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Differentially expressed mRNAs in androgen-independent but not androgen-dependent Shionogi carcinoma

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Abstract Recently, a new and highly effective method termed suppressive subtractive hybridization (SSH) has been introduced to clone differentially expressed mRNAs. Genes expressed in androgen-independent but not in androgen-dependent tumors, and vice versa, are obviously significant to delineate the mechanisms of androgen dependency/independency of these tumors. Mouse mammary cancer (Shionogi carcinoma-115) has been extensively used to analyze the mechanism of androgen-dependent cancer growth. Methods: We cloned androgen-independent and androgen-dependent Shionogi carcinoma-115 specific mRNAs by the SSH method. Cloned sequences were compared with known sequences using NCBI BLAST across the Internet. Two clones were positive for cDNA insert when androgen-independent cDNA was used as tester cDNA, while no clones were positive using the androgen-dependent tester cDNA. One of the former was mouse protein kinase C beta-II while the other was a new DNA sequence. Mouse protein kinase C beta-II mRNA and the new mRNA were shown to be differentially expressed by RT-PCR analysis in androgen-independent but not androgen-dependent Shionogi carcinoma. Two mRNA species differentially expressed in androgen-independent but not androgen-dependent Shionogi carcinoma were cloned by the SSH method. The significance of these mRNAs for androgen-dependency/independency of Shionogi carcinoma should be explained in future studies.

Key words Cloning · cDNA · Androgen · Tumor

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Introduction

Recently, a new and highly effective method termed suppressive subtractive hybridization (SSH) has been introduced to clone differentially expressed mRNAs [1]. This method is based primarily on the recently described technique of suppression PCR, and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes sequences common to both the target and driver populations. The SSH technique enriches for rare sequences by over 1000-fold in one round of subtractive hybridization. Genes expressed in androgen-independent but not in androgen-dependent tumors, and vice versa, are obviously significant to delineate the mechanism of androgen dependency/independency of tumors, and for the development of treatment for androgen-independent tumors. Mouse mammary cancer (Shionogi carcinoma-115) has been extensively used to analyze the mechanism of androgen-dependent cancer growth. These tumors exhibit marked androgen-dependent growth in vivo [2]. Therefore, we cloned androgen-independent and androgen-dependent Shionogi carcinoma-specific mRNAs by the SSH method.

Materials and methods

Shionogi carcinoma

Shionogi carcinoma-115 and Shionogi mice were provided by Shionogi Research Laboratories (Osaka, Japan). Tumor fragments were inoculated into the flank of male and female Shionogi mice. When the Shionogi carcinoma grew to about 1–2 cm in diameter in males, the tumor was excised, fragmented, and re-inoculated into male and female Shionogi mice. Androgen-dependency or -independency was determined by tumor non-growth or tumor growth, respectively, in female mice. After 3–4 passages, Shionogi carcinoma tended to be androgen-independent.

RNA preparation

Total RNA was extracted as previously reported [3] from androgen-dependent (AD) and androgen-independent (AI) Shionogi carcinoma. Poly-A RNA was purified from total RNA using a mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Suppressive subtractive hybridization

Suppressive subtractive hybridization (SSH) was performed essentially as reported [1]. Double-strand cDNA (ds-cDNA) was synthesized from poly-A RNA (5 μg) using a TimeSaver cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). These cDNAs were extracted with phenol, precipitated with ethanol, resuspended in dH_2O, and digested with RsaI in a total volume of 20 μl followed by heat-inactivation of RsaI at 95 °C, yielding ADcDNA and AIcDNA. Oligos 1.1, 1.2, 2.1 and 2.2 were resuspended in dH_2O at a concentration of 100 pmol/μl. Oligos 1.1 and 1.2 as well as oligos 2.1 and 2.2 (150 μl for each oligo) were mixed, heated at 95 °C for 10 min, and allowed to cool to room temperature slowly. These hybrids (adapter1 for oligos 1.1 and 1.2; adapter2 for oligos 2.1 and 2.2) of two oligos were extracted with phenol/chloroform, ethanol-precipitated, and resuspended in 5 μl of dH_2O. One microliter of adapter1 or adapter2 was ligated to 1 μl of RsaI-digested ds-cDNAs (ADcDNA and AIcDNA) from androgen-dependent and androgen-independent Shionogi carcinomas using T4 ligase in 10 μl reaction mixtures, yielding ADcDNA-adapter1, ADcDNA-adapter2, AIcDNA-adapter1, and AIcDNA-adapter2. The ligation products were diluted in 1 × TE buffer and purified using a cDNA Spun Column (Pharmacia Biotech). One-tenth (25–35 μl) of the column effuluent (tester cDNA) was co-precipitated with 3 μl of driver ds-cDNA (AIcDNA or ADcDNA) in ethanol (ADcDNA-adapter1 and ADcDNA-adapter2 with AI-cDNA; AIcDNA-adapter1 and AIcDNA-adapter2 with AD-cDNA). These co-precipitated samples were resuspended in 5 μl of hybridization-buffer [50 mM Hepes, pH 8.3/ 0.5 M NaCl/ 0.02 mM EDTA, pH 8.0/ 10% (wt/vol) PEG 8000], heated at 98 °C for 3 min under mineral oil, and incubated at 68 °C for 10 h. Thereafter, ADcDNA-adapter1/AIcDNA and ADcDNA-adapter2/AIcDNA, or AIcDNA-adapter1/ADcDNA and AIcDNA-adapter2/ADcDNA were mixed together. One microliter of AI-cDNA or ADcDNA (driver cDNA) heated at 98 °C for 3 min then cooled on ice was added to the former or the latter mixture, respectively, followed by further incubation at 68 °C for 10 h. After incubation, 200 μl of dilution buffer (20 mM Hepes, pH 8.3/ 50 mM NaCl/ 0.2 mM EDTA, pH 8.0) heated at 72 °C was added to each sample.

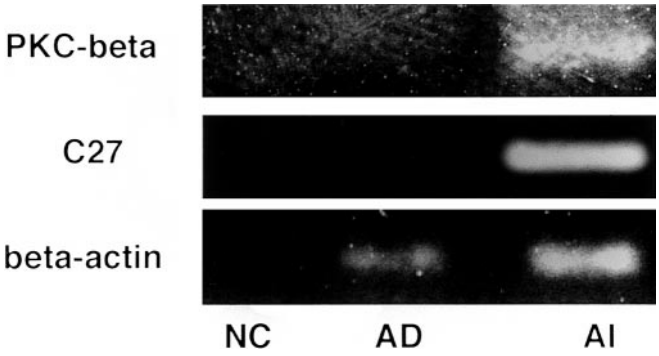

Fig. 1 Expression of protein kinase C β-II and C27 mRNAs in Shionogi carcinoma by RT-PCR. *AD* androgen-dependent, *AI* androgen-independent, *NC* negative control (no RNA added)

PCR amplification and cloning of the subtracted cDNAs

Aliquots of 1 μl of the SSH samples described above were used as the first PCR templates. The first round of PCR (denaturation at 91 °C for 30 s, annealing at 68 °C for 30 s, extension at 72 °C for 2.5 min) was performed for 30 cycles in the presence of 5 μM of P1 and P2 primers, 200 μM of each dNTP, and 2.5 U of Taq polymerase (Boehringer-Mannheim, Germany) in 25 μl volume. Aliquots of 1 μl of the first PCR products diluted 10-fold were used as the template for the second PCR amplification (denaturation at 91 °C for 30 s, annealing at 68 °C for 30 s, extension at 72 °C for 2.5 min) for 10 cycles in 5 μM of PN1 and PN2 primers, 200 μM of each dNTP, and 2.5 U of Taq polymerase. Aliquots of the second PCR products were directly ligated into the pGEM-T vector (Promega) using T4 ligase at 15 °C overnight. Competent XL1-Blue cells (Stratagene) were transformed with the ligation products by heat shock at 42 °C and plated on LB plates containing ampicillin using IPTG/X-Gal color selection. The white colonies were picked up and used as templates for 30 cycles of PCR (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min) in the presence of 50 pmol/μl of T7/SP6 primers, 100 μM of each dNTP, and 2.5 U of Taq polymerase in the supplied buffer to assess inserts in the pGEM-T vector. Colonies transformed with plasmids containing inserts were grown in LB medium, and plasmids were isolated by the alkaline lysis method [4]. Sequences of the inserts were determined with a DSQ 500 autosequencer (Shimadzu, Kyoto, Japan) and compared with registered DNA sequences using NCBI BLAST via the Internet.

Table 1 Oligonucleotides used

Oligonucleotide	Oligonucleotide structure
Oligo 1.1	5′-GTAATACGACTCACTATAGGGTCGAG-CGGCCGCCCGGGCAGGT-3′
Oligo 1.2	3′-CCCGTCCA-5′
Oligo 2.1	5′-TGTAGCGTGAAGACGACAGAAAGGGC-GTGGTGCGGAGGGCGGT-3′
Oligo 2.2	3′-GCCTCCCGCCA-5′
P1	5′-GTAATACGACTCACTATAGGGC-3′
P2	5′-TGTAGCGTGAAGACGACAGAA-3′
PN1	5′-TCGAGCGGCCGCCCGGGCAGGT-3′
PN2	5′-AGGGCGTGGTGCGGAGGGCGGT-3′
T7	5′-TAATACGACTCACTATAGGG-3′
SP6	5′-GATTTAGGTGACACTATAG-3′
mPKC-betaII sense	5′-AGAAGAACGTGCACGAGGTG-3′
mPKC-betaII antisense	5′-TTGCTCCGGGGGTCATCAGA-3′
C27 sense	5′-CAAATGGCTAGAGACAGAGA-3′
C27 antisense	5′-TGAGATTCACATTGGGAACCT-3′
Mouse beta-actin sense	5′-TGGAATCCTGTGGCATCCATGGAAAC-3′
Mouse beta-actin antisense	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

Fig. 2. Partial cDNA sequence of C27 (GenBank Accession Number: AF077762)

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5'AGGGCGTGGTGCGGAGGGCGGTACAAATGGCTAGAGACAGAGATATTACAGTGTCATCTCTGC
AGAATACACTTCCAAGTGCCATGGGAAAGGGAGAATTATCCTTCAGGACCAATCCAGAGACGTGC
AAGTGGCATTCCTATGGATTCTAGGGATATTACAGTCTTCATATGAATACTGGGTGTGTCTCAAG
CTGCTGATAACCCAAGATCCCCTCTCCTCTGACCACTCTTGTCTCCCCAGGTTCCCAATGTGAAT
CTCATTCGCTTATCTTTCTTTCATTGTTTCTGTCTTGAGACTGATTCGCACAGGTTTTCCTGGCC
CTCATATTCATTCTCACTGTTGGGTCTGCTGGTGTATATCTTTGAATAGAAGACGTTCTTTCTGT
CGTCTTCACGCTACAA3'
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RT-PCR: RT-PCR analysis was performed for 30 cycles essentially as described previously [3] using 5 μg of total RNA. Sense/antisense primers for mouse protein kinase C β-II, C27, and mouse β-actin cDNA sequences are shown in Table 1. Electrophoresis of PCR products (10 μl) was carried out in 1.5% agarose gels.

Results

Cloning of differentially expressed mRNAs in androgen-independent Shionogi carcinoma

Approximately 20 white colonies grew on each LB plate. Two clones were positive for cDNA insert when AI-cDNA was used as the tester cDNA, while no clones were positive for cDNA insert when ADcDNA was used as the tester cDNA (data not shown). The insert sequence of one of the former corresponded to the mouse protein kinase C β-II gene (from 1951 to 2105; reference 5) containing the coding region, while the other (C27) had a novel DNA sequence (Fig. 1). RT-PCR analysis demonstrated that mouse protein kinase C beta-II and C27 mRNAs were differentially expressed in androgen-independent (AI) Shionogi carcinoma but not in androgen-dependent (AD) Shionogi carcinoma (Fig. 2).

Discussion and conclusion

Two mRNA species differentially expressed in androgen-independent but not in androgen-dependent Shionogi carcinoma were identified by the SSH method. One was the β-II isozyme of mouse protein kinase C, while the other was a new mRNA species. Activation of protein kinase C, especially α isozyme, has been reported to enhance tumor growth and invasiveness [6, 7, 8, 9, 10, 11]. In contrast, there have been reports that PKC alpha isozyme suppresses tumor growth by inducing apoptosis [12, 13, 14]. Suppression of rat prostatic AT3 tumors by progestational steroid (dMGA) was mediated by suppression of PKC activity [11]. Linkage of the PKC beta isozyme to growth, apoptosis and androgen-independence has not been reported previously. Whether mouse protein kinase C beta-II isozyme and a new species of mRNA (C27, only part of the sequence is known.) are actually linked to androgen-dependency/independency of Shionogi carcinoma is not known and should be demonstrated in future studies.

With the SSH method, a given mRNA in a test sample is eliminated by an excess of the same mRNA in a driver (control) sample. Only when a given mRNA is expressed the most in a test sample as compared with a control, will the cDNA clone remain in the subtracted sample as a candidate for the final PCR-amplification. It is possible that if a given mRNA is expressed ten times more in a test sample than in a control, then the mRNA will not be lost in the hybridization process. Additionally, even if a relatively small amount of a given cDNA recombinant remains in the final sample, this may be lost in the transforming process. These are characteristic of the SSH method and quite different from the differential display method that may identify several times a difference in the mRNA expression. This may be the reason for only two clones being obtained with the current study.

In summary, two mRNA species differentially expressed in androgen-independent but not in androgen-dependent Shionogi carcinoma were cloned by the SSH method. Linkage of these transcripts to androgen-dependency/independency of Shionogi carcinoma should be defined in future studies.

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