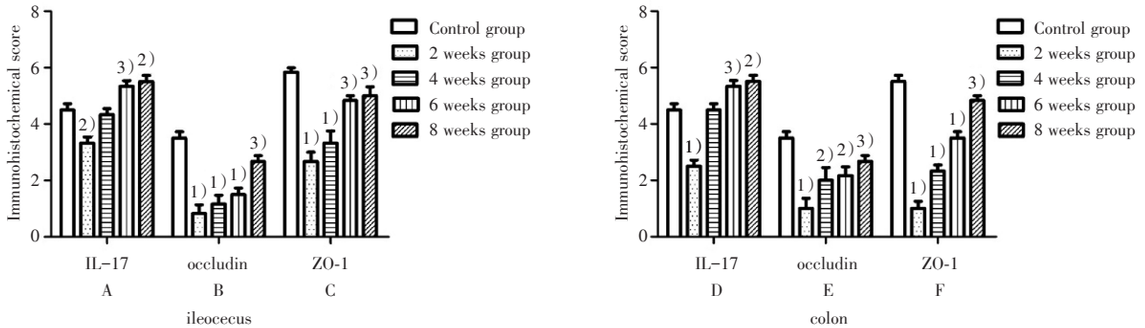
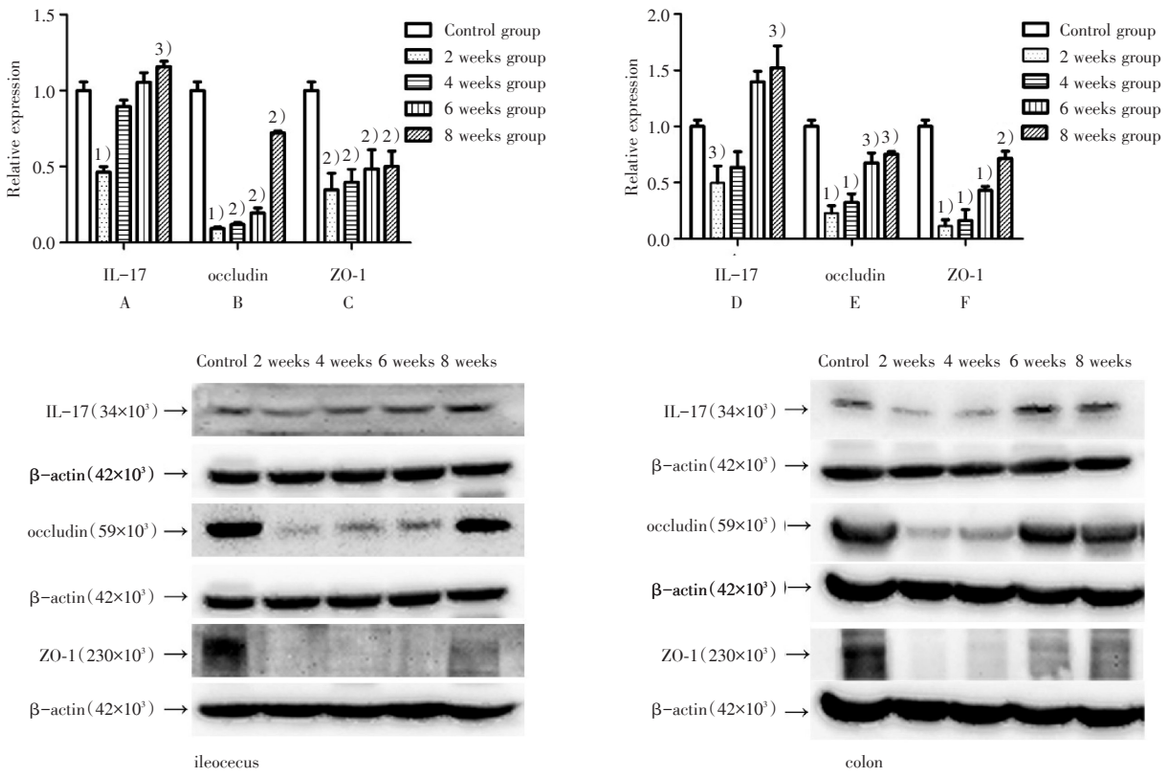


One-Way ANOVA and LSD or Dunnett's T3, n=6/group. A: F=16.250, P=0.000; B: F=18.667, P=0.000; C: F=26.458, P=0.000; D: F=29.091, P=0.000; E: F=8.059, P=0.000; F: F=69.942, P=0.000. *vs* control group: 1)P=0.000; 2)P<0.01; 3)P<0.05

图7 各组回盲部和结肠中IL-17、occludin，和ZO-1表达

Fig.7 The expressions of IL-17, occludin, and ZO-1 in ileocecus and colon in different groups



One-Way ANOVA and LSD or Dunnett's T3, n=3/group. A: F=43.113, P=0.000; B: F=545.808, P=0.000; C: F=7.508, P=0.005; D: F=11.426, P=0.001; E: F=27.734, P=0.000; F: F=39.735, P=0.000. *vs* control group: 1)P=0.000; 2)P<0.01; 3)P<0.05

图8 Western blotting检测各组回盲部和结肠中IL-17、occludin和ZO-1的表达

Fig.8 The expressions of IL-17, occludin, and ZO-1 in ileocecus and colon in different groups by Western blotting

2.6 Western blotting检测回盲部和结肠组织中IL-17、occludin和ZO-1的表达

与对照组相比，感染后2周组回盲部和结肠中IL-17显著降低(P<0.05)，随后逐渐增高，感染后8周组增高具有统计学意义(P<0.05)；感染后2周组回盲部和结肠中occludin和ZO-1显著降低，随后逐渐升高，但至感染后8周组仍低于对照组(P<0.05；图8)。

3 讨 论

本实验中，旋毛虫感染后第2周小鼠回盲部和结肠间质充血水肿，可见中性粒细胞浸润，提示肠道有急性炎症反应。至感染后第8周肠黏膜炎症基本恢复至正常，但AWR评分及粪便含水百分数仍高于对照组。上述结果提示旋毛虫感染C57BL/6小鼠8周后虽然肠黏膜炎症基本恢复至正常，但内脏高敏感及肠道通透性增加持续存在，说明该模型适合作为PI-IBS的动物模型。

本实验中，免疫组化和Western blotting检测均显示感染后2周组回盲部和结肠中IL-17显著降低。Kang等[12]发现在旋毛虫感染的第2周IL-17降低，推测IL-17的降低可能与蠕虫感染诱导的Th2优势免疫应答有关，进而抑制Th1和Th17的免疫应答，从而有助于机体排虫。随着旋毛虫离开肠道进入肌肉组织，肠黏膜炎症逐渐减轻，感染后第4周IL-17逐渐恢复。

本实验中，旋毛虫感染后第6、8周IL-17的表达高于对照组。IL-17的增加可能与PI-IBS肠黏膜屏障受损及感染造成的肠道菌群失调导致肠黏膜免疫激活有关[13-15]。从肠道急性感染至PI-IBS形成的过程中IL-17出现先降低后低度增高的动态变化，至感染后第8周小鼠造模成功时AWR评分和粪便含水百分数仍高于对照组，提示IL-17的动态改变可能参与了PI-IBS的发生发展。

肠上皮之间的紧密连接蛋白是肠黏膜机械屏障的重要组成部分，主要包括claudins（CLDN）、occludin（OCLN）、junctional adhesion molecule（JAM）、tricellulin和zonula occludens（ZO），在保护肠黏膜屏障的完整性及调节肠道通透性中起着重要作用[16]。本实验发现旋毛虫感染小鼠后第2周肠黏膜出现急性炎症反应，occludin和ZO-1的表达显著降低，随着肠黏膜炎症减轻，occludin和ZO-1的表达逐渐增高，感染后第8周肠黏膜炎症虽然已基本恢复，但occludin和ZO-1的表达仍低于对照组。提示PI-IBS模型小鼠存在肠黏膜屏障完整性的缺失，这可能是PI-IBS肠黏膜通透性增加的原因，且可能是小鼠粪便含水百分数显著增加的原因之一。

本实验中，旋毛虫感染后第2周，occludin、ZO-1和IL-17显著降低，随着肠道炎症的恢复，occludin、ZO-1和IL-17逐渐增高，occludin、ZO-1和IL-17呈同步改变。Hönzke等[17]研究发现Th2类细胞因子可以破坏紧密连接蛋白的表达，旋毛虫感染可造成肠道出现Th2反应，这可能是旋毛虫感染后第2周肠黏膜occludin和ZO-1的表达显著降低的原因之一。旋毛虫感染早期出现了以Th2占优势的免疫失衡，可能是引起感染后第2周肠黏膜IL-17和紧密连接蛋白均降低的原因。旋毛虫感染后期肠道感染恢复，肠黏膜IL-17和紧密连接蛋白逐渐恢复，但至感染后第8周紧密连接蛋白仍未完全恢复至正常。肠黏膜紧密连接蛋白的减少，肠黏膜通透性增加，肠腔内的食物、微生物、毒素等抗原易通过肠黏膜屏障激活肠黏膜免疫反应，使免疫细胞活化并释放多种细胞因子，导致肠黏膜低度炎症的形成，这可能是后期IL-17低度升高的原因；而激活的免疫细胞及释放的细胞因子可参与紧密连接蛋白的调控，使紧密连接蛋白不能完全恢复至正常，从而造成肠黏膜通透性增加、持续低度炎症的发生[7,18-19]。本实验中感染后第8周，肠黏膜炎症基本恢复至正常，但紧密连接蛋白的减少仍未恢复正常，IL-17呈低度升高状态，可能是肠黏膜屏障与肠黏膜免疫相互作用的结果。以紧密连接蛋白为代表的肠黏膜屏障受损和以IL-17为代表的肠黏膜免疫激活两者的相互作用，可能是PI-IBS粪便含水百分数增高、内脏高敏感性长期持续存在的原因之一。

综上所述，本实验观察了旋毛虫急性感染到PI-BS形成过程中IL-17、occludin和ZO-1的动态改变，发现PI-IBS同时存在肠黏膜屏障的破坏和肠黏膜低度炎症的形成，二者的改变可能是PI-IBS小鼠内脏敏感性和粪便含水百分数增高的原因之一，参与了PI- IBS的发生。但是在PI-IBS中IL-17的主要来源细胞及IL-17的具体信号传导机制，目前尚不清楚，需要进一步研究。

参考文献

[1] Lacy BE, Mearin F, Lin C, et al. Bowel disorders [J]. Gastroenterology, 2016, 150(6): 1393-1407.

[2] Porter CK, Gormley R, Tribble DR, et al. The incidence and gastrointestinal infectious risk of function-

al gastrointestinal disorders in a healthy US adult population [J]. Am J Gastroenterol, 2011, 106(1): 130-138.

[3] Holtmann GJ, Ford AC, Talley NJ. Pathophysiology of irritable bowel syndrome [J]. Lancet Gastroenterol Hepatol, 2016, 1(2): 133-146.

[4] 苏斌涛,徐宏,关飞,等. 日本血吸虫感染小鼠IL17IL23动态变化研究 [J]. 中国血吸虫病防治杂志, 2012, 24(2): 189-192.
Su BT, Xu H, Guan F, et al. Dynamic expressions of IL-17 and IL-23 in mice infected with Schistosoma ja-ponicum [J]. Chin J Schi Contl, 2012, 24(2): 189-192.

[5] 刘丹,杨波,蓝程,等. 感染后肠易激综合征小鼠肠黏膜和血中细胞因子的表达变化[J]. 中山大学学报(医学科学版), 2015, 36(6): 841-845.
Liu D, Yang B, Lan C, et al. Altered expression of intestinal mucosa and blood cytokines in post-infectious irritable bowel syndrome mouse [J]. J Sun Yat-sen Univ (Med Sci), 2015, 36(6): 841-845.

[6] 代迎欢,蓝程,刘丹,等. 感染后肠易激综合征患者临床特征和细胞因子的表达 [J]. 中山大学学报(医学科学版), 2017, 38(2): 260-266.
Dai YH, Lan C, Liu D, et al. Clinical features and expression of cytokine in post- infectious irritable bowel syndrome patients [J]. J Sun Yat-sen Univ (Med Sci), 2017, 38(2): 260-266.

[7] GonzálezCastro AM, Martínez C, SalvoRomero E, et al. Mucosal pathobiology and molecular signature of epithelial barrier dysfunction in the small intestine in Irritable Bowel Syndrome [J]. J Gastroenterol Hepatol, 2017, 32(1): 53-63.

[8] Martínez C, Vicario M, Ramos L, et al. The jejunum of diarrhea- predominant irritable bowel syndrome shows molecular alterations in the tight junction signaling pathway that are associated with mucosal pathobiology and clinical manifestations [J]. Am J Gastroenterol, 2012, 107(5): 736-746.

[9] 杨波,蓝程,周旭春. 感染后肠易激综合征小鼠Cajal间质细胞改变对肠道动力和内脏敏感性的影响[J]. 上海交通大学学报(医学版), 2014, 34(7): 978-983.
Yang B, Lan C, Zhou XC. Effects of changes of interstitial cells of Cajal from mice with post-infection irritable bowel syndrome on intestinal motility and visceral sensitivity [J]. J Shang Hai Jiao Tong Univ (Med Sci), 2014, 34(7): 978-983.

[10] Al-Chaer ED, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development [J]. Gastroenterology, 2000, 119(5): 1276-1285.

[11] Yang B, Zhou XC, Lan C. Impact of the alterations in the interstitial cells of Cajal on intestinal motility in post-infection irritable bowel syndrome [J]. Mol Med Rep, 2015, 11(4): 2735-2740.

[12] Kang SA, Cho MK, Park MK, et al. Alteration of helper T-cell related cytokine production in splenocytes during Trichinella spiralis infection [J]. Vet Parasitol, 2012, 186(3-4): 319-327.

[13] Piche T. Tight junctions and IBS-the link between epithelial permeability, lowgrade inflammation, and symptom generation? [J]. Neurogastroenterol Motil, 2014, 26(3): 296-302.

[14] 毕胜,齐宗春,马淑霞,等. 旋毛虫感染小鼠肠道菌群变化的研究 [J]. 中国微生态学杂志, 2012, 24(8): 707-710.
Bi S, Qi ZC, Ma SX, et al. Changes of intestinal flora in mice with trichinella spiralis infection [J]. Chin J Microecol, 2012, 24(8): 707-710.

[15] Jin Y, Ren X, Li G, et al. Beneficial effects of Rifaximin in post-infectious irritable bowel syndrome mouse model beyond gut microbiota [J]. J Gastroenterol Hepatol, 2018, 33(2): 443-452.

[16] Lee JS, Tato CM, Joyce-Shaikh B, et al. Interleukin-23-independent IL-17 production regulates intestinal epithelial permeability [J]. Immunity, 2015, 43(4): 727-738.

[17] Hönzke S, Wallmeyer L, Ostrowski A, et al. Influence of Th2 cytokines on the cornified envelope, tight junction proteins, and β-defensins in filaggrin-deficient skin equivalents [J]. J Invest Dermatol, 2016, 136(3): 631-639.

[18] Vanuytsel T, Vanormelingen C, Vanheel H, et al. From intestinal permeability to dysmotility: The biobreeding rat as a model for functional gastrointestinal disorders [J]. PLoS One, 2014, 9(10): 1-12.

[19] Farré R, Vicario M. Abnormal barrier function in gastrointestinal disorders [J]. Handb Exp Pharmacol, 2017, 239(23): 193-217.

(编辑 孙慧兰)

MiR-192/-215 靶向 *BIVM* 基因调控胃癌细胞生物学功能

林慧娟[1]，张江宇[1]，杨梦婷[2]，陈文静[1]，李辉斌[1]，王　健[1]，张小静[2]
(1. 广州医科大学附属广东省妇儿医院病理科，广东 广州 510000；2. 深圳大学医学部基础医学院病理学系，广东 深圳 518060)

摘　要：【目的】研究 miR-192/-215 通过靶基因 *BIVM* 调控人胃癌发生发展的机制。【方法】首先利用靶基因预测网站、基因芯片、实时荧光定量 PCR 技术筛选出 miR-192/-215 的靶基因 *BIVM*，然后构建双荧光素酶报告质粒，验证 *BIVM* 为 miR-192/-215 靶基因。随后通过转染 *BIVM*-siRNA 干扰 *BIVM* 表达后，检测其对胃癌细胞增殖、凋亡的作用。8 只裸鼠随机分为 *BIVM*-siRNA 实验组和 NSC-siRNA 对照组，将转染 *BIVM*-siRNA 的胃癌细胞种植于裸鼠皮下，观察 *BIVM* 对胃癌细胞体内成瘤能力的影响。【结果】基因芯片及荧光定量 PCR 筛选出 *BIVM* 为 miR-192/-215 的靶基因，双荧光素酶报告系统确认 miR-192/-215 靶定 *BIVM*。细胞增殖实验发现 *BIVM* 可促进胃癌细胞增殖($P<0.05$)，流式细胞凋亡实验显示 *BIVM* 能抑制胃癌细胞凋亡($P<0.05$)，小鼠成瘤实验证实 *BIVM* 可促进胃癌细胞体内生长($P<0.05$)。【结论】*BIVM* 在胃癌中发挥致癌基因的作用，miR-192/-215 调控 *BIVM* 表达从而促进胃癌的发展进程。

关键词：胃癌；miR-192/-215；*BIVM*
中图分类号：R735.2　　**文献标志码**：A　　**文章编号**：1672-3554(2018)03-0349-07

MiR-192/-215 Regulates the Biological Functions of Gastric Cancer Cells by Targeting *BIVM*

LIN Hui-juan[1], ZHANG Jiang-yu[1], YANG Meng-ting[2], CHEN Wen-jing[1], LI Hui-bin[1], WANG Jian[1], ZHANG Xiao-jing[2]
(1. Department of Pathology, Guangdong Women and Children Hospital of Guangzhou Medical University, Guangzhou 510000, China; 2. Department of Pathology, School of Medicine, Health Science Center, Shenzhen University, Shenzhen 518060, China)
Corresponding to: ZHANG Jiang-yu, E-mail: superchina2000@foxmail.com

Abstract: 【Objective】To investigate the molecular mechanism of miR-192/-215 targeting *BIVM* in human gastric cancer. 【Methods】First, *BIVM* was the target gene of miR-192/-215 screened by the target gene prediction in solico and gene microarrays. Real-time quantitative PCR verified the results of the microarrays. Then the double-luciferase reporter plasmids were constructed to test that *BIVM* was the target gene of miR-192/-215. Subsequently, *BIVM*-siRNA was transfected, to know the effects of *BIVM*-siRNA on proliferation and apoptosis of gastric cancer cells. 8 nude mice were randomly divided into two groups: *BIVM*-siRNA experimental group and NSC-siRNA control group. Gastric cancer cells transfected with BIVM-SiRNA were implanted under the skin of nude mice to observe the effect of *BIVM* on the tumorigenicity of gastric cancer cells. 【Results】*BIVM* was screened as miR-192/-215 target gene by gene microarrays and quantitative PCR. Double-luciferase reporting assays were performed to identify the *BIVM* as miR-192/-215 target gene. The cell proliferation assays showed that *BIVM* promoted the proliferation of gastric cancer cells ($P<0.05$). Test of flow cytome-

收稿日期：2018-02-26
基金项目：广东省自然科学基金(2017A030313479)
作者简介：林慧娟，在读研究生，研究方向：肿瘤病理，E-mail:linhuijuan21@163.com；张江宇，通信作者，博士，教授，主任医师，研究方向：肿瘤病理，E-mail:superchina2000@foxmail.com

try showed that *BIVM* inhibited the apoptosis of gastric cancer cells (P<0.05). Mouse tumorigenesis test confirmed that BIVM could promote gastric cancer cells growth in vivo (P<0.05).【Conclusion】*BIVM* plays a role in the carcinogenesis of gastric cancer, and miR-192/-215 targeting *BIVM* promotes the development of gastric cancer.

Key words: gastric cancer; miR-192/-215; *BIVM*

[J SUN Yat-sen Univ(Med Sci),2018,39(3):349-355]

WHO全球报告统计,胃癌的发病率排恶性肿瘤第五,病死率居第三,每年造成72万多人死亡,其中一半的病例发生在亚洲东部国家[1]。在我国胃癌的发病率和死亡率均高居第二,仅次于支气管肺癌[2],给患者家庭、社会带来沉重的经济负担。胃癌的发生与HP感染、饮食、吸烟等等密切相关。然而,其发病机制尚未完全明确,进一步探索胃癌的发生、发展过程,寻找与疾病的早期发现、诊断、治疗、预后判断相关的分子标志物,将对人类产生重要意义。微小RNA(microRNAs,miRs)最早发现于新秀丽小杆线虫[3],是一类大小为17~25个碱基的内源性非编码小RNA。miRs通过与靶基因mRNA 3′UTR区完全或不完全互补结合后,降解mRNA或者抑制蛋白质翻译从而负向调控蛋白质表达[4-5]。miRs参与细胞生命的诸多过程,包括细胞增殖、分化、凋亡等等,越来越多的证据表明miRs调节肿瘤细胞多种重要的生物学行为,与肿瘤的发生、进展和预后密切相关,是肿瘤基因诊断与治疗的潜在分子。大量文献报道发现miRs在人类癌症中存在差异性表达,提示miRs可能发挥致癌或抑癌作用。miR-192和miR-215为同源RNA,含有高度同源序列[6]。研究发现,miR-192/-215在胃癌组织中表达明显上调,促进肿瘤细胞增殖和迁移[7-9]。但是,其具体的作用机制和途径尚不清楚。碱性免疫球蛋白样可变区域(basic, immunoglobulin-like variable motif containing, *BIVM*)定位于人类第13号染色体的q32-q33区域,编码由503个氨基酸组成的碱性蛋白质[10]。*BIVM*广泛分布于人体正常组织,其5′UTR末端含一个CpG岛,因此被认为是“管家基因”[10],但是有关*BIVM*的表达、功能知之甚少,其与胃癌的关系也不甚明确。在本文中,我们提出假设,miR-192/-215通过调控靶基因*BIVM*促进胃癌细胞生长。

1 材料与方法

1.1 细胞培养与主要试剂

人正常胃黏膜上皮细胞HFE145,人胃癌细胞BGC-823、SGC-7901均为本实验室自存。胃细胞株用含10%血清(Gibco,美国)的DMEM高糖(Hyclone,美国)培养基置于37 ℃,5%CO_2恒温箱中培养。所有培养基均加入100 U/mL青霉素和100 mg/mL链霉素(Invitrogen,美国)。miR-192/-215拟似物(miR-192/-215 mimics, miR-192/-215 mim)、miR-192/-215抑制剂(miR-192/-215 inhibitors, miR-192/-215 inh)及其相应的非特异性对照均购自美国Dharmacon公司;Lipofectamine RNAiMAX、*BIVM*-siRNA、NSC-siRNA购自美国Invitrogen公司;蛋白裂解液2× Laemmli sample buffer购自美国BIO-RAD公司;*BIVM*和GAPDH抗体购自美国Cell Signaling Technology公司;逆转录、实时荧光定量PCR试剂盒、*Not*Ⅰ酶、*Xho*Ⅰ酶、*E.coli* JM109感受态细胞均购自日本TaKaRa公司;psiCHECK-2质粒为本实验室前期自存;CCK-8试剂盒购自日本Dojindo公司。

1.2 Western Blotting

细胞转染前一天,按照30%密度将细胞均匀接种至6 cm皿并于恒温箱中培养24 h。按60 nmol/L的浓度将miR-192/-215 mim、NSC-mim转染至HFE145细胞,miR-192/-215 inh、NSC-inh转染至BGC-823细胞。48 h后收集细胞,置于冰上以2× Laemmli sample buffer裂解细胞10~20 min,离心后取上清,荧光酶标仪(TECAN)测定总蛋白质浓度。取30 μg蛋白质样品加入10%聚丙烯酰胺凝胶上样孔,110 V恒压电泳,随后将蛋白质转至PVDF膜。封闭后一抗*BIVM*(1∶1 000)和GAPDH(1∶3 000)4 ℃摇床孵育过夜。TBST洗膜后二抗(1∶3 000)室温孵育1 h,发光工作液显色,凝胶成像仪读取图像。

1.3 qRT-PCR

按上述转染方法处理HFE145、BGC-823和NCI-N87细胞48 h后，TRIzol提取总RNA并测量其浓度和纯度，按照试剂盒说明书进行逆转录反应。以cDNA为模板，采用SYBR Green II荧光染料法检测*BIVM* mRNA相对表达量，GAPDH作为内参。*BIVM*正向引物：5′-GATTGAAAGATGAATCGCTGGCTTA-3′；*BIVM*反向引物：5′-TCCCCTGCTGAATGCTTTATTAGC-3′；GAPDH正向引物：5′-GACTCATGACCACAGTCCATGC-3′；GAPDH反向引物：5′-AGAGGCAGGGATGATGTTCTG-3′，重复3次实验。

1.4 质粒构建

从正常胃黏膜上皮细胞HFE145中提取基因组DNA，以正向引物：5′-ccgctcgagCATTAAATAGGAGCAGATCTTGTGG-3′及反向引物：5′-aaggaaaaaaagcggccgcATCTCCAGGATCAGTTTCTTGAGTC-3′进行PCR反应扩增大小为526 bp的野生型*BIVM*-3′UTR，产物电泳、切胶回收。*Not*I、*Xho*I同时37 ℃过夜双酶切*BIVM*-3′UTR产物和psiCHECK-2双荧光素酶报告质粒，T4连接酶16 ℃过夜连接*BIVM*-3′UTR及psiCHECK-2。连接产物转化感受态大肠杆菌，摇菌、提质粒及基因测序，得到野生型*BIVM*-3′UTR质粒（psi-BIVM-wt）。随后以突变型*BIVM*-3′UTR正向引物：5′-AATTGGGGCCTTCGATTTCTAGTTTTCACTCACGGTTAACAC-3′，反向引物：5′-TTTAAATGCGTGTTAACCGTGAGTGAAAACTAGAAATCGAAG-3′按上述方法构建突变型*BIVM*-3′UTR质粒（psi-BIVM-mut）。

1.5 细胞增殖实验

实验前一天将BGC-823和SGC-7901胃癌细胞按照30%密度均匀接种于6孔细胞培养板，并设置5个复孔，共3板。细胞过夜贴壁后取其中1板，更换100 μL新鲜培养基后向每个实验孔加入11 μL CCK-8试剂，置于37 ℃培养箱中孵育合适时间后取出，使恢复常温后酶标仪测定读数作为空白组。另外2板按上述方法转染60 nmol/L *BIVM*-siRNA（实验组）和NSC-siRNA（对照组）后，37 ℃分别培养48 h、72 h后更换100 μL新鲜培养基，加入11 μL CCK-8试剂于酶标仪测定读数。实验重复3次，计算细胞存活率 =［（实验组-空白组］/（对照组-空白组）］×100%，作出生长曲线图。

1.6 细胞凋亡检测

依据前述方法将*BIVM*-siRNA、NSC-siRNA均分别转染至HFE145和BGC-823细胞。48 h后，ACCUTASE消化贴壁细胞并离心，1×Annexin-binding buffer重悬细胞至1×10^6个/ mL。取5 μL Annexin V-FITC染液及1 μL 100 μg/mL PI染液加入100 μL细胞悬液，混匀后室温静置15 min。分别设置空白对照管、Annexin V-FITC单染对照管、PI单染对照管、双染阴性对照管，准备好样品后流式细胞仪上机检测。实验重复3次。

1.7 小鼠体内成瘤实验

4～5周龄健康雌性BALB/c-nu裸鼠8只购买自北京维通利华实验动物技术有限公司，实验动物生产许可证编号为SCXK（京2012-0001），本实验获得了深圳大学医学部实验动物伦理委员会批准。全部裸鼠饲养于深圳大学实验动物中心SFP级动物房，随机分成NSC-siRNA组、*BIVM*-siRNA组，每组各4只。按照前述方法分别转染60 nmol/L NSC-siRNA和*BIVM*-siRNA至SCG-7091胃癌细胞，48 h后收集细胞，预冷PBS稀释成5×10^6个/mL单细胞悬液，半小时内将细胞悬液注射至裸鼠右下背侧皮下，细胞总数约为1×10^6个。每隔3～4 d测量瘤体大小，按公式“长径×短径×短径/2”计算肿瘤体积，连续观察30 d。

1.8 统计学处理

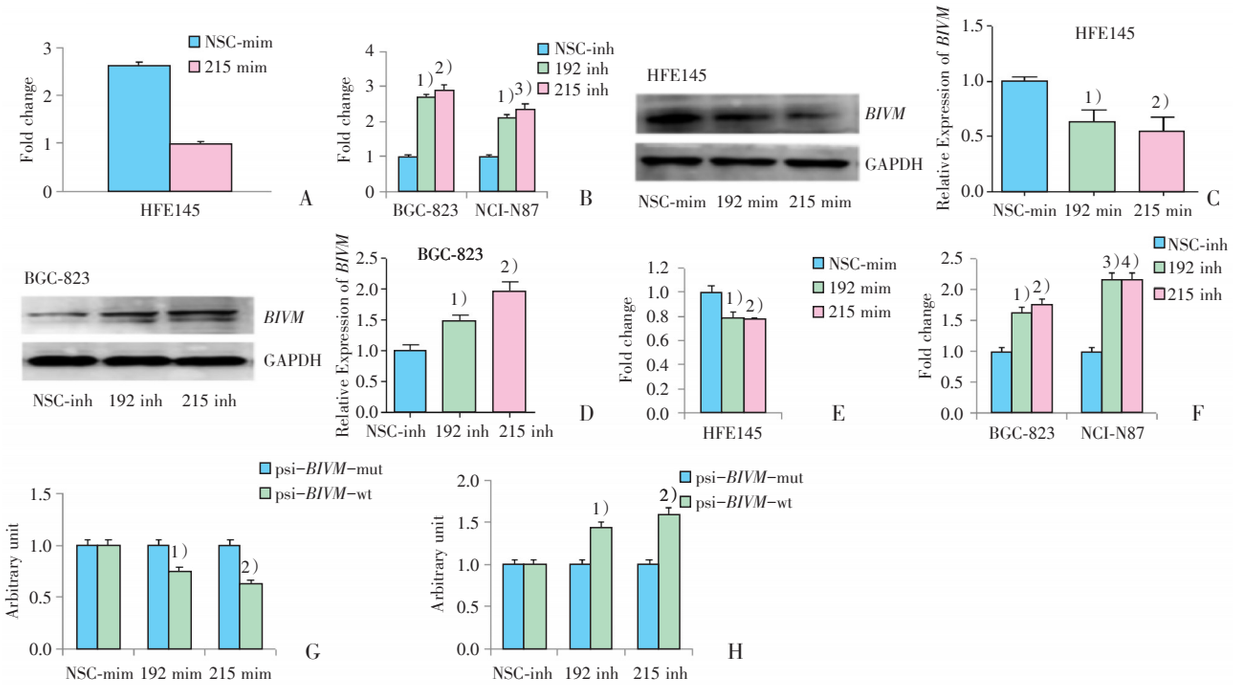
采用SPSS 16. 0和GraphPad Prism 5统计软件分析数据，两组间均数比较先进行正态分布和方差齐性检验，方差齐采用独立样本t检验，方差不齐采用校正的t检验。动物实验采用析因设计资料的方差分析，检验水准α=0.05。qRT-PCR结果以Folds=$2^{-\Delta\Delta Ct}$比较目的基因表达水平，其中ΔΔCt=（$Ct_{(目的基因)}$-$Ct_{(内参)}$）实验组-（$Ct_{(目的基因)}$-$Ct_{(内参)}$）对照组。

2 结　果

2.1 *BIVM*是miR-192/-215靶基因

本课题组首先在miRBase（http://microrna.sanger.ac.uk/cgi-bin/targets/v4/search.pl），TargetScan（http://www.targetscan.org/vert_40/），PicTar（http://pictar.bio.nyu.edu/），microRNA.org（http://www.microrna.org/microrna/home.do）等相关搜索引擎预测miR-192/-215靶基因。随后，分别转染miR-192/-215 mim至HFE145、转染miR-192/-215 inh至BGC-823和NCI-N87细胞中，利用基因表达谱芯片测试比较胃细胞株中*BIVM* mRNA表达水平，

发现转染miR-215 mim后*BIVM* mRNA表达下调，差异具有统计学意义（*P*=0.004；图1A），转染miR-192 mim后*BIVM* mRNA表达无明显变化（数据未显示），而转染miR-192/-215 inh后*BIVM* mRNA表达水平均明显提高，差异具有统计学意义（*P*<0.05图1B），由此初步筛选出*BIVM*为miR-192/-215的潜在靶基因。此外，通过分别转染miR-192/-215 mim至HFE145、转染miR-192/-215 inh至BGC-823和NCI-N87细胞中检测*BIVM*蛋白质与mRNA表达量，我们发现与对照组相比，转染miR-192/-215 mim后，*BIVM*蛋白质及mRNA表达水平均明显下调，而转染miR-192/-215 inh后，*BIVM*蛋白质和mRNA表达均显著增加，差异均具有统计学意义（*P*<0.05；图1C-F）。这些结果说明miR-192/-215可调控*BIVM*蛋白质和RNA的表达。随后，我们构建了双荧光素酶报告质粒以进一步验证*BIVM*是miR-192/-215靶基因。结果显示，在HFE145细胞中共转染psi-*BIVM*-wt和miR-192/-215 mim后，miR-192/-215能抑制*BIVM*蛋白表达，从而使荧光素酶活性均显著降低，而共转染psi-*BIVM*-mut及miR-192/-215 mim后荧光素酶活性改变不明显，差异具有统计学意义（*P*<0.05；图1G）。在BGC-823细胞中共转染psi-*BIVM*-wt和miR-192/-215 inh后，miR-192/-215对*BIVM*的抑制作用减弱，从而使荧光素酶活性均明显增高，共转染psi-*BIVM*-mut及miR-192/-215 inh后荧光素酶活性则无明显改变，差异具有统计学意义（*P*<0.05；图1H）。上述结果说明miR-192/-215可直接结合*BIVM* 3′UTR进而调控*BIVM* mRNA表达，即*BIVM*是miR-192/-215的靶基因。



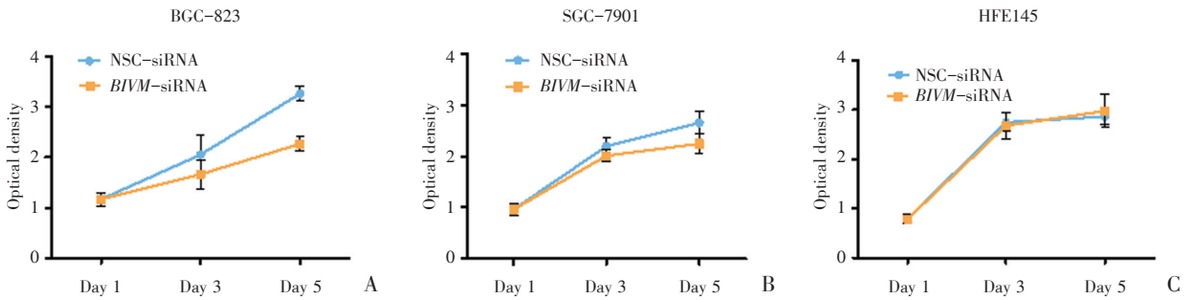
A：*t*=-16.541，*P*=0.004 *vs.* NSC-mim. B：BGC-823 cells：*t*=9.735，1）*P*=0.001 *vs.* NSC-inh；*t*=-22.620，2）*P*<0.001 *vs.* NSC-inh；NCI-N87 cells：*t*=-9.673，1）*P*=0.001 *vs.* NSC-inh；*t*=-7.542，3）*P*=0.002 *vs.* NSC-inh. C：*t*=4.724，1）*P*=0.042 *vs.* NSC-mim；*t*=5.081，2）*P*=0.037 *vs.* NSC-mim. D：*t*=-62.321，1）*P*<0.001 *vs.* NSC-inh；*t*=-8.788，2）*P*=0.013 *vs* .NSC-inh. E：*t*=4.623，1）*P*=0.01 *vs.* NSC-mim；*t*=8.20，2）*P*=0.001 *vs.* NSC-mim. F：BGC-823 cells：*t*=-6.395，1）*P*=0.024 *vs.* NSC-inh；*t*=-3.467，2）*P*=0.026 *vs.* NSC-inh；NCI-N87 cells：*t*=-5.968，3）*P*=0.004 *vs.* NSC-inh；*t*=-8.696，4）*P*=0.001 *vs.* NSC-inh. G：192 mim，*t*=2.864，1）*P*=0.011 *vs.* psi-*BIVM*-mut；215 mim，*t*=3.875，2）*P*=0.001 *vs.* psi-*BIVM*-mut. H：192 inh，*t*=-3.257，1）*P*=0.005 *vs.* psi-*BIVM*-mut；215 inh，*t*=-5.604，2）*P*<0.001 *vs.* psi-*BIVM*-mut. *n*=3

图1 *BIVM*是miR-192/-215靶基因

Fig.1 *BIVM* is the target gene of miR-192/-215

2.2　*BIVM*促进胃癌细胞增殖

*BIVM*在胃细胞中的生物学功能尚不清楚，本课题组通过在HFE145、BGC-823、SGC-7901细胞株中转染*BIVM*-siRNA干扰*BIVM*的正常表达，探究*BIVM*在胃细胞中扮演的角色。结果显示，与对照组NSC-siRNA相比，下调*BIVM*表达使BGC-823（P=0.005；图2A）和SGC-7901（P=0.036；图2B）胃癌细胞增殖明显受抑。在正常胃黏膜上皮细胞HFE145中，下调*BIVM*表达对细胞增殖无显著影响（P>0.05；图2C）。因此我们初步认为*BIVM*在胃癌中是一个促癌基因。



A：BGC-823 cells，t=11.257，P=0.005；B：SGC-7901，t=3.362，P=0.036；C：HFE145 cells，t=-0.534，P > 0.05. n=3

图2　CCK-8试剂盒检测*BIVM*对胃细胞增殖能力的影响

Fig.2　Effect of *BIVM* on the proliferation of gastric cells by CCK-8 kit

2.3　*BIVM*抑制胃癌细胞凋亡

为了研究*BIVM*在细胞凋亡方面所起的作用，本课题组在BGC-823和HFE145细胞中分别转染60 nmol/L *BIVM*-siRNA和NSC-siRNA 48 h后利用流式细胞仪检测细胞凋亡情况。我们发现转染*BIVM*-siRNA后，BGC-823细胞中早期凋亡和晚期凋亡的细胞数比对照组显著增多，差异具有统计学意义（P=0.001；图3A），而在HFE145细胞中转染*BIVM*-siRNA与NSC-siRNA后，细胞凋亡数无明显差异（P > 0.05；图3B）。这些结果提示*BIVM*可通过抑制胃癌细胞凋亡，进而发挥致癌的作用。

2.4　*BIVM*促进体内成瘤

既然体外实验显示*BIVM*可促进胃癌的发生、发展，那其在体内是否也产生同样的作用呢？本课题组将*BIVM*-siRNA和NSC-siRNA分别转染至SCG-7091细胞，收集细胞并移植于裸鼠皮下，记录小鼠肿瘤体积（表1）。析因设计资料的方差分析结果显示：NSC-siRNA和*BIVM*-siRNA两组间肿瘤体积有显著性差异（F=44.905，P<0.001），生长时间水平有显著性差异（F=23.045，P<0.001），时间与分组之间交互效应显著（F=8.203，P<0.001）。两两比较后发现，*BIVM*-siRNA组裸鼠肿瘤的生长速度较NSC-siRNA组偏慢，且体积明显减小，差异具有统计学意义（P<0.05；图4）。结果说明*BIVM*-siRNA能抑制胃癌细胞在裸鼠体内的皮下成瘤能力，进一步证明*BIVM*促胃癌形成的作用。

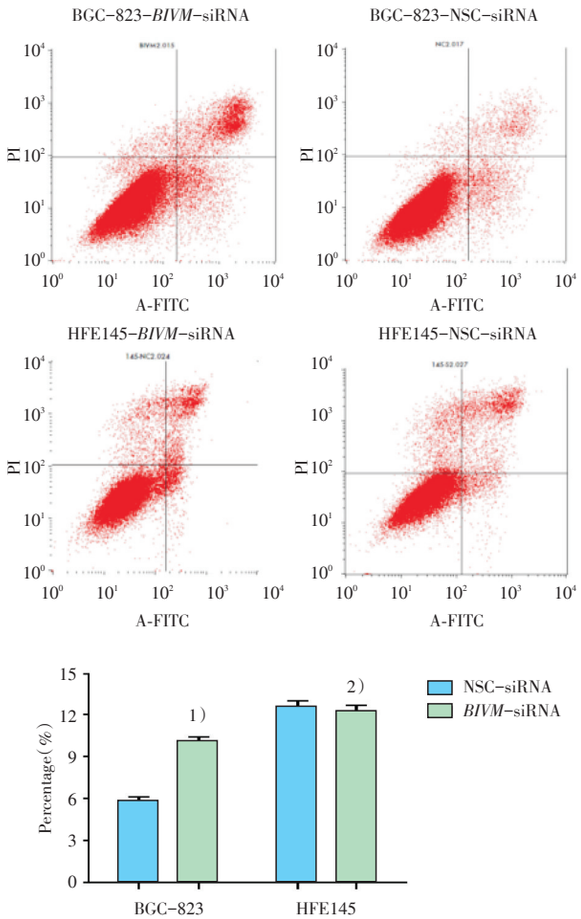
表1　不同处理组比较裸鼠肿瘤体积的差异

Table 1　Comparison of tumor volume between different treatment groups in nude mice　（$\bar{x} \pm s$）

Day	NSC-siRNA	*BIVM*-siRNA	*P* value
1	0	0	/
7	7.834±3.756	0.8445±0.07	<0.0001
11	26.71±13.00	3.515±1.04	0.0017
15	57.96±21.26	10.95±3.89	0.0196
19	163.7±79.57	25.73±7.56	0.0029
23	296.1±128.42	74.06±24.62	0.0225
28	615.3±345.30	140.3±49.31	0.0095
32	911.4±324.41	231.5±87.39	0.041

3　讨　论

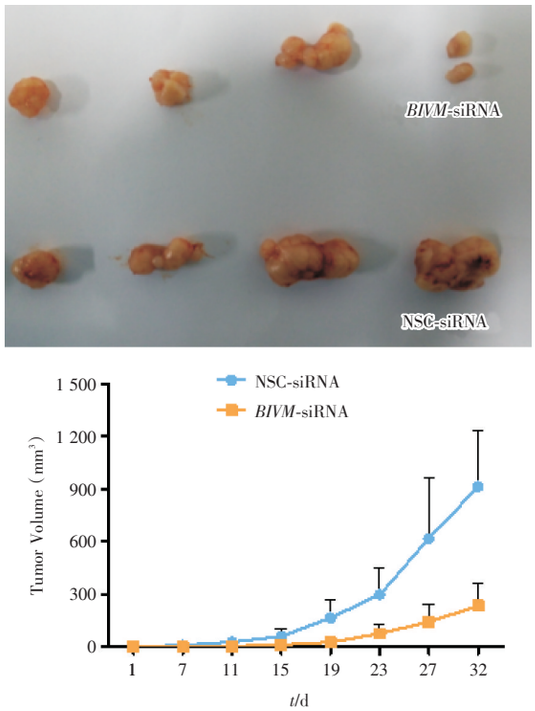
miRs是肿瘤发病机制研究中的一大发现，深入研究miRs的生物学功能使我们更全面了解肿瘤发生发展，为寻找新的生物标志物和治疗靶点提供基础。一个miRs家族可以靶定多个基因，同一个mRNA也可以被一个或多个miRs靶向结合[11]。



BGC-823 cells, t=-7.814, 1) P=0.001 *vs.* NSC-siRNA; HFE145 cells, t=0.543, 2) P>0.05 *vs.* NSC-siRNA. n=3

图3 流式细胞仪检测*BIVM*对细胞凋亡的影响

Fig.3 The effects of *BIVM* on the apoptosis of cells by flow cytometry



BIVM-siRNA vs. NSC-siRNA, n=4, t=3.265, P<0.05

图4 动物实验观察*BIVM*对胃癌细胞体内成瘤能力的影响

Fig.4 Effect of *BIVM* on the tumorigenicity of gastric cancer cellsin vivo

miR-192和miR-215同属于miR-192家族，其基因序列仅相差2个碱基[6]。miRs的表达具有组织特异性，在不同组织中可以扮演致癌miRs或者抑癌miRs的角色。研究报道miR-192/-215在食管鳞状细胞癌[12]、神经胶质瘤[13]、肝癌[14]、胰腺导管腺癌[15]中异常升高，具有促癌作用；在结肠癌[16]、肾细胞癌[17]、膀胱癌[18]、多发性骨髓瘤[19]中则表达下调，抑制肿瘤进程。在胃癌研究方面，多个课题组[7,9,20]报道miR-192/-215在胃癌组织中高表达，可促进胃癌生长。本课题组首先通过靶基因预测引擎及芯片技术初步筛选出*BIVM*为miR-192/-215潜在靶基因。在上调miR-192/-215表达后可发现*BIVM*蛋白质及mRNA表达均受到抑制，相反，下调miR-192/-215使*BIVM*蛋白质和mRNA表达显著增加，这些结果强烈提示miR-192/-215可调控*BIVM*的表达。最后，双荧光素酶报告系统证实*BIVM*为miR-192/-215调控靶点。目前对*BIVM*的研究甚少，Yoder等[10]在2002年报道*BIVM*为“管家基因”，其蛋白定位于细胞核和细胞浆，提示它可能通过与其他蛋白质或DNA相互作用而调控细胞生物过程。由此我们推测miR-192/-215通过靶基因*BIVM*调控胃细胞生物学功能。在细胞增殖实验中我们发现，下调*BIVM*表达可抑制胃癌细胞生长，而对正常胃黏膜上皮细胞无明显作用，这可能是由于*BIVM*在胃癌细胞与正常细胞中的表达量不一致导致的。同样，细胞凋亡实验也显示抑制*BIVM*表达可使胃癌细胞凋亡明显增加而不影响正常胃黏膜上皮细胞。另外，我们还检测了*BIVM*对细胞周期的作用，结果表明*BIVM*并不影响细胞周期(数据未显示)。综合上述体外细胞实验，我们认为*BIVM*在胃癌中发挥了促癌基因的作用。最后通过构建小鼠成瘤模型，从体内实验进一步验证了我们的猜想，即miR-192/-215通

过调控靶基因*BIVM*促进胃癌的发生发展。

尽管现代医疗水平不断提高，但胃癌对人类健康造成的危害较大，胃癌的治疗及预后并不乐观，探索胃癌早期发现、有效治疗、判断预后的方法是我们不断前进的目标。本课题组通过一系列实验提出一个新的胃癌发病机制，即miR-192/-215通过调控靶基因*BIVM*促进胃癌发生、进展，将为寻找胃癌分子标志物提高新的思路与方向。

参考文献

[1] Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in Globocan 2012 [J]. Int J cancer, 2015, 136(5): e359-386.

[2] Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015 [J]. CA, 2016, 66(2): 115-132.

[3] Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14[J]. Cell, 1993, 75(5): 843-854.

[4] Bartel DP. MicroRNAs: Target recognition and regulatory functions [J]. Cell, 2009, 136(2): 215-233.

[5] Garzon R, Calin GA, Croce CM. MicroRNAs in cancer [J]. Annu Rev Med, 2009, 60(1): 167-179.

[6] Sun Y, Koo S, White N, et al. Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs [J]. Nucleic Acids Res, 2004, 32(22): 37-42.

[7] Chen Q, Ge X, Zhang Y, et al. Plasma miR-122 and miR-192 as potential novel biomarkers for the early detection of distant metastasis of gastric cancer [J]. Oncol Rep, 2014, 31(4): 1863-1870.

[8] Jin Z, Selaru FM, Cheng Y, et al. MicroRNA-192 and -215 are upregulated in human gastric cancer in vivo and suppress ALCAM expression in vitro [J]. Oncogene, 2011, 30(13): 1577-1585.

[9] Xu YJ, Fan Y. MiR-215/192 participates in gastric cancer progression [J]. Clin Transl Oncol, 2015, 17(1): 34-40.

[10] Yoder JA, Hawke NA, Eason DD, et al. BIVM, a novel gene widely distributed among deuterostomes, shares a core sequence with an unusual gene in Giardia lamblia [J]. Genomics, 2002, 79(6): 750-755.

[11] Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer rationale, strategies and challenges [J]. Nat Rev Drug Discov, 2010, 9(10): 775-789.

[12] Li S, Li F, Niu R, et al. Mir-192 suppresses apoptosis and promotes proliferation in esophageal aquamous cell caicinoma by targeting Bim [J]. Int J Clin Exp Pathol, 2015, 8(7): 8048-8056.

[13] Tong YQ, Tan BH, Liu B, et al. MiR-215, an activator of the CTNNBIP1_β-catenin pathway, is a marker of poor prognosis in human glioma [J]. Oncotarget, 2015, 6(28): 25024-25033.

[14] Gui J, Tian Y, Wen X, et al. Serum microRNA characterization identifies miR-885-5p as a potential marker for detecting liver pathologies [J]. Clin Sci, 2011, 120(5): 183-193.

[15] Zhao C, Zhang J, Zhang S, et al. Diagnostic and biological significance of microRNA-192 in pancreatic ductal adenocarcinoma [J]. Oncol Rep, 2013, 30(1): 276-284.

[16] Braun CJ, Zhang X, Savelyeva I, et al. p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest [J]. Cancer Res, 2008, 68(24): 10094-10104.

[17] Khella HW, Bakhet M, Allo G, et al. miR-192, miR-194 and miR-215: A convergent microRNA network suppressing tumor progression in renal cell carcinoma [J]. Carcinogenesis, 2013, 34(10): 2231-2239.

[18] Jin Y, Lu J, Wen J, et al. Regulation of growth of human bladder cancer by miR-192 [J]. Tumour Biol, 2015, 36(5): 3791-3797.

[19] Pichiorri F, Suh SS, Rocci A, et al. Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development [J]. Cancer cell, 2010, 18(4): 367-381.

[20] Deng Y, Huang Z, Xu Y, et al. MiR-215 modulates gastric cancer cell proliferation by targeting RB1 [J]. Cancer letters, 2014, 342(1): 27-35.

（编辑　孙慧兰）

·临床研究·

复合声治疗对不同类型听阈正常耳鸣患者的疗效观察

李昌武[1,2,3]，熊观霞[1,2]，王仙仁[1,2]，姜鸿彦[1,2,3]
(1. 中山大学附属第一医院耳鼻咽喉头颈外科，广东 广州 510080；2. 中山大学附属第一医院耳鼻咽喉科研究所，广东 广州 510080；3. 海南省人民医院，海南 海口 570311)

摘　要：【目的】探讨复合声对听阈正常耳鸣患者的疗效，为纯音听阈正常耳鸣患者治疗提供参考。【方法】收集 2014 年 9 月 1 日-2015 年 12 月 30 日间中山大学附属第一医院门诊就诊的纯音测听听阈正常的 46 例耳鸣患者，耳鸣持续时间>3 个月。详细收集患者病例资料如年龄、性别、耳鸣特点、伴随症状等，并行纯音测听、耳鸣测听检查，以及耳鸣致残量表(THI)评分和视觉模拟评分(VAS)评分。给予复合声治疗，选取主频与耳鸣频率一致的自然声，并挑选音乐主频覆盖耳鸣频率中心的音乐片段，以选出的掩蔽自然声与音乐材料片段进行叠加重合，对耳鸣患者进行声治疗并评估疗效。【结果】46 例耳鸣患者中 32 例后效抑制阳性，其治疗前 THI 评分平均为(33.38±16.23)分，VAS 评分平均为(4.38±1.62)分；经复合声治疗后 THI 评分为(21.75±11.67)分，VAS 评分为(2.97±1.06)分；14 例后效抑制阴性的患者，治疗前 THI 评分平均为(29.86±20.15)分，VAS 评分平均为(3.93±1.69)分；复合声治疗后 THI 评分为(23.43±16.29)分，VAS 评分为(3.36±1.2)分。后效抑制阳性组声治疗前后 THI 评分和 VAS 评分显著降低，后效抑制阴性组声治疗也有效，效果不如后效抑制阳性组。【结论】复合声对听力正常耳鸣患者有效，后效抑制阳性的患者复合声治疗效果较好，而后效抑制阴性的患者声治疗效果不明显。

关键词：复合声治疗；耳鸣；后效抑制
中图分类号：R764.45　　**文献标志码**：A　　**文章编号**：1672-3554(2018)03-0356-07

Tailor-made Complex Sound Alleviates both Residual-Inhibition Positive and Negative Tinnitus Patients with Normal Hearing Audiogram

LI Chang-wu[1,2,3], XIONG Guan-xia[1,2], WANG Xian-ren[1,2], JIANG Hong-yan[1,2,3]
(1.Department of Otorhinolaryngology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China; 2.Institute of Otorhinolaryngology, Sun Yat-sen University, Guangzhou 510080, China; 3.Hainan General Hospital, HaiKou 570311, China)
Corresponding to: JIANG Hong-yan; E-mail: jhongy@mail.sysu.edu.cn

Abstract: 【Objective】To evaluate the efficacy of sound therapy and provide a kind of treatment for tinnitus patients with normal audiogram. 【Methods】From the database of the Audiometric Center of the First Affiliated Hospital, Sun Yat-sen University, we identified 46 outpatients with normal hearing thresholds in the conventional pure-tone audiometry suffering subjective tinnitus over 3 months. The clinical information were collected with respect of age, gender, tinnitus severity, pitch, laterality and duration, comorbid symptoms. All the patients received the tinnitus test, including loudness and frequency of the tinnitus and residual inhibition, Tinnitus Handicap Inventory (THI) and Visual Analogue Scale (VAS) scores pre- and post- complex sound therapy. The complex sound were remixed with natural sounds and music materials, while the masking frequency of natural sounds is consistent with the tinnitus frequency of patients and the main frequency of music materials covers the tinnitus frequency. 【Results】32 of the 46 patients showed residual inhibition (RI) positive. The average THI score before treatment was 33.38±16.23 and the VAS score was 4.38±1.62. The THI score

收稿日期：2018-01-14
基金项目：国家自然科学基金青年项目(81200748)
作者简介：李昌武，硕士研究生，研究方向：耳鼻咽喉科学，E-mail：lichw6@163.com；姜鸿彦，通信作者，主任医师，博士生导师，E-mail：jhongy@mail.sysu.edu.cn

decreased to 21.75±11.67 and the VAS score was 2.97±1.06 after sound therapy. The rest 14 patients were residual inhibition (RI) negative. The THI score were 29.86±20.15 and 23.43±16.29 pre- and post- complex sound therapy, while the VAS score were 3.93±1.69 and 3.36±1.2. The THI and VAS scores were significantly decreased after the treatment in both RI positive and negative patients. The RI positive group showed better results than RI negative group.【Conclusions】Both RI positive and negative tinnitus with normal audiometry shown adaptation with complex sound therapy, RI positive patients acquired more benefits from tailor-made complex sound therapy.

Key words: complex sound therapy; tinnitus; residual inhibition

[J SUN Yat-sen Univ(Med Sci),2018,39(3):356-362]

Subjective tinnitus is the perception of a sound in the absence of a corresponding external sound source, and is therefore considered as an auditory phantom percept[1]. It is a common problem for millions of individuals and have serious negative effects on their quality of life. Approximately 10% of adult suffered from tinnitus in the Unit State[2]. It has been said that tinnitus has emerged mainly from the auditory nerve to the brain associated with hearing loss[3-4], but many tinnitus patients also present with a normal audiogram which represent with no direct signs of hearing impairment. It have been supposed that there might be different tinnitus generating mechanisms between hearing loss and normal hearing[5]. The relationship between hearing loss and tinnitus has been extensively researched[6], many difference treatment methods were developed for these part patients. There is rarely research focus on the methods for treatment of the tinnitus patients with normal audiogram, especially sound therapy, even do not know which factors related the treatment outcomes.

A lot of methods were developed for tinnitus therapy, such as sound music therapy[7], Cognitive behavioral therapy[8-9], Transcranial magnetic stimulation[10-11] and medical therapy. However, most of these are focus on the tinnitus with hearing loss, only very few concerns about of normal audiogram tinnitus therapy[12]. Sound therapy has a lot of forms, such as noise, pure tone, music, notched-music, TRT and so on. Here are many different opinions on tinnitus with sound therapy, someone suggest only optional for bothersome tinnitus, the others recommend for mild tinnitus[13-14]. There are much fewer articles talking about sound therapy on tinnitus patients with normal audiogram.

Damage of partly hair cells and/or auditory nerve fibers may affect the auditory system without causing hearing loss. In this study, we wanted to explore the relationship between effects of therapy and residual inhibition. Whether there is a connection between hair cells and/or auditory nerve fibers and residual inhibition needs to research further.

1 Methods

1.1 Participants

Clinical and audiometric data were obtained from the Audiometric Center of the First Affiliated Hospital, Sun Yat-sen University. Data were analyzed from patients with normal pure-tone audiometry (PTA) experiencing persistent subjective tinnitus from 2014.09-2015.12, who had been informed consent for data analyzing. All patients wereable to communicate and cooperate, no trauma and ear surgery history, no central nervous system diseases and diabetes, hypertension and other cardiovascular and cerebrovascular diseases. Inclusion criteria were (1) normal otoscopic finding; (2) middle ear status of type A tympanograms; (3) no significant air-bone gap at tested frequencies (between 0.25 and 8 kHz).

1.2 Audiological examination

Conventional pure-tone audiometry was performed in the examining room with a Madsen Conera audiometer (GN Otometrics, Denmark) with a Sennheiser HDA200 supra-aural headphones (Sennheiser, Wademark, Germany), the test ambient noise

< 30 dB SPL. The hearing threshold for all frequencies was determined by a standard Hughson-Westlake procedure (steps: 10 dB down, 5 dB up; 2 out of 3). The mean hearing level (dB HL) was calculated by averaging thresholds ≥25 dB HL for both ears measured in PTA. The threshold of each frequency of PTA ≤25 dB HL is defined as the normal hearing threshold.

1.3 Tinnitus matching

Tinnitus detection using micro-Digital Technology Co., Ltd. TTS-1000 tinnitus diagnosis and treatment instrument, the application of Bo Chi medical technology TinniFit tinnitus detection template for tinnitus test. The tinnitus matching includes the tinnitus frequency, the tinnitus threshold, the tinnitus loudness, and the minimum masking level, the residual inhibition test and the Feldmann masking curves.

1.4 Tinnitus questionnaire

The patients were required to fill up the Tinnitus Handicap Inventory (THI) questionnaire and visual analogue scale (VAS) truthfully according to their own feelings (see schedules). The Tinnitus Handicap Inventory questionnaire has 25 questions and is divided into three sub-modules, respectively, for functional F, emotional E, and severity C. Each question has option of "yes" (4 points), "sometimes" (2 points), "no" (0 points), up to 100 points.

1.5 Sound therapy

First to determine the pitch frequency of tinnitus patients by tinnitus matching. Then select a different natural sound, such as rain, birds, buzz, etc. using the sound templates of TinniFit tinnitus diagnosis and treatment instrument. Natural sounds have been processed and can be divided into low frequency, intermediate frequency, high frequency and multi-band sound. The masking frequency of natural sounds should consistent with the tinnitus frequency of patients. Second, a variety of music materials were available, refer to each patient's education background, lifestyle, personal preferences, etc., or allow patients to provide their own favorite music type, which the music material frequency was analyzed. Three music clips that the main frequency covers the tinnitus frequency were picked up. Each piece of music is about 20~30 minutes. Then superimpose the masked natural sound on the three music clips in the first ten minutes in a fade in-out way, each superimposed last no more than a minute. The remaining part of the music pieces add no more natural sound. The synthetic music files use Adobe Audition CS6 software to measure root-mean-square values (RMS) for uniform sound pressure level. The loudness of synthetic music files was about 10~15 dB above the tinnitus loudness threshold refer to tinnitus matching results. The maximum sound intensity should less than 60dB so that to be able to mask tinnitus and do not cause discomfort to patients. Wearing a headphone to listen to three synthetic music files completely is a cycle, three times or more each day, lasting three months.

Recording beginning and ending time of each cycle as well as test times. The follow-up treatment should continue to retest the tinnitus matching including tinnitus frequency, tinnitus threshold, tinnitus loudness, the minimum masking level, residual inhibition test and the Feldmann masking curves. To compare the difference between the two groups.

1.6 Statistical analysis

Independent samples *t*-tests, chi-square testsand paired *t*-tests were used to determine whether differences were significant though SPSS 18.0.Data in the text and tables are given as the mean ± the standard deviation. A value of < 0.05 was used to determine statistical significance.

2 Results

2.1 The general characteristics of tinnitus patients with normal audiogram

A total of 46 patients with normal hearing threshold were enrolled in this study. Among them, 26 were male, with an average age of 35.73±11.15 years and 20 females with an average age of 28.95 ± 13.38 years. 20 patients with unilateral tinnitus of right ear, 20 patients with tinnitus of left, 6 patients with bilateral tinnitus. 32 of the 46 patients showed RI

positive and the rest 14 patients were RI negative. These two groups were compared with aspect of age, gender, tinnitus characteristics such as laterality, pitch, threshold, loudness, duration, tinnitus severity and comorbidities of tinnitus. The average tinnitus frequency of RI positive group was 6172±604 Hz, the average threshold was 29.88±12.89 dB HL, the average loudness was 3.06±1.54 dB SL, and the average THI score before treatment was 33.38±16.23, The sub-items E, F, C were 15.63±8.78, 11.88±6.85, 5.88±3.13 points, VAS score of 4.38±1.62 points on average. The RI negative group showed average tinnitus frequency of 5964±1824 Hz, threshold of 29.14±14.86 dB HL, and loudness of 2.57±0.76 dB SL, the THI score before therapy was 29.86±20.15, E, F and C were 12.29±8.03, 10.86±7.09 and 6.43±5.50, respectively, and the average score of VAS was 3.93±1.69. There was no significant difference in the characteristics and severity of tinnitus between the two groups (P>0.05).(see Table 1).

2.2 Sound therapy

46 patients were followed up after 2 months of sound therapy. The 32 RI positive patients had a THI score of 21.75±11.67 and a VAS score of 2.97±1.06. While the 14 RI negative patients showed a THI score of 21.75±11.67 points, VAS score of 2.97±1.06 points. Two groups of THI score as shown in Table 2, 3. Comparison of the two groups before and after treatment as shown in Figure1, THI sub-score changes in Figure 2, 3. The THI score and the VAS score were significantly different before and after the treatment within RI positive patients, and the THI score and the VAS score of the patients with RI negative also decreased.

Table 1　Clinical characteristics of the 46 patients with normal audiogram

	N[1]	RI+	RI−	Group comparison	
				P value	
Age	46(32/14)	36.4±11.9	22.4±7.2	t=4.073	<0.001[1)]
Gender(M/F)	46(32/14)	19/13	7/7	χ^2=0.348	0.555
Tinnitus characteristics					
Laterality(R/L/B, ear)	46(32/14)	16/15/3	4/5/3		
Pitch(Hz)	46(32/14)	6172±604	5964±1824	t=0.583	0.563
Threshold(dB HL)	46(32/14)	29.88±12.89	29.14±14.86	t=0.167	0.868
Loudness(dB SL)	46(32/14)	3.06±1.54	2.57±0.76	t=1.127	0.266
Duration(in months)	46(32/14)	9.59±2.95	9.85±2.59	t=0.285	0.777
Tinnitus severity					
THI	46(32/14)	33.38±16.23	29.86±20.15	t=0.628	0.533
E	46(32/14)	15.63±8.78	12.29±8.03	t=1.217	0.230
F	46(32/14)	11.88±6.85	10.86±7.09	t=0.460	0.648
C	46(32/14)	5.88±3.13	6.43±5.50	t=0.431	0.668
VAS	46(32/14)	4.38±1.62	3.93±1.69	t=1.712	0.094
Uncomfortable	46(32/14)	6.05±2.52	6.90±2.24	t=1.087	0.283
Annoying	46(32/14)	4.67±2.69	5.92±2.40	t=1.496	0.142
Unpleasant	46(32/14)	4.83±2.74	6.05±2.70	t=1.396	0.169
Ignoring	46(32/14)	5.08±3.07	6.04±2.90	t=0.992	0.327
Comorbidities(no/yes in %)					
Headache	46(32/14)	63/37	71/29	χ^2=0.280	0.804
Dizziness or vertigo	46(32/14)	59/41	57/43	χ^2=0.02	0.887
Other syndromes	46(32/14)	78/22	86/14	χ^2=0.319	0.847

Results from independent samples t-tests, chi-square tests for group comparisons. N[1]:46 patients involved in this study, 32patients of RI+ group and 14patients of RI− group. 1): α<0.001

Table 2 The score of 32 RI positive patients before and after sound therapy

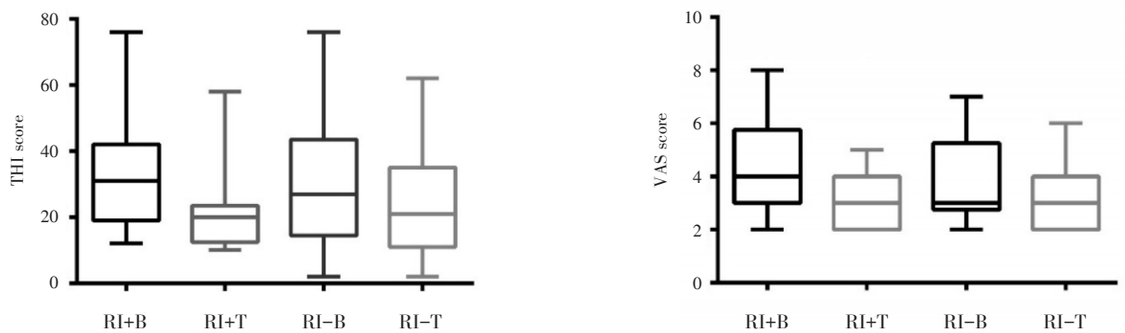
RI positive(32 cases)	THI	THI sub-items			VAS
		E	F	C	
Before therapy	33.38±16.23	15.63±8.78	11.88±6.85	5.88±3.13	4.38±1.62
After therapy	21.75±11.67	10.38±5.85	7.56±4.97	3.81±1.93	2.97±1.06
t value	8.882	6.808	6.246	5.203	7.212
P value[1)]	<0.001	<0.001	<0.001	<0.001	<0.001

Results from paired *t*-tests before and after therapy for RI+ group comparisons; 1): α<0.001

Table 3 The score of 14 RI negative patients before and after sound therapy

RI negative(14 cases)	THI	THI sub-items			VAS
		E	F	C	
Before therapy	29.86±20.15	12.29±8.03	10.86±7.09	6.43±5.50	3.93±1.69
After therapy	23.43±16.29	10.00±6.61	8.29±6.17	5.14±4.20	3.36±1.22
t value	3.798	3.889	3.347	1.979	2.828
P value[1)]	0.002	0.002	0.005	0.070	0.014

Results from paired *t*-tests before and after therapy for RI+ group comparisons; 1): α<0.001

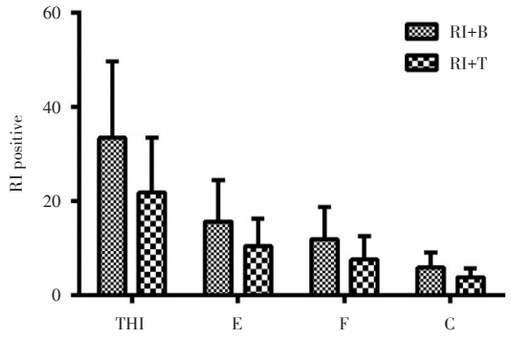


RI+B refers to RI positive patients before sound therapy; RI+T refers to RI positive patients after sound therapy; RI-B refers to RI negative patients before sound therapy; RI-T refers to RI negative patients after sound therapy.

Fig.1. The comparison of THI score and VAS score between 32 RI positive patients and 14 RI negative patients before and after sound therapy

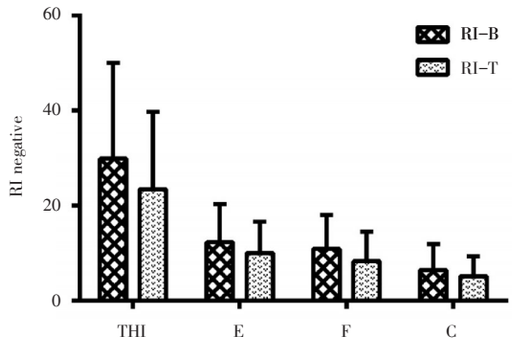
3 Discussion

We found that tinnitus patients with normal audiogram had a reduced THI and VAS value after receiving tailor-made complex music therapy. And the alleviated degree was affected by residual inhibition, the residual inhibition positive group shown better result than negative group. There are various forms and effects of sound therapy, however it still be recommended by many tinnitus clinical guideline for conveniences and non-traumatic. In the pass decades, many sound therapy methods were developed base on the different tinnitus hypothesis. Sound-based therapies function on four general mechanisms of action, masking, distraction, habituation and Neuromodulation[7, 15-16]. There are many devices that offer different levels of sound therapy. The noise generated by sound machines can partially or fully mask a patient's perception of tinnitus, providing relaxation and temporary respite from the condition. Sound masking devices are typically only effective during or



RI+B：residual inhibition positive tinnitus patients before sound therapy；RI+T：residual inhibition positive tinnitus patients postal sound therapy.

Fig.2　Comparison of THI score before and after sound therapy of RI positive



RI-B：residual inhibition negative tinnitus patient before sound therapy. RI-T：residual inhibition negative tinnitus patient postal sound therapy.

Fig.3　Comparison of THI score before and after sound therapy of RI negative

immediately after active use；they have very limited longer-term effectiveness in reducing overall perception of tinnitus. Unlike standard white noise machines，a variety of medical-grade devices provide more customized sounds and generate specific frequencies and tones modified-sounds. By facilitating habituation these products may alleviate the perceived burden of tinnitus and even "tune out" the perception of tinnitus. Almost all the sound therapy results come from tinnitus patients with hearing loss. Just few research work focus on the tinnitus patients with normal audiogram. Teja Deepak Dessai and her colleagues found sound masking therapy did not show significant effects to the normal audiogram tinnitus patients[17]. In the present，we shown that complex music therapy could alleviate perception of tinnitus，both THI and VAS scores are reduced. The complex sound consists of customized music and natural sounds which mainly pitch covered tinnitus pitch. Customized music can work at limbic system and relax the tinnitus patients[18]，meanwhile the natural sounds matched to the patient′s tinnitus to provide cortical stimulation and partial residual inhibition of tinnitus，so all the tinnitus patients show effective to the complex sound.

Residual inhibition is one of the few procedures known to reduce or eliminate tinnitus for brief periods. The relationship between RI and sound therapy did not extensively explore. In our results show that all patients shown effectiveness to the complex sound therapy no matter its residual test positive or negative. Except catastrophic subscale no change pre-and post-treatment in the RI negative group，all other subscales in RI negative group and all subscale in the RI positive group significant reduced the scores after complex sound therapy[19-21]. RI negative patients have higher catastrophic item scores than RI positive patients and may have more severity psychological symptoms. These data also suggest that RI and complex sound therapy has some intrinsic connection but there also maybe have different function mechanism. RI may function as a masker while complex sound function as distress and plastic of cortex. Of course，we should concern the factor of treatment course，RI just receive temporal noise masking，while our complex sound therapy last for at least 2 months. There are need more data to prove and uncover the relationship between RI and sound therapy.

Summarily，this present indicated that tailor-made complex sound alleviated the scale of THI and VAS on normal audiogram tinnitus patients. RI could predicate the benefit acquired from complex sound therapy on these patients，RI positive have better results，however RI negative tinnitus patient also could acquire some benefit from sound therapy. Complex sound has potential function to most normal audiogram tinnitus patient.

Reference:

[1] LockwoodAH, Salvi RJ, and Burkard RF. Tinnitus [J]. N Engl J Med, 2002. 347(12): 904-910.

[2] BhattJM, LinHW, BhattacharyyaN. Prevalence, severity, exposures, and treatment patterns of tinnitus in the United States [J]. JAMA Otolaryngol Head Neck Surg, 2016, 142(10): 959-965.

[3] Sereda MM, EdmondsonJ, HallDA. Relationship between tinnitus pitch and edge of hearing loss in individuals with a narrow tinnitus bandwidth[J]. Int J Audiol, 2015. 54(4): 249-256.

[4] SalviR. Inner hair cell loss disrupts hearing and cochlear function leading to sensory deprivation and enhanced central auditory gain[J]. Front Neurosci, 2016, 10(7): 621-634.

[5] Vanneste S, Ridder DD. Deafferentation- based pathophysiological differences in phantom sound: Tinnitus with and without hearing loss[J]. NeuroImage, 2016, 129(8): 80-94.

[6] LangguthB.Different patterns of hearing loss among tinnitus patients: A latent class analysis of a large sample[J]. Front Neurol, 2017, 8(1): 46-56.

[7] Hoare DJ. Sound therapy for tinnitus management: practicable options[J]. J Am Acad Audiol, 2014. 25(1): 62-75.

[8] Sweetow RW. The evolution of cognitive-behavioral therapy as an approach to tinnitus patient management[J]. Int Tinnitus J, 1995, 1(1): 61-65.

[9] Jastreboff PJ, Hazell JW. A neurophysiological approach to tinnitus: clinical implications[J]. Br J Audiol, 1993, 27(1): 7-17.

[10] NohTS. Comparison of treatment outcomes following either prefrontal cortical-only or dual-site repetitive transcranial magnetic stimulation in chronic tinnitus patients: A double- blind randomized study [J]. Otol Neurotol, 2017, 38(2): 296-303.

[11] Mennemeier M, GeorgeM. The case for a definitive multisite, randomized clinical trial of repetitive transcranial magnetic stimulation for tinnitus[J]. JAMA Otolaryngol Head Neck Surg, 2017, 143(5): 441-442.

[12] Lee K, MakinoK, YamaharaK. Evaluation of tinnitus retraining therapy for patients with normal audiograms versus patients with hearing loss[J]. Auris Nasus Larynx, 2007, 17(1): 332-339.

[13] Fuller TE. Different teams, same conclusions? a systematic review of existing clinical guidelines for the assessment and treatment of tinnitus in adults [J]. Front Psychol, 2017, 8(7): 206-213.

[14] Tunkel DE. Clinical practice guideline: tinnitus [J]. Otolaryngol Head Neck Surg, 2014. 151 (2 Suppl): S1-s40.

[15] Hobson J, Chisholm E, and El RefaieA. Sound therapy (masking) in the management of tinnitus in adults[J]. Cochrane Database Syst Rev, 2012, 11 (9): Cd006371.

[16] Neff P. 10 Hz Amplitude modulated sounds induce short- term tinnitus suppression [J]. Front Aging Neurosci, 2017, 9(2): 130-140.

[17] Dessai TD. Effectiveness of residual inhibition therapy[J]. Int Tinnitus J, 2014, 19(1): 63-67.

[18] Koelsch S. A Neuroscientific Perspective on Music Therapy[J]. Annals of the New York Academy of Sciences, 2009, 1169(1): 374-384.

[19] Newman CW, SandridgeSA, JacobsonGP. Psychometric adequacy of the Tinnitus Handicap Inventory (THI) for evaluating treatment outcome[J]. J Am Acad Audiol, 1998. 9(2): 153-160.

[20] Newman CW, JacobsonGP, SpitzerJB. Development of the tinnitus handicap inventory[J]. Arch Otolaryngol Head Neck Surg, 1996, 122(2): 143-148.

[21] Schaette R, McAlpineD. Tinnitus with a normal audiogram: physiological evidence for hidden hearing loss and computational model[J]. JNeuroscience, 2011, 38(8): 13452-13457.

(编辑 王晓鹰)