携带线粒体tRNA^{Thr} 15910C>T突变和12S rRNA 1555A>G突变的非综合征型耳聋家系的线粒体功能研究

姚 娟[1] 林 枝[1] 王 辉[1] 高应龙[3] 范文露[1] 王 恒[1] 姜 丰[1]
薛 凌[1] 唐霄雯[2] 郑斌娇[1] 管敏鑫[1,3]*

([1]温州医科大学Attardi线粒体生物医学研究院, 温州 325035; [2]温州医科大学仁济学院, 温州 325035;
[3]浙江大学医学院, 遗传研究所, 杭州 310058)

摘要 该研究旨在探讨一个非综合征型耳聋(nonsyndromic hearing impairment, NSHI)家系中线粒体tRNA^{Thr} 15910C>T和12S rRNA 1555A>G基因突变共同作用对线粒体功能的影响。该研究建立了同时携带线粒体tRNA^{Thr} 15910C>T和12S rRNA 1555A>G基因突变(双突变组)、仅携带12S rRNA 1555A>G基因突变(单突变组)和对照组的永生化淋巴细胞, 这3组细胞系的线粒体DNA单体型均属于R单体型。对该家系的临床资料进行分析, 当包括使用氨基糖苷类抗生素(aminoglycoside antibiotics, AmAn)的药物性耳聋家系成员时, 此家系耳聋外显率为37.5%; 当排除用药的耳聋成员时, 耳聋外显率是25.0%; 相比之下, 在用药和未用药的情况下, 已报道的14个m.1555A>G的耳聋家系的平均外显率只有12.8%和6.1%。通过对双突变组、单突变组和对照组永生化细胞系的线粒体功能进行研究, 结果发现与对照组相比, 双突变和单突变组细胞系的ROS水平分别上升了19.08%(P=0.005 4)和9.05%(P=0.003 7); $\Delta\Psi$m水平分别下降了47.78%(P=0.006 3)和35.39%(P=0.024 5); 复合体II活力分别下降了8.26%(P=0.721 1)和19.48%(P=0.004 9), 复合体IV活力分别下降了32.75%(P=0.033 5)和27.44%(P=0.180 5)。m.1555A>G与m.15910C>T共同作用, 导致ROS生成量升高, $\Delta\Psi$m水平下降以及线粒体呼吸链复合体IV活力降低等线粒体功能缺陷, 提示m.15910C>T可能是m.1555A>G导致耳聋的继发性突变。

关键词 非综合症型耳聋; 线粒体tRNA^{Thr}基因; 突变; 功能障碍

The Study of Mitochondrial Function in A Nonsyndromic Hearing Loss Family Carrying Mitochondria tRNA^{Thr} 15910C>T and 12S rRNA 1555A>G Mutations

Yao Juan[1], Lin Zhi[1], Wang Hui[1], Gao Yinglong[3], Fan Wenlu[1], Wang Heng[1], Jiang Feng[1], Xue Ling[1], Tang Xiaowen[2], Zheng Binjiao[1], Guan Minxin[1,3]*

([1]*Attardi Institute of Mitochondrial Biomedicine, Wenzhou Medical University, Wenzhou 325035, China*; [2]*Renji College, Wenzhou Medical University, Wenzhou 325035, China*; [3]*Institute of Genetics, School of Medicine, Zhejiang University, Hangzhou 310058, China*)

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*通讯作者。Tel: 0571-88206916, E-mail: gminxin88@gmail.com
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*Corresponding author. Tel: +86-571-88206916, E-mail: gminxin88@gmail.com
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Abstract　In this study, we investigated the mitochondrial function of 12S rRNA 1555A>G and mitochondria tRNA^{Thr} 15910C>T mutations in a nonsyndromic hearing loss (NSHL) family. We established three groups of lymphoblastoid cell lines in this study, including the double mutations group, the single mutation group and the controls, which all belong to the Eastern Asian haplogroup R. The double mutations group are 3 subjects carried both m.1555A>G and m.15910C>T. The single mutation group are 3 subjects carried m.1555A>G mutation only. The controls are 3 subjects with normal auditory sense. To analyze the clinical data of the family, the penetrance of deafness is 37.5% or 25.0% which contains or excludes the family members who have used AmAn drugs; while the penetrance of deafness is 12.8% or 6.1% which contains or excludes the family members who have used AmAn drugs in the 14 reported deafness families with m.1555A>G mutation. Compared with controls, the ROS level in the double-mutations group has been raised 19.08% (P=0.005 4) and the single-mutation group only raised 9.05% (P=0.003 7). Compared with controls, the mitochondrial membrane potential decreased 47.78% (P=0.006 3) and 35.39% (P=0.024 5) in the double-mutations group and the single-mutation group, respectively. It showed that 8.26% (P=0.721 1) and 19.48% (P=0.004 9) decrease in the activity of respiratory chain complex II while 32.75% (P=0.033 5) and 27.44% (P=0.180 5) decrease in the activity of respiratory chain complex IV in the double-mutations group and the single-mutation group compared with controls. These results showed that m.1555A>G and m.15910C>T led to mitochondrial dysfunction, included ROS raise up, mitochondrial membrane potential and the activity of respiratory chain complex IV decreased. Thus, m.15910C>T worsens the mitochondrial function associated with m.1555A>G, and is probably a secondary mutation of m.1555A>G caused deafness.

Keywords　NSHL; mitochondrial tRNA^{Thr} gene; mutant; dysfunction

耳聋是影响人类身心健康和生活质量的最常见感觉系统障碍之一[1-2]。根据是否伴有其他症状, 耳聋可分为非综合征型耳聋(nonsyndromic hearing impairment, NSHI)和综合征型耳聋(syndromic hearing impairment, SHI)。线粒体DNA(mitochondrial DNA, mtDNA)突变是造成听力损失的重要原因之一[3-5], 现已证实, 氨基糖苷(aminoglycoside)类抗生素致聋和非综合征型耳聋与线粒体12S rRNA 1494C>T和1555A>G突变有关[2,6]。线粒体tRNA基因突变是导致综合症型耳聋和非综合征型耳聋的重要原因之一, 有些tRNA突变可直接造成耳聋的发生, 称之为原发突变, 如tRNA$^{Leu(UUR)}$ 3243A>G等突变与综合征型耳聋相关, 而tRNA$^{Ser(UCN)}$ 7511T>C等突变则与非综合征型耳聋相关[7]。此外, 继发突变如tRNA^{Thr} 15927G>A则对原发突变起协同作用, 影响耳聋的表型表达[7-8]。

m.15910C>T突变破坏了tRNA^{Thr} DHU-loop结构上十分保守的C-G碱基对, 这可能加重由m.1555A>G突变造成的线粒体功能缺陷。目前, 国内外尚无关于m.15910C>T与m.1555A>G共同作用导致耳聋的文献报道。本研究建立了一个同时携带线粒体tRNA^{Thr} 15910C>T和12S rRNA 1555A>G的非综合征型耳聋家系的永生化淋巴细胞系, 通过对该家系永生化淋巴细胞系的细胞内活性氧(reactive oxygen species, ROS)生成量、线粒体膜电位(mitochondrial membrane potential, MMP)水平、呼吸链复合体酶活力的研究, 从线粒体功能水平对该位点的致病性进行了评估, 为耳聋的早期诊断、预防和遗传咨询提供理论依据。

1　材料与方法

1.1　材料

本研究通过EB(Epstein-Barr)病毒诱导建立永生化的外周血类淋巴母细胞系。外周血来源于参加此项研究的试验者, 所有试验者按照温州医科大学伦理委员会管理规定的方法, 签署知情同意书。随后, 对研究对象进行了详细的听力学和临床体格检查, 排除综合征型耳聋, 同时深入细致地询问了患者及其家系成员的耳聋病史、是否有氨基糖苷类抗生素使用史以及是否存在其他致聋因素等。听力学检查包括纯音测听(pure-tone hearing threshold, PTA)、听觉脑干反应(auditory brainstem responses, ABR)、

声导抗以及畸变产物耳声反射(distortion product oto-acoustic emission, DPOAE)。耳聋以听力水平的分贝(dBHL)数为单位进行测量, 并以语言频率(500、1 000、2 000、4 000和8 000 Hz)的听阈平均值计算, 将听力损失的严重程度分成5级, 正常<26 dBHL、轻度聋26~40 dBHL、中度聋41~70 dBHL、重度聋71~90 dBHL及极重度聋>90 dBHL。此外, 选取342名汉族健康人群作为正常对照。

1.2 线粒体基因组全序列分析

从外周血或永生化淋巴母细胞中按照Universal Genomic DNA Extraction Kit Ver.4.0(TaKaRa公司)的使用说明提取基因组DNA, −20 ℃保存备用。以提取的外周血全基因组DNA为模板, 通过PCR扩增先证者、家系成员及对照组成员线粒体DNA, 使用24对引物进行线粒体全基因组扩增, PCR产物纯化后, 送华大基因测序公司进行双向测序, 测序结果与校正的剑桥标准序列[9]进行比对, 以确定是否含有耳聋相关热点基因突变。

1.3 细胞培养

永生化类淋巴母细胞系可以进行无限繁殖和生存的特性弥补了外周血淋巴细胞在体外寿命有限的缺点, 使人类珍贵的耳聋家系遗传资料得以永久保存。通过EB病毒诱导可以建立永生化的外周血类淋巴母细胞系, 其原理为EB病毒感染并与人外周血B淋巴细胞膜上的EB病毒受体结合后转化细胞, 产生可在体外长期传代生长的永生性类淋巴母细胞系, 使得在体外研究细胞功能和分子遗传机制变为可能。

本研究建立的永生性类淋巴母细胞系包括3个携带1555A>G和15910C>T突变的个体(WZD85 IV-2、WZD85 III-3、WZD85 III-2), 3个携带1555A>G突变的个体(WZD83 IV-1、WZD83 IV-3、WZD84 III-1), 3个听力正常的个体(A24、A25和A26)。所有的细胞系采用10%胎牛血清的RPMI 1640培养基进行培养, 置于37 ℃、5% CO_2及饱和湿度恒温培养箱中培养。

1.4 细胞内活性氧检测

采用上海碧云天生物技术有限公司ROS检测试剂盒(reactive oxygen species assay kit S0033), 通过流式细胞术对细胞内的ROS水平进行检测[10-12]。每个细胞系取2×10^6细胞, 重悬于含有100 μmol/L的2′,7′-二氯二乙酸酯(2′,7′-dichloroethyl acetate, DCFH-DA)的PBS中, 然后在于37 ℃孵育20 min; H_2O_2刺激组用PBS洗涤2次后重悬于新鲜制备的含有2 mmol/L H_2O_2的PBS中, 室温孵育5 min。最后, 将细胞用PBS洗涤1次, 重悬于1 mL的PBS。正常和H_2O_2刺激的样品通过BD-Accuri-C6流式细胞仪进行分析, 分别在488 nm和525 nm时激发和发射。

1.5 线粒体膜电位检测

使用上海碧云天生物技术有限公司的JC-1荧光染料, 通过流式细胞术对线粒体膜电位水平进行检测[13]。在24孔板中接种约5×10^5细胞。在37 ℃、5% CO_2条件下加入JC-1(碧云天)染液孵育30 min。阳性对照在JC-1染料染色前用10 μmol/L解偶联剂羰氰化3-氯苯(CCCP)37 ℃、5% CO_2条件预处理30 min。使用BD-Accuri-C6流式细胞仪Ex/Em=585/590 nm和Ex/Em=514/529 nm测定为JC-1聚合物和JC-1单体的荧光强度。

1.6 线粒体酶活性检测

收集1×10^8处于对数生长期的细胞, 蔗糖密度梯度离心法提取线粒体, 液氮/30 ℃水浴反复冻融3次(1 min/次), 利用SpectraMax M5多功能酶标仪分别检测5个线粒体呼吸链复合体的酶活力。线粒体酶活性的测定参照Birch-Machin和Turnbull修订后的实验过程。将复合体I、复合体II、复合体III和复合体IV的酶促反应速率以柠檬酸合酶的酶促反应速率为标准进行标准化, 计算不同复合体的相对酶活力[14-15]。

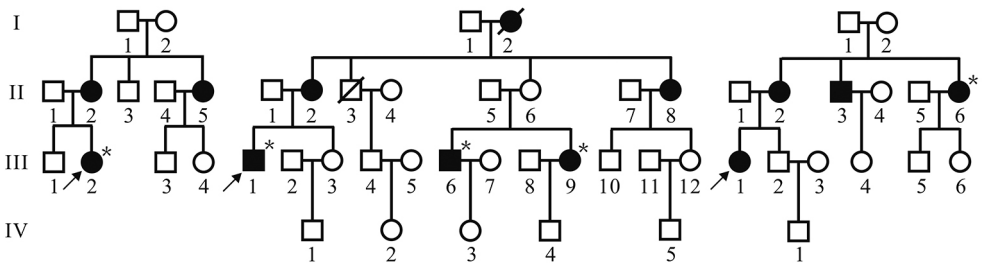
柠檬酸合酶催化乙酰辅酶A和草酰乙酸产生柠檬酰辅酶A, 进一步水解产生柠檬酸, 该反应促使无色的5,5′-二硫代双-2-硝基苯甲酸[5,5′-dithio bis-(2-nitrobenzoic acid, DTNB]转变成黄色1,3,5-三硝基苯(1,3,5-Trinitrobenzene, TNB), 通过对412 nm处的特征吸光值检测, 分析柠檬酸合酶活性。NADH的氧化速率通过340 nm处的吸光值的减少来测定, 吸光值的减少与线粒体复合体I的活性成正比, 通过测定线粒体复合体I催化的NADH的氧化速率来反映线粒体复合体I的活性。在线粒体复合体II催化的琥珀酸氧化反应中, 使用2,6-二氯吲哚酚(2,6-dichlorophenolindophenolsodium salt, DCPIP)作为染色剂, 600 nm处吸光值随着DCPIP的减少而减少, 线粒体复合体II的活性通过检测DCPIP的减少速率来测定。还原型细胞色素C在550 nm处吸光值有特征光吸收, 通过测定550 nm处光吸收增加速率反映线粒体复合体III的活性。线粒体复合体IV催化还原型细胞色素C生成氧化型细胞色素C, 通过测定

细胞色素C的氧化速率(550 nm处吸光值)反映线粒体复合体IV的活性[14-15]。

2　结果

2.1　听力学及临床检查结果分析

在WZD85家系中, 先证者IV-2, 女, 9岁。发病年龄2岁, 诱因为脑外伤, 臀部有肌注射氨基糖苷类抗生素药物史, 具体药物不详。ABR结果表明, 其听力损失为极重度感音神经性聋(左耳听阈110 dBHL, 右耳听阈90 dBHL)。对该家系其他成员进行临床和遗传学的评估发现, 该家系主要成员生活在江西省瑞昌市清岭村, 共3代11人(图1), 家系中所有耳聋病人均为母系成员后代, 符合母系遗传方式; 该家系共有3位听力下降的母系成员(III-2、III-5及IV-2), 均为女性, 听力损失程度从轻度到极重度; 听力图结构主要为斜坡型(III-2、III-5)(图2和表1)。

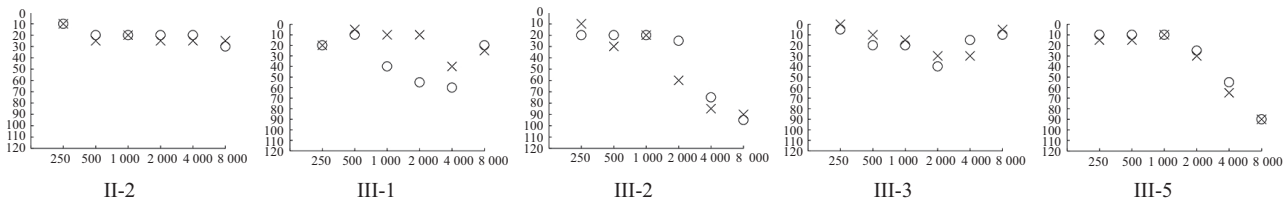


WZD85为同时携带1555A>G和15910C>T突变的家系, WZD83和WZD84为携带1555A>G突变的家系。黑色符号表示听力下降者; 箭头所指为先证者; *表示有AmAn用药史。

WZD85 is Chinese family with 1555A>G and 15910C>T, WZD83 and WZD84 are 2 Chinese families with 1555A>G. Hearing impaired individuals are indicated by filled symbols; arrow denotes proband, * denotes the individuals who had a history of exposure to aminoglycosides.

图1　三个耳聋家系的家系图

Fig.1　Three Chinese families with hearing loss



×: 左耳; ○: 右耳。

×: left ear; ○: right ear.

图2　WZD85家系中5位家系成员听力图

Fig.2　Air conduction audiogram of five members in a Chinese family (WZD85)

表1　携带m.1555A>G和m.15910C>T突变的家系中6位成员临床资料表

Table 1　Summary of the clinical data of six maternal members of a Chinese family carrying m.1555A>G and m.15910C>T mutations

编号 Code	性别 Sex	年龄 Age (years)		氨基糖苷类药物史 Use of aminoglycosides	纯音测听(dBHL) PTA (dBHL)		听力损失程度 Level of hearing impairment	听力曲线类型 Audiometric configuration
		检测年龄 At testing	发病年龄 At onset		右耳 Right ear	左耳 Left ear		
II-2	F	–	–	No	22	24	Normal	Flat
III-1	M	34	–	No	34	18	Mild	Valley
III-2	F	29	–	No	42	50	Moderate	Slope
III-3	M	–	–	No	18	15	Normal	Flat
III-5	F	–	–	No	33	40	Mild	Slope
IV-2^{ABR}	F	9	2	Yes	90	110	Profound	–

ABR:听性脑干反应, IV-2的听力情况为ABR检测结果。

ABR: auditory brainstem response, the hearing condition of IV-2 is the testing result of ABR.

检查发现, 该家系成员以双侧对称的听力障碍为唯一的临床症状, 未发现其他临床症状, 如糖尿病、肌病等。

2.2 线粒体基因组突变分析

该家系呈现母系遗传特征, 提示线粒体DNA 突变可能是致病的分子基础。我们首先对先证者线粒体12S rRNA 基因进行测序分析, 发现了存在m.1555A>G突变(图3), 而其他家系成员不含有该突变。

为了确定线粒体多态性在m.1555A>G突变的临床表型中的作用, 我们对先证者线粒体全基因组进行了扩增和测序, 测序结果和修正后的剑桥序列做比较[9], 这个家系除了存在同质性的1555A>G突变外, 还存在多个多态性位点(表2)。其中, 5个是新发现的变异位点。 包括tRNA^{Thr} 15910C>T和4个同义突变*MT-ND1* 3535T>C、*MT-ND2* 4802T>C、*MT-CO3* 9947G>A和*MT-ND5* 13044C>T。

2.3 膜电位检测结果

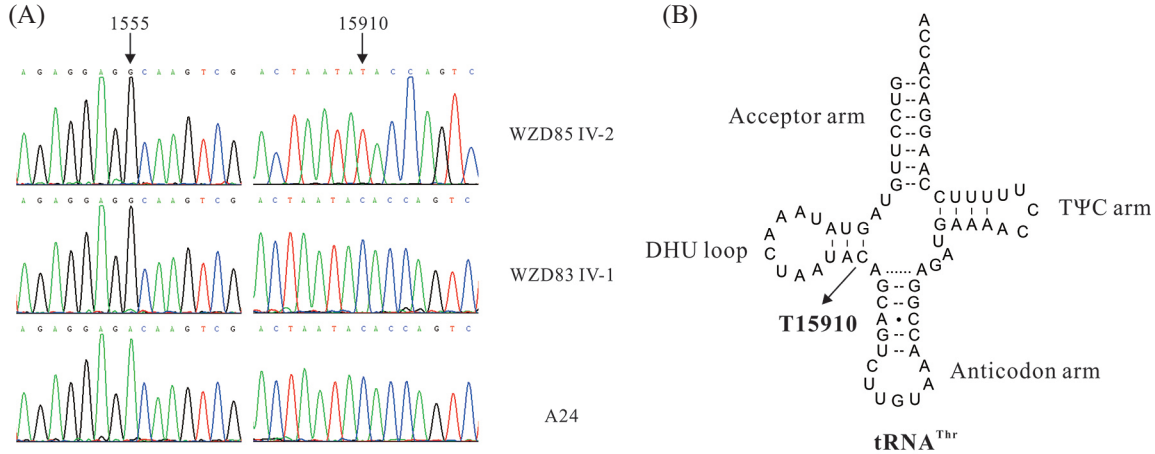
线粒体膜电位($\Delta\Psi$m)在控制呼吸速率, ATP合成和活性氧的产生中起着重要作用[12-13]。我们使用JC-1荧光染料检测双突变组、单突变组和对照组细胞系的$\Delta\Psi$m水平。Ex/Em=490/590 nm和Ex/Em=490/529 nm的荧光强度比(FL590/FL529)反映每个样本的$\Delta\Psi$m水平。对照组与单突变组, 对照组与双突变组的FL590/FL529相对几何平均值代表平均$\Delta\Psi$m水平。

如图4所示, 与对照组相比, 单突变组的$\Delta\Psi$m水平下降35.39%(P=0.024 5), 而双突变组的细胞系的$\Delta\Psi$m水平下降47.78%(P=0.006 3)。双突变组比单突变组降低更为明显, 说明m.15910C>T突变可能使得m.1555A>G对线粒体膜电位的影响变得更加严重。

2.4 线粒体复合体酶活性检测结果

为了解m.1555A>G和m.15910C>T突变在氧化磷酸化过程中的作用, 提取对照组、单突变组和双突变组的永生淋巴细胞系中的线粒体。分别测定每个样本的柠檬酸合酶、复合体I、复合体II、复合体III和复合体IV的活力[15]。将复合体I、复合体II、复合体III和复合体IV的酶促反应速率以柠檬酸合酶的酶促反应速率为标准进行标准化, 计算不同复合体的相对酶活力[16]。

如图5所示, 与对照组相比, 单突变组的复合体II活性水下降19.48%(P=0.004 9), 具有显著差异。复合体IV活性水下降27.44%(P=0.180 5), 无显著差异。双突变组的细胞系的线粒体复合体II活性下降8.26%(P=0.721 1), 无显著差异。复合体IV活性下降32.75%(P=0.033 5), 具有显著差异。而复合体I和复合体III的活力, 各组间无显著性差异。这表明以下观点, (1)单突变组和双突变的复合体II的酶活力降低, 单突变组降低较为明显, 其中双突变组的复合体II下降无统计学意义可能是由于样本间存在差异。(2)单突变组和双突变的复合体IV的酶活力降低, 双



A: mtDNA 1555位点和15910位点的测序峰图。箭头指示mtDNA 1555位点和15910位点碱基变化。B: 15910位点位于tRNA^{Thr}的D茎, 箭头指示15910位点由C突变为T。

A: partial sequence chromoatograms of mtDNA 1555 and 15910. Arrows indicate the location of the base changes. B: 15910 site is located in the D-stem of tRNA^{Thr} . Arrow indicates m.15910C>T.

图3 患者与正常人中m.1555A>G位点和m.15910C>T位点的测序峰图结果和m.15910C>T位点在tRNA^{Thr}二级结构中的位置

Fig.3 Partial sequence chromatograms of the fragments carrying the m.1555A>G or m.15910C>T mutation from affected individuals and controls from the family and the location of the m.15910C>T mutation in the mitochondrial tRNA^{Thr}

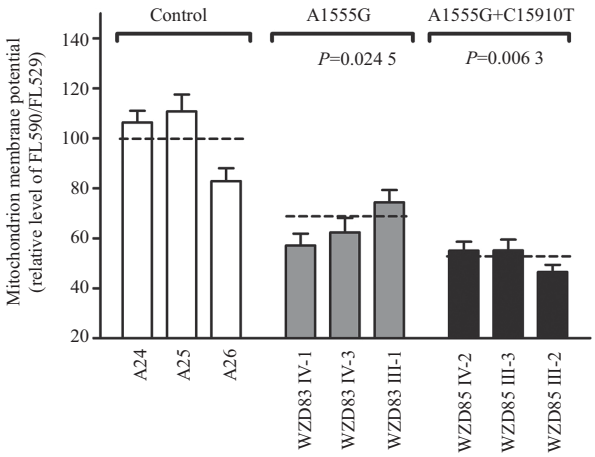
表2　WZD85家系的线粒体DNA全序列分析

Table 2　mtDNA variants in one Han Chinese subject (WZD85) with hearing loss

基因 Gene	位点 Position	碱基替换 Replacement	保守性(%)[a] Conservation (%)	是否已报道[b] Previously reported
D-loop	73	A to G		Y
	152	T to C		Y
	249	del A		Y
	263	A to G		Y
	310	T to CTC		Y
	16 220	A to C		Y
	16 298	T to C		Y
	16 362	T to C		Y
	16 519	T to C		Y
12S rRNA	750	A to G	100	Y
	1 438	A to G	100	Y
	1 555	A to G	88	Y
16S rRNA	2 392	T to C	35	N
	2 706	A to G	88	Y
ND1	3 434	A to G (Thr to Cys)	71	Y
	3 535	T to C	100	N
	3 970	C to T	100	Y
ND2	4 769	A to G	35	Y
	4 802	T to C	94	N
NC3	5 585	G to A		Y
CO1	5 913	G to A (Asp to Asn)		Y
	5 978	A to G	100	Y
	6 392	T to C	100	Y
	7 028	C to T	100	Y
NC7	8 271-8 279	9 bp del		Y
ATP6	8 860	A to G (Thr to Ala)	82	Y
CO3	9 947	G to A	100	N
ND3	10 310	G to A	88	Y
	10 320	G to A (Val to Ile)	24	Y
ND4	11 065	A to G	100	Y
	11 719	G to A	94	Y
ND5	13 044	C to T	100	N
	13 928	G to C (Ser to Thr)	12	Y
CytB	14 766	C to T (Thr to Ile)	41	Y
	15 326	A to G (Thr to Ala)	59	Y
tRNA^{Thr}	15 910	C to T	94	N

a: 多肽中氨基酸或rRNAs中核苷酸的保守性包含17个物种, 分别是白额卷尾猴、东非黑白疣猴、大猩猩、智人、白掌长臂猿、环尾狐猴、普通狨猴、地中海狨猴、间蜂猴、倭黑猩猩、黑猩猩、狒狒、阿拉伯狒狒、婆罗洲猩猩、苏门答腊猩猩、马来跗猴、郁乌叶猴、绿猴。b: 参考http//www.mitomap.org和http://www.genpat.uu.se/mtDB/。*ND1*: 线粒体NADH脱氢酶亚基1基因; *NC3*: noncoding position 3; *CO1*: 线粒体细胞色素c氧化酶亚基1基因; *CytB*: 线粒体细胞色素b基因。N: 此变异尚无文献报道; Y: 此变异已经有文献报道。

a: conservation of amino acid for polypeptides or of nucleo tide for rRNAs in 17 primary species including *Cebus albifron*, *Colobus guereza*, *Gorilla gorilla*, *Homo sapiens*, *Hylobates lar*, *Lemur catta*, *Macaca mulatta*, *Macaca sylvanus*, *Nycticebus coucang*, *Pan paniscus*, *Pan troglodytes*, *Papio hamadryas*, *Pongo pygmaeus*, *Pongo pygmaeus abelii*, *Tarsius bancanus*, *Trachypithecus obscurus*, *Chlorocebus sabaeus*. b: see from http//www.mitomap.org and http://www.genpat.uu.se/mtDB/. *ND1*: mitochondrially encoded NADH dehydrogenase 1; *NC3*: noncoding position 3; *CO1*: mitochondrially encoded cytochrome c oxidase 1; *CytB*: mitochondrially encoded cytochrome b. N: the variation hasn't been reported; Y: the variation has been reported.

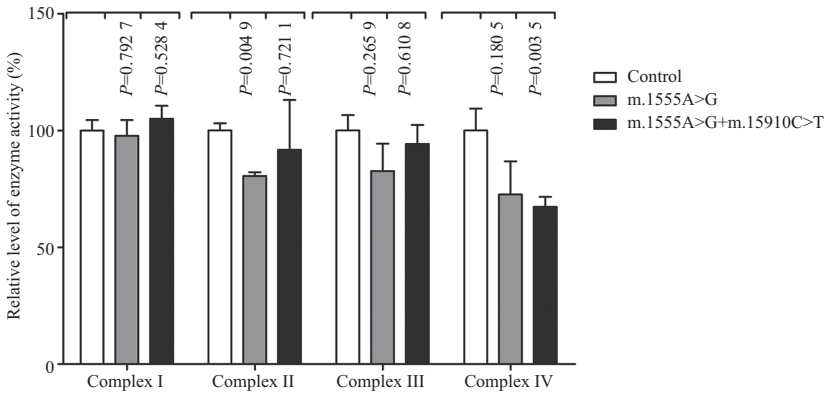


使用JC-1荧光染料对突变组和对照组样品进行染色后, 应用BD-Accuri-C6流式细胞仪分析线粒体膜电位, Ex/Em=490/590 nm和Ex/Em=490/529 nm的荧光强度比(FL590/FL529)表示每个样本的$\Delta\Psi$m水平。突变组与对照组的FL590/FL529相对比率反映了膜电位的相对水平。实验独立重复3~5次, 数据以mean±S.D.形式表示。

The mitochondrial membrane potential ($\Delta\Psi$m) in mutant and control cell lines were analyzed by BD-Accuri-C6 flow cytometer using a fluorescence probe JC-1 assay. The ratio of fluorescence intensities Ex/Em=490/590 nm and Ex/Em=490/529 nm (FL590/FL529) were recorded to delineate the $\Delta\Psi$m level of each sample. Relative ratio of JC-1 fluorescence intensities at Ex/Em=490/529 nm and Ex/Em=490/590 nm. Each point represents the mean±S.D. of 3-5 independent experiments.

图4 线粒体膜电位水平分析

Fig.4 Mitochondrial membrane potential analysis



从细胞系中提取线粒体, 通过酶活性测定以示线粒体中呼吸链复合体I、II、III和IV的活性。结果根据3次独立检测结果进行计算, 数据以mean±S.D.表示。

The activities of respiratory chain complexes were investigated by enzymatic activities assay on complexes I, II, III and IV in mitochondria isolated from various cell lines. Each point represents the mean±S.D. of three independent experiments.

图5 线粒体呼吸链复合体酶活性

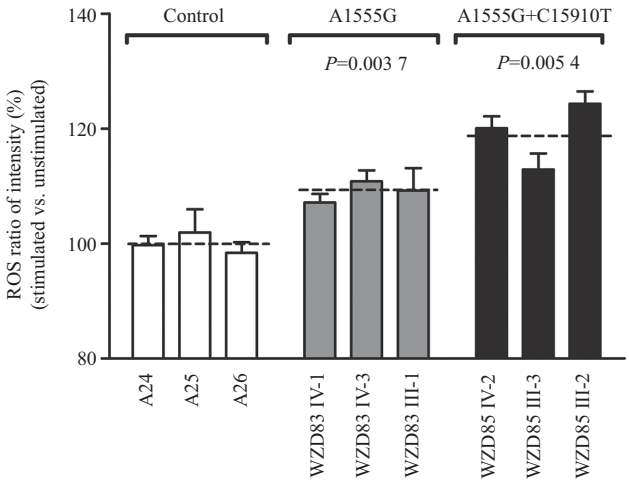
Fig.5 Enzymatic activities of respiratory chain complexes

突变组降低尤为明显。这说明, m.15910C>T突变可能加重了m.1555A>G对复合体IV活性的影响。

2.5 活性氧检测结果

在正常情况下和H_2O_2刺激条件下使用流式细胞术检测双突变组、单突变组和对照组细胞系的细胞内ROS生成水平, 记录检测到的每个样本的ROS几何平均荧光强度。每个样本的刺激组与非刺激组的几何平均荧光强度之比表示氧胁迫下ROS的增加水平[2,10,17]。

如图6所示, 与对照组相比, 仅携带m.1555A>G突变的单突变组细胞ROS上升9.05%(P=0.003 7), 而同时携带m.1555A>G和m.15910C>T突变的双突变组细胞ROS上升19.08%(P=0.005 4)。这说明, 在氧胁迫下, 双突变组细胞系的ROS水平高于单突变组,



通过BD-Accuri-C6流式细胞仪对有或没有H_2O_2刺激的突变组和对照组样品进行ROS分析。计算相对强度(刺激组/非刺激组)。实验独立重复3次, 数据以mean±S.D.形式表示。

The rates of production in ROS from mutant cell lines and control cell lines were analyzed by BD-Accuri-C 6 flow cytometer system with or without H_2O_2 stimulation. The relative ratio of intensity (stimulated versus unstimulated with H_2O_2) was calculated. Each point represents the mean±S.D. of three independent experiments.

图6　细胞内活性氧水平比较

Fig.6　Relative levels of ROS production

提示m.15910C>T可能加重了m.1555A>G对细胞造成细胞的损伤。

3　讨论

根据家系调查, 排除了综合征型耳聋和其他临床症状后, 发现WZD85家系中3位母系成员(IV-2、III-2和III-5)均出现不同程度的听力下降, 并且除听力下降外无其他临床症状。家系中其他成员听力表现正常, 提示该家系的耳聋表型为母系遗传的可能性。对该家系母系成员的线粒体DNA全序进行分析, 发现该家系除了携带同质性m.1555A>G外, 还有一个未报道的m.15910C>T位点变异。

m.1555A>G突变是过去已经报道的有关母系遗传药物性耳聋的一个药物敏感性位点[2,6]。携带m.1555A>G突变的耳聋家系有着不同的外显率和表现度, 提示可能有其他的修饰因子参与m.1555A>G的表型表达, 如氨基糖苷类抗生素、核修饰基因、线粒体单体型和线粒体DNA突变[18-20]。当包括使用AmAn的药物性耳聋家系成员时, 此家系耳聋外显率为37.5%; 当排除用药的耳聋成员时, 耳聋外显率是25.0%。相比之下, 已报道的14个汉族家系的平均外显率则非常低, 在用药和未用药的情况下, 外显率只有17.5%和9.5%[21-23]。此外, 曾经报道过的6个高外显率的汉族家系在用药和未用药的情况下, 平均外显率分别是56.7%和36.3%[24-25]。我们以前的研究表明, 线粒体tRNA变异位点, 如tRNA^{Glu} 14693A>G, tRNA^{Thr} 15908T>C, tRNA^{Thr} 15927G>A, tRNA^{Arg} 10454T>C, tRNA$^{Ser(UCN)}$ 7444G>A和tRNA^{Cys} 5821G>A可能是这6个携带m.1555A>G突变的中国家系耳聋外显率较高的原因[19,24-26]。以上提示, m.15910C>T可能也参与了m.1555A>G表型的修饰作用。

目前, 还未有关于m.1555A>G与m.15910C>T共同作用导致临床上听力下降的文献报道。m.15910C>T是本研究发现的一个新的突变位点, 该突变发生在tRNA^{Thr}的D茎, 它破坏了本来具有高度保守性的C-G碱基对, 该位点突变仅发现于该家系的母系成员, 而在其他342名正常人群中并没有检测到。该位点保守性高达94%突变后可能会导致tRNA^{Thr}代谢障碍, 进而加重由m.1555A>G突变引起的线粒体功能障碍。

本文构建了同时携带m.1555A>G与m.15910C>T的WZD85家系, 只携带m.1555A>G的WZD83、WZD84家系和对照组的永生化淋巴细胞系(均属于R单体型), 利用构建成功的细胞系进行了细胞内ROS水平、线粒体膜电位水平和线粒体氧化呼吸链复合体酶活性检测等功能研究。

正常的$\Delta\Psi m$是维持线粒体功能和进行氧化磷

酸化、产生ATP的先决条件, $\Delta\Psi m$水平的下降将会导致线粒体氧化呼吸链异常[12,27]。与对照组相比, 双突变组比单突变组降低更为明显, 说明m.15910C>T突变可能使得m.1555A>G对线粒体膜电位的影响变得更加严重。

我们对呼吸链复合体的酶活性进行检测, 其复合体II、IV酶活性下降提示我们, 双突变和单突变组均发生了氧化磷酸化损伤, 双突变组可能更为严重。氧化磷酸化的损伤可导致更多的电子从电子传递链漏出, 并反过来使突变细胞细胞内ROS的产生增加[28]。

与对照组相比, 氧胁迫下双突变组细胞系的ROS水平高于单突变组, 提示m.15910C>T可能加重了m.1555A>G对细胞造成细胞的损伤。ROS的主要来源是线粒体氧化过程, 正常情况下, 会有0.2%的O_2被转变为活性氧[29]。线粒体DNA突变会导致电子传递链中电子流受到损伤, 生成过量的ROS[30-31]。

细胞内过量积累的ROS会造成线粒体的氧化应激恶性循环, 对线粒体蛋白质、核酸和脂类等生物大分子造成损伤, 进而造成一系列的线粒体功能障碍[32-34]。ROS积累是耳蜗毛细胞的受到损失的重要机制, 也是各种类型听力损失的重要病理过程, 而毛细胞损伤是听力损失的重要病理基础[31,35-39]。

综上所述, 家系先证者及母系成员均存在同质性m.1555A>G与m.15910C>T突变, 呈母系遗传特征。使用m.1555A>G与m.15910C>T双突变组, m.1555A>G单突变组和对照组的永生淋巴细胞系进行线粒体功能研究, 结果表明, m.1555A>G与m.15910C>T共同作用, 导致线粒体呼吸链复合体IV活力降低, ROS生成量升高, $\Delta\Psi m$水平下降等线粒体功能缺陷, 且双突变组的ROS生成明显高于单突变组, $\Delta\Psi m$水平、复合体IV活力低于单突变组, 提示m.15910C>T可能是m.1555A>G导致耳聋的继发性突变, 与m.1555A>G相互作用, 使得耳聋的外显率增加。本研究将为非综合征型耳聋的早期诊断、治疗和预防以及遗传咨询提供重要的理论依据。

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