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Original Article

Increased expression of OCIA domain containing 2 during stepwise progression of ovarian mucinous tumor

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Ovarian cancer immunoreactive antigen domain containing 2 (OCIAD2) has been reported to show cancer-specific expression in early invasive lung adenocarcinoma. OCIAD2 shows high homology with OCIAD1, which was originally immunoscreened from ascites of a patient with ovarian cancer and found to be a tumor-specific protein. Therefore, like OCIAD1, OCIAD2 is expected to show high immunoreactivity in ovarian tumors. In this study, we examined the expression pattern of OCIAD2 in 117 ovarian mucinous tumors, and confirmed that it was more highly expressed in borderline tumor and carcinoma (51/74 cases, 69%) than in adenoma (6/43 cases, 14%). The immunoreactivity of OCIAD2 in borderline tumor and carcinoma was more specific than that of OCIAD1 (adenoma, 21/43 cases, 49%), and more sensitive than that of CEA (borderline tumor and carcinoma, 35/74 cases, 47%). Like OCIAD1, OCIAD2 is a cancer-related protein and its expression level increases during the course of malignant progression and is thought to be a very useful marker for evaluating the malignancy of ovarian mucinous tumors.

Key words: OCIAD2, ovarian mucinous tumor, malignancy

Ovarian carcinoma is still a major cause of death among women in Japan and western countries. Ovarian tumors are classified histologically into various categories such as surface epithelial tumors, sex-cord stromal tumors and germ cell tumors. Among them, surface epithelial tumors constitute the major category and have four subgroups: serous tumor, mucinous tumor, endometrioid tumor and clear cell tumor. These surface epithelial tumors are further subclassified into benign (adenoma), borderline malignancy and malignant (carcinoma) forms. Among the ovarian surface epithelial tumors, the concept of multistep carcinognenesis has been accepted, especially in the development of mucinous carcinoma.[1,2] Based on an increased frequency of KRAS mutation, mucinous adenoma is thought to develop into mucinous carcinoma through a mucinous borderline lesion.[3–6] In terms of prognosis, mucinous borderline tumor is defined as an ovarian tumor of low malignant potential exhibiting epithelial proliferation of mucinous-type cells more pronounced than that seen in its benign counterpart, but without evidence of stromal invasion.

We have previously reported genes that are overexpressed in early invasive adenocarcinomas of the lung in comparison to *in situ* adenocarcinomas, based on cDNA microarray analysis of their gene expression profiles.[7] Among the genes selected, OCIAD2 (ovarian cancer immunoreactive antigen domain containing 2) showed significantly higher expression in early invasive than in *in situ* carcinoma. Immunohistochemically, OCIAD2 was expressed in most invasive pulmonary adenocarcinomas but completely negative in normal lung tissue. OCIAD2 belongs to a family of genes that contain the ovarian carcinoma immunoreactive antigen (OCIA) domain and related eukaryotic sequences, and are especially expressed in ovarian carcinoma. The OCIAD gene family includes OCIAD1 and OCIAD2. OCIAD1, showing high homology with OCIAD2, was originally immunoscreened by Luo *et al.* from ascites of a patient with ovarian cancer and found to be a tumor-specific protein and immunoreactive antigen.[8] They reported that patients who had tumors expressing OCIAD1 might develop an antibody against it. This means that OCIAD1 could be a cancer-specific protein potentially applicable as a marker for detection of carcinoma. Over the past few years, several studies have focused on OCIAD1, and it has become apparent that OCIAD1 overexpression is related to progression of ovarian cancer and plays a role in the formation of metastatic foci by affecting cancer cell adhesion.[9,10] So far, however, the role of OCIAD2 has not been studied. On the basis of the available evidence, the purpose of

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the present study was to analyze the expression pattern of OCIAD2 in ovarian mucinous tumors in comparison with known tumor markers such as CEA and OCIAD1, and to discuss the multistep development of ovarian mucinous tumors.

MATERIALS AND METHODS

Patients and tissue specimens

All the cases studied were taken from samples that had been surgically resected between 1996 and 2009 at Tsukuba University Hospital (Tsukuba, Japan) and between 2004 and 2009 at Kasumigaura Medical Center Hospital (Tsuchiura, Japan). We extracted 40 mucinous borderline tumors, including 30 of the intestinal type and 10 of the endocervical type, and 34 mucinous carcinomas, including 8 of the infiltrative invasion type and 26 of the expansile invasive type. In addition, 43 mucinous adenomas were selected from 842 adenomas that had been resected during the same period in the two hospitals as control cases. The patient age distribution was matched for the borderline tumors and the carcinomas (Fig. 1). All patients had given informed consent to the use of their materials before surgery. The specimens were formalin-fixed and paraffin-embedded, and subjected to hematoxylin-eosin (HE) and immunohistochemical staining. Four independent investigators (CN, HK, AS and KS), including one gynecological pathologist (AS), reviewed the sections and confirmed the diagnoses. In this study, we followed the 2003 WHO classification for tumors of the female genital organs.[11–13] For diagnosis of the mucinous borderline tumors, we mainly examined papillary proliferation and epithelial atypia, as there was no definite stromal invasion to differentiate them from true benign adenomas and real invasive adenocarcinomas.[14–18] Therefore, all of the mucinous borderline tumors were classical (typical) ones, and tumors with microinvasion and intraepithelial carcinoma were excluded from this study. One case was diagnosed as a borderline tumor with the features of pseudomyxoma peritonei. We then selected representative sections for each of the cases. Clinical data on the patients were collected in an anonymised manner (Table 1).

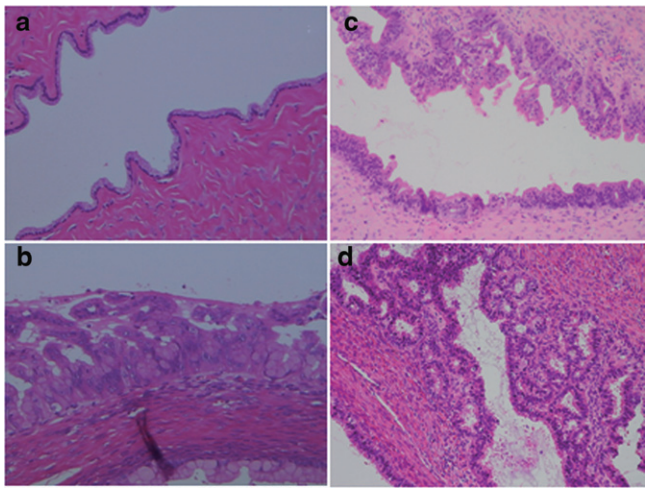

Figure 1 Histological features (HE staining) of mucinous ovarian tumors. Adenoma:1a, borderline lesion (endocervical type);1b, borderline lesion (intestinal type);1c, and adenocarcinoma;1d.

Immunohistochemical staining

Cut sections were deparaffinized and rehydrated, then autoclaved in 10 mmol/L citrate buffer (pH 6.0) at 121°C for

Table 1 Clinicopathological features of the patients

	Mucinous adenoma	Mucinous borderline tumor	Mucinous adenocarcinoma
Patient age (yr)	18–76 (mean 45.7)	17–90 (mean 49.6)	22–77 (mean 54.8)
Laterality			
Right	20	14	14
Left	23	24	20
Unknown	0	2	0
Maximum diameter (cm)	1.5–28 (mean 10.5)	4–40 (mean 13.6)	5–26 (mean 14)
FIGO stage		Ia 34	Ia 15
		Ic 1	Ib 1
		IIb I	Ic 8
		IIIc 2	IIa 1
		IIIb 1	IIb 2
		IV 1	IIc 1
			IIIa 1
			IIIc 5
Follow-up period (months)	1–108	7–156	1–144
Patients' outcome			
NED	43	38	26
Dead	0	1	5
Unknown	0	1	3

10 min for antigen retrieval. Immunohistochemical staining was then performed with rabbit polyclonal anti-OCIAD2 antibody diluted 1:1000 (Takara Bio, Shiga, Japan) using a HISTOSTAINER 36A autostainer (Nichirei, Tokyo, Japan). Using the same sections, immunohistochemical staining was also performed with a mouse monoclonal anti-CEA (carcinoembryonic antigen, clone CEM010) antibody diluted 1:200 (Takara Bio), without antigen retrieval treatment, and with a rabbit polyclonal anti-OCIAD1 antibody diluted 1:400 (Proteintech Group Inc, Chicago, IL, USA) with antigen retrieval using TE buffer at 105°C for 15 min.

Evaluation of immunoreactivity and statistical analysis

For evaluation of OCIAD2 and OCIAD1 staining, we used a case of lung adenocarcinoma as a positive control. This case had been confirmed to show high expression of OCIAD2 by RT-PCR and *in situ* hybridization (data not shown). The cytoplasmic granular staining pattern of the tumor cells was judged to be positive. For evaluation of CEA staining, we used a case of colonic adenocarcinoma as a positive control. This case revealed a significantly higher serum CEA level (20 ng/ml) and showed a diffuse cytoplasmic staining pattern (not apical staining showing polarity) that was judged as positive.

As all of the benign adenomas showed less than 30% staining for CEA per case, we defined positivity in 30% or more of the tumor area as positive. The intensity of staining with each antibody in positive cases was easily judged, and we did not use the staining intensity as a positive criterion. To examine the association between OCIAD2, OICIAD1, CEA and tumor malignancy, we used the χ^2 test. To evaluate the correlation between OCIAD2, OCIAD1 and CEA positivity, we used Spearman's rank correlation test.

RESULTS

Immunohistochemical staining of OCIAD2, OCIAD1 and CEA

OCIAD2 was detected in the cytoplasm of the tumor cells of ovarian mucinous tumor. They showed a granular staining pattern, similar to the cases of lung adenocarcinoma that were positive for OCIAD2. Unlike lung cancer, however, secreted mucin was stained as background.

Among the 43 cases of mucinous adenoma, 6 (14%) showed positive staining for OCIAD2 (Table 2, Fig. 2a). In the tumor cell cytoplasm, OCIAD2 showed a granular staining pattern that was separated from intracytoplasmic mucin. Secreted mucin was also stained in intracystic areas and the brush border. Sometimes, nuclei of tumor cells were also stained, but this staining was judged as negative. Among the 40 cases of mucinous borderline tumor, 25 (63%) including 8 of 10 of the endocervical type (80%) showed positive staining for OCIAD2 (Table 2, Fig. 2b, c). There was also background positive staining for secreted mucin in intracystic areas and the brush border. The nuclei of tumor cells showed nonspecific staining, but this was judged as negative. The cytoplasm of the tumor cells showed a granular OCIAD2 staining pattern like that of adenoma, and also positive staining for mucin when mucin secretion was abundant. Areas of papillary proliferation showed strongly positive staining for OCIAD2 in comparison to flat lining tumor cells (Fig. 3a). Among the 34 cases of mucinous carcinoma, 26 (74%) (6 of the infiltrating invasive type and 20 of the expansile invasive type) showed positive staining for OCIAD2 (Table 2, Fig. 2d). Areas of stromal invasion showed strongly positive staining for OCIAD2 in comparison with flat lining tumor cells (Fig. 3b).

The stainability of OCIAD1 was similar to that of OCIAD2, and positive staining for secreted mucin at the brush border was also evident. However, staining for intracystic secreted mucin and staining of nuclei were unremarkable. Among the 43 cases of mucinous adenoma, 21 (49%) were positive for OCIAD1 (Table 2, Fig. 4a). In the tumor cell cytoplasm, OCIAD1 showed a granular staining pattern that was separated from intracytoplasmic mucin like that of OCIAD2. On the other hand, unlike OCIAD2, positive staining was evident in the stromal cells. Among the 40 cases of mucinous borderline tumor, 26 (65%) including 9 among 10 of the endocervical type (90%) were positive for OCIAD1 (Table 2, Fig. 4b, c). Similarly to adenoma, OCIAD1 showed a granular staining pattern in the cytoplasm of the tumor cells. The tumor stroma also showed staining in the stromal cells. Several cases showed positive staining only in the stromal cells, whereas the tumor cells were negative. Among the 34 cases of mucinous carcinoma, 30 (86%) (7 of the infiltrating invasive type and 23 of the expansile invasive type) were positive

Table 2 Immunohistochemical results of OCIAD2, OCIAD1 and CEA

	N		OCIAD2		OCIAD1		CEA	
Mucinous adenoma	43		6 (14)		21 (49)		0 (0)	
Mucinous borderline tumor	Endocervical 10	40	8 (80)	25 (63)	9 (90)	26 (65)	5 (50)	10 (25)
	Intestinal 30		17 (57)		17 (57)		5 (17)	
Mucinous adenocarcinoma	Infiltrating 8	34	6 (75)	26 (76)	7 (88)	30 (88)	5 (63)	25 (74)
	Expansile 26		20 (77)		23 (88)		20 (77)	
Total	117		57 (49)		77 (66)		35 (30)	

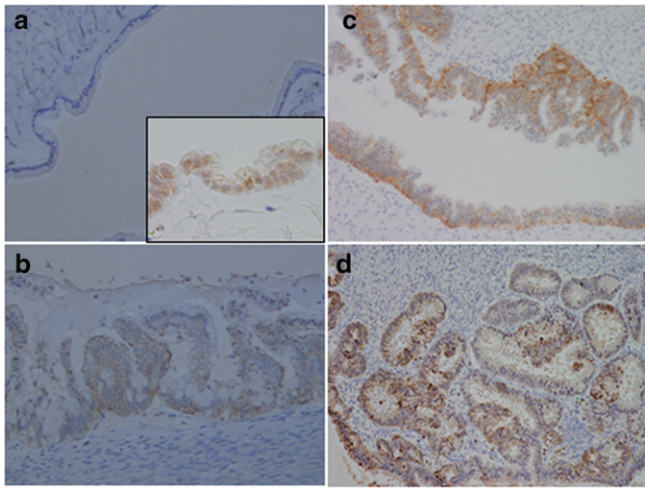

Figure 2 Immuohistocheical staining of ovarian cancer immunoreactive antigen domain containing 2 (OCIAD2) in mucinous ovarian tumors. Immunohistochemistry of OCIAD2 for adenoma (2a), borderline lesion (endocervical type) (2b), borderline lesion (intestinal type) (2c), and adenocarcinoma (2d). OCIAD2 is granularly positive in borderline tumor and carcinoma.

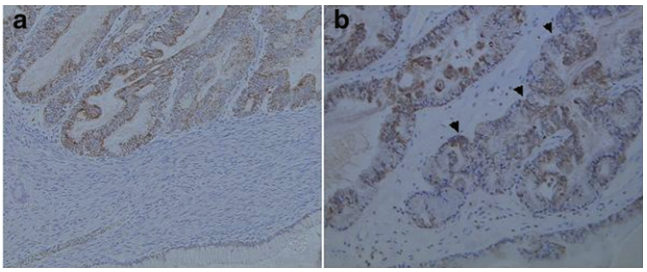

Figure 3 Histological staining pattern of ovarian cancer immunoreactive antigen domain containing 2 (OCIAD2) for OCIAD2 in mucinous borderline tumors and invasing mucinous adenocarcinoma. (a) Immunohistochemial staining of OCIAD2 for the case of mucinous borderline tumor. Areas of papillary proliferation showed more intense reactivity for OCIAD2 than flat lining tumor cells. (b) Immunohistochemical staining of OCIAD2 in a case of mucinous adenocarcinoma. Areas of stromal invasion (arrow heads) showed more intense reactivity for OCIAD 2 than flat lining tumor cells.

for OCIAD1 (Table 2, Fig. 4d). As was the case in adenocarcinoma, OCIAD1 showed a granular staining pattern that was separated from intracytoplasmic mucin. Stromal cells also showed positive background staining.

With regard to CEA, tumor cells showed a diffuse cytoplasmic staining pattern, as had been observed in colonic adenocarcinoma, and also positive staining for mucin. Among the 43 cases of mucinous adenoma, 0 (0%) were positive for CEA (Table 2, Fig. 5a). Background staining for intracystic and brush border secreted mucin was evident, but this was judged as negative. Among the 40 cases of mucinous borderline tumor, 10 (25%) including five among ten of the endocervical type (50%) were positive for CEA (Table 2, Fig. 5b, c). There was strongly positive background staining

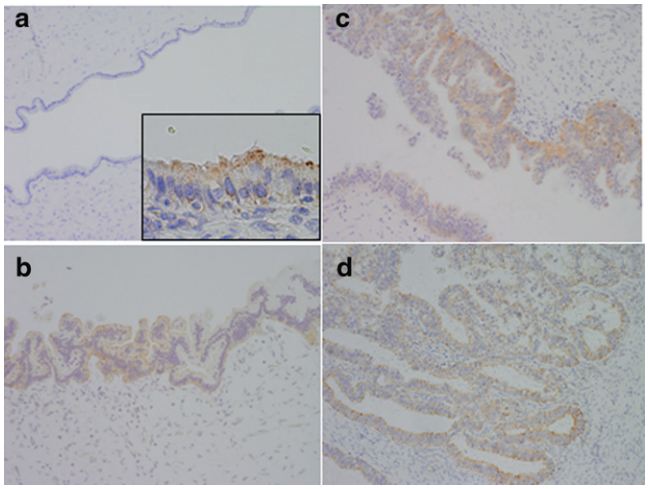

Figure 4 Immuohistocheical staining of ovarian cancer immunoreactive antigen domain containing 1 (OCIAD1) in mucinous ovarian tumors. Immunohistochemistry of OCIAD1 for adenoma (4a), borderline lesion (endocervical type) (4b), borderline lesion (intestinal type) (4c), and adenocarcinoma (4d). OCIAD1 is granularly positive in borderline tumor and carcinoma.

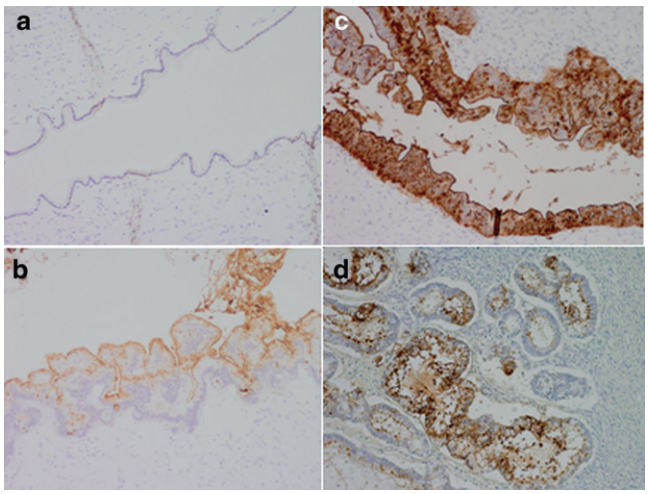

Figure 5 Immuohistocheical staining of CEA in mucinous ovarian tumors. Immunohistochemistry of CEA for adenoma (5a), borderline lesion (endocervical type) (5b), borderline lesion (intestinal type) (5c), and adenocarcinoma (5d). CEA is granularly positive in borderline tumor and carcinoma.

for intracystic and brush border secreted mucin. Non-specific positive staining was evident in the nuclei of tumor cells and in the tumor stroma. Among the 34 cases of mucinous carcinoma, 25 (71%) (5 of the infiltrating invasive type and 20 of the expansile invasive type) were positive for CEA (Table 2, Fig. 5d). Strongly positive background staining for intracystic and brush border secreted mucin was evident, similarly to adenoma and borderline lesions. Diffuse cytoplasmic staining was judged as positive. The tumor cell nucleoli and tumor stroma showed non-specific positive staining.

The one case of borderline tumor showing pseudomyxoma peritonei was stained with OCIAD2, but negative for OCIAD1 and CEA.

Statistical analysis

Positivity for OCIAD2 increased gradually with tumor progression, and more than 70% of the mucinous carcinomas were positive (Table 2). A similar tendency was seen for CEA and OCIAD1, more than 70% of the carcinomas also being positive. The expression of OCIAD2, CEA and OCIAD1 increased significantly as the malignancy of the tumor increased ($P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively). Table 2 compares the positivity ratio of adenoma vs borderline lesion and adenocarcinoma. Among the cases of adenoma, 6 (14%), 21 (49%), and 0 (0%) were positive for OCIAD2, OCIAD1, and CEA, respectively, whereas among the cases of borderline lesion and adenocarcinoma, 51 (68%), 56 (75%), and 35 (47%) were positive, respectively.

DISCUSSION

The ovarian cancer immunoreactive antigen domain (OCIAD) family comprises OCIAD1 and OCIAD2. In 2001, Luo *et al*. immunoscreened an ovarian carcinoma cDNA expression library using ascites from a patient with ovarian cancer and detected an antibody against OCIAD1.[8] Therefore, OCIAD1 is thought to be a cancer-specific protein that could be applicable for detection of carcinoma, and several reports have stressed the association between ovarian carcinoma malignancy and OCIAD1 expression. On the other hand, OCIAD2 was identified in 2001 by Strausberg *et al*. on the basis of its sequence similarity to OCIAD1 through the National Institute of Health Mammalian Gene Collection project.[19] Although OCIAD2 has high homology with OCIAD1, to date, no reports have examined the relationship between its expression and carcinoma or autoantibody against human tissue. Ishiyama T. first reported the association between lung adenocarcinogenesis and OCIAD2 on the basis of a cDNA microarray study.[7] In the present study, we examined the expression of OCIAD2 in ovarian carcinoma, especially ovarian mucinous tumors, since the diagnosis of their malignancy is based on histology, and no biomarkers for these tumors have been characterized.

In order to examine the relationship between the OCIAD family and other immunohistochemical biomarkers, we also examined the expression of CEA. As Table 2 shows, OCIAD2 expression was detected in 74% of mucinous ovarian carcinomas and 63% of borderline tumors (i.e. 69% of ovarian mucinous tumors with malignancy), but in only 14% of mucinous adenomas (benign counterpart) ($P < 0.01$). Expression of OCIAD2 was associated with the malignancy of ovarian mucinous tumors. OCIAD2 showed a granular staining pattern in the cytoplasm of the tumor cells, but interestingly its positivity was stronger in areas of papillary proliferation and stromal invasion than in flat tumor cells, suggesting an association between OCIAD2 expression and tumor malignancy.

With regard to borderline tumors, it was of considerable interest that positivity for all of the markers examined (OCIAD2, OCIAD1, and CEA) had a tendency to be higher in the endocervical type than in the intestinal type. As the tumor cells of intestinal-type tumors contain much mucin in their cytoplasim, relative to those of endocervical-type tumors, it might be difficult to judge the staining positivity of intestinal-type tumors. On the other hand, in the carcinomas, the rates of positivity for the three proteins showed no significant difference between the infiltrating invasive type and the expansile invasive type.

The staining patterns of OCIAD1 and CEA were similar to that of OCIAD2, but several differences were evident. OCIAD1 was positive in 86% of carcinomas and 65% of borderline tumors (i.e. 75% of all ovarian mucinous tumors with malignancy), whereas 49% of adenomas were also positive for OCIAD1. These results indicated that positivity for OCIAD1 increased in the earlier stage of malignant progression. Although we are unable to verify whether the staining was non-specific, stromal cells also showed positive staining for OCIAD1. However, the results suggested that OCIAD2 is a marker more associated with malignancy of ovarian mucinous tumor cells than is OCIAD1.

CEA was immunopositive in 71% of carcinomas and 25% of borderline tumors (i.e. 47% of all ovarian mucinous tumors with malignancy). The specificity of CEA for diagnosis of malignancy in ovarian mucinous tumors was highest among the three markers we examined, thus confirming that CEA is a very useful biomarker for detection of ovarian tumors. The CEA positivity rate mainly increased during the course from borderline tumor to carcinoma. Therefore, CEA might be a marker of more advanced-stage mucinous tumors in comparison with OCIAD2.

The association between OCIAD2 stainability and tumor malignancy was of considerable interest; papillary proliferating and invasive tumor cells were positive, whereas flat lining tumor cells were negative. Six cases of adenoma gave a positive reaction with anti-OCIAD2 antibody. These tumors were histologically diagnosed as adenoma, but may have had the potential to progress to borderline tumors. As it has been suggested that OCIAD2 may be localized to the tumor cell membrane, OCIAD2 protein in exfoliated tumor cells or cell fragments in ascites or blood could be detectable and applicable as a new biomarker of ovarian mucinous tumors.

In summary, we have demonstrated specific expression of OCIAD2 in ovarian mucinous tumors. Immunohistochemi-

cally. OCIAD2 appeared to be more specific than OCIAD1 and more sensitive than CEA. Examination of OCIAD2 expression is thus expected to become a new immunohistochemical biomarker of the malignancy of ovarian mucinous tumors. OCIAD2 is a membrane-localized protein expressed in several malignant tumors including lung cancer and ovarian cancers, but its function is still unclear. As normal tissue is unreactive with a specific antibody against OCIAD2, it appears that OCIAD2 is not a basic protein required for the survival of human cells. However, the biological implications of OCIAD2 should be examined extensively and utilized for the detection or treatment of malignant tumors expressing it.

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