

肝癌相关 cDNA 片段的快速克隆和表达

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Rapid cloning and expression of hepatocellular carcinoma associated cDNA fragments

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Abstract

AIM: To clone and characterize novel genes that might be involved in hepatocellular carcinoma.

METHODS: An EST fragment differentially expressed between hepatocellular carcinoma (HCC) and its adjacent nontumorous liver tissues had been cloned using suppression subtractive hybridization (SSH). With this approach, we identified a novel EST that consisted of 447bp. In order to gain its full-length cDNA fragment, two gene-specific primers were designed for 3'-rapid amplification of cDNA end RACE. One HCC cell line, HepG2, was maintained in the RPMI1640 media and recommended culture conditions. Total RNA was extracted from HepG2 by the SV Total RNA Isolation System (Promega). RACE reactions were prepared using the SMART™ RACE cDNA amplification kit (Clontech). Initial amplification was carried out with gene-specific primer 3'GSP2(5'-CGCATAGT ACCAGTATCGACAA AGG-3'), followed by nested PCR using gene-specific primer 3'NGSP2(5'-TCCACATTACGGACCCGACGGATT-3'). These amplified cDNA fragments obtained from RACE were subcloned into the PMD18-T vector (TaKaRa) and sequenced by ABI PRISM377 DNA sequencer. Basic local alignment search tools were carried out using BLASTN and dbEST and nr database. Northern blot was applied to detect the expression of these cDNA fragments between HCC and its adjacent nontumorous liver tissues. A total of 3 liver specimens were collected from the Southwest Hospital of Chongqing in China. The final diagnosis of HCC was confirmed by histological examination. Total RNA was extracted from either HCC or its adjacent nontumorous liver tissues

in the same way. These cDNA fragments were excised from the PMD18-T vector, purified and ³²P-labeled as cDNA probes using the random primed labeling method. Northern blot was prepared by using the ExpressHyb™ hybridization solution (Clontech) according to the protocol provided by the manufacturer. Combination of Northern blot and virtual Northern (<http://www.ncbi.nlm.nih.gov/SAGE>) (series analysis of gene expression, SAGE), expression of these cDNA fragments in multiple carcinoma and normal tissues were analyzed.

RESULTS: Five EST fragments (694 447-3, 724 447-3, 697 447-3, 711 447-3, 692 447-3) were cloned, including two EST fragments with ploy (A) tail (694 447-3, 724 447-3). Compared with ESTs in GeneBank, the two EST fragments with ploy (A) tail represented novel genes with a common sequence. Five EST fragments accession numbers in Genebank were CK730344, CK730345, CK730346, CK730347, CK730348, respectively. Northern blot revealed that 694 447-3, 724 447-3 presented higher expression in HCC than that in its adjacent nontumorous liver tissues. Virtual Northern blot (SAGE) revealed that 694 447-3, 724 447-3 presented higher expression in multiple cancers that contained in brain, colon, breast, lung and stomach than that in their normal tissues.

CONCLUSION: Two novel human hepatocellular carcinoma-associated cDNA fragments are identified. RACE technique is a rapid and effective method for seeking for disease related genes in specific tissues.

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摘要

目的: 克隆原发性肝癌相关新基因。

方法: 利用抑制消减杂交法(suppression subtractive hybridization, SSH)已经发现了1条新的肝癌相关基因片段表达序列标签(EST), 长 447 bp, 经 Genebank 检索, 90% 无同源性。在其保守序列区设计了2条用于3' RACE扩增的寡聚核苷酸引物(3' GSP2: 5' -CGCATAGTACCAGTATCGACAAAGG-3', 3' NGSP2: 5' -TCCACATTACGGACCCGACGGATT-3'), 利用 cDNA 末端快速扩增法(RACE)进一步克隆该基因的全长cDNA序列。人原发性肝癌细胞株 HepG2, 体外传代培养, 培养基为 RPMI1640 培养基。提取 HepG2 总 RNA, 方法参照 SV Total RNA Isolation System 的说明进行。RACE 法扩增采用 Clontech 公司的 Smart™ Race cDNA Amplification Kit。将 3' RACE-PCR 扩增的目的片段以 Race 自带的产物纯化试剂盒进行纯化、回

收, 然后将其克隆到 PMD 18-T Vector 中, 提纯质粒后进行酶切鉴定, 确认质粒内有插入片段, 由宝生物工程(大连)有限公司协助完成测序. 将克隆所得 cDNA 片段用 NCBI 提供的 BLASTN 与 GeneBank 的 dbEST、nr 数据库进行同源性比较, 确认代表新基因的 EST 并且登录 GeneBank (<http://www.ncbi.nlm.nih.gov/submit>). 3 例病理标本取自西南医院肝胆科, 病理证实均为原发性肝细胞肝癌, 分别提取肝癌及远端正常肝组织总 RNA. 将酶切回收的克隆插入片段分别进行同位素标记获得 cDNA 探针, 利用 Clontech 公司的 ExpressHyb™ 杂交液通过 RNA 印记法检测克隆片段在肝癌及正常肝组织中的表达, 方法参照 ExpressHyb™ Hybridization Solution user manual 说明进行. 同时, 利用互联网的基因表达分析序列数据库(<http://www.ncbi.nlm.nih.gov/SAGE>)(series analysis of gene expression, SAGE)对基因的表达及其表达水平进行分析, 从而确定其组织的分布.

结果: 得到 5 条 3' EST(694 447-3, 724 447-3, 697 447-3, 711 447-3, 692 447-3; 大小 500-550 bp), 5 条 3' EST 均已登录 GeneBank(登录号: CK730344, CK730345, CK730346, CK730347, CK730348). 对其中 2 条带有 poly-A 尾的 3' EST(694 447-3, 724 447-3), 进行序列分析后, 发现他们是代表新基因或不同剪接体的 EST, 且具有共同的保守序列. RNA 印记分析显示 694 447-3, 724 447-3 在 3 例肝癌组织中的表达强度明显高于对应的正常肝组织. 通过 SAGE 文库分析基因的表达谱, 发现 694 447-3 和 724 447-3 在神经系统肿瘤、结肠癌、胃癌、乳腺癌肿瘤文库的表达高于对应的正常组织文库.

结论: 克隆所得的 2 条带有 poly-A 尾的 3' EST 可能是新的肝癌多基因家族成员. 利用 RACE 技术可以快速、高效的克隆疾病相关基因.

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<http://www.wjgnet.com/1009-3079/12/1785.asp>

0 引言

通过抑制消减杂交法(suppression subtractive hybridization, SSH)我们已经发现了 1 条新的肝癌相关基因片段 EST^[1], 长 447 bp, 经 Genebank 检索, 90% 无同源性. 在此基础上, 我们采用 RACE 技术, 从肝癌组织中钓取肝癌相关的多个 cDNA 片段^[2-3].

1 材料和方法

1.1 材料 人原发性肝癌细胞株 HepG2, 由西南医院消化科司遂海同学惠赠. 体外传代培养, 培养基为 RPMI1640 培养基(Hyclone 公司), 内含抗生素及 100 mL/L 的小牛血清. 细胞总 RNA 提取采用 Promega 公司的 SV Total RNA Isolation System. RACE 法扩增采用 Clontech 公

司的 Smart™ Race cDNA Amplification Kit.

1.2 方法 肝癌细胞株 HepG2 总 RNA 的提取参照 SV Total RNA Isolation System 的说明进行^[4]. Race 法扩增采用 Clontech 公司的 Smart™ Race cDNA Amplification Kit 中介绍的方法^[5-7], 严格按说明操作. 3' Race ready-cDNA: RNA 1 μL (3 μL)、3' -CDS 引物 1 μL、ddH₂O 1 μL, 终体积 5 μL. 在 PCR 仪(Bio-Rad) 70 °C 孵育 2 min, 冰上冷却 2 min, 之后加入: 5× First -strand buffer 2 μL、DTT 1 μL、dNTP mix 1 μL、powerscript reverse transcriptase 1 μL, PCR 仪 42 °C 孵育 1.5 h, Tricine-EDTA buffer 稀释反应产物至 100 μL, PCR 仪 72 °C 孵育 7 min. 以 3' Race ready-cDNA 为模板, 以试剂盒提供的通用引物为下游引物, 合成的 3' Race 引物为上游引物, PCR 法扩增 Race 的 3' 末端. 在保守序列设计了 2 条用于 3' Race 扩增的寡聚核苷酸引物(3' GSP2, 3' NGSP2). 通用引物(UPM)序列为: long 5' -CTAATACGACTCAC TATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' (0.2 μmol/L); short 5' -CTAATACGACTCACTATAGGG C-3' (1 μmol/L); 3' GSP2 引物(序列为: 5' -CGCATA GTACCAGTATCGACAAAGG-3', 10 μmol/L). 3' RACE-PCR 循环参数为: 94 °C 30 s, 72 °C 3 min → 5 cycles; 94 °C 30 s, 70 °C 30 s, 72 °C 3 min → 5 cycles; 94 °C 30 s, 68 °C 30 s, 72 °C 3 min → 30 cycles. 将第 1 次的产物稀释后作为模板用 3' NGSP2 引物(序列为: 5' -TCCACATTACGGACC CGACGGATT-3', 10 μmol/L)与巢式引物 NUP(序列为: 5' -AAGCAGTGGT AACAAACG CAGAGT-3', 10 μmol/L) 进行第 2 次扩增, 条件为: 94 °C 30 s, 68 °C 30 s, 72 °C 3 min → 20 cycles. 5 μL 的 PCR 产物于 1.2% 琼脂糖凝胶上电泳与 DNA Marker (DL2000) 比较判断产物的大小. 将 3' RACE-PCR 扩增的目的片段以 Race 自带的产物纯化试剂盒进行纯化、回收, 然后将其克隆到 PMD 18-T Vector 中(购于 Takara 公司), 转染入感受态 DH5α 细菌, 经过 X-gal/IPTG 蓝白斑的筛选后, 挑取多于 10 个的白色菌落进行质粒扩增, 提纯质粒后进行酶切鉴定, 确认质粒内有插入片段, 采用 PCR、四色荧光、双脱氧终止法, 应用 PE 公司的 ABI Prism™ 377XL DNA Sequencer 进行测序, 引物为载体上的通用测序引物 F Prime(M13-47)序列, 由宝生物工程(大连)有限公司协助完成. 将测序所得 5 条 cDNA 片段用 NCBI 提供的 BLASTN 与 GeneBank 的 dbEST、nr 数据库进行同源性比较^[8-9], 确认代表新基因的 EST 并且登录 GeneBank(<http://www.ncbi.nlm.nih.gov/submit>). 3 例病理标本取自西南医院肝胆科新鲜手术标本, 病理证实均为原发性肝细胞肝癌, 分别提取肝癌及远端正常肝组织总 RNA, 具体方法同前^[4]. RNA 转膜、固定, 通过对酶切回收的克隆插入片段分别进行同位素标记获得 cDNA 探针(Promega 公司), 利用 Clontech 公司的 ExpressHyb™ 杂交液进行预杂交、杂交, 洗膜、曝光及显影^[10-11]. 用试剂盒中提供的 β-actin

作为对照探针进行 RNA 印记, 操作方法同前. 用 Image J 软件扫描各转录本灰度, 用公式 $\text{Mean A} = \log_{10}(255 / \text{Mean grey})$ 计算强度, 以 Mean A 作为量化指标, 分析各转录本的表达丰度. 利用互联网的基因表达分析序列数据库 (<http://www.ncbi.nlm.nih.gov/SAGE>) (series analysis of gene expression, SAGE) 对特定基因的表达及其表达水平进行分析, 从而确定其组织分布^[12-13].

2 结果

2.1 3' RACE-PCR 产物的序列 设计 3' GSP2 和 3' NGSP2 进行 Race, 共得到 5 条大小不等的片段, 分别命名为 694 447-3, 724 447-3, 697 447-3, 711 447-3, 692 447-3; 大小 500-550 bp (图 1, 2), 对扩增的重组质粒进行酶切鉴定 (图 3). 对这些序列进行分析, 有 2 条是带有 poly(A) 尾的 cDNA 的片段, 1 条片段可见加尾信号 AATAAA, 将这些序列在计算机软件上进行比对 (align) 发现: 除靠近 5' 端的共有序列外, 在 3' 端序列有明显的不同, 而且这些序列不能够互相的囊括, 提示可能是不同基因的 3' 端^[14-15].

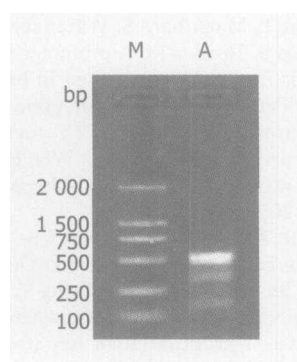


图 1 3' RACE-PCR 产物. M: marker; A: 3' RACE-PCR.



图 2 3' RACE-nest-PCR 产物. M: marker; B: 3' RACE-nest-PCR.



图 3 酶切鉴定重组质粒. M: marker; 1: 692 447-3; 2: 711 447-3; 3: 697 447-3; 4: 724 447-3; 5: 694 447-3; 6: 质粒对照.

2.2 cDNA 片段核苷酸序列的同源性 在 5 个测序的克隆中, 与 GenBank 的 nr 数据库进行同源性的比较显示, 2 条带有 poly(A) 尾的 cDNA 的片段 (登录号为: CK730344, CK730345) 与基因编码序列的同源性均小于 50%, 因而相应的基因及功能均不清楚, 可能代表新的未知基因^[16-20]. 5 条 3' EST 于 2004-2-16 呈递 GeneBank, 现已登录, 登录号: CK730344, CK730345, CK730346, CK730347, CK730348. RNA 印迹 (图 4) 显示 2 条 cDNA (694 447-3 和 724 447-3) 在 3 例肝癌组织中的表达强度高于其对应的正常肝组织.

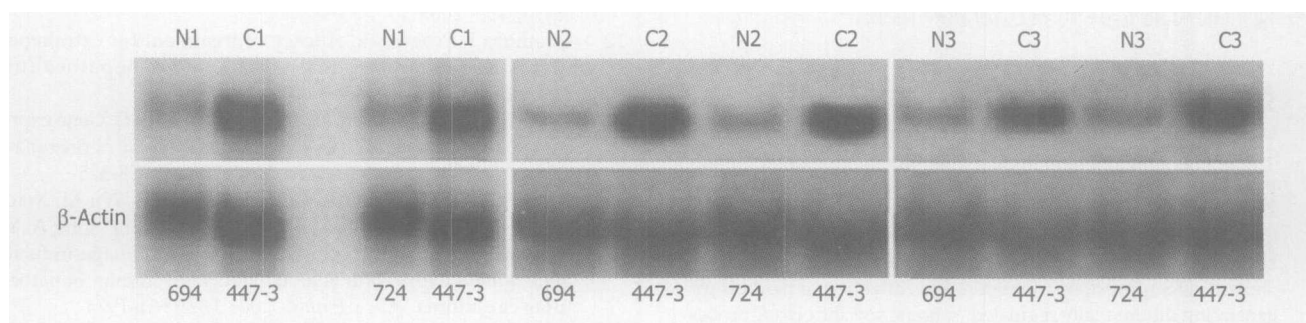


图 4 3 对肝癌及正常对照组织 RNA 印迹结果. N: normal; C: cancer.

2.3 基因电子表达谱分析 694 447-3 和 724 447-3 在肿瘤和正常 Sage 文库中的表达, Sage 文库总 tags 数 (见表 1).

表 1 cDNA 在肿瘤和正常 Sage 文库中的基因电子表达谱

Probe	Sage libraries									
	Brian		Colon		Breast		Lung		stomach	
	N	C	N	C	N	C	N	C	N	C
694 447-3	15	49	11	20	0	17	0	0	0	0
724 447-3	0	11	0	0	0	0	0	0	0	19

N: normal; C: cancer.

3 讨论

原发性肝癌是常见的恶性肿瘤之一,居恶性肿瘤死因的第2位,且有逐年增高趋势^[21-22]。在肝癌的研究中^[23-24],虽然已经肯定了很多在肝癌中异常变化的基因,对肝癌的诊断与治疗有一定的参考价值,但他们最初并不是通过直接研究肝癌所得到的,所以从肝癌组织出发,进行肝组织癌变过程中基因型变化的研究,可以为明确肝癌的发生机制及早期诊断提供进一步的线索。

Race 技术是一种快速、灵敏、短时间内即可以获得新基因 cDNA 全长序列的方法,而且 Race 能够产生大量的独立克隆^[25-26]。我们在进行差异片段的克隆时发现,用基因特异性的引物在肝癌组织中进行 3' Race 的扩增,同时扩增出 5 条高度同源的 ESTs 的片段,他们在 5' 端的一致性很好,蛋白质的编码序列可能有高度的保守性,但是 3' 端差异却很明显,这与多基因家族的特点是吻合的,而且癌基因与抑癌基因多以基因家族的形式出现^[27-30]。通过测序分析,他们有 2 条是带有 poly(A) 尾的 3' EST,经用 NCBI 提供的 BLASTN 对 GeneBank 的 nr 数据库进行电子信息杂交,这是代表新基因的 EST,已经在 GeneBank 上注册。

为了对得到的 2 条候选同源基因与肝癌的关系做进一步的确定,使用 RNA 印记分析 694 447-3, 724 447-3 显示其在 3 例肝癌组织中的表达强度明显高于其对应的正常肝组织;为了更全面的检测克隆所得到的基因在其他肿瘤中的表达丰度及对其功能进行预测,通过 SAGE 文库来分析基因的表达谱,表明 694 447-3 和 724 447-3 在肿瘤文库表达高于正常文库,这些肿瘤文库来源于神经系统肿瘤、结肠癌、胃癌、乳腺癌,结合他们在肝癌中的表达情况,提示在肝癌的发生、发展中他们很可能是共同表达的新基因簇。

4 参考文献

- Li J, Han BL, Huang GJ, Qian GS, Liang P, Yang TH, Chen J. Screening and identification for cDNA of differentially expressed genes in human primary hepatocellular carcinoma. *Zhonghua Yixue Yichuanxue Zazhi* 2003;20:49-52
- Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD. Suppression subtractive hybridization: a method for generating differentially regulated or tissue specific cDNA probes and libraries. *Proc Natl Acad Sci USA* 1996;93:6025-6030
- Yamamoto Y, Sakamoto M, Fujii G, Kanetaka K, Asaka M, Hirohashi S. Cloning and characterization of a novel gene, DRH1, down-regulated in advanced human hepatocellular carcinoma. *Clin Cancer Res* 2001;7: 297-303
- Technical manual & SV total RNA isolation system. Available from: URL: <http://www.promega.com/tbs/tm048/tm048.html>
- Smart™ Race cDNA amplification kit user manual. Available from: URL: <http://www.bdbiosciences.com/clontech/techinfo/manuals/index.shtml>
- 张学敏,王宜强. 靶向新基因的分子克隆策略-理论与方法. 第1版. 北京: 军事医学科学出版社, 1999:113-116
- Forhman MA, Dush MK, Martin GR. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* 1988;85:8998-9002
- Wang ZX, Wang HY, Wu MC. Identification and characterization of a novel human hepatocellular carcinoma-associated gene. *Br J Cancer* 2001;85:1162-1167

- Zeng JZ, Wang HY, Chen ZJ, Ullrich A, Wu MC. Molecular cloning and characterization of a novel gene which is highly expressed in hepatocellular carcinoma. *Oncogene* 2002;21: 4932-4943
- Fu Z, Wang J. The expression of epidermal growth factor receptor mRNA in the laryngeal squamous cell carcinoma with northern blot. *Linchuang Erbiyanhouke Zazhi* 2002;16: 648-649
- Ozturk M. Genetic aspects of hepatocellular carcinogenesis. *Semin Liver Dis* 1999;19:235-242
- Li H, Wang MW, Shao Y, Wang GS, You WD. Cloning and tissue expression analysis of up-regulated cDNA fragment in human gastric cancer. *Zhonghua Yixue Yichuanxue Zazhi* 2003;20:12-14
- Shostak K, Labunskyy V, Dmitrenko V, Malisheva T, Shamayev M, Rozumenko V, Zozulya Y, Zehetner G, Kavsan V. HC gp-39 gene is upregulated in glioblastomas. *Cancer Lett* 2003;198:203-210
- Takeda K, Saito T, Tanaka T, Morio T, Maeda M, Tanaka Y, Ochiai H. A novel gene trap method using terminator-REMI and 3' rapid amplification of cDNA ends (RACE) in Dictyostelium. *Gene* 2003;312:321-333
- Rippe V, Drieschner N, Meiboom M, Escobar HM, Bonk U, Belge G, Bullerdiek J. Identification of a gene rearranged by 2p21 aberrations in thyroid adenomas. *Oncogene* 2003;22: 6111-6114
- Kondoh N, Wakatsuki T, Ryo A, Hada A, Aihara T, Horiuchi S, Goseki N, Matsubara O, Takenaka K, Shichita M, Tanaka K, Shuda M, Yamamoto M. Identification and characterization of genes associated with human hepatocellular carcinogenesis. *Cancer Res* 1999;59:4990-4996
- Tomizawa M, Yu L, Wada A, Tamaoki T, Kadomatsu K, Muramatsu T, Matsubara S, Watanabe K, Ebara M, Saisho H, Sakiyama S, Tagawa M. A promoter region of the midkine gene that is frequently expressed in human hepatocellular carcinoma can activate a suicide gene as effectively as the alpha-fetoprotein promoter. *Br J Cancer* 2003;89:1086-1090
- Qian C, Drozdick M, Caselmann WH, Prieto J. The potential of gene therapy in the treatment of hepatocellular carcinoma. *J Hepatol* 2000;32:344-351
- Srinivas PR, Kramer BS, Srivastava S. Trends in biomarker research for cancer detection. *Lancet Oncol* 2001;2:698-704
- Shao GZ, Zhou RL, Zhang QY, Zhang Y, Liu JJ, Rui JA, Wei X, Ye DX. Molecular cloning and characterization of LPTM4B, a novel gene upregulated in hepatocellular carcinoma. *Oncogene* 2003;22:5060-5069
- 汤钊猷. 肝癌转移复发的基础与临床. 第1版. 上海: 上海科技教育出版社, 2003:1
- Imamura I. Prognostic efficacy of treatment for extrahepatic metastasis after surgical treatment of hepatocellular carcinoma. *Kurume Med J* 2003;50:41-48
- Bonin S, Pascolo L, Croce LS, Stanta G, Tiribelli C. Gene expression of ABC proteins in hepatocellular carcinoma, perineoplastic tissue, and liver diseases. *Mol Med* 2002;8: 318-325
- Qiu W, David D, Zhou B, Chu PG, Zhang B, Wu M, Xiao J, Han T, Zhu Z, Wang T, Liu X, Lopez R, Frankel P, Jong A, Yen Y. Down-regulation of growth arrest DNA damage-inducible gene 45beta expression is associated with human hepatocellular carcinoma. *Am J Pathol* 2003;162:1961-1974
- Wang ZZ, Jiao CZ, Zhang XJ, Xiang JH. Molecular cloning and sequence analysis of full length cDNA encoding molt-inhibiting hormone from *Fennropenaeus chinensis*. *Yichuan Xuebao* 2003;30:128-134
- Song H, Peng Y, Hu R. New full length cDNA cloned from normal pituitary and pituitary tumors. *Zhonghua Yixue Zazhi* 2000;80:890-892
- Hardiman G, Kastelein RA, Bazan JF. Isolation, characterization and chromosomal localization of human WNT10B. *Cytogenet Cell Genet* 1997;77:278-282
- Allen JF. Bioinformatics and discovery: induction beckons again. *Bioessays* 2001;23:104-107
- Koonin EV. Cell cycle and apoptosis: possible roles of Gadd45 and MyD118 proteins inferred from their homology to ribosomal proteins. *J Mol Med* 1997;75:236-238
- Furnes B, Feng J, Sommer SS, Schlenk D. Identification of novel variants of the flavin-containing monooxygenase gene family in African Americans. *Drug Metab Dispos* 2003;31:187-193