Screening for genetic abnormalities involved in ovarian carcinogenesis using retroviral expression libraries

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Abstract. The purpose of this study was to screen for genes involved in ovarian carcinogenesis in an attempt to develop an effective molecular-targeted therapy for ovarian cancer. We constructed retroviral expression libraries for the human ovarian cancer cell lines SHIN-3 and TYK-CPr, and performed a focus formation assay with 3T3 cells. As a result, *proteasome subunit beta-type 2 (PSMB2), ubiquitin-specific protease 14 (USP14)*, and *keratin 8 (KRT8)* were identified from SHIN-3, and *polymerase II RNA subunit (POLR2E), chaperonin containing T-complex polypeptide 1 subunit 4 (CCT4), glia maturation factor beta (GMFB)*, and *neuroblastoma ras viral oncogene homolog (NRAS)* from TYK-CPr. *NRAS* gene analysis revealed a CAA→AAA substitution at codon 61, resulting in a Glu→Lys change at position 61. When the mutant *NRAS* was introduced into fibroblasts for its expression, many transformed foci were generated, confirming the transforming ability of the mutant *NRAS*.

Introduction

Ovarian cancer is the fifth most common cause of death from gynecologic malignancies in the USA. Approximately 25,000 women are affected by ovarian cancer every year, and about 14,000 die of this disease (1). In recent years, debulking surgery followed by multidrug therapy with platinum and taxine drugs have been used, with some improvement in prognosis (2); however, the 5-year survival rate remains at about 50% (3). This is due to the lack of effective therapy for treatment-resistant or recurrent ovarian cancer.

A series of recent studies have reported that STI571, which targets the *BCR-ABL* gene responsible for chronic myelogenous leukemia, is effective for this disease, and that the anti-CD20 antibody rituximab is highly effective for B-cell lymphocytic leukemia (4). These observations have demonstrated that it is clinically very important to elucidate the pathogenesis of malignancies and thereby develop molecular-targeted therapy for them. To improve the prognosis of patients with ovarian cancer, it is important to define genetic abnormalities involved in the onset of this disease, and to develop effective molecular-targeted therapies for ovarian cancer.

To date, several studies have reported that mutations in the *p53* gene (5) or deletions in the *BRCA1* and *BRCA2* genes (6) are common in ovarian cancer. However, there is no evidence that these genetic abnormalities are directly involved in the development of ovarian cancer.

In a recent study, we constructed a full-length cDNA expression library of non-small cell lung cancer (NSCLC) using a retroviral vector, and demonstrated by functional screening that a fusion gene, composed of portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase (*ALK*) genes, was involved in the development of NSCLC (7). In this study, we aimed to screen for genes responsible for ovarian carcinogenesis using similar techniques.

Materials and methods

Cell culture. Ovarian serous cystadenocarcinoma cell line SHIN-3 (8) and ovarian undifferentiated carcinoma cell line TYK-CPr (JCRB0234.1, Health Science Research Resources Bank: HSRRB, Osaka, Japan) (9) cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). The BOSC23 packaging cell line for ecotropic retroviruses (10) and mouse 3T3 fibroblasts (American Type Culture

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Collection: ATCC) were maintained in DMEM/F12 supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and 1% penicillin-streptomycin.

Construction of a retrovirus library. Total RNA was extracted from SHIN-3 and TYK-CPr cells with the use of an RNeasy Mini column and RNase-free DNase (Qiagen, Valencia, CA, USA), and first-strand cDNA was synthesized from the RNA with PowerScript reverse transcriptase, a SMART IIA oligonucleotide, and CDS primer IIA (Clontech, Palo Alto, CA, USA). The resulting cDNA molecules were then amplified for 15 cycles with the 5'-PCR primer IIA and a SMART PCR cDNA synthesis kit (Clontech), with the exception that LA Taq polymerase (Takara Bio, Shiga, Japan) was substituted for the Advantage 2 DNA polymerase provided with the kit. The PCR products were treated with proteinase K, rendered blunt-ended with T4 DNA polymerase, and ligated to a *BstXI* adapter (Invitrogen). Unbound adapters were removed with a cDNA size fractionation column (Invitrogen), and the modified cDNAs were ligated into the pMX retroviral plasmid (11) that had been digested with *BstXI*. The pMX-cDNA plasmids were introduced into ElecroMax DH10B cells (Invitrogen) by electroporation.

Focus formation assay. BOSC23 cells ($1.8x10^6$) were seeded onto 6-cm culture plates, cultured for 1 day, and then transfected with a mixture comprising 2 μg of retroviral plasmids, 0.5 μg of pGP plasmid (Takara Bio), 0.5 μg of pE-eco plasmid (Takara Bio), and 18 μl of Lipofectamine reagent (Invitrogen). Two days after transfection, polybrene (Sigma, St. Louis, MO, USA) was added at a concentration of 4 μg/ml to the culture supernatant, which was then used to infect 3T3 cells for 48 h. For the focus formation assay, the culture medium of 3T3 cells was changed to DMEM-high glucose (Invitrogen) supplemented with 5% calf serum and 2 mM L-glutamine. Transformed foci were isolated after 3 weeks of culture.

Recovery of cDNAs from 3T3 cells. Each 3T3 cell clone was harvested with a cloning syringe and cultured independently in a 10-cm culture plate. Genomic DNA was subsequently extracted from the cells and subjected to PCR with the 5'-PCR primer IIA and LA Taq polymerase for 50 cycles of 98˚C for 20 sec and 68˚C for 6 min. Amplified genomic fragments were purified by gel electrophoresis and ligated into the pT7Blue-2 vector (EMD Biosciences, San Diego, CA, USA) for nucleotide sequencing. The cDNAs obtained were introduced into the pMXS plasmid to prepare a recombinant retrovirus, which was used again to test its 3T3-transforming ability.

Analysis of the NRAS gene in the TYK-CPr cell line. Using as the substrate the cDNA that had been synthesized to construct the retroviral library, along with NRAS primers [5' primer (GTGGAGCTTGAGGTTCTTGC) and 3' primer (GCAG CTTGAAAGTGGCTCTT)], the *NRAS* gene was amplified by PCR for 30 cycles of 98˚C for 30 sec, 62˚C for 30 sec, and 68˚C for 30 sec. The amplified DNA fragments were separated by electrophoresis, then purified, and inserted into the pGEM-T Easy Vector (Promega Corp., Madison, WI, USA) for sequencing.

Results

Construction of full-length cDNA expression libraries for SHIN-3 and TYK-CPr cells. cDNAs from SHIN-3 and TYK-CPr cells were inserted into the pMXS retroviral plasmid, which was then introduced into DH10B cells by electroporation. As a result, we obtained plasmid libraries of cDNA clones from SHIN-3 ($1.1x10^6$ colony-forming units, or cfu) and TYK-CPr ($1.2x10^6$ cfu) cells. From each of these plasmid clone libraries, 24 clones were picked up at random. In addition, to ascertain whether the cDNA inserts were full-length (complete reading frame), 10 clones were selected from each library, and ~500 bp of both ends of the cDNA insert were sequenced. The identified sequences were compared with the University of California-Santa Cruz Genome Browser Database (http://genome.ucsc.edu) by BLAST search (12). As a result, 7 of the 10 TYK-CPr-derived clones and 7 of the 10 SHIN-3-derived clones contained full-length cDNA inserts (data not shown). We therefore concluded that the retroviral cDNA expression libraries were of sufficient complexity and adequately enriched in full-length cDNAs for the present study.

Screening for transformed clones. 3T3 cells were transfected with retroviral cDNA expression libraries, and, after 3 weeks of culture, transformed clones were isolated. As a result, 17 and 15 transformed clones were identified for TYK-CPr and SHIN-3, respectively (Fig. 1).

Transformed clones were isolated using a cloning syringe, and cultured in separate dishes to extract genomic DNA from each clone. The genomic DNA was amplified by PCR using the same primers as those employed in cDNA amplification for library construction. As shown in Fig. 2, a single sharp cDNA band per clone was identified in about half of the clones.

Analysis of cDNAs recovered from transformed cells. Transformed clones, whose genomic DNA amplified by PCR gave a single sharp cDNA band, were selected, and their respective cDNAs were sequenced for gene identification. As a result, *proteasome subunit beta-type 2 (PSMB2), ubiquitin-specific protease 14 (USP14)*, and *keratin 8 (KRT8)* were identified from SHIN-3, and *polymerase II RNA subunit (POLR2E), chaperonin containing T-complex polypeptide 1 subunit 4 (CCT4), glia maturation factor beta (GMFB)*, and *neuroblastoma ras viral oncogene homolog (NRAS)* from TYK-CPr. All cDNAs contained complete open reading frames (ORF).

Analysis of the NRAS gene in the TYK-CPr cell line. Focusing on *NRAS* among the genes identified in this study, we examined the TYK-CPr-derived cDNA for point mutations, and found a codon 61 mutation (CAA→AAA, Glu→Lys) (Fig. 3).

Furthermore, the resulting mutant NRAS ($NRAS^{Q61K}$) cDNA was inserted into the pMX plasmid to construct a recombinant retrovirus, which was transfected into 3T3 cells for a focus formation assay. As shown in Fig. 4, the identified $NRAS^{Q61K}$ generated many transformed foci, confirming its transforming ability.

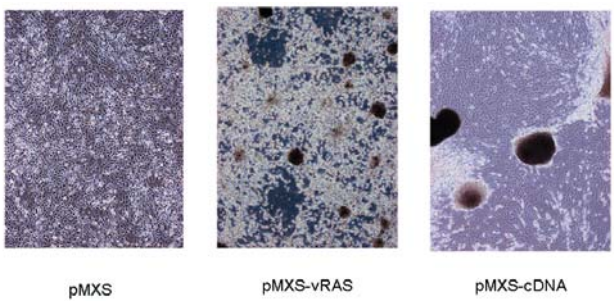

Figure 1. Focus formation assay with a retroviral library derived from TYK-CPr cells. Mouse 3T3 cells were infected with the empty virus (pMXS), a retrovirus expressing v-Ras as a positive control (pMXS-vRAS), or retroviruses from the TYK-CPr cell library (pMXS-cDNA). The cultures were photographed 3 weeks after infection.

Figure 4. A recombinant retrovirus encoding NRAS^{Q61K} was used to infect 3T3 cells. The cells were photographed after culture for 2 weeks.

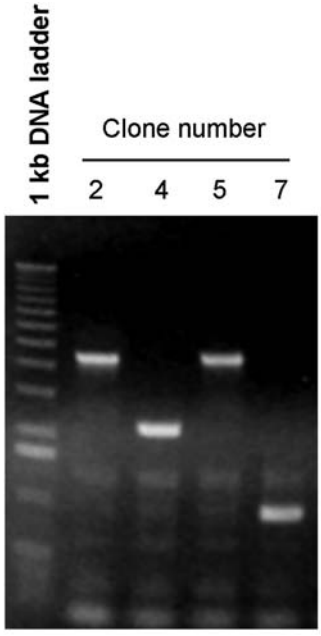

Figure 2. Genomic DNA isolated from transformed 3T3 cell foci (clone numbers 2, 4, 5, and 7) was subjected to PCR for amplification of the DNA inserts. The left lane contains DNA size markers (1-kbp DNA ladder; Invitrogen).

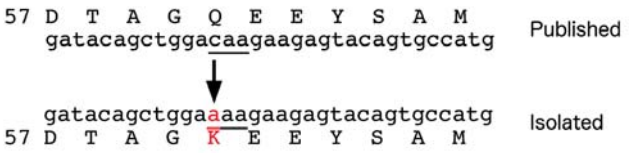

Figure 3. The amino acid sequence in the vicinity of the amino acid residue at position 61 of NRAS protein and the corresponding NRAS cDNA (NM_002524) codon sequence are shown in the upper row (published). Similarly, the amino acid sequence and the corresponding NRAS cDNA (isolated) codon sequence identified in this study are shown in the lower row. In the screened cDNA, the glutamine-encoding codon (caa) at position 61 had been converted to the lysine-encoding codon (aaa). The site of the nucleotide substitution is indicated in red.

To confirm that the NRAS^{Q61K} mutation did not arise during the process of library construction or PCR, we sequenced the *NRAS* gene in 10 randomly selected TYK-CPr cell clones, and found a CAA→AAA substitution at codon 61 in 7 clones. This indicates that the TYK-CPr cell line has a wild-type allele and a mutant allele (NRAS^{Q61K}).

Discussion

The focus formation assay in mouse 3T3 fibroblasts has been widely used to identify oncogenes (13). The conventional 3T3 focus formation assay involves the introduction of cancer cell-derived genomic DNA, followed by screening for focus-forming, transformed clones of 3T3 cells. To date, many oncogenes such as *RAS, ABL*, and *RAF* have been identified using this assay. However, the assay involving the introduction of genomic DNA alone has the major disadvantage of a lower screening ability, that is, the expression of oncogenes is controlled by their own enhancer/promoter region. However, the enhancer/promoter region of oncogenes functioning in ovarian cancer is not necessarily active in 3T3 fibroblasts. Therefore, the possibility of the successful identification of ovarian cancer-related oncogenes by the classical focus formation assay involving the introduction of genomic DNA alone is small. To ensure that all genes introduced into 3T3 cells are expressed at sufficient levels, it is necessary that their transcription be regulated by exogenous promoters and enhancers.

The retroviral vector is a type of vector most commonly used for gene introduction, and has advantages in that the cDNA inserted between the left and right long terminal repeats (LTR) is integrated directly into the chromosomes of infected cells, and viral vectors can be produced at high titers using packaging cells (14,15).

In this study, to screen for genes involved in ovarian carcinogenesis, we attempted to construct cDNA-expressing recombinant retroviral libraries that were engineered to express cDNA from retroviral LTR, and succeeded in constructing libraries with a sufficient complexity and mean insert size, derived from the ovarian cancer cell lines SHIN-3 and TYK-CPr. Using these libraries, we performed a focus formation assay in 3T3 cells, and were able to recover the cDNA inserts easily from the transformed clones employing the primers used for cDNA synthesis. From each of 7 clones, a single cDNA was identified and sequenced. As a result, *PSMB2, USP14*, and *KRT8* were screened from SHIN-3 cells, and *POLR2E, CCT4, GMFB*, and *NRAS* from TYK-CPr

cells. Focusing on *NRAS*, known as an oncogene, we analyzed it, and identified a CAA→AAA substitution at codon 61, resulting in a Glu→Lys change at position 61. When the mutant *NRAS* was transfected into fibroblasts for its expression, many transformed foci were generated, confirming the transforming ability of the mutant *NRAS*.

The RAS gene family is a group of three oncogenes, KRAS, HRAS, and NRAS, which are most commonly activated in human malignant neoplasms (16). For RAS activation, an amino acid substitution at position 12, 13, 59, or 61 is important. It was reported in thyroid tumors that a CAA→CGA substitution at codon 61 of NRAS resulted in a Glu→Arg change at position 61 and NRAS activation (17). It was also noted in the neuroblastoma cell line SK-N-SH that a Glu→Lys mutation at position 61 of NRAS resulted in its activation (18).

Furthermore, KRAS mutations were reported to occur in about 50% of ovarian mucinous carcinomas (19,20) and 30% of serous borderline ovarian tumors (21). In addition, HRAS mutations were reported in about 6% of ovarian cancers (22). However, no NRAS activation in ovarian cancers has been reported to date (22). Since the cell proliferation function of the HRAS, KRAS, and NRAS gene products overlaps with one another in many cases, the reason for selective KRAS activation in specific histological types of ovarian cancer is unclear.

This study is the first in the world to identify *NRAS* gene activation by point mutation in an ovarian cancer cell line. However, no *NRAS* gene activation has been reported in ovarian cancer patients. In the future, it will be necessary to analyze clinical samples for NRAS mutations, particularly amino acid substitutions at position 61. TYK-CPr, in which an activated NRAS gene was identified in this study, is a cell line derived from an undifferentiated ovarian cancer with a clinically poor prognosis. This suggests that NRAS activation is associated with specific histological types of ovarian cancer.

Recently, sorafenib, a molecular-targeted therapeutic drug targeting activated RAS, has been developed and used clinically. The drug inhibits Raf kinase downstream of RAS, thereby blocking the RAS/MEK/ERK signaling pathway and exerting antitumor effects (23). Such a molecular-targeted drug may be effective for ovarian cancer patients with the NRAS mutations reported here.

Among the genes screened in this study, proteasome has been reported to be involved in cell cycle regulation and apoptosis (24). In addition, *keratin 8* has been reported to be involved in malignant transformation and cancer cell invasion (25). These genes may be ovarian cancer-related oncogenes, although further studies are needed.

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