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Expression of EBAG9/RCAS1 is associated with advanced disease in human epithelial ovarian cancer

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Oestrogen receptor-binding fragment associated gene 9, EBAG9, is an oestrogen-responsive gene that was identified in MCF-7 human breast carcinoma cell line. It is identical to RCAS 1, a cancer cell surface antigen possibly involved in immune escape. In the present study, we examined the expression of EBAG9/RCAS1 in human epithelial ovarian cancer using immunohistochemistry, immunoblotting and reverse transcription–polymerase chain reaction (RT–PCR). A total of 90 epithelial ovarian cancer cases were examined immunohistochemically by means of the antibodies for EBAG9 and ERα. The correlation between EBAG9 immunoreactivity and clinicopathological parameters was examined. mRNA expression of EBAG9 and ERα were evaluated by RT–PCR in 22 cases. The expression for EBAG9 and ERα was examined by immunoblotting in 12 ovarian cancer cell lines. EBAG9 immunoreactivity was detected in the surface and cytoplasm of carcinoma cells in 46 out of 90 cases (51.1%). EBAG9 expression was significantly higher in serous histology ($P=0.0402$) and advanced disease ($P=0.0206$). No significant relationship was detected between EBAG9 immunoreactivity and overall survival ($P=0.689$). There was a highly significant correlation between EBAG9 and ER immunoreactivity ($P<0.0001$). The EBAG9 mRNA was detected in 20 out of 22 cases. In all of the cases that were positive for ERα mRNA, they were also positive for EBAG9 mRNA. Immunoreactive band corresponding to EBAG9 was detected in 11 out of 12 of ovarian cancer cell lines, and was consistent with ERα expression. In conclusion, the wide distribution of EBAG9 and its relation to advanced disease suggest that this protein may play important roles in epithelial ovarian cancer.
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Epithelial ovarian cancer is the leading cause of death from gynaecological malignancies in the great majority of developed countries (Akahira J *et al*, 2001; Akahira JI *et al*, 2001,). Sex steroid hormones have been implicated in the aetiology and/or progression of some epithelial ovarian cancers. Both oestrogen (ER) and progesterone receptors (PR) have been reported in human epithelial ovarian cancer (Rao and Slotman, 1991; Akahira *et al*, 2002). In endometrial and breast carcinomas, steroid hormone receptor status correlates well with response to hormonal manipulation and prognosis (McGuire, 1978; Benraad *et al*, 1980; Ehrlich *et al*, 1981; Kaupilla, 1984). However, in epithelial ovarian carcinoma, the prognostic significance of tumour ER status among patients still remains controversial (Bizzi *et al*, 1988; Masood *et al*, 1989; Sevelda *et al*, 1990; Rao and Slotman, 1991; Hempling *et al*, 1998).

Recently, oestrogen receptor-binding fragment associated gene 9 (EBAG9) has been identified as an oestrogen-responsive gene from a cDNA library of MCF-7 human breast cancer cell line (Watanabe *et al*, 1998). Oestrogen receptor-binding fragment associated gene 9 is identical to the receptor-binding cancer antigen expressed in SiSo cells (RCAS1) (Nakashima *et al*, 1999). The EBAG9/RCAS1 is a membrane molecule that acts as a ligand for a putative receptor present in cells (Nakashima *et al*, 1999). *In vitro* studies have also demonstrated that EBAG9/RCAS1 inhibits growth of activated $CD3^+$ T lymphocytes, suggesting a possible involvement in the immune escape of neoplastic cells (Nakashima *et al*, 1999). Endocrine-immune interactions are considered to play an important role in the development and/or progression of various hormone-dependent neoplasms, but the details of these interactions remain unclear.

Oestrogen receptor-binding fragment associated gene 9 is demonstrated to be widely distributed in human breast carcinoma, and may play an important role in the development of this oestrogen-dependent cancer (Suzuki *et al*, 2001). Others reported that RCAS1 is associated with poor prognosis and/or advanced stage in various human cancers (Sonoda *et al*, 1996; Kaku *et al*, 1999; Izumi *et al* (2001); Nakakubo *et al*, 2002). However, the expression of EBAG9 and its clinical significance have not been examined in epithelial ovarian cancer. Therefore, in this study, we examined the expression of EBAG9 in human epithelial ovarian cancer using immunohistochemistry, reverse transcription–polymerase chain reaction (RT–PCR) and immunoblotting. We also evaluated the correlations of EBAG9 immunoreactivity with various clinicopathological parameters and ER status.

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MATERIALS AND METHODS

We studied a total of 90 cases of common epithelial ovarian carcinoma. Information regarding age, performance status on admission, histology, stage, grade, residual tumour after primary surgery and overall survival was retrieved from the review of patient charts. The median follow-up time of the patients in this study was 54 months (18–112 months). Of 90 patients, 76 (84.4%) received platinum-containing chemotherapy after operation. Performance status was defined according to WHO criteria (World Health Organization, 1979). Histology, stage and grade were determined according to FIGO (International Federation of Gynecology and Obstetrics) criteria (Shimizu *et al*, 1998). Residual disease was determined by the amount of unresectable tumour left following primary cytoreductive surgery. Optimal cytoreduction was defined as no gross residual tumour greater than 2 cm in diameter, whereas suboptimal cytoreduction was defined as any gross residual disease remaining greater than 2 cm in diameter. Overall survival was calculated from the time of initial surgery to death, or the date of last contact. Survival times of patients still alive or lost to follow-up were censored in December 2002. All of these archival specimens were retrieved from the surgical pathology files at Tohoku University Hospital, Sendai, Japan. These specimens were all fixed in 10% formalin and embedded in paraffin. Among these 90 cases, 22 cases were available for examination by RT-PCR analysis. These specimens were dissected immediately into small pieces following gross dissection, quickly transferred to liquid nitrogen, and then stored at −80°C until further use. The research protocol was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan.

OVCAR3, Caov3, SKOV3, TOV112D, TOV21G, OV90 and ES2 (adenocarcinoma: OVCAR3, SKOV3; serous adenocarcinoma: Caov3, OV90; clear cell adenocarcinoma: TOV21G, ES2; endometrioid adenocarcinoma: TOV112D) cell lines were purchased from American Type Culture Collection. JHOS2, JHOS3, HTOA, OMC3 and JHOC5 (serous adenocarcinoma: JHOS2, JHOS3, HTOA; mucinous adenocarcinoma: OMC3; clear cell adenocarcinoma: JHOC5) cell lines were purchased from Riken cell bank (Tsukuba, Japan). Cell lines were maintained in DMEM/F12 (Invitrogen, CA), supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin (Invitrogen, CA), and incubated in 5% CO_2 at 37°C.

Immunohistochemistry

Immunohistochemical analysis was performed using the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan), and have been previously described in detail (Akahira JI *et al*, 2001, Akahira J *et al*, 2001). Oestrogen receptor-binding fragment associated gene 9 antibody was a rabbit polyclonal antibody against a GST-EBAG9 fusion protein (Tsuchiya *et al*, 2001). The characterisation of this antibody was confirmed by Western blotting (Suzuki *et al*, 2001). Monoclonal antibody for ERα was purchased from Immunotech (Marseille, France). For antigen retrieval, the slides were heated in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dyhydrate, pH 6.0). The dilutions of primary antibodies for EBAG9 and ERα were 1:200 and 1:2, respectively. The antigen-antibody complex was visualised with 3,3′-diaminobenzidine (DAB) solution (1 mm DAB, 50 mM Tris-HCl buffer (pH 7.6) and 0.006% H_2O_2), and counterstained with haematoxylin. The ER-positive breast carcinoma tissue was used as a positive control for EBAG9 (Suzuki *et al*, 2001). As negative controls, 0.01 M phosphate-buffered saline (PBS) and normal mouse IgG were used in place of primary antibodies. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunostaining

For statistical analyses of EBAG9 immunoreactivity, carcinomas were classified independently by two of the authors (MA and JA) into two groups: +, positive carcinoma cells; and −, no immunoreactivity. Cases with disconcordant results among the observers were re-evaluated. For evaluation of ERα immunoreactivity, labeling index (LI) was obtained in carcinoma cells as described by Sasano *et al* (1996). In brief, two of the authors (JA and TM) independently evaluated at least 500 carcinoma cells microsopically and the percentage of immunoreactivity was determined. In the present study, interobserver differences were less than 5%, and the mean of the two values was obtained.

Reverse transcription-PCR

Total RNA was isolated from tissues by phenol-chloroform extraction using Isogen (Nippon Gene, Japan), and was treated by DNase I (Roche, Germany). The RT-PCR kit (SUPERSCRIPT Preamplification system, Invitrogen) was employed and cDNA synthesis was carried out according to the instructions. cDNAs were synthesised from 5 μg of total RNA using random hexamer and RT was carried out for 50 min at 42°C with SUPERSCRIPT II reverse transcriptase. After an initial 1-min denaturation step at 94°C, 35-cycle PCRs were carried out on a DNA thermal cycler (PTC-200 DNA Engine, MJ Research, Inc., USA) under the following conditions: 1-min denaturation at 94°C, 1-min annealing at 58°C for EBAG9, 62°C for ERα and 2-min extension at 72°C. Primers for PCR reactions were as follows: EBAG9: 5′sense – GCTACACAAGATTCTGCCT and 3′ antisense – CTTCTTCATT AGCCGTTGTG (680–892, 213 bp); ERα: 5′ sense – AAGAGCTGCC AGGCCTGCC and 3′ antisense – TTGGCAGCTCTCATGTCTCC (702–869, 168 bp); β-actin: 5′ sense – CCAACCGCGAGAAGAT GAC and 3′ antisense – GGAAGGAAGGCTGGAAGAGT (382–841, 459 bp). In initial expreriments, following amplification, PCR products were purified and subjected to direct sequencing to verify amplification of the correct sequences (ABI prism 310 Genetic Analyzer, Applied Biosystems, CA, USA). β-Actin primers were utilised as positive controls. Negative controls without RNA and without reverse transcriptase were also performed.

Immunoblotting

Cells were grown to 70% confluence in 10-cm plates and after removal of culture medium with PBS, whole-cell protein was extracted by conventional method. The protein concentration was measured by Model 680 microplate reader (Biorad, USA) using BradFord reagent (Biorad). In all, 20 μg of protein of each sample was mixed with an equal volume of 2× concentrated SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled, and then electrophoresed on 7% ready-made gels containing SDS (Mini Protian II Western blotting system, Biorad). Proteins were then transferred to nitrocellulose membrane (Hybond PDVF, Biorad). The membranes were incubated in blocking solution (PBS containing 5% nonfat milk and 0.05% Tween-20), and then incubated in 1:200 dilution of EBAG9 antibody (1:2 for ERα and 1:1000 for Actin) in blocking solution overnight at 4°C. After incubation with HRP-rabelled anti-rabbit IgG (Vector Laboratories, Inc., USA), the antigen-antibody complex was visualised with ECL system (Amersham, Germany). MCF-7 breast cancer cell line was used as a positive control (Watanabe *et al*, 1998). Actin (Ab-1, Oncogene) was used as internal positive controls.

Statistical analysis

Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc., NC, USA) software. The statistical significance

between EBAG9 and characteristics of the patients was evaluated using Mann–Whitney *U*-test, Kruskal–Wallis and Scheffe analysis. The correlation between EBAG9 and ERα immunoreactivity was also assessed using Mann–Whitney *U*-test. Univariate analysis of prognostic significance for prognostic factors was performed using a log-rank test, after each survival curve was obtained by the Kaplan–Meier method. All patients who could be assessed were included in the intention-to-treat analysis. A result was considered significant when the *P*-value was less than 0.05.

RESULTS

Results of immunohistochemistry and their correlation with clinicopathological parameters are summarised in Table 1. Immunoreactivity for EBAG9 was detected on the surface and in the cytoplasm of epithelial ovarian cancer tissues. Oestrogen receptor α immunoreactivity was confined exclusively to the nuclei of tumour cells (Figure 1). The number of cases immunopositive for EBAG9 was 46 out of 90 cases (51.1 %). The median LI for ERα was 12.8 % (0–85.2%). As shown in Table 1, EBAG9 expression was significantly higher in serous histology ($P=0.0402$) and advanced disease ($P=0.0206$). There was no significant relationship between EBAG9 immunoreactivity and patient age, histological grade, residual tumour or performance status. There was a highly significant correlation between EBAG9 immunoreactivity and ERα LI ($P<0.0001$).

Results of univariate analysis of prognostic significance for each variable, with respect to survival, are summarised in Table 2. Among the clinicopathological factors examined, those significantly associated with overall survival were histology, grade, stage and residual tumour. No significant relationship was detected between EBAG9 immunoreactivity and overall survival ($P=0.689$).

Relationships between EBAG9 immunoreactivity and EBAG9, ERα mRNA in 22 cases are summarised in Table 3. Oestrogen receptor-binding fragment associated gene 9 mRNA was positive in 20 cases. In four cases, EBAG9 immunoreactivity was not detected although its mRNA was present. In all of the cases that

Table 1 Association between EBAG9 immunoreactivity and clinicopathological parameters in human ovarian cancer

	EBAG9 immunoreactivity		
	+($n=46$)	−($n=44$)	*P*-value
Age (years)	52.8 ± 1.8	49.5 ± 1.5	NS
Histological type			
Serous	27	14	
Mucinous	7	9	
Endometrioid	3	10	
Clear cell	9	11	0.0402
Histological grade			
Grade 1	15	16	
Grade 2	11	15	
Grade 3	12	6	NS
Stage			
I,II	17	27	
III,IV	29	17	0.0206
Residual tumour			
<2 cm	27	34	
>2 cm	19	10	NS
PS			
0,1	30	32	
2,3,4	16	12	NS
ER LI	18.8	11.4	<0.0001

Performance status score: 0 = asymptomatic and fully active; 1 = symptomatic, fully ambulatory, restricted in physically strenous activity; 2 = symptomatic, ambulatory, capable of self-care, more than 50% of waking hours are spent out of bed; 3 = symptomatic, limited self-care, spends more than 50% of time in bed, but not bedridden; 4 = completely disabled, no self-care, bedridden.

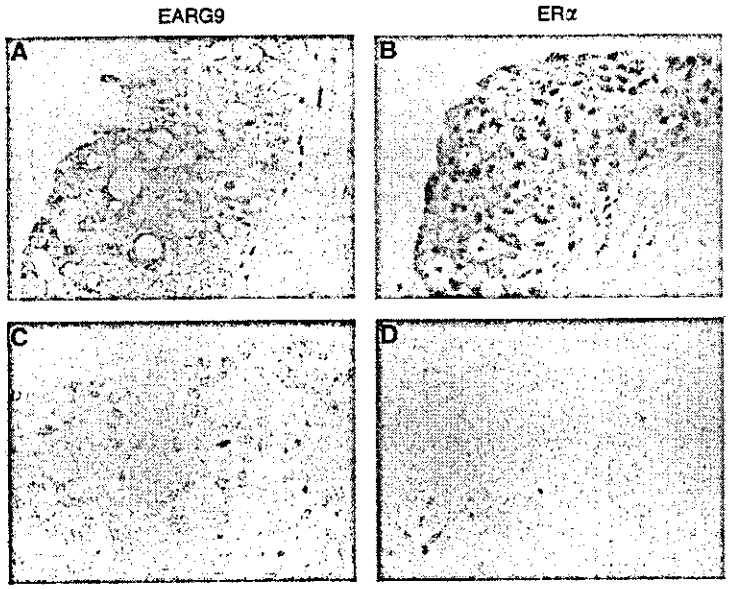

Figure 1 Serial sections of positive and negative cases of immunohistochemistry for EBAG9 and ERα in epithelial ovarian carcinoma (**A**,**B**) a case of serous adenocarcinoma, positive for both EBAG9 and ERα; (**C**,**D**) a case of clear-cell adenocarcinoma, negative for both EBAG9 and ERα). Immunoreactivity for EBAG9 was detected on the surface, and in the cytoplasm of epithelial ovarian cancer tissues. ER-α immunoreactivity was confined exclusively to the nuclei of tumour cells.

Table 2 Univariate analysis of overall survival

Variable	P-value
EBAG9 immunoreactivity (+ vs −)	0.689
Histological type	0.0177
Histological grade	0.0085
Stage	<0.0001
Residual tumour	<0.0001

Table 3 Association between EBAG9 immunoreactivity and RT–PCR

		EBAG9 immunoreactivity	
		+	−
EBAG9 mRNA	+	16	4
	−	0	2
ERα mRNA	+	9	2
	−	0	6

were positive for ERα mRNA ($n=11$), they were also positive for EBAG9 mRNA (Figure 2).

The result of immunoblotting is shown in Figure 3. Immunoreactive bands corresponding to EBAG9 and ERα were detected in 11 out of 12 and 10 out of 12 of ovarian cancer cell lines, respectively. JHOS2, which was negative for EBAG9, was also negative for ERα.

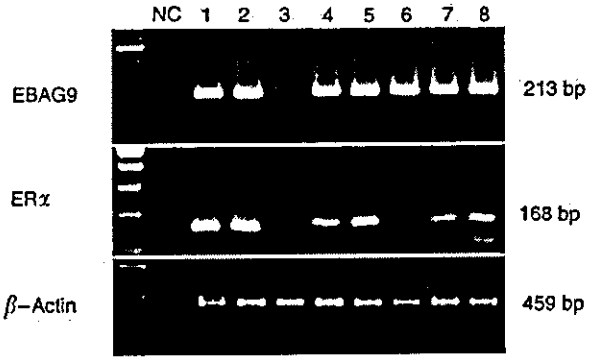

Figure 2 Representative results of RT–PCR for total RNA extracted from epithelial ovarian carcinoma tissues. Bands of the correct size for EBAG9 (213 bp), ERα (168 bp) and β-actin (459 bp) were detected in each histological subtype of ovarian cancer (1,2: serous; 3,4: mucinous; 5,6: endometrioid; 7,8: clear cell). β-Actin was used as a positive control and NC as a negative control. Note that all of the cases that were positive for ERα mRNA were also positive for EBAG9 mRNA.

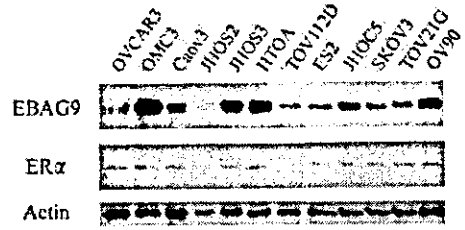

Figure 3 Results of immunoblotting of ovarian cancer cell lines are shown. Actin was used as internal positive controls.

DISCUSSION

In the present study, EBAG9 immunoreactivity was detected in 46 out of 90 epithelial ovarian carcinomas (51.1%). The expression of EBAG9 was significantly associated with advanced disease, although it turned out not to be correlated with prognosis.

It is well recognised that human cancer tissues are infiltrated with tumour-infiltrating lymphocytes (TIL) (Balch *et al*, 1990), a phenomenon known to be a manifestation of the host immune reaction to cancer cells (Rosenberg, 1996). Tumour-infiltrating lymphocytes has been reported to be associated with improved prognosis of some carcinomas, including lung (DiPaola *et al*, 1977) and colon (Naito *et al*, 1998) carcinomas. In ovarian tumour, the degree of lymphocyte infiltration was reported to be associated with the patients' survival rate, clinical stage, grade and histological type (Ma and Gu, 1991). Nakashima *et al* (1999) recently reported that activated CD3+ T lymphocytes express a putative receptor for EBAG9/RCAS1. This receptor expression was enhanced by activation of the lymphocytes, and when these receptor-positive cells were cultured with EBAG9/RCAS1 peptides, their growth was strongly suppressed, and they were eventually led to cell death by apoptosis (Nakashima *et al*, 1999). In breast carcinoma, EBAG9 immunoreactivity was inversely associated with the degree of intratumoral infiltration of mononuclear cells or CD3+ T lymphocytes (Suzuki *et al*, 2001). These results suggested that tumour cells might have evaded immune surveillance by expressing EBAG9/RCAS1, which suppressed clonal expansion and induced apoptosis of receptor-positive immune cells. Although the precise mechanism of immune evasion remains uncertain, the expression of EBAG9/RCAS1 may be a factor related to the escape mechanism of cancer cells from the host immune system.

Several authors reported that expression of RCAS1 was associated with poor prognosis and/or advanced disease in human cancer. Izumi *et al* (2001) reported that RCAS1 expression was positive for 48 of 102 non-small-cell lung carcinoma patients (47.1%) and was significantly correlated with advanced stage, poor differentiation and poor prognosis. Ito *et al* (2003) reported that RCAS1 overexpression was more frequently observed in anaplastic carcinomas than well-differentiated carcinoma in thyroid cancer. In pancreatic ductal adenocarcinoma, RCAS1 expression was demonstrated in 77 of 80 cases (96%) and was an independent prognostic factor (Hiraoka *et al*, 2002). In gynaecological malignancies, Kaku *et al* (1999) reported that patients with high RCAS1 expression showed significantly worse overall survival than those with low expression in adenocarcinoma of uterine cervix. In addition, Sonoda *et al* (1998) reported that RCAS1 was not detected in the normal uterine cervix or ovarian tissue, but strongly expressed in uterine endometrial adenocarcinomas, ovarian adenocarcinomas (Sonoda *et al*, 1996; Sonoda *et al*, 2000) and cervical squamous cell carcinomas. Recently, Suzuki *et al* (2001) reported that EBAG9 immunoreactivity was detected in 82 of 91 in breast carcinoma (90.1%), although it was not associated with clinicopathological parameters. Others reported that EBAG9 gene was consistently expressed in breast cancer cell line, and might play a specific role in early stages of breast carcinogenesis (Tsuneizumi *et al*, 2001). To our knowledge, this is the first report that evaluated the relationships between EBAG9/RCAS1 and clinicopathological parameters in epithelial ovarian cancer. Our results, together with previous reports, suggest that ovarian cancer that expresses EBAG9 may have invasive and progressive characteristics.

There was a strong correlation between EBAG9 and ERα immunoreactivity in ovarian cancer tissues ($P<0.0001$) and cell lines in this study. Also, EBAG9 immunoreactivity was associated with serous histology. Moreover, all of the cases that were positive for ERα mRNA, were also positive for EBAG9 mRNA, suggesting

that the regulation of EBAG9 may be under oestrogen control in ovarian epithelial carcinoma. Oestrogen receptor-binding fragment associated gene 9 was isolated utilising a genomic-binding site cloning method from a cDNA library of MCF-7 human breast cancer cell (Watanabe *et al*, 1998), which expresses ERα and low level of ERβ (Vladusic *et al*, 2000). Transfection analyses have demonstrated that the nucleotide sequences between −86 and −36 contains an ERE in the 5′-promoter region of the EBAG9 gene (Ikeda *et al*, 2000). mRNA levels of EBAG9 in MCF-7 cells are significantly increased within 6 h of oestrogen treatment, an effect that is mediated by the binding of ERα to the ERE in the promoter of the EBAG9 gene (Ikeda *et al*, 2000). On the other hand, Quinn *et al* (1982) reported that serous tumours were more frequently ER-positive than other types of cancers. Results from our present study are consistent with these previous reports, and suggest that EBAG9 is widely distributed in carcinoma cells of human epithelial ovarian carcinoma tissues and cells, maybe especially in serous histology, as a result of oestrogen actions through ER.

In conclusion, the wide distribution of EBAG9 and its relation to advanced disease suggest that this protein may play important roles in epithelial ovarian cancer. Further investigations are required to clarify the precise functions of EBAG9 in epithelial ovarian cancer.

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Secondary Cytoreductive Surgery for Recurrent Epithelial Ovarian Carcinoma; Proposal for Patients Selection.

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Running Title: Secondary cytoreduction in ovarian carcinoma

ABSTRACT

The value of secondary cytoreductive surgery (SCS) for recurrent ovarian cancer is still controversial. The aim of this study was to clarify candidates for SCS. Between January 1987 and September 2000, we performed SCS in 44patients with recurrent ovarian cancer, according to our selection criteria; disease-free interval (DFI)>6months, PS<3, no apparent multiple diseases, age<75years and no progressive disease during preoperative chemotherapy, if undertaken. The variables were investigated by univariate and multivariate analyses. Twenty-six of 44 (59.1%) patients achieved complete removal of all visible tumors at SCS. SCS outcome, complete or incomplete resection, was significantly related to overall survival (p=0.0019). As for variables determined before SCS, DFI>12months, no liver metastasis, solitary tumor and tumor size<6cm were independently associated with favorable overall survival after recurrence in the multivariate analysis. Patients with three or all four variables (n=31) had significantly better survival compared with the other patients (n=13) (47months vs. 20months in median survival, p<0.0001). In these patients, fairly good median survival (40months) was obtained even in patients with incomplete resection. SCS had a large impact on survival of patients with recurrent ovarian cancer when they had three or all of above-mentioned four factors at recurrence. These patients should be considered as ideal candidates for SCS.

Key words: ovarian cancer, recurrence, secondary cytoreductive surgery, prognosis

INTRODUCTION

Since Griffiths (Griffiths, 1975) first demonstrated the inverse relationship between residual tumor size after primary debulking and survival of ovarian cancer patients in 1975, many investigators have reproduced and confirmed this observation (Hacker et al., 1983; Vogl et al., 1983; Delgado et al., 1984; Conte et al., 1985; Louie et al., 1986; Neijt et al., 1987; Hainsworth et al., 1988; Sutton et al., 1989). Thus, the value of debulking of large tumor masses in the primary surgery of ovarian cancer has been generally accepted, and primary cytoreductive surgery followed by chemotherapy is considered to be a standard treatment procedure for patients with advanced ovarian cancer.

The cytoreduction contributes to removal of the tumor burden and relief of symptoms caused by tumors or massive ascites. In addition, the cytoreduction has another important effect on the sensitivity to postsurgical chemotherapy. By removing bulky tumors, the decreased growth fractions should increase (Norton and Simon, 1977) and poorly perfused anoxic cells should decrease. By reducing the number of cancer cells, the chance for cancer cells to undergo spontaneous mutations resulting in drug resistance should decrease (Goldie and Coldman, 1979). All these effects are believed to enhance the sensitivity to chemotherapy.

Theoretically, the favorable effects of cytoreduction may also be expected in patients with recurrent ovarian cancer. Recently, several investigators have reported the significant value of secondary cytoreductive surgery (SCS) in a subset of patients with recurrent ovarian cancer (Jänicke et al., 1992; Vaccarello et al, 1995; Eisenkop et al., 1995 and 2000; Cormio et al., 1999; Zang et al., 2000 and 2004; Munkarah et al., 2001; Scarabelli et al., 2001; Tay et al., 2002). The value of complete resection at the time of SCS for highly selected patients is in consensus in these recent reports. They reported a considerable number of factors related to good prognosis including longer disease-free interval (DFI), smaller size of residual tumor at primary cytoreductive surgery, good response to first line chemotherapy, younger age at recurrence, and smaller size of maximum tumor at recurrence. However, there is limited information regarding the ideal candidates for SCS. Though only preoperative or intraoperative variables before starting SCS should be analyzed for selection of the candidate, these variables have been analyzed together with

SCS outcome in most previous studies. In addition, the follow-up periods of living patients were rather short (the median or average follow-up periods were between one and four years) (Jänicke et al., 1992; Vaccarello et al, 1995; Cormio et al., 1999; Zang et al., 2000 and 2004; Munkarah et al., 2001; Scarabelli et al., 2001) in most of the previous reports.

Since 1987 we have performed SCS according to our criteria of patient selection in 44 out of 70 ovarian cancer patients who had recurrence after DFI. In the present study, the median follow-up period of living patients is 60 months after initiation of treatment, SCS or chemotherapy before SCS, for recurrence. Using univariate and multivariate analyses of variables before starting SCS, we planned to clarify the ideal candidates for SCS among patients with recurrent ovarian cancer.

PATIENTS AND METHODS

Patient Selection

Between January 1984 and December 1999 we treated 236 patients with stage I to IV epithelial ovarian cancer at the Department of Obstetrics and Gynecology, University of Tokyo Hospital. Our standard surgical procedures for ovarian cancer consist of total abdominal hysterectomy, bilateral salpingo-oophorectomy, infracolic or total omentectomy, and in advanced cases, debulking of tumor masses with maximum efforts. Patients with no or small intra-peritoneal residual tumors (less than 2cm in diameter) also underwent systematic retroperitoneal lymphadenectomy. The extent of retroperitoneal lymphadenectomy is pelvic lymph nodes only (1984-1986) or both pelvic and aortic lymph nodes (1987-1999). All but stage Ia patients underwent at least 6 cycles of cisplatin based chemotherapies following surgery as described previously (Onda et al., 1998). Of the 236 patients, 204(86%) achieved complete clinical remission after primary treatment.

By September 2000, 70 of the 204 (34%) patients had recurrence and, from January 1987 through September 2000, 44 of the 70 (63%) patients underwent SCS prior to or following chemotherapy. Administration of chemotherapy before SCS was decided based on various clinical factors including short disease free interval (DFI < 12 months) and poor performance status (PS 3) defined by ECOG (Eastern Cooperative Oncology Group). Our selection criteria for SCS were as follows; 1) DFI >6months, 2) age at recurrence < 75 years, 3) PS 0-2 just before the surgery, 4) absence of apparent extensive intra-peritoneal dissemination or multiple distant metastases and 5) no progressive disease during presurgical chemotherapy, if undertaken. There were three exceptions to the above-mentioned criteria for SCS. One patient with DFI <6 months (5 months) underwent SCS, because the recurrent site was expected to be limited to a solitary aortic lymph node by CT. The other two patients had PS 3 at surgery. One patient with three metastatic brain tumors underwent emergent brain surgery followed by γ-knife radiosurgery to one residual tumor (Kawana et al., 1997), and one patient underwent ileo-cecal resection because of acute bowel obstruction. Before the treatment, informed consent was obtained from all of the patients.

Chemotherapy

Twenty-one of 44 (47.7%) patients received chemotherapy before SCS and all of 44 patients were treated with chemotherapy after SCS. One to 8 (median; 2) cycles of presurgical chemotherapy were performed in 8 of 13 (61.5%) patients with DFI<12 months and 13 of 31 (41.9%) patients with DFI >12 months. Forty-four patients received 2 to 9 (median; 4) cycles of postsurgical chemotherapy.

Two to 4 cycles of presurgical chemotherapy were generally administered until beneficial response (partial or minor response) was observed. In two patients, second line chemotherapy showed no beneficial response, and SCS was performed after successful third line chemotherapy (7 and 8 cycles in total). One patient received only a cycle of presurgical chemotherapy, because SCS could not be scheduled immediately after diagnosis of recurrence.

The number of postsurgical chemotherapy given was determined by SCS outcome and response to chemotherapy, evaluated by CT scan and serum level of CA125. Generally, 3 to 4 cycles of chemotherapy were planned for patients with no residual tumor and 5 to 6 cycles of chemotherapy were planned for patients with any residual disease. In principle, we gave at least 2 cycles of chemotherapy after the serum level of CA125 was normalized. Thus, 3 patients were treated with more than 6 cycles of chemotherapy after SCS. On the contrary, chemotherapy was discontinued before accomplishment of the planned cycles in 5 patients because rapid disease progression or severe adverse effects were observed during the planned cycles.

In presurgical and postsurgical chemotherapies, a platinum-based combination, CAP, EP or TJ, was used. The CAP regimen consisted of 600mg/m^2 of cyclophosphamide, 30mg/m^2 of doxorubicin and 50-75 mg/m^2 of cisplatin. The EP regimen consisted of 80 mg/m^2 of etoposide during day 1 to 5 and 75 mg/m^2 of cisplatin. Paclitaxel was introduced in Japan in 1998 and, thereafter, a TJ regimen consisting of paclitaxel (175 mg/m^2 over 3-hours infusion) and AUC 5 of carboplatin was used as second-line chemotherapy.

Statistical Methods

Survival was measured from the day of starting treatment for recurrence, i.e. the day of starting presurgical chemotherapy or the day of performing SCS. The survival curves were

determined by the Kaplan-Meier product limit method (Kaplan et al., 1958). Factors influencing survival were analyzed using the Log-rank test (univariate) and Cox proportional-hazards regression analysis (multivariate). These analyses were performed using a JMP program (SAS Institute Inc., USA). Contingency table analysis was performed using the chi-square test or chi-square test for trend.

RESULTS

Patient characteristics

The number of patients was 3 in stage I, 2 in stage II, 36 in stage III and 3 in stage IV according to the International Federation of Gynecology and Obstetrics (FIGO). Histology was serous type in 35, clear cell type in 3, endometrioid type in 3, transitional cell type in 2 and mixed epithelial type in 1. Median DFI was 18.5 months with a range of 5-58 months; one patient (2.3%) had 5 months, 12 (27.3%) had 6-12 months and 31 (70.5%) had >12 months. Median age at recurrence was 52 years with a range of 37-74 years. Median follow-up period of patients, excluding those who died, was 60 months with a range of 17-199 months from initiation of treatment for recurrence.

Surgery

Our attempt to perform SCS resulted in exploratory laparotomy in 4 patients (9.1%) due to presence of unexpected extensive peritoneal tumors. Various debulking surgeries classified into 4 categories such as 1) gastrointestinal resection, 2) resection of other organs, 3) lymph node dissection and 4) other tumor debulking was performed with maximum efforts in the remaining 40 patients (90.9%). Among these patients, gastrointestinal resection (category 1) was required in 11 patients (25.0%); large bowel resection in 9 patients (20.5%), small bowel resection in 3 patients (6.8%), partial gastrectomy in 1 patient and ileocecal resection in 1 patient (2.3%), and one of the patients (2.3%) underwent sigmoid colostomy. Three patients had category 1 surgeries at two sites. Resection of other organs (category 2) was required in 6 patients (13.6%); splenectomy in 3 patients (6.8%), distal pancreatectomy in 2 patients (4.5%), partial liver resection in 1 patient, hysterectomy in 1 patient and brain tumor resection in 1 patient (2.3%). Two patients had category 2 surgeries at two sites. Regional or distant lymph node dissection (category 3) was performed in 12 patients (27.3%). Five patients (11.4%) underwent systematic aortic lymphadenectomy and one (2.3%) underwent both systematic pelvic and aortic lymphadenectomies. Selective dissections of the following lymph nodes were performed in 6 patients; aortic nodes in 1 patient, pelvic nodes in 1 patient, axillary nodes in 1 patient, portal nodes in 1 patient, inguinal

nodes in 1 patient and mesenteric nodes in 1 patient (2.3%). Other tumor debulking (category 4) including removal of tumors in the remnant omentum, the diaphragmatic muscles and vaginal stump, and tumors on the visceral or parietal peritoneum including the undersurface of the diaphragm, was performed in 22 patients (50.0%); omentectomy in 7 patients, partial full-thickness diaphragm resection in 1 patient, resection of tumors around the vaginal stump in 4 patients (9.1%), peritoneum resection of disseminated tumors on the undersurface of the diaphragm and other peritoneal surfaces in 16 patients (36.4%). Six patients were counted twice because they underwent two types of category 4 surgeries. Ten patients underwent 2 or 3 out of the above 4 categories of debulking surgery. No patients died within a month following SCS.

Cytoreductive outcome and survival of patients

Among a total of 44 patients, complete resection of visible tumors was achieved in 26 patients (59.1%), largest residual tumors <1cm in diameter were left in 11 patients (25.0%) and largest residual tumors ≥1cm in diameter were left in 7 patients (15.9%). The median survival and 5-year survival of all patients who underwent cytoreductive surgery were 32 months and 33.2% (Figure 1), whereas the median survival and 5-year survival of 26 patients who had recurrence after complete remission achieved by primary treatment and did not undergo the surgery were 11 months and 3.9%. Figure 2 shows the survival of patients after initiation of treatment for recurrence according to the outcome of SCS (SCS outcome). The median survival and 5-year survival after recurrence of the patients with largest residual tumors 0cm, <1cm, ≥1cm were 52months and 47.6%, 23months and 18.2% and 20months and 0%, respectively (p=0.0007, Log-rank). The overall survival of patients with no residual tumor was much better than that of patients with residual tumors (22 months in median survival and 12.0% in 5-year survival, Figure not shown) with statistical significance (p=0.0019). There was no statistical difference in overall survival between patients with residual tumors <1cm and ≥1cm (p=0.1314).

Factors influencing survival in univariate analyses

Factors influencing overall survival after recurrence were analyzed using univariate analyses. Factors analyzed and the results of univariate analyses are listed in Table 1 and Table 2. As for prognostic factors determined during primary therapy, univariate analyses revealed that peritoneal

tumor spread (p=0.039), FIGO stage (p=0.045) and aortic lymph node metastasis (p=0.009) were significantly associated with overall survival after recurrence. Regarding prognostic factors determined at recurrence, univariate analyses revealed that DFI (p=0.002), presence of liver metastasis (p=0.005), number of recurrent tumors (p=0.007), size of maximum tumor (p<0.001) and SCS outcome (p=0.002) had significant associations with overall survival after recurrence.

Factors influencing survival in multivariate analysis

To determine patient selection for the surgery, we performed multivariate analysis using statistically significant prognostic factors in univariate analyses. Out of eight significant factors, SCS outcome was omitted in the multivariate analysis because SCS outcome is not yet known on considering indications for the surgery, though SCS outcome had a statistically significant correlation with number of recurrent tumors (p<0.001, chi-square test). The multivariate analysis using the remaining seven factors revealed that four factors determined at recurrence, specifically DFI, presence of liver metastasis, number of recurrent tumor, and size of maximum tumor, were independently and significantly associated with survival after recurrence (Table 3). Additionally, the multivariate analysis using only these four factors confirmed that all four factors were independently and significantly associated with survival after recurrence. The relative risk (95% confidence interval) was 0.37(0.20-0.68) for DFI >12 months, 0.23(0.10-0.65) for absence of liver metastasis, 0.26(0.12-0.48) for a solitary tumor and 0.20(0.09-0.42) for size of maximum tumor < 6 cm.

Grouping of patients determined by number of favorable prognostic factors

According to the number of favorable statuses among the above-mentioned four prognostic factors, i.e. DFI > 12 months, no liver metastasis, solitary tumor and tumor size < 6cm, patients were divided into four groups as follows; patients with all four favorable factors (Group 4, n=10), patients with three favorable factors (Group 3, n=21), patients with 2 favorable factors (Group 2, n=11) and patients with only one favorable factor (Group 1, n=2). There were no patients with zero favorable factors. Complete resection of visible tumors was achieved in 100%(10/10), 62%(13/21), 18%(2/11) and 50%(1/2) of patients in Group 4, Group 3, Group 2 and Group1, respectively. Apparently, a higher rate of complete surgical resection was achieved in patients

with a larger number of favorable factors, and the distribution was statistically significant by contingency table analysis ($p<0.001$, chi-square test for trend). Five-year survival of Group 4 was 88.9% and median survival was not reached. Five-year survivals and median survivals of Group 3, Group 2 and Group1 were 26.0%, 0% and 0%, and 37 months, 20 months and 10 months, respectively (Figure not shown). The differences of overall survival were also statistically significant among the four groups ($p<0.001$, Log-rank) and between them (e.g. $p<0.007$ in Group 1 vs. Group 2, $p<0.001$ in Group 2 vs. Group 3 and $p<0.001$ in Group 3 vs. Group 4, Log-rank). Figure 3 shows the combined survival of Group 4 and Group 3 and that of Group 2 and Group 1. Patients with three or all four favorable factors (Group 3/4) (n=31) had significantly better survival compared with those with less than three favorable factors (Group 1/2) (n=13) (median and 5-year survival; 47months and 45.9% vs. 20months and 0%, $p<0.001$).

Survival of patients determined by number of favorable prognostic factors and SCS outcome

Patients with three or all four favorable prognostic factors (Group 3/4) had better survival when complete surgical resection was achieved at the time of SCS (n=23) (64 months in median survival, 53.8% in 5-year survival). However, even when SCS left residual tumors, survival of the Group 3/ 4 patients (n=8) was fairly good (40 months in median survival, 25% in 5-year survival). On the other hand, Group 1/2 patients had poorer survival both in completely resected cases (n=3) and in incompletely resected cases (n=10) (23 months and 18 months in median survival, and 0% and 0% in 5-year survival) (Figure 4).

DISCUSSION

We achieved surgical removal of all visible tumors in 59.1% of patients at the time of SCS. Residual tumors <1 cm or ≥1 cm in diameter were present in 25.0% and 15.9%, respectively. In line with previous reports, removal of all visible tumors at SCS contributed to long-term survival (Figure 2). The rate of complete resection (59.1%) in our series was a little lower than the rates reported by Eisenkop et al. (Eisenkop et al., 2000), Landoni et al. (Landoni et al., 1998) and Cormio et al. (Cormio et al., 1999). However, in Landoni's study, the subjects were restricted to those patients who were sensitive to first line chemotherapy and chemotherapy before SCS. Cormio et al. also restricted the subjects to patients with apparently isolated and resectable tumors and without ascites. Our criteria for patient selection were similar to those of Eisenkop et al. and their subjects were patients with DFI > 6months and without liver metastases. They achieved an 82% complete resection rate by using argon beam laser to remove disseminated cancer foci and reported 44 months in median survival and approximately 35% in 5-year survival in the completely resected cases. In our experience, median survival and 5-year survival in completely resected cases were 52 months and 47.6%, respectively, being much better than previous reports. Our rate of optimal cytoreduction, 84.1% (if defined as residual tumor <1cm), was similar to the rate of complete resection in Eisenkop's report. In our series, optimally resected cases had 40 months in median survival and 38.6% in 5-year survival (Figure not shown), in keeping with the survival of completely resected cases in Eisenkop's study. These findings suggest that the debulking efforts performed at SCS in our cases are comparable to those of previous reports.

Univariate analyses revealed that three factors during primary treatment (peritoneal spread, aortic lymph node metastasis, FIGO stage) and five factors at recurrence (DFI, liver metastasis, number of tumors, size of maximum tumor, SCS outcome) were significantly related to overall survival after recurrence. In the multivariate analysis excluding SCS outcome, the significance of all the three factors during primary treatment disappeared. Four factors determined at recurrence, i.e. DFI, presence of liver metastasis, number of tumors and size of maximum tumor, were revealed to be independent prognostic factors.

DFI is the most important prognostic factor after recurrence, as described in many previous

reports. In most studies, the cut-off period of DFI was set to 12 months. Two cut-off periods were set in Eisenkop's study (Eisenkop et al., 2000) (12 and 36 months) and in Tay's study (Tay et al., 2002) (12 and 24 months), and patients were divided into three groups. Although we also analyzed our patients with DFI > 12 months using cut-off periods such as 24 and 36 months, there were no significant differences between patients with and without DFI > 24 or 36 months (data not shown). Recently, Zang et al. (Zang et al., 2004) performed SCS even in patients with DFI of three months and reported negative influence of DFI on overall survival. However, their follow-up period was only 16 months. This might be too short to detect a statistical difference.

Size of maximum tumor was also identified by Eisenkop (Eisenkop et al., 2000) as an independent prognostic factor. Eisenkop et al. used 10cm as the cut-off size, whereas we used 6cm. The difference may be due to our earlier detection of recurrent tumors by using ultrasonography or CT scan within a 3 month interval. In our cases, there were only 2 patients in whom maximum tumor size exceeded 10cm in diameter. At all events, tumor size seems to be an important factor reflecting biological aggressiveness of recurrent tumors.

Number of recurrent tumors has not been previously highlighted as a prognostic determinant. One reason is that some studies restricted the subjects for SCS to patients with isolated tumors or a solitary tumor (Cormio et al., 1999; Munkarah et al., 2001; Scarabelli et al., 2001). Another possible reason is that Eisenkop et al. (Eisenkop et al., 2000) and Tay et al. (Tay et al., 2002) did not analyze number of recurrent tumors as a factor influencing survival, although they pointed out that this factor may influence SCS outcome. In concordance with our results, Zang et al. (Zang et al., 2004), reported that the number of recurrent tumors influenced both overall survival and SCS outcome.

The current study revealed that liver metastasis is another important prognostic determinant. Vaccarello et al. (Vaccarello et al., 1995) examined the relationship between site of recurrence and survival, and reported that liver metastasis had a negative influence on survival. In most studies, patients with liver metastasis were excluded from subjects for SCS. In our series, two patients with solitary liver metastasis were included; one patient underwent hepatic resection and the other patient did not undergo hepatic resection because of the presence of unresectable metastatic portal

lymph nodes. They did not achieve good survival (20months and 14months, respectively).

From the results of the multivariate analysis, we propose the following criteria for patient selection for SCS. Patients with recurrent ovarian cancer should be considered as ideal candidates for SCS when they have three or all of the following four factors at recurrence; 1) DFI>12 months, 2) no liver metastasis, 3) a solitary tumor and 4) tumor size < 6cm. Considering our original patient selection, we should propose exclusion criteria including 1) age at recurrence ≥75 years, 2) PS 3 or 4 just before SCS, and 3) progressive disease during presurgical chemotherapy, if undertaken. Although we used intraoperative findings for the number and size of tumors, size of maximum tumor was consistent between intraoperative findings and imaging in available cases. Therefore, we can accurately evaluate all these factors, except number of tumors, before SCS. As for number of tumors, ultrasonography or CT scan before SCS cannot always identify multiple peritoneal disseminated tumors. When the patient meets the criteria for SCS preoperatively, it is recommended to decide whether SCS should be accomplished after reconfirming the criteria at the time of laparotomy.

In the previous studies, several prognostic factors were shown to have significant correlation with overall survival of the patients. However, these factors were obtained from SCS inselected patients in most of the previous studies. In addition, how to use several significant prognostic factors to select good candidates for SCS was not fully analyzed. To our knowledge, generally accepted or recommended selection criteria are "patients with longer DFI" (Roberts, 1996; Bristow et al., 1996; Rose, 2000; Sijmons and Heintz, 2000). Thus, it was sometimes difficult to decide whether or not SCS should be performed in patients who have some favorable factors and a few unfavorable factors. We believe that our selection criteria for SCS should be helpful in deciding whether SCS should be performed.

In conclusion, our data suggest that patients with three or all four of the above-mentioned favorable factors are ideal candidates for SCS and that the final decision should be made at laparotomy in borderline cases. It seems that SCS has a large impact on survival of patients with recurrent ovarian cancer when the patients are selected by the new criteria (47 months in median survival and 45.9% in 5-year survival). However, these patients were likely to have good