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·临床研究·

KRAS基因突变与分化型甲状腺癌^{131}I放疗耐受性及预后的关系

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[摘 要] **目的**:探究KRAS基因突变与分化型甲状腺癌(differentiated thyroid carcinoma,DTC)^{131}I放疗疗效和预后的相关性,并阐明其可能的机制。**方法**:收集经^{131}I放射治疗DTC临床组织样本,聚合酶链反应-单链构象分析法(single strand conformation polymorphism analysis of polymerase chain reaction products,PCR-SSCP)检测KRAS的遗传突变;采用qPCR和Wb检测p21蛋白的表达水平;亚致死剂量的^{131}I放射治疗DTC细胞系,采用CCK-8、流式细胞术(FCM)、Transwell实验检测细胞活力的变化,并通过动物模型验证。**结果**:^{131}I放射治疗耐受DTC患者的KRAS基因突变增加(P<0.01),KRAS基因突变导致p21蛋白表达下调(P<0.05),且与DTC临床分期及预后较差相关(P<0.05,P<0.01)。体内外实验证明,亚致死剂量的^{131}I放射治疗导致DTC细胞KRAS基因的突变率增加、p21蛋白的表达水平降低,导致DTC细胞产生^{131}I放射耐受,而超表达KRAS基因显著提高p21的表达,抑制肿瘤增长及转移。**结论**:KRAS基因突变与DTC临床分期及^{131}I放射耐受相关,亚致死剂量^{131}I放射治疗DTC促进KRAS基因突变产生放射耐受,而超表达KRAS基因能够提高DTC对^{131}I放射治疗的敏感性。

[关键词] 分化型甲状腺癌;^{131}I放射治疗;KRAS基因;突变;放疗耐受性
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Correlation between KRAS gene mutation and DTC resistance to ^{131}I radiotherapy and prognosis

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[Abstract] Objective: To investigate the correlation between KRAS gene mutation and differentiated thyroid carcinoma (DTC) treatment effect and prognosis, and to explore the mechanism. **Methods:** Clinical tissue samples from DTC patients undergoing ^{131}I Radiotherapy were collected. Then single strand conformation polymorphism analysis of polymerase chain reaction products (PCRC-SSCP) was used to detect KRAS mutation rate in thyroid cancer patients of different TNM stages; p21 protein expression level was detected by real-time quantitative polymerase chain reaction (qPCR) and western blotting. DTC cells were treated by sub-lethal dose of ^{131}I Radiotherapy, and then CCK-8 assay, transwell assay and flow cytometry (FCM) were used to evaluate the changes of cells viability. Animal models were then constructed for verification. **Results:** The results showed that KRAS gene mutants were increased in ^{131}I-resistant DTC patients; KRAS gene mutation suppressed p21 protein expression and was associated with clinical stage and poor prognosis. *In vivo* and *in vitro* experiments proved that sub-lethal dose of ^{131}I increased KRAS gene mutation rate, suppressed p21 expression level, and caused ^{131}I radiotherapy resistance. Reversely, over-expression of KRAS gene could significantly increase p21 expression, and inhibit tumor proliferation and metastasis. **Conclusion:** KRAS gene mutations were associated with DTC TNM stages and ^{131}I resistance in DTC patients. Sub-lethal dose of ^{131}I treatment could improve ^{131}I resistance in DTC cells line, inversely, over-expressed KRAS gene could increase the sensitivity to ^{131}I radiotherapy in DTC patients.

[Key words] differentiated thyroid carcinoma; ^{131}I radiation therapy; KRAS gene; mutant; radiotherapy resistant
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分化型甲状腺癌(differentiated thyroid carcinoma,DTC)严重危害人类健康[1],^{131}I放射治疗是DTC的有效治疗方法[2],但是DTC患者对^{131}I放射治疗产生的耐受性严重限制了其在临床上的应用。由于不同患者肿瘤存在异质性[3],导致^{131}I放射治疗产生不同的疗效,甚至产生DTC对^{131}I放射

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治疗的耐受。KRAS是一种原癌基因，能够通过编码p21蛋白调控细胞凋亡[4-6]。KRAS基因突变与人类肿瘤的发生、发展关系密切[7-8]，也与肿瘤对放疗产生耐受性和患者预后相关[9-10]。57.4%的DTC癌患者为KRAS突变型，并且KRAS的突变与DTC患者的预后呈负相关[11-12]，目前尚未有KRAS基因突变与肿瘤细胞对^{131}I放射治疗产生耐受性关系的报道。^{131}I放射治疗能够通过破坏细胞基因结构，诱导细胞凋亡治疗癌症[13]，而p21蛋白也被报道与放疗诱导的细胞凋亡相关，其功能缺失会导致细胞异常增殖和凋亡失控[14-15]。基于以上研究推测，KRAS基因突变可能导致p21蛋白介导的细胞凋亡功能丧失，进而抑制^{131}I放射治疗对肿瘤细胞凋亡的诱导作用，最终导致KRAS突变的DTC患者对^{131}I放射治疗产生耐受性，使患者产生较差的预后。本研究对此进行实验验证，以期寻找DTC预后的分子标志，为DTC治疗提供新的思路。

1　资料与方法

1.1　患者资料

选取2017年1月至2017年12月在昆明医科大学第三附属医院参与^{131}I放射治疗的DTC患者72例，其中^{131}I放射治疗敏感的患者37例，^{131}I放射治疗耐受的患者35例。经过规范^{131}I治疗后仍出现复发及转移或已知病灶进展的DTC患者，符合以下条件之一认为其为^{131}I放射治疗耐受：（1）肿瘤组织或转移病灶不摄碘（在清甲成功后的首次诊断性或治疗性^{131}I全身显像未出现甲状腺床以外的碘摄取）；（2）曾经摄碘的病灶在^{131}I治疗后逐渐散失摄碘能力；（3）^{131}I治疗后仅部分病灶摄碘，部分病灶不摄碘；（4）尽管病灶存在碘摄取，但经^{131}I治疗后仍出现进展。TNM分期I/II期41例、III/IV期31例，男性38例、女性34例；年龄45~78岁，中位年龄61岁。手术切除甲状腺癌组织及对应的癌旁组织于－80℃冰箱冷冻保存备用。所有患者术前均未接受其他放化疗，均签署知情同意书。

1.2　细胞系、主要试剂与仪器

人DTC细胞系TPC-1、K1和BCPAP购买于上海中国科学院细胞库，分别用含有10%胎牛血清、100 U/ml青霉素和100 μg/ml链霉素的DMEM培养基在37 ℃、5% CO_2培养箱中传代培养，待单细胞生长汇合到80%时，用0.25%胰酶消化，收集细胞备用。

^{131}I耐受细胞模型是通过将甲状腺癌细胞常规接种于6孔板，给予持续的半致死剂量的^{131}I处理：1.0~2.2 mCi/孔的剂量处理TPC-1及K1细胞，0.5~1.1 mCi/孔剂量处理BCPAP细胞；正常传代至第10代，即可获得^{131}I-耐受甲状腺癌细胞模型。

胎牛血清、DMEM培养液、0.25%胰酶、TRIzol试剂、Lipofectamine^{TM}2000均购自Invitrogen公司，兔抗人p21抗体购自Abcam公司，CCK-8试剂盒购自索莱宝公司，多功能酶标仪（SynergyTM2）购自BioTek公司，ABI PRISM 7500型荧光定量PCR扩增仪购自Thermo公司，化学发光仪购自BD公司，KRAS基因过表达载体pcDNA3.1 KRAS及用于聚合酶链反应-单链构象分析法（single strand conformation polymorphism analysis of polymerase chain reaction products，PCR-SSCP）检测的引物由上海吉玛基因设计并合成。

1.3　PCR-SSCP检测DTC组织中KRAS基因突变率

将收集到的临床标本置于固定液，进行预处理。提取石蜡切片组织内DNA，提取方法完全按照试剂盒说明书进行，将提取得到的DNA置于－80 ℃冰箱中冻存备用。参照NCBI数据库中基因序列，根据要检测的位点设计并合成KRAS引物序列。KRAS上游引物（F）：5'-GGCTGCAAAATGACTGA-3'；KRAS下游引物（R）：5'-GTCCTGCACCAGTA-ATATGC-3'。进行PCR反应，反应条件为：95 ℃ 30 s，95 ℃ 15 s，58 ℃ 30 s, 72 ℃ 90 s, 进行35个循环，72 ℃ 7 min。采用1%琼脂糖凝胶电泳检测KRAS基因的突变情况，并采用凝胶成像仪拍照记录，Image J软件分析灰度值，计算基因相对表达量。

1.4　qPCR检测DTC和癌旁组织中p21 mRNA的表达

使用TRIzol试剂提取DTC癌组织和癌旁组织标本及细胞总RNA。测定总RNA浓度及纯度后，用逆转录试剂盒逆转录合成cDNA，置于－80℃冰箱冷冻保存备用。以GAPDH作为内参，qPCR引物见表1。用荧光定量PCR扩增仪检测p21 mRNA表达水平，反应体系及条件：2×SYBR Premix 10 μl，0.8 μl ddH_2O，1 μl cDNA，PCR上游和下游引物各0.5 μl，95℃预变性30 s，95℃ 20 s，60℃ 30 s，共40个循环。采用$2^{-\triangle\triangle Ct}$计算基因的相对表达量，每个样本设3个复孔，实验重复3次。

表1 qPCR引物序列

Tab.1 Primer sequences for qPCR

Gene	Primer
p21	F 5'-AGCTGTTAACATCGGTGCCCTCT-3'
	R 5'-TGCACACGCCAGGATTGCAGGGTTCA-3'
GAPDH	F 5'-GTCCACCCCAAATGCTCTA-3'
	R 5'-TGCTGTCACCTTCACCGTTC-3'

1.5 Wb检测DTC组织、^{131}I耐受和敏感TCP-1细胞中p21蛋白表达的水平

使用RIPA细胞裂解液提取甲状腺癌和癌旁组织样本及^{131}I耐受及^{131}I敏感TCP-1细胞总蛋白，BCA法检测蛋白浓度。8%SDS-PAGE分离蛋白，半干转法将蛋白转至PVDF膜，浸入5%脱脂奶粉中室温封闭2 h。TBST洗膜3次，加入抗体p21（1∶1 000）和GAPDH（1∶1 000），4℃孵育过夜，次日移去一抗，TBST洗膜3次，加入HRP标记的鼠抗兔二抗（1∶10 000），室温孵育1 h。加入ECL发光液，利用凝胶成像系统（美国Bio-Rad公司）观察蛋白条带，采用Image J软件进行灰度分析并计算蛋白相对表达量。

1.6 KRAS过表达TCP-1细胞模型的构建以及CCK-8法检测细胞增殖能力

将处于对数生长期的^{131}I耐受及敏感TCP-1细胞分别接种于6孔板中，每孔2×10^6个细胞，置于37 ℃，5%CO_2培养箱中培养，细胞汇合度达70%~80%时进行转染。按照说明书将转染物pcDNA3.1 KRAS与Lipofectamine™2000混合后转染细胞48 h之后，采用qPCR检测转染效率，Wb检测各组p21蛋白的表达水平。将转染细胞以100 μl/孔（约1×10^4个细胞）接种于96孔板中，采用^{131}I放射治疗（使用放射剂量为1.0 ~ 2.2 mCi/孔），分别于0、24、48、72、96 h后向每孔加入10 μl CCK-8，继续孵育4 h后使用酶标仪测定450 nm波长下光密度（*D*）值。

1.7 流式细胞术检测过表达KRAS对TCP-1细胞凋亡的影响

将经^{131}I放射治疗的转染后不同组^{131}I耐受及敏感TCP-1细胞继续培养6 h，0.25%的胰酶消化，PBS清洗3次，稀释细胞密度为1×10^6个/ml，向流式检测管中加入100 μl的细胞悬液，分别加入PI和Annexin V染料，轻轻混匀，室温避光孵育15 min，采用流式细胞仪检测细胞凋亡情况。

1.8 Transwell侵袭实验检测过表达KRAS对TCP-1细胞迁移能力的影响

预先用Matrigel胶包被transwell小室内膜，向每个小室内加入50 μl含10 g/L BSA的无血清培养基，37 ℃孵育30 min。将转染后的^{131}I耐受及敏感TCP-1细胞胰酶消化后计数，调整细胞浓度为5×10^5 cells/ml，向上层小室加入200 μl细胞悬液，下层加入400 μl含10%胎牛血清的完全培养基，用^{131}I放射治疗处理细胞。培养24 h后弃去培养基，PBS洗2遍，使用棉签轻轻拭去微孔膜上层的细胞。4%多聚甲醛溶液固定30 min，PBS洗2次，1%结晶紫染色15 min后，PBS洗2次。倒置显微镜（日本奥林巴斯公司）下选取5个视野计数转移至微孔膜下层的细胞。

1.9 DTC小鼠移植瘤模型的建立与治疗

实验所需BALB/c裸鼠购买于中国科学院昆明动物研究所，实验动物使用许可证号：SYXK（滇）K2017-0009，4周龄雄性裸鼠14只，体质量为16~20 g，动物购买后于本单位SPF级动物实验室适应饲养一周，用于后于实验。取5周龄雄性BALB/c裸鼠随机分为两组，TCP-1细胞移植前分别导入KRAS基因超表达载体或空载体，按照每只小鼠1×10^6个细胞的细胞量移植至小鼠右背，每周对小鼠肿瘤直径进行测量，按照V=最短直径^2×最长直径×0.5，并记录小鼠生存情况，28 d后处死小鼠，测量并计算肿瘤体积。

1.10 统计学处理

采用SPSS 18.0统计软件和Graph Pad Prism 7.0作图软件。所有实验重复3次，计量资料以$\bar{x}\pm s$表示；两组间差异比较采用student's-*t*检验，多组间差异比较采用单因素方差分析；使用Kaplan-Meier作生存曲线，并通过Log-rank检验进行分析；生存数据采用Cox回归分析方法进行分析。以P<0.05或P<0.01表示差异具有统计学意义。

2 结 果

2.1 ^{131}I放射治疗耐受DTC患者KRAS基因突变率高且预后较差

对参与^{131}I放射治疗的甲状腺癌患者KRAS基因突变与^{131}I放射治疗耐受、TNM分期、性别之间的关系进行卡方检验分析，并通过PCR-SSCP对KRAS基因的突变情况进行检测，结果（表2）显示，^{131}I放射治疗耐受患者的KRAS基因突变率显著高于^{131}I放射治疗敏感患者（91.43% *vs* 27.03%，P<0.01），TNM分期（III/IV）患者的KRAS基因突变率显著高于TNM分期（I/II）患者（93.55% *vs* 36.58%，P<0.01）。Kaplan-Meier生存分析结果（图1）显示，处于同一分期阶段的患者经^{131}I放射治疗后，产生^{131}I耐受的患者预后较差。qPCR检测结果显示KRAS基因突变的样本中，p21的表达较未发生突变的样本显著下调（1.01±0.0003 *vs* 0.64±0.0004, t=21.4, P<0.01）。

2.2 KRAS基因突变导致甲状腺癌细胞^{131}I放射治疗耐受

成功构建^{131}I-耐受甲状腺癌细胞（图2A），选择耐受剂量最高的TPC-1细胞进行后续实验。PCR-SSCP检测结果（图2B）显示，^{131}I耐受TPC-1细胞的KRAS基因突变率为83%，^{131}I敏感TPC-1细胞的KRAS基因突变率为18.6%（P<0.01）。Wb检测结果（图2C、D）显示，p21在^{131}I耐受TPC-1细胞中的表达水平显

著低于未接受^{131}I治疗及^{131}I敏感TPC-1细胞（P<0.05、P<0.01）；而过表达KRAS后，p21的表达水平则显著上调（P<0.05、P<0.01）。过表达KRAS后，^{131}I敏感及耐受TPC-1细胞的增殖明显受到抑制（t=2.85，2.66，均P<0.05）；^{131}I敏感及耐受TPC-1细胞的迁移受到抑制（t=112.58，118.80，均P<0.01，图2E）；^{131}I敏感及耐受TPC-1细胞的凋亡增加（t=−40.68，−47.26，均P<0.01，图2F）。

2.3　KRAS基因突变导致荷瘤小鼠^{131}I放射治疗耐受

建立小鼠移植瘤模型，使用半致死剂量的^{131}I持续治疗，同时导入KRAS基因超表达载体，结果显示，半致死剂量的^{131}I持续治疗小鼠的KRAS基因的突变率显著高于阴性对照组（t=− 82.06，P<0.05，图3A），^{131}I持续治疗与未接受治疗小鼠的肿瘤大小无显著差异，而同时超表达KRAS基因的小鼠肿瘤生长明显受到抑制（均P<0.01，图3B和C），通过生存分析发现，超表达KRAS基因的小鼠存活率较高（图3D）。

表2　DTC患者KRAS基因突变检测 [n(%)]

Tab.2　Detection of KRAS gene mutation in DTC patients [n(%)]

Variable	N	Mutation	χ^2	P
^{131}I scan			30.692	<0.0001
Sensitive	37	10(27.03)		
Resistant	35	32(91.43)		
TNM			24.102	<0.0001
I/II	41	15(36.58)		
III/IV	31	29(93.55)		
Sex			0.005	0.944
Male	38	12(31.58)		
Female	34	11(32.35)		

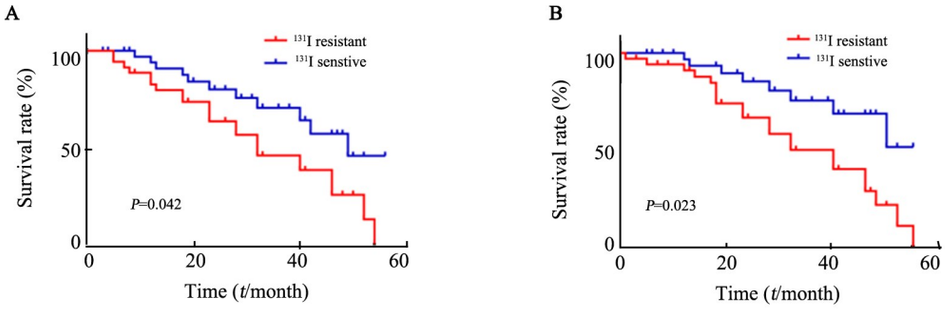


图1 ^{131}I放射治疗敏感及耐受DTC患者生存曲线

Fig.1 Survival curve of ^{131}I radiation sensitive or resistance DTC patients

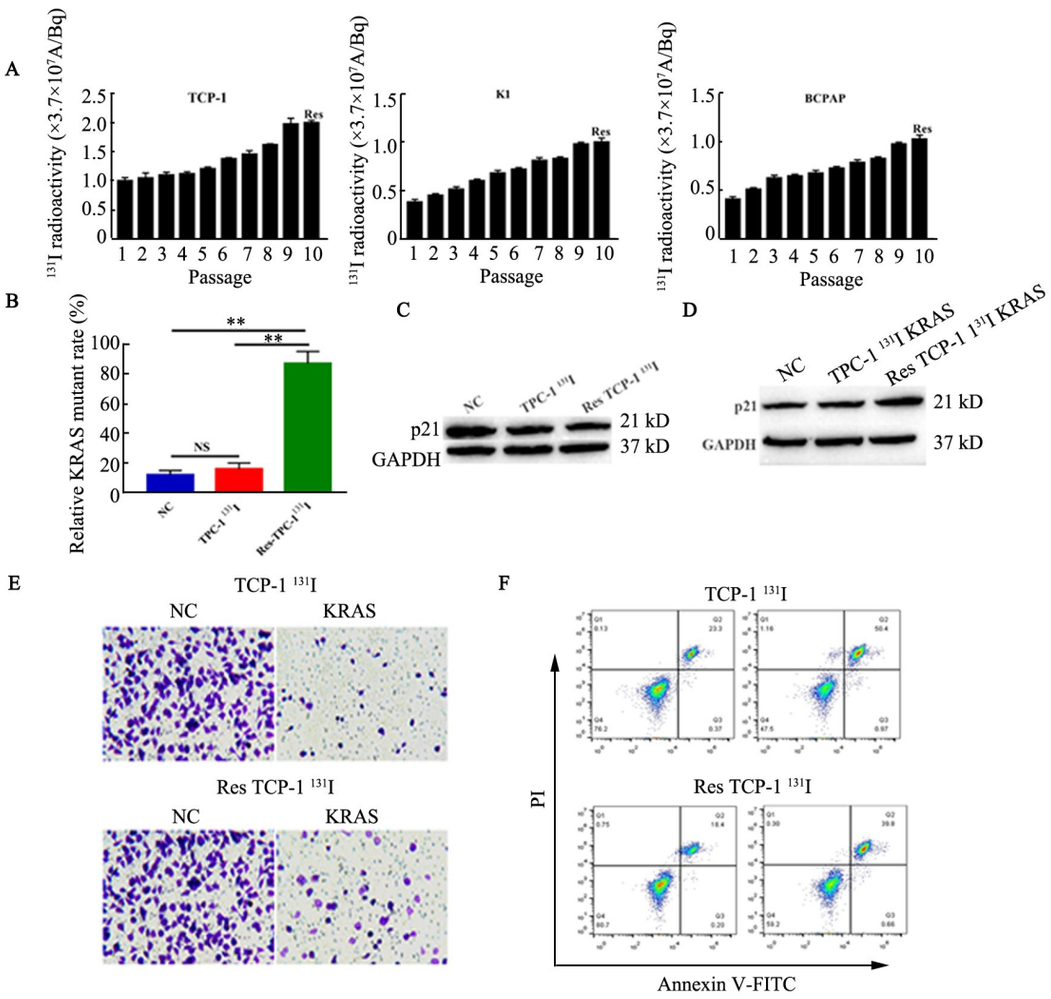
3　讨　论

DTC是一种常见的内分泌恶性肿瘤[16]，发病率逐年上升，目前的治疗手段主要以外科手术切除、促甲状腺激素抑制治疗及^{131}I放射治疗，然而许多DTC患者在接受^{131}I放射治疗后无明显效果[17-18]，由于不同患者肿瘤存在异质性，患者对^{131}I放射治疗产生的耐受性，严重限制^{131}I放射在DTC临床上的应用。本研究通过对临床DTC^{131}I放射治疗敏感及耐受的患者进行研究，发现^{131}I放射治疗耐受的患者中KRAS基因突变率高，且KRAS基因突变率高的患者预后较差。

KRAS是一种原癌基因，属于RAS基因家族，RAS基因可通过活化GTP调节细胞的增殖、分化及凋亡[19]，此外，RAS基因在促进肿瘤生长及促血管生成中发挥重要作用。KRAS基因发生突变能够持续激活AF-MAPK及PI3K/AKT信号通路促进肿瘤细胞大量增殖及转移等，与多种肿瘤的转移、恶化及耐药等密切相关。如在非小细胞肺癌中KRAS基因通过抑制RAF-ERK及激活AKT通路促进癌细胞的增殖[20]，在结直肠癌中可通过检测KRAS基因的突变预测结直肠癌细胞的转移[21]；此外，研究发现KRAS基因的突变与结直肠癌EGFR治疗及西妥昔单抗耐药相关[22-23]，非小细胞肺癌中KRAS基因的突变也会对吉非替尼及奥西替尼等产生耐药[24-25]。在甲状腺癌中，RAS基因的突变与预后不良相关[26]，KRAS基因的高表达水平与甲状腺癌的恶化密切相关[27]。然而，^{131}I放射治疗耐受是否与KRAS基因突变相关未见报道，因此本研究通过对临床样本^{131}I放射治疗耐受及敏感的患者进行研究发现^{131}I放射治疗耐受的患者中KRAS基因突变率高，且KRAS基因突变率高的患者p21的表达较低。

据报道[28],KRAS基因可通过编码p21蛋白调控细胞凋亡。p21属于CIP1家族是近年来发现的细胞周期蛋白依赖性激酶抑制剂,是Ras基因家族编码的一种蛋白质[29],p21表达与肿瘤抑制作用密切相关,发挥抑癌基因作用,p21蛋白与肿瘤的分化、浸润、增殖及转移密切相关[30-31],既能直接参与抑制肿瘤,又能通过抑制周期素依赖激酶复合物活性,协调细胞周期,通过诱导细胞G1期停滞,抑制细胞的生长并促进其分化[32]。通过细胞及动物实验验证,^{131}I放射治疗耐受性与KRAS基因突变的相关性,检测结果与临床检测一致,^{131}I放射治疗耐受性细胞及小鼠中KRAS突变率高,且p21的表达较低,然而在细胞及小鼠体内超表达KRAS基因,p21的表达水平上调,细胞及小鼠对^{131}I放射敏感性增强,且小鼠的生存时间也延长,表明KRAS基因与^{131}I放射治疗的敏感性密切相关。

综上,KRAS基因可能通过调控p21的表达影响甲状腺癌细胞对^{131}I放射治疗的敏感性。因此,KRAS基因突变的研究可为^{131}I放射耐受的分化型甲状腺癌患者的治疗提供新的解决思路及治疗靶点。

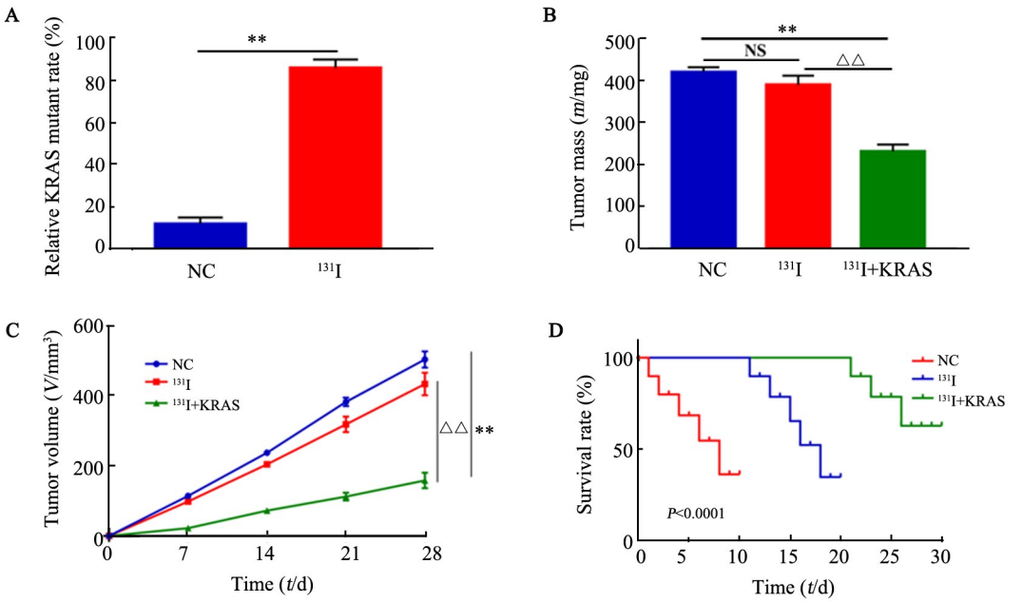


$^{**}P$<0.01

A: Treatment with sub-lethal dose of ^{131}I on continuously passaged thyroid cancer cell lines, and the 10th generation cells were considered as ^{131}I-resistant cells; B: PCR-SSCP detected KRAS gene mutation in TCP-1 cells; C, D: p21 expression in TCP-1 cells was evaluated by Wb; E: TCP-1 cells migration was analyzed by transwell assay (×100); F: The apoptosis of TCP-1 cells was analyzed by flow cytometry

图2 KRAS基因突变导致TCP-1细胞^{131}I放射治疗耐受

Fig.2 KRAS mutant induced ^{131}I resistant in TCP-1 cells



**P<0.01, △△P<0.01

A: PCR-SSCP detected KRAS gene mutant in DTC xenograft tumors nude mice; B,C: The mice tumor mass and size were measured; D: Kaplan-Meimer survival curve analysis the survival of DTC xenograft tumors nude mice

图3 KRAS基因突变导致DTC荷瘤小鼠^{131}I放射治疗耐受

Fig.3 ^{131}I resistant induced by KRAS gene mutating in DTC xenograft tumors nude mice

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