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World Chin J Digestol, 2003 January;11(1):6-9
世界华人消化杂志 ISSN 1009-3079 CN 14-1260/R

胃癌癌前病变相关基因的筛查及表达研究

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国家重点基础研究发展规划项目（973 项目），G1998051203
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收稿日期:2002-11-12 接受日期:2002-11-28

Screening and expression of associated genes in gastric dysplasia

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Received:2002-11-12 Accepted:2002-11-28

Abstract

AIM:To explore molecular mechanism of gastric carcinogenesis, we screened associated genes of gastric dysplasia and further investigated their expression in gastric carcinomas with different stages.

METHODS:Relatively pure dysplasia and normal tissue were procured by manual microdissection, amplified by cDNA-PCR, and then used to carry out forward (dysplasia as tester, normal tissue as driver) and reverse (normal tissue as tester, dysplasia as driver) SSH. Subtracted cDNA fragments were cloned into vector, screened, sequenced, and made homologous analysis. The expression of differentially expressed fragments was detected and verified by Dot hybridization and reverse transcription-PCR.

RESULTS:Two subtracted cDNA libraries were constructed. Twenty-one of 26 sequenced clones were verified to be expressed differentially. It was noted that differentiall expressions of 4 genes (P125,cytochrome c oxidase subunit I, meprin A,acidic calponin)were detected simultaneously in dysplasia, early cancer and advanced cancer.

CONCLUSION:Four new associated genes have been identified in dysplasia. Further studies are necessary to determine their roles in gastric carcinogenesis.

Hao DM, Sun XJ, Zheng ZH, He G, Ma MC, Xu HM, Wang MX, Sun KL. Screening and expression of associated genes in gastric dysplasia. Shijie Huaren Xiaohua Zazhi 2003;11(1):6-9

摘要

目的:胃黏膜异型增生是一种公认的重要癌前病变，筛查癌前病变相关基因并研究这些基因在胃癌不同阶段的表达，探讨胃癌发生分子机制.

方法:手工显微切割取胃异型增生和正常组织,应用 cDNA PCR方法对少量组织全基因组扩增后进行双向抑制性消减杂交，消减后片段与载体连接、克隆、筛选、测序及同源性检索．应用斑点杂交检测基因在胃癌不同阶段的表达，并用半定量 RT-PCR 方法进一步验证检测结果.

结果:正常和异型增生组织互为 tester 和 driver 成功构建了两个 cDNA 消减文库，测序的 26 个克隆中 21 个片段在胃癌不同阶段有表达异常，特别是其中 4 个基因（P125，cytochrome coxidase subunit I,meprin A，acidic calponin)在异型增生、早癌、进展期胃癌中皆有表达改变，可能是重要的胃癌癌前病变相关基因.

结论:发现 4 个新的与胃癌发生相关基因,其具体机制有待进一步研究.

郝冬梅,孙秀菊,郑志红,贺光,马鸣超,徐惠绵,王梅先,孙开来.胃癌癌前病变相关基因的筛查及表达研究. 世界华人消化杂志 2003;11(1):6-9
http://www.wjgnet.com/1009-3079/11/6.htm

0 引言

胃癌是我国发病率和死亡率最高的恶性肿瘤之一，其发病率呈明显的上升趋势[1,2]，胃黏膜异型增生是公认的重要癌前病变，但由于异型增生细胞取材困难，局部分布，数量少，该阶段是胃癌发生分子机制研究的重点也是难点，基因表达变化研究刚刚起步，而且局限于应用免疫组化、原位杂交或显微切割方法研究单个基因的表达，系统化研究较少，尚未发现重要的相关基因[3-15].应用抑制性消减杂交技术（suppression subtractive hybridization, SSH）从整体水平筛选差异表达基因[16,17]，寻找胃癌相关基因，是研究胃癌发生分子机制的重要途径, 对胃癌的早期诊断、治疗和预防有重要意义，迄今未见报道．本研究借鉴代表性差

异显示技术(RDA)首先对少量组织cDNA进行全基因组扩增，然后应用抑制性消减杂交技术进行双向杂交，把三种方法结合创建了显微切割- cDNA PCR-SSH法．应用此方法成功构建了胃异型增生和正常组织间cDNA消减文库，为少量组织消减文库的构建提供新途径．此外，应用斑点杂交和半定量RT-PCR方法检测差异基因在胃癌不同阶段的表达情况，初步筛选胃异型增生相关基因，为阐明胃癌发生分子生物学机制提供依据．

1 材料和方法

1.1 材料 由中国医科大学附属一院肿瘤科提供.抑制性消减杂交所用标本由显微切割取材，将胃癌患者癌旁组织，切成若干块，分别做冰冻切片，HE染色后置于倒置显微镜上在10倍镜下用玻璃细针手工显微切割取病理诊断为异型增生处组织[18,19]，胃正常组织取自切缘处,方法同上.斑点杂交用标本为早癌1例,进展期癌4例，大体标本；异型增生1例，同SSH．RT-PCR实验标本为进展期癌,30例,大体标本．

1.2方法 (1)cDNA PCR扩增:正常和异型增生组织cDNA分别用Mbo Ⅰ酶切，连接头,接头序列为：R-Bgl-24: 5’-AgCACTCTCCAgCCTCTCACCgCA-3’ R-Bgl-12: 5’-gATCTgCggTg-3’ 然后以R-Bgl-24为引物做PCR扩增，循环条件:72 ℃ 5 min→25 ×(95 ℃ 1 min→ 72 ℃ 3 min)→ 72 ℃ 7 min,合并20管扩增产物,再用Mbo Ⅰ酶切，利用Gel Extraction Kit（上海华舜）除去接头，参照cDNA-RDA方法[20,21]，酶切产物用T4 DNA聚合酶补齐粘末端后分别做tester和driver进行双向SSH．(2)SSH:以正常组织做tester，异型增生组织做driver进行SSH，简称NT；以异型增生组织做driver，正常组织做tester进行SSH，简称PT，应用Clontech PCR select™ cDNA Subtraction Kit，具体步骤同说明书．(3)cDNA消减文库构建、筛选和序列鉴定:消减杂交产物经PCR Purification kit（上海华舜）纯化，与pMD-18载体连接，转化受体菌JM109进行蓝白斑筛选（含x-gal,IPTG,Amp），分别随机挑取30个白色克隆，以巢式PCR引物1和2R 进行PCR扩增，再分别将其中20个有清晰单条带的克隆送上海博亚生物技术公司测序，测序结果在GenBank(http://www.ncbi.nlm.nih.gov/BLAST)进行blastN和EST-human同源性比较，如果为新序列，送交GenBank．(4)斑点杂交：阳性克隆PCR扩增后应用96 well dot-blot system制备杂交膜，β-actin做内对照．应用随机引物法（Takara）标记正常和异型增生组织cDNA PCR扩增产物，应用逆转录反应体系标记癌组织和配对正常组织中RNA，探针标记，杂交，洗膜，放射自显影同分子克隆．压片同时放两张X-片，48 h后洗第一张，10 d时洗第二张．杂交结果用安莱图像分析仪扫描和Chemilmager 5500软件分析．(5)半定量RT-PCR:CFDP1引物序列:F 5’-AggCATTggATCAgAggATg-3’ R 5’-ATggATggCCAgTTCTTCAC-3’扩增片段长度为499 bp; β-actin做内对照,引物序列:F 5’-AgAgCTACgAgCTgCCTgAC-3’R 5’-AgTACTTgCgCTCAggAggA,扩增片段长度为300 bp，利用Primer 3.0软件设计．PCR扩增条件：95 ℃ 5 min→30 ×(94 ℃ 45 ”→ 56 ℃45 ” 72 ℃ 45 ”)→ 72 ℃ 7 min．

2 结果

2.1 SSH实验cDNA测序结果 从两个cDNA消减文库中各自挑取30个克隆，经PCR扩增验证其中NT 25个、PT 26个克隆有插入片段，分别选取20个阳性克隆测序，测序结果与GenBank数据库进行同源性分析，结果显示:含有26个不同克隆,其中15个克隆与已知基因高度同源，3个与已知EST（expressed sequence tags）高度同源，8个为未知EST，已被GenBank收录，登录号为BQ164614- BQ164616，BQ291516-BQ291520，可能代表新基因．

2.2斑点杂交结果 应用斑点杂交技术检测26个阳性克隆在异型增生(1例)、早癌(1例)和进展期胃癌(4例)中表达水平，结果显示：21个克隆在至少1例标本中有表达差异，其中10个克隆在异型增生(图1)，8个克隆在早癌，20个克隆在进展期癌中表达改变．5个克隆在异型增生、早癌、进展期癌中皆有表达改变，其中4个克隆为已知基因（P125，cytochrome c oxidase subunit I，meprin A，acidic calponin),1个克隆为EST，3个克隆表达上调，2个克隆表达下调，统计结果见表1.

2.3 半定量RT-PCR结果 进展期胃癌及配对正常组织内皆有CFDP1基因表达，但其中9例胃癌标本表达显著下降，阳性率30 %(9/30),与斑点杂交结果基本一致(1/4，25 %),进一步验证上述实验数据，见图2．

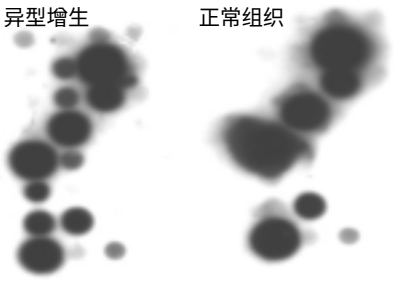

图1 正常和异型增生组织斑点杂交结果(曝光48 h).

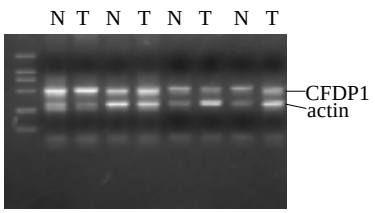

图2 RT-PCR检测结果 N:正常组织 T:胃癌组织.

表1 阳性克隆在胃癌不同阶段中的表达

克隆	基因名称	差异表达 *n*	差异表达标本类型	阳性率(%)
PT1	AL713668 (hypothetical protein)	1	进展期	17
PT2	NM_007190 (Sec 23-interacting protein p125)	3	异型增生 早期进展期	50
PT3	AI339219 (EST)	1	早期	17
PT4	FLJ10417 (hypothetical protein)	2	异型增生 进展期	33
PT6	NM_004190.1 (gastric lipase, LIPF)	1	进展期	17
PT10	BM840901 (EST)	2	早期 进展期	33
PT11	XM_044902 (differentially expressed in hematopoietic lineages)	3	异型增生 进展期	50
PT17	NM_004859 (clathrin, heavy polypeptide, CLTC)	1	进展期	17
PT18	XM_008731 (meprin A, beta)	3	异型增生 早期进展期	50
PT19	AF382013 (cytochrome C oxidase subunit I)	3	异型增生 早期进展期	50
PT21	BQ291520 (EST)	2	早期 进展期	33
PTB1	NM_021804 (angiotensin I converting enzyme 2)	3	异型增生 进展期	50
PTB4	AB023058 (chromosome 6p21.3, HLA class I region)	1	进展期	17
NT4	BQ291517 (EST)	1	进展期	17
NT8	BQ291516 (EST)	1	进展期	17
NT12	NM_017455 (stromal cell derived factor receptor 1)	2	进展期	33
NT13	S80562 (acidic calponin)	3	异型增生 早期进展期	50
NT14	XM_058493 (similar to pepsin A precusor)	3	异型增生 进展期	50
NT15	BQ291519 (EST)	3	异型增生 早期进展期	50
NT17	NM_006324 (CFDP1)	2	异型增生 进展期	33
NT21	BQ291518 (EST)	1	进展期	17

3 讨论

肿瘤学研究中，分析微小癌前病变非常重要，他是正常细胞演变为癌细胞的中间过程，是癌症发生的第一个关键步骤. 胃黏膜上皮异型增生是胃癌的一种重要癌前病变，但由于肉眼无法辨别，细胞数量少，细胞异质性干扰严重，需要利用显微切割技术在显微镜下定点准确切取组织，取材非常困难，研究受到严重限制. 我们借鉴cDNA RDA方法首先对显微切割获取的少量组织进行全基因组（cDNA PCR）扩增，然后再进行消减杂交，将显微切割、RDA和SSH的优点结合，有效保证了实验的精确性. 抑制性消减杂交技术是Diatchenko et al [22]1996年建立起来的一种以PCR为基础的cDNA消减杂交技术,其归一化(normalization)过程可使高、低丰度的差异基因都能有效分离，从整体水平系统化筛选差异表达基因，研究该阶段基因表达变化和特异性标志物. 获得的26个不同基因片段中21个经斑点杂交方法证实在异型增生或胃癌组织中差异表达，这提示:利用本室建立的显微切割-cDNA PCR-SSH法从少量组织构建cDNA消减文库，是一种简便、快速、高效克隆鉴定差异表达基因的方法.

基因芯片技术是高通量研究基因表达谱的新方法[23-25]，国外文献报道将消减杂交获得的差异片段制成芯片进行基因的初步筛查及验证，替代传统的Northern杂交方法，省时省力效率高[26,27]，但费用昂贵，本实验应用斑点杂交技术代替基因芯片进行小规模的表达谱检测，国内尚无报道. 胃癌发生发展过程中保留了绝大多数早期的遗传学改变，由于无法直接应用斑点杂交检测筛查基因在异型增生大体标本中的表达水平，我们研究了这些基因在胃癌不同阶段的表达状况，筛选频繁表达异常的基因，从而反推法间接寻找重要的癌前病变相关基因. 获得的21个差异片段中有5个片段在异型增生、早癌、进展期胃癌中皆有表达改变，3个表达上调，2个表达下调，其中4个为已知基因，1个为EST，可能是重要的胃癌发生相关基因，尚无这些基因在胃癌方面研究报道. 已知基因中， cytochrome c oxidase subunit I（细胞色素c氧化酶亚单位I）编码呼吸链蛋白，参与细胞能量代谢，其缺陷可引起肌肉、神经等退行性病变. Weber et al [28]应用差异显示方法发现该基因在低侵袭能力的上皮肉瘤细胞系中表达增强. P125蛋白有磷脂酶活性，与Cop II包被成分Sec23P相互作用，参与将蛋白质从内质网运输到高尔基体[29]. 这两种基因表达上调,我们认为可能与细胞过度增生时需要提供大量营养能量有关.meprin A编码上皮细胞分泌的金属蛋白酶，能够裂解细胞外基质蛋白[30].Lottaz et al [31]证明人结肠癌组织中meprin A表达增加，酶活性增强，呈现肿瘤特异性分布，与肿瘤转移有关. Acidic calponin在平滑肌细胞和非平滑肌细胞中皆大量表达，功能未知，可能与细胞骨架运动有关，已证明Basic calponin是抑癌基因[32,33]. meprin A表达上调和Acidic calponin表达下调可促进癌细胞向远处扩散.

本室曾经应用RDA方法在胃肠上皮化生阶段筛选出CFDP1基因，表达下调，本实验又证明该基因在异型增生组织和进展期癌中表达显著降低，其正常生理功能尚无报道，进一步的分析和功能鉴定正在进行中，推测CFDP1基因在维持胃黏膜细胞功能形态方面起重要作用，这也提示了胃癌由肠化生到异型增生再到癌的发生发展顺序[34]. 本实验首次成功建立胃异型增生组织消减文库，并研究了这些基因在异型增生、早癌、进展期等胃癌不同阶段的表达，初步筛选癌前病变相关基因，作者将深入研究这些基因的功能及其在胃癌发生、发展过程中的作用机制.

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