miR-300通过负向调控垂体肿瘤转化基因1抑制骨肉瘤细胞MG63的侵袭和转移

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摘要:目的 研究miR-300及垂体肿瘤转化基因1(PTTG1)对骨肉瘤侵袭转移的影响,探讨引起骨肉瘤侵袭转移的分子机制。**方法** Western blot检测人成骨细胞hFOB1.19及骨肉瘤细胞MG63中PTTG1的表达情况,并检测转染敲低PTTG1质粒后细胞的转染效率;Transwell侵袭实验和CCK8实验检测敲低PTTG1及过表达miR-300对骨肉瘤细胞MG63侵袭和增殖能力的影响;网上预测并筛选与PTTG1互补结合的microRNAs(miRNAs);qRT-PCR检测人成骨细胞hFOB1.19及骨肉瘤细胞MG63中miR-300的表达情况;Western blot检测转染过表达miR-300质粒后PTTG1在骨肉瘤细胞MG63的表达情况;双荧光素酶实验检测miR-300和PTTG1的靶向结合情况;Transwell侵袭实验和CCK8实验检测过表达miR-300和过表达PTTG1质粒共转后对骨肉瘤细胞MG63侵袭和增殖能力的影响。**结果** PTTG1在骨肉瘤细胞MG63中表达较高(P=0.0002);敲低PTTG1可抑制骨肉瘤细胞MG63的侵袭(P=0.0002)和增殖(P=0.0039)能力;网上预测软件预测可能与PTTG1互补结合的miRNAs,NCBI数据库下载数据集分析确定研究对象为miR-300;qRT-PCR结果表明miR-300在骨肉瘤细胞MG63中表达降低(P=0.0004);采用过表达质粒过表达骨肉瘤细胞MG63中的miR-300,qRT-PCR结果提示转染成功(P<0.0001);Western blot发现过表达miR-300后PTTG1的表达明显降低(P=0.0007);双荧光素酶实验结果显示miR-300可与PTTG1靶向结合(P=0.0010);细胞共转实验显示过表达PTTG1可逆转miR-300对骨肉瘤细胞侵袭(P=0.0003)和增殖(P=0.0077)能力的影响。**结论** miR-300通过靶向结合PTTG1抑制骨肉瘤细胞MG63的侵袭转移能力。

关键词:骨肉瘤;miR-300;垂体肿瘤转化基因1;侵袭;转移;增殖

MiR-300 inhibits invasion and metastasis of osteosarcoma cell MG63 by negatively regulating PTTG1

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Abstract: Objective To investigate the effects of miR-300 and PTTG1 on osteosarcoma invasion and metastasis and explore the molecular mechanism of osteosarcoma invasion and metastasis. **Methods** Western blot was used to detect the expression of PTTG1 in human osteoblasts hFOB1.19 and osteosarcoma cell MG63 and to detect the transfection efficiency of cells transfected with PTTG1-knockdown plasmid; Transwell invasion assay and CCK8 assay detected the effects of knockdown of PTTG1 and overexpression of miR-300 on the invasion and proliferation of osteosarcoma cell MG63. On-line prediction and screening of microRNAs (miRNAs) with complementary PTTG1 binding was conducted. qRT-PCR was performed to examine the expression of miR-300 in hFOB1.19 and MG63 cells, and Western blotting was used to detect the expression of PTTG1 in MG63 cells after transfection with a miR-300 plasmid. Double luciferase assay was used to detect the targeted binding of miR-300 and PTTG1, Transwell invasion assay and CCK8 assay were used to detect the effects of overexpression of miR-300 and overexpression of PTTG1 plasmid on invasion and proliferation of osteosarcoma cell line MG63. **Results** PTTG1 was highly expressed in MG63 cells (P=0.0002). PTTG1 knockdown significantly inhibited the invasion (P=0.0002) and proliferation (P=0.0039) of MG63 cells. Based on the results of online prediction of complementary miRNAs to PTTG1 and analysis of the data from NCBI database, miR-300 was determined as the target miRNA in this study. qRT-PCR results showed a significantly decreased expression of miR-300 in MG63 cells (P=0.0004). Overexpression of MiR-300 in MG63 cells significantly decreased the expression of PTTG1 (P=0.0007), and the expressions of miR-300 and PTTG1 were negatively correlated. Dual luciferase assay showed that miR-300 could specifically bind to PTTG1 (P=0.001). Overexpression of PTTG1 could significantly reverse the effect of miR-300 overexpression on invasion (P=0.0003) and proliferation (P=0.0077) of MG63 cells. **Conclusion** Overexpression of miR-300 can inhibit the invasion and metastasis of osteosarcoma cell MG63 by targeting PTTG1.

Keywords: osteosarcoma; miR-300; PTTG1; invasion; metastasis; proliferation

骨肉瘤目前仍是儿童和青少年恶性肿瘤死亡率很高的疾病,患者的5年生存率一直保持在60%~70%[1]。约18%的患者在临床表现时可以发现转移,大多数转移发生在肺部[2]。早期转移是骨肉瘤治疗的难点。因此,有必要了解骨肉瘤的发病机制,以制定有效的治疗策略。

垂体肿瘤转化基因1(PTTG1)是一种参与促进中

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期姐妹染色单体分离的安全蛋白[3]，在有丝分裂过程中通过保护染色体稳定性发挥重要作用[4]。据报道，PTTG1除了具有保护蛋白功能外[5]，还有促进恶性细胞增殖和促进肿瘤生长的作用[6]。PTTG1通过整合素局灶性粘附激酶信号转导上皮-间质转化和MMPs的改变直接参与肺癌细胞的侵袭[7]。PTTG1在人乳腺癌组织和细胞中表达上调[8]。然而，PTTG1是否能通过其他方式影响骨肉瘤细胞的侵袭和增殖还有待于进一步研究。

MiRNA通过抑制mRNA翻译或促进mRNA降解而充当基因表达的负调节剂[9]。研究表明miRNA的异常表达与骨肉瘤发生发展相关[10-12]。为了研究PTTG1影响骨肉瘤细胞侵袭和增殖的机制，我们通过生物信息学预测网站starbase和miRmap发现miR-300和PTTG1可能结合。且在GEO数据集GSE28423中，我们发现与正常的骨组织相比miR-300在骨肉瘤细胞中表达降低。MiR-300与PTTG1在骨肉瘤中相互作用未见有报道，因此我们选定miR-300继续下一步研究。MiRNA作为基因表达的关键调节剂，是生物标志物开发的有希望的候选者。因此，充分了解miR-300在骨肉瘤发生发展中的具体作用及其与PTTG1之间的关系至关重要。

1 材料和方法

1.1 材料

逆转录试剂盒及SYBR Green real-time PCR Master Mix（博日科技有限公司）；PTTG1、β-Actin抗体均（艾博抗贸易有限公司）；双荧光素酶报告基因载体、PTTG1敲减质粒及其对照质粒、PTTG1过表达质粒及其对照质粒、miR-300过表达及其对照质粒由吉凯基因构建；实验细胞系hFOB1.19、293T、MG63（ATCC）。

1.2 网上在线microRNA分析工具

用starBase、miRmap预测网站预测可能与PTTG1互补结合的miRNA；NCBI下载骨肉瘤中miRNA表达的GEO数据集GSE28423。GSE28423数据集包含19个骨肉瘤细胞系，4个正常骨组织的miRNA的表达情况，利用正常骨组织作为对照，进行骨肉瘤细胞的差异miRNA表达分析。选定可以与PTTG1互补结合而且在骨肉瘤组织中表达降低的miR-300为研究对象；在线预测miR-300与PTTG1存在的结合位点。利用miRpath（http://diana.imis.athena-innovation.gr/DianaTools/index.php）进行miR-300的信号相关通路筛选。

1.3 细胞培养

hFOB1.19使用含有10% FBS的DMEM/F12培养基，293T、MG63使用含有10% FBS的DMEM培养基。细胞转染依照Lipofectamine 2000说明书进行操作。PTTG1敲低对照质粒序列由吉凯基因构建合成，PTTG1敲低序列5'-GACCCUGGAUGUUGAAUUG-3'。细胞经转染不同质粒后分组。MG63组：正常培养，不做任何处理；Scr/MG63组：转入PTTG1敲低对照质粒；SiPTTG1/MG63组：转入PTTG1敲低质粒；MG63/NC组：转入miR-300过表达对照质粒；MG63/miR-300组：转入miR-300过表达质粒；MG63/miR-300+PTTG1组：共转染miR-300过表达及PTTG1过表达质粒。

1.4 qRT-PCR

RNA提取及逆转录参考本课题组已发表文献[13]。通过得到的逆转录产物进行qRT-PCR。U6的上游引物为GCTTCGGCAGCACATATACTAAAAT，下游引物为CGCTTCACGAATTTGCGTGTCAT；miR-300上游引物：AGGGTATACAAGGGCAGACT，下游引物：GAGAGGAGAGGGAGAGGAGA，茎环结构：GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTGTG；miR-300表达使用U6作为内参，使用$2^{-\Delta\Delta Ct}$分析的方法进行分析。

1.5 Western blot检测

将各组细胞提取总蛋白质，检测蛋白质浓度并进行凝胶电泳、转膜、封闭、一抗4 ℃过夜、TBST洗膜、二抗孵育、TBST洗膜、显影、曝光。实验重复3次。抗体配制如下：β-Actin（1∶1 000）、PTTG1（1∶500）。

1.6 Transwell侵袭实验

制备含有基质胶的Transwell小室，细胞接种后培养48 h，用棉签取出内侧未侵入的细胞。用4%多聚甲醛固定，吉姆萨试剂染色，在光学显微镜下随机选取3个区域进行计数。所有实验均重复3次，取均值作为最终结果。

1.7 CCK8细胞增殖实验

制备细胞悬液接种到96孔板中，每孔约100 μL、2×10^3细胞，培养24 h后每孔加入含10%CCK8的培养基10 μL。分别测定转染24、48、72、96 h后的细胞吸光度值$A_{450\,nm}$，计算结果。

1.8 双荧光素酶检测实验

将miR-300过表达质粒及其对照质粒分别与PTTG1的3'UTR野生型（pGL3-PTTG1 3'UTR-WT）报告载体和突变型（pGL3-PTTG1 3'UTR-MUT）报告载体共转染入293T细胞中，转染报告载体48 h，吸取培养基上清，再用PBS洗涤细胞；向培养孔中加入PLB裂解液，裂解15 min；收集裂解液并进行荧光素酶活性测定，以萤火虫荧光素酶活性值与海肾荧光素酶活性值的比值作为相对荧光素酶活性。

1.9 统计学方法

采用SPSS22.0软件进行统计学分析，计量结果使用均数±标准差表示，两组间均数比较采用独立样本t检验，$P<0.05$为差异具有统计学意义。

2 结果

2.1 PTTG1在骨肉瘤细胞MG63中高表达以及PTTG1在骨肉瘤细胞中敲减成功

用Western blot检测人成骨细胞hFOB1.19及骨肉瘤细胞MG63中PTTG1的表达情况，结果显示，PTTG1在骨肉瘤细胞MG63中表达(1.53±0.08)较高，在人成骨细胞hFOB1.19中表达(1.00±0.07)较低，且差异具有统计学意义(P=0.0002，图1A)。RNA干扰技术敲低PTTG1后，通过Western blot检测骨肉瘤细胞MG63中PTTG1的表达情况，结果显示，与Scr/MG63组(1.00±0.10)相比，PTTG1在SiPTTG1/MG63组中表达(0.40±0.06)明显降低，且差异具有统计学意义(P=0.0007，图1B)。

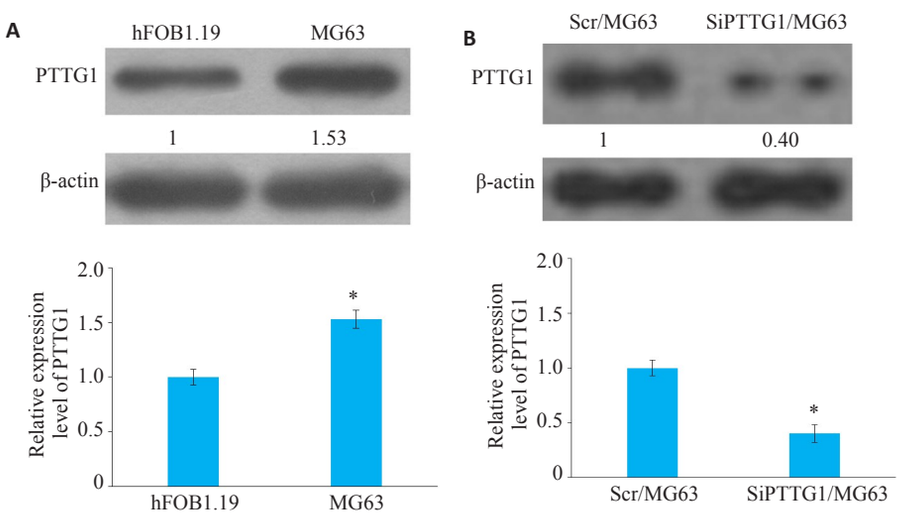

图1 PTTG1在骨肉瘤细胞MG63中高表达以及PTTG1在骨肉瘤细胞中敲减成功

Fig.1 Detection of PTTG1 expression of in MG63 cells and PTTG1 was successfully knocked down in osteosarcoma cells. **A**: PTTG1 expression was higher in MG63 cells and lower in hFOB1.19 cells, *P<0.05 *vs* hFOB1.19 group. **B**: After the knockdown of PTTG1, the expression of PTTG1 in MG63 cell was significantly reduced, *P<0.05 *vs* Scr/MG63 group.

2.2 敲低PTTG1后，骨肉瘤细胞MG63的体外侵袭和增殖能力下降

敲低PTTG1后，Transwell侵袭实验检测各组细胞的侵袭能力，结果显示，与对照组穿过下室的细胞数量(466.67±18.80)相比，敲低PTTG1组的穿过下室的细胞数量(267.00±9.09)明显降低(图2A、B)。运用CCK8细胞增殖实验检测各组细胞的增殖能力，结果显示，与对照组在72 h、96 h(72 h：0.66±0.04，96 h：0.95±0.03)的增殖能力相比，敲低PTTG1组在72 h、96 h(72 h：0.44±0.04，96 h：0.67±0.06)的增殖能力明显降低(图2C)。

2.3 miR-300是PTTG1的靶基因

网上预测软件starBase与miRmap预测可能与PTTG1互补结合的miRNA，NCBI下载骨肉瘤中miRNA表达的GEO数据集GSE28423并筛选出log2FC<-1、P<0.05的miRNA，三者取交集，分析选定可以与PTTG1互补结合而且在骨肉瘤组织中表达降低的miR-300为研究对象(图3A、B)。qRT-PCR检测人成骨细胞hFOB1.19及骨肉瘤细胞MG63中miR-300的表达情况，发现miR-300在骨肉瘤细胞MG63中表达(0.48±0.06)较低，在人成骨细胞hFOB1.19中表达(1.00±0.06)较高(P=0.0004，图3C)。转染miR-300过表达质粒后，qRT-PCR检测骨肉瘤细胞MG63中miR-300的表达情况，结果显示，与MG63/NC组(1.00± 0.10)相比，MG63/miR-300组miR-300的表达(2.30± 0.12)明显升高，且差异有统计学意义(P<0.0001，图3D)。

2.4 miR-300在多种癌相关信号通路富集并且和PTTG1靶向结合

KEGG通路富集分析表明miR-300主要富集于Wnt信号通路、TGF-β信号通路等多种癌相关信号通路(图4A)。转染miR-300过表达质粒后，Western blot检测骨肉瘤细胞MG63中PTTG1的表达情况，结果显示，与MG63/NC组(1.00±0.10)相比，PTTG1在MG63/miR-300组中的表达(0.48±0.08)明显降低(P=0.0007，图4B)。在线预测miR-300与PTTG1存在的结合位点，采用双荧光素酶检测miR-300能否与PTTG1靶向结合。结果显示，miR-300过表达质粒与pGL3-PTTG1 3'-UTR-WT报告载体共转染后，细胞的荧光素酶活性显著降低。miR-300与PTTG1 mRNA的3'UTR可靶向结

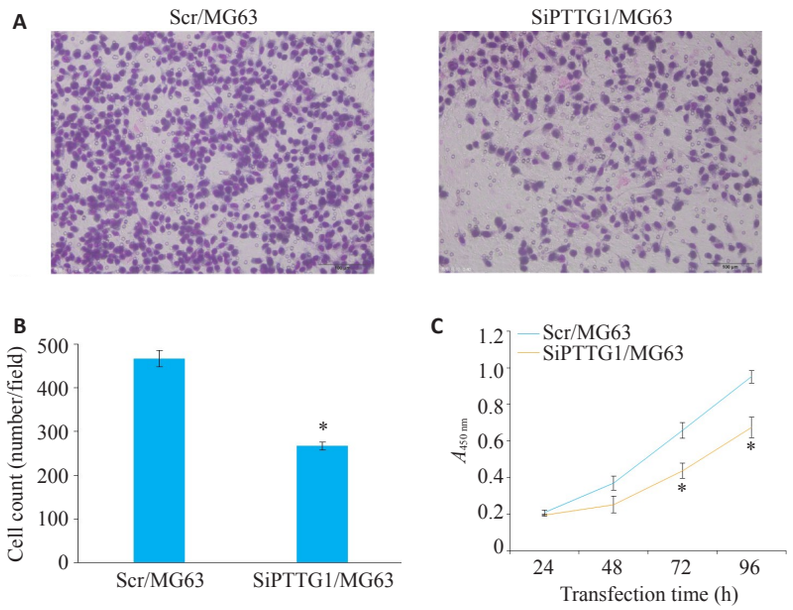

图2 敲低PTTG1抑制MG63细胞的体外侵袭和增殖能力

Fig.2 Knockdown of PTTG1 inhibits invasion and proliferation of MG63 cells. **A**, **B**: Invasion of transfected PTTG1 knockdown control group and knockdown group were determined by Transwell, Giemsa staining, scale bar=100 μm, *Mean±SD*, *n*=3, **P*<0.05 *vs* Scr/MG63 group; **C**: CCK8 assay detected the proliferation ability of each group, **P*<0.05 *vs* Scr/MG63 group.

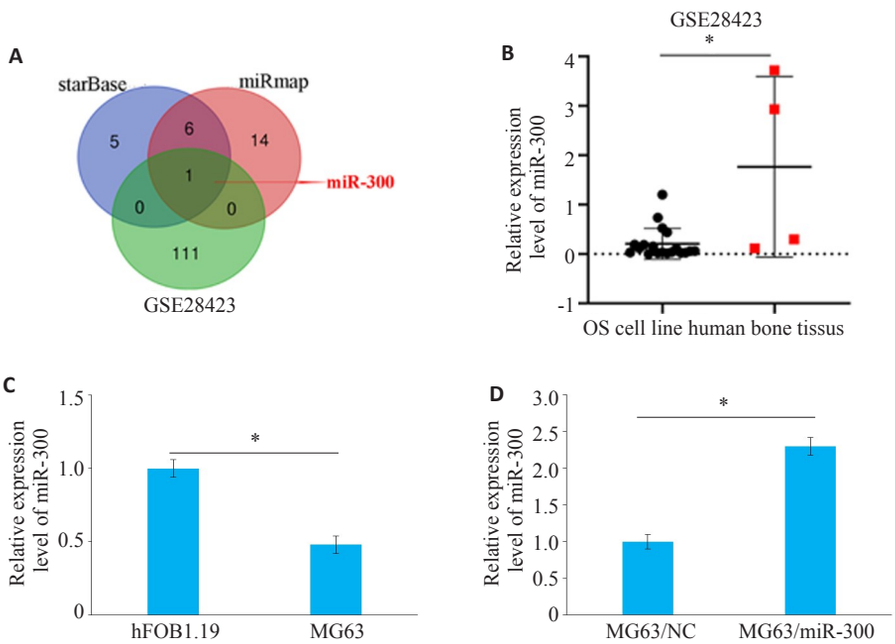

图3 miR-300是PTTG1的靶基因

Fig.3 MiR-300 is the target gene of PTTG1. A: Venny plot of predicted genes; **B**: Expression of miR-300 in OS cell line and Human Bone tissue; **C**: Expression of miR-300 in hFOB1.19 and MG63 cells; **D**: Transfection efficiency of miR-300 in MG63 cells. **P*<0.05.

合(图4C)。

2.5 miR-300靶向调控PTTG1抑制骨肉瘤细胞MG63体外侵袭和增殖能力

Transwell侵袭实验结果显示,与转染过表达miR-300质粒(126.00±17.57)相比,共转染过表达miR-300和过表达PTTG1质粒后,骨肉瘤细胞MG63的穿膜细胞数(320.00±12.47)明显增多(*P*=0.0003,图5A、B)。CCK8检测结果显示与转染过表达miR-300

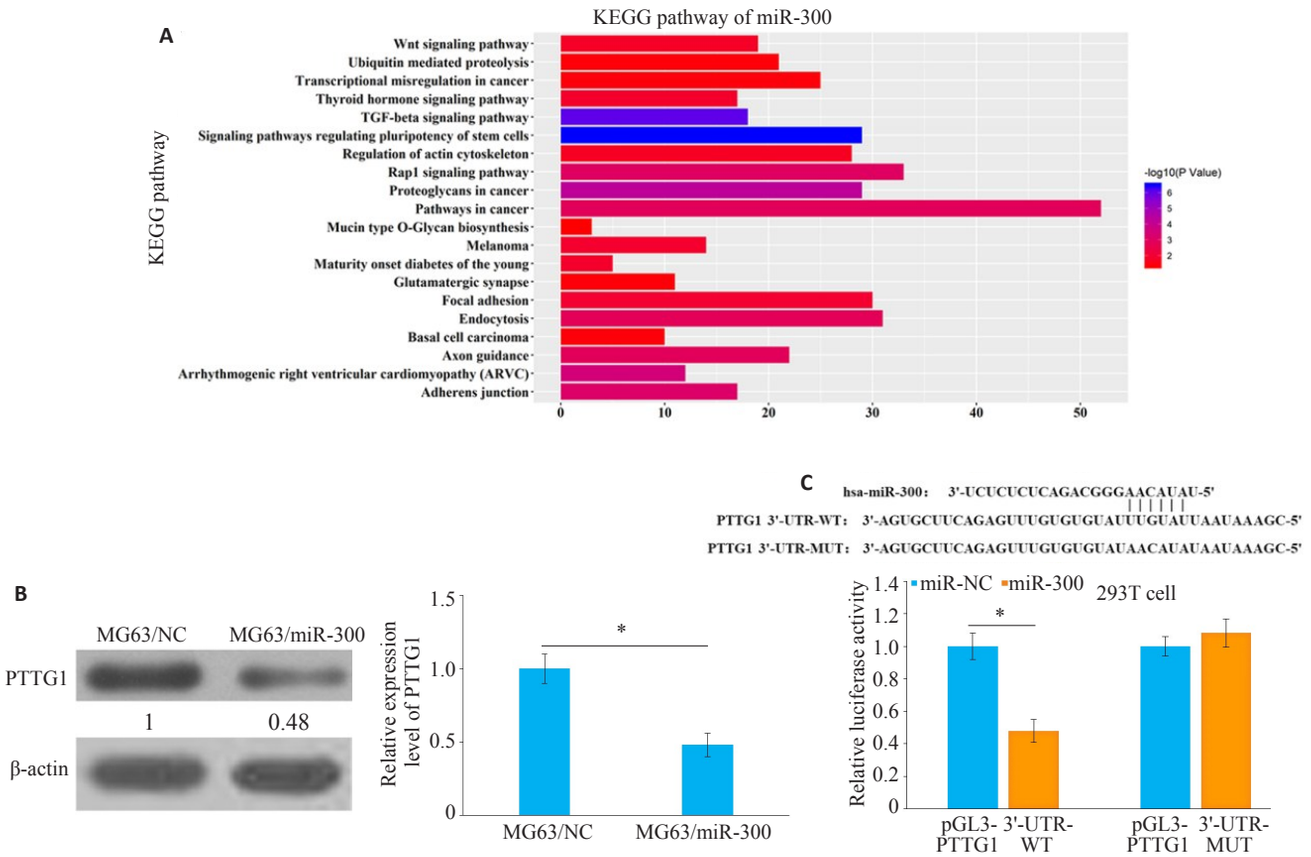

图4 miR-300与PTTG1靶向结合

Fig.4 Targeted binding of MiR-300 to PTTG1. **A**: KEGG analysis of miR-300; **B**: After overexpression of miR-300, PTTG1 expression in MG63 cells was low; **C**: The binding sites of miR-300 and PTTG1. *P<0.05.

(48 h: 0.37±0.03; 72 h: 0.43±0.02; 96 h: 0.55±0.02)相比,共转染过表达miR-300和过表达PTTG1质粒后,骨肉瘤细胞MG63的增殖能力(48 h: 0.48±0.02; 72 h: 0.66±0.04; 96 h: 0.79±0.06)明显升高,差异具有统计学意义(P=0.0077,图5C)。

3 讨论

骨肉瘤是一种来源于间充质组织的恶性骨肿瘤[14]。尽管骨肉瘤的治疗方法有手术切除,辅助化疗和放疗,但是对于转移性或复发性疾病的患者生存率仍然低于20%[15-16]。因此,研究骨肉瘤的侵袭转移的分子机制,控制肿瘤转移的发生,有助于提高患者生存率,对骨肉瘤的诊断、治疗和预后评估等具有重要的临床意义[17]。

人类PTTG家族至少含有3种同源蛋白PTTG1、PTTG2和PTTG3,其中PTTG1已被详细研究[18]。PTTG1是一种多功能蛋白,在控制有丝分裂、细胞转化、DNA修复和胎儿发育等方面发挥作用[19]。最重要的是,PTTG1在大多数正常组织中的表达受到限制。相反,它在各种内分泌相关肿瘤中大量表达,例如垂体、乳腺肿瘤和甲状腺,以及非内分泌相关的癌症,包括消化、呼吸和神经系统[20,1]。本研究通过Western blot、Transwell侵袭和CCK8实验得出,PTTG1基因可以促进MG63的侵袭和增殖能力,使得肿瘤细胞的自我更新和分化能力增强。前期研究发现通过抑制PTTG1的表达能够抑制骨肉瘤的发展[21],与本研究得出的结论一致。

越来越多的证据表明,miRNA在调节癌细胞的发展中起着重要的作用[22]。例如:miR-26a、miR-29b、miR-155-5p、miR-148a-3p等miRNA在调节骨肉瘤进展中具有重要作用[23-25];本研究还证明了miR-300可与PTTG1靶向结合,进而对骨肉瘤细胞的侵袭,转移和增殖能力产生影响。而且有研究发现miR-300表达水平的改变与肿瘤进展相关,miR-300能够通过靶向cullin 4B抑制胰腺癌的发生发展[26];miR-300可通过靶向Twist抑制头,颈鳞状细胞癌和乳腺癌细胞的上皮向间充质转化和转移[27];miR-300能够通过靶向ROS1抑制肺鳞状细胞癌细胞的增殖和侵袭[28],表明miR-300具有在多种肿瘤中均具有抑制作用[29]。本研究可以作为miR-300在肿瘤中发挥作用的一个有效补充。

综上所述,本研究证实,miR-300通过负向调控PTTG1影响骨肉瘤细胞MG63的侵袭,转移和增殖。

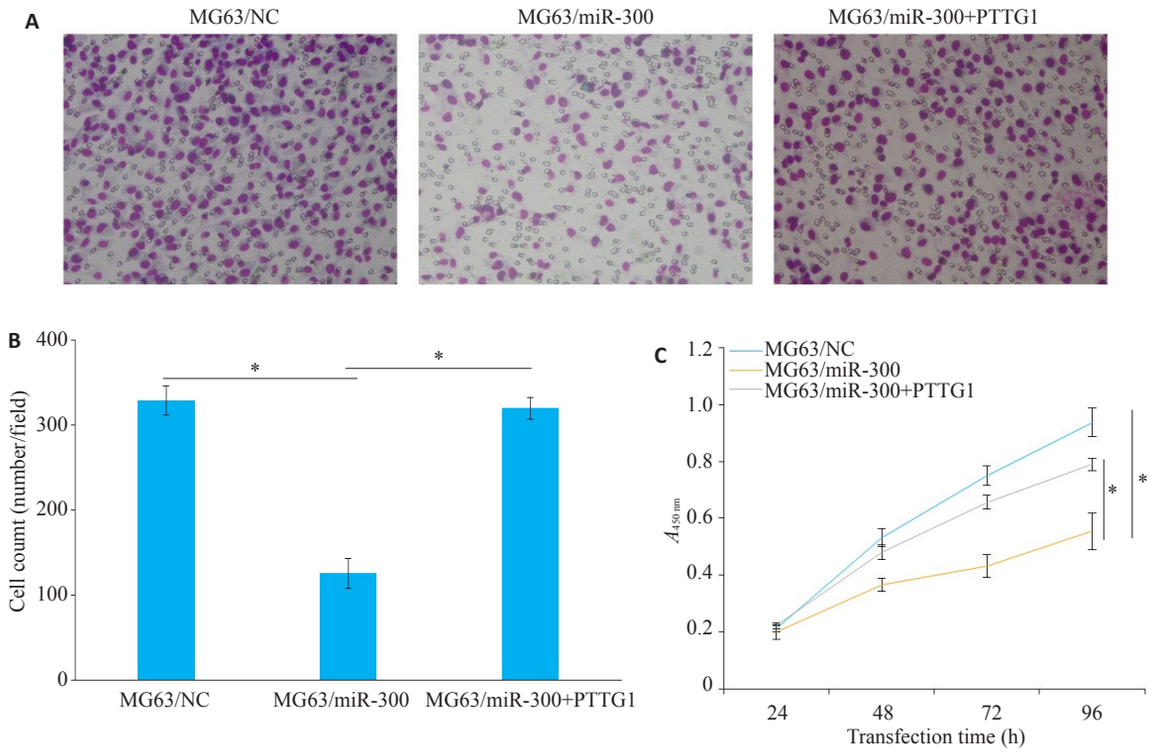

图5　miR-300靶向调控PTTG1并抑制骨肉瘤细胞MG63体外侵袭和增殖能力

Fig.5 miR-300 targets PTTG1 and inhibits the invasion and proliferation of MG63 cells. **A**, **B**: Invasion ability in different transfected cells, Giemsa staining, scale bar=100 μm, *Mean*±*SD*, *n*=3; **C**: CCK8 assay for detecting the proliferation and statistical analysis of each group. **P*<0.05.

我们的研究结果为骨肉瘤发生和发展的分子机制提供了新的见解，为临床提供了可能治疗骨肉瘤的新靶点。

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