Gene amplification and protein overexpression of MET are common events in ovarian clear-cell adenocarcinoma: their roles in tumor progression and prognostication of the patient

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The aim of this study was to assess protein overexpression and gene copy number alterations of MET in ovarian clear-cell adenocarcinoma, and to assess its potential as a novel therapeutic target. Ninety cases of clear-cell adenocarcinoma were analyzed for MET protein overexpression and copy number alterations of the *MET* gene by immunohistochemistry and brightfield double *in situ* hybridization, respectively. In addition, 101 cases of the non-clear-cell type ovarian carcinomas at advanced stages were also evaluated for comparison. MET overexpression was assigned when complete membrane staining with moderate or strong intensity was observed in at least 10% of the tumor cells examined. Double *in situ* hybridization was determined as positive when the tumor exhibited high-level polysomy ($\geq$ 4 copies in $\geq$ 40% of tumor cells) or *MET* gene amplification. MET overexpression was detected in 20 of 90 clear-cell adenocarcinomas (22%) and none of 111 non-clear-cell type ovarian carcinomas. Double *in situ* hybridization was positive in 21 of 89 informative clear-cell adenocarcinomas (24%) and only 3 non-clear-cell type ovarian carcinomas (3%). In the whole population, true amplification of the *MET* gene was detected only in the clear-cell adenocarcinoma histology (five cases, 6%). In clear-cell adenocarcinomas, double *in situ* hybridization positivity was highly correlated with the presence of MET overexpression and a poorly differentiated histology of tumors ($P = 0.0105$ and 0.00038, respectively). For the patients with clear-cell adenocarcinomas, MET overexpression, as well as advanced clinical stage and the poorly differentiated histology of tumors, was identified as an independent unfavorable prognostic factor for overall survival. In conclusion, among ovarian carcinomas, the amplification of the *MET* proto-oncogene is highly selective and commonly occurs in clear-cell adenocarcinoma. MET could serve as a biomarker for the prognostication of patients with clear-cell adenocarcinoma and tumor progression, and has potential as a novel therapeutic target for this carcinoma.
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Clear-cell adenocarcinoma accounts for 5–10% of all epithelial ovarian cancers in Western countries, whereas in Japan, for unknown reasons, it accounts for > 20%.[1–3] Among the ovarian carcinomas, clear-cell adenocarcinoma has been recognized as a form of lethal histological subtype, mainly resulting from its highly chemoresistant nature.[1–7] Surgical

debulking of the tumor is thought to be the only effective treatment for improving the prognosis of clear-cell adenocarcinoma, and some studies have reported a poor prognosis for that carcinoma even when detected at an early stage.[4–7] Despite progress in surgical techniques and modalities of chemotherapy for ovarian cancer, the mortality rate of patients with clear-cell adenocarcinoma has remained largely unchanged. Hence, there is a need to improve our understanding of its pathobiology in order to develop new therapeutic strategies and optimize currently available treatments.

A unique member of the receptor tyrosine kinase family, MET, has attracted much attention in recent years, representing an intriguing target for cancer therapy, although that has not yet been established in a clinical setting. MET, located on chromosome 7q31, encodes some functional domains, including the ligand-binding domain, regulatory juxtamembrane domain, and the receptor tyrosine kinase domain.[8] Physiologically, on binding of its ligand, hepatocyte growth factor (HGF, also known as scatter factor), the MET receptor, undergoes dimerization and autophosphorylation at specific tyrosine residues within the cytoplasmic domain, creating docking sites for intracellular signal transducers that activate the Ras-mitogen-activated protein kinase, the phosphatidylinositol 3-kinase (PI3K), and the signal transducers and activators of transcription signaling pathways.[8,9]

The overexpression of MET has been reported in several epithelial and mesenchymal cancers, and in general, a high expression level of MET is associated with cancer progression and an adverse outcome for these patients.[8,9] In contrast to non-neoplastic cells, MET in tumor cells can be activated in a ligand-independent manner through activating mutation, amplification, and overexpression of the MET gene.[8–10] However, to date, it is thought that somatic mutation of the MET gene is quite a rare event in sporadic primary carcinomas of adults, including ovarian carcinomas,[11,12] with papillary carcinoma of the kidney being the exception.[13] On the other hand, MET gene amplification was identified in 5–10% of gastric cancers,[14–16] 4% of esophageal cancers,[17] 3–4% of lung cancers,[18,19] and 10% of colorectal cancers.[20]

The MET receptor is found to be expressed in approximately 70% of human ovarian carcinomas, and overexpressed in 30% of cases and cell lines;[21–26] MET overexpression was reported to be correlated with adverse outcomes for the patients.[24,25] Blocking the effects of HGF by neutralizing antibodies, the HGF antagonist, or knocking out MET expression by small interfering RNA has been shown to inhibit peritoneal dissemination and ascites formation of ovarian cancer cells in vivo.[26] However, only a small number of cases with clear-cell adenocarcinoma were enrolled in those studies, and little is known about the status of copy number alterations of MET in ovarian carcinomas.

In the present study, using immunohistochemistry and brightfield double in situ hybridization, we sought first to determine the frequencies of MET protein overexpression and copy number alterations of the MET gene in ovarian carcinomas, especially focusing on clear-cell adenocarcinoma; second, to clarify the relationship between that protein overexpression and gene copy number alterations; and third, to examine the clinicopathological significance of MET overexpression and its altered gene copy number in the patients with clear-cell adenocarcinoma. This information will not only lead to better understanding of the pathobiology of ovarian clear-cell adenocarcinoma, but also provide insight into potential treatment options for this highly lethal malignancy.

Materials and methods

Patients and Tissue Samples

Primary ovarian carcinomas—201 cases as a consecutive series, including 90 clear-cell adenocarcinomas and 111 non-clear-cell type ovarian carcinomas—were identified from the files of the Department of Clinical Laboratory, National Defense Medical College Hospital, Japan. These 201 patients underwent surgical resection between 1987 and 2007, and none had undergone chemotherapy or radiation therapy before surgery. All specimens were formalin fixed and paraffin embedded, and tumors were classified according to the criteria of the World Health Organization.[4] For clear-cell adenocarcinoma cases, tumors at all clinical stages (stages I–IV) were included. For non-clear-cell type ovarian carcinoma cases, tumors at the advanced stages (stage III/IV) —as defined by the International Federation of Gynaecology and Obstetrics (FIGO) staging system—were selected, including 89 cases of serous adenocarcinoma, 12 of mucinous adenocarcinoma, and 10 endometrioid adenocarcinomas.

For the patients with clear-cell adenocarcinoma, clinicopathological details such as patient age, clinical stage of disease, residual tumor after initial cytoreductive surgery, clinical response to chemotherapy, regional lymph node status, histological grade of the tumor, and overall survival were assessed. These clinicopathological features are listed in Table 1. Of the 90 patients with clear-cell adenocarcinoma, 88 (98%) received post-operative platinum-based chemotherapy after initial surgery. The chemotherapeutic regimens comprised irinotecan and cisplatin in 39 patients; cyclophosphamide, doxorubicin, and cisplatin in 30 patients; etoposide and cisplatin in 8 patients; paclitaxel and carboplatin in 5 patients; cyclophosphamide and cisplatin in 3 patients; irinotecan and carboplatin in 2 patients; and paclitaxel and cisplatin in 1 patient. A second-look operation or second reductive surgery was performed, depending on the surgeon's preference. Clinical response to chemotherapy was assessed for

Table 1 Clinical characteristics of the patients with ovarian clear-cell adenocarcinoma enrolled

Characteristics	Number of cases (%)
Age (years); median (s.d.)	52.9 (8.1)
FIGO stage	
I	49 (54)
II	9 (10)
III	27 (30)
IV	5 (6)
Residual tumor after initial surgery	
Absent	63 (70)
Present	27 (30)
Response to first-line chemotherapies	
Complete response or Partial response	10 (37)
Stable disease or Progressive disease	17 (63)
Lymph node status	
pN0[a]	46 (79)
pN1[a]	12 (21)
pNx (not assessable)	32
Poorly differentiated histology in clear-cell adenocarcinoma	
Absent	68 (76)
Present	22 (24)

FIGO, International Federation of Gynaecology and Obstetrics.
[a]pN0, no lymph node metastases; pN1, one or more lymph node metastasis found in pelvic or paraaortic lymph nodes.

the 27 patients with a measurable residual tumor (regardless of size) after initial surgery, evaluated by ultrasonography or computed tomography, and classified into complete response, partial response, stable disease, or progressive disease according to the new Response Evaluation Criteria for Solid Tumours guidelines.[27] Follow-up was calculated from the date of initial definitive surgery to the date of either last follow-up or death. The average follow-up period after initial surgery was 52.0 months, ranging between 2 and 182 months. Thirty (33%) of 90 patients died because of their tumor burden, and 1 patient died because of another cause.

The research protocol was approved by the ethics committee of the National Defense Medical College, Tokorozawa, Japan.

Poorly Differentiated Histology in Clear-Cell Adenocarcinoma

To assess the relationship between altered expression of MET and histological progression/dedifferentiation of clear-cell adenocarcinoma, we have set criteria for the poorly differentiated histology of that carcinoma. Since clear-cell adenocarcinoma is a subtype of adenocarcinoma, the following architectures were defined as poorly differentiated histology of that carcinoma: tumor cells growing as solid masses, cords, or individual tumor cells infiltrating

toward surrounding stromal tissue, without easily discernible glandular differentiation. Small foci may show gland formation, but there should be one gland or less in a low-power field of view (5.51 mm^2 using $\times 10$ objective lens). When these poorly differentiated components occupying 5.51 mm^2 or more, the component was judged as present. The solid appearance of glands growing in a cribriform or fusiform papillary pattern, where the latter contain easily discernible fibrovascular cores of papillae in solid growth, was not considered a poorly differentiated component.

Construction of Tissue Microarray

To construct tissue microarray blocks, we selected formalin-fixed paraffin-embedded cancer tissue blocks from all 90 clear-cell adenocarcinoma cases and all 111 non-clear-cell type ovarian carcinomas. Two core specimens, 2.0 mm in diameter, for each case were taken from these blocks and transferred to recipient blocks using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD, USA). For the cases with clear-cell adenocarcinoma, if the tumor area contained foci of poorly differentiated histological components, at least 1 core was punched out from these components. These tissue microarray blocks were then cut into 4-ím-thick sections and subjected to both immunohistochemistry and brightfield double *in situ* hybridization analyses.

Immunohistochemistry

Sections were subjected to a BenchMark® XT automated slide processing system (Ventana Medical Systems, Tucson, Arizona). The primary antibody used was a rabbit monoclonal antibody against the carboxyl region of the transmembrane human c-Met (MET) protein (CONFIRM anti-Total c-MET (SP44), ready for use; Ventana). The immunoreaction was visualized by using an ultraView DAB Detection kit (Ventana) according to the manufacturer's instructions.[28] Endothelial cells in the tumor tissues served as positive controls for the antibody examined. Sections without the primary antibody were used as negative controls.

Brightfield Double *In Situ* Hybridization

The BenchMark® XT automated slide processing system was used for the optimization of the double *in situ* hybridization assay for copy number alteration of the *MET* gene. A detailed protocol of these methods was described by Nitta *et al*.[28] Briefly, deparaffinized tissue sections were pretreated with a combination of heat treatment with reaction buffer (Tris-based pH 7.6 solution; Ventana) and ISH Protease 3 (Ventana) to unmask DNA targets. For the *MET* gene detection, the INFORM® *MET* DNA Probe (Ventana), a dinitrophenyl (DNP)-labeled probe, was applied to the tissue sections, denatured

at 95°C and hybridized at 52°C for 6 h. After wash steps with $2 \times SCC$ (Ventana), tissue sections were incubated with monoclonal rabbit anti-DNP antibody (Ventana) for 20 min and then with HRP-conjugated goat anti-rabbit antibody for 16 min at 37°C. The metallic silver deposit for *MET* ISH signal was developed using the *ultra*View™ SISH Detection kit (Ventana). For CEN 7 detection, the INFORM Chromosome 7 Probe (Ventana), a DNP-labeled oligoprobe, was applied to the tissue sections, denatured at 95°C, and hybridized at 44°C for 2 h. Then, after wash steps with $2 \times SSC$, tissues were incubated with monoclonal rabbit anti-DNP antibody for 20 min and then with an alkaline phosphatase (AP)-conjugated goat anti-rabbit antibody for 12 min at 37°C. The signal for CEN 7 was visualized with a fast red and naphthol phosphate reaction using an *ultra*View Red ISH Detection kit. Finally, counterstain with hematoxylin was performed.

Interpretation of the Immunohistochemistry and Double *In Situ* Hybridization Data

The data of immunohistochemistry and double *in situ* hybridization were evaluated independently by two observers (SY and KM). In the interpretation of immunohistochemistry, any discrepancies between the two observers were resolved by discussion, and using a multiviewer microscope.

The intensity of the immunoreaction was scored using a four-tier system with the same criteria as used for assessing HER2/neu immunoreaction in breast cancer (the HercepTest™ criteria):[29] negative, no discernible staining or background type staining; $1+$, definite cytoplasmic staining and/or equivocal discontinuous membrane staining; $2+$, unequivocal membrane staining with mild to moderate intensity; $3+$, strong and complete membrane staining. MET overexpression was defined as moderate ($2+$) or strong ($3+$) when complete membrane staining was observed in at least 10% of the tumor cells. For each individual case, the extent (%) of overexpressing cells was determined in a percentage of immunoreactive tumor cells on the tissue microarray cores from each case.

For double *in situ* hybridization analysis, the number of dark brown and red dot signals, corresponding to the copies of the *MET* gene and those of CEN 7, respectively, were counted in 80 interphase tumor cell nuclei (40 cells by 1 observer) using a $\times 100$ oil immersion objective lens. When the tentative averages of counted gene copy numbers per nucleus in a tumor significantly differed between the observers, the two observers counted a further 80 nuclei. All cases were arranged in four categories as follows: disomy (≤ 2 *MET* copies in $>90\%$ of cells), low genomic gain (≥ 3 *MET* copies in $\geq 10\%$ of cells and ≥ 4 copies of the *MET* gene in $<40\%$ of cells), high-level polysomy (≥ 4 copies of the *MET* gene in $\geq 40\%$ of cells), or gene amplification (presence of tight gene clusters, a ratio *MET* gene/CEN7 per cell ≥ 2, or ≥ 15 copies of the *MET* gene in $\geq 10\%$ of cells examined) using the reported criteria.[30] Then, the two former and the two latter

Table 2 MET overexpression and copy number alterations in ovarian clear-cell adenocarcinomas and non-clear-cell type ovarian carcinomas

Histological types		Number of tumors (%)					P-value
	Total	Negative	Overexpression				
			1+	2+	3+		
(a) MET immunoreaction							
Clear cell	90	31 (34)	39 (43)	17 (19)	3 (3)		$<0.00001^a$
Non-clear cell	111	88 (79)	23 (21)	0	0		
Serous	89	75 (84)	14 (16)	0	0		
Mucinous	12	6 (50)	6 (50)	0	0		
Endometrioid	10	7 (70)	3 (30)	0	0		

Histological types		Number of tumors (%)				P-value
	Total[b]	Negative		Positive		
		Disomy	Low gain	High polysomy	Amplification	
(b) Copy number alterations by double in situ hybridization						
Clear cell	89	20 (22)	48 (54)	16 (18)	5 (6)	$<0.0001^a$
Non-clear cell	106	47 (44)	56 (53)	3 (3)	0	
Serous	86	35 (41)	48 (56)	3 (3)	0	
Mucinous	11	7 (64)	4 (36)	0	0	
Endometrioid	9	5 (56)	4 (44)	0	0	

[a]Comparison between clear-cell adenocarcinomas and non-clear-cell carcinomas.
[b]Number of informative cases for double *in situ* hybridization analysis.

were classified as double *in situ* hybridization negative and double *in situ* hybridization positive, respectively.

Statistical Analysis

Statistical analyses were performed using StatMate III software (ATMS, Tokyo, Japan). Comparisons between parameters were computed by the χ^2 test. For survival analysis, Kaplan–Meier curves were drawn and differences between the curves were calculated by the log-rank test. Independent prognostic significance was computed by the Cox proportional hazards general linear model. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Frequent Protein Overexpression and Copy Number Alterations of the MET Gene in Clear-Cell Adenocarcinomas, Compared with Non-Clear-Cell Type Ovarian Carcinomas

MET immunoreaction was detected ($\geq 1+$) in 59 out of 90 clear-cell adenocarcinomas (66%) and 23 out of 111 non-clear-cell type ovarian carcinomas (21%), respectively (Table 2a). MET overexpression ($\geq 2+$) was assigned in 20 out of 90 clear-cell adenocarcinomas (22%) and none of 111 non-clear-cell type ovarian carcinomas (Figure 1). In the former, 17 and 3 cases were scored as $2+$ and $3+$, respectively. Consequently, when compared with non-clear-cell type ovarian carcinomas, the frequency of both MET immunoreaction ($\geq 1+$) and MET overexpression ($\geq 2+$) was significantly higher in the clear-cell adenocarcinoma ($P < 0.00001$, respectively).

Results of double *in situ* hybridization analysis for copy number alteration of the *MET* gene in clear-cell adenocarcinomas and non-clear-cell type ovarian carcinomas were summarized in Table 2b. Information about the copy number alteration of the *MET* gene and CEN7 was obtained from 89 (99%) of 90 clear-cell adenocarcinomas and 106 (95%) of 111 non-clear-cell type ovarian carcinomas. In one clear-cell adenocarcinoma and five non-clear-cell type ovarian carcinomas, double *in situ* hybridization was performed, but dot signals were not visible, or cells with countable signals were <40 in the tissue microarray cores even though the assays were repeated. In the informative cases, 20 out of 89 clear-cell adenocarcinomas (22%) and 47 out of 106 non-clear-cell type ovarian carcinomas (44%) were considered as disomy (Figure 2a). Low genomic gain of the *MET* gene (Figure 2b) was detected in 48 clear-cell adenocarcinomas (54%) and in 56 non-clear-cell type ovarian carcinomas (53%); the latter included 48, 4, and 4 cases with serous (56%), mucinous (36%), and endometrioid (44%) adenocarcinomas, respectively. A high-level polysomy (Figure 2c) was
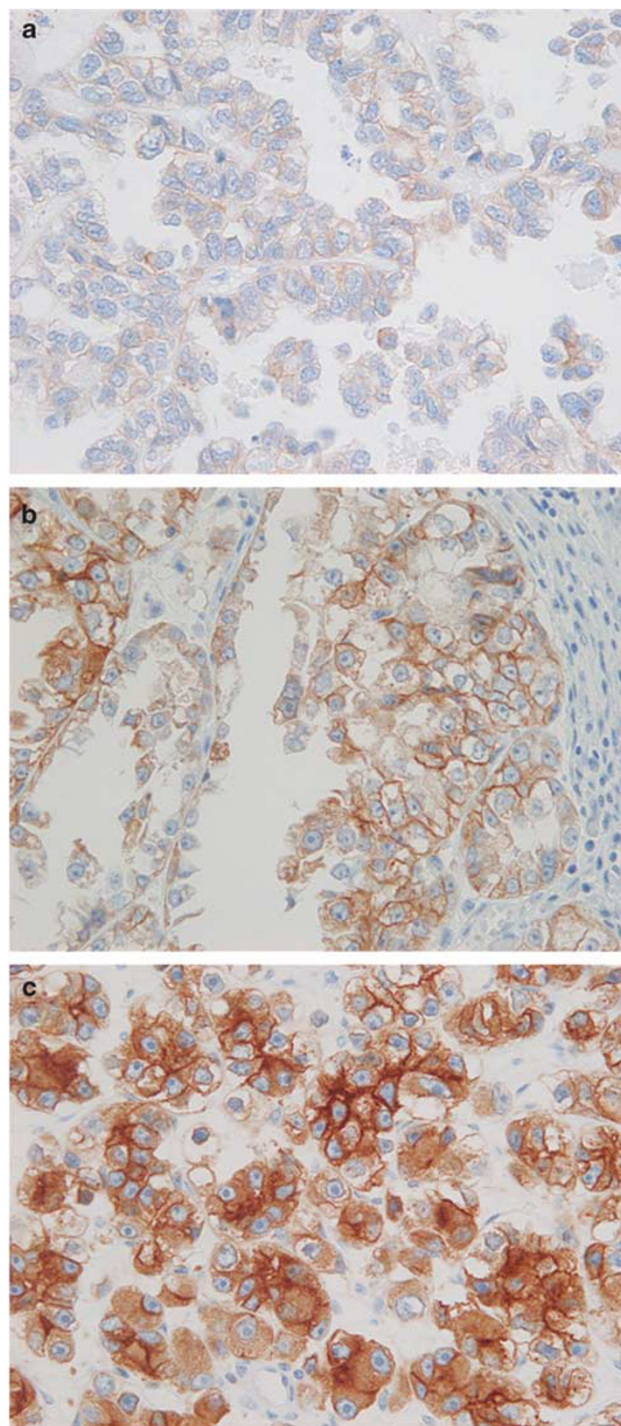

Figure 1 Representative immunohistochemistry for MET. Clear-cell adenocarcinomas exhibiting scores $1+$, $2+$, and $3+$ are shown in (**a**), (**b**), and (**c**), respectively. (**a**) Weak but definite cytoplasmic immunoreaction is noted, and membrane staining is discontinuous. (**b**) Continuous membrane immunoreaction with moderate intensity is noted. (**c**) Tumor cells show the strong membrane and cytoplasmic immunoreaction. Original magnification: $\times 400$.

detected in 16 clear-cell adenocarcinomas (18%) and 3 non-clear-cell type ovarian carcinomas (3%); all of the latter were serous adenocarcinomas (3%, 3 out of 86). True gene amplification of the *MET* gene
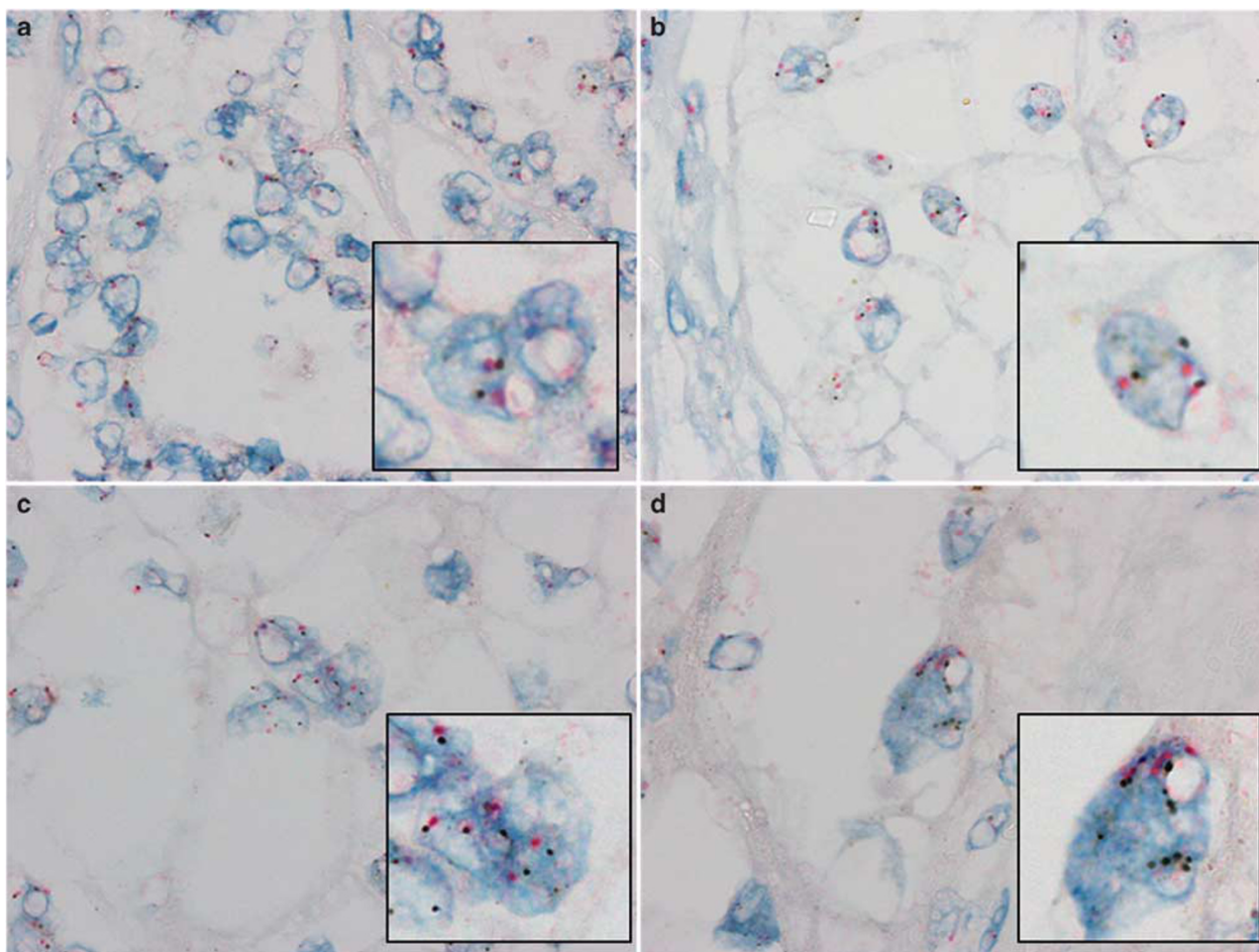
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Figure 2 Copy number status of the *MET* gene determined by brightfield double *in situ* hybridization in ovarian clear-cell adenocarcinomas. (**a**) A tumor defined as disomy. Most ($\geq 90\%$) of the tumor cells in this microphotograph show 1 to 2 dark brown (corresponding to the *MET* gene) and red (corresponding to CEN7) signals. (**b**) A tumor defined as low genomic gain. About half of the tumor cells in this microphotograph show three pairs of *MET* and CEN signals. (**c**) A tumor defined as high-level polysomy. Tumor cells show four to five pairs of *MET* and CEN7 signals. (**d**) A tumor defined as true gene amplification. In the focused tumor cell, *MET* signals form tight gene clusters, and a ratio of *MET* gene/CEN7 per cells is clearly > 2. According to the described criteria, the tumors shown in (**a**, **b**) were defined to be double *in situ* hybridization negative and tumors in (**c**, **d**) were defined to be double *in situ* hybridization positive. Insets indicate the representative tumor cells in each microphotograph.

(Figure 2d) was observed in five cases with clear-cell adenocarcinoma (6%), but none of the non-clear-cell type ovarian carcinomas. Consequently, double *in situ* hybridization-positive cases were more frequent in clear-cell adenocarcinomas than in non-clear-cell type ovarian carcinomas with a statistical significance (24 *vs* 3%, $P < 0.0001$), and also in the comparison between clear-cell adenocarcinomas and serous adenocarcinomas ($P = 0.00011$).

Relationships Between MET Overexpression and Double *In Situ* Hybridization Positivity in Clear-Cell Adenocarcinomas

Among the 20 clear-cell adenocarcinomas assigned as MET overexpression, 9 (45%) and 11 (55%) were double *in situ* hybridization positive and negative, respectively. Among the 70 clear-cell adenocarcinomas without MET overexpression, 12 (17%) and 57 (81%) were double *in situ* hybridization positive

and negative, respectively. The remaining case was non-informative for double *in situ* hybridization analysis. Consequently, there was a significant correlation between MET overexpression and double *in situ* hybridization positivity ($P = 0.0105$).

Clinicopathological Significance of MET Overexpression in Clear-Cell Adenocarcinomas

The cases assigned as MET overexpression showed significantly shorter survival periods in comparison with the cases not assigned overexpression (5-year survival rates, 33.0% *vs* 75.7%, $P = 0.00012$ by log-rank test) (Figure 3a). On the other hand, with regard to mean patient age, distribution of FIGO stage (stages I/II *vs* III/IV), presence of the residual tumor after initial surgery, frequency of lymph node metastasis, response to first-line chemotherapy of tumors, and the extent of histological differentiation of tumors (poorly differentiated tumors *vs* relatively
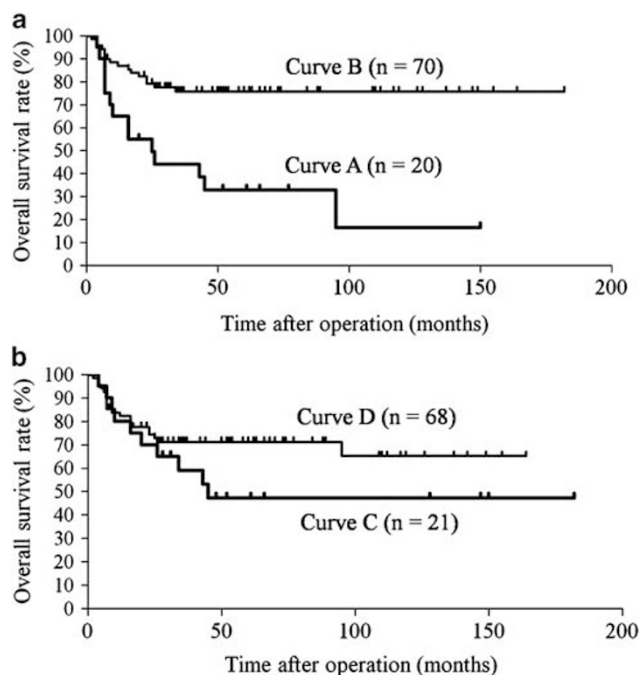
Figure 3 Overall survival curves for the patients with clear-cell adenocarcinoma, stratified by (**a**) MET overexpression detected by immunohistochemistry, and (**b**) positivity of double *in situ* hybridization for copy number alteration of the *MET* gene. (**a**) Curve A for the group with MET overexpression ($n = 20$), and curve B for the group without MET overexpression ($n = 70$). These two curves were significantly different ($P = 0.00012$, by log-rank test). (**b**) Curve C for the double *in situ* hybridization-positive group ($n = 21$), and curve D for the double *in situ* hybridization-negative group. Although the double *in situ* hybridization-positive group shows a trend of shorter overall survival than the negative group, this difference is not statistically significant ($P = 0.192$, by log-rank test).

differentiated tumors), there were no significant differences between the two groups (Table 3).

In comparison with the double *in situ* hybridization-negative cases ($n = 68$), the double *in situ* hybridization-positive cases ($n = 21$) tended to be poorly differentiated histologically (52% (11 of 21) *vs* 15% (10 of 68), $P = 0.00038$) (Table 3). On the other hand, there were no significant differences between the two groups with regard to mean patient age, distribution of FIGO stage (stages I/II *vs* III/IV), presence of the residual tumor after initial surgery, frequency of lymph node metastasis, and response to first-line chemotherapy of tumors (Table 3). Survival analyses showed that patients with double *in situ* hybridization-positive tumors tended to have a poorer outcome than those with double *in situ* hybridization-negative tumors (the 5-year survival rates were 47.3 and 71.1%, respectively), but the difference was not statistically significant ($P = 0.192$ by log-rank test) (Figure 3b).

A multivariate analysis using Cox's model identified that the presence of MET overexpression had an independent impact on overall survival ($P = 0.0176$, relative risk (RR) = 2.42) as well as advanced stages of disease ($P = 0.0011$, RR = 5.76) and a poorly

differentiated histology of tumors ($P = 0.00051$, RR = 4.58) (Table 4).

Discussion

In recent years, structural alteration of the *MET* gene, especially in the form of gene amplification, has received increased attention. Accumulated evidence suggests that MET can be an exciting and novel drug target, because of the success observed *in vitro*, *in vivo*, and in preclinical studies. Specifically, it has been shown that lung cancer and gastric cancer cell lines with *MET* gene amplification display significantly increased sensitivity to MET tyrosine kinase inhibitors,[19,31,32] suggesting that patients with tumors containing amplified *MET* may have clinical responses to the MET inhibitors. Moreover, recent studies have demonstrated that *MET* amplification contributes to the acquired drug resistance to EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib, in non-small cell lung cancers.[31] Additionally, *in vitro*, gefitinib sensitivity by cell lines can be restored by blocking MET signaling.[31] These findings indicate that amplified *MET* could serve as a biomarker for targeted therapy, similar to *HER2* gene amplification in breast cancer and *EGFR* gene mutation and/or amplification in lung cancer.

This study demonstrated that, among the ovarian carcinomas enrolled, overexpression of MET and the positivity of double *in situ* hybridization (high-level polysomy or true amplification of the *MET*) are highly characteristic for clear-cell adenocarcinoma: 22 and 24% in clear-cell adenocarcinomas compared with 0 and 3% in non-clear-cell type ovarian carcinomas, respectively. The frequency of MET overexpression shown in this study may be somewhat lower than the reported frequencies of MET expression in non-clear-cell type ovarian carcinomas,[21–25] although weak immunoreaction (scored as 1 +) for MET was seen in 21% of non-clear-cell type ovarian carcinomas enrolled in this study. These discrepancies would result from the criteria for evaluating the immunoreactions as well as the antibodies and the detection techniques used. However, we thought the criteria used in this study (the criteria was the same as that of the HercepTest™ for HER2/neu immunoreaction in breast cancer) were appropriate because the MET overexpression in clear-cell adenocarcinomas was highly correlated with patient's outcome being an independent prognostic factor for overall survival, probably reflecting true protein expression levels, and because MET overexpression defined was highly correlated with double *in situ* hybridization positivity. As well as the strong prognostic impact of MET overexpression in the patients with clear-cell adenocarcinoma, double *in situ* hybridization positivity was significantly correlated with the poorly differentiated histology of tumors. In the carcinomas of other

Table 3 Correlation of MET overexpression and *MET* copy number alteration with clinicopathological parameters

Parameters	Number of cases (%)					P-value
	MET overexpression		P-value	*Double* in situ *hybridization-positive*		
	Yes (n = 20)	No (n = 70)		Yes (n = 21)	No (n = 68)	
Age (years); median (s.d.)	52.9 (8.9)	52.9 (7.9)	0.997	51.1 (9.8)	53.2 (7.5)	0.306
FIGO stage						
I–II	10 (17)	49 (83)	0.097[a]	13 (22)	45 (78)	0.719[a]
III–IV	10 (32)	21 (68)		8 (26)	23 (74)	
Residual tumor after initial surgery						
Absent	13 (21)	50 (79)	0.580	16 (26)	46 (74)	0.457
Present	7 (26)	20 (74)		5 (19)	22 (81)	
Response to chemotherapy						
Complete or partial response (n = 10)	1 (10)	9 (90)	0.148	1 (10)	9 (90)	0.382
Stable disease or progressive disease (n = 17)	6 (35)	11 (65)		4 (24)	13 (76)	
Lymph node status						
pN0	8 (17)	38 (83)	0.953	11 (24)	34 (76)	0.968
pN1	2 (17)	10 (83)		3 (25)	9 (75)	
pNx (not assessed)	10	22		7	25	
Tumor differentiation in histology						
Relatively differentiated	13 (19)	55 (81)	0.213	10 (15)	58 (85)	**0.0004**
Poorly differentiated	7 (32)	15 (68)		11 (52)	10 (48)	
5-Year survival (%)	33.0	75.7	**0.00012[b]**	47.3	71.1	0.192[b]

FIGO, International Federation of Gynaecology and Obstetrics; pN0, no lymph node metastases; pN1, one or more lymph node metastasis found in pelvic or paraaortic lymph nodes.
[a]Comparison between stages I–II and stages III–IV tumors.
[b]Calculated by log-rank test.
Bold values indicate statistical significance.

Table 4 Cox's proportional hazards model estimates of the significance of prognostic factors for patients with ovarian clear-cell adenocarcinoma

Variables	P-value	RR (95% CI)
(a) Univariate Cox regression model		
Age (≥53 years *vs* <53 years)[a]	**0.014**	0.35 (0.15–0.81)
FIGO stage (III–IV *vs* I–II)	**<0.0001**	7.76 (3.49–17.25)
Residual tumors (present *vs* absent)	**<0.0001**	5.99 (2.86–12.58)
Poorly differentiated tumor (present *vs* absent)	**0.0055**	2.79 (1.35–5.76)
MET overexpression (yes *vs* no)	**0.0053**	2.84 (1.37–5.92)
Double in situ *hybridization for MET gene*		
(Positive *vs* negative)	0.197	1.65 (0.77–3.52)
(b) Multivariate Cox regression model		
Age (≥53 years *vs* <53 years)[a]	0.058	0.44 (0.19–1.03)
FIGO stage (III–IV *vs* I–II)	**0.0011**	5.76 (2.02–16.41)
Residual tumors (present *vs* absent)	0.055	2.51 (0.98–6.42)
Poorly differentiated tumor (present *vs* absent)	**0.00051**	4.58 (1.94–10.78)
MET overexpression (yes *vs* no)	**0.018**	2.42 (1.17–5.03)

FIGO, International Federation of Gynaecology and Obstetrics; RR, relative risk; CI, confidence interval.
[a]Mean values.
Bold values indicate a statistical significance.

primary sites, MET becomes increasingly overexpressed as the tumors become poorly differentiated, invasive, and metastatic.[26,33] *In vitro* and *in vivo* MET receptor elicits a unique biological program leading to 'invasive growth,' resulting from the activation of proliferation, motility, cell dissociation, and protection from apoptosis.[10,14] As shown in the uni- and multivariate analyses in this study, the

poorly differentiated histology of tumors was found to be a significant prognostic factor for overall survival in the patients with clear-cell adenocarcinoma. Consequently, our present study suggests that, primarily by increased gene copy number (including true amplification), structural alteration of the *MET* gene causes MET protein overexpression and histological progression (dedifferentiation), and acts as an oncogene in the clear-cell adenocarcinoma.

Herein, with regard to the overexpression (or potentially activation) of MET receptor in clear-cell adenocarcinomas, we must discuss the possible mechanisms that may cause MET receptor activation other than gene amplification. Although a strong relationship between MET overexpression and double *in situ* hybridization positivity was statistically supported, more than half (55%) of the clear-cell adenocarcinoma cases with MET overexpression were defined as double *in situ* hybridization negative, and 57% (12 of 21) of double *in situ* hybridization-positive cases were assigned as no or weak (1 +) immunoreaction for MET. These findings suggest that *MET* gene copy number alterations may potentiate but not always be required, or not be sufficient, for MET protein overexpression.

The absence of structural abnormality of the gene (ie double *in situ* hybridization-negativity defined in this study) in MET-overexpressing clear-cell adeno-carcinomas suggests that the overexpression is secondary to the alteration of other signaling pathways. In addition to gene amplification, MET activation can occur through alternative mechanisms such as selected *MET* mutations, ligand-independent constitutive dimerization, pathway activation mediated by the hypoxia inducible factor (HIF1)-α under hypoxic conditions, transactivation by other receptors including EGFR, and loss of negative regulators such as the von Hippel–Lindau (VHL) tumor suppressor.[8,9] Although somatic mutation of *MET* was not found in primary ovarian carcinomas,[11,12] alternative expression of HIF1-α, allelic losses on chromosome 3q25–q26 (containing *VHL* locus), and downregulation of VHL protein frequently occur in ovarian clear-cell adenocarcinoma.[34–36] Moreover, Nam *et al*[37] demonstrated that aberrant HGF/MET signaling induces centrosome amplification and chromosomal instability via the PI3K/Akt pathway. Among ovarian carcinomas, clear-cell adenocarcinoma is unique in that it has a high percentage (46%) of *PIK3CA*-activating mutations.[38] These mechanisms may either partially or completely eliminate the dependence of MET activation.

In summary, among ovarian carcinomas, MET overexpression and increased copy number (including true amplification) are highly characteristic and common events in clear-cell adenocarcinoma. MET acts as an oncoprotein in ovarian clear-cell adenocarcinoma, and its overexpression is highly correlated with prognosis of the patients with that

carcinoma and associated with tumor progression/dedifferentiation. These results contribute to the understanding of the pathogenesis of this highly lethal malignancy and support the development of targeted therapies that inhibit MET activation. As well as gene amplification, further studies are needed to evaluate the alternative MET activation mechanisms in clear-cell adenocarcinoma, including autocrine/paracrine loops of HGF-MET, hypoxia-related signaling pathways (ie HIF1-α, VHL), dysregulated PI3K/AKT signaling pathway, and the potential of activating mutation of *MET*.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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