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World J Gastroenterol 2006 November 14; 12(42): 6893-6897
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Identification of differentially expressed genes in mouse hepatocarcinoma ascites cell line with low potential of lymphogenous metastasis

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Received: 2006-07-24 Accepted: 2006-09-12

Abstract

AIM: To identify genes differentially expressed in mouse hepatocarcinoma ascites cell line with low potential of lymphogenous metastasis.

METHODS: A subtracted cDNA library of mouse hepatocarcinoma cell line with low potential of lymphogenous metastasis Hca-P and its synogenetic cell line Hca-F with high metastatic potential was constructed by suppression subtracted hybridization (SSH) method. The screened clones of the subtracted library were sequenced and GenBank homology search was performed.

RESULTS: Fifteen differentially expressed cDNA fragments of Hca-P were obtained which revealed 8 known genes, 4 expressed sequence tags (ESTs) and 3 cDNAs showed no homology.

CONCLUSION: Tumor metastasis is an incident involving multiple genes. SSH is a useful technique to detect differentially expressed genes and an effective method to clone novel genes.

Key words: Suppression subtracted hybridization; Liver neoplasm; Metastasis suppression genes

Cui XN, Tang JW, Hou L, Song B, Ban LY. Identification of differentially expressed genes in mouse hepatocarcinoma ascites cell line with low potential of lymphogenous metastasis. *World J Gastroenterol* 2006; 12(42): 6893-6897

http://www.wjgnet.com/1007-9327/12/6893.asp

INTRODUCTION

Tumor metastasis is an incident involving multiple genes. However, the number of metastasis related genes available nowadays is very limited to elucidate the puzzling process of metastasis. Therefore, more attentions have been paid to screen candidate genes responsible for metastasis by high throughput technique. Hca-P and Hca-F are a pair of synogenetic mouse hepatocarcinoma ascites cell lines, possessing a specific potential of lymphogenous metastasis when inoculated subcutaneously into 615 mice, Hca-P showing a low metastatic potential (< 30%), while Hca-F showing a high potential (> 80%)[1]. In the current study, we employed suppressive subtracted hybridization (SSH) technique to identify differentially expressed genes specific for Hca-P in an effort to obtain candidate genes related to lymphogenous metastasis of hepatocarcinoma in mice.

MATERIALS AND METHODS

Hca-F and Hca-P have been established and maintained by our laboratory[1]; inbred 615-mice were provided by the experimental animal center of our university.

Determination of lymph node metastatic rates of Hca-P and Hca-F

Sixty inbred 615-mice were randomly divided into 2 groups. The Hca-P and Hca-F tumor cell lines were inoculated at 2×10^6 tumor cells of approximately 0.1 mL cell suspension into each mouse subcutaneously in each group. The mice were decapitated on the 28th day post-inoculation. The implanted tumor and the regional lymph nodes were removed and paraffin sections of tissues were HE stained and examined under microscope. The lymph node metastatic rates of Hca-F and Hca-P tumor cells were calculated.

Construction of a subtracted cDNA library by SSH

Preparation of total RNA and mRNA: Isolation of total RNA was performed by TRIZOLTM(GIBCOBRL) and that of mRNA was carried out according to the

protocol of oligotex mRNA spin column purification kit (Qiagen). The quantity and integrity of mRNA were detected by ultraviolet spectrometer and by electrophoresis on a denaturing formaldehyde agarose stained by EB. mRNA of Hca-P served as tester and mRNA of Hca-F as driver. SSH was performed between tester and driver by a PCR select^{TM} cDNA subtraction kit and 50 × PCR enzyme kit (Clontech, Heidelberg, Germany) following the instructions of the manufacturer.

dscDNA synthesis and digestion with *Rsa* I : Briefly, 2 μg aliquots of each of poly (A^+) mRNA from the tester and the pooled driver were subjected to dscDNA synthesis. Thereafter, they were purified by passing through Chroma spin-400 columns (Clontech, USA). Each purified dscDNA was digested with *Rsa* I .

ligation to adaptor 1 and 2R: The tester cDNAs were subdivided into 2 equal groups and then ligated to adaptor 1 and 2R in separate ligation reactions. Ligation efficiency analysis was performed by amplifying ligation products with G3PDH 3' primer/PCR primer 1 and G3PDH 3' primer/G3PDH 5' primer, respectively, and their intensity was compared.

Subtractive hybridization: Subtractive hybridization was performed by annealing an excess of driver cDNAs with each sample of adaptor-ligated tester cDNAs. The cDNAs were heat-denatured and incubated at 68℃ for 8 h. After the first hybridization, the 2 samples were mixed together and hybridized again with freshly heat-denatured driver cDNAs for 20 h at 68℃. Two rounds of hybridization would generate a normalized population of tester-specific cDNAs with different adaptors at each end. After filling in the ends, 2 rounds of PCR amplification were performed to enrich desired cDNAs containing both adaptors by exponential amplification of these products[2]. The optimized cycles for the first and second PCRs were 27 and 13 respectively to increase representation and reduce redundancy of subtracted cDNA libraries.

Analysis of subtractive efficiency: Secondary PCR products were used as templates for PCR amplification of human G3PDH for 18, 23, 28 and 33 cycles respectively to assure subtraction efficiency. PCR products were run on 1.8% agarose gel.

Ligation of the subtracted library into a TA vector

Products of the secondary PCR reactions were cloned into a pT Adv vector (Clontech) and the resultant ligation products were then transformed into DH5α *E. coli* competent cells. The bacteria were subsequently grown in 800 μL of liquid Luria-Bertani medium and allowed to incubate for 45 min at 37℃ with shaking at 150 rpm. Thereafter, the cells were plated onto agar plates containing ampicillin (50 μg/mL), 5-bromo-4-chloro-3-indoly-b-D-galactoside (X-gal; 20 μg/cm^2) and iso-ploprl-b-D-thiogalactoside (IPTG; 12.1 μg/cm^2) and incubated overnight at 37℃. Individual recombinant white clones were picked and grown in single line pattern onto Luria-Bertani agar solid medium containing ampicillin and allowed to incubate at 37℃ for 6-7 h before single clone was picked from single-line pattern agar medium and allowed to grow in Luria-Bertani liquid medium containing ampicillin overnight at 37℃ with shaking at 150 r/min.

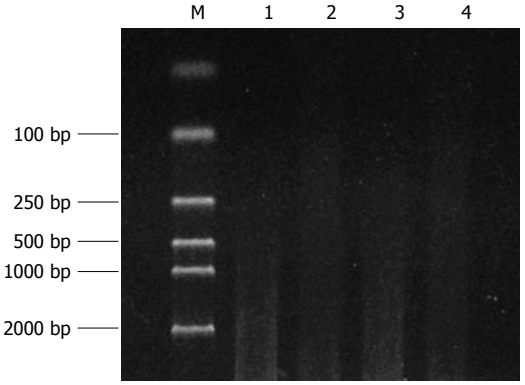

Figure 1 The effect of *Rsa* I digestion. Lane 1, 3: cDNA of Hca-F and Hca-P cells; Lane 2, 4: cDNA of Hca-F and Hca-P cells after *Rsa* I digestion; M: DNA marker DL2000.

Identification of the subtracted clones

Plasmids of candidate positive clones from subtracted cDNA library were isolated and amplified by PCR with nested primer 1 and primer 2. Meanwhile the product of PCR was detected by agarose gel electrophoresis.

Sequencing and BLAST homology search

Randomly screened 14 positive clones from the subtracted cDNA library were sequenced by T7/SP6 chain termination reaction in TaKaRa (DaLian, China). Nucleic acid homology searches were subsequently performed at the National Center of Biotechnology Information (National Institutes of Health, Bethesda, Md., NCBI).

RESULTS

Determination of lymph node metastatic rates of Hca-P and Hca-F

Implanted tumors of both Hca-P tumor-bearing mice and Hca-F tumor-bearing mice were palpable on 7th day post-inoculation. On the 28th day post-inoculation, 10% Hca-P cells bearing mice developed metastatic regional lymph nodes (3/30), while 80% Hca-F cells bearing mice developed metastatic regional lymph nodes (24/30).

Total RNA and mRNA analysis

The RNA samples electrophoresed on 1% agarose/EB gel exhibited 2 typical bands, corresponding to ribosomal 28s and 18s RNA, respectively, with a ratio of intensities > 2:1 and 1.9, ideal A260/A28 ratios of both samples obtained, indicating high integrity and purification of the total RNA we obtained. mRNA samples appeared as a smear with weak ribosomal RNA band: a high-quality mRNA was purified.

Rsa I *digestion*

Both the digested cDNA and undigested cDNA usually presented as smears. However, their patterns were different. The digested cDNA fragments became shorter after Rsa I digestion (Figure 1).

Ligation efficiency analysis

Intensity of the PCR product amplified using one gene-

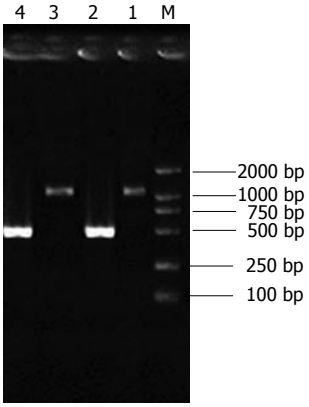

Figure 2 Ligation efficiency analysis. Lane 1: Tester-1 as template, G3PDH 3'Primer and PCR Primer1; Lane 2: Tester-1 as template, G3PDH 3'Primer and G3PDH 5'Primer; Lane 3: Tester-2R as template, G3PDH 3'Primer and PCR Primer1; Lane 4: Tester-2R as template, G3PDH 3'Primer and G3PDH 5'Primer.

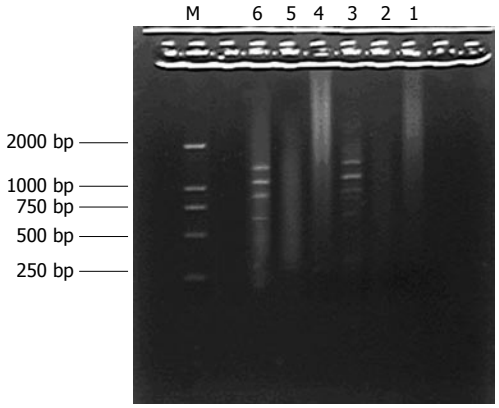

Figure 3 The results of secondary PCR amplification. Lane 1-3: Product of primary PCR amplification, Lane 4: secondary PCR amplification product of unsubtracted cDNA, Lane 5: secondary PCR amplification product of subtracted cDNA, Lane 6: secondary PCR amplification product of PCR control cDNA, M: DNA Marker DL2000.

specific primer (G3PDH 3' primer) and PCR primer 1 was 25% more than that of PCR product amplified using two gene-specific primers (G3PDH 3' primer and 5' primer). Ligation efficiency was > 25%, ensuring enough tester cDNA in the following hybridization (Figure 2).

Construction of subtracted cDNA library by SSH

PCR products of the subtracted and unsubtracted usually looked like smears with or without discrete bands. However, the patterns between them were different (Figure 3).

Analysis of subtractive efficiency

Subtraction efficiency analysis showed the effectively reduced amount of non-differentially expressed genes. In unsubtracted cDNA libraries, housekeeping gene G3PDH PCR products were visible after 23 cycles of amplification and became saturated after 23-28 cycles. However, subtracted libraries required 33 cycles for G3PDH to be detected (Figure 4).

Differential screening of subtracted cDNA libraries

The subtracted cDNA libraries were composed of 995 positive clones, of which 200 clones were randomly picked up and plasmids of the candidate positive clones were isolated and amplified by PCR with nested primer 1 and

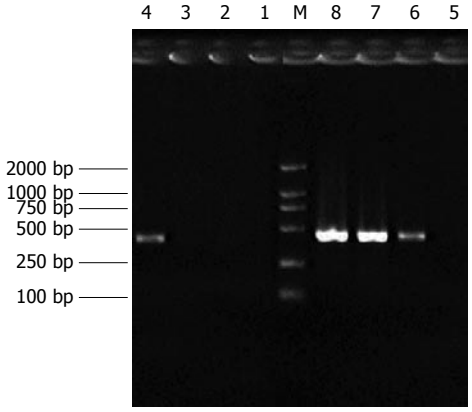

Figure 4 Analysis of subtraction effect. PCR was performed on subtracted (Lane 1-4) or unsubtracted (Lane 5-8) secondary PCR product with G3PDH 5'Primer and 3'primer. Lanes 1, 5: 20 cycles, Lanes 2, 6: 25 cycles, Lanes 3, 7: 30 cycles, Lanes 4, 8: 35 cycles. M: DNA marker DL2000.

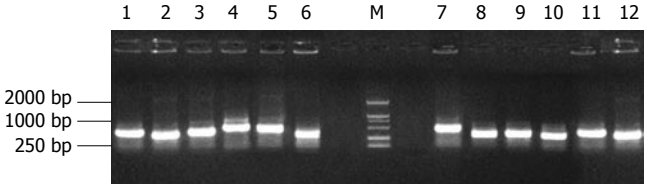

Figure 5 The results of clone PCR amplification. There was an average insert size of 300-1000 bp. M: DNA marker DL2000.

primer 2. As a result, 189 positive clones showed PCR products of a size of 300-1000 bp (Figure 5).

Sequencing and homology search

Fifteen screened clones randomly selected were sequenced and homology search (http://www.ncbi.hlm.nih.gov/BLAST/) revealed 8 known genes and 4 expressed sequence tags (ESTs). Three cDNAs showed no homology and presumably represented novel genes (Table 1).

DISCUSSION

Tumor metastasis, as the leading cause of tumor related death, is a process involving multiple genes and their products. Elucidation of the gene expression profiles specific for tumor cells with different potential of metastasis might help in the understanding of the molecular mechanisms of metastasis. As one of the high throughput screening techniques, SSH technique has two distinct advantages: (1) it boasts a high subtraction efficiency; (2) it harbors an equalized representation of differentially expressed sequences which can separate effectively both high and low copy expressed genes mainly because of normalization[2]. von Stein *et al*[3] found about 94% positive rate in their research. Thus they considered confirmation of differentially expressed genes by Northern blot analysis for each clone obtained was probably unnecessary.

For a long time, studies have focused on the angiogenesis of tumors, but the roles of lymphatic vessels

Table 1 Homologue searching of the sequenced cDNA fragments from SSH library

Clone seriel number	Size (bp)	Sequence identity
1-3	508	Mus musculus Telomere repeat binding factors TRF1
3-5	543	Mus musculus Telomere repeat binding factors TRF2,
9-6	489	Mus musculus maspin
15-4	335	Mouse chromosome 3 clone RP 6-126M1
11-7	386	Mouse 7 d embryo whole body cDNA RIKEN full-length enrich library, clone 2210102k3
16-3	503	Mouse 5 d liver cells cDNA RIKENfull -length library enriched library, clone E330462F4
10-1	549	Mouse chromosome 17 clone RP 26-122M3
13-7	502	Mouse chromosome 4 clone RP13-110N10
9-8	340	EST-mouse
6-6	411	EST-mouse
11-2	390	EST-mouse
20-9	399	EST-mouse
23-3	470	Unknown
17-6	486	Unknown
12-3	344	Unknown

in tumor growth and metastasis were neglected. However, it is well known that lymphatic metastasis is mainly responsible for the spread of epithelial malignant tumors, and is closely related to the prognosis of patients.

Hca-P and Hca-F are a pair of synogenetic mouse hepatocarcinoma ascites cell lines presenting a specific potential of lymphogenous metastasis with a significant difference in their potential of metastasis[1]. Candidate genes involved in lymphogenous metastasis are supposed to be among the differentially expressed genes.

Using Hca-P as a tester, Hca-F as a driver, and we employed SSH technique to identify differentially expressed genes specific for Hca-P(low metastatic potential) so as to obtain candidate suppressor genes of lymphogenous metastasis. Fifteen screened clones randomly selected were sequenced and homology search revealed 8 known genes as TRF_1, TRF_2(telomere repeat binding factor 1, 2) ; maspin; mouse 7 days embryo whole body cDNA, RIKEN full-length enriched library clone 2210102k3; mouse 5 days liver cells cDNA, RIKEN full-length enriched library, clone E330462F4; mouse chromosome 3 clone RP 6-126M1;mouse chromosome 17 clone RP 26-122M3 and mouse chromosome 4 clone RP13-110N10. Studies showed TRF_1, and TRF_2 play important roles in genome stabilization [4-11] and are down-regulated in some malignant cell lines and tumor tissues[12-18]. In hematopoietic carcinogenesis, gene expression of telomerase suppressors such as TRF and TIN2, is decreased. mRNA encoding TRF_1 and TRF_2 when gastric cancer becomes more deeply invaded**,** is significantly decreased, indicating a negative association with tumor progression. Of the 8 known fragments, one showed high homology to mouse Maspin gene. Serving as one of the few p53-targeted genes involved in tumor invasion and metastasis, Maspin, a member of the serpin family, has been reported to suppress metastasis and angiogenesis in breast and prostate cancers, and is closely correlated with their clinical manifestations[19-31]. It indicates that SSH in our study is capable of enriching metastasis related genes.

Another 5 known fragments were attributed to embryo genes. Embryo genes AFP and CEA are overexpressed in hepatocarcinoma and other malignant tumors, indicating a possible association between embryo development and tumor. Embryo genes were also found in our previously established SSH library which contains candidate tumor metastasis genes[32]. These data showed that up-regulated expression of embryo genes during metastasis is not a casual event. Their roles in tumor metastasis need to be clarified. Moreover, 4 cDNA fragments demonstrated homology with 4 ESTs-mouse and 3 cDNA fragments showed no homology and presumably represented novel genes[33].

In summary, the findings of our study suggest that the lymphatic invasiveness of tumor cells is determined by multiple genes and co-factors with complex cellular signal pathways. Further functional study of the candidate novel genes might provide clues to molecular mechanism of tumor metastasis.

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