Expression of the Bric-a-Brac Tramtrack Broad Complex Protein NAC-1 in Cervical Carcinomas Seems to Correlate with Poorer Prognosis

Shamima Yeasmin, Kentaro Nakayama, Masako Ishibashi, Atsuko Katagiri, Kouji Iida, Indri Nuryani Purwana, Naomi Nakayama, and Kohji Miyazaki

Abstract

Purpose: Recent studies have suggested a novel oncogenic role of a bric-a-brac tramtrack broad complex (also known as POZ) domain gene, NAC-1, in ovarian carcinomas. The aim of this study was to clarify the functional role of NAC-1 in human cervical carcinomas.

Experimental Design: NAC-1 expression in cervical cancer was assessed by immunohistochemistry, and data on clinical variables were collected by retrospective chart review. NAC-1 gene knockdown using small interfering RNA and a NAC-1 gene transfection system were used to assess NAC-1 function in cervical cancer in vivo.

Results: Immunohistochemical and gene expression analysis revealed that NAC-1 is significantly overexpressed in cervical adenocarcinomas and adenosquamous carcinomas compared with squamous cell carcinomas. Patients with squamous cell carcinomas positive for NAC-1 expression who received radiotherapy had significantly shorter overall survival than peers whose tumors did not express NAC-1, and multivariate analysis showed that NAC-1 expression was an independent prognostic factor for overall survival after radiotherapy. Overexpressions of the NAC-1 gene stimulated cell proliferation in cervical carcinoma cells of the TCS, CaSki, and HeLa P3 lines, which do not have endogenous NAC-1 expression. NAC-1 gene knockdown inhibited cell growth and induced apoptosis in HeLa, HeLaTG, and ME180 cells, all of which overexpressed NAC-1.

Conclusions: Our findings suggest that NAC-1 may play an important role in cervical carcinomas; moreover, these findings provide a rationale for future development of NAC-1-based therapy for cervical carcinomas that overexpress this candidate oncogene.

Uterine cervical cancer is the second most common malignancy among women worldwide (1). Although early diagnosis has decreased the death rate, cervical cancer is still a leading cause of cancer deaths in Japanese women. This is partly because some patients continue to be diagnosed with advanced stage disease, for which conventional therapy is less effective. As a result, many cervical cancer patients in Japan develop and eventually succumb to recurrent disease. New, effective therapeutic agents are urgently needed to improve outcome in these patients.

The bric-a-brac tramtrack broad complex [BTB, also known as POZ] gene family is composed of several proteins that share a conserved BTB/POZ protein-protein interaction motif at the NH2 terminal that mediates homodimer or heterodimer formation (2–4). These proteins have been shown to participate in a wide variety of cellular functions, including regulation of transcription, cellular proliferation, apoptosis, cell morphology, ion channel assembly, and protein degradation through ubiquitination (2). A subset of BTB/POZ proteins have been implicated in human cancer; they include BCL-6 (5, 6), promyelocytic leukemia zinc finger (5, 7), leukemia/lymphoma related factor/Pokemon (8, 9), hypermethylated in cancer-1, and Kaiso (10, 11). Based on analyzing gene expression levels in all 130 deduced human BTB/POZ genes using serial analysis of gene expression data, we recently identified NAC-1 as a carcinoma-associated BTB/POZ gene (12). NAC-1 is a transcription repressor that is involved in self renewal and maintenance of pluripotency in embryonic stem cells (13). In human cancer, our previous study revealed that NAC-1 is significantly overexpressed in several types of carcinomas (12).

To extend these observations to cervical carcinomas, we measured NAC-1 expression levels in cervical carcinoma tissue samples and cell lines and sought to determine the role of NAC-1 in cervical cancer.

Human Cancer Biology
Materials and Methods

Tissue samples and immunohistochemistry. A total of 108 paraffin-embedded tumor tissue and normal cervical tissue samples were obtained from the Department of Obstetrics and Gynecology at Shimane University Hospital, consisting of 84 cervical squamous cell carcinomas, 7 adenocarcinomas, 5 adenosquamous cell carcinomas, 3 cervical intraepithelial neoplasm samples, and 9 normal cervical tissue samples. Patients had received appropriate therapy at Shimane University Hospital between January 1994 and December 2003. Acquisition of tissue specimens and clinical information was approved by the institutional review board of Shimane University. Cervical carcinomas were classified according to the staging system of the International Federation of Gynecology and Obstetrics (14). Invasive carcinomas were divided into 32 cases of stage I disease, 24 of stage II disease, 20 of stage III disease, and 8 of stage IV disease. All tumors were classified histologically according to WHO criteria. Median patient age was 60 y (range, 26-84 y). Primary therapy for the 96 patients with invasive carcinomas was as follows: total hysterectomy in nine patients; radical or modified radical hysterectomy in 18; radical hysterectomy + radiation in 12; radiation in 28; radiation + cisplatin-based chemotherapy in 20; and cisplatin-based concurrent chemoradiotherapy in nine. Patients with incomplete response to radiotherapy or patients with recurrent tumors were treated with a variety of salvage chemotherapy agents, including cisplatin, pepleomycin, and paclitaxel. All 96 patients with invasive cervical carcinoma were followed for survival analysis. The follow-up period ranged from 5 to 90 mo, with a median of 39 mo. NAC-1 mouse monoclonal antibody was a kind gift from Dr. Ie-Ming Shih (Johns Hopkins Medical Institutions). Immunohistochemistry was done on deparaffinized sections using the NAC-1 antibody at a dilution of 1:100 and an EnVision + System peroxidase kit (DAKO). Immunoreactivity was scored by two investigators as follows: 0, undetectable; 1+, weakly/moderate positive; 2+, intensely positive. NAC-1 immunoreactivity was not detectable (immunointensity score, 0) in normal cervical epithelium and cervical intraepithelial neoplasms (CIN1-CIN3).

Western blot analysis was done using the same antibody (1:100) on seven cervical carcinoma cell lines. Quantitative PCR analysis. A total of 16 frozen cervical cancer samples (2 adenocarcinomas, 2 adenosquamous carcinoma, 12 squamous cell carcinomas) and six samples of normal cervix were analyzed for NAC-1 transcript expression by quantitative real-time PCR using an ABI 7000 (Applied Biosystems) with SYBR Green dye (Molecular Probes). Averages in the threshold cycle number (Ct) of duplicate measurements were obtained. The results were expressed as the difference between the Ct of the gene of interest and the Ct of a control gene (e.g., APP), for which expression is relatively constant among serial analysis of gene expression libraries analyzed (15).

Cell culture, apoptosis counting, and Western blot analysis. Human cervical adenocarcinoma cell lines (e.g., HeLa, HeLa TG, and HeLa P3) were obtained from Tohoku University. Human cervical epidermoid carcinoma cell lines ME180 and CaSki were also obtained from Tohoku University. The human cervical squamous cell carcinoma line TCS and SKGIIIa were obtained from Riken Bioresource Center and Tohoku University, respectively. All cell lines were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The apoptotic index was expressed as the percentage of apoptotic figures obtained by counting at least 300 of 4′,6-diamidino-2-phenylindole–stained cells under a fluorescence microscope.

Western blot analysis was done using the same antibody (1:100) on seven cervical carcinoma cell lines: HeLa, HeLa TG, HeLa P3, ME180, CaSki, SKGIIIa, and TCS. Similar amounts of total proteins from each lysate were loaded and separated on 10% Tris-glycine-SDS polyacrylamide gels and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes (Millipore). Western blots were developed by chemiluminescence (Pierce). To assess whether NAC-1 overexpression affected growth activity, we constructed a mammalian expression vector, pCMV/NAC-1 with V5 tag at the COOH terminus. The cDNA of NAC-1 was prepared from a clinical sample that overexpressed NAC-1, underwent PCR, and was cloned to the mammalian expression vector pCDNA6/V5-His A (Invitrogen). The clone was sequenced to ensure the wild-type coding sequence of NAC-1. The pCMV/NAC-1 vector was...
stably transfected into TCS, CaSki, and HeLa P3 cells using the Nucleofector II electroporator (Amaxa). Selection of pCMV/NAC-1 stable clones was done with use of minimal dilutions in selection medium containing 3 to 6 \( \mu \)g/mL blasticidine (Invitrogen).

Small interfering RNA knockdown of NAC-1 gene expression. Two small interfering RNA (siRNA) sequences that targeted NAC-1 were designed with sense sequences of UGAUGUACACGUUGGUGCCUGU-CACCA and GAGGAAGAACUCGGUGCCCUCCAU. Control siRNA (off-target control) was purchased from IDT. Cells were seeded into 96-well plates and transfected with siRNAs using oligofectamine (Invitrogen). Cell number was determined indirectly by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay 72 h after transfection of siRNA (16). Apoptotic cells were detected using 4',6-diamidino-2-phenylindole staining. Data were expressed as mean ± 1 SD from triplicates. To confirm the presence of apoptotic cells, 4',6-diamidino-2-phenylindole–stained cells were also stained with Annexin V dye.

Cell proliferation assay. Cells were seeded in 96-well plates at a density of 3,000 cells per well. The cell number was determined indirectly by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay (16). Data were expressed as mean ± 1 SD from triplicates.

Statistical methods for clinical correlation. Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. Patients whose tumors showed NAC-1 expression and those whose tumors were without expression had similar age and performance status distributions. Data were plotted as Kaplan-Meier curves, and statistical significance was determined by the log-rank test. Multivariate prognostic analysis was done using a Cox proportional hazards model. Data were censored when patients were lost to follow-up. Student's t test was used to examine statistical significance in differences of growth assay data.

Results

NAC-1 expression is higher in adenocarcinomas/adenosquamous cell carcinomas than in squamous cell carcinomas. In contrast to normal cervical tissue and CINs, both squamous cell carcinomas and adenocarcinomas/adenosquamous cell carcinomas showed higher NAC-1 immunoreactivity, with 10% and 50% of cases showing 1+ and 2+ positivity, respectively (\( \chi^2 \) test, Table 1).

### Table 1. Association between NAC-1 expression and clinicopathologic factors in cervical tumors

#### A. NAC-1 immunoreactivity in the cervical tumor and normal cervix

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Immunointensity, n (% )</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cervix</td>
<td>9</td>
<td>9 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CIN</td>
<td>3</td>
<td>3 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCC</td>
<td>84</td>
<td>73 (87)</td>
<td>11 (13)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AC/ASC</td>
<td>12</td>
<td>6 (50)</td>
<td>3 (25)</td>
<td>3 (25)</td>
</tr>
</tbody>
</table>

#### B. The relationship between NAC-1 expression and histologic subtype of cervical carcinoma

<table>
<thead>
<tr>
<th>Negative</th>
<th>Positive</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC</td>
<td>73 (87%)</td>
<td>11 (13%)</td>
</tr>
<tr>
<td>AC/ASC</td>
<td>6 (50%)</td>
<td>6 (50%)</td>
</tr>
</tbody>
</table>

#### C. Univariate and multivariate analysis of prognostic factors in patients with stage II to stage IV cervical squamous cell carcinoma

<table>
<thead>
<tr>
<th>Factors</th>
<th>Patients</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients’ age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;60 y</td>
<td>35</td>
<td>1.5 (0.4-5.1)</td>
<td>0.564</td>
</tr>
<tr>
<td>&lt;60 y</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, II</td>
<td>41</td>
<td>7.4 (1.5-35.0)</td>
<td>0.011</td>
</tr>
<tr>
<td>III, IV</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Status of NAC-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>7.5 (2.2-22.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AC, adenocarcinomas; ASC, adenosquamous carcinomas; SCC, squamous cell carcinomas; FIGO, International Federation of Gynecology and Obstetrics; 95% CI, 95% confidence interval.
P < 0.001; Fig. 1A-D; Table 1A). Positive NAC-1 staining intensity (1+ and 2+) was more frequently found in cells of adenocarcinomas/adenosquamous cell carcinomas than in squamous cell carcinomas (P < 0.01, x² test; Table 1B). To validate the immunohistochemistry results, we did quantitative real-time PCR to assess the correlation between NAC-1 gene expression level and histologic type. We found that increased NAC-1 gene expression level is significantly correlated with adenocarcinomas/adenosquamous cell carcinoma types (Fig. 1E).

Expression of NAC-1 correlates with poor prognosis in patients with squamous cell carcinoma who received radiation therapy. Expression of NAC-1 correlated with clinical outcome in patients with squamous cell carcinoma. A total of 69 patients received radiation as primary therapy. Among them, the nine patients with NAC-1 expression had a shorter overall survival than the peers whose tumors did not express NAC-1 (P = 0.001; log-rank test; Fig. 2). Univariate analysis showed that stage III or stage IV disease and positive NAC-1 expression correlated with shorter overall survival. When data were stratified for multivariate analysis, stage III or stage IV disease and positive NAC-1 expression remained significant (P = 0.022 and P = 0.005, respectively) for shorter overall survival (Table 1C).

Functional analysis of NAC-1 expression. NAC-1 is essential for cell growth and survival in cervical cancer cell lines that overexpress NAC-1. NAC-1 siRNA was applied to the culture medium of seven cervical cancer cell lines, including two adenocarcinoma lines and one epidermoid carcinoma line that had overexpression of NAC-1 (e.g., HeLa, HeLa TG, and ME180) and four cervical carcinoma cell lines (HeLa P3, CaSkI, SKGIIa, and TCS) that did not express NAC-1 (Fig. 3A). siRNA treatment significantly reduced NAC-1 protein expression compared with control siRNA treatment (Fig. 3B). NAC-1 siRNA reduced cell number most significantly in HeLa, HeLa TG, and ME180 cells, which overexpressed NAC-1 compared with reduction in cell number of the other lines (P < 0.001, Student’s t test; Fig. 3C). Inhibition of cell growth after repressing NAC-1 expression in HeLa, HeLaTG, and ME180 cells was likely a result of induced apoptosis: the percentage of apoptotic cells identified using 4',6-diamidino-2-phenylindole staining was significantly increased for NAC-1 siRNA-treated cells compared with control siRNA-treated cells (Fig. 4A and B). Similar results were obtained from Annexin V staining in an apoptotic assay (Supplementary Fig. S1).

Next, to confirm the results of NAC-1 knockdown experiments, we randomly selected two clones from TCS, CaSkI, and HeLa P3 cells without endogenous NAC-1 expression that were stably transfected with a NAC-1 expression vector. When compared with vector-transfected controls, all TCS, CaSkI, and HeLa P3 clones with NAC-1 expression had higher cellular proliferation based on growth assay results (Fig. 5A and B and Supplementary Fig. S2).

Discussion

NAC-1 was first discovered and cloned as a novel transcript induced in the nucleus accumbens of rats treated with cocaine (17). Recently, we reported that NAC-1 is a tumor recurrence–associated gene with oncogenic potential in ovarian cancer (12). Except for its role in ovarian tumorigenesis, the function...
of NAC-1 in other carcinomas is unknown. In this study, we showed that some cervical carcinomas expressed NAC-1 using immunohistochemistry and gene expression analysis. No expression of NAC-1 protein could be detected in normal cervical tissue and in CINs. This finding, taken together with our previous study showing that activation of NAC-1 leads to cell transformation, suggests that NAC-1 may have an oncogenic role in cervical carcinoma (12).

Uterine cervical cancer is an example of a tumor with a clinically important histologic variant. Squamous cell carcinoma accounts for the majority of tumors. Adenocarcinomas and adenosquamous carcinomas comprise 10% to 18% of all cervical malignant tumors and seem to be increasing in absolute number of cases, as well as in relative percentage of invasive cervical tumors diagnosed (18, 19). The observed survival rate for patients with adenocarcinoma/adenosquamous carcinoma has generally been poorer than the survival rate for patients with squamous cell carcinoma (19, 20). In this study, the significantly higher frequency of NAC-1 expression in adenocarcinoma/adenosquamous carcinoma compared with squamous cell carcinoma is of great interest, suggesting that adenocarcinoma/adenosquamous carcinoma and squamous cell carcinoma have distinct gene expression profiles and thus may have a different pathway for development of carcinoma (21). In this hypothetical model, adenocarcinomas/adenosquamous carcinomas and squamous cell carcinomas develop independently and are characterized by different molecular genetic changes and gene expression profiles (21). The result in this study presents new insight into the biology of cervical adenocarcinoma/adenosquamous carcinoma and may lead to development of a new therapeutic strategy for these types based on use of a NAC-1 inhibitor.

Prognostic markers that can predict clinical outcome, including treatment response and overall survival, have substantial clinical effect on management of patients with cervical cancer (22). To further explore the clinical relevance of NAC-1 expression in cervical carcinomas, we correlated NAC-1 expression and overall survival in our population of patients with cervical carcinoma. Adenocarcinomas/adenosquamous carcinomas are usually more refractory to conventional radiotherapy than squamous cell carcinomas, and overall survival rates for these two types are worse than for squamous cell carcinomas (19, 23, 24). Therefore, we analyzed the correlation between NAC-1 expression and survival only in patients with squamous cell carcinomas. Interestingly, we found a positive correlation between poor prognosis and NAC-1 expression in patients who received radiotherapy. The mechanism underlying the association between NAC-1 expression and shorter survival is not known; however, because mortality of cervical cancer patients is directly related to recurrence of disease after radiotherapy, it is conceivable that

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**Fig. 4.** Detection of apoptotic cells. A, the experiment was done 72 h after NAC-1 siRNA and control siRNA treatment. Many of the NAC-1-siRNA-treated cells but not the control siRNA-treated cells show morphologic features typical of apoptosis. B, treatment with NAC-1 siRNA increases apoptosis of HeLa, HeLa TG, and ME180 cells, as measured by 4',6-diamidino-2-phenylindole staining.

**Fig. 5.** Functional analysis of NAC-1 expression. A and B, Western blot analysis shows that NAC-1 transfected TCS and CaSki clones (C1 and C2) express NAC-1 protein. Anti-V5 antibody was used to detect NAC-1 protein. C and D, the NAC-1 clones of TCS and CaSki cells show higher proliferative activity than the vector-only controls, as evidenced by a time-dependent increase in cell number in low (0.5%) serum-containing medium.
NAC-1 expression may confer resistance to radiotherapy and/or enhance cell proliferation in radiotherapy-resistant recurrent tumors. Because the etiology of tumor recurrence is multifactorial, it is likely that NAC-1 expression confers a growth advantage to tumor cells by providing them with higher proliferative and lower apoptotic activity, as was shown in this study. Because the current study identifies the previously undescribed pattern of NAC-1 expression in cervical carcinomas, further studies are required to elucidate the relation between NAC-1 expression and tumor sensitivity to radiation. This study had another limitation: patients whose disease recurred after primary therapy were also treated with various chemotherapy agents, including cisplatin, peplomycin, and paclitaxel. Therefore, it is not entirely clear that the effect of NAC-1 on sensitivity to radiotherapy plays the primary role in the survival patterns seen in this study. To address this limitation, additional studies that analyze NAC-1 function in large numbers of patients treated with chemotherapeutic agents, such as cisplatin and paclitaxel, should be done.

Although siRNA is an effective and convenient approach to assess functional roles of genes by silencing their expression, it is essential to ensure that the phenotypic changes seen after siRNA treatment are the results of specific gene knockdown rather than control siRNA effects associated with the siRNA approach itself (25). In this study, we used two NAC-1 siRNAs and control siRNA that we had previously designed (12). The phenotypic changes were only seen in cervical cancer cells with NAC-1 expression and not in those without detectable NAC-1 expression. Reduction of NAC-1 expression resulted in apoptosis in the NAC-1–expressing cell lines HeLa, HeLa TG, and ME180, indicating that NAC-1 is essential for proliferation and survival of these cell lines. These results are similar to results obtained in our previous experiments in ovarian cancer cell lines (12). Besides its critical role in maintaining cell proliferation and survival, NAC-1 overexpression could also enhance cellular proliferation. These findings suggest that NAC-1 is a gene with significant cell growth and survival effects in cervical carcinomas.

In conclusion, we showed that NAC-1, a member of the BTB/POZ family, is expressed in a subset of cervical carcinomas. Of special interest is the finding that expression is more common in adenocarcinomas/adenosquamous carcinomas that are more refractory to conventional radiotherapy. Furthermore, NAC-1 expression is a prognostic factor for patients whose squamous cell carcinomas were treated with radiotherapy. Therefore, NAC-1 expression may be considered a predictive marker of resistance to radiotherapy in patients with squamous cell carcinomas. We further predict that a drug targeting NAC-1 might be useful in combination with radiotherapy for improvement of prognosis of patients with cervical carcinomas.

References
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