Jikeikai Med J 2011 ; 58 : 57-62

Expression of the Wild Type Rearranged during Transfection Protooncogene in Ovarian Cancer

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ABSTRACT

Background : The rearranged during transfection (RET) protooncogene is expressed in a variety of cancers. The pathogenesis of ovarian cancer is poorly understood. The aim of this study was to determine whether the RET protooncogene is expressed in ovarian cancer.

Materials and Methods : The reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemical methods were used to confirm the expression of the RET protooncogene in two ovarian cancer cell lines and ovarian tumor samples.

Results : The PCR products of the RET protooncogene were 300 bp in both ovarian cancer cell lines. On immunohistochemical analysis using an anti-RET polyclonal antibody, positive signals were observed in 59 of 82 cases of ovarian cancer (72.0%). The rates of RET expression in ovarian cystadenomas and ovarian cystadenomas with borderline malignancy were 20.7% and 53.3%, respectively.

Conclusion : The wild type RET protooncogene is expressed in ovarian cancer. This result suggests that the RET protooncogene is involved in the pathogenesis of ovarian cancer.
(Jikeikai Med J 2011 ; 58 : 57-62)

Key words : RET protooncogene, ovarian cancer, immunohistochemistry

INTRODUCTION

Ovarian cancer is a leading cause of death among gynecologic malignancies. Although survival rates have increased somewhat owing to adjuvant chemotherapy with paclitaxel and carboplatin, the overall survival rate in patients with ovarian cancer remains poor[1], because ovarian cancer is diagnosed at an advanced stage in most patients and because effective therapies are not available to prevent recurrence in patients who have shown a complete response to chemotherapy. Recently, new therapeutic approaches, such as targeted therapy, have been explored to improve the prognosis of patients with ovarian cancer. To develop a targeted therapy for ovarian cancer, a tumor-specific antigen must be identified.

The rearranged during transfection (RET) protooncogene encodes a receptor tyrosine kinase. The receptor tyrosine kinase controls cell growth and differentiation. It is also known to be activated as oncogenes in human tumors. In addition, RET is a characteristic protooncogene found in several hereditary and nonhereditary diseases, such as multiple endocrine neoplasia (MEN) 2A, 2B,

Received for publication, April 14, 2011
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Hirschsprung's disease, and papillary thyroid carcinoma[2-4]. In our previous study RET was expressed in all neuroblastomas developed from 11 tumor cell lines and 29 fresh tumor cells[5]. Glial cell line-derived neurotrophic factor (GDNF) knock-out mice show phenotypes similar to those of RET knock-out mice[6-8], implying that RET is a functional receptor for GDNF. Because the GDNF receptor itself has no cytoplasmic domain serving as a signal transducer, the tyrosine kinase domain of RET has been proposed to function as a transmitter of the biological signals triggered by GDNF[9].

Moreover, we already obtained a monoclonal antibody to the RET extracellular domain (NBL-1)[10]. For tumor-cell targeting, monoclonal antibodies are frequently used. In addition, several novel peptides that can bind specific molecules and cells have been identified[11-13].

Park et al. have reported that a rationally designed anti-HER2/neu peptide can inhibit the growth of breast cancer cells *in vitro* and *in vivo*[13], suggesting that targeting peptides can mimic the activities of monoclonal antibodies. In the treatment of breast cancer trastuzumab (trade name : Herceptin) is a humanized recombinant monoclonal antibody that recognizes the extracellular domain of the HER2 transmembrane protein. Trastuzumab was the first clinically applied immunologic target specific drug. Its development represents a model for integrating new agents[14,15]. Therefore, the identification of tumor-associated cell surface antigens is important for the development of tumor-targeted antibody therapy[15].

The aim of this study was to confirm the expression of the RET protooncogene in patients with ovarian cancer and to establish the effective ovarian tumor targeting diagnosis and therapy using the anti-RET monoclonal antibody.

MATERIALS AND METHODS

Cell lines

Two cell lines derived from human ovarian cancer were used. The 2008 cell line[16] was kindly provided by Dr. Howell, and the A2780 cell line[17] was provided by Dr. Ozds. Both NB-39-nu cells (human neuroblastoma cells)[18] and HL60 cells (human promyelocytic leukemia cells) were used as RET positive controls.

Tissue samples

Ovarian cancer tissue samples were resected from 82 patients (mean age, 48±11 years) who had been admitted to The Jikei University School of Medicine from 1989 through 1999.

The clinicopathologic diagnoses of ovarian tumors were ovarian cystadenoma (87 cases ; mean age, 38±13 years) and ovarian cystadenomas with borderline malignancy (15 cases ; mean age, 40±15 years). All histological diagnoses were reviewed according to established morphological classification criteria of the World Health Organization. Overall survival was defined by the interval from the first surgery to death.

Reverse transcriptase-polymerase chain reaction

Total tissue RNA was isolated from 2 ovarian cancer cell lines by using the RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Two micrograms of each RNA sample was subjected to complementary DNA synthesis with an Omniscript RT kit (Qiagen) according to the manufacturer's protocol. The polymerase chain reaction (PCR) was subsequently performed to analyze the expression of RET by using 5′<AGATCCTGGAGGATCCAAAG<3′ and 5′<GTATTTGGCGTACTCCACGA<3′ as forward and reverse primers, respectively. The PCR was performed using 1 μl of template, 0.5 μl of each 10 μM primer, 0.1 μl of Taq DNA polymerase (TaKaRa, Tokyo, Japan), 0.5 μl of 10 mM deoxyribonucleoside triphosphate, 2.5 μl of 10×buffer, and 19.9 μl of purified and ionized water (MilliQ, Millipore Corp., Billerica, MA, USA), in a total volume of 25 μl. The thermal cycler conditions used were 94°C for 5 minutes followed by 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR products were visualized with ethidium bromide staining after separation over a 1% agarose gel.

Characterization of the RET protooncogene in ovarian cancer cell lines

Exons 10, 11, and 13 to 16 of the RET protooncogene, which are the most common mutation sites of the RET protooncogene in other diseases, were analyzed in cells of the 2008 cell line[19]. Briefly, each exon was amplified with PCR and reported primers[20] and then cloned into the pTOPO

vector (Invitrogen, Tokyo, Japan). Several independent clones were subjected to sequence analysis with an automated sequencer (Prism 370, Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

Immunohistochemical staining was performed as follows. The tissues were fixed immediately with buffered 10% formalin for 24 to 72 hours, dehydrated, and embedded in paraffin. Four-micrometer-thick sections were cut and deparaffinized in ethanol and xylene. Immunohistochemical staining was performed with an immunoperoxidase avidin-biotin conjugate system, with diaminobenzidine and hydrogen peroxide as the substrate and with hematoxylin as the counterstain. Slides were rinsed in a phosphate buffer (pH 7.4) for examination. The sections were incubated with a primary polyclonal anti-RET antibody[21]. All primary antibodies were titrated by dilution (1 : 500 with anti-RET polyclonal antibodies) to obtain optimal intensity of specific staining with minimal nonspecific background reactivity. The secondary antibody was a biotinylated horse anti-mouse immunoglobulin (Zymed, South San Francisco, CA, USA) for use with primary polyclonal antibodies. Negative controls initially consisted of tissue processed without inclusion of the primary antibody. The slides were examined with light microscopy and the intensity of immunostaining was evaluated. The intensity of immunostaining in all slides was classified into 4 levels : level 0 for negative staining, level 1 for lower intensity, level 2 for moderate intensity, and level 3 for the highest intensity. To simplify the results, intensity levels 0 and 1 were defined as negative staining, and levels 2 and 3 were defined as positive staining, except for cases in which the number of stained cells in levels 2 and 3 were less than 50% of all cells in a slide.

Statistical analysis

Fisher's exact test were used to compare the RET-positivity rate among patients with different histologic diagnoses (cystadenoma, cystadenoma with borderline malignancy, and cancer). All statistical analyses were performed with a statistical software program (SAS version 9.1, SAS Institute, Cary, NC, USA). A p value of <0.05 was considered to indicate significance.

Results

Expression and characterization of the RET protooncogene in ovarian cancer cell lines

Synthesized complementary DNAs from 2 cell lines

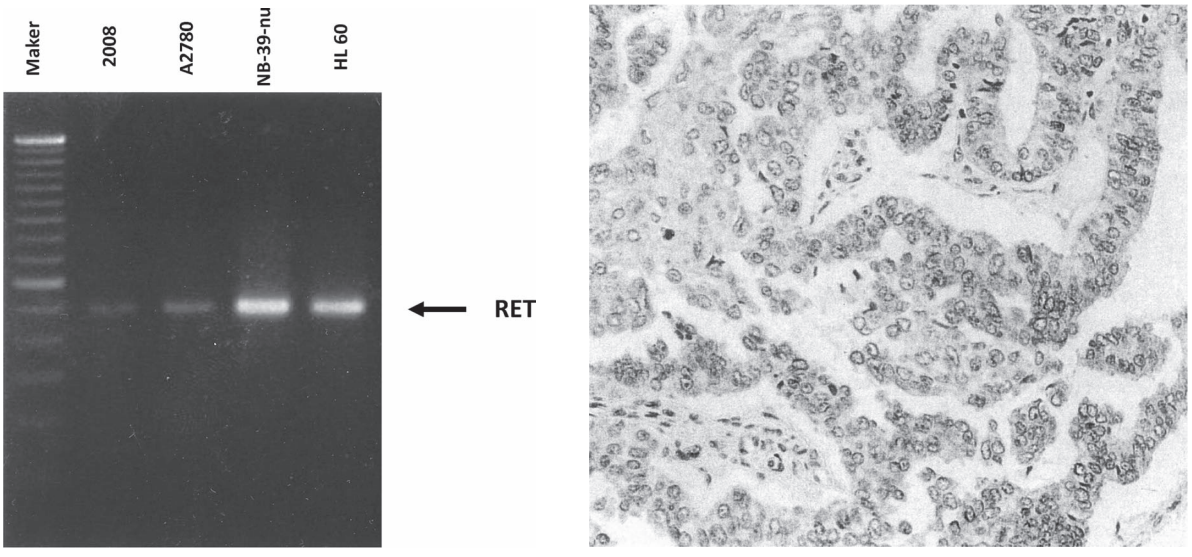

Fig. 1. RET protooncogene expression in ovarian cancer. A, Reverse trascriptase-PCR with primers RET yielded an expected band of 300 bp in 2008 cells and A2780 cells. Both NB-39-nu cells and HL 60 cells served as positive controls. B, Immunohistochemical analysis of ovarian cancer showing cytoplasmic staining for RET.

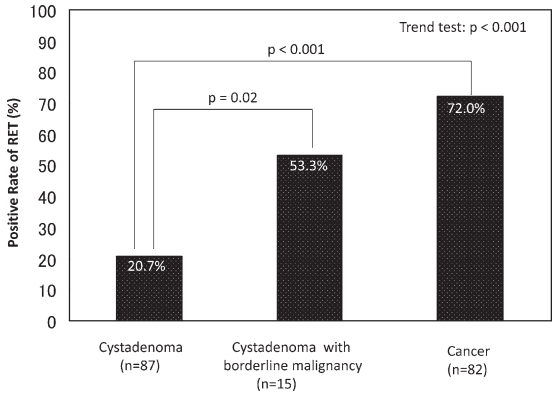

Fig. 2. RET expression in relation to histological type. The rates of RET staining in 3 histologic types : ovarian cystadenoma, cystadenoma with borderline malignancy, and ovarian cancer.

Table 1. Correlation between RET expression and clinicopathologic features in Ovarian Cancer (n=82)

Clinicopathologic Parameters	No. of Patients	RET positive rate
FIGO stage		
I	45 (54.9%)	84.4%
II	7 (8.5%)	71.4%
III	26 (31.7%)	53.9%
IV	4 (4.9%)	50.0%
Histologic type		
Serous	25 (30.5%)	48.0%
Endometrioid	10 (12.2%)	70.0%
Mucinous	18 (21.9%)	100%
Clear cell	29 (35.4%)	75.9%
Grade		
1	24 (29.3%)	79.2%
2	46 (56.1%)	71.7%
3	12 (14.6%)	58.3%
Age		
50 >	45 (54.9%)	77.8%
50 ≦	37 (45.1%)	64.9%

(2008 cells, A2780 cells) were successfully amplified with RET primers. The size of PCR products was 300 bp (Fig. 1A). To characterize the RET protooncogene in 2008 cells, PCR amplification followed by nucleotide sequencing was peformed. Analysis of exons 10, 11, and 13 to 16 revealed no mutations (data not shown).

Immunohistochemical staining of ovarian tumors for RET

Immunohistochemical staining for RET proteins was found predominantly in the cytoplasm, but also in the nucleus (Fig. 1B). Among the 82 cases of ovarian cancer (Table 1), the frequency of RET expression was high in mucinous cystadenocarcinoma (18 of 18 cases ; 100%), clear cell adenocarcinoma (22 of 29 cases ; 75.9%), and endometrioid adenocarcinoma (7 of 10 cases ; 70.0%). According to disease stage, the frequency of RET-positive cases was 84.4% (38 of 45 cases) in stage I, 71.4% (5 of 7 cases) in stage II, 53.9% (14 of 26 cases) in stage III, and 50% (2 of 4 cases) in stage IV. According to histological grade, the rate of RET-positive staining was 79.2% in grade 1, 71.7% in grade 2, and 58.3% in grade 3. According to histological type, the rate of RET expression was 72.0% (59 of 82 patients) in ovarian cancer, 53.3% (8 of 15 patients) in ovarian cystadenoma with borderline malignancy, and 20.7% (18 of 87 patients) in ovarian cystadenoma (Fig. 2).

Correlation of RET expression with clinicopathologic characteristics

The rate of RET expression (Fig. 2) was significantly higher in ovarian cancer (p<0.001) or ovarian cystadenoma with borderline malignancy (p=0.02) than in ovarian cystadenoma.

DISCUSSION

The prognosis of advanced ovarian cancer is poor, with a 5-year survival rate of 30% to 35%, because most cases are not diagnosed until the advanced stage. Furthermore, if the first-line chemotherapy is not effective against ovarian cancer, cancer will continue to grow and spread. Mucinous cystadenocarcinoma and clear cell adenocarcinoma have been reported to show chemoresistance. Our present study has found high expression of the RET protooncogene in mucinous cystadenocarcinoma and clear cell adenocarcinoma. However, the relation between chemoresistance and expression of the RET protooncogene is unclear.

Several research efforts have focused on the identification of new biological markers of prognosis in ovarian cancer. Activation of oncogenes, such as HER-2, and inactivation of onco-suppressor genes, such as p53, have been used in an attempt to assess the prognosis of ovarian cancers[22]. Moreover, *in vitro* and *in vivo* studies have ob-

tained some evidence concerning genes related to cellular apoptosis in ovarian carcinogenesis, such as Bcl-2 and p53, and the resistance to chemotherapy in ovarian cancer[23,24]. Aberrations of the proteins produced by these genes are frequently observed in ovarian cancer. However, the degree of protein aberration does not correlate with prognosis.

Ovarian cancer, especially endometrioid adenocarcinoma and clear cell adenocarcinoma, often co-exist with endometriosis. The potential for carcinogenesis, such the change to endometrioid adenocarcinoma or clear cell adenocarcinoma, cannot be ruled out in any case of endometriosis, because no marker is available for identifying this change. The present study is, to our knowledge, the first to examine the expression of RET in a wide spectrum of ovarian tumors. In this study we have found that RET is frequently expressed in endometrioid adenocarcinoma (70%) and clear cell adenocarcinoma (75.9%). Thus, RET expression may be useful for assessing the potential for carcinogenesis and for deciding the follow-up period after surgery for endometriosis. We are studying a larger number of cases of endometriosis to further validate our findings. We hypothesized that RET expression would be a good maker to assess the malignant potential of ovarian tumor. This study suggests that, in the future, RET may be useful for recognizing whether a patient has an ovarian cystadenoma or a potential malignant tumor before operation. We hope that the findings of our study will also lead to new targeted therapies for ovarian cancer.

Acknowledgments : We express great thanks to Dr. Yasuko Shiraishi (Oak Clinic, Chiba), Dr. Loreto B. Feril, Jr. (Fukuoka University School of Medicine, Fukuoka), and Professor Masahide Takahashi (Nagoya University Graduate School of Medicine, Nagoya) for their comments that helped us in the completion of this work.

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