Expression of NLRP7 (PYPAF3, NALP7) Protein in Endometrial Cancer Tissues

SATOSHI OHNO[1,2*], TAKESHI KINOSHITA[3*], YUMIKO OHNO[2], TOSHINARI MINAMOTO[4], NOBUTAKA SUZUKI[1,2], MASAKI INOUE[2] and TAKASHI SUDA[3]

Departments of [1]Complementary and Alternative Medicine Clinical R&D and [2]Obstetrics and Gynecology, Kanazawa University, Graduate School of Medical Science, Ishikawa; Divisions of [3]Immunology and Molecular Biology and [4]Translational and Clinical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa, Japan

Abstract. *Background: Nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 7 (NLRP7) (pyrin-containing apoptotic protease activating factor-1-like protein 3; PYPAF3, NACHT domain-, leucine-rich repeat, and pyrin domain-containing 7; NALP7) has been thought to contribute to innate immunity and inflammation. Although expression of NLRP7 in human seminoma tissues and several cancer cell lines has been demonstrated, the pathophysiological and prognostic importance in cancer tissues has not been defined. Materials and Methods: A series of 70 endometrial cancer cases that had undergone curative resection was studied to determine the correlation between NLRP7 expression and clinico-pathological characteristics in human endometrial cancer tissue. Tissue specimens were evaluated for NLRP7 by immunohistochemistry. Results: NLRP7 expression was positive in cancer cells in 7 cases (10%). There was a statistical relationship between the depth of tumor invasion and NLRP7 expression (p=0.0326). NLRP7 expression showed a trend for being associated with poor prognosis. Conclusion: Tumor-produced NLRP7, associated with myometrial invasion, might provide additional prognostic information in endometrial cancer patients.*

Endometrial cancer is the most common gynecological malignancy in the United States. In Japan, it is the second most common gynecological cancer, but its frequency has dramatically increased in the last decade. Although there are well-established surgical and chemotherapeutic treatments for endometrial cancer, the need for molecular-target therapy has increased, especially for recurrent disease that has acquired radio- or chemoresistance. Thus, there is a need for a better understanding of the molecular pathways of endometrial carcinogenesis.

Nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 7 (NLRP7) (pyrin-containing apoptotic protease activating factor-1-like protein 3; PYPAF3, NACHT domain-, leucine-rich repeat, and pyrin domain-containing 7; NALP7) is a member of the recently emerged NLR (CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats; CATERPILLER, PYPAF, nucleotide-binding and oligomerization domain; NOD, NALP) family, that is thought to be involved in innate immunity and inflammation (1, 2). Several NLR proteins such as NLRP3 (cryopyrin, PYPAF1) and NLRP1 (NALP1) function as a platform to activate caspase-1 that in turn catalyzes proteolytic maturation of interleukin-1β (IL-1β). In contrast, NLRP7 has been found to inhibit caspase-1-mediated IL-1β maturation in a genetic reconstitution study using the human embryonic kidney (HEK) 293 cell line (3), suggesting an anti-inflammatory function of NLRP7. Mutation in the maternal *NLRP7* gene causes recurrent hydatidiform moles and reproductive wastage in humans (4). On the other hand, overexpression and an oncogenic role of NLRP7 in human testicular seminomas have been reported (5). Thus, NLRP7 plays important roles in both physiological and pathological processes in humans.

The expression of *NLRP7* mRNA has also been observed in several cancer cell lines (3). However, to date little has been reported on the clinicopathological significance of NLRP7 in human cancer tissues. Whether or not localization of NLRP7 expression correlated with clinicopathological factors was therefore examined in a series of endometrial cancer tissues. The prognostic significance of NLRP7 protein in endometrial cancer patients was also assessed.

*Both authors contributed equally to this work.

Correspondence to: Satoshi Ohno, Department of Complementary and Alternative Medicine Clinical R&D, Kanazawa University, Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa, 920-8640, Japan. Tel: +81 76 265 2147, Fax: +81 76 234 4247, e-mail: satoshio@med.kanazawa-u.ac.jp

Key Words: NLRP7, immunohistochemistry, endometrial cancer, clinicopathological factors, prognosis.

Materials and Methods

Patients. This study included 70 primary endometrial carcinoma patients who had been consecutively admitted, treated and followed-up at the Department of Obstetrics and Gynecology, Kanazawa University Hospital from January 1995 to December 2002. All the patients had received no pre-surgical treatment and undergone a total abdominal or radical hysterectomy plus bilateral salphingo-oophorectomy. At the time of laparotomy, peritoneal fluid samples were obtained for cytological testing. Systemic pelvic lymphadenectomy was performed in 51 (72.9%) patients. Paraaortic lymph node sampling was performed in two patients because of visible or palpable enlarged lymph nodes. All the patients were classified by the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system (1988). No patients had remaining macroscopic tumors or known distant metastasis immediately after surgery. The high-risk patients (*e.g.* deep myometrial invasion, cervical involvement, special histology, peritoneal cytology) underwent external radiotherapy and/or six cycles of chemotherapy (paclitaxel: 180 mg/m^2, carboplatin: according to Chatelut's formula [AUC=5 mg min/ml]) as post-operative adjuvant therapy. The treatment was followed by a gynecological examination, recording of laboratory data, transvaginal/abdominopelvic ultrasonography and a radiological investigation. The data from regular follow-up visits to the outpatient department were stored in a database specifically designed for endometrial carcinoma patients. A telephone inquiry to update the present status of all surviving patients was made in July 2003. The exact date of disease recurrence was obtained from the referring physicians or from the physicians who attended the patient for the initial diagnosis of the recurrence. All the treatments and clinical research were conducted with written informed consent.

Monoclonal antibody. To examine the expression of NLRP7 protein in tissues, a monoclonal antibody (mAb) was established. A part of human NLRP7 (amino acids 1-110) fused to His6-tag was expressed in *Escherichia coli*, purified using a Chelating Sepharose Fast Flow column (Amersham, Uppsala, Sweden) and used as the antigen. Splenocytes from a mouse immunized with the purified antigen were isolated and fused with P3U1 mouse myeloma cells. Hybridomas were selected in hypoxanthine, aminopterin, thymidine (HAT) medium. The culture supernatants of the hybridomas were screened for mAbs specific for the immunized protein by ELISA. The specificity of the mAbs was further confirmed by Western blotting against the lysate from HEK293 cells transfected with human NLRP7 cDNA using mock transfectants as a negative control. Monoclonal antibody purified using a protein G column from the hybridoma clone (6) was used in this study.

Plasmids. Expression plasmids for FLAG- or hemagglutinin (HA)-tagged NLRP3, NLRP2 (PYPAF2) and NLRP7 have been described previously (3).

Western blotting. HEK293T cells were transfected with the various plasmids using polyethyleneimine as described previously (3). Western blotting was performed as described previously (7) except that anti-human NLRP7 mAb was used in this study.

Immunofluorescence confocal microscopy. COS7 cells (African Green Monkey SV40-transformed kidney fibroblast cell line) were transfected with various plasmids using Lipofectamine and PLUS reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Immunofluorescence confocal microscopy was performed as described previously (8) except that anti-human NLRP7 mAb and FITC-labeled sheep anti-mouse IgG antibodies (Cappel, West Chester, PA, USA) were used in this study.

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissues from 70 tumors were retrieved with informed consent from archive sources at Kanazawa University Hospital. The histological diagnosis of each tumor was confirmed on the hematoxylin and eosin-stained sections. Representative sections containing both the normal endometrium and the invasive front of the tumor tissue were selected for immunohistochemical staining. The slides were deparaffinized, and rehydrated in graded alcohols. Epitope retrieval was conducted by microwave heating (5 minutes × 2) using Taget Retrieval Solution (Dako Co. Carpinteria, CA, USA). Endogenous peroxidase activity was quenched by dipping in 3% hydrogen peroxide for 30 minutes. Nonspecific staining was blocked by treating the slides with normal serum for 30 minutes. The slides were incubated with mouse mAbs (clone 273 against NLRP7) at a concentration of 14 μg/ml for 2 hours at room temperature. The subsequent steps were carried out according to the manufacturer's instructions by the EnVision+ ® System-Horseradish Peroxidase (HRP) Labelled Polymer (Dako Cytomation. Carpinteria, CA, USA). Color development was carried out with the peroxidase substrate 3-amino-9-ethylcarbazole (AEC). All the slides were counterstained with Mayer's hematoxylin. Formalin-fixed, paraffin-embedded sections of human seminoma were used as positive controls for NLRP7.

Evaluation of staining. For the assessment of NLRP7 expression, the extent of immunohistochemical staining within the carcinomas was categorized as negative, focally positive (fewer than 20% of carcinoma cells stained) or diffusely positive (more than 20% of carcinoma cells stained). In this study, tumors with focally or diffusely stained cancer cells were considered to exhibit positive expression.

All the histological slides were examined by two observers (S.O. and Y.O.) who were unaware of the clinical data or the disease outcome.

Statistical analysis. The Chi-square test for 2×2 tables was used to compare the categorical data. Mortality and probability of relapse after surgery were compared by Kaplan-Meier analysis and the log-rank statistic. In the analysis of relapse-free survival rates, those who died of causes unrelated to endometrial cancer and those who had no detected evidence of disease recurrence were considered to be relapse-free. A p-value of <0.05 was considered to indicate statistical significance. All the statistical analyses were performed using the statistical package StatView version 5.0 for Macintosh (Abacus Concepts, Berkeley, CA, USA).

Results

Characteristics of the patients. The patients' average age at the time of surgery was 57.3 years (range, 26-78); 22 had premenopausal status, 4 had perimenopausal status and 44 had postmenopausal status. The patients' mean preoperative body mass index (BMI) was 24.0 (range, 16.9 - 32.9). Among the 70 patients, 12 (17.1%) had relapses of

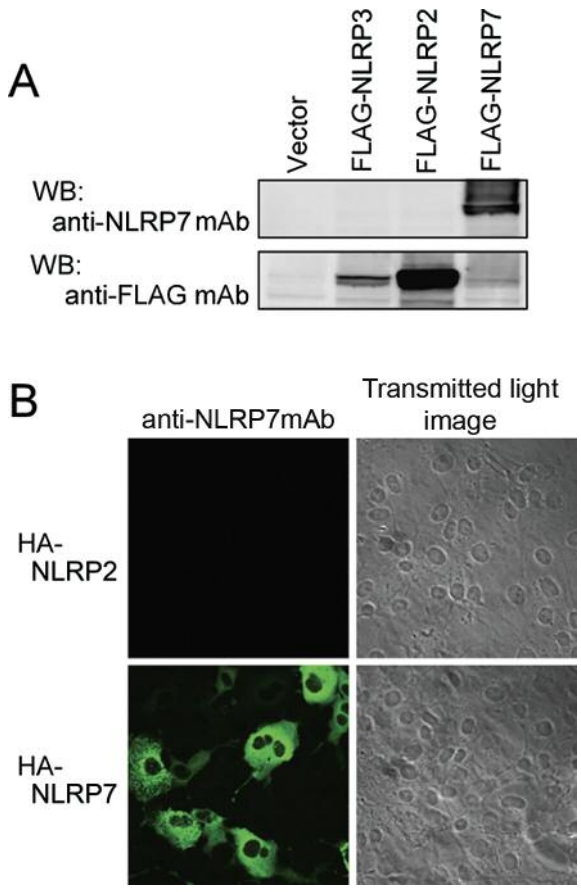

Figure 1. *Specificity of anti-human NLRP7 mAb. (A) Cell lysates of HEK293T cells transfected with empty vector or an expression vector for Flag-tagged NLRP3, NLRP2 or NLRP7 were subjected to Western blot analyses using anti-NLRP7 (upper panel) or anti-FLAG mAb (lower panel). (B) COS7 cells transfected with N-terminally HA-tagged NLRP2 (upper panels) or NLRP7 (lower panels) were grown on glass coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton® X-100 and stained with anti-NLRP7 mAb followed by FITC-labeled anti-mouse antibodies. Fluorescence (left) and transmitted light images (right) are shown.*

endometrial cancer at the time of the last follow-up. The median follow-up time for all the patients was 3.28 years (range, 0.15-8.50 years).

Specificity of the anti-NLRP7 mAbs. The mAb from the hybridoma clone (6) specifically detected an approximately 120 kDa protein in the lysate from the NLRP7, but not the mock, NLRP3 or NLRP2-transfected HEK293 cells (Figure 1A). In addition, the NLRP7, but not the mock-transfected HEK293 cells were specifically stained by immunocytochemistry using this mAb (Figure 1B). These results indicated that this mAb was useful for detecting human NLRP7 specifically using multiple immunochemical techniques.

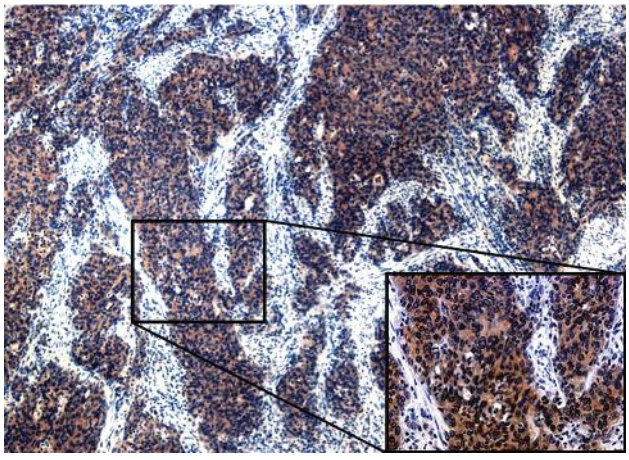

Figure 2. *Representative sections of endometrial cancer with immunohistochemical staining of NLRP7. Strong cytoplasmic staining is observed in the invasion front of the tumor (×40; inset, ×200).*

NLRP7 expression in endometrial cancer. The expression of the NLRP7 antigen within the carcinomas was positive (diffusely positive: 1 case; focally positive: 6 cases) in 7 patients (10%) and negative in 63 patients (90%). The typical NLRP7 expression in endometrial cancer cells is shown in Figure 2. The staining of NLRP7 was heterogeneous in advanced tumors and NLRP7 was frequently located at the invasion front of the tumor. The association between NLRP7 expression and clinicopathological variables is shown in Table I. There was a significant relationship between the depth of tumor myometrial invasion and NLRP7 expression (p=0.0326), indicating up-regulation of NLRP7 expression with tumor progression in this study. Although there were no statistically significant associations between NLRP7 expression and other clinicopathological characteristics, NLRP7-positive cases were found 3.5 times more frequently in postmenopause than pre/peri-menopause cases.

Prognostic impact of NLRP7 expression in endometrial cancer. Although there was no clear statistical significance, NLRP7 expression was a factor negatively influencing the overall and relapse-free survival rate by univariate analysis (Figure 3A and B).

Discussion

In this study, immunohistochemical analysis revealed that the NLRP7 expression was correlated with myometrial invasion in the human endometrial cancer tissues. Moreover, the expression of NLRP7 showed a trend for assosiation with poor prognosis of the endometrial cancer patients. Although further study is required to confirm the statistical significance

Table I. *NLRP7 expression and clinicopathological characteristics.*

Variable	NLRP7 expression		*P*-value (χ^2 test)
	Positive (n=7)	Negative (n=63)	
Age (years)			
<60 (n=43)	4	39	0.8060
≥60 (n=27)	3	24	
FIGO stage			
I (n=52)	5	47	0.8553
II, III, IV (n=18)	2	16	
Lymph node metastasis			
Negative (n=65)	6	59	0.4392
Positive (n=5)	1	4	
Depth (myometrial invasion)			
a, b (a; n=17, b; n=36)	3	50	0.0326
c (n=17)	4	13	
Histopathology-degree of differentiation			
Grade 1 (n=38)	3	35	0.5223
Grade 2, 3 (n=32)	4	28	
Menopause			
Peri, pre (n=26)	1	25	0.1871
Post (n=44)	6	38	
Body mass index			
<25 (n=45)	3	42	0.2123
≥25 (n=25)	4	21	

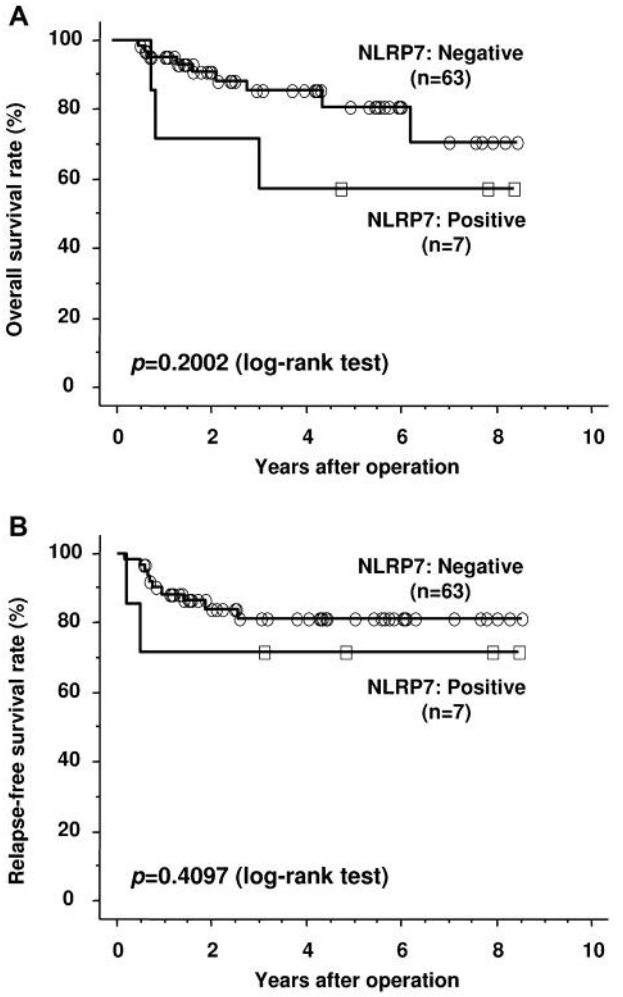

Figure 3. *The Kaplan-Meier survival curves of 70 patients with endometrial carcinoma in relation to NLRP7 expression are shown. (A) Overall survival rate. (B) Relapse-free survival rate.*

of this observation, this trend is likely to be real because myometrial invasion is an independent predictor of poor prognosis in endometrial carcinoma (9). NLRP7-positive cases were also found more often in the postmenopause group which may indicate that the hormonal environment influences NLRP7 expression in endometrial cancer cells. To the best of our knowledge, this is the first report showing a pathophysiological role of NLRP7 expression in endometrial cancer tissues. We also investigated the NLRP7 expression in primary tumors removed from ten patients with stomach cancer and another ten patients with colorectal cancer by immunohistochemistry using the anti-NLRP7 mAb. However, no apparent expression of NLRP7 was found in these tumors (data not shown).

In a previous report (5), overexpression of NLRP7 was observed in a seminoma tissue. NLRP7 protein is also overexpressed in the Tera-1 human embryonal carcinoma cell line, and knockdown of NLRP7 expression using siRNA inhibited proliferation of this cell line *in vitro* (5). Similarly, overexpression of NLRP7 in endometrial cancer may promote proliferation of cancer cells and thus causes myometrial invasion and poor prognosis. Alternatively, because NLRP7 has an anti-inflammatory potential (3) and NLRP10, another NLRP protein, has both anti-inflammatory and anti-apoptotic activities (7), such functions of NLRP7 may also be involved in this outcome. The expression of NLRP7 was frequently observed at the invasive front of the cancer tissues. This fact may indicate that NLRP7 expression is involved in the invasiveness of cancer cells. Alternatively, NLRP7 expression in the cancer cells may be a result of interaction between the cancer cells and the surrounding tissues or immune cells.

Malignant solid tumors usually develop into compact masses with a peripheral vital zone between cancer cells and the adjacent normal tissue. It is now becoming clear that vital microenvironments in the invasive margin of tumors contribute to cancer growth and spread, and to the suppression of antitumor immunity (10, 11). Earlier, we showed that NLRP7 inhibits caspase-1-dependent interleukin-1β processing (3). We have also reported that the large numbers of immune cells along the tumor margin stay

idle at the periphery of the tumor and are unable to exert an antitumor effect (12). There is a possibility that the expression of NLRP7 in the invasive front of cancer provides malignant tissue with a suitable milieu for their growth and spread, through inducements to immunosuppression.

In conclusion, tumor-produced NLRP7, associated with myometrial invasion, might provide additional prognostic information in endometrial cancer patients. Further study is required to determine the precise role of NLRP7 in the development and progression of endometrial cancer and its value in cancer therapy.

Acknowledgements

This work was supported by a Grant-in-Aid for Young Scientists (B) (No. 19791140, for S.O.), and a Grants-in-Aid for Scientific Research on Priority Areas (Cancer) (No. 18013022, for T. S.) from the Ministry of Education, Culture, Sports, Science and Technology, of the Japanese Government. We are grateful to the staff at the Pathology Section, Kanazawa University Hospital, for collecting the samples and providing paraffin-embedded tissues.

References

1 Inohara N and Nunez G: NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol *3*: 371-382, 2003.

2 Tschopp J, Martinon F and Burns K: NALPs: a novel protein family involved in inflammation. Nat Rev Mol Cell Biol *4*: 95-104, 2003.

3 Kinoshita T, Wang Y, Hasegawa M, Imamura R and Suda T: PYPAF3, a PYRIN-containing APAF-1-like protein, is a feedback regulator of caspase-1-dependent interleukin-1beta secretion. J Biol Chem *280*: 21720-21725, 2005.

4 Murdoch S, Djuric U, Mazhar B, Seoud M, Khan R, Kuick R, Bagga R, Kircheisen R, Ao A, Ratti B, Hanash S, Rouleau GA and Slim R: Mutations in *NALP7* cause recurrent hydatidiform moles and reproductive wastage in humans. Nat Genet *38*: 300-302, 2006.

5 Okada K, Hirota E, Mizutani Y, Fujioka T, Shuin T, Miki T, Nakamura Y and Katagiri T: Oncogenic role of NALP7 in testicular seminomas. Cancer Sci *95*: 949-954, 2004.

6 Stehlik C, Lee SH, Dorfleutner A, Stassinopoulos A, Sagara J and Reed JC: Apoptosis-associated speck-like protein containing a caspase recruitment domain is a regulator of procaspase-1 activation. J Immunol *171*: 6154-6163, 2003.

7 Wang Y, Hasegawa M, Imamura R, Kinoshita T, Kondo C, Konaka K and Suda T: PYNOD, a novel Apaf-1/CED4-like protein is an inhibitor of ASC and caspase-1. Int Immunol *16*: 777-786, 2004.

8 Hasegawa M, Imamura R, Kinoshita T, Matsumoto N, Masumoto J, Inohara N and Suda T: ASC-mediated NF-kappa B activation leading to IL-8 production requires caspase-8 and is inhibited by CLARP. J Biol Chem *280*: 15122-15130, 2005.

9 Prat J: Prognostic parameters of endometrial carcinoma. Hum Pathol *35*: 649-662, 2004.

10 Balkwill F and Mantovani A: Inflammation and cancer: back to Virchow? Lancet *357*: 539-545, 2001.

11 Coussens LM and Werb Z: Inflammation and cancer. Nature 420: 860-867, 2002.

12 Ohno S, Tachibana M, Fujii T, Ueda S, Kubota H and Nagasue N: Role of stromal collagen in immunomodulation and prognosis of advanced gastric carcinoma. Int J Cancer *97*: 770-774, 2002.

Received January 28, 2008
Revised April 22, 2008
Accepted May 5, 2008