

• GASTRIC CANCER •

Applying a highly specific and reproducible cDNA RDA method to clone garlic up-regulated genes in human gastric cancer cells

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Abstract

AIM: To develop and optimize cDNA representational difference analysis (cDNA RDA) method and to identify and clone garlic up-regulated genes in human gastric cancer (HGC) cells.

METHODS: We performed cDNA RDA method by using abundant double-stranded cDNA messages provided by two self-constructed cDNA libraries (Allitridi-treated and paternal HGC cell line BGC823 cells cDNA libraries respectively). *Bam*H I and *Xho* I restriction sites harbored in the library vector were used to select representations. Northern and Slot blots analyses were employed to identify the obtained difference products.

RESULTS: Fragments released from the cDNA library vector after restriction endonuclease digestion acted as good marker indicating the appropriate digestion degree for library DNA. Two novel expressed sequence tags (ESTs) and a recombinant gene were obtained. Slot blots result showed a 8-fold increase of glia-derived nexin/protease nexin 1 (GDN/PN1) gene expression level and 4-fold increase of hepatitis B virus x-interacting protein (XIP) mRNA level in BGC823 cells after Allitridi treatment for 72h.

CONCLUSION: Elevated levels of GDN/PN1 and XIP mRNAs induced by Allitridi provide valuable molecular evidence for elucidating the garlic's efficacies against neurodegenerative and inflammatory diseases. Isolation of a recombinant gene and two novel ESTs further show cDNA RDA based on cDNA libraries to be a powerful method with high specificity and reproducibility in cloning differentially expressed genes.

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INTRODUCTION

cDNA representational difference analysis (cDNA RDA), with high specificity by eliminating those fragments present in two compared populations and leaving only the differences, has been employed with some degree of success^[1-6]. While cDNA RDA, like other PCR-

based difference screening methods, is prone to produce false positive results^[7,8]. In our study, we used cDNA RDA method to isolate garlic inducible differentially expressed genes in human gastric cancer (HGC) cells. Allitridi is a critical constituent of garlic oil, mainly containing diallyl trisulfide (DATS) and diallyl disulfide (DADS), which is widely used in cancer chemoprevention and anti-cardiovascular disease research^[9-16]. Differences between two double-stranded cDNAs populations derived from Allitridi-treated and paternal HGC cell line BGC823 cells cDNA libraries were identified by using cDNA RDA. *Bam*H I and *Xho* I restriction sites harbored in the library vector were employed to select representations. We found another major source of false positives in cDNA RDA, which was inappropriate enzyme digestion of sample DNAs, and introduced improvements to minimize their production.

MATERIALS AND METHODS

Allitridi-treated and paternal HGC cell line BGC823 cells cDNA libraries

Allitridi is a critical constituent of garlic oil, containing 97.98% diallyl trisulfide (DATS) and 0.85% diallyl disulfide (DADS) in concentration. BGC823 cells were incubated in medium containing 25mg·L⁻¹ Allitridi for 72h. Total RNA isolated from paternal and Allitridi-treated BGC823 cells were extracted, followed by synthesis of double stranded cDNAs using λZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene). cDNAs derived from Allitridi-treated and paternal BGC823 cells, with *Eco* I cutting site at 5'-end and *Xho* I site at 3'-end, were unidirectionally cloned into λZAP II vector for cDNA library construction.

cDNA RDA based on cDNA libraries: generation of representations

Difference between Allitridi-treated BGC823 (Alli823) cDNA library DNA (used as tester) and paternal cell (BGC823) library DNA (used as driver) was identified by using cDNA RDA to isolate Allitridi up-regulated genes. Library DNAs of the two cDNA libraries were prepared and digested with *Bam*H I respectively in a mixture containing 40μg library DNAs, 8μL *Bam*H I (10kU·L⁻¹, Promega), and 10μL 10×Buffer H in a final volume of 100μL. The digestions were carried out at 37°C for 2, 4, and 12h respectively to obtain the most appropriately digested fragments. After phenol extraction and ethanol precipitation, digested DNAs (about 24μg) were then ligated to 18μL R-Bam-24 (10μmol·L⁻¹) and 18μL R-Bam-12 (10μmol·L⁻¹) adapters. PCR reactions were set up to generate the initial representations by using R-Bam-24 as primer.

In order to expand the content of messages in the representations, we further employed *Bam*H I and *Xho* I together to digest the library DNAs. Library DNAs (about 40μg) of Alli823 and BGC823 cDNA libraries were digested with *Xho* I respectively. Digested DNAs were then ligated to *Xho* I Linker-15 (5'-TCGAGGATCCATTCA-3') and *Xho* I Linker-13 (5'-ACTGAATGGATCC-3'). Resulting ligations were digested with *Bam*H I and then ligated to R-Bam-12/24 adapters (10μmol·L⁻¹),

followed by PCR amplification to generate amplicons.

Using *Bam*H I alone or *Bam*H I and *Xho* I together to digest library DNAs and to prepare representations is showed schematically in Figure 1.

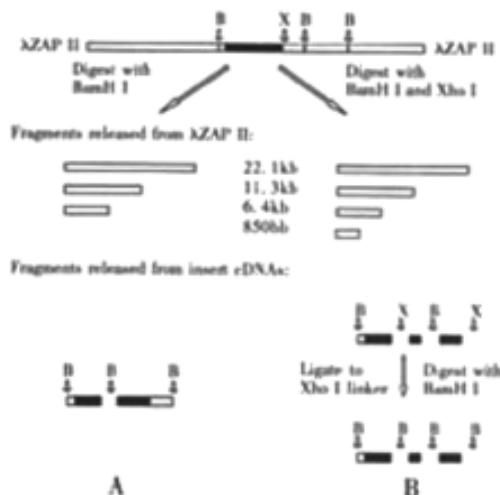


Figure 1 Schematic diagram of preparing representations for cDNA RDA based on cDNA libraries. Using *Bam*H I alone (A) or *Bam*H I and *Xho* I together (B) to digest library DNAs. Black boxes represent insert cDNAs of cDNA libraries. White boxes represent two arms of cDNA library vector (λ ZAP II vector). "B" represents *Bam*H I and "X" *Xho* I.

The followed three rounds of hybridizations and selective amplifications were performed according to the protocol supplied by typical cDNA RDA. Sequences of adapters used here are as follows: R-Bam-24 5'-AGCACTCTCCAGCCTCTCACCGAG-3'; R-Bam-12 5'-GATCCTCGGTGA-3'; J-Bam-24 5'-ACCGACGTCGACTATCC ATGAACG-3'; J-Bam-12 5'-GATCCGTTTCATG-3'; N-Bam-24 5'-AGGCAACTGTGCTATCCGAGGGAG-3'; N-Bam-12 5'-GATCCTCCCTCG-3'.

Slot and Northern blots analyses

Reamplified difference products (100ng each) were mixed with 2.5 μ L 3mmol·L⁻¹ NaOH respectively and incubated for 1h at 65°C, and then transferred onto Nitrocellulose filters (S&S Com) by using Slot Minifold® II (Schleicher&Schull). Two same filters were prepared and then cross-linked using a UV Stratalinker. Reverse transcription products (the first stranded cDNAs) of 5 μ g total RNA of Allitridi-treated and paternal BGC823 cells were used as probes respectively. Probes were labeled with ³²P using a Random-Primer labeling kit (Promega) and hybridization carried out in 1mmol·L⁻¹ EDTA, 0.25mmol·L⁻¹ Na₂HPO₄ and 70g·L⁻¹ SDS solution for 16h at 60°C. Following hybridization, the filters were washed twice in 1mmol·L⁻¹ EDTA, 40mmol·L⁻¹ Na₂HPO₄ and 50g·L⁻¹ SDS for 25min at 60°C and twice in 1mmol·L⁻¹ EDTA, 40mmol·L⁻¹ Na₂HPO₄ and 10g·L⁻¹ SDS for 25min at 60°C again. Filters were exposed to a phosphor screen for 48h and analyzed.

Total RNA (10 μ g each) isolated from HGC cell lines BGC823, MGC803, PAMC82, SGC7901 and MKN45 were transferred onto Nitrocellulose filters. Probe (the third difference product, DP3) was labeled by random primer extension and Northern blots hybridization carried out in the same manner described above.

Sequencing and Database Searching

Difference products were amplified and cloned into pGEM-T Easy Vector (Promega). Double stranded plasmid DNAs were prepared using miniprep columns (Promega) and sequenced with Ultra VGI 1280 (applying User Manual version 3.0). Resulting sequences were compared to the GenBank database by using the BLAST program.

RESULTS

Generation of representations for cDNA RDA based on cDNA libraries

Double stranded cDNAs, with *Eco* I cutting site at 5'-end and *Xho* I site at 3'-end, were unidirectionally cloned into λ ZAP II vector for cDNA library construction. Library vector (λ ZAP II vector) has three *Bam*H I restriction sites with one site located very near to 5'-end of insert cDNAs. Thus *Bam*H I adapters can be introduced into the 5'-end of insert cDNAs. With the same copies three distinct fragments appeared in the products of library DNA digested with *Bam*H I at 37°C for 4h (about 22.1, 11.3 and 6.4kb in size respectively), which indicated appropriate digestion degree. However, library DNAs were not fully digested at 37°C for 2h, and the smears of library DNAs after digestion for 12h showed excessive reactions. When *Bam*H I and *Xho* I were used together to digest library DNAs (1 cut/2 kb insert cDNAs in average), another vector-derived fragment (850 bp in size) was released from 3'-end of insert cDNAs. We further designed *Xho* I Linker-15/13 to convert *Xho* I cutting site to *Bam*H I site. Thus the content of cDNA messages available for difference analysis was expanded. The results for library DNAs after digestion with different enzymes or different conditions are showed in Figure 2. Subsequently, PCR reactions were set up to generate the initial representations (amplicons) (Figure 3).

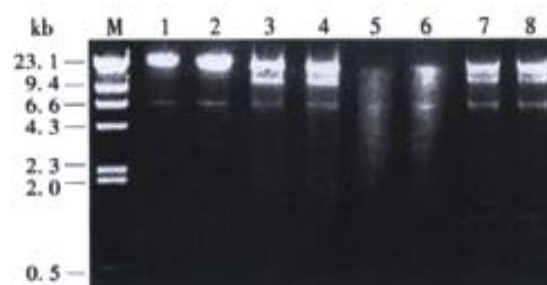


Figure 2 Agarose gel electrophoresis of digestion products of Alli823 (lanes 1,3,5) and BGC823 (lanes 2,4,6) cDNA library DNAs digested with *Bam*H I at 37°C for 2 (lanes 1,2), 4 (lanes 3,4), and 12h (lanes 5,6) respectively. The digestion products of Alli823 (lane 7) and BGC823 (lane 8) library DNAs digested with *Bam*H I and *Xho* I together (A fragment, 850 bp in size, appeared in the digestion products). ePhage/*Hind* III size marker (lane M).

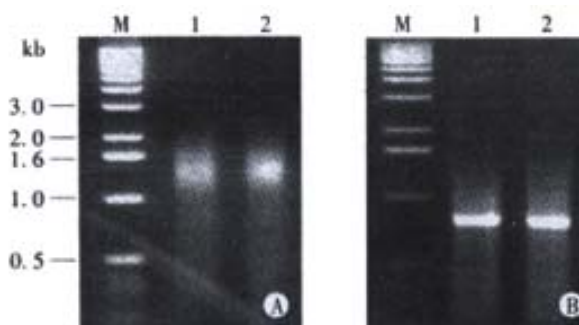


Figure 3 Agarose gel electrophoresis of amplicons derived from different enzyme digestions. A: The amplicons obtained by using *Bam*H I to digest Alli823 (lane 1) and BGC823 (lane 2) cDNA library DNAs respectively; B: The amplicons obtained by using *Bam*H I and *Xho* I together to digest Alli823 (lane 1) and BGC823 (lane 2) library DNAs respectively. 1kb size marker (lane M).

Identification of differentially expressed genes in Allitridi-treated BGC823 cells

Results of Slot blots analysis showed that expression of 4 cDNAs was up-regulated by Allitridi treatment and the degree to which each cDNA was up-regulated ranged from 3- to 8-fold (Figure 4). Sequencing and GenBank database searching results showed that two isolated

difference fragments (SH2 and SH3) had remarkable homology over 97% with glia-derived nexin/protease nexin 1 (GDN/PN1) mRNA and hepatitis B virus x-interacting protein (XIP) mRNA respectively. No homology was found in fragment SH1, which indicated SH1 to

be a novel EST. SH4 showed remarkable homology as high as 98% with a piece of human DNA sequence on chromosome 20 and was also showed to be a novel EST. The sequences of SH1 and SH4 were showed in Figure 5.

SH1 (434bp)

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TAGGCAACTGTGCTATCCGAGGGAGCAACGCCCTTCGTGCGCCACATGACGCATACTACCTGCCACATCTCAC
GCTCGTCCACCGTCGGCGCGCACACACTTGACAGCCCTTGCGGTACATCGCCGTACGACGAGCGGCATCATG
TTTTCCAGCACACCGCGCGCTGACCCGCCCTACCGCCGTGTCGATACCGGCAAGGTGGAGCCATCGCCCTT
GCCCCAGATCAAACGTTTCGGTGCCAGTGGGCATCAGCTTGCGGTGCTTGACCACCAGGCCGCCAGACGGTTTCG
AAATACAGCACGGTGCAATACAGGGTGCTGCCACTGCGCTCGATCACACCCAGGACCAGGCTCGCGCCGGTTTCG
GGCCGACAACCCCGCCAGCGCCTCGGTCTTGGTGCCCGCCTTCCTTGGATAGCACAGTTGCCTA
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SH4 (400bp)

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TAGGCAACTGTGCTATCCGAGGGAGATCCTTTTGCCTTAATCTCAGTGCTCGTTACTTTCATGGTCCCAAGATGGCT
GCTGTATCCCAAGAATCATGTCTGCGTTCAAGGAAGGAGGGGTGGAGGAAGAGGAAGGGCCAAACTAGCTGG
ACCCGTACCTTCTATCAGAAAGTAGAACCTCGTCAGAAAGTCTGTTTCTGCTCTCTCCCTCTGCATATCTTCACT
TAGATGCCCTTGGCCCCAGCCAGCTACCATTCACCTCTAGCTGCAAACAAAGCTAAGACAGCAGGGAACAGGA
TTGTCATGGCTGAATAGACCAATCGTGTTCATCTACTGAGACTGGCACACTGCCTCTGCAATAAACTGGGAT
CTCCCTCGGATAGCACAGTTGCCTA
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Figure 5 Sequences of two novel ESTs (SH1 and SH4) isolated by cDNA RDA based on cDNA libraries.

Northern blots analysis was employed to detect the expression level of another obtained difference product (derived from calyculin mRNA) and a novel transcript about 2 kb in size appeared only in the hybridization result of BGC823 total RNA (Figure 6). Further cDNA library screening and database searching showed this novel transcript to be a recombinant gene merged from calyculin gene and homo sapiens heterogeneous nuclear ribonucleoprotein A0 (HNRPA0) gene.

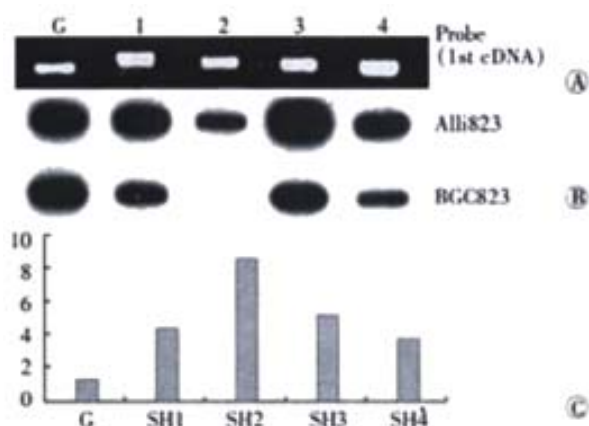


Figure 4 A: Agarose gel electrophoresis of reamplified difference products SH1-4 (lanes 1-4 respectively). PCR product of GAPDH (400bp in size) used as quantity control (lane G). B: Slot blots analysis showing differentially expressed cDNAs. Reverse transcription products (first stranded cDNA) of total RNA of BGC823 and Alli823 cells used as probe respectively. C: The degree to which each cDNA was up-regulated.

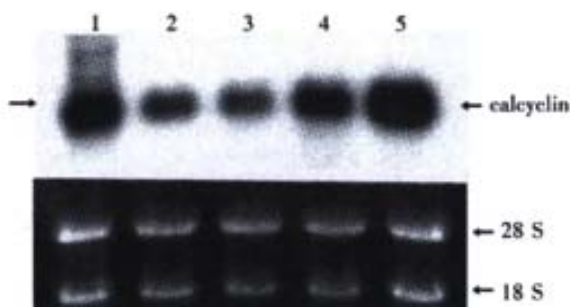


Figure 6 Northern blotting result showing the expression level of calyculin gene in human gastric cancer cell lines BGC823, MGC803, PAMC82, SGC7901 and MKN45 (lanes 1-5) respectively. The arrow shows the novel transcript derived from the recombinant gene merged from calyculin gene and HNRPA0 gene.

DISCUSSION

When cDNA RDA^[1-6] or RDA^[17-19] is performed, sample cDNAs or genome DNAs are digested into fragmented populations to prepare representations, and the digestion products showed as smears by agarose gel electrophoresis. Therefore, it is difficult to determine the most appropriate digestion degree. However, deficient or excessive enzyme digestion of sample DNAs can undoubtedly result in false positive results. In our study, cDNA RDA was performed based on cDNA libraries. Our data showed that time for enzyme digestion affected the digestion degree obviously. With the same copies, three or four fragments released from library vector after enzyme digestion acted as good marks indicating appropriate digestion, which increased the specificity and reproducibility of cDNA RDA. Moreover, when *Bam*H I and *Xho* I were used together to digest library DNAs, the content of cDNA messages available for difference analysis was expanded and more specific products were obtained.

Thrombin, as the principal component of the blood coagulation cascade, also prevents neurite outgrowth and modulates morphologic changes in both neurons and astrocytes^[20-22]. Recent studies have showed that thrombin mediates polynuclear synapse elimination, both *in vivo* and *in vitro*^[23]. Glia-derived nexin/protease nexin 1 (GDN/PN1) is the most potent vertebrate inhibitor for thrombin, which, acting as neurite-promoting factor, plays an important role in neurotrophic and neuroprotective properties^[24-29]. Garlic extract contains antioxidant phytochemicals that prevent the oxidant-mediated brain cell damage and produce neurotrophic effects^[30-33]. Our results showed the potential effect of garlic on reducing the risk of neurodegenerative disease through up-regulation of GDN/PN1 expression.

The hepatitis B virus (HBV) has been reported to be a risk factor in the development of hepatocellular carcinoma^[34-42]. The HBV X protein (HBx) is a small transcriptional activator and has been showed capable of transactivating many different viral and cellular promoters. It is essential for virus infection and is implicated in the development of hepatocellular carcinoma^[43-49]. An HBx-interacting protein that specifically binds to the carboxy terminus of wild-type HBx was identified in 1998 and designated as HBx interacting-protein (XIP), which could inhibit HBx activity and thus decrease HBV replication^[50]. Elevated level of XIP mRNA induced by garlic extract provided valuable molecular evidence for elucidating the garlic's efficacies against inflammatory diseases.

Identification of recombinant gene in our study showed the feasibility of employing cDNA RDA method to the discovery of probes for anonymous loci that suffered genetic rearrangements. Cloning of two novel ESTs further showed cDNA RDA based on cDNA libraries to be a powerful method in isolating novel genes.

REFERENCES

- 1 Yoon DY, Buchler P, Saarikoski ST, Hines OJ, Reber HA, Hankinson O. Identification of genes differentially induced by hypoxia in pancreatic cancer cells. *Biochem Biophys Res Commun* 2001;288:882-886
- 2 Graveel CR, Jatko E, Madore SJ, Holt AL, Farnham PJ. Expression profiling and identification of novel genes in hepatocellular carcinomas. *Oncogene* 2001;20:2704-2712
- 3 Seidita G, Polizzi D, Costanzo G, Costa S, Di Leonardo A. Differential gene expression in P53-mediated G(1) arrest of human fibroblasts after gamma-irradiation or N-phosphoacetyl-L-aspartate treatment. *Carcinogenesis* 2000;21:2203-2210
- 4 Xu W, Wang S, Wang G, Wei H, He F, Yang X. Identification and characterization of differentially expressed genes in the early response phase during liver regeneration. *Biochem Biophys Res Commun* 2000;278:318-325
- 5 Davenport J, Neale GA, Goorha R. Identification of genes potentially involved in LMO2-induced leukemogenesis. *Leukemia* 2000;14:1986-1996
- 6 Yamashita Y, Yokoyama M, Kobayashi E, Takai S, Hino O. Mapping and determination of the cDNA sequence of the Erc gene preferentially expressed in renal cell carcinoma in the Tsc2 gene mutant (Eker) rat model. *Biochem Biophys Res Commun* 2000;275:134-140
- 7 Kim S, Zeller K, Dang CV, Sandgren EP, Lee LA. A strategy to identify differentially expressed genes using representational difference analysis and cDNA arrays. *Anal Biochem* 2001;288:141-148
- 8 Bole-Feysot C, Perret E, Roustan P, Bouchard B, Kelly PA. Analysis of prolactin-modulated gene expression profiles during the Nb2 cell cycle using differential screening techniques. *Genome Biol* 2000;1:RESEARCH0008
- 9 Nakagawa H, Tsuta K, Kiuchi K, Senzaki H, Tanaka K, Hioki K, Tsubura A. Growth inhibitory effects of diallyl disulfide on human breast cancer cell lines. *Carcinogenesis* 2001;22:891-897
- 10 Wu CC, Sheen LY, Chen HW, Tsai SJ, Lii CK. Effects of organosulfur compounds from garlic oil on the antioxidant system in rat liver and red blood cells. *Food Chem Toxicol* 2001;39:563-569
- 11 Gupta N, Porter TD. Garlic and garlic-derived compounds inhibit human squalene monooxygenase. *J Nutr* 2001;131:1662-1667
- 12 Hong YS, Ham YA, Choi JH, Kim J. Effects of allyl sulfur compounds and garlic extract on the expression of Bcl-2, Bax, and P53 in non small cell lung cancer cell lines. *Exp Mol Med* 2000;32:127-134
- 13 Cho BH, Xu S. Effects of allyl mercaptan and various allium-derived compounds on cholesterol synthesis and secretion in Hep-G2 cells. *Comp Biochem Physiol C Toxicol Pharmacol* 2000;126:195-201
- 14 Munday R, Munday CM. Low doses of diallyl disulfide, a compound derived from garlic, increase tissue activities of quinone reductase and glutathione transferase in the gastrointestinal tract of the rat. *Nutr Cancer* 1999;34:42-48
- 15 Li XG, Xie JY, Lu YY. Suppressive action of garlic oil on growth and differentiation of human gastric cancer cell line BGC-823. *Huaren Xiaohua Zazhi* 1998;6:10-12
- 16 Singh SV, Pan SS, Srivastava SK, Xia H, Hu X, Zaren HA, Orchard JL. Differential induction of NAD(P)H:quinone oxidoreductase by anti-carcinogenic organosulfides from garlic. *Biochem Biophys Res Commun* 1998;244:917-920
- 17 Toder R, Grutzner F, Haaf T, Bausch E. Species-specific evolution of repeated DNA sequences in great apes. *Chromosome Res* 2001;9:431-435
- 18 Cummings M, Brown KW. Low frequency of genetic lesions in Wilms tumors by representational difference analysis. *Cancer Genet Cytogenet* 2001;127:155-160
- 19 Endoh D, Cho KO, Tsukamoto K, Morimura T, Kon Y, Hayashi M. Application of representational difference analysis to genomic fragments of Marek's disease virus. *J Clin Microbiol* 2000;38:4310-4314
- 20 Smirnova IV, Citron BA, Arnold PM, Festoff BW. Neuroprotective signal transduction in model motor neurons exposed to thrombin: G-protein modulation effects on neurite outgrowth, Ca(2+) mobilization, and apoptosis. *J Neurobiol* 2001;48:87-100
- 21 Fritsche J, Reber BF, Schindelhof B, Bandtlow CE. Differential cytoskeletal changes during growth cone collapse in response to hSema III and thrombin. *Mol Cell Neurosci* 1999;14:398-418
- 22 Turgeon VL, Houenou LJ. Prevention of thrombin-induced motoneuron degeneration with different neurotrophic factors in highly enriched cultures. *J Neurobiol* 1999;38:571-580
- 23 Kim S, Nelson PG. Transcriptional regulation of the prothrombin gene in muscle. *J Biol Chem* 1998;273:11923-11929
- 24 Hengst U, Albrecht H, Hess D, Monard D. The phosphatidylethanolamine-binding protein is the prototype of a novel family of serine protease inhibitors. *J Biol Chem* 2001;276:535-540
- 25 Docagne F, Nicole O, Marti HH, MacKenzie ET, Buisson A, Vivien D. Transforming growth factor-beta1 as a regulator of the serpins/t-PA axis in cerebral ischemia. *FASEB J* 1999;13:1315-1324
- 26 Mbebi C, Hantai D, Jandrot-Perrus M, Doyennette MA, Verdiere-Sahuque M. Protease nexin I expression is up-regulated in human skeletal muscle by injury-related factors. *J Cell Physiol* 1999;179:305-314
- 27 Kariko K, Harris VA, Rangel Y, Duvall ME, Welsh FA. Effect of cortical spreading depression on the levels of mRNA coding for putative neuroprotective proteins in rat brain. *J Cereb Blood Flow Metab* 1998;18:1308-1315
- 28 Kim S, Buonanno A, Nelson PG. Regulation of prothrombin, thrombin receptor, and protease nexin-1 expression during development and after denervation in muscle. *J Neurosci Res* 1998;53:304-311
- 29 Lee P, Spector JG, Derby A, Roufa DG. Effects of thrombin and protease nexin-1 on peripheral nerve regeneration. *Ann Otol Rhinol Laryngol* 1998;107:61-69
- 30 Youdim KA, Joseph JA. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. *Free Radic Biol Med* 2001;30:583-594
- 31 Sumi S, Tsuneyoshi T, Matsuo H, Yoshimatsu T. Isolation and characterization of the genes up-regulated in isolated neurons by aged garlic extract (AGE). *J Nutr* 2001;131:1096S-1099S
- 32 Borek K. Antioxidant health effects of aged garlic extract. *J Nutr* 2001;131:1010S-1015S
- 33 Iqbal M, Athar M. Attenuation of iron-nitritotriacetate (Fe-NTA)-mediated renal oxidative stress, toxicity and hyperproliferative response by the prophylactic treatment of rats with garlic oil. *Food Chem Toxicol* 1998;36:485-495
- 34 Wang ZX, Hu GF, Wang HY, Wu MC. Expression of liver cancer associated gene HCCA3. *World J Gastroenterol* 2001;7:821-825
- 35 Tang ZY. Hepatocellular Carcinoma Cause, Treatment and Metastasis. *World J Gastroenterol* 2001;7:445-454
- 36 Rabe C, Pilz T, Klostermann C, Berna M, Schild HH, Sauerbruch T, Caselmann WH. Clinical characteristics and outcome of a cohort of 101 patients with hepatocellular carcinoma. *World J Gastroenterol* 2001;7:208-215
- 37 Arbutnot P, Kew M. Hepatitis B virus and hepatocellular carcinoma. *Int J Exp Pathol* 2001;82:77-100
- 38 Wang Y, Liu H, Zhou Q, Li X. Analysis of point mutation in site 1896 of HBV precore and its detection in the tissues and serum of HCC patients. *World J Gastroenterol* 2000;6:395-397
- 39 Yu MC, Gu CH. Mutation of hepatitis B virus and its association with liver diseases. *Shijie Huaren Xiaohua Zazhi* 1999;7:978-979
- 40 Wang HY, Yan RQ, Long JB, Wu QL. Cyclin D1 amplification is associated with HBV DNA integration and pathology in human hepatocellular carcinoma. *Shijie Huaren Xiaohua Zazhi* 1999;7:98-100
- 41 Tang RX, Gao FG, Zeng LY, Wang YW, Wang YL. Detection of HBV DNA and its existence status in liver tissues and peripheral blood lymphocytes from chronic hepatitis B patients. *World J Gastroenterol* 1999;5:359-361
- 42 Wu GY, Wu CH. Gene therapy and liver diseases. *World J Gastroenterol* 1998;4:18-19
- 43 Kim YC, Song KS, Yoon G, Nam MJ, Ryu WS. Activated ras oncogene collaborates with HBx gene of hepatitis B virus to transform cells by suppressing HBx-mediated apoptosis. *Oncogene* 2001;20:16-23
- 44 Diao J, Khine AA, Sarangi F, Hsu E, Iorio C, Tibbles LA, Woodgett JR, Penninger J, Richardson CD. X protein of hepatitis B virus inhibits Fas-mediated apoptosis and is associated with up-regulation of the SAPK/JNK pathway. *J Biol Chem* 2001;276:8328-8340
- 45 Guo SP, Wang WL, Zhai YQ, Zhao YL. Expression of nuclear factor- κ B in hepatocellular carcinoma and its relation with the X protein of hepatitis B virus. *World J Gastroenterol* 2001;7:340-344
- 46 Chen WN, Oon CJ, Leong AL, Koh S, Teng SW. Expression of integrated hepatitis B virus X variants in human hepatocellular carcinomas and its significance. *Biochem Biophys Res Commun* 2000;276:885-892
- 47 Klein NP, Bouchard MJ, Wang LH, Kobarg C, Schneider RJ. Src kinases involved in hepatitis B virus replication. *EMBO J* 1999;18:5019-5027
- 48 Yu DY, Moon HB, Son JK, Jeong S, Yu SL, Yoon H, Han YM, Lee CS, Park JS, Lee CH, Hyun BH, Murakami S, Lee KK. Incidence of hepatocellular carcinoma in transgenic mice expressing the hepatitis B virus X protein. *J Hepatol* 1999;31:123-132
- 49 Lee YH, Yun Y. HBx protein of hepatitis B virus activates Jak1-STAT signaling. *J Biol Chem* 1998;273:25510-25515
- 50 Melegari M, Scaglioni PP, Wands JR. Cloning and characterization of a novel hepatitis B virus x binding protein that inhibits viral replication. *J Virol* 1998;72:1737-1743

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