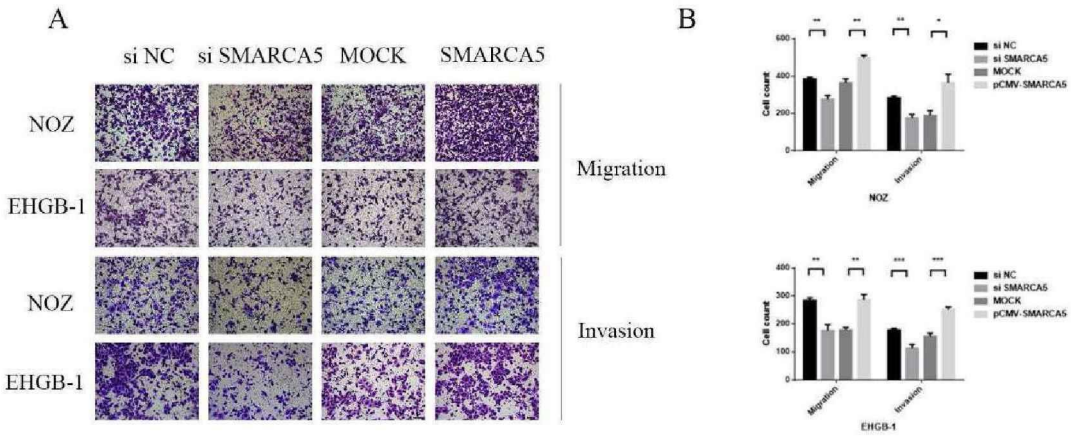


图 3-8：敲减和过表达 SMARCA5 后检测胆囊癌细胞系侵袭转移能力。

Figure 3-8: Evaluation of the invasion and metastasis ability of GBC cell lines after knockdown and overexpression of SMARCA5.

*: $P<0.05$，**: $P<0.01$，***: P<0.001.

3.7 miR-99a-5p 通过直接调控 SMARCA5 影响胆囊癌细胞的侵袭转移能力

考虑到 SMARCA5 的蛋白表达水平可以由 miR-99a-5p 调控，结合上节结果，我们不禁产生疑问： miR-99a-5p 是否能够通过与 SMARCA5 相互作用来实现抑制胆囊癌细胞侵袭和转移的过程？于是我们通过在过表达 SMARCA5 和空质粒组的胆囊癌细胞中分别转染 miR-99a-5p 的模拟物，然后评估胆囊癌细胞侵袭和转移的功能。图 3-9 显示，在 NOZ 细胞系中，过表达 SMARCA5 可以使 miR-99a-5p 所抑制的肿瘤细胞侵袭和转移的功能得到部分恢复。上述结果共同说明了 miR-99a-5p 能够通过靶定 SMARCA5 实现抑制胆囊癌细胞侵袭和转移的功能。

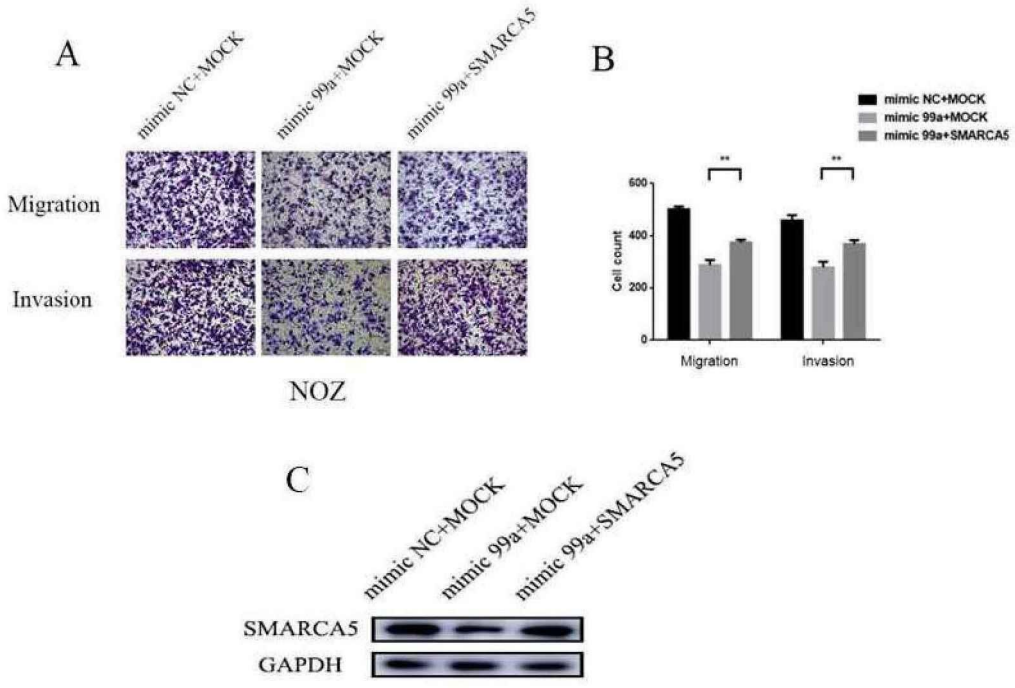


图 3-9：SMARCA5 回复 miR-99a-5p 造成的细胞侵袭转移能力下降。

Figure 3-9: Overexpression of SMARCA5 rescued the miR-99a-5p-induced inhibition of migration and invasion ability.

**: P<0.05.

4. 讨论

学者们通过研究发现，miRNA 的生成需要经过一系列复杂的过程[42]。首先，在细胞核内会转录生成原始 pri-miRNA，经过一次加工后形成前体 miRNA(pre-miRNA)。Pre-miRNA 在 Dicer 酶的酶切作用下形成双链 miRNA，解旋后根据在 pre-miRNA 的位置分别形成 5p 和 3p 单体。miRNA 单体可以参与形成 RNA 诱导沉默复合物(RNA-induced silencing complex, RISC)，通过 RISC 对下游靶基因的 mRNA 产生降解或影响翻译的作用。

本部分研究采用生物信息学的方法对 miR-99a-5p 进行靶基因预测。结合四个数据库的结果可以发现，SMARCA5 是出现频率最高且评分排名均较为靠前的候选靶基因。另外，MTOR 也是各个数据库中均有出现的基因。许多学者曾报道过恶性肿瘤中 MTOR 与 miR-99a-5p 之间的相互关系[43-44]，故我们也将其定为一个候选的靶基因。通过双荧光素酶报告基因试验和蛋白免疫印迹试验，我们可以明确 MTOR 在胆囊癌中也是 miR-99a-5p 的一个靶基因，但根据实时荧光定量 PCR 试验的结果，miR-99a-5p 不能使 MTOR 的 mRNA 水平下调。该结果说明 miR-99a-5p 对 MTOR 的作用为抑制其翻译过程，而并非直接降解其 mRNA。PI3K/AKT 信号通路在恶性肿瘤中研究广泛，被认为与细胞转化、增殖、转移、凋亡、周期阻滞、耐药等功能相关，也成为了目前许多肿瘤综合治疗的研究靶点[45-47]。其在胆囊癌中的研究也较为广泛，目前普遍认为该通路在胆囊癌中起着促进增殖转移、细胞周期阻滞等功能[48-50]。

SMARCA5 是 SWI/SNF 蛋白家族的一个成员，作为一种染色质重塑因子，它能够通过调控组蛋白去乙酰化来参与细胞信号转导、DNA 损伤修复等功能。目前对 SMARCA5 的研究主要集中在染色质重塑、DNA 的损伤修复及细胞自我更新的领域[51-54]。Jin 等[55]报道了 SMARCA5 在乳腺癌中能促进肿瘤的增殖转移功能，但相关机制仍然有待挖掘。本研究是首次在胆囊癌中发现 SMARCA5 具有促进侵袭转移能力的功能，SMARCA5 高表达与胆囊癌患者的淋巴结转移相关，能减少患者生存期。说明 SMARCA5 有望成为未来胆囊癌诊断治疗中一个全新的分子靶标。但其详细作用机制仍不明确，我们需要更深层次地对其上下游互作蛋白、相关信号通路等方面进行研究。

5. 小结

1. 通过生物信息学预测，我们筛选出了 SMARCA5 与 MTOR 作为 miR-99a-5p 的候选靶基因，同时通过双荧光素酶报告基因和蛋白免疫印迹实验验证了 SMARCA5 和 MTOR 确实是 miR-99a-5p 的直接靶基因。

2. miR-99a-5p 可以通过直接抑制 PI3K/AKT 通路发挥其抑癌功能。

3. SMARCA5 与胆囊癌患者的淋巴结转移密切相关，其高表达预示着胆囊癌患者的不良预后。

4. SMARCA5 能促进胆囊癌细胞的侵袭转移功能，miR-99a-5p 可以直接与 SMARCA5 作用，通过抑制其蛋白翻译过程来降低表达，从而起到抑制胆囊癌侵袭转移的功能。

全文小结及展望

本研究通过高通量 miRNA 芯片检测胆囊癌组织以及其对应的癌旁组织中差异表达的 miRNAs，结合 TCGA 数据库分析，从中筛选出了在胆囊癌组织中显著低表达的 miR-99a-5p 作为研究对象。接着我们通过 qRT-PCR 的方法检测了 41 例胆囊癌组织以及其癌旁组织中 miR-99a-5p 的表达量，进一步验证了 miR-99a-5p 在胆囊癌组织中显著低表达，同时通过临床病理资料与生存分析发现 miR-99a-5p 的低表达预示着胆囊癌患者的不良预后。根据体外和体内的功能学实验验证，miR-99a-5p 能抑制胆囊癌细胞侵袭转移的能力，并可能通过 EMT 过程介导。为了探究 miR-99a-5p 功能的作用机制，我们用生物信息学网站预测与双荧光素酶报告基因的方法发现了 SMARCA5 和 MTOR 是 miR-99a-5p 的直接靶基因。miR-99a-5p 能够通过与 3'UTR 区结合，抑制二者的蛋白翻译过程。miR-99a-5p 还可直接影响 PI3K/AKT 通路发挥作用，同时也能通过靶向结合 SMARCA5 来抑制胆囊癌的侵袭转移功能。

综上所述，miR-99a-5p 的表达水平的检测对胆囊癌患者的预后评估有着重要的意义，miR-99a-5p 也有成为胆囊癌诊断与治疗潜在靶点的可能性。我们需要更加深入地对其进行研究，深刻阐明其行使抑癌功能的作用机理，从而能将理论研究转化为临床应用，为提高胆囊癌诊断与治疗水平做出贡献。

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综述

精准医学在胆囊癌领域的研究进展

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【摘要】胆囊癌是恶性程度极高、预后差的恶性肿瘤，早期诊断困难，手术根治率低。精准医学是以个体化治疗为基础而发展起来的新型医学理念与医疗模式。生物医学及生物信息学研究的兴起使精准地对肿瘤患者进行诊断以及个体化治疗成为可能。本文对精准医学理念在胆囊癌领域的基础研究、靶向治疗及根治性手术中的应用进行了综述。新型分子靶点的发现以及新技术的应用能够推动胆囊癌精准诊疗的发展，改善患者的预后。

【关键词】胆囊癌；精准医学；个体化治疗；靶向治疗；根治性手术

胆囊癌(GBC)是一种发病率相对较低、恶性程度极高的肿瘤，其发病率在消化道肿瘤中排在第六位，占胆道系统肿瘤的 2/3[1]。如能早期发现，行根治性手术可以达到比较满意的 5 年生存率。但胆囊癌起病隐匿、早期无特异性症状，一经发现，许多患者已经进展至中晚期，此时手术治疗、放化疗均难以达到理想的治疗效果。

精准医学(precision medicine)计划是美国总统 Barack Obama 于 2015 年初在国情咨文中首次提出的。它是建立在基因组测序的基础上，根据个体化治疗的理念，依托大数据科学及生物信息学等工具而得到发展的新型医学治疗模式。精准医学概念一经提出就在国际舆论引起了广泛关注，学者们也希望通过这样的医学模式，能够提高胆囊癌的治疗效果，因此我们对精准医学理念在胆囊癌基础研究、靶向治疗以及根治性手术中的应用进行简要综述。

1 精准医学理念指导下的胆囊癌基础研究

精准医学的本质是通过蛋白组学、基因组学等技术和基因测序等前沿技术，对于大样本人群中特定类型的疾病采取特异性生物标志物的鉴定、分析和应用，从而准确找到内在病因，为诊断和治疗提供靶向目标，最终实现对特定患者的个体化治疗[2]。随着人类肿瘤基因组计划的不断进展，许多癌症相关基因的突变和异常调控机制已被证实，这是精准医学在癌症研究领域得以实施的有力保障。

1.1 基因组学及信号转导

具体到胆囊癌，不同的基因型及其突变会对胆囊癌的肿瘤特性、发生发展产生不同的影响，且关于一些信号通路的研究对未来可能的分子靶向治疗有着重要意义。Li 等[1]运用全外显子测序和靶基因的深度测序技术，在国际上首次系统描述了胆囊癌的基因突变图谱，发现了肿瘤抑制蛋白 p53(TP53)、鼠类肉瘤病毒癌基因(KRAS)、erb-b2 受体酪氨酸激酶 3(ERBB3)等基因在胆囊癌患者中存在着高频突变，另外也明确了驱动胆囊癌发生发展的关键基因以及信号通路，证实了 ERBB 信号通路上的突变在胆囊癌患者中最为显著，与胆囊癌患者的不良预后存在显著的相关性。Deshpande 等[3]对 77 例胆道恶性肿瘤患者的手术标本进行基于高通量质谱法的基因突变分析，发现了磷脂酰肌醇 3 激酶催化亚基 α (PIK3CA)的激活突变与胆囊癌患者的不良预后密切相关，PI3K/AKT 通路的异常调控可以诱导正常胆囊上皮细胞发生癌变，磷脂酰肌醇 3 激酶(PI3K)的抑制剂作为潜在的靶向治疗药物，可能对胆囊癌的个体化治疗存在一定价值[4]。此外，也有 Hedgehog 信号通路调控胆囊癌增殖转移的报道。研究表明 Hedgehog 信号通路的活性在胆囊癌细胞中明显上调，使胆囊癌出现高度恶性的表型，可能是一个胆囊癌治疗的潜在靶点[5]。

1.2 非编码 RNA

近年来关于非编码核糖核酸(RNA)在肿瘤领域的研究不断深入，为研究者寻找胆囊癌新的诊疗靶点提供了突破口。非编码 RNA 是一类不编码蛋白质的 RNA，包括小核仁 RNA (snoRNAs)、微小 RNA (miRNAs)、长链非编码 RNA (lncRNAs)等[6]。其中 miRNA 在肿瘤中的研究已较为广泛，在胆囊癌中也发现了较多具有生物学功能的 miRNA。miR-27a、miR-570 以及 miR-181a 在胆囊癌中的表达能够影响化疗药物的疗效，从而给胆囊癌患者带来不良预后[7]。另外一些研究发现，许多 miRNA 在组织样本中的表达量与胆囊癌患者的预后密切相关，通过改变它们在胆囊癌细胞中的表达量，可以使其表型及功能发生改变，从而证明这些 miRNA 与肿瘤发生的关系。Chang 等[8]的研究指出，miR-20a 和转化生长因子-β1（TGF-β1）高表达的胆囊癌患者的生存率较低，外源性表达 miR-20a 在体外试

验中能促进胆囊癌细胞的上皮-间质转化(EMT)，而在体内能够促进胆囊癌细胞的转移。还有学者提出，miR-146b-5p 在胆囊癌组织样本中含量较低的观点，在胆囊癌细胞系 SGC996 中过表达 miR-146b-5p 能够通过促进凋亡和 G1 期阻滞来抑制胆囊癌细胞的生长，同时它也能够调节表皮生长因子受体(EGFR)的表达[9]。在消化道肿瘤中，lncRNA 能够介导信号转导、调控细胞周期、影响肿瘤的转移能力、干扰细胞的凋亡进程[10,11]。lncRNA 在胆囊癌中的研究较少，但随着人们对其认识逐渐深入，lncRNA 越来越受到肿瘤研究人员的关注。Wu 等[12]发现肺腺癌转移相关转录子 1(MALAT1)作为促癌的 lncRNA 在胆囊癌患者肿瘤组织中普遍高表达，在胆囊癌细胞系 GBC 中用干扰慢病毒敲减 MALAT1 能够通过失活 ERK/MAPK 信号通路，显著抑制 GBC 细胞的增殖和转移能力。另有研究表明鸟类髓细胞病毒癌基因同源物(c-Myc)可与其特异性的应答元件相结合，激活下游的 lncRNA 分子同源盒基因转录反义 RNA(HOTAIR)，继而增强胆囊癌细胞的恶性程度[13]。同时，miRNA-130a 能够通过负性调控作用减弱 HOTAIR 的功能。

1.3 蛋白组学

利用蛋白组学技术，尤其是比较蛋白组学分析蛋白质的差异表达也是目前研究肿瘤发展机制的一种方法之一[14,15]。Sahasrabuddhe 等[16]利用同位素标记相对和绝对定量（iTRAQ）技术结合质谱分析了 2575 个蛋白，找出了一系列在胆囊癌组织中表达上调与下调的蛋白，分别包括鞘脂激活蛋白原(prosaposin)和转凝蛋白(transgelin)等，是国际上首次报道的大规模胆囊癌蛋白组学的研究，为今后寻找胆囊癌新的分子靶标提供了丰富资料。Huang 等[17]采用双向凝胶电泳结合质谱方法对原发性胆囊癌、胆囊炎及正常胆囊组织进行对比，并用免疫组化证实了膜联蛋白 A4(ANXA4)、热休克蛋白 90β(HSP90β)和胞质动力蛋白 1 重链 1(Dync1h1)三种差异表达蛋白，可能成为潜在的胆囊癌肿瘤标志物。

2 精准医学时代的胆囊癌靶向治疗研究

以特异性生物标志物为基础的肿瘤靶向药物在临床上得到了广泛应用，取得了一定疗效，如曲妥珠单抗针对人类表皮生长因子受体 2 (HER2)阳性乳腺癌患者的治疗、维罗非尼应用于 B 型 Raf 激酶(BRAF)基因突变的转移性黑色素瘤患

者等[18]。这是精准医学在临床上成功的典范。对于进展期胆囊癌，目前临床上的一线治疗药物仍然是吉西他滨联合顺铂，有时辅以卡培他滨、5-氟尿嘧啶等[19]。由于胆囊癌恶性程度高，具有很强的增殖、侵袭和转移能力，传统的化疗以及放疗对胆囊癌的治疗效果十分有限，而靶向治疗为胆囊癌的精准治疗提供了新思路。以下介绍几类靶向抑制剂的代表药物在胆囊癌治疗中的研究进展。

2.1 EGFR 抑制剂

厄洛替尼是一种酪氨酸激酶抑制剂，它能可逆性地与 EGFR 的三磷酸腺苷(ATP)结合域进行结合，从而使其无法激活下游效应通路。此前，厄洛替尼被视为一种有一定疗效的单药治疗胆囊癌，不过之后的研究发现，与其它药物联用时，其效果不甚理想[20]。在所有报道中，只有 Lee 等[21]的III期临床试验提出，吉西他滨联合奥沙利铂加用厄洛替尼能够短暂延长胆管癌患者的无进展生存期。除此之外，其他研究均显示加用厄洛替尼不能延长已接受其它化疗药物的包括胆囊癌在内的胆道恶性肿瘤患者的生存期[22]。尽管如此，厄洛替尼作为单药的有效性以及良好的耐受性，使它成为胆囊癌靶向治疗的潜在的理想药物。目前有许多正在进行的关于厄洛替尼的研究，其中有将其与化疗药物联用，也有研究将其作为其它靶向药物的辅助用药[23]。

2.2 VEGF 抑制剂

贝伐单抗是一种人造的单克隆抗体，它能与血管内皮生长因子(VEGF) 特异性结合，令后者无法与其受体结合，使下游通路失活。Riley 等[24]报道称在一名 76 岁的广泛转移的男性胆囊癌患者身上，贝伐单抗与帕尼单抗联用获得了较好的疗效。Zhu 等[25]的 II 期临床试验发现，吉西他滨联合奥沙利铂加用贝伐单抗能在一定程度上延长进展期胆囊癌患者的生存期，中位无进展生存期和中位生存期分别为 6.1 月和 8.5 月。大部分患者能够良好地耐受贝伐单抗，但仍有一部分患者出现中性粒细胞减少、神经病变、高血压等不良事件，也有心肌缺血和胆囊穿孔等个别不良反应的报道[26]。另外，该研究缺少吉西他滨联合奥沙利铂的对照组，难以说明贝伐单抗真正带来的获益。我们需要更多的研究来证明贝伐单抗的作用效果，以及对其副作用的评价。

2.3 HER2 抑制剂

HER2 抑制剂在乳腺癌和胃癌的临床治疗上被证明有显著效果，但在胆囊癌的研究中并不能令人满意。拉帕替尼作为一种酪氨酸激酶抑制剂，能够同时抑制 EGFR 和 HER2 的活性。虽然有临床前试验发现拉帕替尼能够增强吉西他滨的疗效，但并没有相关的临床试验报道[27]。Sorscher[28]曾报道过一例肝转移及腹腔转移的胆囊癌患者，其活检结果显示 HER2 强阳性，便对其采用了紫杉醇联合曲妥珠单抗的治疗方案。结果这名患者在影像学和糖类抗原 19-9(CA19-9)水平均取得了显著缓解。目前已有学者开展曲妥珠单抗治疗胆囊癌的Ⅱ期临床研究，其作用效果仍有待观察。

此外，如吉非替尼、克唑替尼、帕尼单抗、西妥昔单抗等药物已有胆囊癌相关Ⅱ期临床试验正在进行中。与在其它疾病中类似，药物毒性和药物抵抗也是靶向药物应用与胆囊癌临床治疗的巨大挑战。我们亟待更有效，特异性更强，但毒性更低的新型靶向药物，也需要进一步研究来解决药物抵抗问题，使对靶向治疗药物反应较好的患者能够长期获益。另外，一些免疫疗法，如程序性死亡受体 1(PD-1)抗体等或许是解决药物抵抗的良好途径[29]。

3 精准医学时代的胆囊癌手术治疗

在现阶段，根治性切除仍然是唯一能够使胆囊癌患者获得长期生存的治疗方法，其目标是在患者能够耐受的情况下获得 R0 切除。但盲目扩大切除范围会导致创伤增大，提高术后并发症的发生率，并不能给患者带来生存获益。其次，遵循整块切除原则对于胆囊癌的精准根治也是十分必要的，包括胆囊连同肝脏及其它受累器官的整块切除和肝十二指肠韧带的整块清扫。所以合理地实施精准的根治性手术就显得尤为重要。

3.1 术前评估

在肝脏外科领域，随着影像学技术的不断发展，精准肝切除的理念已被临床医师所广泛接受。精准手术切除不仅讲究外科技术的精细和准确，而且强调以疾病相关的病理生理状态为依据进行术前评估和围术期处理[30]。而对于胆囊癌患

者，需要基于其病史、辅助检查以及肝脏和其他重要脏器功能进行分期和综合评估，以初步决定手术方案。根据肿瘤不同的 T 分期决定肝切除范围，如需联合大范围肝切除者，术前应量化肝功能储备及残肝体积，从而确定安全肝切除量。吲哚氰绿(ICG)排泄试验是定量评估肝功能储备较为准确的方法，在肝切除手术中已得到广泛应用[31]。利用该系统可帮助指导胆囊癌根治术中大范围肝切除的范围，以保证剩余肝功能可以代偿。而肝体积测量可以通过对肝脏电子计算机断层扫描(CT)或核磁共振成像(MRI)扫描的断层图像进行手工测算或三维重建软件来实现[32]。

3.2 术中再次分期评估

对于进展期的胆囊癌根治术，淋巴结清扫范围一直以来存在着争议。D2 切除仍是胆囊癌根治术最为推荐的术式，盲目地扩大淋巴结清扫范围并不能有效提高淋巴结清扫率，对患者预后无益[33]。目前最主流的观点认为，13a 组淋巴结为胆囊癌第一站与第二站淋巴结的分界，其阳性提示癌细胞已转移至第二站淋巴结[34,35]。术中应常规对 13a 组淋巴结行快速冰冻切片活检，若结果为阳性则应行扩大淋巴结清扫。但冰冻切片病理诊断准确率受术者及病理科医师等因素的影响较大，存在一定的假阳性及假阴性可能。如果术中能够对淋巴结转移情况进行准确定位，将会为术者的决策提供极大的便利。ICG 近红外荧光成像对腹腔镜胆囊切除术(LC)术中提示血管及胆管的位置十分便利，且在胃癌、乳腺癌、妇科肿瘤手术中已有关于前哨淋巴结显像的应用，将来有望应用于胆囊癌根治术[36,37]。另外，在精准根治手术中，为了保证 R0 切除，对胆囊癌标本进行三维切缘冰冻病理检查是十分必要的，这能够改善患者预后。只要相关技术得到合理开发和应用，胆囊癌也将更加贴近精准切除的理念。

4 展望

精准医学理念迎合了 21 世纪生物信息学和大数据挖掘和开发的潮流，具有不可忽视的发展潜力。整合不同组学研究成果，推动多中心合作交流，收集珍贵的临床病例资料，并建立标准化模型，根据患者的肿瘤遗传学特征实现个体化治疗，对于达到精准医学的目标具有重要意义。我们期待在精准医学理念的指导下，

未来能够出现更多帮助明确诊断胆囊癌的方法，通过多样化、有效的综合治疗手段，显著改善胆囊癌患者的临床预后。

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攻读学位期间发表的论文

- 张亦弛，王许安，龚伟 等. 精准医学在胆囊癌领域的研究进展.中华实验外科杂志. (已录用).
- 张亦弛，王许安，刘颖斌. 放射治疗在胆囊癌综合治疗中的研究进展. 肝胆胰外科杂志.(已录用)
- 张亦弛，王许安，刘颖斌. 快速康复理念在外科围手术期的应用. 上海医药.(已录用).
- 徐驿，张亦弛，王许安 等. miR-490-5p 抑制人肝内胆管细胞癌 RBE 侵袭和迁移的研究.肿瘤.(已录用)
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英语全文翻译

Introduction

Gallbladder cancer (GBC) is a digestive tumor with relatively low incidence and high malignancy, which is the most common malignant tumor in biliary tract system, also with the sixth incidence in the gastrointestinal tumors. GBC often occurs in the Far East and Chile. Its mortality rate ranks the tenth in the malignant tumors worldwide, which has a serious impact on human health. In China, the incidence of GBC is 3.9 cases per 100,000 people [1]. If with early detection, radical surgery can achieve a satisfactory 5-year survival rate. But the GBC is more concealed, and due to the lack of specific early symptoms, many patients have progressed to the advanced phase once diagnosed, who lost the chance of radical resection. Moreover, the current radiotherapy, chemotherapy and other methods cannot achieve satisfactory treatment effect, so the overall 5-year survival rate is only less then 5% [2]. Therefore, early diagnosis is the key to improving the prognosis of GBC. There are several studies finding that more than 90% of deaths are caused by recurrence and metastasis in GBC [3]. So the study on the pathogenesis of GBC and the mechanism of recurrence can help to find new GBC tumor markers and targeted therapies, which is of great significance for improving the diagnosis, the treatment effect and the prognosis of GBC patients.

With the rapid development of molecular biologic technology, the human genome project has been completed. Scientists have found that the number of non-coding RNA is far more than that of RNA encoding, of which microRNA is the most widely studied. MicroRNA (miRNAs) is a kind of small molecular non-coding RNA with a length of about 18 to 25 bases. It can be combined with the 3'UTR of the target genes to participate in the regulation of the target genes. By direct degradation of the target gene mRNA or inhibition of the protein translation, the expression of the target genes is regulated at the post-transcriptional level, which plays an important role in the functions of proliferation, invasion, adhesion, metastasis and apoptosis by influencing downstream molecules and signaling pathways [4,5]. Nowadays, more and more studies have pointed out that the abnormality of miRNAs expression in tissues is closely related to the development of tumors. For example, some researchers have found that miR-26a is highly expressed in lymph node metastases of malignant tumors and activates the PI3K / AKT pathway by targeting PTEN [6].

What's more, MiR-199a-3p has been shown to regulate inflammation and ERK-MAPK pathway by directing on multiple genes, such as MET, IKKβ and ERK-2, which are closely associated with ischemia-reperfusion injury [7].

In recent years, with the deep study of the tumor nature, the important role of miRNA in the regulation of tumor development has been confirmed. Although various studies have shown that many miRNAs are upregulated or downregulated in different tumors, the specific regulatory mechanisms are not fully understood. Moreover, the etiology of GBC has not yet been cleared. The prognosis of GBC patients has often associated with tumor site, preoperative jaundice, tumor cell differentiation, lymph node metastasis, etc. [8]. There are several studies based on microarrays and bioinformatics that have found miRNAs are closely related to the development of GBC and have initially elucidated a number of related mechanisms. For example, Chang et al [9] found several valuable miRNAs by high-throughput miRNA screening, and their expression was closely related to the staging and prognosis of GBC, in which miR-20a was highly expressed. What's more, the expression of Smad7 protein was significantly decreased in the GBC cell lines transfected with miR-20a, and the proliferation and invasion of GBC cell lines were inhibited, which was identified by Western blot. Therefore, miR-20a might be a new tumor marker and therapeutic target of GBC. In addition, miR-20a was stimulated by TGF-β, which promoted epithelial-mesenchymal transitions (EMT) and further affected the invasion and metastasis of GBC.

miR-99a-5p is located at position 21 of chromosome q21. It has been proved that it is closely related to the development of many tumors, such as hepatocellular carcinoma, prostate cancer, head and neck squamous cell carcinoma, bladder cancer and ovarian cancer [10-14]. And researchers have confirmed that it is involved in the proliferation, invasion, metastasis, differentiation and apoptosis of tumor cells. Its low expression in the tumors is considered to be poor prognostic factor. However, there is no relevant research of miR-99a-5p in GBC. Therefore, the role of miR-99a-5p in GBC and the exploration of related mechanisms are in urgent need.

PART 1: Screening and validation of differential miRNAs in gallbladder cancer tissue samples

1.Introduction

GBC, which is the most common malignant tumor in biliary tract, is a highly malignant digestive tract tumor, of which the incidence rate is about 3.9 cases per 100,000 people. It ranks No. 6 in the digestive tract tumors [1]. As the onset of GBC is hidden and due to the lack of specific early symptoms, many patients have progress to the advanced phase and lost the chance of radical surgery once diagnosed. The current radiotherapy, chemotherapy and other methods cannot achieve satisfactory treatment effect, so the overall 5-year survival rate is only less than 5% [2]. Therefore, early diagnosis is the key to improve the prognosis of GBC. And the study finds that more than 90% of GBC deaths are caused by tumor recurrence and metastasis [3]. Therefore, the study on the pathogenesis of GBC and the mechanism of recurrent invasion and metastasis can help to find new targets of GBC tumor markers and targeted therapy, which is of great significance for improving the diagnosis, treatment effect and the prognosis of GBC patients.

MicroRNA (miRNAs) is a kind of small molecular non-coding RNA with a length of about 18 to 25 bases. It can be involved in the regulation of target gene expression by binding to the target genes. By direct degradation of the mRNA or inhibition of the protein translation of target genes, the expression of the target genes is regulated at the post-transcriptional level, which plays an important role in the functions of proliferation, invasion, adhesion, metastasis and apoptosis by influencing downstream molecules and signaling pathways [4,5]. Nowadays, more and more studies have pointed out that the abnormality of miRNAs expression in tissues is closely related to the development of tumors. Therefore, the study of differential expression of miRNAs in malignancies, which can reveal the occurrence and development of tumors and provide the better diagnosis and treatment of malignant tumors, is of great significance.

In order to find and screen out important differentially expressed miRNAs in GBC patients, our group has previously adopted a high-throughput miRNA microarray to investigate and detect the expression of miRNAs in 4 cases of GBC tissues and their corresponding peritumoral tissues [15].

In addition, we combined the analysis of the Cancer Genome Atlas (TCGA) database and the qRT-PCR method for large-scale validation, and finally specifically targeted miR-99a-5p in GBC study. At the same time, the relationship between the expression level of miR-99a-5p and the clinicopathological characteristics and prognosis of GBC patients was analyzed.

2 Materials and Methods

2.1 Materials

2.1.1 Patients and specimens

All of the GBC specimens were collected from 41 cases who underwent radical surgery in department of general surgery of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine from 2011 to 2014 treatment of due to GBC not receiving neoadjuvant therapy. All specimens were diagnosed by histopathological examination. The corresponding peritumoral normal tissues of these patients was collected at the same time. The specimens were frozen in liquid nitrogen after immediate release for the extraction of total RNA. After that, qRT-PCR was performed after reverse transcription. Peritumoral tissues referred to the normal gallbladder mucosa tissue at least 2 cm from the edge of GBC tissues, in which tumor cells could not found microscopically. Informed consent of all patients was obtained and the study was approved by the Ethics Committee of Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine.

2.1.2 qRT-PCR reagents

Product	Manufacturer
Trizol reagent	Invitrogen Corporation
PrimeScript® RT reagent Kit with gDNA Eraser	Takara Company
Mir-X™ miRNA First-Strand Synthesis Kit	Takara Company
SYBR *Premix Ex Taq* (Tli RNaseH Plus)	Takara Company
SYBR Premix Ex Taq II (Tli RNaseH Plus)	Takara Company
isopropanol	Sinopharm Group Chemical Reagent Co., Ltd.
chloroform	Shanghai Runjie Chemical Reagent Co., Ltd.

2.1.3 Main instruments and reagents

Product	Manufacturer
PCR instrument	LifePro Co., Ltd
Step One Plus Real Time PCR instrument	Applied Biosystems company
MDF-382E(CN) ultra-low temperature refrigerator	Sanyo company
High speed low temperature centrifuge 5424R	Eppendorf company
Clean bench	Shanghai Experimental Equipment Co., Ltd
NanoDrop2000 Ultra-micro spectrophotometer	Thermo Fisher Scientific
200μL/1.5ml Eppendorf tubes	Axygen brand product

2.2 Methods

2.2.1 Total RNA extraction from GBC tissues and their corresponding peritumoral tissues

The frozen GBC tissues and their corresponding peritumoral tissues were cut into knobs and placed into 1.5ml EP tubes. 1 mL of Trizol reagent was added, and then the steel beads were added, homogenized at 4℃. The obtained mixture was centrifuged at 12000 rpm for 5 min at 4℃ then absorbing the supernatant for extraction of total RNA.

Protocol for total RNA extraction:

1. Add 200 μL of chloroform to the treated sample, vortex the EP tube for 15 s, and place the EP tube on ice for 5 min.

2. Centrifuge at 12000 rpm for 15 min.

3. Absorb the appropriate amount of the supernatant into the new clean 1.5ml EP tube, add an equal volume of isopropyl and mix, then put the tube at 4℃ for 10min.

4. Centrifuge at 12000 rpm for 10 min.

5. Gently wash the white RNA pellet after discarding the supernatant, then add 1 mL of 75% ethanol.

6. Centrifuge at 7500 rpm for 5 min.

7. The supernatant was discarded and dried in air for 15 min. RNA was precipitated with 20 μL of DEPC water.

8. A260 / A280 and RNA concentration were measured using NanoDrop2000 spectrophotometer. A260 / A280 should be between 1.9 and 2.1.

9. Total RNA was reverse transcribed according to the instructions of the mRNA reverse transcription kit. Reaction conditions:

Step 1: 42℃ 3 min (erase gDNA)

Step 2: 37°C 15 min

85°C 5 s

10. MiRNA was reverse transcribed according to the instructions of the miRNA reverse transcription kit. Reaction conditions:

Step 1: 37°C 60 min

Step 2: 85°C 5 min

11. The remaining RNA is frozen at -80 ° C standby.

2.2.2 Verifying the miRNA microarray result by qRT-PCR

Primer of miR-99a-5p:

Forward primer: 5'-AACCCGTAGATCCGATCTTGTG-3'

The primer was synthesized by Tiangen biotech (Beijing) Co., Ltd. The primers of U6 were provided from Mir-X™ miRNA First-Strand Synthesis Kit.

Preparing the PCR reaction solution as follows:

Reagent	Volume	Final Concentration
SYBR Premix Ex Taq II(2×)	10μl	1x
Forward Primer (10μM)	0.8μl	0.2μM
Uni-miR Reverse Primer (10μM)	0.8μl	0.2μM
ROX Reference Dye (50×)	0.4μl	1x
Template（cDNA solution）	2μl	*2
ddH_2O	6μl	
Total	20μl	*4

Reaction conditions:

95°C 30s

95°C 5s
60°C 34s
} 40 cycles

The relative expression levels of miR-99a-5p in GBC tissues and their corresponding peritumoral tissues were determined by $2^{-\Delta Ct}$ method. Ct value is the number of amplification cycles experienced when the fluorescence signal intensity of the PCR amplification product reaches the threshold value.

ΔCt = mean Ct of miR-99a-5p in the tissue - mean Ct of U6 in the tissue. The relative expression levels of miR-99a-5p in tissues of GBC patients were determined by the $2^{-\Delta\Delta Ct}$ method. Formula: ΔΔCt = ΔCt in GBC tissues – ΔCt in peritumoral tissues.

2.3 Statistical analysis

Data analysis was performed by SPSS 21.0 software. The comparison of expression levels of miR-99a-5p in GBC and its corresponding peritumoral tissues was operated by paired student t test. And the relationship between the miR-99a-5p expression and clinicopathological characteristics was analyzed by Pearson $\chi 2$ test. Survival analysis was performed using the Kaplan-Meier method, and the survival curve was examined by Log-rank method. Univariate and multivariate analysis were performed by Cox regression. The measurement datum are expressed in the form of (mean ± standard deviation). $P < 0.05$ was considered statistically significant.

3.Results

3.1 Screening of differentially miRNA in GBC tissues

In order to find and screen out the important differentially expressed miRNAs in GBC, our group used Aglient human miRNA microarray (v19.0) to detect four cases of GBC patients, including GBC tissues and their corresponding peritumoral tissues [15]. The microarray contained a total of 2588 homo sapiens mature miRNA probes which had been identified. U6 was used as internal reference miRNA during detection. Results showed that there were 2 miRNAs that were significantly overexpressed and 11 miRNAs with significant low expression in the GBC tissues (fold change value> 2, P value<0.05) (Figure 1-1) .

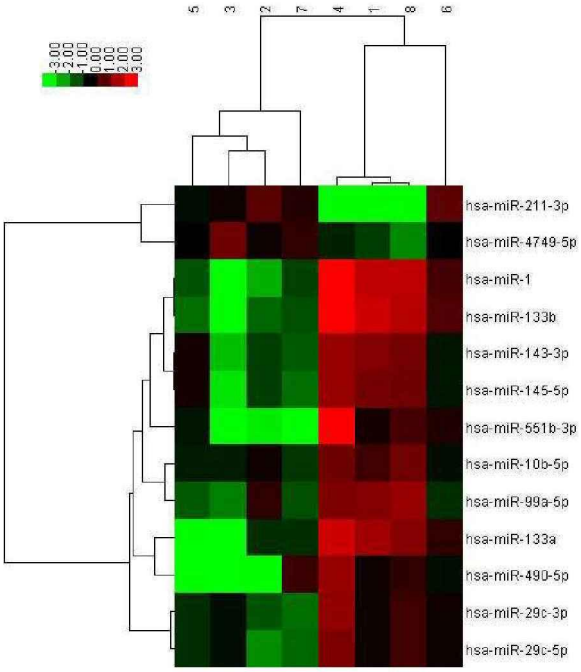

Figure 1-1：Heat map diagram of differentially expressed miRNAs in GBC tissues by clustering analysis [15]. The right side of the map is the name of the selected miRNAs. The numbers of GBC sample are listed on the top. The left four samples are for the GBC tissues, the right four samples are for the normal peritumoral tissue. The red region represents a relatively high expression, and the blue region represents a relatively low expression.

The results suggested that there were plenty of differentially expressed miRNAs valuable for research in GBC, which may also prove that the abnormalities of miRNA expression were probably important for the GBC development. According to the screening functional test we performed previously, miR-99a-5p was initially targeted as the main research object among the 13 differentially expressed miRNAs.

3.2 miR-99a-5p expression analysis in TCGA database

In order to further verify the rationality of screening results, we searched the TCGA database to determine whether miR-99a-5p was valuable for research in GBC. As GBC-related case data were not included in the TCGA database, we chose hepatocellular carcinoma and intrahepatic cholangiocarcinoma as the analytical objects, the source of which were similar to GBC. Figure 1-2 showed that miR-99a-5p was significantly overexpressed in the peritumoral tissues of hepatocellular carcinoma and intrahepatic cholangiocarcinoma, and generally low in the tumor tissues. The results of TCGA database indirectly demonstrated that miR-99a-5p may have potential research value in GBC.

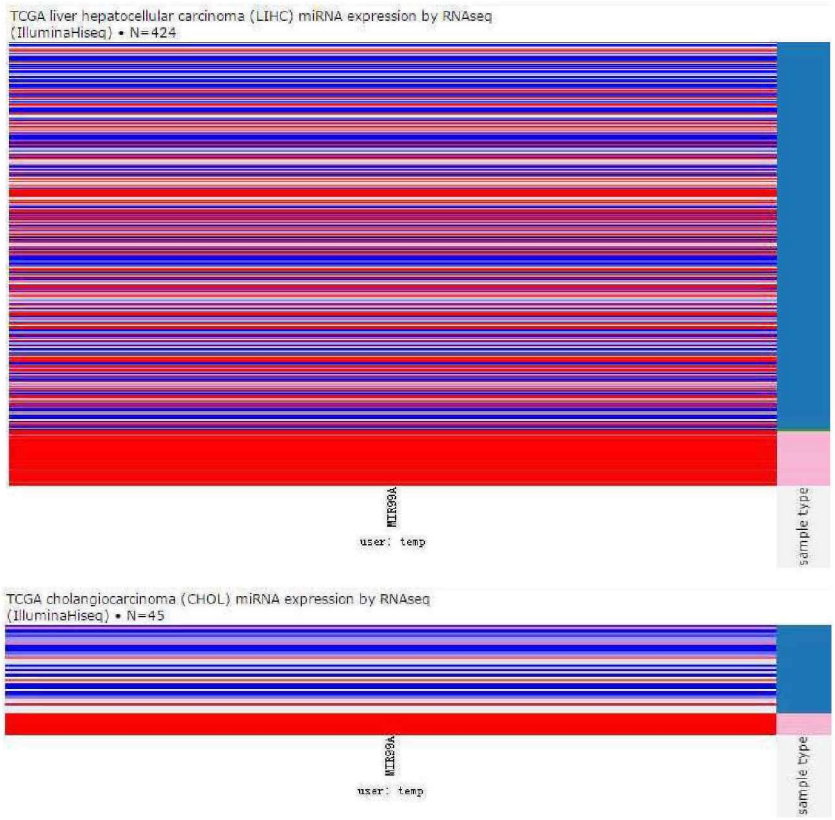

Figure 1-2：Comparison of miR-99a-5p relative expression in hepatocellular carcinoma (upper) and intrahepatic cholangiocarcinoma (lower) tumor tissues and peritumoral tissues in TCGA database. (Left column represents miR-99a-5p expression level. High expression marks as red and low expression marks as blue. Right column represents sample type. Tumor tissues mark as blue and peritumoral tissues mark as pink.)

3.3 miR-99a-5p expression in GBC tissues

To verify the results of the above data, we chose to detect the miR-99a-5p expression of 41 cases in GBC tissues and their corresponding peritumoral tissues by qRT-PCR. Figure 1-3 showed that miR-99a-5p expression in GBC tissues was significantly lower than that in peritumoral tissues, and the difference was statistically significant. The average expression of miR-99a-5p in peritumoral tissues was 2.79 times higher than that in tumor tissues

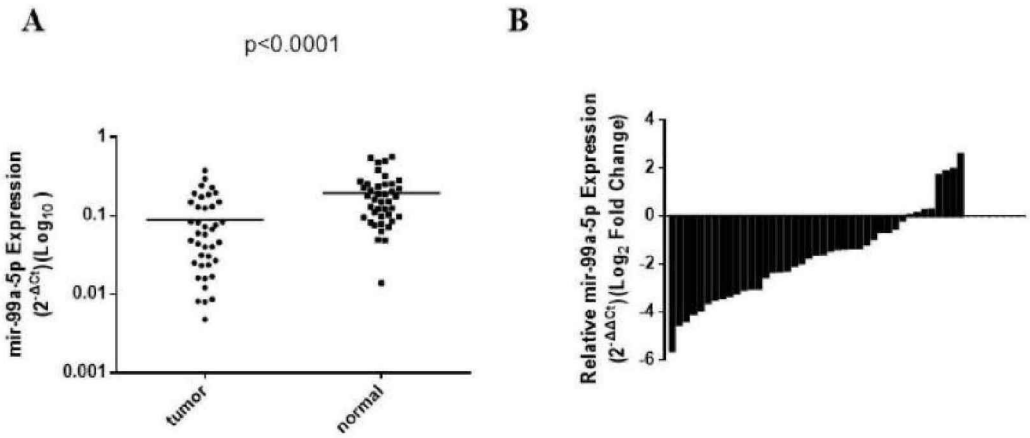

Figure 1-3: Evaluatioin of miR-99a-5p relative expression levels. (A) miR-99a-5p in 41 cases of GBC and their corresponding peritumoral tissues. The expression level of miR-99a-5p in peritumoral tissues was 2.79 times higher than that in tumor tissues (P<0.0001). (B) The Comparison of expression levels of miR-99a-5p in tumor tissues and peritumoral tissues.

3.4 Correlation analysis of miR-99a-5p expression with clinicopathological characteristics and prognosis in patients with GBC

In order to study the potential relationship between the expression of miR-99a-5p and clinicopathological characteristics, we divided 41 GBC cases into two groups by miR-99a-5p expression in the tumor tissues of these patients. The results showed that the expression of miR-99a-5p was only correlated with lymph node metastasis (P = 0.009), not with other characteristics (Table 1-1).

Table 1-1: Association between miR-99a-5p expression and the clinicopathological characteristics in GBC

Variable/Category	n	Relative miR-99a-5p expression		χ2	*P*
		Low (n=27)	High (n=14)		
Gender					
Male	13	8 (29.6%)	5(35.7%)	0.158	0.691
Female	28	19 (70.4%)	9(64.3%)		
Age(year)					
<60	10	6 (22.2%)	4 (28.6%)	0.158	0.691
≥60	31	21 (77.8%)	10(71.4%)		
Tumor size(cm)					
<3	15	9(33.3%)	6 (42.9%)	0.360	0.548
≥3	26	18(66.7%)	8 (57.1%)		

Histology differentiation					
Well/moderate	32	21(77.8%)	11(78.6%)	0.003	0.954
poor	9	6 (22.2%)	3(21.4%)		
Tumor invasion(AJCC)					
Tis-T_2	16	9 (33.3%)	7 (50.0%)	1.076	0.300
T_3-T_4	25	18 (66.7%)	7(50.0%)		
Lymph node metastasis					
Yes	14	13 (48.1%)	1(7.1%)	6.894	0.009
No	27	14 (51.9%)	13 (92.9%)		
TNM stage(AJCC)					
I-II	15	8 (29.6%)	7 (50.0%)	1.649	0.199
III-IV	26	19 (70.4%)	7 (50.0%)		

Then we found that there was a significant difference in the survival time between the low expression group and the high expression group by survival analysis (median survival was 10.5 months and 20.0 months, P = 0.014, respectively). The 1-year survival rates were 48.4% and 76.0%, respectively (Figure 1-4).

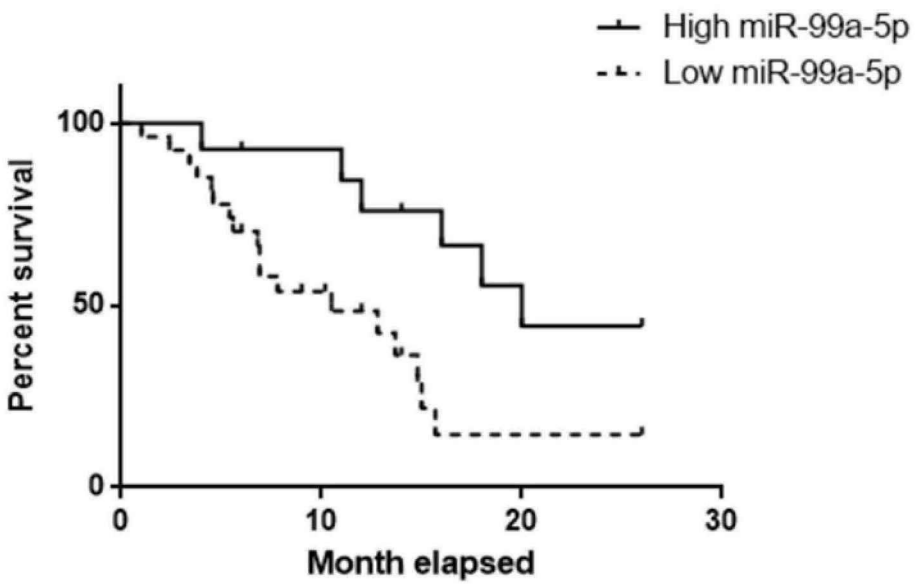

Figure 1-4: The relationship between the expression of miR-99a-5p and the survival time of GBC patients

Furthermore, univariable and multivariable Cox regression analysis confirmed miR-99a-5p expression, including lymph node metastasis, to be independent indicators of overall survival (Table 1-2).

Table 1-2 Univariate and multivariate analysis between clinicopathologic characteristics and prognosis in

GBC patients

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*p*	HR (95%CI)	*p*
Age (＜60 vs. ⩾60)	0.680 (0.311-1.591)	0.365	-	-
Sex (Male vs.Female)	0.383 (0.124-1.049)	0.069	-	-
Tumor size (cm) (⩾3 vs.＜3)	0.506 (0.164-1.522)	0.233	-	-
Histology differentiation (Well or moderate vs.Poor)	2.314 (0.850-6.262)	0.085	-	-
Tumor invasion(AJCC) (Tis-T2 vs.T3-T4)	0.698 (0.366-1.316)	0.506	-	-
Lymph node metastasis (Yes vs.No)	**3.278 (1.345-7.969)**	**0.006**	**1.310 (0.418-5.384)**	**0.022**
TNM stage(AJCC) (I-II vs.III-IV)	0.421 (0.087-1.911)	0.245	-	-
Type of surgery (Curative resection vs.Palliative)	1.663 (0.619-4.498)	0.357	-	-
miR-99a-5p expression in tumor (Low vs.High)	**2.469 (1.027-6.167)**	**0.040**	**1.952 (0.165-5.103)**	**0.014**

CI, Confidence intercal; HR, Hazard ratio.

4. Discussion

With the deepening of research on malignant tumors, non-coding RNA in cancers has gradually become a hot spot to study for researchers. Many studies have found that non-coding RNA plays a crucial role in the development of malignant tumors [16-20], of which miRNA is an important part. There are many evidence that abnormal expression of miRNAs is common in a large number of tumors, and the number of research on its function and mechanism of regulating malignant tumor is also increasing [21-23]. In this part, we studied the miRNAs which were differentially expressed in the tissues of GBC by high-throughput miRNA microarray. 11 low-expression miRNAs and 2 highly expressed miRNAs in GBC tumor tissues were obtained. For these miRNAs, our group has preliminarily screened according to the cellular functional test in GBC cell lines, and miR-99a-5p was found to have anti-cancer function in GBC cell lines. Then, we found data of hepatocellular carcinoma and intrahepatic cholangiocarcinoma which were histoembryologically similar to GBC in TCGA database. It was found that miR-99a-5p was significantly lower in tumor tissues of hepatobiliary tumors. Therefore, we selected miR-99a-5p as our research object.

The miR-99 family is one of the oldest miRNA families in the evolutionary process, including miR-99a, miR-99b and miR-100 [24]. MiR-99a, located at chromosome 21, has been shown to be inhibited in a variety of tumor tissues and can affect tumor-related functions [25-27]. Lee et al. [28] retrospectively analyzed the relationship between miRNA and the survival of 221 cases of pancreatic

cancer patients. They found that miR-99a-5p was closely related with tumor margin, tumor staging, pathological differentiation, postoperative CA199 level. And its expression level was significantly reduced in pancreatic cancer tissues, which was an independent factor affecting the prognosis of patients. Yu et al. [29] found that miR-99a-5p was significantly associated with advanced stage and tumor metastasis in non-small cell lung cancer, suggesting a poor prognosis, and subsequent functional studies found that overexpression of miR-99a-5p was able to inhibit cell proliferation, invasion and metastasis function in vitro in non-small cell lung cancer through AKT1. Feng et al. [30] detected the expression of miR-99a-5p in 100 cases of bladder cancer and peritumoral tissues by stem-loop Taqman probe qRT-PCR. Moreover, they also detected the expression level of miR-99a-5p in serum of bladder cancer patients and normal population, showed that miR-99a-5p was usually low expression in tumor tissues and serum in bladder cancer patients, and its low expression was related to invasive phenotype. What's more, overexpression of miR-99a-5p in bladder cancer cells could inhibit its proliferation.

In this part, we compared the expression of miR-99a-5p in 41 cases of GBC and the corresponding peritumoral tissues. The results showed that miR-99a-5p was significantly lower in GBC tissues, which was consistent with the relevant studies. It is concluded that miR-99a-5p may play an anti-cancer effect in GBC, which needed further functional test in vitro to confirm. On the basis of this, we analyzed the relationship between the expression of miR-99a-5p and the clinicopathological data of GBC patients. The results showed that the low expression of miR-99a-5p was significantly correlated with lymph node metastasis, which indicated that miR-99a-5p might affect the invasion and metastasis of GBC. We found that many studies had confirmed miR-99a-5p could inhibit the invasion and metastasis of tumors, and the a few studies also supported that miR-99a-5p could inhibit the proliferation of malignant tumors. Therefore, we will focus on whether miR-99a-5p can affect proliferation and invasion/metastasis in GBC in the next step of our study. At the same time, we can see that the low expression of miR-99a-5p is closely related to the poor prognosis through survival analysis. So miR-99a-5p may be a potential diagnostic and therapeutic target in GBC.

5. Conclusions

1. miR-99a-5p was found to be low expressed in GBC tumor tissues and may be associated with

lymph node metastasis of GBC by miRNA microarray analysis combined with qRT-PCR.

2. The low expression of miR-99a-5p had a significant correlation with the poor prognosis of GBC.

PART 2: Functional study of miR-99a-5p in GBC

1. Introduction

GBC, which is the most common malignant tumor in biliary tract, is a highly malignant digestive tract tumor, of which the incidence rate is about 3.9 cases per 100,000 people. It ranks No. 6 in the digestive tract tumors [1]. As the onset of GBC is hidden and due to the lack of specific early symptoms, many patients have progress to the advanced phase and lost the chance of radical surgery once diagnosed. The current radiotherapy, chemotherapy and other methods cannot achieve satisfactory treatment effect, so the overall 5-year survival rate is only less than 5% [2]. Therefore, early diagnosis is the key to improve the prognosis of GBC. And the study finds that more than 90% of GBC deaths are caused by tumor recurrence and metastasis [3]. Therefore, the study on the pathogenesis of GBC and the mechanism of recurrent invasion and metastasis can help to find new targets of GBC tumor markers and targeted therapy, which is of great significance for improving the diagnosis, treatment effect and the prognosis of GBC patients.

At present, many studies have pointed out that tumorigenesis and tumor progression are affected by abnormal expression of miRNAs in malignant tumors, and miRNAs can affect multiple tumor pathways, which influences tumor progression, invasion and metastasis, apoptosis and cycle arrest. In tumor tissues low expressed miRNAs is usually manifested as the anti-cancer function by negatively regulating some targeted oncogenes to achieve inhibition of tumor proliferation, invasion and metastasis and other functions, such as miR-204, miR-140-5p, etc. [31-32]. While the other miRNAs can show cancer-promoting function which are overexpression in tumor tissues. For example, Liu et al. [33] pointed out that miR-19b can promote the development of breast cancer by inhibiting the tumor suppressor gene PTPRG.

In the last part of the study, miR-99a-5p was screened out as a low expressed miRNA in GBC tissues and verified by qRT-PCR. It was initially suggested that miR-99a-5p might be a suppressor gene in GBC. Since the relationship between miR-99a-5p and GBC has not been reported, we will focus on the effect of miR-99a-5p on GBC cellular function and further confirm whether miR-99a-5p plays a role in suppression of GBC progression.

2. Materials and Methods

2.1 Materials

2.1.1 Cell lines

GBC cell lines GBC-SD, NOZ, EHGB-1 and OCUG were purchased from Institute of Cell Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.2.2 Laboratory animals

4-6 weeks old SPF grade nude mice for in vivo assays were purchased from the Shanghai Animal Experimental Center, Chinese Academy of Sciences. Nude mice were fed in the SPF environment according to the guidance of the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

2.1.3 Main reagents

Reagents for cell culture

Product	Manufacturer
DMEM medium	Gibco company
William's medium	Gibco company
FBS	Gibco company
Trypsin-EDTA	Gibco company
Penicillin-streptomycin	Gibco company

Reagents for cell proliferation and metastasis experiment

Product	Manufacturer
CCK8 reagent	Shanghai Yeason Biological Technology Co., Ltd
Transwell chamber	Corning Corporation
Transwell chamber with matrigel	Corning Corporation
4% paraformaldehyde	Sinopharm Group Chemical Reagent Co., Ltd.
Crystal violet staining solution	Beyotime Biotechnology

Reagents for transfection and qRT-PCR

Product	Manufacturer
Trizol reagent	Invitrogen Corporation
PrimeScript® RT reagent Kit with gDNA Eraser	Takara Company
Mir-X™ miRNA First-Strand Synthesis Kit	Takara Company
SYBR *Premix Ex Taq* (Tli RNaseH Plus)	Takara Company

SYBR Premix Ex Taq II (Tli RNaseH Plus)	Takara Company
isopropanol	Sinopharm Group Chemical Reagent Co., Ltd.
chloroform	Shanghai Runjie Chemical Reagent Co., Ltd.
RFect small RNA Transfection Reagent	Changzhou Biogen Biological Technology Co., Ltd

Reagents for western blot

Product	Manusfacturer
Ammonium persulfate	Beyotime Biotechnology
RIPA lysis buffer	Beyotime Biotechnology
Acrylamide	Beyotime Biotechnology
TEMED	Beyotime Biotechnology
6×loading buffer	Beyotime Biotechnology
Trisbase	Shanghai Yeason Biological Technology Co., Ltd
Glycine	Shanghai Yeason Biological Technology Co., Ltd
SDS	Shanghai Yeason Biological Technology Co., Ltd
Skim milk powder	Shanghai Yeason Biological Technology Co., Ltd
20×TBS	Shanghai Yeason Biological Technology Co., Ltd
Tween-20	Shanghai Yeason Biological Technology Co., Ltd
PVDF membrane	Bio-rad company
E-cadherin, N-cadherin Vimentin monoclonal antibody	CST company
Rabbit anti-human GAPDH antibody	Shanghai Abways Biological Technology Co., Ltd
Rabbit anti-human MTOR antibody	CST company
Rabbit anti-human SMARCA5 antibody	Abcam company
Goat anti-rabbit/mouse HRP-secondary antibody	Beyotime Biotechnology
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific
ECL kit	Thermo Fisher Scientific
BCA Protein Assay Kit	Beyotime Biotechnology

2.1.4 Instruments and equipment

Product	Manusfacturer
1300-A2 Biological safety cabinets	Thermo Fisher Scientific
501A Digital constant temperature water bath	Shanghai Shen'an medical equipment factory
LDZX-40 Vertical automatic electric pressure steam sterilization pot	Shanghai Pudong Rongfeng Science Instrument Co., Ltd
High speed low temperature centrifuge 5417R	Eppendorf company
DMI3000B Inverted fluorescence microscope	Leica company
Temperature control shaker	Shanghai Precision & Scientific Instruments Co.,Ltd
High speed centrifuge	Hitachi company
DK-420BS Electric thermostat water bath	Shanghai Hede Experimental Equipment Co., Ltd
FA2004A Electronic analytical balance	Shanghai Precision & Scientific Instruments Co.,Ltd
90-1A Magnetic stirrer	Shanghai pigeon Electronic Technology Co., Ltd
SAGA-10T Ultra-pure water system	Merck Millipore company
YX280A Portable stainless steel steam sterilizer	Shanghai Sanshen Medical Devices Co., Ltd
-20℃/4°C refrigerator	Haier company
YDS-10 Storage liquid nitrogen biological containers	Chengdu Jinfeng Liquid Nitrogen Container Co., Ltd
D1008E Palm centrifuge	Scilogex company
MDF-382E(CN) ultra-low temperature refrigerator	Sanyo company
2.5μl, 20μl, 200μl, 1 ml Pipettes	Eppendorf company
AF100 Ice maker	Scotsman company
550 Microplate reader	Bio-Rad company
Blood cell count plate	Shanghai Pharmaceutical Industry Research Institute
0.22μm micro pores nylon filter	Shanghai Pharmaceutical Industry Research Institute
HERAcell 150i CO_2 cell incubator	Thermo Fisher Scientific
96, 24, 6 wells cell culture plate	Nest company
Step One Plus Real Time PCR System	Applied Biosystems company

Steady flow regulator electrophoresis	Shanghai Precision & Science Instrument Co., Ltd
SDS-PAGE Biological protein electrophoresis	Bio-rad company
Mini Trans-Blot Transfer slot	Bio-rad company
Transfer machine	Bio-rad company
Amersham Imager600	Bio--rad company

2.2 Methods

2.2.1 Cell culture

Human GBC cell lines NOZ and EHGB-1 were purchased from cell bank of type culture collection of Chinese Academy of Sciences. NOZ cells were cultured in william's medium containing 10% fetal bovine serum (FBS). EHGB-1 cells were cultured in DMEM medium containing 15% FBS. GBC-SD and OCUG cells were cultured in DMEM medium containing 10% FBS. All of the culture medium contain 100U/ml of penicillin and streptomycin. Usually the cells were incubated in a 100 mm cell culture dish in 5% CO_2 at 37℃and harvested with a solution of Trypsin-EDTA during the logarithmic growth phase.

2.2.2 Overexpression and knockout reagents' synthesis and construction

MiR-99a-5p mimics and inhibitors were synthesized by Guangzhou Ruibo Biotechnology Co., Ltd. MiR-99a-5p overexpression lentivirus PGMLV-CMV-MCS-EF1-ZsGreen1-T2A-Puro-miR99a was constructed and concentrated by Shanghai Genechem Co., Ltd.

2.2.3 Detection of intracellular miRNA expression by qRT-PCR

The methods of total RNA Extraction, reverse transcription system and reaction conditions are according to Section 2.2.1. Part 1. qRT-PCR system and reaction conditions are according to Section 2.2.2. Part 1. The primer sequence of MiR-99a-5p is shown in Section 2.2.2. Part 1.

2.2.4 Cell counting

After the clean coverslip was placed in the center of the blood cell count plate and the cell suspension was thoroughly mixed, 10μL of the cell suspension was aspirated with a 10μL pipette, and the cell suspension was slowly added along one side of the coverslip, covering the gap between the coverslip and the blood cell count plate. No bubbles were generated and the cell suspension cannot overflow from the coverslips. The count plate was stewed for 1min until the cells settle on the counting plate. Then we observed cells on the counting plate under a microscope and counted the total number of

cells in the four corners. For the cells press on the lines, only the cells pressed on the left and upper lines were counted, and the cell mass was counted as a single cell. The density of the cell suspension was calculated according to the following formula: cell density = (the number of cells in 4 large squares / 4) × 10^4 cells / ml.

2.2.5 Examination of cell proliferation in vitro by CCK8 assay

The main component of CCK8 is a water-soluble tetrazolium salt (2- (2-methoxy-4-nitrophenyl) -3- (4-nitrophenyl) -5- (2,4-disulfobenzene)-2H-tetrazole monosodium salt), which is a MTT-like compound. In the presence of an electron-coupling reagent, it can be reduced by some of the dehydrogenase in the mitochondria to produce orange-yellow water-soluble product formazan. The reagent is reduced by the biodegradation of the dehydrogenase in the cell, and the formazan can be directly dissolved in the medium. The more the number of living cells, the deeper the color of the product. For the same cell, the absorbance of the product solution is linearly related to the total number of cells. Compared with the MTT method, the CCK8 assay has the advantages of higher sensitivity, wider linear range and less cytotoxicity.

Protocol:

1. Cells were grouped according to transfection or infection, and 5 sub wells were set for each group of cells. The cells were labeled with d0 ~ d5 on the 96-well cell culture plates. Every group of cells were plated with 30 wells. The examining times were 6h, 24h, 48h, 72h, 96h and 120h, respectively.
2. The cells in the logarithmic growth phase were digested, centrifuged and resuspended to form a cell suspension before counting.
3. Cells were seeded in 96-well plates at a density of 1000 cells/well and at a volume of 100μL per well and 100μL of PBS was placed on each well surrounding and incubated in a 5% CO2 incubator at 37℃.
4. The CCK8 reagent was diluted with complete medium at a ratio of 10: 1.
5. The corresponding 96-well plates were removed at each test point. Then 100μL of the diluted CCK8 reagent was added to each well and incubated at 37 ° C for 1-4 h.
6. The OD value at 450 nm was measured by a microplate reader, and the cell growth was calculated and plotted.

2.2.6 Colony formation assay

Colony formation assay was commonly used to examine the ability of single cells to form colonies.

Single cells in vitro growth in the environment for more than 6 generations of cells, composing a group, called a colony. Each colony had more than 50 cells, which could be observed after staining. There were plate and soft agar colony formation assays. As the GBC cells used in this experiment were adherent growth cells, we took the plate assay for our experiment. Colony formation ability was an important indicator of population dependency and proliferation of cells. The key points of this assay were the density of cell suspension and the uniformity of cell clonal distribution. If the cell inoculation density was too large, adjacent clones would be easy to confluence. Conversely, if the density is too small, it was difficult to observe cell colonies. The process was to incubate cells at a certain density in the culture plate, regular checking the cell status, and changing the medium if necessary. When a single cell colony grew up to more than 50 cells, the culture should be terminated. After staining, the number of clones could be visually observed and the growth of individual colonies was observed under microscope.

Protocol:

1. The corresponding experimental and control cells are washed once with PBS and harvested with trypsin-EDTA for 3 min. Then resuspend the cells with FBS-containing medium. Centrifugation is performed at 1500 rpm for 3 min. The cells are then resuspended in complete medium and counted with a cell count plate.

2. 1.5 ml/well of the corresponding complete medium is added to the six well plates and the cell suspension volume is calculated according to the cell count results. And cells are seeded in a six well plate at a density of 1000 cells/well. Resuspend the cells with the pipette gently, so that single cells scatter evenly. The plates are placed in a 5% CO2 cell incubator and incubate at 37° C.

3. Regularly observe the status of inoculated cells, and change the medium if necessary. When the number of cells in a single colony in the control group is more than 50, the medium should be aspirated. Then the cells are washed with PBS solution for once. After that, the cells are fixed in 4% paraformaldehyde for 15 min, and stained with 0.1% crystal violet solution for 10 minutes. The number of colonies is counted and the cells in a single clone were observed with an inverted microscope. Record the total number of colonies with more than 50 cells.

2.2.7 Wound healing assay

The basic principle of wound healing assay is to create scratches in monolayer cells to produce a blank area for cell healing, and then observe the process of cells moving from the surrounding to

the middle. The narrower scratches are formed in the same time, the higher migration ability the cells possess.

Protocol:

1. The cells are harvested and counted, then inoculate into 6-well plates at a density of 300,000 cells per well, incubating overnight.

2. Draw the horizontal lines behind the 6-well plate using a marker pen with the help of a ruler. Each line is separated by 1cm across the well. Each well has at least three horizontal lines.

3. Scratch in the cell growth plane with sterilized 200μL pipette tip as far perpendicular to the horizontal line as possible. The tip should be kept as far vertical as possible to the bottom of the board, so that each scratch will be into a straight line.

4. After scratching, wash the cells 3 times with PBS to remove the exfoliated cells.

5. 1.5ml of serum-free DMEM medium is added to each well and the plate is placed in a 5% CO2 incubator at 37°C.

6. Observe, take pictures and measure the width of scratches under a microscopy after 0,24 and 48 hours of scratching, respectively.

2.2.8 Transwell cell migration/invasion assay

Transwell chamber is a container with a layer of semipermeable membrane. There are two kinds of transwell chamber with or without Matrigel, respectively. The medium containing FBS or chemokine is added in the lower chamber. After that, the cells are diluted with serum-free medium and then added to the upper chamber. After incubation for a certain period of time, the cells penetrate into the lower chamber through the semipermeable membrane. By staining with crystal violet, the number of cells passing through the semipermeable membrane can be observed under the microscope to determine the invasion or migration ability of the cells.

Protocol:

1. Before the experiment, the transwell chamber with Matrigel need to be placed in 24-well cell culture plate with 500μL of serum-free medium in the upper and lower chambers, respectively. Then incubate the plate in a 37°C incubator for 2 hours. This step is omitted when performing cell migration assay.

2. The cells are harvested with trypsin, washed twice with PBS and resuspended in the corresponding serum-free medium. Then count the cells.

3. Put 20,000 cells into the upper chamber of each chamber and adjust the cell suspension volume to 200μL with serum-free medium. 500μl of the corresponding complete medium containing 20% FBS is added to the lower chamber of each chamber. Place the plate in 5% CO2 incubator and incubate it at 37°C for 24 hours.

4. After 24 hours, discard the liquid in upper chamber and wash the membrane with PBS once. Add 700μl 4% paraformaldehyde in lower chamber and the cells are fixed for 20min. Then the lower chamber is stained with 0.1% crystal violet solution for 10min. After staining, the lower chamber is gently washed three times with PBS and gently wiped with a cotton swab. Five different fields were randomly observed under a 400-fold microscope to count cells. After the end of the assay, the film is gently removed and immersed in a 33% acetic acid solution. The OD value of the eluate at 570 nm is measured by a microplate reader. The number of reaction cells is quantitatively determined.

2.2.9 Subcutaneous xenograft model of nude mice

To explore the effects of miR-99a-5p on tumor growth in vivo, stable expression miR-99a-5p cells were subcutaneously injected into the left axilla of the mice (five mice/group). Tumor growth was monitored every week and measured in two dimensions. The tumor volume was calculated using the following formula: tumor volume = (length × width^2)/2, where the width and length were the shortest and longest diameters, respectively. After 4 weeks, the mice were sacrificed and the tumors were dissected out, weighed, and for IHC.

2.2.10 Splenic vein-liver metastasis tumor model of nude mice

BAL B/C nude mice were randomly divided into control group and miR-99a-5p mimic group. The mice in the treated group received a splenic vein injection of pretreated NOZ cells, whereas the mice in the control group received a splenic vein injection of NOZ cells without any treatments. On day 45, the mice were sacrificed and individual organs were removed, at which time metastatic tissue was pictured.

2.2.11 Western blot assay

The corresponding cells were washed with PBS and scraped with a cell scraper, then collected and resuspended with RIPA lysis buffer. After that, the lysate was put on ice for 20 minutes. The lysate was centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was aspirated and the protein concentration was measured using the BCA Protein Quantification Kit. The protein suspension was mixed with the loading buffer and heated at 100°C for 5 minutes to denature the protein and then

stored at -20°C. For western blot analysis, bromophenol blue (0.01%) was added to the samples and equal amounts of proteins were loaded in each lane for SDS-PAGE and blotting onto PVDF membranes. The membrane was blocked in blocking buffer (5% non-fat dry milk) for 1 h at room temperature, and then incubated with appropriate primary antibodies in blocking buffer overnight at 4°C. The blot was then incubated with the appropriate secondary antibody and then detected in ECL buffer at room temperature for several seconds. Photographs were taken, and the optical densities of the bands were scanned and quantified with the Amersham Imager600. GAPDH was used as a loading control.

2.2.12 Statistical analysis

Each experimental value was expressed as the mean ± standard deviation (SD). All of the statistical analyses were performed using SPSS21.0 software. The differences between groups were considered significant when the p value was less than 0.05. All data points represented the mean of triplicate data points.

3. Results

3.1 Effects of miR-99a-5p on proliferation of GBC Cell lines

First, we detected the expression level of miR-99a-5p in four GBC cell lines by qRT-PCR. The result showed that the expression levels of miR-99a-5p in NOZ and EHGB-1 cell lines were relatively low, and the expression levels of miR-99a-5p were higher in GBC-SD and OCUG cell lines. Therefore, we used NOZ and EHGB-1 cell lines for overexpression treatment, and GBC-SD cell line for inhibitor treatment.

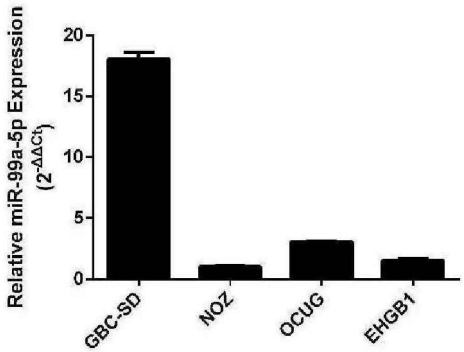

Figure 2-1: Relative expression levels of miR-99a-5p in 4 GBC cell lines. The expression level of miR-99a-5p in NOZ cell line was set as 1.

In order to verify the overexpression and inhibition effect of miR-99a-5p expression, we used qRT-PCR to determine the expression levels of miR-99a-5p after NOZ and EHGB-1 cells were infected with miR-99a-5p overexpression lentivirus. The expression level of miR-99a-5p in the cell line can be found to be significantly up-regulated in Fig 2-2. Similarly, miR-99a-5p Inhibitor, an inhibitor of chemical synthesis, was able to significantly down-regulate the expression of miR-99a-5p in GBC-SD cell line.

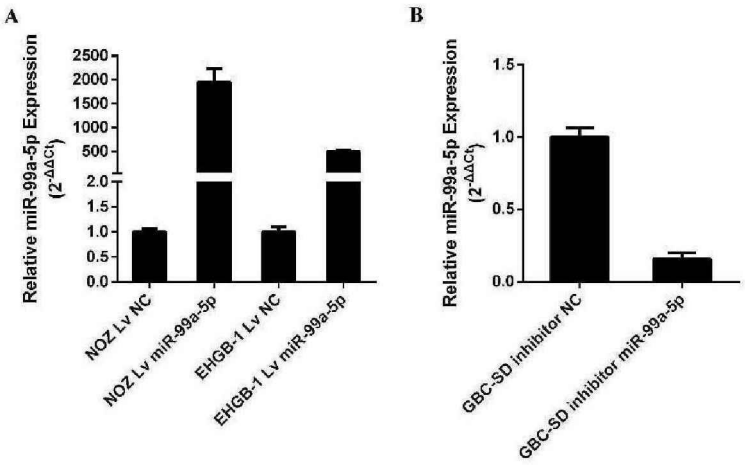

Figure 2-2: Relative expression levels of miR-99a-5p after treatment in GBC cell lines. miR-99a-5p relative expression levels after (A) NOZ and EHGB-1 infected by miRNA overexpression lentivirus and (B) GBC-SD transfected by miRNA inhibitor.

Then we carried out the CCK8 assay to evaluate the effect of miR-99a-5p on proliferation in GBC cell lines in vitro. We found that the overexpression of miR-99a-5p had a very weak inhibitory effect on NOZ and EHGB-1 cell lines, although P value < 0.001, whereas inhibition of miR-99a-5p expression had no effect on GBC cell proliferation (Figure 2-3).

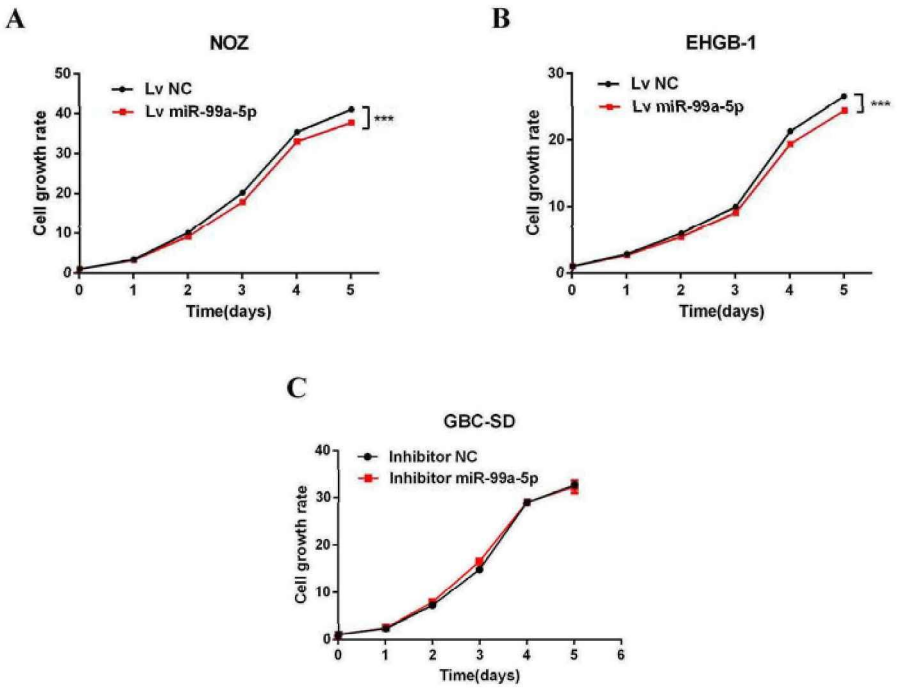

Figure 2-3: Effect of MiR-99a-5p on proliferation of GBC cell lines.

（A:NOZ cell line; B:EHGB-1 cell line; C:GBC-SD cell line. 0 point of abscissa is on behalf of 6h.Set the 6h OD450 value as 1 for vertical axis。***: P<0.001）

The colony formation assay was able to detect the ability of a single cell to form a colony, and the GBC cells that had been treated with lentivirus and inhibitor were seeded in a six-well plate with 1000 cells per well and the number of cells in the control group was greater than 50. The number of clones formed was observed by the naked eye, and then the number of cells in a single clone was observed under the microscope. The results were shown in Figure 2-4, overexpression and inhibition of expression levels of miR-99a-5p could not change the colony formation ability of GBC cell lines.

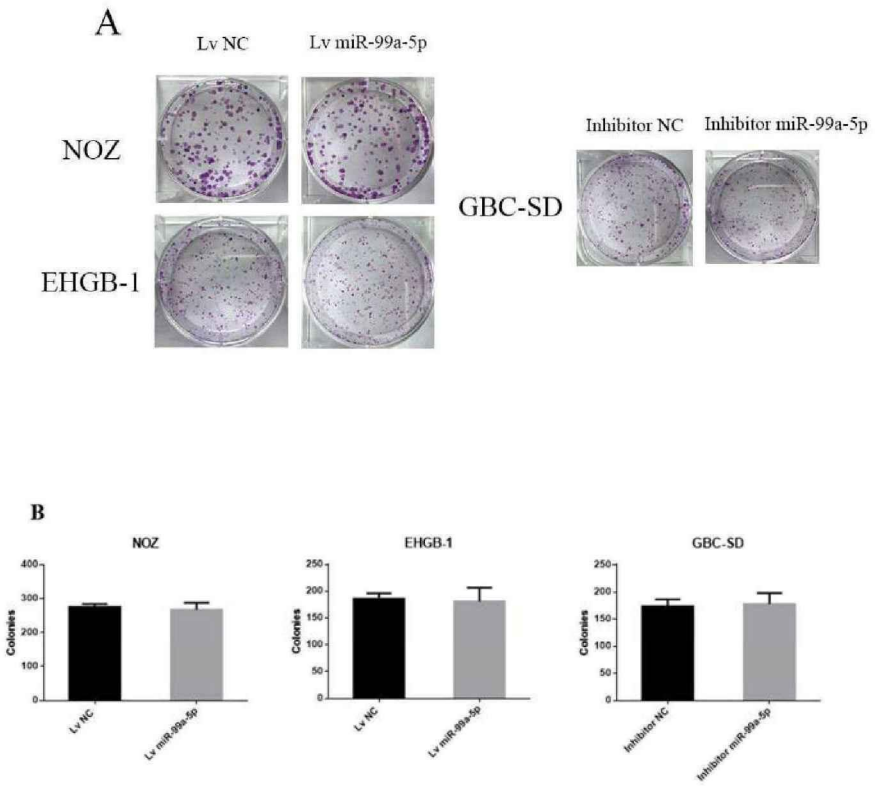

Figure 2-4: The effect of miR-99a-5p on the proliferation of GBC single cells in vitro. (A) colonies of GBC cells treated with inhibitor and lentivirus; (B) GBC cell lines colonies bars. No statistical significance exists.

In order to further clarify whether miR-99a-5p had an effect on the proliferation of GBC cells, we infected NOZ cells with lentivirus to overexpress miR-99a-5p, and then injected the cell suspension into the auxiliary of nude mice. After 4 weeks, the nude mice were sacrificed. As shown in Figure 2-5, overexpression of miR-99a-5p only slightly inhibited subcutaneous tumor growth capacity in vivo. The above experiments showed that miR-99a-5p could not significantly affect the proliferation of GBC cells.

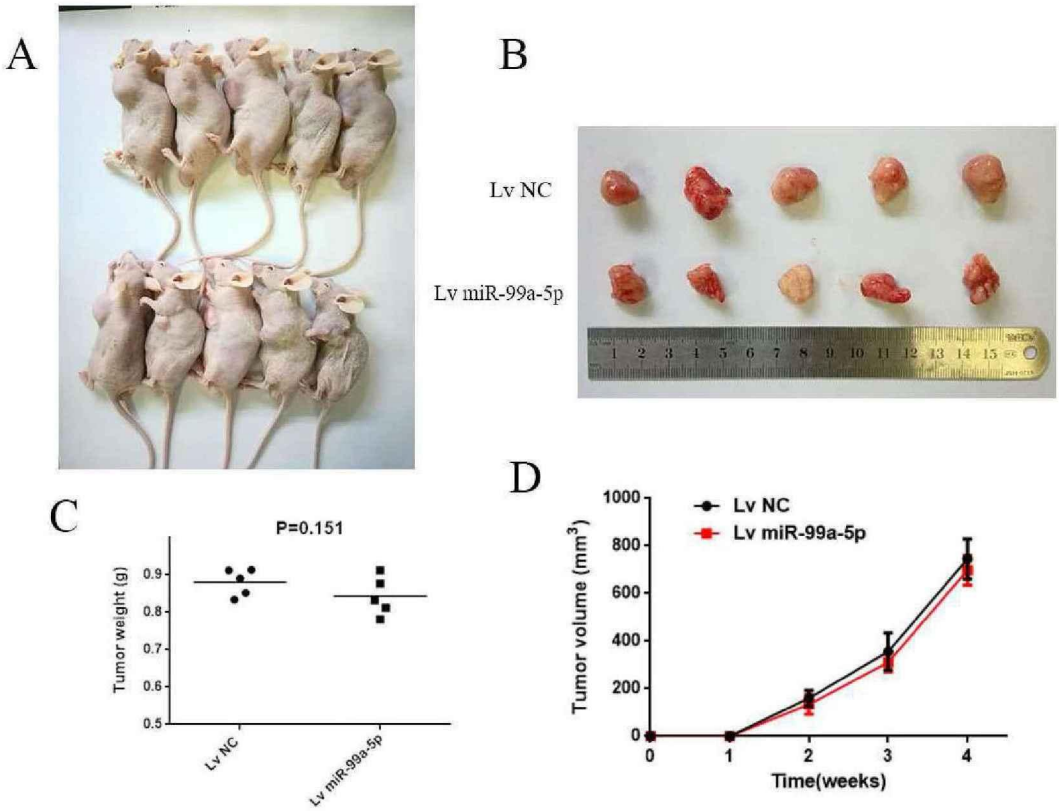

Figure 2-5: Effect of miR-99a-5p on the growth of NOZ cells in vivo.

(A) The picture of xenograft nude mice; (B) The picture of the subcutaneous xenografts; (C) The scatter plot of subcutaneous xenografts' weight; (D) Subcutaneous xenografts growth curve. The results were not statistically significant.

3.2 Effects of miR-99a-5p on the invasion and metastasis of GBC cells

On the basis of the research above, in order to continue to study the effect of miR-99a-5p on the invasion and metastasis of GBC cells, we carried out Transwell invasion/migration assay and wound healing assay in vitro. Using the Matrigel and the Matrigel-free Transwell chamber, we were able to evaluate the invasion and metastasis ability of GBC cells in vitro. As shown in Figure 2-6, the Transwell invasion/migration assay showed that overexpression of miR-99a-5p significantly inhibited the invasion and metastasis of GBC cell lines. In the contrast, the number of cells passing through the chamber was significantly increased after treating with miR-99a-5p inhibitor.

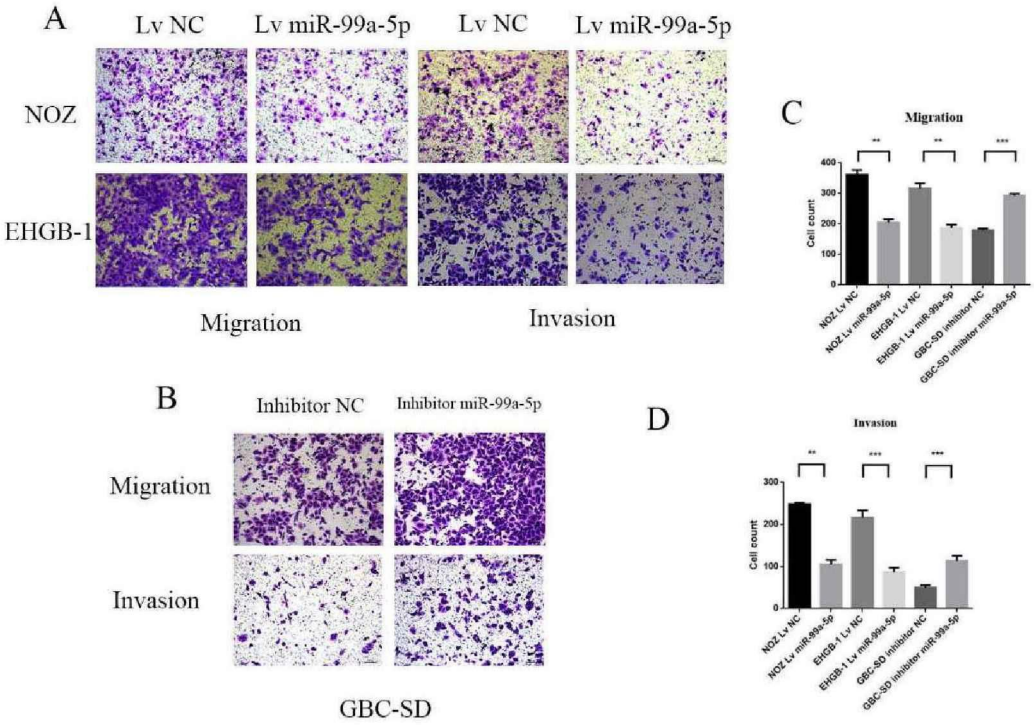

Figure 2-6: Tranwell chamber invasion/metastasis assay in GBC cell lines.

(A) NOZ, EHGB-1 cell lines in migration/invasion assay; (B) GBC-SD cell line in migration/invasion assay; (C) The number of cells transmembrane migration assay ; (D) The number of cells transmembrane of invasion assay. **: P <0.01, ***: P <0.001.

Wound healing assay were also performed and achieved the same result. In the three cell lines, the width of the cells treated with miR-99a-5p was significantly larger than that of the control group after 48h, which indicated that the healing was the slowest and the cell migration ability was weakened. On the contrary, miR-99a-5p inhibitor treatment made cells healing speed increased and the width of wound was significantly reduced. The results above showed that miR-99a-5p could significantly inhibit the invasion and metastasis ability of GBC cells in vitro.

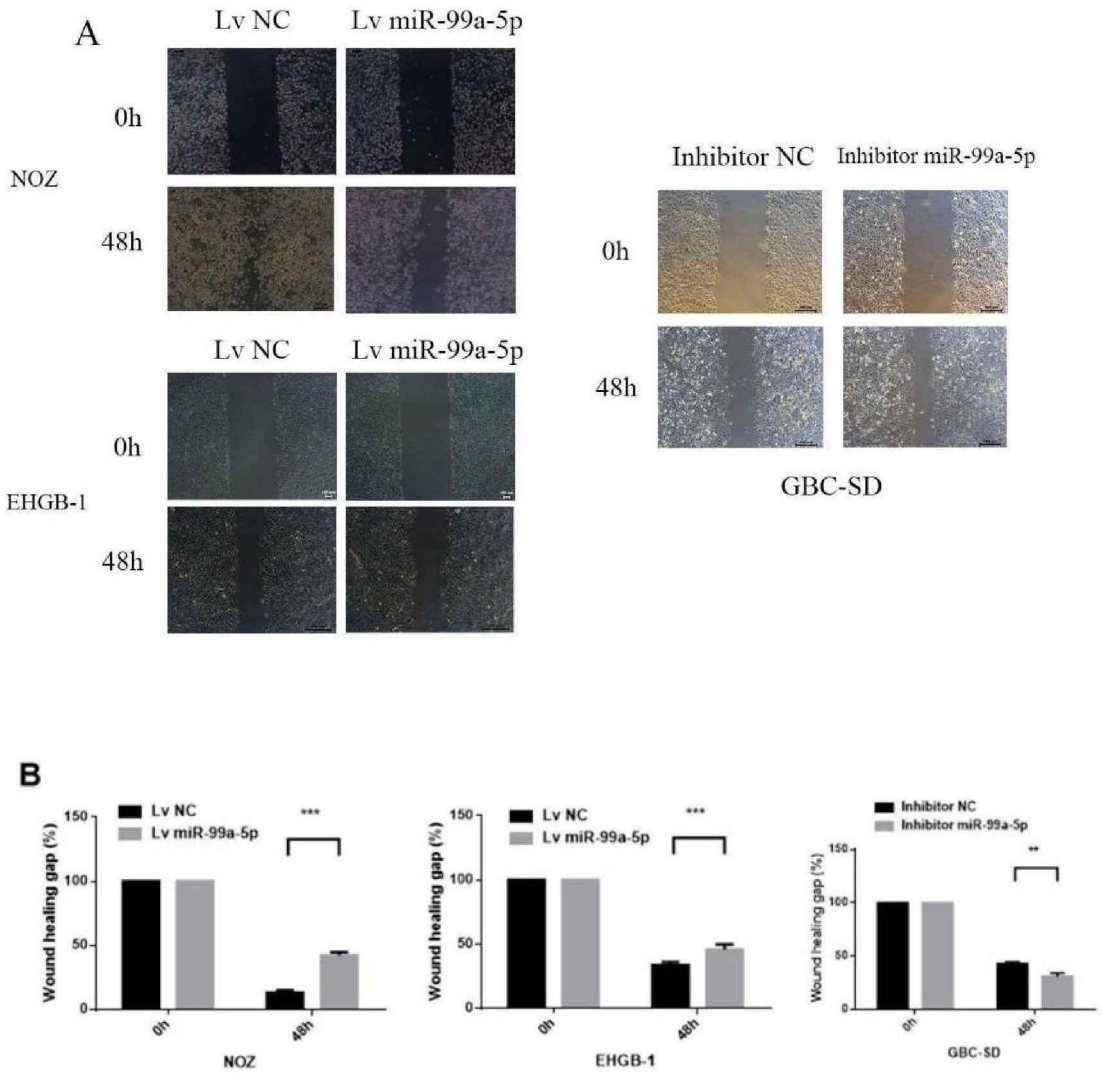

Figure 2-7: wound healing assay in GBC cell lines.

(A) Microscopic observation of wound healing of GBC cell lines within 48 hours; (B) Quantitative analysis of the wound healing distance. **: P<0.01; ***; P<0.001.

Nude mice splenic vein-liver metastases was an animal model that could reflect the ability of tumor cells to metastasize in vivo, commonly used in models of digestive tract malignancies. We evaluated the effect of miR-99a-5p on the metastasis of GBC cells in vivo by injecting miR-99a-5p lentivirus infected NOZ cell line into the spleen of nude mice. We found that the control group had a higher incidence of liver metastases in the same time. Liver HE staining confirmed the presence of metastatic nodules (Figure 2-8). The above results indicate that miR-99a-5p can inhibit the metastasis of GBC cells in vivo.

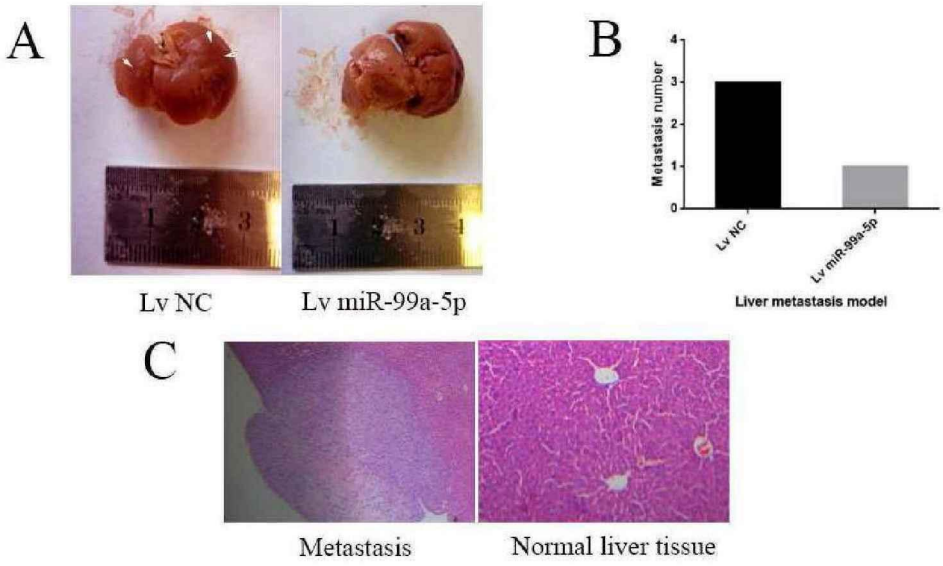

Figure 2-8: Splenic-liver metastasis model of nude mice.

(A) The general picture of typical liver metastases.The arrows pointed to the metastases; (B)The number of metastatic nude mice; (C) HE staining of liver metastasis and normal liver tissue.

In addition, the occurrence of invasion and metastasis was directly related to EMT in many tumors. Therefore, the expression level of EMT-related proteins after miR-99a-5p treatment was also evaluated by western blot assay. We could see that overexpression of miR-99a-5p significantly increased the expression level of E-cadherin associated with epithelial cells, whereas the expression levels of N-cadherin and Vimentin, which represented interstitial cell levels, were down-regulated (Figure 2-9). The results showed that miR-99a-5p could inhibit the occurrence of EMT process, so as to reduce the ability of invasion and metastasis of GBC.

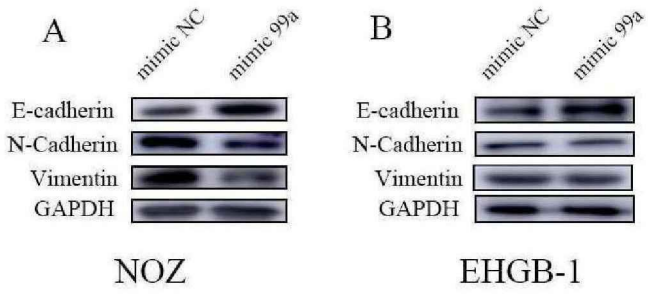

Figure 2-9: The expression of EMT-related proteins was detected by WB after overexpression

of miR-99a-5p in NOZ and EHGB-1 cell lines.

4. Discussion

In recent years, GBC has got a lot of progression in the diagnosis and treatment. However, the early lymph node metastasis, direct invasion to the liver, and radical surgery after local recurrence and lymph node metastasis are still limitations for GBC patients to obtain long-term disease-free survival [34]. Therefore, how to inhibit the invasion and metastasis function of GBC is the key to improve the therapeutic effect of GBC. But currently, the causes and mechanism of GBC invasion and metastasis have not yet been elucidated.

MiRNAs are a kind of single-stranded small molecular non-coding RNA that is evolutionarily relatively conserved and degrade the target genes mRNA or inhibit the translation by binding to the 3'UTR of the specific target genes, which can achieve the gene regulating function. Being similar to other malignancy studies, miRNAs have been studied a lot in the field of GBC in recent years [35-38].

In this part, we investigated the effect of miR-99a-5p on proliferation, invasion and metastasis of GBC by CCK8 assay, colony formation, wound healing assay, Transwell assay and in vivo animal assays by overexpression and knockdown the expression of miR-99a-5p in GBC cell lines. We found that changes in miR-99a-5p expression had little effect on the proliferation of GBC cell lines. However, after treated with lentivirus, the numbers of NOZ and EHGB-1 cells passing through Transwell chamber were significantly reduced, and the time of wound healing was also prolonged. In contrast, with treatment of miR-99a-5p inhibitor in GBC cell lines, GBC cells invasion and metastasis ability had been enhanced. Similar conclusions had been drawn in vivo assays. These results fully demonstrated that miR-99a-5p was involved in the invasion and metastasis of GBC both in vitro and in vitro.

The process of tumor invasion and metastasis is quite complex, involving many aspects of the impact on effect molecules, signaling pathways and tumor microenvironment. Although there are many studies on GBC, which kind of molecules or signaling pathways dominate the process of invasion and metastasis in GBC still has no conclusion. Bao et al [39] found that miR-101 can targeted inhibit the oncogene ZFX to achieve part of the development in GBC functional regulation. In

addition, miR-101 can affect the Smad pathway, inhibiting the TGF-β-mediated EMT process to achieve the inhibition of invasion, metastasis and other functions in GBC. And another study found that miR-29c-5p could negatively regulate the proliferation and metastasis function in GBC by targeting CPEB4. In another way, it could directly inhibit the MAPK pathway, thereby enhancing the tumor suppressor effect of miR-29c-5p [15]. We need further profound study in determining how miR-99a-5p regulates the invasion and metastasis function in GBC.

5. Conclusion

1. MiR-99a-5p had no significant effect on the proliferation of GBC cell lines both in vivo and in vitro;

2. MiR-99a-5p could inhibit the invasion and metastasis of GBC cell lines both in vitro and in vivo;

3. MiR-99a-5p affected the EMT process of GBC cell lines, leading to inhibition of invasion and metastasis in GBC.

PART 3: MiR-99a-5p affects the functions of GBC by targeting MTOR and SMARCA5

1. Introduction

GBC, which is the most common malignant tumor in biliary tract, is a highly malignant digestive tract tumor, of which the incidence rate is about 3.9 cases per 100,000 people. It ranks No. 6 in the digestive tract tumors [1]. As the onset of GBC is hidden and due to the lack of specific early symptoms, many patients have progress to the advanced phase and lost the chance of radical surgery once diagnosed. The current radiotherapy, chemotherapy and other methods cannot achieve satisfactory treatment effect, so the overall 5-year survival rate is only less than 5% [2]. Therefore, early diagnosis is the key to improve the prognosis of GBC. And the study finds that more than 90% of GBC deaths are caused by tumor recurrence and metastasis [3]. Therefore, the study on the pathogenesis of GBC and the mechanism of recurrent invasion and metastasis can help to find new targets of GBC tumor markers and targeted therapy, which is of great significance for improving the diagnosis, treatment effect and the prognosis of GBC patients.

Up to now, we have discovered tens of thousands of miRNA molecules, which constitute a complex network with the downstream target proteins. The data have shown that more than 30% of the proteins are regulated directly or indirectly by miRNAs in a certain extent [40]. Compared with the traditional genome, miRNA-related regulation network of tumor biological behavior is even larger. The miRNA is bound to the 3'UTR of the target genes according to the principle of complementary base pairing, which can directly degrade the mRNA or affect the subsequent protein translation process. In this regard, a miRNA molecule may have a role in multiple downstream target genes, and one protein may also be regulated by multiple miRNAs [41]. Predicting and researching the downstream target genes are important methods of understanding how miRNAs affect tumor mechanisms.

In the previous part, we identified the effect of miR-99a-5p on the invasion and metastasis of GBC cells, which played a role in tumor suppressing. In order to understand its regulatory mechanism, we used bioinformatics tools to find and predict downstream target genes and validate them with dual luciferase reporter assay and western blot assay. Moreover, we would also study in the potential signaling pathway regulating miR-99a-5p functions, so as to improve its mechanism

of action.

2. Materials and Methods

2.1 Materials

2.1.1 Main reagents

Product	Manusfacturer
Dual luciferase reporter assay kit	Promega company
EndoFree Mini Plasmid Kit II	Tiangen biotech (Beijing) Co., Ltd.
Viafect reagent	Promega company
Lipofectamine2000	Invitrogen Corporation
Absolute ethanol	Sinopharm Group Chemical Reagent Co., Ltd.
Dimethylbenzene	Sinopharm Group Chemical Reagent Co., Ltd.
Acetic acid	Sinopharm Group Chemical Reagent Co., Ltd.

For the remaining reagents, see Part 2, "Materials and Methods".

2.1.2 Main instrument

Product	Manusfacturer
Chemiluminescence analyzer	Bio-rad company

2.2 Methods

2.2.1 Prediction of the target genes of miR-99a-5p by bioinformatics

For the bioinformatics prediction of the miR-99a-5p target genes, it was performed using miRDB, TargetScan, picTar and miRanda prediction tools.

2.2.2 Dual luciferase reporter assay

We used dual luciferase reporter assay to verify the direct effect of miR-99a-5p on candidate target genes. The synthesis of dual luciferase reporter plasmids and negative control mutant plasmids containing the 3'UTR of MTOR, SMARCA5 was performed by Shanghai Longqian Biotechnology Co., Ltd. The wild-type plasmid contained the binding site of miR-99a-5p, and the mutant plasmid was randomized at the miR-99a-5p binding site. The PCR product was inserted into the pmirGLO vector containing rennet fluorescence. The MTOR-3'UTR pmirGLO plasmid or the SMARCA5-3'UTR pmirGLO plasmid is co-transfected with the corresponding miRNA mimic or inhibitor in

293T cells. 48 hours later, we used dual luciferase reporter assay kit for firefly luciferase and rennet luciferase activity detection. Standardization of relative fluorescence activity was performed using rennet-luciferase activity.

Protocol of co-transfection:

1. 293T cells are plated into 24-well plates the day before transfection, and 100,000 cells are made in each cell. The cells are incubated at 37 ℃ in 5% CO2 cell incubator, which can make the cell fusion rate 50% at the second day.

2. Each well of cells is transfected with 10 ng of plasmid and 1μL of miRNA mimic or inhibitor, using 1μL Lipofectamin 2000 transfection reagent.

3. Add 50μl of optiMEM in a 1.5ml EP tube. Then add 10ng of the corresponding plasmid and 1μL of the corresponding miRNA mimic or inhibitor before gently mixing them. Another 1.5ml of EP tube is filled with 50μl of optiMEM, then adding 1μL Lipofectamin 2000 transfection reagents. After standby for 5min at room temperature, mix two reagents in one EP tube. The co-incubation time is 20min at room temperature.

4. Take out the cultured cells and all the cells are washed once with PBS. 400μL of DMEM containing FBS and without antibiotics is added to each well.

5. The incubated mixture is added to the corresponding wells and gently mixed, and the cells are kept in incubator. After 6 hours, replace the medium with complete medium. After transfection for 48h, the dual luciferase reporter activity is tested.

2.2.3 Construction and transfection of target genes overexpression plasmid

The full length of the SMARCA5 Coding sequence was inserted into the MCS region of the pCMV plasmid by Shanghai Longqian Biotechnology Co., Ltd. (Figure 3-1) to form plasmid pCMVGFPPuro05(-)SMARCA5 and transfected into the GBC cell lines. Colonies with stable expression of SMARCA5 were screened out using puromycin. The expression level of SMARCA5 was verified by qRT-PCR and Western blot. Empty vector served as blank control group.

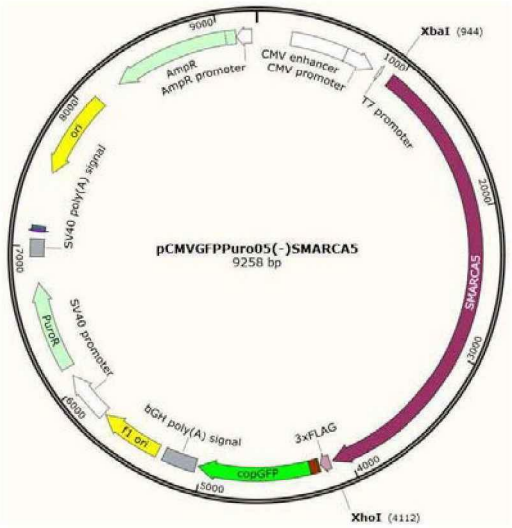

Figure 3-1：Vector map of pCMVGFPPuro05(-)SMARCA5.

Protocol of plasmid transfection:

1. One day before transfection, the cells were plated into a six-well plate and placed in a 5% CO2 cell incubator at 37 ° C to allow the cell fusion to reach 70% at the second day.

2. Each well was transfected with 2μg plasmid, using 6μg of viafect transfection reagent.

3. The corresponding volumes of plasmid and 6μL/well of viafect reagent were co-added to 300μL/well of optiMEM medium, gently mixed and incubated at room temperature for 15 min.

4. The cultured cells were taken out and all the cells were washed once with PBS. Then 1700μL of the corresponding medium containing FBS without antibiotics was added to each well.

5. The incubated plasmid containing optiMEM was added to the corresponding wells at 300μL/ well and gently mixed. The cells were kept in medium and cultured for 6 hours. Then the medium was replaced with complete medium.

6. After 24h of the transfection, we could continue with subsequent experiments.

2.2.4 Small interfering RNA synthesis

Small interfering RNA (siRNA) against SMARCA5 and negative control siRNA were synthesized by Shanghai Tuolan Biology Co., Ltd. and transfected into GBC cells by RFect transfection reagent. The expression level of SMARCA5 was verified by qRT-PCR and Western blot. The sequence of siSMARCA5:5'- GUCAGAGUGUUCCGCUUUA-3'.

2.2.5 Sequences of major qRT-PCR primers

名称	序列

MTOR-F	5'- AGCCGAAGGAGATGCAGAAG -3'
MTOR-R	5'- TTCGGACCAGCTCGTTAAGG -3'
SMARCA5-F	5'- GCATGAGCTGTGGTCACTTC -3'
SMARCA5-R	5'- GCCTTAATTCGACGAAGGAG -3'
GAPDH-F	5'-AGAAGGCTGGGGCTCATTTG-3'
GAPDH-R	5'-AGGGGCCATCCACAGTCTTC-3'

2.2.6 Statistical analysis

SPSS 21.0 software was used for statistical analysis. The t-test was used to compare the measurement data between groups, and the data was expressed by (mean ± standard deviation). $P < 0.05$ was considered statistically significant. All experimental data represent the average of triplicate data.

3.Results

3.1 Prediction of target genes of miR-99a-5p

Through the use of bioinformatics analysis, we can predict the downstream target genes directly interacting with miRNAs, so as to further study their mechanisms of biological behavior in malignant tumors. In this study, we selected online databases such as miRDB, TargetScan, picTar and miRanda as the forecasting tools. Based on the predicted results, we selected SMARCA5 and MTOR, which were both the top-ranked genes in these four databases, as targets for the candidate genes.

Table 3-1: Binding site of miR-99a-5p to candidate target genes SMARCA5 and MTOR predicted by Bioinformatics.

Position 51-57 of SMARCA5 3' UTR	5'...AGUAGUUCUUUAAUUUACGGGUC...
	|||||||
Has-miR-99a-5p	3 GUGUUCUAGCCUAGAUGCCCAA
SMARCA5 3' UTR Mutant	5'...AGUAGUUCUUUAAUUAUGAACAC...

```
Position 295-301 of MTOR 3' UTR   5'  ...CCAUAACUUUAGAAAUACGGGUU...
                                          ||   |||||||
Has-miR-99a-5p                    3'      GUGUUCUAGCCUAGAUGCCCAA
                                          ||   ||    ||
MTOR 3' UTR Mutant                5'  ...CCAUAACUUUAGAAAUAGCCGUU...
```

3.2 Validation of target genes of miR-99a-5p by dual luciferase reporter assay

In order to verify whether miR-99a-5p and the target genes could bind directly, we constructed plasmids of the wild-type and mutant dual luciferase reporter genes with the 3'UTR of the MTOR and SMARCA5, then co-transfected with miR-99a-5p mimic or inhibitor. The activity of luciferase was detected by the dual luciferase reporter kit. The results showed when MTOR and SMARCA5 wild-type 3'UTR plasmids were co-transfected with miR-99a-5p mimics, the corresponding luciferase activity was significantly reduced comparing with mutant plasmid groups (Figure 3-2 A). In addition, we also co-transfected the miR-99a-5p mimic and the control mimic with the MTOR and SMARCA5 wild-type 3'UTR plasmids, respectively. The results showed that miR-99a-5p mimic could significantly down-regulate the activity of luciferase comparing with the control group. Conversely, co-transfection of the miR-99a-5p inhibitor with the wild-type 3'UTR plasmid of the target genes enhances the activity of luciferase (Figure 3-2 B, C).

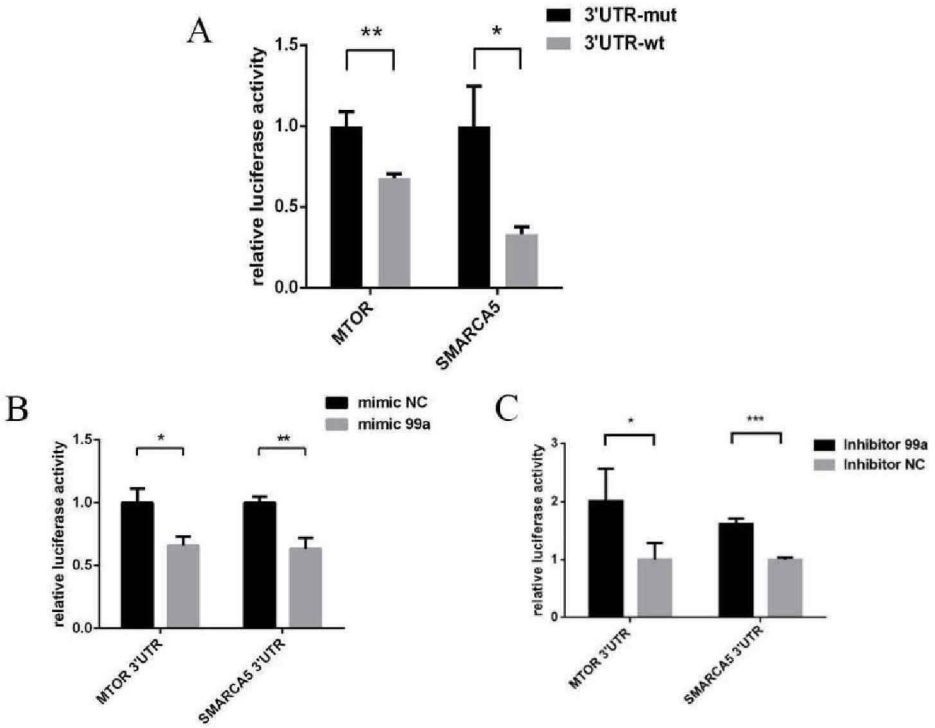

Figure 3-2: Relative activity of luciferase reporters of 3'UTR plasmids of MTOR and SMARCA5 inhibited by miR-99a-5p.

(A) MTOR and SMARCA5 wild-type 3'UTR plasmids were co-transfected with miR-99a-5p mimics; (B) MiR-99a-5p mimic and control mimic were co-transfected with MTOR and SMARCA5 wild-type 3'UTR plasmids, respectively; (C) The miR-99a-5p inhibitor and the control inhibitor were co-transfected with and the wild type 3'UTR plasmid of the target genes, respectively. *: P<0.05; **: P<0.01; ***: p<0.001。

3.3 Effects of miR-99a-5p on the expression of candidate target genes MTOR and SMARCA5

In order to verify the effect of miR-99a-5p on mRNA and protein expression levels of candidate target genes MTOR and SMARCA5, we used qRT-PCR and Western blott to detect the expression of MTOR and SMARCA5 treated by miR-99a-5p at mRNA and protein levels, respectively. As shown in Figure 3-3, overexpression of miR-99a-5p in NOZ and EHGB-1 GBC cells did not result in a change in the expression level of the candidate target genes at the mRNA level, but significantly inhibited the target genes' protein expression levels.

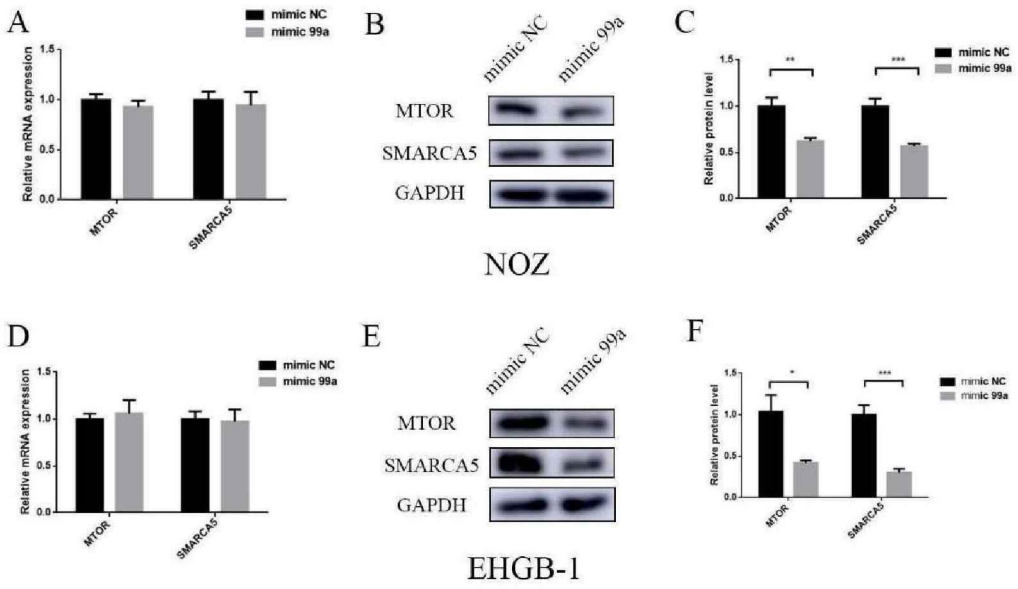

Figure 3-3: mRNA and protein levels of MTOR and SMARCA5 in NOZ and EHGB-1 cell lines after overexpressing miR-99a-5p.

(A) mRNA expression change after overexpression of miR-99a-5p in NOZ cell line; (B) (C)

Protein expression change after overexpression of miR-99a-5p in NOZ cell line; D) mRNA expression change after overexpression of miR-99a-5p in EHGB-1 cell line; (E) (F) Protein expression change after overexpression of miR-99a-5p in EHGB-1 cell line; *: P<0.05; **: P<0.01; ***: p<0.001.

Based on the results above, we could conclude that MTOR and SMARCA5 were the direct target genes of miR-99a-5p.

3.4 Expression of SMARCA5 in gallbladder carcinoma and its correlation with clinical data and prognosis

In order to understand the expression of SMARCA5 in GBC tissues, we used qRT-PCR to detect the expression of SMARCA5 in 30 pairs of GBC tissues and their peritumoral tissues. The results showed that the expression of SMARCA5 in GBC was significantly higher than that in peritumoral tissues (P <0.001).

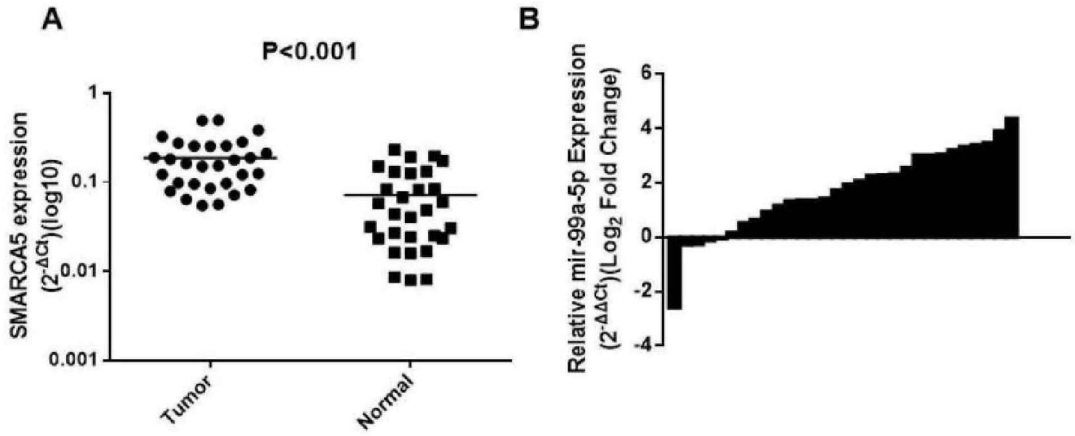

Figure 3-4: Expression of SMARCA5 in GBC tissues.

（A）The expression of SMARCA5 in 30 pairs of GBC tissues and their peritumoral tissues；（B）Comparison of the expression of SMARCA5 in GBC tissues and peritumoral tissues.

In order to further clarify the role of SMARCA5 in GBC, we analyzed the expression of SMARCA5 in close relationship with lymph node metastasis (P = 0.009) and TNM staging (P = 0.02) by clinicopathological characteristics analysis (Table 3-1). Survival analysis confirmed that patients with SMARCA5 overexpression had a poor prognosis (Figure 3-5). The median survival time was 6.9 months and 18 months in the high expression group and low expression group, respectively. The 1 year survival rate was 45.0% and 70.7%, respectively. And lymph node metastasis and SMARCA5 expression could serve as independent prognostic markers for the OS time of GBC patients (Table

3-2).

Table 3-1：Association between SMARCA5 expression and the clinicopathological characteristics in GBC

Clinicopathological characteristics	Cases	Relative SMARCA5 expression		χ2	p
		High(n=11)	Low(n=19)		
Age(Year)					
＜60	9	4 (44.4%)	5 (55.6%)	0.335	0.563
≥60	21	7 (33.3%)	14 (66.7%)		
Gender					
Male	12	6(50.0%)	6 (50.0%)	1.531	0.216
Female	18	5 (27.8%)	13 (72.2%)		
Differentiation degree					
Well/moderate	23	8 (34.8%)	15 (65.2%)	0.151	0.698
pour	7	3 (42.9%)	4 (57.1%)		
Tumor invasion					
Tis-T_2	9	4 (44.4%)	5 (55.6%)	0.335	0.563
T_3-T_4	21	7 (33.3%)	14 (66.7%)		
Lymph node metastasis					
No	22	5 (22.7%)	17 (77.3%)	6.903	0.009
Yes	8	6 (75.0%)	2 (25.0%)		
TNM stage					
0-I	11	7 (63.6%)	4 (36.4%)	5.440	0.02
II-IV	19	4 (21.1%)	15 (78.9%)		

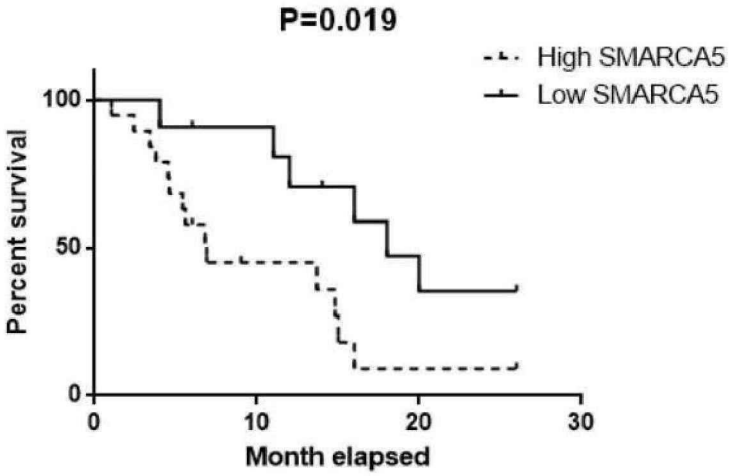

Figure 3-5：Survival analysis of GBC patients correlated with SMARCA5 expression.

Table 3-2 Univariate and multivariate analysis of the relationship between

Variable	Univariable analysis		Multivariable analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (＜60 vs. ≥60)	0.411 (0.213-0.776)	0.226	-	-
Sex (Male vs. Female)	0.522 (0.260-1.071)	0.082	-	-
Histology differentiation (Well or moderate vs.Poor)	1.262 (0.816-1.881)	0.321	-	-
Tumor invasion(AJCC) (Tis-T1 vs.T2-T4)	1.096 (0.595-2.127)	0.808	-	-
Lymph node metastasis (Present vs. Absent)	**6.252 (2.527-14.661)**	**<0.001**	**8.116 (2.564-23.609)**	**<0.001**
TNM stage(AJCC) (0-I vs. II-IV)	**0.326 (0.161-0.639)**	**0.001**	**0.348 (0.134-0.841)**	**0.012**
SMARCA5 expression	**6.248 (3.146-12.651)**	**<0.001**	**2.613 (1.150-6.346)**	**0.036**

CI, confidence interval; HR, hazard ratio.

3.5 MiR-99a-5p affected the invasion and metastasis of GBC cells by PI3K / AKT pathway

In order to further study how miR-99a-5p regulates the invasion and metastasis of GBC cells, we examined the expression of related proteins in PI3K / AKT signaling pathway associated with MTOR by Western blot. As shown in Figure 3-6, overexpression of miR-99a-5p in NOZ and EHGB-1 cell lines reduced the phosphorylation level of AKT, thereby inhibiting the carcinogenic function of PI3K / AKT signaling pathway.

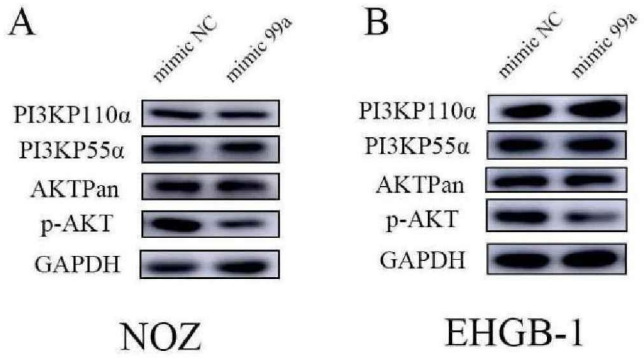

Figure 3-6: Expression levels of proteins involved in PI3K / AKT signaling pathway after overexpression of miR-99a-5p in NOZ and EHGB-1 cell lines.

3.6 Effects of SMARCA5 on proliferation, invasion and metastasis of GBC Cells

The role and function of SMARCA5 in GBC is still unknown. In order to understand whether

SMARCA5 had an effect on the proliferation and metastasis of GBC cells, we used siRNA and overexpression plasmids to knock down and overexpressing GBC cells, respectively (Figure 3-7). Using CCK8 assay, we found whether SMARCA5 were knocked down or overexpressed, it did not significantly affect the proliferation of neither NOZ nor EHGB-1 cell lines (Figure 3-7).

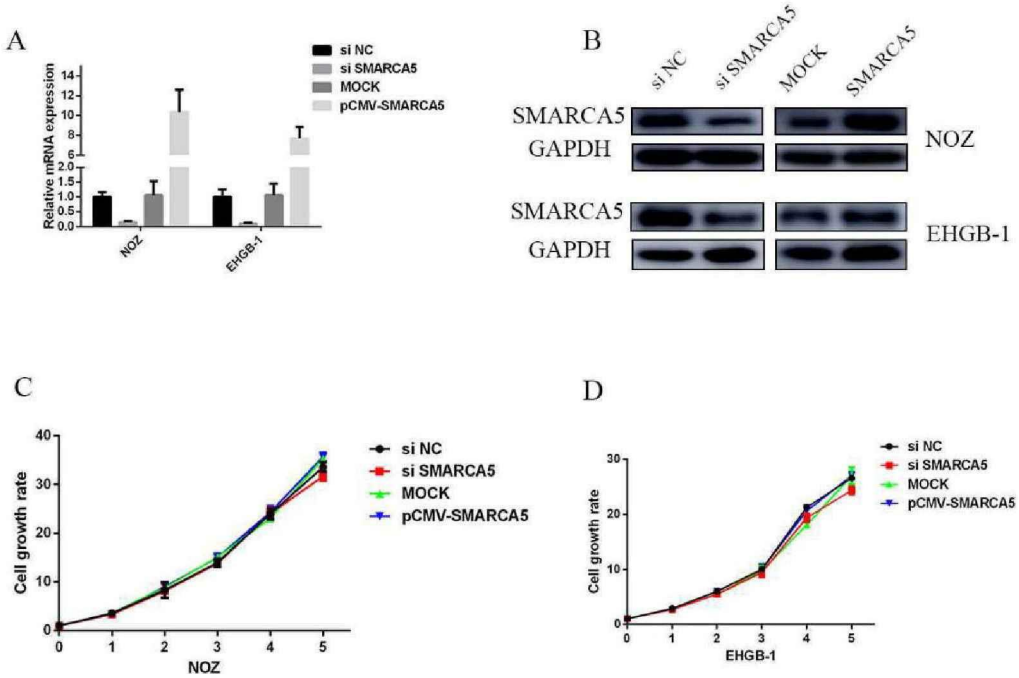

Figure 3-7: Effects on proliferation of GBC cells after knockdown and overexpression of SMARCA5. (A) (B) The effect of knockdown and overexpression of SMARCA5 was detected by qRT-PCR and WB, respectively; (C) (D) CCK8 assay was used to detect the effect of SMARCA5 on proliferation in NOZ and EHGB-1 cells.

According to the results of migration and invasion assay, we could find that the effect of SMARCA5 on the invasion and metastasis of GBC cells was significantly decreased after SMARCA5 was knocked down, while the overexpression of SMARCA5 had the opposite effect (Figure. 3-8). The results above were opposite with and the impact of miR-99a-5p on GBC cell invasion and metastasis. This coincided with the assumption that SMARCA5 was a downstream target gene of miR-99a-5p.

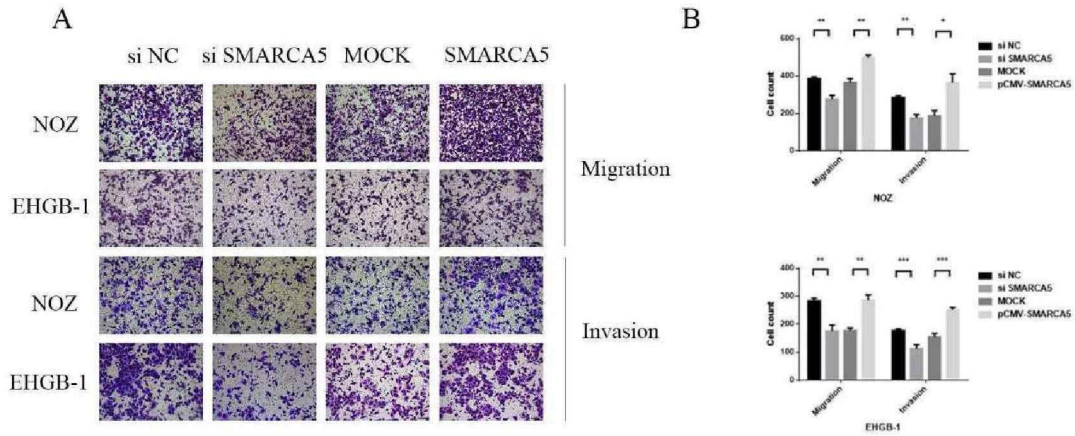

Figure 3-8: Evaluation of the invasion and metastasis ability of GBC cell lines after knockdown and overexpression of SMARCA5.

*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

3.7 MiR-99a-5p affected the invasion and metastasis of GBC cells by direct regulating SMARCA5

Considering that the protein level of SMARCA5 could be regulated by miR-99a-5p, we were doubt whether miR-99a-5p could inhibit the invasion and metastasis of GBC cells by interacting with SMARCA5. Thus, miR-99a-5p mimic was transfected in GBC cells with co-transfection of overexpressing plasmid pCMV-SMARCA5 and empty plasmid, and then evaluated the function of invasion and metastasis of GBC. Figure 3-8 showed that overexpression of SMARCA5 allowed partial recovery of miR-99a-5p-inhibited GBC cell invasion and metastasis. These results suggested that miR-99a-5p could inhibit the invasion and metastasis of GBC cells by targeting SMARCA5.

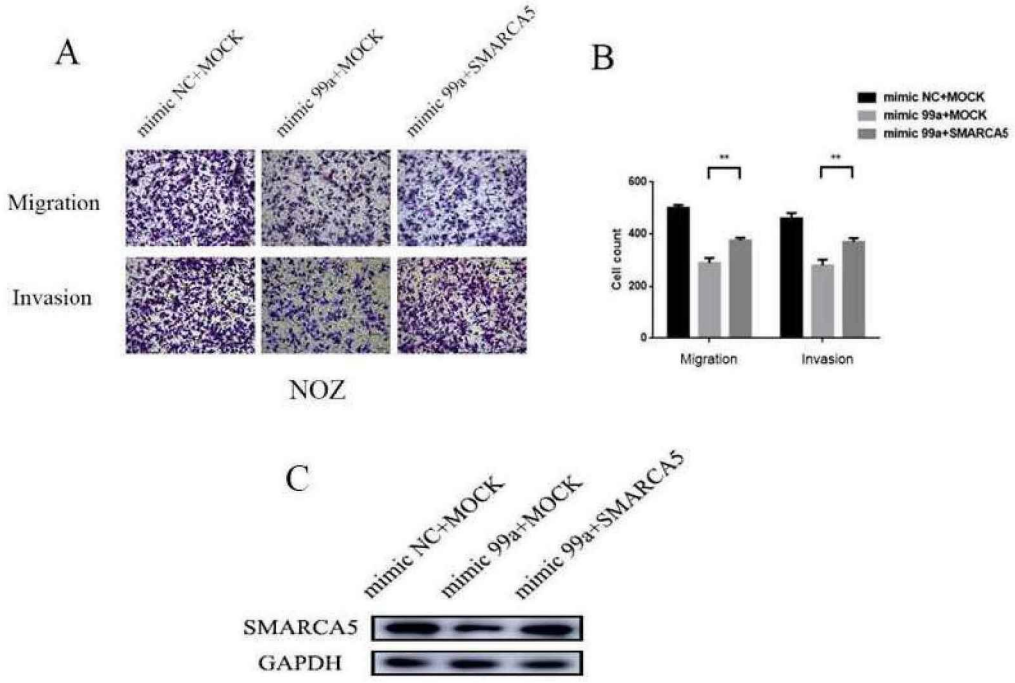

Figure 3-9: Overexpression of SMARCA5 rescued the miR-99a-5p-induced inhibition of migration and invasion ability.

**: P<0.05.

4. Discussion

Scholars have found that the generation of miRNAs requires a series of complex processes [42]. First, the original pri-miRNA is transcribed in the nucleus, and the precursor miRNA (pre-miRNA) is formed after one processing. Pre-miRNAs form double-stranded miRNAs under the digestion of Dicer enzymes, and 5p and 3p monomers are formed according to the position of the pre-miRNAs after the solution. MiRNAs can participate in the formation of RNA-induced silencing complex (RISC), which can affect the downstream target gene by mRNA degradation or inhibiting translation.

In this part, we studied the target gene prediction of miR-99a-5p by bioinformatics. Combined with the results of the four databases, it could be found that SMARCA5 is the candidate gene with the highest frequency and the highest ranking. In addition, MTOR was also appeared in all of the databases. Many scholars had reported the relationship between MTOR and

miR-99a-5p in malignant tumors [43-44], so we also defined it as a candidate target gene. We showed that MTOR was also a target gene for miR-99a-5p in GBC by dual luciferase reporter assay and western blo assay. However, according to the results of qRT-PCR, miR-99a-5p cannot downregulate MTOR mRNA level. The results suggested that miR-99a-5p acted on MTOR to inhibit its translation process, rather than directly degrading its mRNA. PI3K / AKT signaling pathway is widely studied in malignant tumors and is thought to be related to cell transformation, proliferation, metastasis, apoptosis, cycle arrest and drug resistance. It has also become a research target for many tumor comprehensive treatments [45- 47]. Its research in GBC is also plenty. It is generally believed that this pathway in GBC plays an important role in promoting proliferation and metastasis, cell cycle arrest and other functions [48-50].

SMARCA5 is a member of the SWI / SNF protein family as a chromatin remodeling factor that participates in cell signaling, DNA damage repair, and other functions by controlling histone deacetylation. SMARCA5 research is currently focused on chromatin remodeling, DNA damage repair and cell self-renewal [51-54]. Jin et al. [55] reported that SMARCA5 can promote tumor proliferation and metastasis in breast cancer, but the mechanism remains to be excavated. This study is the first time found that SMARCA5 has the ability to promote invasion and metastasis in GBC. SMARCA5 overexpressed in GBC patients and related to lymph node metastasis, which could reduce the survival of patients. These results indicate that SMARCA5 is expected to be a new molecular target in the future diagnosis and treatment of GBC. But its detailed mechanism of action is still not clear. We need a deeper level of research on aspects of its upstream and downstream interaction proteins and the relevant signal pathway.

5.Conclusion

1. SMARCA5 and MTOR were selected as candidate target genes for miR-99a-5p by bioinformatics prediction. It was confirmed by dual luciferase reporter assay and western blot.

2. miR-99a-5p could directly inhibit the PI3K / AKT pathway to play its anti-cancer function.

3. SMARCA5 was closely related to lymph node metastasis in patients with GBC, and its high expression indicated a poor prognosis in GBC patients.

4. SMARCA5 could promote the invasion and metastasis of GBC cells. MiR-99a-5p could directly interact with SMARCA5 and inhibit the invasion and metastasis of GBC cells by

inhibiting the SMARCA5 protein translation process.

Summary and prospect for this paper

In this study, differentially expressed miRNAs were detected by high-throughput miRNA microarray in GBC tissues and their corresponding peritumoral tissues. The expression of miR-99a-5p, which was significantly lower in GBC tissues, was screened by TCGA database. Then we examined the expression of miR-99a-5p in 41 cases of GBC and its corresponding peritumoal tissues by qRT-PCR, further confirmed that miR-99a-5p was significantly lower in GBC tissues. Pathological data and survival analysis found that low expression of miR-99a-5p indicated a poor prognosis in GBC patients. MiR-99a-5p was able to inhibit the invasion and metastasis of GBC cells and could be mediated by EMT process, according to the functional experiments in vitro and in vivo. In order to explore the mechanism of miR-99a-5p function, we found that SMARCA5 and MTOR were the direct target genes of miR-99a-5p by bioinformatics prediction and it was confirmed by dual luciferase reporter assay. MiR-99a-5p could inhibit the protein translation process by binding to the 3'UTR of MTOR and SMARCA5. What's more, miR-99a-5p counld also directly affect the PI3K / AKT pathway, but also through the combination with SMARCA5 to inhibit the invasion and metastasis of GBC function.

In summary, the detection of miR-99a-5p expression has important implications for the prognosis of patients with GBC. And miR-99a-5p has the potential to be a target for the diagnosis and treatment of GBC. We need to study it more deeply, and elucidate the mechanism of its anti-cancer function, which can transform the theoretical research into clinical application and make contributions to improve the diagnosis and treatment of GBC.

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八年制学位论文要求

八年制高等医学教育遵循“八年一贯，整体优化，加强基础，注重临床，培养能力，提高素质”的办学原则，培养适应我国经济社会发展需要的、具有崇高的思想品德和社会责任感、高尚的职业道德、良好的敬业精神、伦理行为和法律意识；具有较宽厚的人文社会科学和自然科学知识及扎实的医学科学基础理论、较强的临床实践能力和交流协调能力；具备基本科研能力、终生自主学习能力和创新意识、良好的中、外文沟通能力和信息获取、管理、应用能力；具有较大发展潜能和团队协作精神的高素质临床医学人才，其培养目标定位于临床医学博士专业学位。

根据八年制医学博士学位培养方案总则、应用型学位的特点和上级的有关精神，八年制学生学位论文的要求：

1. 博士学位论文应在导师或指导小组的指导下，由学生本人独立完成，实行导师负责制。

2. 在博士生导师的指导下，在查阅大量文献资料并结合本人工作的基础上确定学位研究课题。选题必须紧密结合临床实际，可以是病例分析报告，或总结临床经验，或改进临床技术方法，也可以是临床和实验研究相结合的研究工作，研究结果对临床工作具有一定的应用价值或应用前景。

3. 表明学生具有运用所学知识解决临床实际问题和从事临床科学研究的能力。

4. 撰写学位论文应恪守学术道德和学术规范,要求概念清楚、立论正确、分析严谨、数据可靠、计算精确、图表清晰、层次分明、文字简练、格式规范。

5. 学位论文内容一般应不少于 2 万字。中文摘要 800 字以内，英文摘要 1500-3000 字符。

6. 学位论文全文要求中英（或法）文对照。

上海交通大学
学位论文原创性声明

本人郑重声明：所呈交的学位论文，是本人在导师的指导下，独立进行研究工作所取得的成果。除文中已经注明引用的内容外，本论文不包含任何其他个人或集体已经发表或撰写过的作品成果。对本文的研究做出重要贡献的个人和集体，均已在文中以明确方式标明。本人完全意识到本声明的法律结果由本人承担。

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日期：2011年 5月 30日