Oncogenic Transformation by SEI-1 Is Associated with Chromosomal Instability

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Abstract

Amplification of SEI-1, a cell cycle regulatory gene at 19q13.1, is commonly detected in ovarian cancer, suggesting a role in the pathogenesis of ovarian cancer. In the present study, the oncogenic potential of SEI-1 was shown by anchorage-independent growth and tumor formation in nude mice with SEI-1–transfected NIH 3T3 mouse fibroblast cells. Silencing of SEI-1 gene expression by small interfering RNAs in ovarian cancer cell line SKOV3 could inhibit cell growth as well as colony formation on soft agar. Chromosomal alterations including the formation of double minutes were observed in tumor cells derived from SEI-1–transformed NIH 3T3 cells. Micronuclei formation, which is an indicator of nuclear abnormality and genomic instability, was markedly increased in SEI-1–transfected cells. These data suggest that the oncogenic role of SEI-1 might be mediated at least in part via an effect on genomic instability. Furthermore, overexpression of SEI-1 was associated with higher tumor grades and late Federation Internationale des Gynecologistes et Obstetristes (FIGO) stages in ovarian carcinomas. These data strongly suggest that SEI-1 plays an important role in the development and progression of ovarian cancer. (Cancer Res 2005; 65(15): 6504-8)

Introduction

Early stages of ovarian cancer are usually asymptomatic. Thus, the disease often presents late and is a leading cause of cancer death in women. Effective diagnosis and treatment of ovarian cancer would then hinge on understanding the molecular mechanisms in the progression of ovarian carcinogenesis. However, the molecular events at different stages of ovarian carcinogenesis remain the least understood among all major human cancers. Several susceptibility genes have been identified, including BRCA1 and BRCA2 (1), which were initially discovered to be associated with breast cancer. But mutations in these high-risk genes not only account for a small percentage of ovarian cancer cases. P53, HER2/neu, and PCK34 genes are also found to be involved in ovarian carcinogenesis (1). For a better understanding of the molecular mechanism underlying, it is highly desirable to identify novel oncogenes and tumor suppressor genes that contribute to ovarian cancer development.

Cytogenetic studies have revealed a gain of chromosomal region 19q13.1-13.2 in >30% of ovarian cancers (2, 3). Among these patients, 19q amplification seems an early event and persists through out later stages of the carcinogenesis (3). Our previous studies identified two overlapping minimal amplification regions that contain a number of genes including AKT2 and SEI-1 (4). AKT2, a member of a subfamily of protein serine/threonine kinases, has been implicated as a putative oncogene at 19q13. However, AKT2 amplification and overexpression was only observed in a small subset (~12%) of ovarian carcinomas (5, 6). Therefore, amplification and overexpression of one or more yet-unidentified oncogenes in these regions may also contribute to the development of ovarian cancer.

SEI-1 gene was recently identified by two research groups using yeast two-hybrid system. Sugimoto et al. showed that SEI-1 is a CDK4 binding protein which antagonizes the inhibitory effect of p16 on cell cycle progression by stimulating cyclin D1-CDK4 complex formation and CDK4 activity (7). Using a different experimental strategy, Hsu et al. found the same protein which they termed TRIP-Br1. TRIP-Br1 makes physical contact with E2F-1/DP-1 and functions as a transcriptional coactivator to facilitate the transcriptional activity of E2F-1 (8). SEI-1/TRIP-Br1 is therefore recognized as a novel cell cycle regulatory protein. Furthermore, SEI-1 gene is mapped to chromosome 19q13, a region frequently amplified in ovarian carcinomas as well as a variety of other tumors including pancreatic carcinomas (9) and lung cancer (10), suggesting SEI-1 is potentially an oncogene. Indeed, we showed previously that SEI-1 gene is amplified and overexpressed in several ovarian cancer cell lines as well as in ovarian carcinomas (4). However, a direct link between SEI-1 and cancer pathogenesis is still absent. In the present study, we investigated the tumorigenic potential of SEI-1 in NIH 3T3 cells and examined its protein expression pattern in human ovarian cancer specimens.

Materials and Methods

Tumorigenic ability of SEI-1. To evaluate the tumorigenic ability of SEI-1, SEI-1 was cloned into expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) and transfected into mouse fibroblast cell line NIH 3T3 cells using Lipofectamine (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Stable SEI-1 expressing clones were selected using Geneticin (Life Technologies) at a concentration of 800 µg/ml and the expression level of SEI-1 in each clone was determined by reverse transcription-PCR (RT-PCR).

To test cell growth rate, cells were seeded onto 96-well plate at a density of 1 × 104 cells per well and incubated in low serum (2% FCS) for 1 to 4 days. The cell growth rate was determined using cell proliferation 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt kit (Roche, Mannheim, Germany) according to manufacturer’s instructions. Anchorage-independent growth assay was carried out by growing 1 × 104 cells in 0.4% bacltoagar on a bottom layer of solidified 0.6% bacltoagar in 6-well plates. After 3 weeks, colonies formed in the top layer were counted and colony formation rate was calculated as percentage of total seeded cells.
For tumor xenograft experiments, NIH 3T3 cells (2 × 10^6 cells in the volume of 200 μL) transfected with empty vector or SEI-1 gene were injected s.c. into the left and right hind legs of nude mice, respectively. Tumor formation on nude mice was monitored over a 2-month period.

Small interfering RNA transfection. Ovarian cancer lines SKOV3 was obtained from the American Type Culture Collection (Manassas, VA). SKOV3 cells were transfected with small interfering RNA (siRNA) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instruction. Double-stranded siRNAs were obtained from Ambion, Inc. (Austin, TX). The sequence of SEI-1-si1 was designed according to an antisense DNA oligo that has been published previously (7): sense 5’- GAGCAAGAGCUAGAGGAAAGt-3’ and antisense 5’-CUUGCUCAGCAU- CUUGCUtt-3’. SEI-1-si2 was from Ambion’s predesigned siRNA database: sense 5’-GGGCGUGUUUGGAAAUUt-3’ and antisense 5’-AAUUCUC- CAAAACAGGCCCCtt-3’. Forty-eight hours after transfection, gene-silencing effect was measured by RT-PCR, cell growth and soft agar assay were done as described.

Short-term tissue culture and cytogenetic analysis. Fresh tumor specimens were removed from the nude mice, minced with scalpel blade, and digested with 0.125% collagenase for 2 hours at 37°C. Cell suspension was cultured in DMEM with 20% FCS for 48 hours before harvest. Two hours before harvest, colcemid was added to the culture medium at a final concentration of 0.05 μg/mL. Metaphase chromosomes were harvested and G-banded according to routine procedures.

Chromosome microdissection. Chromosome microdissection and fluorescence in situ hybridization (FISH) were done as described (11). Ten copies of double minutes were dissected and PCR amplified using UN1 primer. The amplified microdissected DNA was labeled with Spectrum-orange dUTP (Vysis, Downers Grove, IL) by PCR and hybridized to metaphases chromosomes obtained from short-term cultured cancer cells with double minutes and normal mouse.

Micronuclei assay. For micronuclei assay, cells grown on coverslips were fixed and stained with 4',6-diamidino-2-phenylindole. And the number of cells with micronuclei was counted. For the analysis, 800 cells were examined for each sample. Data presented are the mean of three independent experiments.

Tissue microarray and immunohistochemistry staining. Paraffin blocks from 275 patients with epithelial ovarian tumors and 10 normal ovary samples were collected at Sun Yat-Sen University, Guangzhou, China. The ovarian tumor cases encompassed 240 cases with histologically confirmed invasive carcinoma, 15 borderline tumors, and 20 cystadenomas. The tissue microarray (TMA) blocks were constructed according to the method described previously (12). Multiple sections (5 μm thick) were cut from the TMA block and mounted on microscope slides.

Immunohistochemistry was done using the standard streptavidin-biotin-peroxidase complex method. Polyclonal anti-SEI-1 antibody was kindly provided by Dr. Eiji Hara (Paterson Institute for Cancer Research, Manchester, United Kingdom). Negative control was done by replacing the primary antibody with blocking serum. Malignant and nonmalignant tissues were scored for SEI-1 expression by assessing the site of positive nuclear staining. Because all normal ovarian surface epithelia (seven informative cases) were observed with <20% of cells positively stained, overexpression of SEI-1 was depicted when >20% of tumor cells were positively stained in the nuclei.

Statistical analysis. For statistical evaluation, χ² test for trend was used to assess the different expression status of SEI-1 in various ovarian lesions. χ² test was used to assess the statistical significance of the association of the expression of SEI-1 with patient’s clinicopathologic variables. P < 0.05 was considered significant.

Figure 1. Tumorigenic ability of SEI-1 in NIH 3T3 cells. A, rate of colony formation in soft agar detected in vector-transfected NIH 3T3 cells (Vec), different clones of SEI-1–transfected NIH 3T3 (S1 and S6), and pooled SEI-1–transfected NIH 3T3 cells. SEI-1 expression level in these cells was determined by RT-PCR (bottom). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. B, the cell growth rates of SEI-1–transfected NIH 3T3 cells were obviously faster than that in vector-transfected NIH 3T3. C, examples of tumors formed in nude mice 2 months after injection of SEI-1–expressing NIH 3T3 cells (right dorsal flank). D, SEI-1 gene silencing by siRNAs. D, both siRNAs (SEI-1-si1 and SEI-1-si2) could efficiently reduce the expression of SEI-1 in SKOV3 cells. Glyceraldehyde-3-phosphate dehydrogenase serves as internal control. Both cell growth (E) and colony formation (F) were decreased significantly in siRNA-treated SKOV3 cells.
Results

Tumorigenic potentials of SEI-1. To investigate whether SEI-1 gene overexpression can induce oncogenic transformation, SEI-1 gene was transfected into NIH 3T3 cells. Anchorage-independent growth in soft agar and tumor formation in nude mice were examined. As shown in Fig. 1A, SEI-1–transfected NIH 3T3 cells were capable of growing in soft agar but not empty vector-transfected cells. Furthermore, SEI-1-overexpressing cells were tested for growth under low serum conditions. Two clonal lines S1 and S6 and pooled transfected cells were tested. Similar to what has been observed in rat fibroblasts (7), NIH 3T3 cells overexpressing SEI-1 gene grew much faster than cells transfected with empty vector (Fig. 1B).

To further explore the transforming ability of SEI-1, tumor formation in nude mice was examined with SEI-1-transfected NIH 3T3 cells. At 4 weeks of time, solid tumors were readily visible on mice injected with SEI-1 transfected NIH 3T3 cells (10 of 10 with pooled SEI-1–transfected cells, 3 of 4 with S1, and 4 of 4 with S6 clonal cells), but no sign of tumor formation on mice injected with vector-transfected NIH3T3 cells. Shown in Fig. 1C are examples of tumor formation in nude mice 2 months after cell inoculation.

Silencing of SEI-1 expression inhibits cell growth and colony formation. To gain further insights into the oncogenic role of SEI-1 in ovarian carcinogenesis, we used siRNAs to knock-down the expression of SEI-1 in an ovarian cancer cell line SKOV3. SKOV3 cells express high level of SEI-1 gene. As shown in Fig. 1D, both SEI-1-si1 and SEI-1-si2 efficiently reduced the expression of SEI-1 in SKOV3 cells. Furthermore, in cells treated with siRNAs, both cell growth and colony formation were decreased significantly (Fig. 1E and F).

Overexpression of SEI-1 may cause genomic instability. After short-term culture, metaphase chromosomes from seven tumors formed in nude mice were G-banded and cytogenetically characterized. Twenty metaphase spreads from SEI-1–transfected NIH 3T3 cells and the parental NIH 3T3 cells, 10 to 20 metaphase spreads from cultured tumor cells were analyzed. Several chromosomal changes were detected by cytogenetic analysis including (a) chromosomal number was increased in tumor cells (average, 75; range, 70-80) and SEI-1–transfected NIH 3T3 cells (average, 72; range, 69-75) comparing with that in parental NIH 3T3 cells (average, 68; range, 65-72); (b) more small unknown marker chromosomes were observed in SEI-1–transfected NIH 3T3 cells (average, 72; range, 69-75) and tumor cells (Fig. 2C-D); and (c) double minutes were detected in two of seven tumors (Fig. 2C). The existence of double minutes was confirmed by FISH using probes generated from microdissected DNA of the double minutes (Fig. 3A). The dissected DNA was then mapped to normal metaphase chromosomal region 9A2-3 (Fig. 3B).

Micronuclei, which have been shown to contain unstable extranucleus chromosomal fragments frequently involved in rearrangement events, are recognized as indicators of genomic instability (13). As shown in Fig. 3C, micronuclei formation in SEI-1–transfected cells almost tripled comparing with parental NIH 3T3 cells (8.5% versus 3.4%). This gives further support that overexpression of SEI-1 may cause genomic instability.
Correlation of SEI-1 overexpression with clinical features of ovarian cancers. To address the question whether there is a correlation between SEI-1 overexpression and clinical features of ovarian cancer, SEI-1 expression level was investigated using a TMA containing 275 epithelial ovarian tumors and 10 normal ovaries. Immunohistochemical staining in the nuclei was scored with anti-SEI-1 antibody and the results were summarized in Table 1. SEI-1 was only weakly expressed in normal ovaries (<20% of cells positively stained). However, overexpression of SEI-1 was observed in 3 of 17 (18%) informative cystadenomas, 3 of 12 (25%) informative borderline tumors, and 102 of 209 (49%) informative ovarian carcinomas, respectively. This increased frequency of SEI-1 overexpression from normal ovarian surface epithelium to benign adenomas, LMP tumors, and invasive carcinoma was significant ($\chi^2$ test for trend, $P < 0.05$). Overexpression of SEI-1 was also correlated with Silverberg grades of ovarian cancers. The frequency of SEI-1 overexpression was significantly higher in ovarian cancers with higher grade G3 (39 of 65 cases, 60%) or G2 (46 of 93, 49%) than that in G1 tumors (17 of 51, 33%; $P < 0.05$). Moreover, the association of SEI-1 overexpression and tumor Fédération Internationale des Gynaecologistes et Obstétristes (FIGO) stages was also observed. The frequency of SEI-1 overexpression increased significantly from carcinomas in FIGO stage I (13 of 43 cases, 30%) to tumors in stages II (25 of 47, 53%), III (58 of 111, 52%), and IV (6 of 8, 75%; $P < 0.05$).

Discussion

One of the key pathways that control cell growth and proliferation in mammalian cells is the CDK4/INK4/RB/E2F cascade. This pathway has been found to be deregulated in most human malignancies by genetic and epigenetic alterations (14). In fact, lost of p16 tumor suppressor protein expression by homozygous deletions or promoter methylation has been implicated in a subset of ovarian cancers (15). As a CDK4 binding protein, SEI-1 gene is able to counteract the inhibitory effect of p16 on CDK4 activity and cell cycle progression (7). Consistent with Sugimoto’s observation in rat fibroblasts, our data showed that overexpression of SEI-1 stimulated NIH 3T3 cell growth under low

Table 1. Correlation of SEI-1 overexpression with clinical features of ovarian cancers

<table>
<thead>
<tr>
<th></th>
<th>Total cases</th>
<th>Informative cases</th>
<th>SEI-1 overexpression (%)</th>
<th>$P$</th>
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<tr>
<td>Normal ovary</td>
<td>10</td>
<td>8</td>
<td>0 (0)</td>
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<tr>
<td>Cystadenoma</td>
<td>20</td>
<td>17</td>
<td>3 (18)</td>
<td></td>
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<td>Borderline tumor</td>
<td>15</td>
<td>12</td>
<td>3 (25)</td>
<td></td>
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<tr>
<td>Invasive carcinoma</td>
<td>240</td>
<td>209</td>
<td>102 (49)</td>
<td></td>
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<tr>
<td>Silverberg grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>55</td>
<td>51</td>
<td>17 (33)</td>
<td>0.015 †</td>
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<tr>
<td>G2</td>
<td>103</td>
<td>93</td>
<td>46 (49)</td>
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<tr>
<td>G3</td>
<td>82</td>
<td>65</td>
<td>39 (60)</td>
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</tr>
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<td>FIGO stage</td>
<td></td>
<td></td>
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<tr>
<td>I</td>
<td>48</td>
<td>43</td>
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<td>0.010 †</td>
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<tr>
<td>II</td>
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<td>47</td>
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<td>IV</td>
<td>11</td>
<td>8</td>
<td>6 (75)</td>
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* $\chi^2$ test for trend.
† $\chi^2$ test.
serum condition. Therefore, overexpression of SEI-1 could contribute to the deregulated cell growth by reducing the requirement for mitogenic stimulation and by overcoming growth inhibitory signals from p16. Interestingly, knock-in mice carrying a CDK4 mutant protein that is resistant to the inhibitory effect of p16 developed a wide spectrum of tumors, including malignancies of epithelial origin (16). Furthermore, SEI-1 was found to function as a transcriptional coactivator to stimulate the transcriptional activity of E2F-1, a critical transcription factor at G1-S transition of cell cycle (8). Therefore, SEI-1 seems an important player with multiple targets in the CDK4/INK4/RB/E2F pathway.

Abnormalities in cell cycle control genes have been reported to induce genomic instabilities (17). For example, p53 mutation can affect genomic stability and induce double minute formation (18). Notably, inactivation of Rb gene that impairs the CDK4/INK4/RB/E2F pathway has recently been shown to promote aneuploidy and genomic instability as a byproduct of defects in cell cycle control (19). In the present study, we detected chromosomal changes including double minutes in short-term cultured cells derived from the tumor formed in nude mice after injection with SEI-1-transfected NIH 3T3 cells. Formation of double minutes is one of the cytogenetic manifestations of gene amplification in malignant tumors. Although the mechanism of double minute formation remains unclear, it has been implicated to be associated with genomic instability. Micronuclei formation is another indicator of genomic instability which can be induced by mutagenic agents such as irradiation or overexpression of an oncoprotein (20). The detection of double minutes formation in tumor grafts and the increase in micronuclei formation, along with other numeric and structural chromosomal changes found in SEI-1 transfected cells, suggest that overexpression of SEI-1 may affect genomic stability in the progression of tumorigenesis, although the exact mechanism remains to be elucidated.

Previously, we have detected the amplification and overexpression of SEI-1 gene in several ovarian cancer cell lines (4). And in the present study, we showed that indeed SEI-1 possesses oncogenic properties to induce cellular transformation of NIH3T3 cells. Therefore, we reasoned that SEI-1 might play a role in ovarian carcinogenesis. Two approaches were taken to investigate the potential role of SEI-1 in ovarian carcinogenesis. First, a tissue microarray was used to detect possible association between SEI-1 overexpression and clinical features of ovarian cancer. Immunohistochemistry analysis showed that SEI-1 overexpression is frequently (>40%) observed in ovarian carcinomas. And overexpression of SEI-1 is found to be associated with higher tumor grades and late FIGO stages. Furthermore, siRNAs were designed to silence SEI-1 gene expression in an ovarian cancer cell line SKOV3. Silencing of SEI-1 gene expression was found to decrease cell growth rate and reduce colony formation in SKOV3 cells. These data strongly suggested that SEI-1 may play an important role in the pathogenesis of ovarian carcinomas.

In summary, our study provides direct evidence, for the first time, that overexpression of SEI-1 is capable of transforming NIH 3T3 cells and inducing tumor formation in nude mice. Oncogenic transformation induced by SEI-1 overexpression is associated with chromosomal instabilities. SEI-1, similar to other cell cycle regulatory proteins, such as Cyclin D1, Cdk4, INK4, and Rb, might therefore be a potential target to be considered for both cancer diagnosis and cancer therapy.

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