Fatty acid synthase expression associated with NAC1 is a potential therapeutic target in ovarian clear cell carcinomas

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BACKGROUND: This study examined the clinical significance of NAC1 and the expression level of its potential downstream target fatty acid synthase (FASN) in ovarian clear cell carcinomas (OCCCs), and evaluated the NAC1/FASN pathway as a potential therapeutic target.

METHODS: NAC1 and FASN expression and NACC1 gene amplification were assessed in ovarian cancers by immunohistochemistry, fluorescence in situ hybridisation, and clinical data collected by a retrospective chart review. C75, a FASN inhibitor, was used to assess whether this pathway represented a therapeutic target in OCCCs.

RESULTS: High NAC1 expression was most frequent in clear cell tumours (40.0%:24/60). NACC1 gene amplification was identified in none of the 58 OCCCs. The frequency of NACC1 gene amplification was significantly higher in the high-grade serous histology than in the clear cell histology (P < 0.01). NAC1 expression was significantly correlated with FASN expression in both OCCC samples and OCCC cell lines. Either high NAC1 expression or high FASN expression significantly correlated with shorter progression-free and overall survival (P = 0.002 and 0.0048). NAC1 overexpression stimulated FASN expression, and NAC1 silencing using siRNA decreased FASN expression in OCCC cell lines. Profound growth inhibition was observed in C75-treated carcinoma cells with FASN overexpression when compared with the response in carcinoma cells without FASN expression.

CONCLUSION: These findings indicate that NAC1/FASN overexpression is critical to the growth and survival of a subset of OCCC. The FASN silencing by the C75-induced phenotypes depends on the expression status of the targeted cell line. Therefore, NAC1/FASN pathway-targeted therapy may benefit selected OCCC patients.

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Ovarian carcinoma is the most lethal gynaecological malignancy worldwide (Wingo et al, 1995). Despite a dramatic improvement in survival following the introduction of platinum-taxane combination chemotherapy, the overall 5-year survival remains around 45%. Ovarian carcinoma is classified into four major histological types (serous, mucinous, endometrioid, and clear cell), which are distinct on a molecular level (Cho and Shih, 2009). Ovarian clear cell carcinoma (OCCC), which constitutes around 25% of ovarian carcinomas (serous, mucinous, endometrioid, and clear cell), which are distinct on a molecular level (Cho and Shih, 2009). Ovarian clear cell carcinoma (OCCC), which constitutes around 25% of ovarian carcinomas, carries a poor prognosis despite 60% being diagnosed at an early stage (Sugiyama et al, 2000). Unlike its serous counterpart, OCCC is more frequently platinum resistant (Winter et al, 2007). This may be a reflection of the fundamental molecular differences between OCCC and serous carcinomas. Therefore, chemotherapeutics targeting genes and pathways unique to OCCC may represent one option for improving survival. One such gene, which has recently been shown to have a role in taxane resistance, is NAC1 (Ishibashi et al, 2008; Jinawath et al, 2009).

NAC1 is a member of the BTB/POZ family of proteins that participate in several cellular functions including proliferation, apoptosis, transcription control, and cell morphology maintenance (Stogios et al, 2005). By serial analysis of gene expression levels in all 130 deduced human BTB/POZ genes, we identified NAC1 as a carcinoma-associated BTB/POZ gene (Nakayama et al, 2006). NAC1 is a transcription repressor that is involved in the self-renewal and maintenance of pluripotency in embryonic stem cells (Wang et al, 2006). NAC1 is significantly overexpressed in several types of human carcinomas (Nakayama et al, 2006; Ishibashi et al, 2008; Yeasmin et al, 2008). The levels of NAC1 expression correlate with tumour recurrence in ovarian serous carcinomas, and intense NAC1 immunoreactivity in primary ovarian tumours predicts early recurrence (Nakayama et al, 2006; Ishibashi et al, 2008). Using a quantitative proteomic method employing tandem mass spectrometry (MS/MS) and spectral counting, Ueda et al (2010) demonstrated that NAC1 regulated fatty acid synthase (FASN) expression. Fatty acid synthase (FAS), the enzyme responsible for the de novo synthesis of fatty acids, has emerged as a potential therapeutic target in human cancers. Fatty acid synthase catalyses the condensation of malonyl-CoA and acetyl-CoA to produce long-chain fatty acids (Wakil, 1989). High levels of FAS expression have
been found in ovarian cancer (Gansler et al, 1997; Alo et al, 2000; Ueda et al, 2010) and in most human solid tumours (Kuhajda, 2006). The upregulation of FAS expression in cancer cells has been linked to both mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways through the sterol regulatory binding element binding protein 1c (Van de Sande et al, 2002; Yang et al, 2002; Yang et al, 2003). Fatty acid synthase protein expression denotes poor prognosis in breast and prostate cancers (Alo et al, 1996; Pizer et al, 2001).

NAC1 and FASN expression has not been investigated with respect to ovarian carcinoma in histological subtypes other than serous (Pizer et al, 2001; Ueda et al, 2010). In this study, we further investigated the clinicopathological role of NAC1-regulated FASN expression in OCCC, as well as the possibility of FASN-based therapeutics in OCCC.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed, paraffin-embedded tissue samples of 144 ovarian cancers including 44 serous carcinomas, 10 mucinous carcinomas, 60 clear cell carcinomas, and 20 endometrioid carcinomas were used in this study. Samples were obtained from the Department of Obstetrics and Gynaecology at the Shimane University Hospital and the Department of Obstetrics and Gynaecology at Seirei Hamamatsu General Hospital. Diagnosis was based on conventional morphological examination of sections stained with haematoxylin and eosin (H&E), and tumours were classified according to the WHO classification. Tumour staging was performed according to the International Federation of Gynaecology and Obstetrics (FIGO) classification. All patients were primarily treated with cytoreductive surgery and adjuvant platinum and taxane or CPT-11 chemotherapy (CBDBC AUC 5 with paclitaxel 175 mg m\(^{-2}\) or docetaxel 70 mg m\(^{-2}\) or CDDP 60 mg m\(^{-2}\) with CPT-11 180 mg m\(^{-2}\)). All patients received 6–12 courses of this combination regimen. Acquisition of tissue specimens and clinical information was approved by an institutional review board (Shimane University and Serrei Hamamatsu General Hospital). The paraffin tissue blocks were organised into tissue microarrays, each was made by removing 3-mm diameter cores of tumour from the block. Selection of the area to core was made by a gynaecological oncologist (KN) and pathology technician (KI), and was based on review of the H&E slides.

Immunohistochemistry

The method for NAC1 immunohistochemistry and evaluation criteria has been detailed in a previous report (Ishibashi et al, 2008). For immunohistochemistry, paraffin sections after deparaffinisation were incubated with a primary mouse FASN antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1 : 100 in a 4 °C moist chamber overnight. Two independent observers scored the FASN immunoreactivity using a categorical scoring system from 0 (not detectable) to 3 (intense) with the mean score recorded from triplicates.

Fluorescence in situ hybridisation

BAC clones (RP11-356L15 and CTD-2508D10) containing the genomic sequences of the 19p13.2 ampiclon were purchased from Bacpac Resources (Childrens’ Hospital, Oakland, CA, USA) and Invitrogen (Carlsbad, CA, USA). Bac clones located at Chr19p13.11 (CTD-251801B) were used to generate reference probes. The method for fluorescence in situ hybridisation (FISH) has been described in detail in a previous report (Nakayama et al, 2007).

Cell culture and cell lines

ES2 (clear cell carcinoma) human ovarian cancer cell line was obtained from the American Tissue Culture Centre (Rockville, MD, USA). JHOC9 (clear cell carcinoma) and JHOC5 (clear cell carcinoma) human ovarian cancer cell lines were also obtained from Riken Bioresource Centre (Ibaragi, Japan). The OCCC cell lines OVISE, OVTOKE, and RMGI were obtained from the Japanese Health Science Research Resources Bank. The OCCC cell line OV207 was a kind gift from Dr Vijayalakshmi Shridhar, Mayo Clinic (Rochester, MN, USA).

Western blot analysis

Cell lysates were prepared by dissolving cell pellets in Laemml sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) supplemented with 5% beta-mercaptoethanol (Sigma, St Louis, MO, USA). Western blot analysis was performed on ovarian cancer cell lines ES2, JHOC9, JHOC5, OVISE, OVTOKE, RMGI, and OV207. Similar amounts of total protein from each lysate were loaded and separated on 10% Tris-Glycine-SDS polyacrylamide gels (Novex, San Diego, CA, USA) and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were probed with NAC1 antibody (1 : 100) (Novus Biologicals, Littleton, CO, USA) or FASN (1 : 1000) (Santa Cruz Biotechnology) followed by a peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (1 : 20 000). Western blots were developed by chemiluminescence (Pierce, Rockford, IL, USA). The same membrane was probed with an antibody that reacted with GAPDH (1 : 10 000) (Cell Signaling Technology, Beverly MA, USA) for loading controls and was developed by chemiluminescence (Pierce).

Silencing RNA knockdown of NAC1 gene expression

Two silencing RNAs (siRNAs) that targeted NAC1 were designed with the following sense sequences: 5′-UAGUGUAUGCUUUGGUGCUCUGUCACCA-3′ and 5′-GAGGAGAAGACUCUGGUGCAGCCUCUCUCCA U-3′. Control siRNA (luciferase siRNA) was purchased from IDT (Corvalville, IA, USA). Cells were seeded into 96-well plates and transfected with siRNAs using Oligofectamine (Invitrogen). Following siRNA transfection, cells were collected at 48 h for quantitative real-time PCR analysis of NAC1 and FASN mRNA expression levels.

Quantitative real-time PCR analysis

Relative transcript expression levels were measured by quantitative real-time PCR using a method described previously (Nakayama et al, 2006). The primers for NAC1 and FASN were 5′-CCAGACAC TGCAGATGGAGA-3′ (forward), 5′-AAGCTGAGAGATCAGTGGGA-3′ (reverse) and 5′-GAGGAGAAGACUCUGGUGCAGCCUCUCUCCA U-3′. Control siRNA (luciferase siRNA) was purchased from IDT (Corvalville, IA, USA). Cells were seeded into 96-well plates and transfected with siRNAs using Oligofectamine (Invitrogen). Following siRNA transfection, cells were collected at 48 h for quantitative real-time PCR analysis of NAC1 and FASN mRNA expression levels.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 3000 cells per well. Cell number was determined indirectly by an MT assay (Nakayama et al, 2001). Data were expressed as the mean ± s.d. of triplicate determinations. An MTT cell growth assay was performed 96 h after treating the cells with C75 (Calbiochem, La Jolla, CA, USA) at 10 μM or with DMSO (control). The data were
expressed as a percentage of the DMSO control. The mean and s.d. were obtained from three experiments.

**Plasmid constructs and the generation of stable clones**
To assess whether NAC1 overexpression induced FASN overexpression in OCCC, we used a mammalian expression vector, pCMV/NAC1, with a V5 tag at the COOH terminus as previously described (Nakayama et al., 2006).

The pCMV/NAC1 vector was stably transfected into the ES2 cell line using the Nucleofector II electroporator (Amaxa, Köln, Germany). Selection of pCMV/NAC1 stable transfected cells was performed in selection medium containing 3 \( \mu \)g/ml 6-mercaptopurine (Invitrogen).

**Statistical methods for clinical correlation**
Progression-free and overall survivals were calculated from the date of diagnosis to the date of first relapse or last follow-up. Age and performance status distributions were similar between patients expressing NAC1 or FASN and those not expressing it. The data were plotted as Kaplan–Meier curves, and the statistical significance was determined by the log-rank test. Data were censored when patients were lost to follow-up. The Student’s t-test (for comparison of two groups) or one-way analysis of variance (for comparison of more than two groups) were used to evaluate numeric data.

**RESULTS**

**Relationship between NAC1 protein expression and histological subtype in ovarian carcinoma**
NAC1 immunoreactivity was detected in tumour cell nuclei (Figure 1A). High expression of NAC1 (NAC1 immunointensity \( 2^+ \) and \( 3^+ \)) was observed in 33.3% (44/132) of all analysed tumours. High expression of NAC1 was identified in 15/43 (35%) serous, 1/11 (9%) mucinous, 4/18 (22%) endometrioid, and 24/60 (40%) of clear cell carcinomas. The relationships between NAC1 protein expression and histological subtypes are summarised in Table 1A. Interestingly, the frequency of high NAC1 protein expression was highest in clear cell type in ovarian carcinomas.

As OCCC most frequently had high expression, we focused on OCCCs for the following experiments.

**NACC1 gene amplification was a rare event in OCCCs**
Previously, we reported that NACC1 was a potential oncogene in ovarian cancer and that NACC1 was amplified in 20% of high-grade serous carcinomas (Nakayama et al., 2006; Shih le et al., 2011). Of the 60 OCCCs, 58 had informative cases. None of the clear cell carcinomas, however (0%: 0/58), had detectable NACC1 gene amplification. A total of 9 out of 43 (20.9%) serous high-grade carcinomas showed significant amplification of NACC1 (\( P < 0.05 \), \( \chi^2 \) test) (Table 1B).

**Relationship between NAC1 protein expression and clinicopathological factors in OCCCs**
High expression of NAC1 (NAC1 immunointensity \( 2^+ \) and \( 3^+ \)) was observed in 40% (24/60) of the analysed tumours. Patients were stratified into one of the two groups depending on the status of NAC1 immunostaining. The relationships between NAC1

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**Table 1A** The relationship between NAC1 expression and histological subtype

|                | Negative | Positive |
|----------------|----------|----------|
| Serous         | 28 (65%) | 15 (35%) |
| Mucinous       | 10 (91%) | 1 (9%)   |
| Endometrioid   | 14 (78%) | 4 (22%)  |
| Clear cell     | 36 (60%) | 24 (40%) |

Mucinous vs clear cell, \( P < 0.05 \)

**Table 1B** The relationship between NACC1 amplification and histological subtype

|                | Normal | Amplification |
|----------------|--------|---------------|
| Serous HG      | 34 (79%) | 9 (21%)       |
| Clear cell     | 58 (100%) | 0 (0%)        |

Abbreviation: HG = high grade. \( P < 0.01 \)

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**Figure 1** Immunoreactivity of NAC1 and FASN in OCCC tissues. (A, D) Showing H&E staining of corresponding tissues in upper and lower panels, respectively. (B) Intense NAC1 immunoreactivity is present in the nuclei of OCCC cells (upper centre panel). (C) Intense FASN immunoreactivity is present in the cytoplasm of OCCC cells (upper centre panel). (E, F) An OCCC case with negative staining of both NAC1 and FASN respectively.
Correlation of NAC1 protein expression and FASN protein expression in OCCC cell lines

NAC1 and FASN expression levels measured with immunohistochemistry were then correlated with protein expression levels by western blot in several OCCC cell lines (Figure 3A). NAC1 protein expression levels significantly correlated with FASN protein expression level in the OCCC cell lines ($r = 0.877$, $P < 0.01$) (Figure 3B).

NAC1 knockdown leads to decreased FASN expression in OCCC cell lines

We previously showed that siRNA knockdown of NAC1 significantly reduced NAC1 expression compared with control siRNA treatment (Nakayama et al, 2010). Reduction of NAC1 gene expression significantly inhibited FASN gene expression in OCCC lines OV207 and JHOC9 (Figure 3C).

Constitutive expression of NAC1 leads to increased FASN expression in OCCC cell lines

Next, to confirm the results of NAC1-knockdown experiments, we generated stable NAC1-expressing cells from ES2 cells, which have low endogenous NAC1 expression. This cell line was stably transfected with a NAC1 pcMV vector. When compared with vector-transfected controls, the ES2 cell line that expressed NAC1 had higher FASN gene expression levels as measured by real-time PCR (Figure 3D).

C75 suppresses growth in OCCC cells

The above findings suggest that FASN is one of the downstream targets of NAC1, a gene overexpressed in a subset of OCCC OCCC. This raises the possibility that FASN expression contributes to the OCCC phenotype. To assess the contribution of FASN expression to OCCC cell growth and survival, OCCC cell lines were treated with the FASN inhibitor C75, a FASN inhibitor, and FASN levels measured and cell growth assessed. The FASN protein expression status tended to be correlated with growth inhibition by the C75 FASN inhibitors (Figure 3E and F).

DISCUSSION

Ovarian clear cell carcinomas, which comprise ~25% of all ovarian carcinomas in Japan, display a distinct gene expression profile relative to other histological types (Schwartz et al, 2002; Yamaguchi et al, 2010). They are more aggressive and carry a worse prognosis than stage-matched serous adenocarcinomas (Goff et al, 1996; Sugiyama et al, 2000). This is likely because OCCC is frequently refractory to platinum-based chemotherapy (Goff et al, 1996; Sugiyama et al, 2000). The current immunohistochemical analysis of a panel of ovarian tumours demonstrated strong expression of NAC1 in 40% of clear cell carcinomas, in comparison with high expression in 9–35% of serous, endome-triodi, and mucinous tumours. Given this, NAC1 may represent a potential molecular target in patients with OCCC. We have demonstrated that high NAC1 expression correlates with shorter survival in patients with ovarian carcinoma treated with...
platinum-based chemotherapy regardless of histological type (Ishibashi et al., 2008; Nakayama et al., 2010). Recently, Zhang et al. (2012) reported that NAC1 modulates sensitivity of ovarian cancer cells to cisplatin by altering the HMGB1-mediated autophagic response. It is plausible that while high NAC1 expression in OCCC is one possible explanation for why some of these tumours have a worse prognosis, clearly this applies to only a subset of tumours; therefore other mechanisms likely exist.

NACC1 gene amplification accounts for the increased expression in many high-grade ovarian serous carcinomas; however, some serous carcinomas did have increased NAC1 expression in the absence of gene amplification (Nakayama et al., 2010; Shih Ie et al., 2011). In contrast, NACC1 gene amplification was undetectable in all clear cell carcinoma specimens tested, which suggests that NAC1 in this histology may be regulated at the transcriptional level.

Recently, Ueda et al. (2010) reported that FASN is a potential downstream target of NAC1 in serous high-grade ovarian carcinoma; however, it is unclear if this is the case in other histological subtypes. Therefore, to assess the relationship between NAC1 and FASN in clear cell histology, we used both knockdown and overexpression systems. We first knocked down NAC1 in OCCC lines, JHOC9, and OV207, using a previously designed siRNA (Yeasmin et al., 2008). Reduction of NAC1 expression resulted in decreased FASN expression in the NAC1-knockdown cell line, indicating that FASN was a likely downstream target of NAC1. Conversely, an ES2 cell line overexpressing NAC1 had significantly increased FASN gene expression. These reciprocal findings suggest that FASN is a potential downstream target of NAC1 in OCCCs. Our observations augment the growing body of evidence suggesting that the transcriptional factor NAC1 regulates FASN in multiple histological types of ovarian carcinomas.

In the present study, we demonstrated that OCCC cell lines with FASN overexpression were more sensitive to a potent FASN inhibitor, C75, suggesting that FASN-targeted therapy may have activity in this subset of OCCC. The mechanism underlying the upregulation of FASN in OCCC is not clear and likely involves multiple pathways. In several types of carcinoma, including ovarian carcinoma, FASN overexpression robustly induces de novo lipogenesis. The generated lipids are integrated into membrane lipid rafts and modulate membrane receptor tyrosine phosphorylation.

Figure 2  (A, B) NAC1 overexpression tended to correlate with shorter progression-free/overall survival in patients with OCCC who received cytoreductive surgery, followed by a standard platinum-based chemotherapy regimen ($P = 0.119$ and $0.318$ respectively, log-rank test). (C) Kaplan–Meier survival analysis showing that high expression of FASN was associated with a shorter progression-free survival in comparison to low FASN expression in OCCCs ($P = 0.021$, log-rank test). (D) Kaplan–Meier survival analysis showing that high FASN expression tended to correlate with a shorter overall survival than low FASN expression in OCCC ($P = 0.075$, log-rank test). (E, F) Either high NAC1 or high FASN expression correlated with shorter progression-free/overall survival in OCCC ($P = 0.004$ and $0.037$ respectively, log-rank test).
kinases (for example, the EGFR family). This, in turn, results in the initiation of oncogenic signalling pathways involving cell survival, proliferation, migration, and invasion (Jackowski et al, 2000; Menendez et al, 2005). The FASN overexpression increases EGFR and HER2 protein expression and tyrosine phosphorylation, and thereby amplifies oncogenic signalling pathways that contribute to tumourigenic transformation (Vazquez-Martin et al, 2008). The effects of the FASN pathway are likely mediated by both the FASN kinase activity itself as well as factors along the pathway, for example, in prostate cancer in which cavelin-1 and KLF5/SREBP-1 function upstream of FASN (Di Vizio et al, 2007; Zhou et al, 2007). Thus, at least in OCCCs, the NAC1 pathway represents another mechanism for controlling FASN expression and pathway activity. Unlike other members of the BTB/POZ family, NAC1 has a BEN domain instead of the zinc-finger DNA-binding domain (Abhiman et al, 2008). The BEN theoretically may function as a DNA-binding domain (Abhiman et al, 2008); however, this has not been directly demonstrated. It does act as a transcription co-repressor with other BTB/POZ proteins (Korutla et al, 2009). Moreover, it has also been shown to interact with nuclear proteins potentially involved in tumourigenesis, including Nanog (Ma et al, 2009), CoREST (Korutla et al, 2007), and HDAC3 and HDAC4 (Nakayama et al, 2010). Thus, it is possible that FASN expression is indirectly controlled by NAC1 through binding with its specific partner(s). Identification of the NAC1/FASN pathway sheds new light on the molecular mechanism by which NAC1 promotes tumour progression in OCCC. Further studies are required to elucidate the transcriptional regulation of FASN by NAC1.

As with other carcinomas of the colon, prostate, and breast (Alo et al, 1996; Rashid et al, 1997; Rossi et al, 2003), FASN overexpression appears to correlate with worse prognosis, and overexpression is found more frequently in the aggressive OCCC and to a lesser-degree serous carcinomas. More importantly, in patients with OCCC, the high level of FASN significantly correlated with poor prognosis, suggesting that FASN contributed to the aggressive phenotype. A similar observation has been reported for NAC1 (Ishibashi et al, 2008; Nakayama et al, 2010). It is therefore plausible that the NAC1/FASN pathway represents one factor driving OCCC progression. How FASN contributes to disease aggressiveness in OCCC remains to be elucidated. Besides endowing drug resistance, FASN may enhance oncogenesis via cellular mechanisms such as enhancing the Wnt (Fiorentino et al, 2008), c-Met (Coleman et al, 2009), and proteosome pathways (Little et al, 2008). Moreover, upregulation of FASN confers a growth and survival advantage by blocking apoptosis under hypoxia, a common condition in solid tumours and malignant effusions (Harris, 2002; Furuta et al, 2008).

In conclusion, we have demonstrated that NAC1, a member of the BTB/POZ family, and its potential downstream target FASN are overexpressed in a subset of OCCC. Of special interest is the finding that NAC1 expression is most common in the clear cell subtype of ovarian carcinomas, which are frequently chemoresistant (Goff et al, 1996; Sugiyama et al, 2000). Furthermore, NAC1/FASN expression is a biomarker of poor outcome for patients treated with conventional platinum-based chemotherapy in OCCCs. New-generation FASN inhibitors deserve consideration in future clinical trials involving OCCC, particularly for patients who are refractory to platinum-based chemotherapy.

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Conflict of interest
The authors declare no conflict of interest.
NAC1 associated FASN expression related to poor outcome in OCCC

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