携带线粒体tRNA^{Thr} 15943T>C协同12S rRNA 1555A>G突变的非综合征性耳聋致病机理的初步研究

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摘要 该研究通过构建携带线粒体tRNA^{Thr} 15943T>C协同12S rRNA 1555A>G突变(双突变组)的永生化淋巴母细胞系, 同时建立仅含12S rRNA 1555A>G突变(单突变组)和正常对照组永生化淋巴母细胞系, 探究线粒体tRNA^{Thr} 15943T>C协同12S rRNA 1555A>G突变与耳聋发病的关系, 以了解线粒体突变致聋的分子机制。对该家系的临床资料进行分析的结果表明, 当包括使用氨基糖苷类抗生素(aminoglycoside antibiotic, AmAn)的药物性耳聋家系成员时, 此家系耳聋外显率为26%; 当排除用药的耳聋成员时, 此家系耳聋外显率是10%; 相比之下, 已报道的14个m.1555A>G的耳聋家系的平均外显率在用药和未用药的情况下分别仅为13%和6%。利用Northern blot和Western blot分别检测三组细胞中线粒体tRNA和多肽的表达量, 结果表明相比于正常对照组, tRNA^{Thr}在双突变组中的表达量显著降低, 而在单突变组中的表达量无显著变化, tRNA^{Trp}、tRNA^{Ala}、tRNA^{Tyr}、tRNA^{Cys}和tRNA^{Pro}的稳态水平在三组细胞中没有显著性差异; CO2、CO3和A6在双突变组中的表达量显著降低, 而在单突变组中的表达量无显著性差异; 其他蛋白多肽在三组细胞中的表达量没有显著差异, 说明m.15943T>C突变降低了tRNA^{Thr}的稳态水平, 致使线粒体部分多肽表达水平下降, 从而影响了线粒体呼吸链复合体的功能和稳定性进而导致了线粒体代谢障碍, 提示线粒体tRNA^{Thr} 15943T>C可能与m.1555A>G突变引起的耳聋相关。

关键词 非综合征性耳聋; 突变; 线粒体tRNA^{Thr}

Preliminary Study of the Mechanism of Non-Syndromic Hearing Loss Carrying Mitochondrial tRNA^{Thr} 15943T>C and 12S rRNA 1555A>G Mutations

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Abstract This study aims to explore the relationship between mitochondrial tRNA^{Thr} 15943T>C and 12S rRNA 1555A>G mutations and non-syndromic hearing loss, and the molecular mechanism of mitochondrial mutations in deafness. Immortalized lymphoblastic cell lines carrying mitochondrial tRNA^{Thr} 15943T>C and 12S rRNA 1555A>G mutations (double mutation group), the same haplotype (R9) with only 12S rRNA 1555A>G mutation (single mutation group), and a normal control group were established. Analysis of the clinical data of this fam-

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ily showed that when the family members of the drug-induced deafness using AmAn (aminoglycoside antibiotic) were included, the penetrance rate of deafness in this family was 26.3%; when the deaf members who used the medicine were excluded, the penetrance rate of deafness was 10%; in contrast, in the reported 14 deaf families with m.1555A>G, the average penetrance rates were only 13% and 6% separately in the treated and untreated conditions. Northern blot and Western blot separately were used to detect the expression of mitochondrial tRNA and polypeptide. Compared with the normal control group, the steady-state level of tRNA^{Thr} was significantly lower in the double-mutation group, but there was no significant change in the single-mutation group. Meanwhile, there was no significant difference in the steady-state level of other tRNAs in the three groups. The expression of CO2, CO3, and A6 in the double-mutation group was significantly reduced, but their expression had no significant difference in the single-mutation group. The expression levels of other protein polypeptides had no significant difference in the three groups. The m.15943T>C mutation reduces the steady-state level of tRNA^{Thr}. It results in a decrease in the expression of some mitochondrial polypeptides, which affects the function and stability of the mitochondrial respiratory chain complex. Eventually, it leads to mitochondrial metabolism disorders, suggesting that mitochondrial tRNA^{Thr} 15943T>C may be related to the deafness with m.1555A>G.

Keywords non-syndromic hearing loss; mutant; mitochondrial tRNA^{Thr}

耳聋作为一种异质性疾病, 严重影响着人们的社交、情感和认知[1]。据报道, 每1 000个新生儿中就有一位患有先天性耳聋, 其中一半以上患儿的听力损失为遗传因素所致[2-3]。遗传性耳聋的群体发病率已超过27/1 000, 在所有耳聋病人中, 遗传性耳聋约占50%。目前研究发现, 线粒体基因突变影响了线粒体功能[4-6]; 作为一种多系统疾病, 线粒体疾病在感觉神经性耳聋中具有组织特异性[7], 这些都表明线粒体基因突变是遗传性耳聋的一个致病因素, 但线粒体基因突变是如何致聋的呢? 研究表明, 线粒体突变引起RNA稳定性的改变是多种线粒体疾病发生的基础[8-13]; 线粒体的能量代谢障碍与多种疾病相关[14-17]; 那么遗传性耳聋是不是通过改变tRNA的稳定性造成线粒体能量代谢障碍, 影响了线粒体功能从而致病的呢? 本研究通过一个同时携带线粒体tRNA^{Thr} 15943T>C突变(m.15943T>C)和线粒体12S rRNA 1555A>G突变(m.1555A>G)的非综合征性母系遗传药物性耳聋的家系, 在分子水平上研究线粒体基因突变与非综合征性耳聋的内在联系, 以为遗传性耳聋的治疗提供新的靶点和策略。

1 材料和方法

1.1 研究对象

将研究对象分为A、B、C三组, 每组选择三例样本进行实验。其中A组(双突变组), 同时携带m.15943T>C突变和m.1555A>G突变的汉族非综合征性耳聋家系样本(A1、A2、A3), 来源于安徽省; B组(单突变组), 仅携带m.1555A>G突变的样本(B1、B2、B3), 来源于浙江省; C组, 正常对照组(C1、C2、C3), 来源于本课题组前期收集的大量正常对照样本的资源库。本研究经温州医科大学伦理委员会批准, 参与本研究的所有试验人员均已签署知情同意书。

1.2 方法

1.2.1 永生化淋巴母细胞的建系和培养 采集实验对象的外周血, 提取淋巴细胞用EB病毒转染构建永生化淋巴细胞, 将细胞培养于RPMI 1640培养基(含10%胎牛血清以及1%青/链霉素), 置于37 ℃、5% CO_2的培养箱中; 定期更换细胞培养液; 当细胞密度达到约80%时, 进行传代。

1.2.2 线粒体基因组序列分析 用手工法提取上述构建的永生化淋巴母细胞中的DNA, 线粒体全序扩增后对PCR产物进行基因测序。利用分析软件Codoncode Aligner将反馈的测序结果与修正的剑桥标准序列(revised Cambridge reference sequence, rCRS)进行比对分析。

1.2.3 tRNA水平的检测 从上述构建的永生化淋巴母细胞中提取线粒体RNA(RNA提取试剂盒, Gibco), 用10%的丙烯酰胺胶于120 V电泳分离RNA, 跑胶结束后依次进行30 V转膜80 min, 3 600 kJ/min紫外交联3 min, 55 ℃预杂交30 min后, 分别加入含有地高辛标记的线粒体tRNA^{Thr}、tRNA^{Trp}、tRNA^{Ala}、tRNA^{Tyr}、tRNA^{Cys}、tRNA^{Pro}和细胞的核基因编码的

5S RNA探针的杂交液, 探针序列见表1, 于37 °C杂交炉中孵育过夜, 洗膜后进行荧光检测, 显影曝光后, 用ImageJ图像软件定量分析每个条带的灰度值。

1.2.4 线粒体多肽水平的检测 收集构建的永生化淋巴母细胞, 加入RIPA裂解液(含1% PMSF), 离心后取上清, 用BCA法测量蛋白质浓度, 调整各样本蛋白浓度。每个样本上样量为30 μg, 用聚丙烯酰胺凝胶于120 V电泳分离蛋白质, 跑胶结束后于250 mA冰上湿转90 min, 然后用5%脱脂奶粉室温封闭1 h, TBST洗膜3遍后分别加入CO2(1:5 000)、CO3(1:1 000)、A6(1:4 000)、ND1(1:1 000)、ND4(1:1 000)、ND5(1:1 000)、CytB(1:500)、Actin(1:6 000)一抗, 于4 °C孵育过夜; 室温孵育对应二抗(1:1 000) 2 h, 洗膜3次后用ECL试剂显影曝光, 用ImageJ图像软件定量分析每个条带的灰度值。

1.3 统计学处理方法

利用SPSS 17.0软件进行统计分析; 计量资料以均值±标准差($\bar{x}\pm s$)表示, 两组间比较采用双尾非配对t检验。$P<0.05$为差异具有统计学意义。

2 结果

2.1 家系资料及临床检测结果分析

对同时携带m.15943T>C突变和m.1555A>G突变的汉族非综合征性耳聋家系进行整理, 该双突变的家系来自于安徽省, 共有4代人, 包括19名家系成员, 其中母系成员有13名, 母系成员中耳聋患者有4人, 从家系图中可以看出该家系带有明显的母系遗传特征(图1)。从本次调查进一步完善的家系资料中发现, 所有患病的母系成员皆为重度到极重度耳聋, 且呈双耳对称性。该家系中的先证者为IV-1, 女, 15岁, 3岁时开始患病, 有用药史, 表现为感应神经性的重度耳聋, 双耳呈对称性听力下降, 且无其他疾病表征, 属于非综合征性耳聋(表2)。

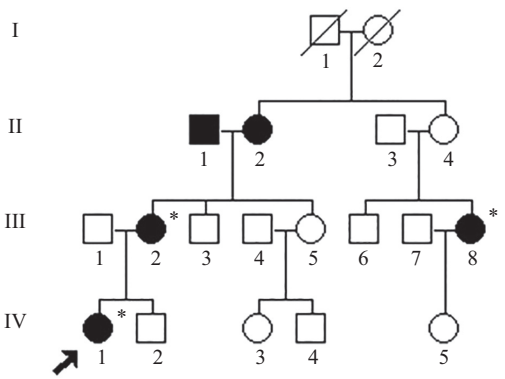
2.2 永生化淋巴母细胞的建系

从三组对象中, 分别选取三例采集外周血以建

表1 DIG标记的tRNA探针

Table 1 tRNA probes labled with DIG

探针名称 Probe name	核苷酸序列(5′→3′) Nucleotide sequence (5′→3′)
$tRNA^{Thr}$	CCT TGG AAA AAG GTT TTC ATC TCC
$tRNA^{Trp}$	CCT TGG AAA AAG GTT TTC ATC TCC
$tRNA^{Ala}$	GCA TCA ACT GAA CGC AAA TCA GCC ACT TTA ATT
$tRNA^{Tyr}$	GGT AAA AAG AGG CCT AAC CCC TGT CTT TAG
$tRNA^{Cys}$	AAG CCC CGG GTT TGA AGC TGC
$tRNA^{Pro}$	CAG AGA AAA AGT CTT TAA CTC CAC CAT TAG
5S RNA	GGG TGG TAT GGC GGT AGA C



箭头表示先证者; 斜线表示死亡, 黑色符号表示听力下降者; *表示有氨基糖苷类药物用药史。

Arrow denotes proband; slash indicates death; hearing impaired individuals are indicated by black symbols; * denotes the individuals who have a history of aminoglycosides exposure.

图1 一个同时携带m.15943T>C和m.1555A>G突变的耳聋患者家系图

Fig.1 A Chinese families with hearing loss carrying m.15943T>C and m.1555A>G mutations

立永生化淋巴母细胞系。转化7天后观察发现细胞体积增大, 周围有刺突, 出现聚团, 肉眼可见白色细胞团块; 培养30天后可见致密的更大的细胞团块, 其折光性强, 说明细胞成功转化为淋巴母样B细胞, 永生化淋巴母细胞建系完成(图2)。

2.3 线粒体基因组测序结果分析

从构建的永生化淋巴母细胞中提取DNA, PCR扩增后进行测序, 利用分析软件Codoncode Aligner将反馈的测序结果与rCRS进行比对分析。患者的tRNA^{Thr}编码区携带m.15943T>C突变, 此突变高度保守, 目前未被相关文献报道(表3); 二级结构51A-63U碱基对突变为51A-63T(图3)。

2.4 线粒体tRNA稳态水平检测

为进一步检测线粒体m.15943T>C突变对tRNA的表达水平的影响, 通过Northern blot杂交实验检测线粒体tRNA稳态水平, 结果显示: 相比于正常对照组, tRNA^{Thr}在双突变组中的表达量显著降低(P<0.05), 而在单突变组中的表达量无显著变化; 同时, 对线粒体中tRNA^{Trp}、tRNA^{Ala}、tRNA^{Tyr}、tRNA^{Cys}和tRNA^{Pro}的稳态水平进行检测, 结果表明, 相比于正常对照组, 双突变组和单突变组都没有显著变化(图4)。

2.5 线粒体多肽表达水平的检测

为了研究m.15943T>C突变是否会在多肽翻译水平上对细胞产生影响, 通过Western blot实验对线粒体基因编码的7个多肽的表达水平进行了检测, 结果表明, 相比于正常对照组, CO2、CO3和A6在双突变组中的表达量显著降低(P<0.05), 而在单突变组中的表达量无显著性差异; 同时, 相比于正常对照组细胞, ND1、ND4、ND5、CytB的表达量在双突变组和单突变组中均没有显著差异(图5)。

表2 家系中部分携带m.15943T>C和m.1555A>G突变的母系成员临床资料统计

Table 2 Summary of the clinical data of part members of a Chinese family carrying m.15943T>C and m.1555A>G mutations

编号 Code	性别 Sex	是否有用药史 Use of AmAn	听力损失情况 Level of hearing impairment	平均纯音测听水平 Average pure tone audiometry level	
				左耳/dB Left ear /dB	右耳/dB Right ear /dB
II-2	Female	No	Severe	74	79
III-2 (A2)	Female	Yes	Extremely severe	98	94
III-8	Female	Yes	Extremely severe	90	95
IV-1 (A1)	Female	Yes	Extremely severe	91	94

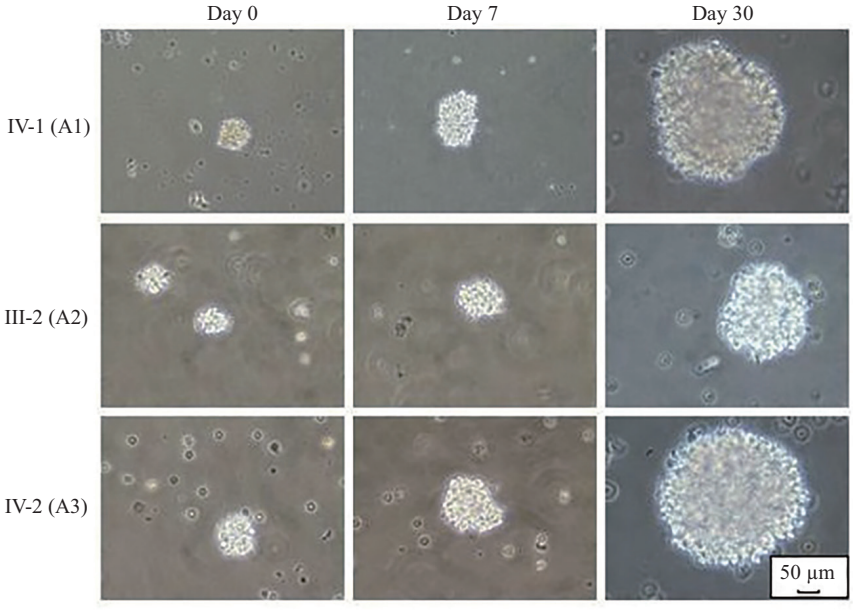


图2 实验组部分成员永生化淋巴母细胞系形态图

Fig. 2 Morphology of immortalized lymphoblastic cell lines in some subjects in experimental group

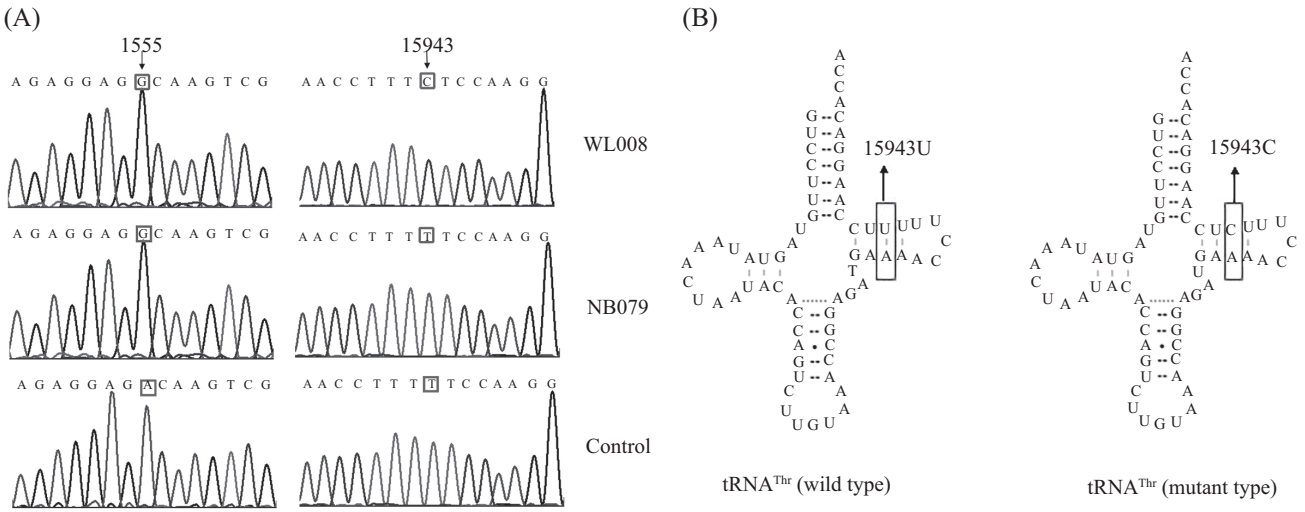
表3 突变组家系中母系成员的线粒体DNA编码区突变汇总

Table 3 Mutation summary of mitochondrial DNA coding region of maternal members in mutation group families

基因 Gene	突变位点 Mutation position	碱基/氨基酸的改变 Base/aminoacid change	保守性 Conservation (H/M/X/B)[a]	标准序列 rCRS	是否报道 Previously reported or not[b]
12S rRNA	709	G to A	G/A/-/A	G	Yes
	750	A to G	A/A/-/A	A	Yes
	1438	A to G	A/A/G/A	A	Yes
	1555	A to G	A/A/A/A	A	Yes
16S rRNA	2706	A to G	A/A/A/G	A	Yes
	3107	delN	N/T/T/T	N	Yes
ND1	3434	A to G (Tyr to Cys)	Y/Y/T/Y	A	Yes
CO1	5913	G to A (Asp to Asn)	D/N/T/N	G	Yes
ATP6	8860	A to G (Thr to Ala)	T/A/T/A	A	Yes
	10320	G to A (Val to Ile)	V/M/I/L	G	Yes
CytB	14766	C to T (Thr to Ile)	T/T/S/S	C	Yes
	15326	A to G (Thr to Ala)	T/I/I/M	A	Yes
$tRNA^{Thr}$	15943	T to C	T/T/T/T	A	No

a: 人(H)、小鼠(M)、非洲爪蟾(X)和牛(B)的相对应位置的氨基酸保守性; b: 参考线粒体基因组数据库(http://www.mitomap.org); -: 位点缺失。

a: conservation of amino acid in human (H), mouse (M), bovine (B), and *Xenopus laevis* (X); b: refer to the mitochondrial genome database (http://www.mitomap.org); -: loss of site.



A: mtDNA 1555位点和15943位点的测序峰图; B: 野生型和突变型$tRNA^{Thr}$的二级结构图。WL008为一个同时携带m.15943T>C和m.1555A>G突变的家系; NB079为只携带m.1555A>G突变的家系; Control为相同单体型的对照组家系。

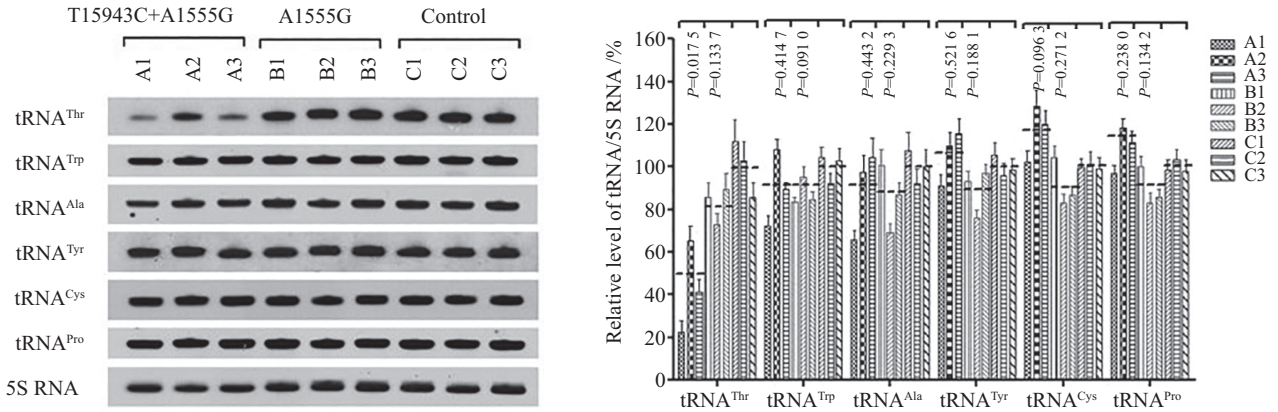
A: sequencing peaks of mtDNA at 1555 and 15943 sites; B: secondary structure of $tRNA^{Thr}$ in the wild type and mutant type. WL008 is a Chinese family with hearing loss carrying m.15943T>C and m.1555A>G mutations; NB079 is a Chinese family with hearing loss carrying m.1555A>G mutation; Control is a normal sample with same haplotype.

图3 永生化淋巴母细胞线粒体12S rRNA和$tRNA^{Thr}$测序峰图和$tRNA^{Thr}$的二级结构图

Fig.3 Sequencing peaks of mitochondrial 12S rRNA and $tRNA^{Thr}$ in immortalized lymphoblasts and the secondary structure of $tRNA^{Thr}$

2.6 m.15943T>C突变对不同多肽的差异性影响分析

为了探究m.15943T>C突变是否会影响线粒体多肽的表达以及对不同多肽的差异性影响的原因，我们对线粒体基因编码的7种多肽总体表达水平进行统计分析，并且对人类线粒体基因编码的多肽中的苏氨酸的个数、苏氨酸所占的比例、双突变组和单突变组分别与正常对照组相比的多肽表达水平的相对值进行了统计汇总。图6结果表明，相比于对照组细胞，双突变组的多肽总体表达水平显著降

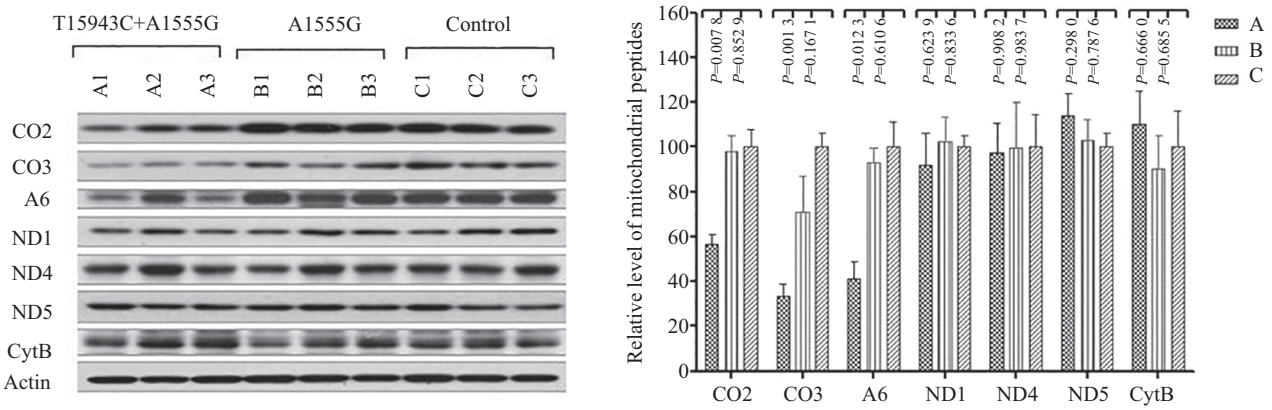


Northern blot分别检测线粒体tRNA^{Thr}、tRNA^{Trp}、tRNA^{Ala}、tRNA^{Tyr}、tRNA^{Cys}和tRNA^{Pro}的表达量, 并以5S RNA为内参进行定量。

The expression levels of mitochondrial tRNA^{Thr}, tRNA^{Trp}, tRNA^{Ala}, tRNA^{Tyr}, tRNA^{Cys} and tRNA^{Pro} were detected by Northern blot, and 5S RNA was used as an internal reference for quantification.

图4 线粒体tRNA稳态水平检测结果

Fig.4 Results of mitochondrial tRNA steady-state detection

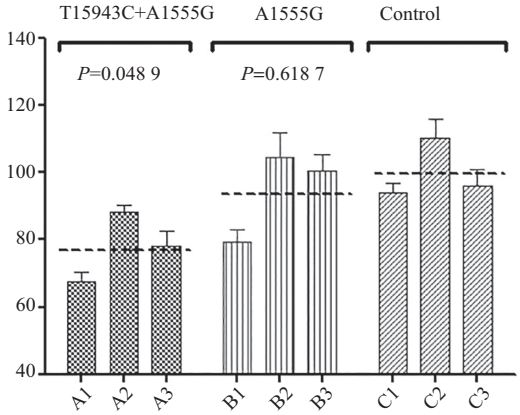


Western blot分别检测线粒体多肽CO2、CO3、A6、ND1、ND4、ND5和CytB的的表达量, 并以Actin为内参进行定量。

Western blot was used to detect the expression of mitochondrial peptides CO2, CO3, A6, ND1, ND4, ND5 and CytB, and Actin was used as an internal reference for quantification.

图5 线粒体多肽表达水平的检测

Fig.5 Results of mitochondrial peptides expression detection



虚线表示组内样本的平均水平。

The dotted line indicates the average level of the samples within the group.

图6 对线粒体基因编码的7种多肽总体表达水平的分析

Fig.6 Analysis of the overall expression levels of 7 polypeptides encoded by mitochondria gene

表4 人类线粒体基因编码的多肽中的苏氨酸比例以及各实验组多肽表达水平

Table 4 The ratio of threonine in the polypeptides encoded by human mitochondria genes and the expression levels of polypeptides in each experimental group

多肽 Polypeptides	编码氨基酸个数 Number of coded amino acids	苏氨酸个数 Number of Threonine	苏氨酸比例 Proportion of Threonine	组间多肽表达水平比较 Comparison of peptide expression between groups	
				Group A/group C	Group B/group C
CO2	225	21	9.3%	57% (P=0.007 8)	97% (P=0.852 9)
CO3	261	24	9.2%	33% (P=0.001 3)	70% (P=0.167 1)
ATP6	227	25	11.0%	41% (P=0.012 3)	92% (P=0.610 6)
ND1	318	35	11.0%	92% (P=0.623 9)	103% (P=0.833 6)
ND4	459	48	10.5%	98% (P=0.908 2)	99% (P=0.983 7)
ND5	604	65	10.8%	114% (P=0.298 0)	103% (P=0.787 6)
CytB	380	30	7.9%	106% (P=0.666 0)	90% (P=0.685 5)

低(P<0.05), 在单突变组中无显著性差异; 比较各个多肽中苏氨酸的含量, 发现其在在各个组中均无显著性差异, 而CO2、CO3、A6中的氨基酸个数分别为225、261和227个, 远远低于ND1、ND4、ND5和CytB中的318、459、604和380个(表4)。

3 讨论

人类线粒体遗传疾病的研究一直受到疾病模型的限制。永生化淋巴细胞模型是通过从患者体内获取携带突变的线粒体, 采用细胞融合技术, 将其与不含线粒体的ρ^0 206细胞融合获得具有相同核背景的线粒体基因突变细胞模型。本研究通过构建携带m.15943T>C突变和m.1555A>G突变的永生化淋巴母细胞系, 在分子水平对其线粒体进行检测, 分析突变致聋的分子机理。对家系临床资料进行分析, 发现患病的母系成员都表现为重度到极重度的耳聋, 表明m.15943T>C协同m.1555A>G突变可能致聋; 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[18-20], m.15943T>C突变是在本实验的前期研究过程中发现的一个新的突变位点。线粒体tRNA稳态水平检测结果发现, 仅存在$tRNA^{Thr}$的水平显著性降低, 说明m.15943T>C突变降低了$tRNA^{Thr}$的稳态水平; 一些研究发现, tRNA突变可能导致其二级结构的变化[21-24], 我们对家系进行保守性分析, 发现$tRNA^{Thr}$在4个物种中高度保守。因此, 我们认为m.15943T>C突变可能通过破坏$tRNA^{Thr}$上具有高度保守性的51A-63U碱基对, 而改变了$tRNA^{Thr}$的二级结构, 降低了$tRNA^{Thr}$的稳态水平。线粒体蛋白表达水平的检测中, CO2、CO3和A6的显著性降低, 结合人类线粒体基因编码的多肽中的苏氨酸比例及数量的结果(线粒体多肽中苏氨酸的比例无显著性差异, 但CO2、CO3、A6中的苏氨酸的数量远远低于ND1、ND4、ND5和CytB), 表明m.15943T>C突变通过影响 $tRNA^{Thr}$的稳态, 进一步影响了CO2、CO3、A6的表达。

综上所述, 我们认为m.15943T>C突变通过破坏$tRNA^{Thr}$上具有高度保守性的51A-63U碱基对, 改变了$tRNA^{Thr}$的二级结构, 降低了$tRNA^{Thr}$的稳态水平, 致使线粒体DNA编码的CO2、CO3和A6表达水平下降; 其中CO2和CO3属于复合体IV上的亚基[25], A6属于复合体V上的亚基[26]; 线粒体主要通过氧化呼吸供能; 复合物IV是线粒体主呼吸链(NADH呼吸链)的重要组成部分, 而复合体V是ATP的主要生成部位, 都与线粒体氧化呼吸的功能密切相关, 因此我们认为CO2、CO3和A6的下调影响了线粒体呼吸链复合体的功能和稳定性, 进而导致线粒体代谢障碍, 最终导致疾病的发生。此研究为遗传性耳聋提供了新的靶点, 也为遗传性耳聋的预防提供了防控重点, 且为疾病的诊断及干预提供了新的方向。

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