·TOPICS ON OTOLARYNGOLOGY· ·耳鼻咽喉疾病专题·

DOI:10.11817/j.issn.1672-7347.2024.230482

PTHrP促进RANKL诱导巨噬细胞分化为破骨细胞参与中耳胆脂瘤骨破坏

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[摘 要] 目的：骨质进行性吸收破坏是中耳胆脂瘤最重要的临床特征之一，可导致一系列颅内外并发症，而目前中耳胆脂瘤骨破坏的机制尚未明确。本研究旨在探究甲状旁腺激素相关蛋白(parathyroid hormone-related protein，PTHrP)参与中耳胆脂瘤骨破坏的机制。**方法：**收集后天性中耳胆脂瘤患者的25例胆脂瘤标本和13例外耳道正常皮肤组织标本。采用免疫组织化学染色方法检测PTHrP、核因子κB受体活化因子配体(receptor activator for nuclear factor-kappa B ligand，RANKL)和骨保护素(osteoprotegerin，OPG)在中耳胆脂瘤和外耳道正常皮肤组织中的表达，抗酒石酸酸性磷酸酶(tartrate-resistant acid phosphatase，TRAP)染色法检测中耳胆脂瘤和外耳道正常皮肤组织中是否存在TRAP阳性多核巨噬细胞。选取小鼠单核巨噬细胞RAW264.7细胞进行干预，分为RANKL干预组和PTHrP+RANKL共同干预组，采用TRAP染色法检测2组破骨细胞的生成情况，实时聚合酶链反应(real-time polymerase chain reaction，real-time PCR)检测干预后2组破骨细胞相关基因*TRAP*、组织蛋白酶K(cathepsin K，*CTSK*)和活化T细胞核因子1(nuclear factor of activated T cell cytoplasmic 1，*NFATc1*)的mRNA表达水平，骨吸收陷窝实验检测2组破骨细胞的骨吸收功能。**结果：**免疫组织化学染色结果显示，PTHrP和RANKL在中耳胆脂瘤组织中的表达均显著增高，OPG表达降低(均$P<0.05$)，且PTHrP的表达与RANKL、RANKL/OPG比值均呈显著正相关，与OPG表达呈显著负相关(分别$r=0.385$、$r=0.417$、$r=-0.316$，均$P<0.05$)。同时，PTHrP、RANKL的表达水平与中耳胆脂瘤的骨破坏程度均呈显著正相关(分别$r=0.413$、$r=0.505$，均$P<0.05$)。TRAP染色结果显示中耳胆脂瘤上皮周围基质中有大量TRAP阳性细胞，并存在细胞核数量为3个或3个以上的TRAP阳性破骨细胞。RANKL或PTHrP+RANKL联合干预5 d后，与RANKL干预组相比，PTHrP+RANKL联合干预组的破骨细胞数量显著增加($P<0.05$)，且破骨细胞相关基因*TRAP*、*CTSK*和*NFATc1*的mRNA表达水平均升高(均$P<0.05$)。骨吸收陷窝扫描电镜结果显示RANKL干预组、PTHrP+RANKL联合干预组的骨片表面均形成骨吸收陷窝；与RANKL干预组相比，PTHrP+RANKL联合干预组的骨片表面骨吸收陷窝数量显著增加($P<0.05$)，面积也更大。**结论：**PTHrP可能通过促进RANKL诱导胆脂瘤组织周围基质中的巨噬细胞分化为破骨细胞，参与中耳胆脂瘤骨破坏。

[关键词] 甲状旁腺激素相关蛋白；中耳胆脂瘤；核因子κB受体活化因子配体；骨保护素；破骨细胞；巨噬细胞

收稿日期(Date of reception)：2023-11-03

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基金项目(Foundation item)：国家自然科学基金(82071036，82000973)；湖南省自然科学基金(2022JJ30821，2019JJ50967)；湖南省创新型省份建设专项(2023SK4030)。This work was supported by the National Natural Science Foundation (82071036, 82000973), the Natural Science Foundation of Hunan Province (2022JJ30821, 2019JJ50967), and the Special Project for the Construction of Innovative Provinces in Hunan Province (2023SK4030), China.

PTHrP participates in the bone destruction of middle ear cholesteatoma via promoting macrophage differentiation into osteoclasts induced by RANKL

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ABSTRACT

Objective: Progressive bone resorption and destruction is one of the most critical clinical features of middle ear cholesteatoma, potentially leading to various intracranial and extracranial complications. However, the mechanisms underlying bone destruction in middle ear cholesteatoma remain unclear. This study aims to explore the role of parathyroid hormone-related protein (PTHrP) in bone destruction associated with middle ear cholesteatoma.

Methods: A total of 25 cholesteatoma specimens and 13 normal external auditory canal skin specimens were collected from patients with acquired middle ear cholesteatoma. Immunohistochemical staining was used to detect the expressions of PTHrP, receptor activator for nuclear factor-kappa B ligand (RANKL), and osteoprotegerin (OPG) in cholesteatoma and normal tissues. Tartrate-resistant acid phosphatase (TRAP) staining was used to detect the presence of TRAP positive multi-nucleated macrophages in cholesteatoma and normal tissues. Mono-nuclear macrophage RAW264.7 cells were subjected to interventions, divided into a RANKL intervention group and a PTHrP+ RANKL co-intervention group. TRAP staining was used to detect osteoclast formation in the 2 groups. The mRNA expression levels of osteoclast-related genes, including *TRAP*, cathepsin K (*CTSK*), and nuclear factor of activated T cell cytoplasmic 1 (*NFATc1*), were measured using real-time polymerase chain reaction (real-time PCR) after the interventions. Bone resorption function of osteoclasts was assessed using a bone resorption pit analysis.

Results: Immunohistochemical staining showed significantly increased expression of PTHrP and RANKL and decreased expression of OPG in cholesteatoma tissues (all $P<0.05$). PTHrP expression was significantly positively correlated with RANKL, the RANKL/OPG ratio, and negatively correlated with OPG expression ($r=0.385$, $r=0.417$, $r=-0.316$, all $P<0.05$). Additionally, the expression levels of PTHrP and RANKL were significantly positively correlated with the degree of bone destruction in cholesteatoma ($r=0.413$, $r=0.505$, both $P<0.05$). TRAP staining revealed a large number of TRAP-positive cells, including multi-nucleated osteoclasts with three or more nuclei, in the stroma surrounding

the cholesteatoma epithelium. After 5 days of RANKL or PTHrP+RANKL co-intervention, the number of osteoclasts was significantly greater in the PTHrP+RANKL co-intervention group than that in the RANKL group (P<0.05), with increased mRNA expression levels of *TRAP*, *CTSK*, and *NFATc1* (all P<0.05). Scanning electron microscopy of bone resorption pits showed that the number (P<0.05) and size of bone resorption pits on bone slices were significantly greater in the PTHrP+RANKL co-intervention group compared with the RANKL group.

Conclusion: PTHrP may promote the differentiation of macrophages in the surrounding stroma of cholesteatoma into osteoclasts through RANKL induction, contributing to bone destruction in middle ear cholesteatoma.

KEY WORDS parathyroid hormone-related protein; middle ear cholesteatoma; receptor activator for nuclear factor-kappa B ligand; osteoprotegerin; osteoclasts; macrophages

中耳胆脂瘤是一种由角化复层鳞状上皮异常增殖形成的囊性病变，具有复发性、过度增殖性、迁移侵袭性等临床特征，当其逐渐生长膨大压迫并破坏周围组织和骨质结构时，可导致耳聋、面神经瘫痪、前庭功能障碍，以及其他颅内外并发症，甚至威胁到患者的生命[1]。然而，中耳胆脂瘤的发病机制至今尚未完全阐明，与其骨破坏相关的分子生物学机制目前也尚不明确。

骨质进行性吸收破坏是中耳胆脂瘤最重要的临床特征。目前学者们[2]认为破骨细胞是胆脂瘤骨破坏的最终作用细胞，是导致中耳胆脂瘤骨破坏的关键细胞。骨吸收和骨质生成分别由破骨细胞和成骨细胞进行，在生理状态下，二者维持着一种动态平衡。在炎症或其他病理状态下，该动态平衡被打破而朝着骨吸收的方向发展[3]。破骨细胞分化因子即核因子κB受体活化因子配体(receptor activator for nuclear factor-kappa B ligand，RANKL)不仅促进破骨前体细胞的分化和融合，也可激活成熟的破骨细胞，被认为是破骨细胞活化的终末环节。骨保护素(osteoprotegerin，OPG)能够抑制由RANKL诱导的破骨细胞活化及骨破坏。因此，破骨细胞的活化受到骨微环境中RANKL和OPG之间平衡的调节[4]。RANKL/OPG比值决定了破骨细胞是被活化还是被抑制，高RANKL/OPG比值可启动并促进破骨细胞活化，在破骨作用发生过程中发挥关键作用。然而，RANKL/OPG通路是如何启动和激活破骨细胞进而导致骨吸收、破坏的过程，目前尚未完全明确。

甲状旁腺激素相关蛋白(parathyroid hormone-related protein，PTHrP)是一种G蛋白偶联受体信号蛋白质，参与骨代谢等正常生理活动，在炎症、肿瘤等病理过程中也发挥重要作用[5-8]。骨细胞是PTHrP最主要的靶细胞，PTHrP是RANKL/OPG通路的上游调控因子，能够激活RANKL诱导破骨细胞前体细胞向成熟破骨细胞分化，并激活破骨细胞的骨吸收活性[9]。

课题组前期研究[2]发现中耳胆脂瘤基质中伴有大量单核细胞及巨噬细胞浸润，笔者推测：PTHrP可能通过RANKL/OPG信号通路促进中耳胆脂瘤基质中的巨噬细胞向破骨细胞分化和成熟，导致胆脂瘤骨破坏。因此，本研究探讨PTHrP参与中耳胆脂瘤骨破坏的机制。

1 材料与方法

1.1 材料

1.1.1 试剂

小鼠单核巨噬细胞RAW264.7细胞、青链霉素混合液、辣根过氧化物酶标记的山羊抗兔/鼠二抗IgG、抗酒石酸酸性磷酸酶(tartrate-resistant acid phosphatase，TRAP)染液、小牛骨片均购自长沙艾碧维生物科技公司；杜尔贝科改良伊格尔培养基(Dulbecco’s modified eagle medium，DMEM)购自美国Gibco公司；胎牛血清(fetal bovine serum，FBS)购自上海富衡生物科技有限公司；重组RANKL购自美国R&D Systems公司；重组PTHrP购自美国PeproTech公司；PTHrP抗体购自英国Abcam公司；RANKL抗体购自美国Proteintech公司；OPG抗体购自北京博奥森生物技术有限公司；RNA提取试剂盒购自美国Thermo公司；反转录试剂盒购自南京诺唯赞生物科技股份有限公司。

1.1.2 标本及分级

收集耳鼻咽喉头颈外科2021年1月至2022年12月收治的25例后天性中耳胆脂瘤患者的胆脂瘤标本和13例外耳道正常皮肤组织标本(术耳耳甲腔成形时外耳道口皮肤组织)。本研究已获中南大学湘雅二医院医学伦理委员会批准(审批号：20230171)。所有患者均被告知标本收集的用途，并签署知情同意书。所有标本均经2位有经验的病理科医生确认。标本收集后立即分成2份：一份即刻浸入4%多聚甲醛中进行固定，另一份迅速放入液氮中。根据患者的术中所见和CT影像资料提示的骨破坏范围将胆脂瘤骨破坏程度分为4级[10]：0级为无骨破坏，无胆脂瘤包绕；1级为有包绕听小骨和/或砧骨部分破坏、周围骨质硬化、盾板破坏；2级为有1~3个听小骨破坏；3级为有外耳道壁、鼓室盖、骨迷路、半规管、面神经骨管、耳蜗等破坏，或邻近器官并发症。

1.2 方法

1.2.1 细胞培养

RAW264.7细胞使用DMEM完全培养基(90% DMEM+10% FBS+1%青链霉素双抗)培养于37 ℃含5% CO_2的细胞培养箱中。每2天换液1次，细胞密度达80%以上时进行传代。

1.2.2 破骨细胞的诱导

将处于对数生长期的RAW264.7细胞以5×10^3/mL接种于6孔板。细胞分为RANKL干预组和PTHrP+RANKL共同干预组，每组设置3个复孔。RANKL组每孔加入2 mL DMEM完全培养基(含50 ng/mL RANKL)，PTHrP+RANKL共同干预组每孔加入2 mL DMEM完全培养基(含50 ng/mL RANKL，100 ng/mL PTHrP)。换液后的6孔板在细胞培养箱中(37 ℃，5% CO_2)继续培养。5 d后收集细胞进行后续实验。

1.2.3 免疫组织化学染色

将石蜡切片依次进行烤片、脱蜡、水化、热修复抗原后，加入一抗：PTHrP(1∶2 000)，RANKL(1∶50)，OPG(1∶50)，于4 ℃下孵育过夜。用PBS冲洗后，加入二抗，于37 ℃下孵育30 min；用PBS冲洗后，滴加辣根酶标记链霉卵白素工作液，于室温孵育10~15 min，以PBS冲洗。经显色、复染、脱水、封片后，在显微镜下观察染色结果。采用平均光密度值进行统计学分析。

1.2.4 TRAP染色

将石蜡切片依次进行脱蜡、水化，于37 ℃避光条件下，将切片放入TRAP染液中孵育1 h。以双蒸水冲洗后，用苏木精复染，二甲苯透明。封片后在显微镜下观察染色结果。分别取RANKL干预组和PTHrP+RANKL共同干预组诱导的破骨细胞，经4%多聚甲醛固定后，加入TRAP染液，于37 ℃下避光孵育1 h。再经双蒸水洗涤、苏木精复染、洗涤后，在显微镜下观察染色结果。阳性细胞染色为酒红色，镜下细胞TRAP染色阳性且细胞核数目≥3个的细胞可认为是破骨细胞，对其进行差异显著性分析。

1.2.5 实时聚合酶链反应

采用RNA提取试剂盒提取样本总RNA，使用反转录试剂盒将纯化后的RNA反转录为cDNA，分别采用TRAP、活化T细胞核因子1(nuclear factor of activated T cell cytoplasmic 1，NFATc1)、组织蛋白酶K(cathepsin K，CTSK)及内参甘油醛-3-磷酸脱氢酶(glyceraldehyde-3-phosphate dehydrogenase，GAPDH)的特异性引物进行实时聚合酶链反应(real-time polymerase chain reaction，real-time PCR)，引物序列见表1。采用$2^{-\Delta\Delta Ct}$值进行统计学分析。

表1 引物序列

Table 1 Sequences of primers

基因名	双向引物序列(5'-3')
TRAP	F: TGTGGCCATCTTTATGCT
	R: GTCATTTCTTTGGGGCTT
NFATc1	F: GAGACCGAGAGGCTCCGAAC
	R: CCTCTCCTTTGCCGACACG
CTSK	F: GCACCCTTAGTCTTCCGCTC
	R: ACCCACATCCTGCTGTTGAG
GAPDH	F: ACAGCAACAGGGTGGTGGAC
	R: TTTGAGGGTGCAGCGAACTT

1.2.6 骨吸收陷窝实验

小牛骨片经消毒灭菌后置于96孔板中，将处于对数生长期的RAW264.7细胞接种于96孔板，细胞分为RANKL组和PTHrP+RANKL共同干预组。RANKL组每孔加入100 μL DMEM完全培养基(含50 ng/mL RANKL)，PTHrP+RANKL共同干预组每孔加入100 μL DMEM完全培养基(含50 ng/mL RANKL，100 ng/mL PTHrP)。干预第6天时取出小牛骨片，依次经冲洗、固定、乙醇脱水、醋酸异戊酯处理、CO_2临界点干燥、镀膜，在扫描电镜下观察并采集图像。通过观察骨表面骨吸收陷窝数目、面积以及骨破坏程度来判断骨吸收强弱。

1.3 统计学处理

采用SPSS 26.0统计软件对数据进行统计分析，计量资料用均数±标准差表示，采用独立样本t检验；

采用Spearman相关分析法分析PTHrP、RANKL和OPG 3种蛋白质与中耳胆脂瘤骨破坏程度的相关性。$P<0.05$为差异有统计学意义。

2 结　果

2.1 PTHrP、RANKL及OPG在中耳胆脂瘤组织中的表达及与骨破坏程度的相关性

免疫组织化学结果(图1)显示：与正常外耳道皮肤组织相比，PTHrP、RANKL在中耳胆脂瘤上皮组织中的表达均显著增高(均$P<0.05$)，OPG的表达显著降低($P<0.05$)。同时，RANKL/OPG比值也显著增高($P<0.05$，表2)。在中耳胆脂瘤上皮组织中，PTHrP的表达与RANKL呈显著正相关($r=0.385$，$P=0.017$)，与RANKL/OPG比值呈显著正相关($r=0.417$，$P=0.009$)，与OPG呈显著负相关($r=-0.316$，$P=0.026$)，而RANKL与OPG无显著相关性($r=-0.062$，$P=0.711$；图2)。

Spearman相关分析结果显示：PTHrP与中耳胆脂瘤的骨破坏程度呈显著正相关($r=0.413$，$P<0.05$)；RANKL与骨破坏程度呈显著正相关($r=0.505$，$P<0.05$)；RANKL/OPG比值亦与骨破坏程度呈显著正相关($r=0.495$，$P<0.05$)；OPG的表达情况与中耳胆脂瘤的骨破坏程度无显著相关性($r=-0.305$，$P=0.138$；表3)。

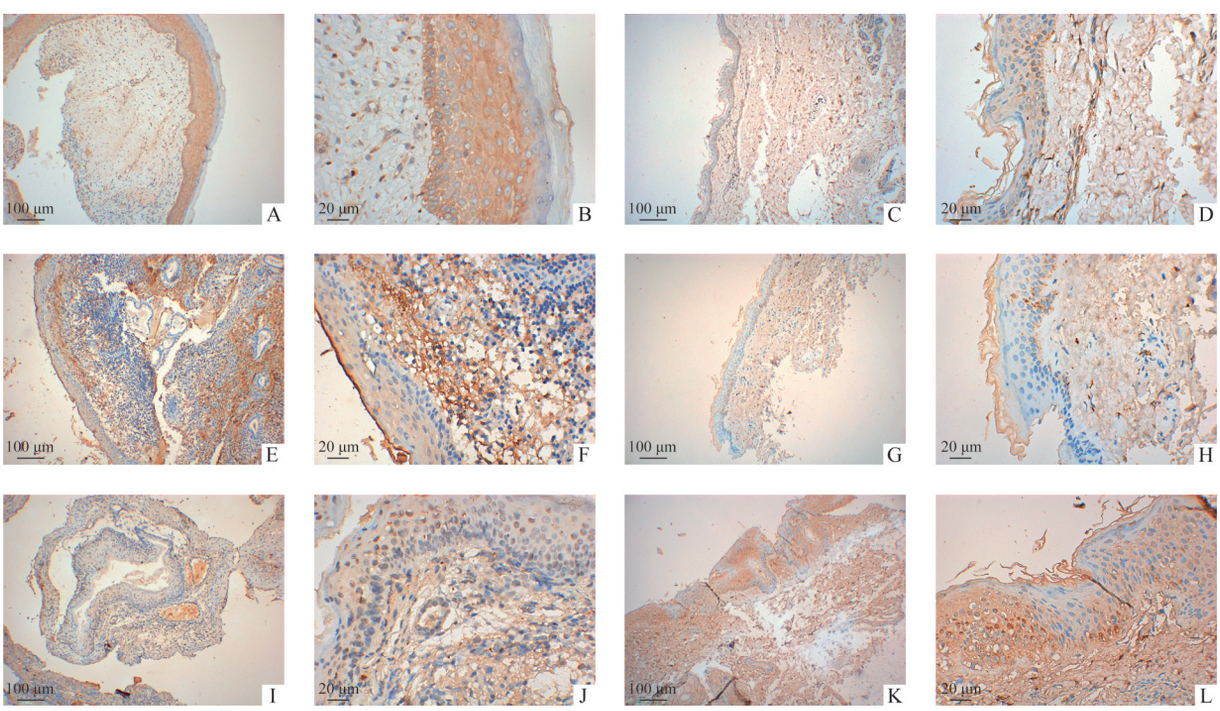

图1　免疫组织化学染色示PTHrP、RANKL、OPG在中耳胆脂瘤和外耳道正常皮肤组织中的表达

Figure 1　Expressions of PTHrP, RANKL, and OPG in the middle ear cholesteatoma and normal skin tissues of the external auditory canal by immunohistochemical staining

A and B: Expression of PTHrP in the middle ear cholesteatoma tissues with the magnification of 100 (A) and 400 (B); C and D: Expression of PTHrP in the normal skin tissues with the magnification of 100 (C) and 400 (D); E and F: Expression of RANKL in the middle ear cholesteatoma tissues with the magnification of 100 (E) and 400 (F); G and H: Expression of RANKL in the normal skin tissues with the magnification of 100 (G) and 400 (H); I and J: Expression of OPG in the middle ear cholesteatoma tissues with the magnification of 100 (I) and 400 (J); K and L: Expression of OPG in the normal skin tissues with the magnification of 100 (K) and 400 (L). PTHrP: Parathyroid hormone-related protein; RANKL: Receptor activator for nuclear factor-kappa B ligand; OPG: Osteoprotegerin.

表2 中耳胆脂瘤和外耳道正常皮肤组织中PTHrP、RANKL和OPG蛋白的表达

Table 2 Expressions of PTHrP, RANKL, and OPG proteins in middle ear cholesteatoma and normal skin tissues of the external ear canal

变量	*n*	PTHrP	RANKL	OPG	RANKL/OPG
中耳胆脂瘤	25	0.054±0.017	0.042±0.017	0.029±0.011	1.570±0.805
正常外耳道皮肤	13	0.027±0.009	0.020±0.010	0.039±0.013	0.545±0.217
t		5.435	4.242	−2.386	4.478
P		<0.001	<0.001	0.022	<0.001

PTHrP：甲状旁腺激素相关蛋白；RANKL：核因子κB受体活化因子配体；OPG：骨保护素。

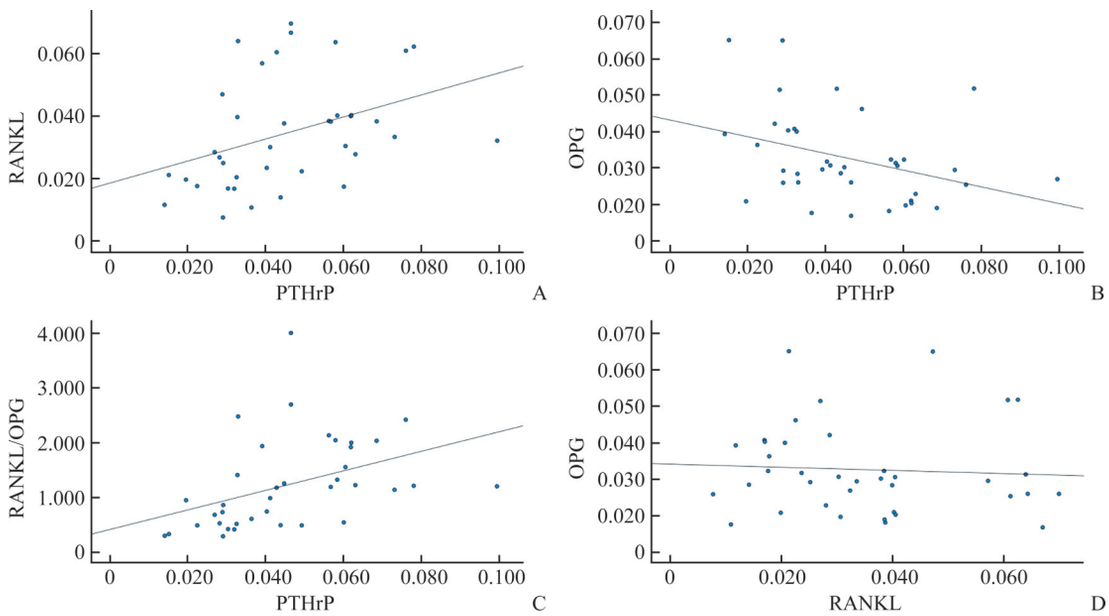

图2 中耳胆脂瘤组织中PTHrP、RANKL、OPG蛋白质表达水平之间的相关性

Figure 2 Correlation between the expression levels of PTHrP, RANKL, and OPG proteins in middle ear cholesteatoma tissues

A: Expression of PTHrP is positively correlated with RANKL. B: Expression of PTHrP is negatively correlated with OPG. C: Expression of PTHrP is positively correlated with the ratio of RANKL to OPG. D: No significant correlation between RANKL and OPG is observed. PTHrP: Parathyroid hormone-related protein; RANKL: Receptor activator for nuclear factor-kappa B ligand; OPG: Osteoprotegerin.

表3 PTHrP、RANKL和OPG表达及PTHrP/OPG比值与中耳胆脂瘤骨破坏程度的相关性

Table 3 Correlation of PTHrP, RANKL, and OPG expressions and PTHrP/OPG ratio with the degree of bone resorption in middle ear cholesteatoma

变量	骨破坏程度分级				*r*	*P*
	0	1	2	3		
PTHrP	0	0.045±0.014	0.044±0.008	0.060±0.017	0.413	0.040
RANKL	0	0.029±0.010	0.046±0.015	0.045±0.017	0.505	0.010
OPG	0	0.038±0.010	0.032±0.014	0.026±0.008	−0.305	0.138
RANKL/OPG	0	0.821±0.434	1.548±0.562	1.809±0.823	0.495	0.012

PTHrP：甲状旁腺激素相关蛋白；RANKL：核因子κB受体活化因子配体；OPG：骨保护素。

2.2 中耳胆脂瘤组织和外耳道正常皮肤组织中破骨细胞检测结果

中耳胆脂瘤组织中有TRAP染色为酒红色的阳性细胞存在，主要位于胆脂瘤组织周围基质中，并可观察到细胞核数量≥3个的TRAP阳性细胞(图3A和3B)；而在外耳道正常皮肤中，未见明显TRAP阳性细胞，且均未发现细胞核数量≥3个的TRAP阳性细胞(图3C和3D)。由于正常皮肤中未检测出破骨细胞存在，因此此处不作统计学分析。

2.3 RANKL干预组和PTHrP+RANKL共同干预组中破骨细胞比较

RAW264.7细胞分别采用RANKL和PTHrP+RANKL干预5 d后，TRAP染色检测结果显示RANKL干预组(图4A和4B)、PTHrP+RANKL共同干预组(图4C和4D)均有多核(≥3个)的TRAP阳性细胞(酒红色)即破骨细胞形成。PTHrP+RANKL共同干预组中破骨细胞数量较RANKL干预组显著增加($P<0.05$，图4E)。

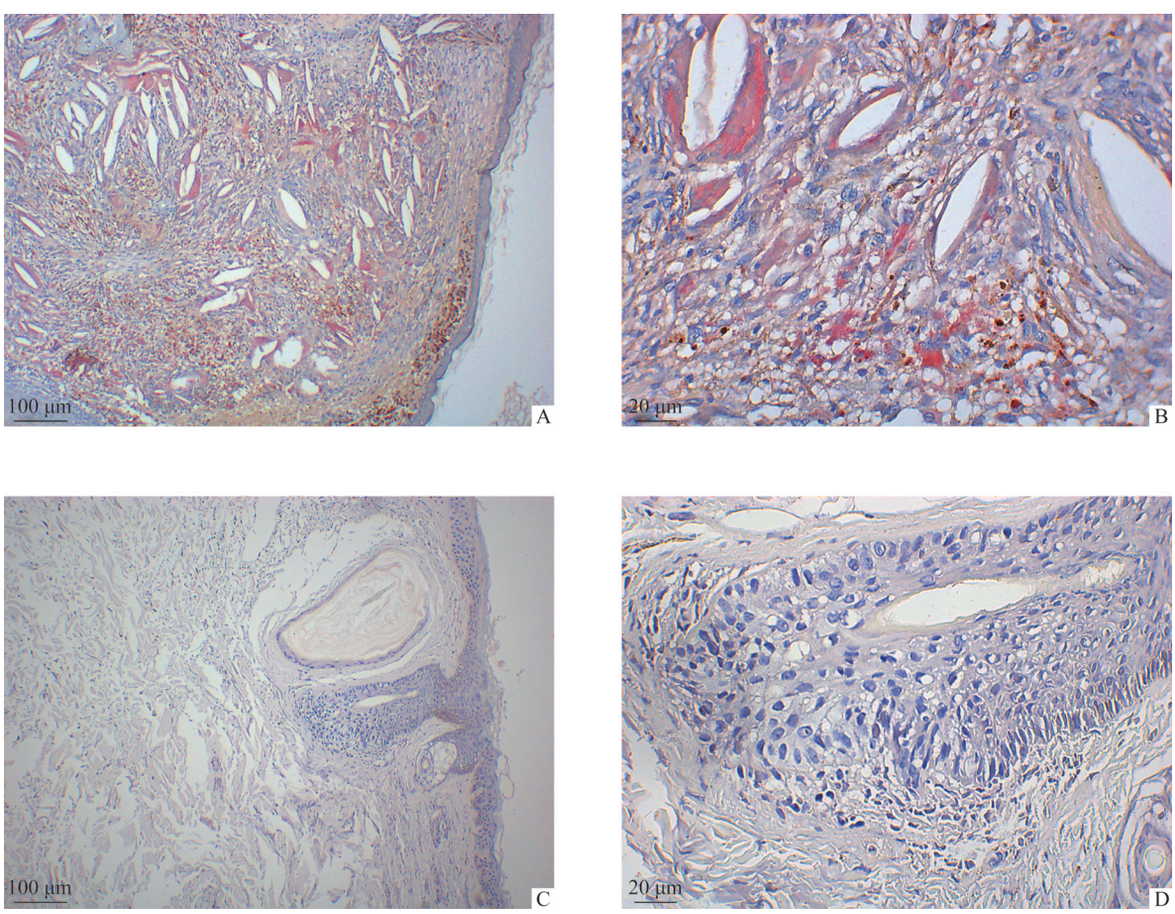

图3　中耳胆脂瘤和外耳道正常皮肤组织的TRAP染色

Figure 3　TRAP staining of middle ear cholesteatoma tissues and normal skin tissues of external auditory canal

A and B: Osteoclasts in middle ear cholesteatoma tissues with the magnification of 100 (A) and 400 (B); C and D: No osteoclasts in normal skin tissue of the external auditory canal with the magnification of 100 (C) and 400 (D). TRAP: Tartrate-resistant acid phosphatase.

2.4 破骨细胞相关基因*TRAP*、*CTSK*和*NFATc1*的mRNA表达

RAW264.7细胞分别采用RANKL和PTHrP+RANKL干预5 d后，real-time PCR检测结果显示：PTHrP+RANKL共同干预组细胞中*TRAP*、*CTSK*和*NFATc1*的mRNA表达水平较RANKL干预组均显著升高(均$P<0.05$，图5)。

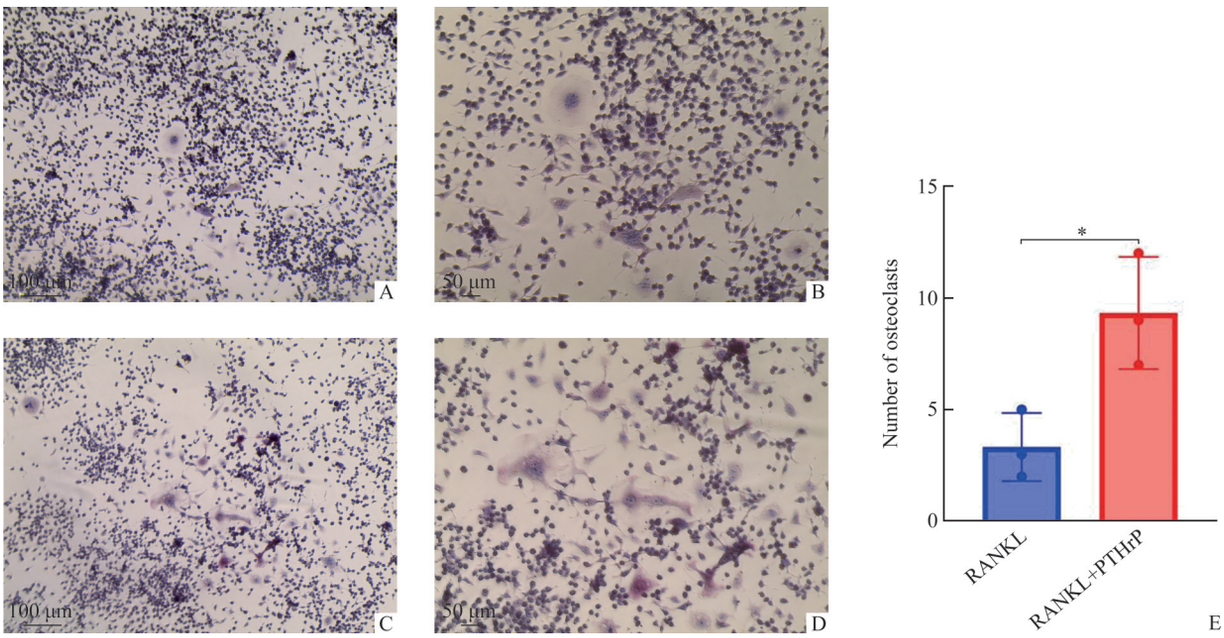

图4 TRAP染色检测RANKL干预组、PTHrP+RANKL共同干预组破骨细胞形成

Figure 4 Osteoclast formation in the RANKL intervention group and the PTHrP+RANKL co-intervention group determined by TRAP staining

A and B: Osteoclast formation in the RANKL intervention group with the magnification of 100 (A) and 200 (B); C and D: Osteoclast formation in the PTHrP+RANKL co-intervention group with the magnification of 100 (C) and 200 (D); E: Comparison of the number of osteoclasts between the PTHrP+RANKL co-intervention group and the RANKL intervention group (n=3). Data are expressed as mean±standard deviation, *P<0.05. TRAP: Tartrate-resistant acid phosphatase; PTHrP: Parathyroid hormone-related protein; RANKL: Receptor activator for nuclear factor-kappa B ligand.

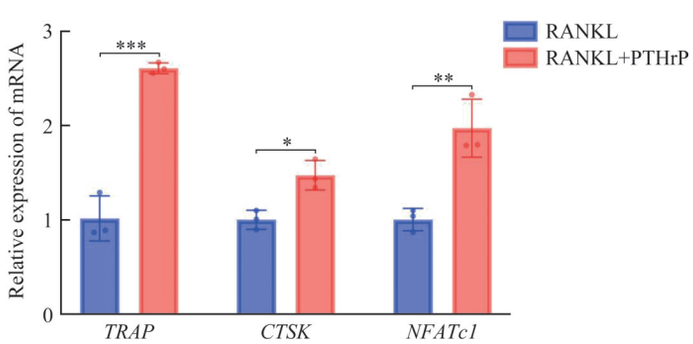

图5 RANKL干预组、PTHrP+RANKL共同干预组破骨细胞相关基因mRNA表达(n=3)

Figure 5 mRNA expressions of osteoclast-related genes in the RANKL intervention group and the PTHrP+RANKL co-intervention group (n=3)

mRNA expressions of *TRAP*, *CTSK*, and *NFATc1* significantly increased in the PTHrP+RANKL co-intervention group. Data are expressed as mean±standard deviation, *P<0.05, **P<0.01, ***P<0.001. PTHrP: Parathyroid hormone-related protein; RANKL: Receptor activator for nuclear factor-kappa B ligand; *TRAP*: Tartrate-resistant acid phosphatase; *CTSK*: Cathepsin K; *NFATc1*: Nuclear factor of activated T cell cytoplasmic 1.

2.5 骨吸收陷窝实验结果

骨吸收陷窝实验结果显示：RANKL组、PTHrP+RANKL共同干预组骨片表面均可观察到骨吸收陷窝形成。与RANKL组相比，PTHrP+RANKL共同干预组骨片表面的骨吸收陷窝数量更多(P<0.05)；PTHrP+RANKL共同干预组骨片表面的骨吸收陷窝面积更大，骨破坏程度更严重(图6)。

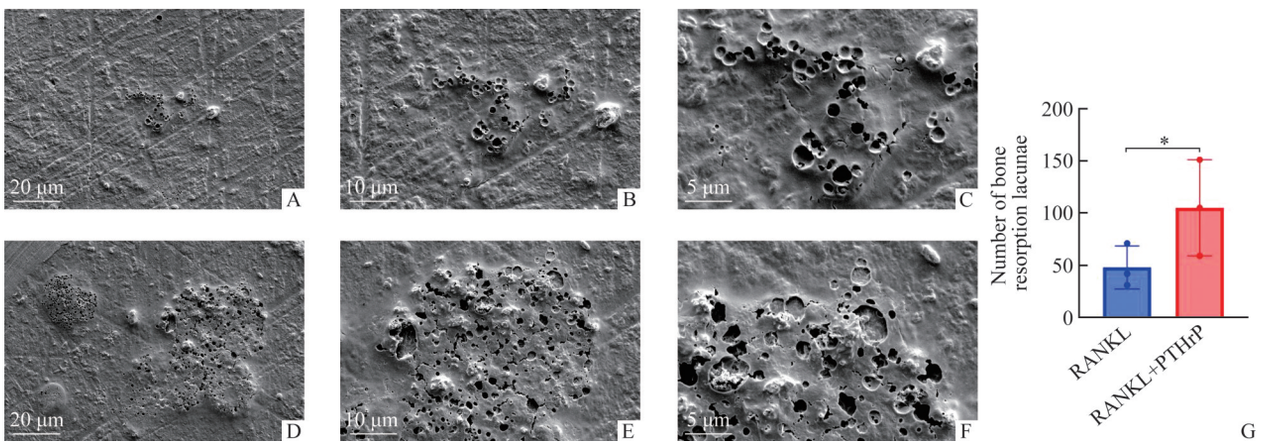

图6　RANKL干预组、PTHrP+RANKL共同干预组破骨细胞骨吸收功能

Figure 6　Bone resorption function of osteoclasts in the RANKL intervention group and the PTHrP+RANKL co-intervention group

A−C: Bone resorption lacunae in the RANKL intervention group under the electron microscope with the magnification of 1 000 (A), 2 000 (B), and 4 000 (C); D−F: Bone resorption lacunae in the PTHrP+RANKL co-intervention group under the electron microscope with the magnification of 1 000 (D), 2 000 (E), and 4 000 (F); G: Number of bone resorption lacunae in the PTHrP+RANKL co-intervention group is significantly higher than that in the RANKL intervention group (n=3). Data are expressed as mean±standard deviation, *P<0.05. PTHrP: Parathyroid hormone-related protein; RANKL: Receptor activator for nuclear factor-kappa B ligand.

3 讨　论

骨吸收、破坏与中耳胆脂瘤的颅内外并发症的发生、发展密切相关。因此，中耳胆脂瘤的骨破坏机制和治疗受到了学者们的广泛关注。不同学者从破骨细胞活化[11]、炎症介质作用[10]、酶介导[12]、酸溶解理论[13]、压力坏死作用[14]等多个方向进行了研究和探讨。

PTHrP是骨重塑和钙稳态过程的重要调节因子，在骨代谢和骨吸收疾病中发挥重要作用。在肺癌、乳腺癌、前列腺癌、多发性骨髓瘤等多种疾病中，PTHrP的表达显著上调，并与疾病的发生、发展密切相关[6, 15-16]。研究[17]发现*PTHrP*基因敲减小鼠的软骨细胞出现异常肥大及骨化。多发性骨髓瘤中高表达的PTHrP通过激活甲状旁腺激素1受体(parathyroid hormone type 1 receptor，PTH1R)，导致细胞内Ca^{2+}内流、环磷酸腺苷(cyclic adenosine monophosphate，cAMP)、RANKL和单核细胞趋化蛋白-1(monocyte chemoattractant protein-1，MCP-1)的表达显著增加，从而激活瘤细胞的破骨活性，导致骨质破坏的发生[6]。RANKL与破骨细胞分化和成熟过程密切相关[4, 18-20]，而OPG作为RANKL的可溶性诱饵受体，发挥抑制骨吸收和破坏的作用[19]。本研究结果证实中耳胆脂瘤上皮组织中PTHrP显著高表达，且与胆脂瘤骨破坏程度呈正相关，提示PTHrP在中耳胆脂瘤骨破坏进程中发挥重要作用。在骨组织局部微环境中，RANKL和OPG的相对表达量决定了破骨细胞是否活化，当RANKL/OPG比值升高时可使破骨细胞活化[21]。本研究结果显示RANKL和RANKL/OPG比值在中耳胆脂瘤上皮组织中升高，可能参与中耳胆脂瘤骨破坏机制。

破骨细胞是发挥骨吸收作用的主要功能细胞，在骨吸收和骨重塑中起关键作用。研究[22]发现：在中耳胆脂瘤周围的破坏骨质中，多核成熟破骨细胞聚集在骨吸收区边缘，形成若干吸收小室，介导骨破坏，且破骨细胞的浸润程度与中耳胆脂瘤骨破坏程度呈显著正相关。巨噬细胞是一种破骨细胞前体细胞，可诱导分化为破骨细胞[23-24]。骨髓、脾、胸腺或外周血等器官和组织的单核巨噬细胞在体外都能分化为TRAP阳性的破骨细胞[18, 25]，因此，有可能形成破骨细胞的单核巨噬细胞被称为破骨细胞前体细胞。研究[26]证实中耳胆脂瘤组织中伴有大量单核巨噬细胞浸润，但目前尚无研究报道其是否向破骨细胞分化和成熟，导致胆脂瘤骨破坏。本研究的TRAP染色结果显示在中耳胆脂瘤组织周围基质中存在大量细胞核数目≥3个的TRAP阳性细胞。因此，笔者推测中耳胆脂瘤中RANKL高表达可能诱导中耳胆脂瘤周围基质中的巨噬细胞分化为破骨细胞，从而介导胆脂瘤的骨吸收和破坏过程。

笔者发现RANKL可诱导RAW264.7巨噬细胞分

化为具有骨吸收功能的成熟破骨细胞，进一步证实巨噬细胞作为破骨细胞前体细胞能够分化为成熟破骨细胞。既往研究[27]发现：RANKL与核因子κB受体活化因子(receptor activator for nuclear factor-kappa B，RANK)结合后激活下游磷脂酰肌醇3-激酶/蛋白激酶B(phosphatidylinositol 3-kinase/protein kinase B，PI3K/AKT)、丝裂原活化蛋白激酶(mitogen-activated kinase，MAPK)、细胞外调节蛋白激酶(extracellular signal-regulated kinase，ERK)、c-Jun氨基末端激酶(c-Jun N-terminal kinase，JNK)信号通路，上调核因子κB(nuclear factor-κB，NF-κB)、c-Fos和c-Jun等转录因子的表达，进而促使NFATc1在破骨细胞形成早期阶段的表达增高。作为破骨细胞生成的主要转录因子，NFATc1能够促使破骨细胞前体细胞分化为成熟的多核破骨细胞[19]，使相关破骨细胞基因表达增高，如*TRAP*和*CTSK*表达上调，最终导致骨吸收和破坏[28-29]。

甲状旁腺激素(parathyroid hormone，PTH)通过结合表达于间充质干细胞、成骨细胞、破骨细胞、T淋巴细胞和巨噬细胞表面的PTH1R后[30]，作用于成骨细胞、破骨前体细胞及骨细胞，在骨代谢的过程中发挥重要作用[9]。PTH被证实可以诱导间充质干细胞和脂肪来源干细胞分化为成骨细胞，促进成骨细胞的增殖，从而促进骨生成。此外，PTH能间接作用于破骨前体细胞及破骨细胞，促进它们的增殖和分化，从而导致骨吸收[9]。T淋巴细胞能表达PTH1R，并可通过Wnt信号通路参与PTH介导的破骨细胞活化[31]，而B淋巴细胞能够分泌RANKL和OPG，并受PTH的间接调控[32]。作为PTH家族中的一员，PTHrP同样能与PTH1R结合，从而激活下游信号通路，调节骨代谢进程[9]。

目前PTHrP在骨吸收和骨破坏中的作用与其促进成骨细胞或骨髓基质细胞表达RANKL等破骨细胞分化相关因子有关[6]。PTHrP可以激活RANKL/RANK/OPG信号通路，上调RANKL的表达，并促进RANKL和表达于破骨前体细胞表面的RANK结合，从而促进破骨前体细胞的分化和成熟，增强其破骨功能[9]。此外，PTHrP还被证实能通过Wnt、cAMP/蛋白激酶A(protein kinase A，PKA)、cAMP/蛋白激酶C(protein kinase C，PKC)等信号通路来调控破骨细胞的活性[9]。在药物治疗乳腺癌骨转移动物实验[5]中，发现模型小鼠体内PTHrP的表达下调和RANKL/OPG比值下降，骨破坏进程同时也被抑制。在前列腺癌骨病变的小鼠实验[33]中，抗肿瘤药物可抑制成骨细胞中RANKL的表达、破骨细胞的生成和PTHrP刺激下的骨吸收过程。在本研究中，中耳胆脂瘤上皮组织中PTHrP的表达与RANKL、RANKL/OPG比值均呈显著正相关，与OPG呈显著负相关，初步推测PTHrP可能通过RANKL介导中耳胆脂瘤的骨破坏进程，进而通过细胞实验发现PTHrP+RANKL共同干预组破骨细胞生成数量更多，且破骨细胞标志性相关基因*TRAP*、*CTSK*和*NFATc1*的mRNA水平较RANKL干预组均上调，提示PTHrP除通过促进RANKL的表达外，对RANKL诱导破骨细胞分化和成熟的其他生物学过程可能也有潜在作用，其具体分子机制有待进一步研究。此外，骨吸收陷窝实验结果显示2组骨片表面均存在骨吸收陷窝，且PTHrP+RANKL共同干预组骨吸收陷窝数量更多，面积更大，证明由RANKL诱导RAW264.7细胞分化成熟的破骨细胞具有骨吸收功能，而PTHrP可能在上游水平调控RANKL介导的骨吸收功能。

综上所述，本研究证实PTHrP、RANKL和RANKL/OPG比值在中耳胆脂瘤组织中高表达，且与胆脂瘤骨破坏程度密切相关，此外还发现中耳胆脂瘤上皮周围基质中存在TRAP染色阳性的破骨细胞；进一步的体外细胞学实验发现PTHrP对RANKL诱导单核巨噬细胞分化为具有骨吸收功能的成熟破骨细胞这一生物学过程具有重要促进作用，证实PTHrP通过促进RANKL诱导胆脂瘤组织周围基质中的单核巨噬细胞分化为破骨细胞，参与中耳胆脂瘤骨破坏。

作者贡献声明：谢淑敏 研究设计，论文撰写；金丽、符金凤、袁秋林 实验实施，数据分析；殷团芳、任基浩 实验指导，论文修改；刘伟 实验指导，论文构思与修改。所有作者阅读并同意最终的文本。

利益冲突声明：作者声称无任何利益冲突。

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（责任编辑 陈丽文）

本文引用：谢淑敏, 金丽, 符金凤, 袁秋林, 殷团芳, 任基浩, 刘伟. PTHrP促进RANKL诱导巨噬细胞分化为破骨细胞参与中耳胆脂瘤骨破坏[J]. 中南大学学报(医学版), 2024, 49(5): 655-666. DOI:10.11817/j.issn.1672-7347.2024.230482

Cite this article as: XIE Shumin, JIN Li, FU Jinfeng, YUAN Qiulin, YIN Tuanfang, REN Jihao, LIU Wei. PTHrP participates in the bone destruction of middle ear cholesteatoma via promoting macrophage differentiation into osteoclasts induced by RANKL[J]. Journal of Central South University. Medical Science, 2024, 49(5): 655-666. DOI:10.11817/j.issn.1672-7347.2024.230482