PCR 直接测序方法及其在肿瘤研究中的应用*

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摘要　PCR 直接测序技术是PCR 扩增与核酸测序技术相结合的一种方法. 根据此技术的原理，建立了一种以 PCR 扩增引物为测序引物，α-^{35}S dATP 直接掺入，Taq DNA 聚合酶直接测序 PCR 扩增产物的方法. 实验表明：该方法简便、快速、稳定. 用此方法对人食管癌组织中的抗癌基因 p53 进行了突变测序分析，发现食管癌组织中 p53 存在点突变，插入、丢失移码突变. 并用此方法对人和恒河猴的 p53 内含子序列进行了测定，发现猴第 5 内含子为 81 个核苷酸，第 8 内含子为 92 个核苷酸.

关键词　聚合酶链反应（PCR），抗癌基因，肿瘤

自 1985 年 PCR 技术建立以来，它已成为分子生物学必不可少的工具[1]，PCR 直接测序技术能简单迅速检测基因突变，诊断遗传性疾病. 对克服传统测序方法的不足，基因表达研究，进化过程中的核酸水平研究等具有非常重要的作用. 本文旨在为实验研究提供一种快速分析基因突变的方法.

1　材料和方法

1.1　材料

1.1.1　试剂　Taq DNA 聚合酶，Taq DNA 聚合酶（测序级），丙烯酰胺，TaqTrack Deaza 测序 kit（购自 Promega 公司）；dATP，dGTP，dCTP，dTTP（Boehringer Mannheim 公司）；α-^{35}S dATP 1250 Ci/mmol（NEN 公司）；琼脂糖（wide Range/standard 3∶1），矿物油（Sigma 公司）.

1.1.2　标本　正常人胎儿食管上皮组织（水囊引产）；食管癌手术标本液氮保存；恒河猴（购自中国医科院昆明生物所）.

1.1.3　仪器　Thermal DNA Circle（Cetus 公司），Gene Machine Ⅱ（Scientific Plastic 公司）.

1.1.4　引物　A：5′-GGAATTCTACTCCCCTGCCCTCAACAAG-3′；B：5′-GGAATTCCTCAGGCGGCTCATAGGGCAC-3′ 中科院微生物所合成. C：5′-CTCCTAGGTTGGCTCTGACT-3′；D：5′-CCCAAGACTTAGTACCTGAA-3′ MWG-Biotech GmbH 西德合成.

1.2　方法

1.2.1　制备双链扩增 DNA 参见文献 [1].

PCR 反应：总体积 50μl，含 0.5—1μg DNA 样品，50mmol/L KCl，10mmol/L Tris（pH 9.0，25℃），1.5mmol/L $MgCl_2$，0.01% gelatin（W/V），0.1% Triton X-100，25 pmol 引物，200μmol/L 4× dNTP，4%—5%甲酰胺，98℃变性 7min，加 1.5—2.0U Taq DNA 聚合酶，40μl 矿物油.

循环参数：变性 94℃ 30s，复性 45—60℃ 30s，延伸 72℃，1min. 循环 25—30 周期，最后 72℃保温 5min，放置 4℃.

纯化产物：1.5%琼脂糖电泳分离产物，切下所需产物凝胶条，75℃加热溶化5min. 酚-氯仿抽提回收扩增片段.

1.2.2　制备单链扩增模板参见文献 [1].

PCR 反应：总体积 100μl，加（0.5—1pmol 双链DNA），50mmol/L KCl，10mmol/L Tris

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(pH 9.0, 25 C), 1.5mmol/L $MgCl_2$, 0.01% gelatin (W/V), 0.1% Triton X-100, 30—50 pmol 单引物, 20μmol/L 4× dNTP, 5%—6% 甲酰胺, 98 C变性 5min 后加 2.5—4U Taq DNA 聚合酶, 60μl 矿物油.

循环参数: 变性 94 C 30s, 复性 50—60 C 30s, 延伸 72 C 1—2min, 循环 15—20 周期, 最后 72 C保温 8min, 放 4 C保存.

纯化产物: 等体积酚-氯仿各抽提一次, 加 4mol/L 乙酸胺 100μl 混匀, 加等体积异丙醇混匀, 室温放置 10min, 离心回收片段. 70% 乙醇洗一次, 真空干燥, 沉淀溶解于 10μl 灭菌三蒸水中, 取 3μl 电泳定量 (见图 1).

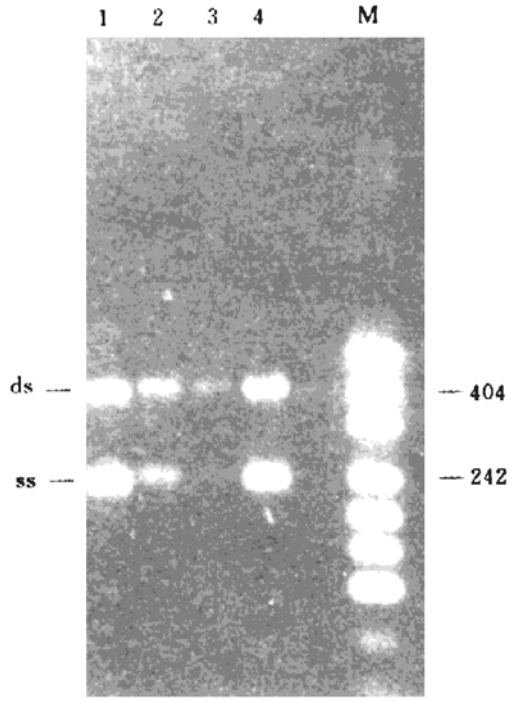

图 1 PCR 扩增单链产物电泳图

1—4 为不同的样品; M: 为 pUC 19-HpaII 酶切片段电泳图谱.

1.2.3 测序反应

模板与引物复性反应: 单链 DNA: 0.5—1.5pmol, 引物 1—2.0pmol, 5×缓冲液 3μl, 延伸/标记混合物 1μl, 加消毒三蒸水至 15μl, (5×缓冲液: 250mmol/L Tris-HCl, pH 9.0, 25 C, 50μmol/L $MgCl_2$; 延伸标记混合物: 7.5μmol/L dGTP, dCTP, dTTP) 混匀, 72 C保温 3min, 42 C保温 10min.

延伸和标记反应: 加 0.5μl α-^{35}S dATP (5μCi) 再加 1.4μl 2.5U/μl Taq DNA 聚合酶 (测序级), 离心混匀, 37 C保温 5min.

终止反应: 分别在红、黄、蓝、绿 4 种彩色 1.5ml 离心管中加入 d/dd NTP (T. C. G. A.) 溶液各 0.8μl. d/dd NTP (T. C. G. A.) 各液浓度如下[2]: 300μmol/L ddTTP, 25μmol/L dTTP, 250μmol/L 7-deaza dGTP, dCTP, dATP; 160μmol/L ddCTP, 25μmol/L dCTP, 250μmol/L 7-Deaza dGTP, dATP, dTTP; 25μmol/L ddGTP, 25μmol/L 7-deaza dGTP, 250μmol/L dATP, dCTP, dTTP; 350μmol/L ddATP, 25μmol/L dATP, 250μmol/L 7-Deaza dGTP, dTTP, dCTP. 当 37 C保温时间完成后, 每个彩色管中加入 4μl 反应液, 混匀, 72 C保温15min, 每个反应管中加入2.8μl终止液 (10μmol/L 氢氧化钠, 95%甲酰胺, 0.05% 溴酚蓝, 0.05%二甲苯胺) 混匀, 电泳上样前, 90 C变性 5min 放冰浴, 取 1—2μl 上样.

1.2.4 测序电泳和放射自显影参见文献 [3].

2 结果与讨论

2.1 本文建立了一种以 PCR 扩增引物为测序引物, α-^{35}S 直接掺入, Taq DNA 聚合酶直接测序扩增产物的方法 (图 2). 实验表明: 该方法简便、快速、稳定.

1988 年 Innis 等[4]首次利用 Taq DNA 聚合酶进行了测序分析. 由于 Taq DNA 聚合酶具有高温反应的特性[4], 能有效消除 DNA 二级结构对测序带来的影响, T7 DNA 聚合酶只能在常温下反应[5], 难以消除 DNA 二级结构的影响, 特别是分析未知基因序列, 用 Taq DNA 聚合酶测序值得优先考虑.

由于 Taq DNA 聚合酶无 3′—5′ DNA 外切酶活性[5]. PCR 产物中有一定比例的掺入错误. 但某一位点的碱基自发突变的产物在总产物中的比例一般不超过 10%, 根据 Chen 等[7]研究报道, 某一位点碱基突变的模板产物低于总产物的 1/6, 直接测序检测不出突变. 说明 PCR 直接测序的结果是可靠的. 如果 PCR 扩

增产物按传统的克隆测序方法分析核酸序列，结果不可靠，必须挑选多个克隆，需反复测序，时间长且不经济.

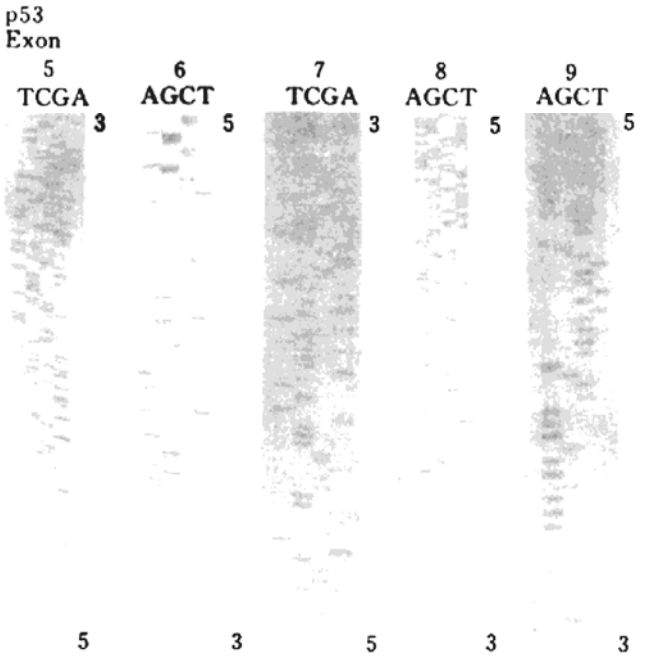

图 2 直接测序放射自显影图

测定的正常人 p53 基因第 5，6，7，8，9 外显子序列放射自显影图.

单链测序效果优于双链测序. 由于 PCR 产物一般小于 1kb，容易复性，增加了双链测序分析的困难，单链测序可读性好，采用 PCR 技术制备单链模板，几个小时就可完成，克服了传统方法制备单链时间长的不足. PCR 制备单链的方法主要有不对称法（asymmetric PCR）和单引物法[8-11]，单引物法是不对称法的特殊形式. 单引物法制备单链有利于产生不含有测序引物互补序列的非测序模板 DNA 片段，特别是在扩增引物作为测序引物特异性不强的情况下，影响更明显，单引物法还能减少扩增周期，降低扩增产物产生的自发突变.

产物的特异性直接影响测序效果，增加 PCR 产物特异性无疑增加测序结果的准确性. 用常规增加特异性方法，如降低引物，核苷酸浓度，提高复性温度，降低延伸时间，减少循环周期等方法使扩增单链产物在没有特定纯化柱情况下达到测序要求较困难，Erlich 等[1]报道二甲基亚砜(DMSO)能有效增加扩增双链产物的特异性，Sharker 等[12]报道甲酰胺能有效增强 PCR 扩增双链产物的特异性. 我们经过实验对比，发现甲酰胺能有效增加 PCR 扩增测序单链模板的特异性，且甲酰胺效果优于 DMSO，明显消除了非特异性小片段产物对测序产生的影响.

2.2 用本方法对正常人和恒河猴 p53 基因第 5 和第 8 内含子序列进行了测定. 发现猴 p53 基因第 5 和第 8 内含子序列为 81 和 92 个碱基（图 3 和图 4），与人的第 5 和第 8 内含子序列进行比较，同源性分别为 96.2%，98.9%.

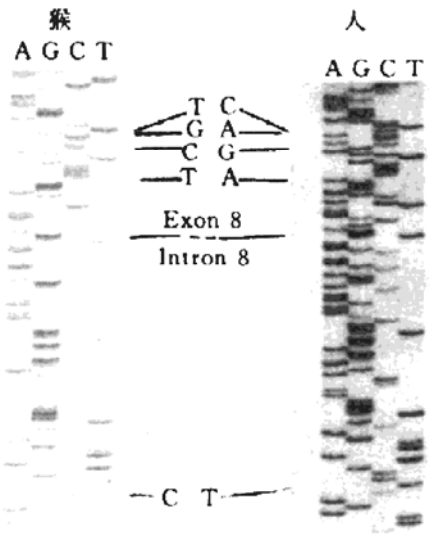

图 3 序列放射自显影图

为正常人和猴 p53 基因测序部分图谱.

Goodrow 等[13]报道了鼠的 p53 基因第 5—8 内含子序列. 序列结果比较表明：人和猴的内含子序列同源性明显高于鼠的同源性，反映他们之间进化上的亲缘关系的远近. 研究不同物种同一基因的内含子序列有利于研究内含子序列的起源，以及内含子与外显子之间的功能关系.

2.3 用本方法对人食管癌组织中抗癌基因 p53 突变热点区域 5，6，7，8，9 外显子和相应内含子进行了突变分析. 检测出点突变，插入、丢失移码突变等变化形式（见图 5）.

p53 基因是一种重要的抗癌基因[14]，主要变化形式为点突变，测定突变的方法很多，但直接测序结果准确性最好，为首选方法.

p53 Intron 5

```
      1          10          20          30          40          50
猴 5′ GTGAGCAGCT GGGACTGGAG AGACGACAGG GCTGGTTGCC CAGGGTCCCC
人 5′ GTGAGCAGCT GGGGCTGGAG AGACGACAGG GCTGGTTGCC CAGGGTCCCC

                60          70          80
猴    AGACCTCTGA TTCCTCACTG ATTTCTCTTA G 3′
人    AGGCCTCTGA TTCCTCACTG ATTGCTCTTA G 3′
```

p53 Intron 8

```
      1          10          20          30          40          50
猴 5′ GTAAGCAAGC AGGACAAGAA GCGGTGGAGG AGACCAAGGG TGCAGTTATG
人 5′ GTAAGCAAGC AGGACAAGAA GCGGTGGAGG AGACCAAGGG TGCAGTTATG

                60          70          80          90
猴    CCCCAGATTC ACTTTTATCA CCTTTCCTTG CCTCTTTCCT AG 3′
人    CCTCAGATTC ACTTTTATCA CCTTTCCTTG CCTCTTTCCT AG 3′
```

图 4 人和猴 p53 基因第 5，第 8 内含子序列比较图

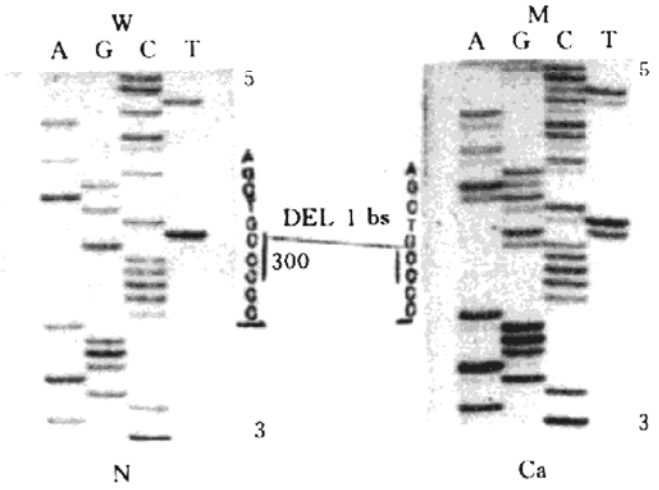

图 5 食管癌 p53 基因突变分析图

N 为正常人食管上皮组织 DNA 样品，第 300 密码子序列为 CCC，Ca 为食管贲门癌组织 DNA 样品，一个等位基因在第 300 密码子的第一个碱基丢失成为-CC，另一个等位基因正常.

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food industry.

Key words superoxide dismutase, chemical modification, enzyme engineering, cosmetics

Phase-Resolved Photoacoustic Spectroscopy and Photoacoustic Phase Spectrum of the Intact Leaf. Du Hao, Fang Jianwen, Zheng Jinju. (*Dept. of Phys., Zhejiang Normal University, Jinhua* 321004). *Prog. Biochem. Biophys.* (China), 1994; **21** (2): 158

The phase-resolved photoacoustic spectroscopy has been used to analyse the pigmental distribution in intact plant leaves and has been compared with the photoacoustic phase spectrum of the leaves. The inverse correlation between the photoacoustic phase spectrum and absorption bands of chloroplasts has been observed. The characteristic valley exists in the photoacoustic phase spectrum of the leaf cuticle. In addition, there are some differences in the photoacoustic phase spectra obtained at different modulation frequency. The phenomena show that photoacoustic phase spectra can also be used for the nondestructive depth-profile analyses of biological sample as good as photoacoustic spectroscopy.

Key words phase-resolved photoacoustic spectroscopy, photoacoustic phase spectrum, phase-resolved method, intact plant leaf, depth-profile analysis

A New HPLC Separation for PTC Derivatives of Amino Acids by Ethanol Elution. Zhu Shudong, Zhao Huiren, Zhao Shenghao. (*Department of Biochemistry, Xuzhou Medical College, Xuzhou* 221002). *Prog. Biochem. Biophys.* (China), 1994; **21** (2): 162

Analysis of phenylthiocarbamyl (PTC) amino acids with ethanol as organic eluent is described. Compared with acetonitrile elution system, ethanol is less toxic, easier to obtain and much cheaper. Under optimized chromatographic conditions, the resolution, sensitivity and accuracy are excellent.

Key words ethanol, PTC amino acid, HPLC

Simultaneous and rapid purification of total cytoplasmic RNA and genomic DNA from small numbers of transfected mammalian cells. Zhang Hongquan, Wang Huixin, Zhou Tingchong, Wang Yunling. (*Inst. Bas. Med. Sci, Acad. Mil. Med. Sci. Beijing* 100850). *Prog. Biochem. Biophys.* (China), 1994; **21** (2): 165

A protocol by using 4 mol/L LiCl phasing the DNA and RNA could lead to simultaneous and rapid purification of total cytoplasmic RNA and genomic DNA from small numbers of transfected mammaliam cells. Comparing with other methods, this protocol shows rapid, easy and economic, and can be used in many aspects especially in the studies of mammalian cell gene expression and regulation.

Key words total cytoplasmic RNA, genomic DNA, gene expression and regulation

The Method of PCR Direct Sequencing and It's Application in Cancer Research. Li Huachuan, Lu Shixin. (*Cancer Institute, Chinese Academy of Medical Sciences, Beijing* 100021). *Prog. Biochem. Biophys.* (China), 1994; **21** (2): 167

PCR direct sequencing is a method which combined PCR amplification with nucleic acid sequencing technique. According to this technique, direct sequencing DNA strand of PCR amplification using PCR primer, α-^{35}S dATP and Taq DNA polymerase. The experiment showed that it is simple, rapid and stable. This method was used to analyze the tumor

suppressor gene p53 mutation in human esophageal cancer. It was found that there were point mutation. insertion and deletion frameshift mutation of p53 gene in human esophageal cancer. Intron 5 and 8 sequences of p53 gene in human and Rhesus monkey were sequenced and in monkey they are 81 and 92 nucleotides respectively.

Key words PCR. tumor suppressor gene. tumor

Quantum Calculation for the Coordination Modes of Substrates Binding on Nitrogenase Active-Center. Liu Aimin. Zhou Taijin. Zhang Hongtu. Wan Huilin. Cai Qirui (Tasi Khirui). (*Department of Chemistry and Institute of Physical Chemistry, Xiamen University, Xiamen* 361005). *Prog. Biochem. Biophys.* (China). 1994: **21** (2): 171

EHMO studies of N_2 and C_2H_2 coordination-activation led to the conclusion that the iron-molybdenum cofactor of nitrogenase might be able to give a special treat to its special substrate. i. e. N≡N. The exogenous substrates except N_2 are apparently not to get into the cage of the active-center and/or to manoeuvre as freely as N≡N inside the cage with the proposed structural settings.

Key words nitrogenase. FeMo-cofactor. EHMO approach. coordination

Measurement of Surface Charge Numbers of Purple Membrane. Wang Guangyu. Hu Kunsheng. Zhang Hengtao. (*Institute of Biophysics, Academia Sinica, Beijing* 100101). *Prog. Biochem. Biophys.* (China). 1994: **21** (2): 173

The pH-dependent surface charge densities of the acetylated and the native purple membrane were determined by the ESR spin lable method. The spin probe is CAT_{12}. The number of surface charges shieded by acetylation was adapted as a criterion to calculate the surface charge numbers on both sides of the purple membrane from surface pH 4—11. The result shows that the total surface negative charge numbers are 9 per bacteriorhodopsin at surface pH 5—9 but increases both above surface pH 9 and below surface pH 5. It supports strongly the model based on five divalent cation binding sites on the surface of purple membrane.

Key words purple membrane. bacteriohodopsin. acetylation. ESR. surface charge number

The Expression of p53. Rb and c-myc Gene mRNA in Human Primary Brain Tumor. Tan Deyong. Lin Xi. Sun Zhilin. (*Department of Biochemistry West China Univeristy of Medical Science, Chengdu* 610041). *Prog. Biochem. Biophys.* (China). 1994: **21** (2): 175

21 human primary brain gliomas and 11 human menigiomas were examined with RNA dot blot hybridization for the expression of p53. Rb and c-myc gene. It was found that the level of p53 gene expression is lower in 48.4% (15/31) of the tumors tested than that of normal brain tissues: the level of Rb gene expression is lower in 21.9% (7/32) for the tumors tested than that of normal tissues; and the level of c-myc gene expression is higher in 71.9% (23/32) for the tumors tested than that of normal tissues. Interestingly. in 13 of the tumors tested. the level of p53 gene expression is lower and the level of c-myc gene expression is higher. These results suggested that the expressive decrease of p53 gene and the expressive increase of c-myc gene are relative to the generation of human primary brain tumor.