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# High Expression of SQSTM1/p62 Protein Is Associated with Poor Prognosis in Epithelial Ovarian Cancer

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High expression of SQSTM1/p62 (p62) protein, which functions as a hub for various cellular signaling pathways, has been detected in several human cancers. However, the clinicopathological impact of high p62 expression is largely unknown in epithelial ovarian cancer (EOC). Here, the expression level of p62 in primary EOCs (*n*=266) was assessed by immunohistochemistry, and its clinical significance was analyzed. Univariate and multivariate analyses were used to determine the impact of p62 expression on overall survival. p62 was expressed in the cytoplasm (Cyto) and/or nucleus (Nuc) in primary EOCs, and an expression subtype ($Cyto^{High}/Nuc^{Low}$), showing high expression in the cytoplasm but low expression in the nucleus, was significantly correlated with serous carcinoma (*P*<0.001), advanced stage (*P*=0.005), presence of residual tumor (*P*<0.001), and low overall survival rate (*P*=0.013). Furthermore, in serous carcinomas (*n*=107), the p62 $Cyto^{High}/Nuc^{Low}$ subtype was significantly correlated with low overall survival rate (*P*=0.019) as an independent factor (*P*=0.044). Thus, our findings suggest that high expression of cytoplasmic p62 may be a novel prognostic biomarker in EOC, particularly in serous carcinoma.

**Key words:** epithelial ovarian cancer, serous carcinoma, immunohistochemistry, p62, prognosis

## I. Introduction

SQSTM1/p62 (hereafter referred to as p62) functions as a signaling hub for various cell survival or cell death pathways [15]. This protein is also known as one of the selective substrates for autophagy, a cellular degradation system by which cytoplasmic components, organelles, and incorporated p62 protein are degraded [1, 8, 15, 22]. Mice lacking *Atg5* or *Atg7*, which are essential components in autophagy pathway, show elevated rates of spontaneous tumor formation accompanied by p62 accumulation, and tumor size is reduced by deletion of p62, suggesting that p62 accumulation may contribute to tumor progression [9, 15, 26]. Furthermore, it has been demonstrated that excess p62 expression may be involved in the activation of various oncogenic signaling pathways, including the NF-κB [4, 15], Wnt [6], mTOR [17], or NRF2 [9, 15, 16] pathways. Moreover, abnormal expression of p62 has been indeed detected in several cancers including prostate [7, 13], kidney [17], liver [9], lung [10], breast [3, 21, 24], and oral [11, 18] cancer cases. Thus, accumulating evidence from previous studies has indicated that excess p62 expression may play an oncogenic role in human cancers.

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Ovarian cancer is the most lethal gynecological malignancy in developed countries [5]. Epithelial ovarian cancer (EOC), comprises 90% of all ovarian cancers, include serous, mucinous, clear cell, and endometrioid carcinomas [2]. Among them, serous carcinoma, particularly high-grade serous carcinoma (HGSC), usually occurs in the advanced stage and spreads beyond the ovary at diagnosis [23]. Therefore, it is necessary to identify novel and efficient biomarker to use for prognosis for human EOC. In the present study, we examined the expression of p62 protein in EOC patients and analyzed the clinicopathological implications of the p62 expression status. While p62 protein was detected in the cytoplasm (Cyto) and/or nucleus (Nuc) in primary EOCs, we found that an expression subtype ($Cyto^{High}/Nuc^{Low}$; high expression in the cytoplasm but low expression in the nucleus) was associated with aggressive phenotypes and poor clinical outcome. Furthermore, this expression subtype of p62 protein was an independent prognostic factor in serous carcinomas. Thus, our findings in the present study indicate that p62 may serve as a biomarker for the prognostic prediction of EOC, especially for patients with serous carcinoma.

## II. Materials and Methods

***Patients and tumor specimens***

Formalin-fixed paraffin-embedded tissue blocks of primary epithelial ovarian cancers (EOCs) from 266 consecutive patients were used to construct tissue microarrays (TMAs). All patients underwent for in surgery at the National Defense Medical College (NDMC) Hospital (Saitama, Japan) from 1986 to 2006 with a ten year period of follow-up starting from the initial surgery. The average follow-up period was 59 months (range, 1–120 months). Of the 266 patients, 93 patients (35.0%) died due to their cancer, with a median follow-up period of 20 months (range, 2–108 months). The tumor histological types were classified according to the WHO criteria. Clinical stages of the disease were classified according to the International Federation of Gynecology and Obstetrics (FIGO) system in 1988. All patients gave their written informed consent in formal style before the study. This study was approved by the ethics committees of the National Defense Medical College, Keio University, and Tokyo Medical and Dental University.

***Immunohistochemical analysis***

We constructed TMAs from tissue blocks prepared from the 266 EOC tumors using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA) as previously described [14], and immunohistochemistry was performed on TMA sections. The tissue sections were deparaffinized in xylene, and rehydrated with graded ethanol (100%, 90%, 80%, 70%, and 50%) to water. After the retrieval of antigens by boiling in 10 mM citrate buffer (pH 6.0), the sections were treated with 0.3% hydrogen peroxide in methanol to inactivate the endogenous peroxidase activity. Non-specific binding was blocked by incubation in horse serum in PBS. Slides were incubated with mouse anti-p62 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilution: 1/2000) overnight at room temperature. The bound antibody was visualized using diaminobenzidine as a chromogen (VECTASTAIN Elute ABC kit, Vector Laboratories), and the sections were lightly counterstained with hematoxylin.

***Evaluation of immunohistochemistry***

Immunohistochemical evaluation was performed by three researchers (R.I., Ju.I., and H.T.), and cases with discrepant grades were re-evaluated by discussion until consensus was achieved. The intensity score of cytoplasmic p62 expression was defined as 0 (no expression), 1+ (weak), and 2+ (strong). Specimens with 10% or more immunoreactive tumor cells with an intensity score of 2+ were considered "high expression", and specimens with less than 10% of 2+ cells or specimens where almost all tumor cells had an intensity score of 1+ or 0 were considered "low expression". Nucleus staining of p62 was classified as either "high expression" or "low expression" when stained with greater or less than 5% of tumor cells, respectively. Based on the distribution (cytoplasm; Cyto or nucleus; Nuc) and the expression level (High or Low), specimens were classified into four subtypes: Type-A: $Cyto^{Low}/Nuc^{Low}$, Type-B: $Cyto^{Low}/Nuc^{High}$, Type-C: $Cyto^{High}/Nuc^{High}$, and Type-D: $Cyto^{High}/Nuc^{Low}$.

***Statistical analysis***

Correlation between p62 expression in primary EOCs and the clinicopathological variables were analyzed by the chi-square or Fisher's exact test. Survival data were analyzed by the Kaplan-Meier method, and compared with the expression status of p62 by log-rank (Cox-Mentel) test. Calculated $P$ values lower than 0.05 were considered as statistically significant.

## III. Results

***Immunoreaction of p62 in epithelial ovarian cancer***

To assess the clinical significance of p62 expression in EOC, we performed immunohistochemical analysis using TMAs from 266 primary EOCs. The expression of p62 protein was detected in the cytoplasm and/or nucleus of EOC tumor cells (Fig. 1). High cytoplasmic immunoreaction for p62 protein was found in 42 (15.8%) of 266 cases (Fig. 1D–F). Nuclear immunoreaction for p62 protein was shown in some population (5–90%) of tumor cells in 50 (18.8%) of 266 cases (Fig. 1G–I). Based on the expression level (Low or High) and the distribution (Cyto or Nuc), we classified tumors into four subtypes; $Cyto^{Low}/Nuc^{Low}$ as Type-A ($n$=178), $Cyto^{Low}/Nuc^{High}$ as Type-B ($n$=46), $Cyto^{High}/Nuc^{High}$ as Type-C ($n$=4), and $Cyto^{High}/Nuc^{Low}$ as Type-D ($n$=38). Interestingly, the staining intensity of cytoplasmic

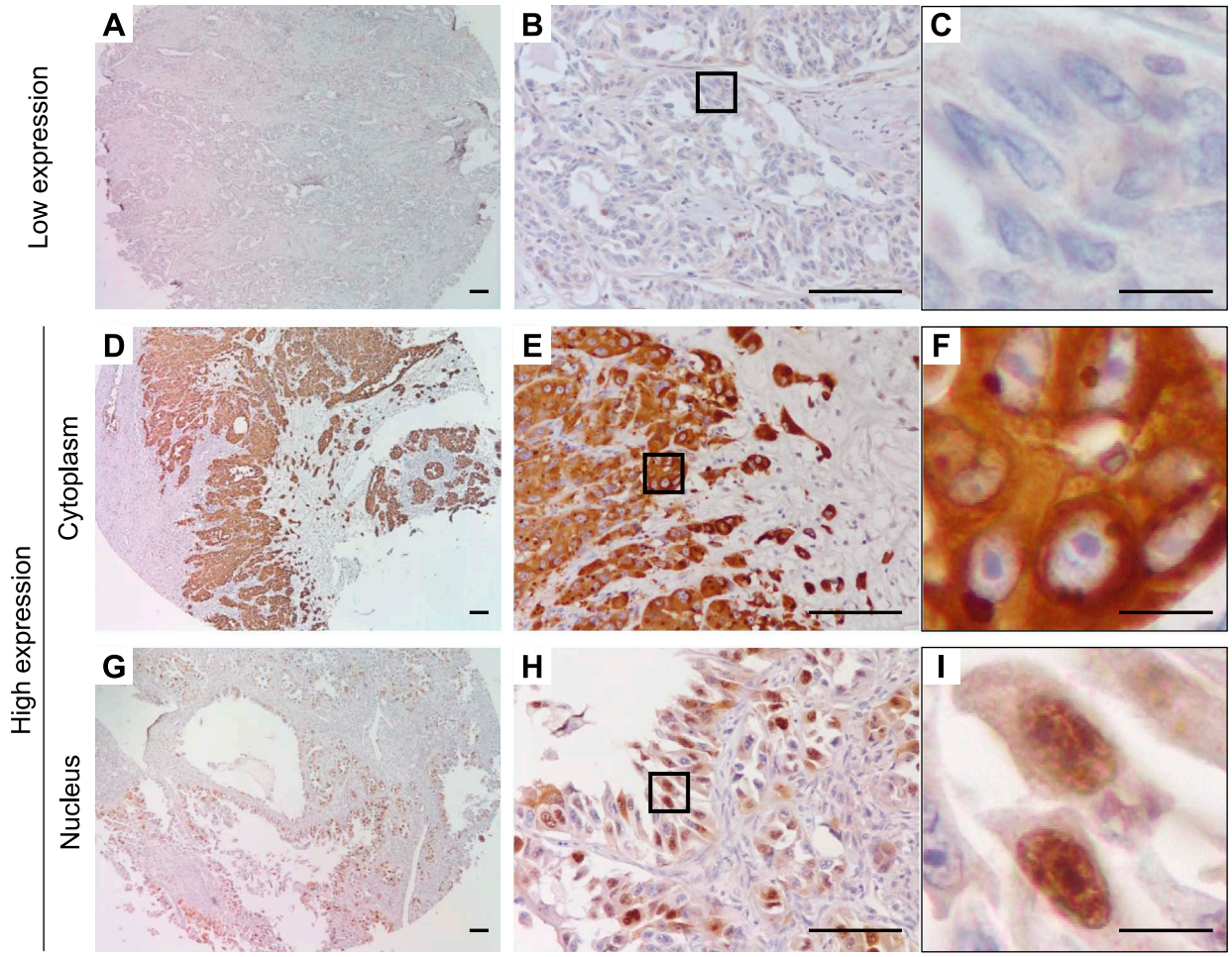


**Fig. 1.** Immunostaining of p62 expression in epithelial ovarian cancer. Representative images for tumors with low p62 expression (**A–C**), high cytoplasmic p62 expression (**D–F**), and high nuclear p62 expression (**G–I**). Bar=100 μm (**A, B, D, E, G**, and **H**) or 10 μm (**C, F**, and **I**).

p62 seemed to be stronger in the invasive front area than in the intratumoral area within p62-highly expressing tumors. Furthermore, in the invasive front area, EOC tumor cells with p62-positive aggregate-like structures were occasionally observed, but the frequency was very low (4 of 42 cytoplasmic p62-highly expressing cases, 9.5%; Fig. 1E and 1F).

### *Clinicopathological and prognostic implications of p62 expression for epithelial ovarian cancers*

To examine the clinicopathological and prognostic implications of p62 expression status in EOC, we performed the chi-square or Fisher's exact test, as appropriate. High expression of cytoplasmic p62 and the Type-D ($Cyto^{High}/Nuc^{Low}$) expression subtype were significantly associated with aggressive phenotypes, including serous histology ($P=1.32\times10^{-6}$ and $P=1.46\times10^{-7}$, respectively), advanced FIGO stage ($P=7.85\times10^{-3}$ and $P=4.99\times10^{-3}$, respectively), and presence of residual tumor ($P=7.14\times10^{-4}$ and $P=3.74\times10^{-4}$, respectively) (Table 1). Overall survival time of EOC patients was significantly shorter in the high cytoplasmic p62 expression group compared with the group with low expression ($P=9.13\times10^{-3}$, Fig. 2A) or in the group with the expression subtype of Type-D compared with the group with other subtypes ($P=1.30\times10^{-2}$, Fig. 2B).

In the present cohort included 107 cases with serous carcinoma, among them 9 cases were low-grade, 65 were HGSC, and the others were not determined. Although there was no significant correlation between the clinicopathological characteristics and the expression status of p62 (Table 2), Kaplan-Meier survival curves revealed that overall survival time was significantly shorter in the Type-D expression subtype compared with the other subtypes ($P=1.91\times10^{-2}$, Fig. 2C). Additionally, even in the 65 patients with HGSC, the Type-D expression subtype was significantly correlated to shorter overall survival time ($P=1.20\times10^{-2}$, Fig. 2D).

### *Multivariate analysis for poor prognosis marker in epithelial ovarian cancers*

In all 266 patients, univariate analysis showed that advanced FIGO stage, presence of residual tumor, and the Type-D expression subtype ($Cyto^{High}/Nuc^{Low}$) were significantly correlated with overall survival, however the p62

**Table 1.** *Correlation between p62 expression and clinicopathological variables in 266 patients with epithelial ovarian cancer*

| | All | Expression level of Cytoplasmic p62 [a] | | | | *P* [b] | Subtypes of p62 expression status [a] | | | | *P* [b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Low | | High | | | Type-A, -B, -C | | Type-D | | |
| | *n* | *n* | (%) | *n* | (%) | | *n* | (%) | *n* | (%) | |
| Number | 266 | 224 | (84.2) | 42 | (15.8) | | 228 | (85.7) | 38 | (14.3) | |
| Age, years [c] | | | | | | | | | | | |
| ≤50 | 107 | 87 | (81.3) | 20 | (18.7) | 0.287 | 88 | (82.2) | 19 | (17.8) | 0.184 |
| >50 | 159 | 137 | (86.2) | 22 | (13.8) | | 140 | (88.1) | 19 | (11.9) | |
| Histological type (serous vs. others) | | | | | | | | | | | |
| Serous | 107 | 76 | (71.0) | 31 | (29.0) | **<0.001** | 77 | (72.0) | 30 | (28.0) | **<0.001** |
| Mucinous | 30 | 27 | (90.0) | 3 | (10.0) | | 27 | (90.0) | 3 | (10.0) | |
| Endometrioid | 42 | 38 | (90.5) | 4 | (9.5) | | 38 | (90.5) | 4 | (9.5) | |
| Clear cell | 87 | 83 | (95.4) | 4 | (4.6) | | 86 | (98.9) | 1 | (1.1) | |
| FIGO stage (I–II vs. III–IV) | | | | | | | | | | | |
| I | 96 | 89 | (92.7) | 7 | (7.3) | **0.008** | 91 | (94.8) | 5 | (5.2) | **0.005** |
| II | 30 | 25 | (83.3) | 5 | (16.7) | | 25 | (83.3) | 5 | (16.7) | |
| III | 104 | 86 | (82.7) | 18 | (17.3) | | 88 | (84.6) | 16 | (15.4) | |
| IV | 36 | 24 | (66.7) | 12 | (33.3) | | 24 | (66.7) | 12 | (33.3) | |
| Residual tumor | | | | | | | | | | | |
| Absent | 127 | 117 | (92.1) | 10 | (7.9) | **<0.001** | 119 | (93.7) | 8 | (6.3) | **<0.001** |
| Present (>0 cm) | 139 | 107 | (77.0) | 32 | (23.0) | | 109 | (78.4) | 30 | (21.6) | |

[a] Expression status of p62 was assessed by IHC and classified into four subtypes based on expression level (Low or High) and distribution (cytoplasm; Cyto or nucleus; Nuc): Type-A ($Cyto^{Low}/Nuc^{Low}$), Type-B ($Cyto^{Low}/Nuc^{High}$), Type-C ($Cyto^{High}/Nuc^{High}$), and Type-D ($Cyto^{High}/Nuc^{Low}$).
[b] Chi-square or Fisher's exact test, as appropriate. Statistically significant values are in boldface type.
[c] Median age was 53 years (range, 20–81 years).

**Table 2.** *Correlation between p62 expression and clinicopathological variables in 107 patients with serous ovarian carcinoma*

| | All | Expression level of Cytoplasmic p62 [a] | | | | *P* [b] | Subtypes of p62 expression status [a] | | | | *P* [b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Low | | High | | | Type-A, -B, -C | | Type-D | | |
| | *n* | *n* | (%) | *n* | (%) | | *n* | (%) | *n* | (%) | |
| Number | 107 | 76 | (71.0) | 31 | (29.0) | | 77 | (72.0) | 30 | (28.0) | |
| Age, years [c] | | | | | | | | | | | |
| ≤50 | 39 | 26 | (66.7) | 13 | (33.3) | 0.287 | 26 | (66.7) | 13 | (33.3) | 0.356 |
| >50 | 68 | 50 | (73.5) | 18 | (26.5) | | 51 | (75.0) | 17 | (25.0) | |
| Histological grade (1 vs. 2 or 3) | | | | | | | | | | | |
| 1 | 9 | 9 | (100.0) | 0 | (0.0) | 0.102 | 9 | (100.0) | 0 | (0.0) | 0.102 |
| 2 or 3 | 65 | 45 | (69.2) | 20 | (30.8) | | 45 | (69.2) | 20 | (30.8) | |
| Unknown | 33 | 22 | (66.7) | 11 | (33.3) | | 23 | (69.7) | 10 | (30.3) | |
| FIGO stage (I–II vs. III–IV) | | | | | | | | | | | |
| I | 11 | 8 | (72.7) | 3 | (27.3) | 0.655 | 8 | (72.7) | 3 | (27.3) | 0.583 |
| II | 7 | 4 | (57.1) | 3 | (42.9) | | 4 | (57.1) | 3 | (42.9) | |
| III | 63 | 48 | (76.2) | 15 | (23.8) | | 49 | (77.8) | 14 | (22.2) | |
| IV | 26 | 16 | (61.5) | 10 | (38.5) | | 16 | (61.5) | 10 | (38.5) | |
| Residual tumor | | | | | | | | | | | |
| Absent | 19 | 15 | (78.9) | 4 | (21.1) | 0.401 | 15 | (78.9) | 4 | (21.1) | 0.455 |
| Present (>0 cm) | 88 | 61 | (69.3) | 27 | (30.7) | | 62 | (70.5) | 26 | (29.5) | |

[a] Expression status of p62 was assessed by IHC and classified into four subtypes based on expression level (Low or High) and distribution (cytoplasm; Cyto or nucleus; Nuc): Type-A ($Cyto^{Low}/Nuc^{Low}$), Type-B ($Cyto^{Low}/Nuc^{High}$), Type-C ($Cyto^{High}/Nuc^{High}$), and Type-D ($Cyto^{High}/Nuc^{Low}$).
[b] Chi-square or Fisher's exact test, as appropriate. Statistically significant values are in boldface type.
[c] Median age was 54 years (range, 29–81 years).

expression status was not found to be an independent prognostic factor by multivariate analysis (Table 3). On the other hand, in 107 patients with serous cancer, multivariate analysis following univariate analysis revealed that the Type-D expression subtype was only an independent prognostic factor for overall survival ($P=4.36\times10^{-2}$, Table 3). These findings suggest that high expression of p62 protein in the cytoplasm may be a molecular marker of poor prog-

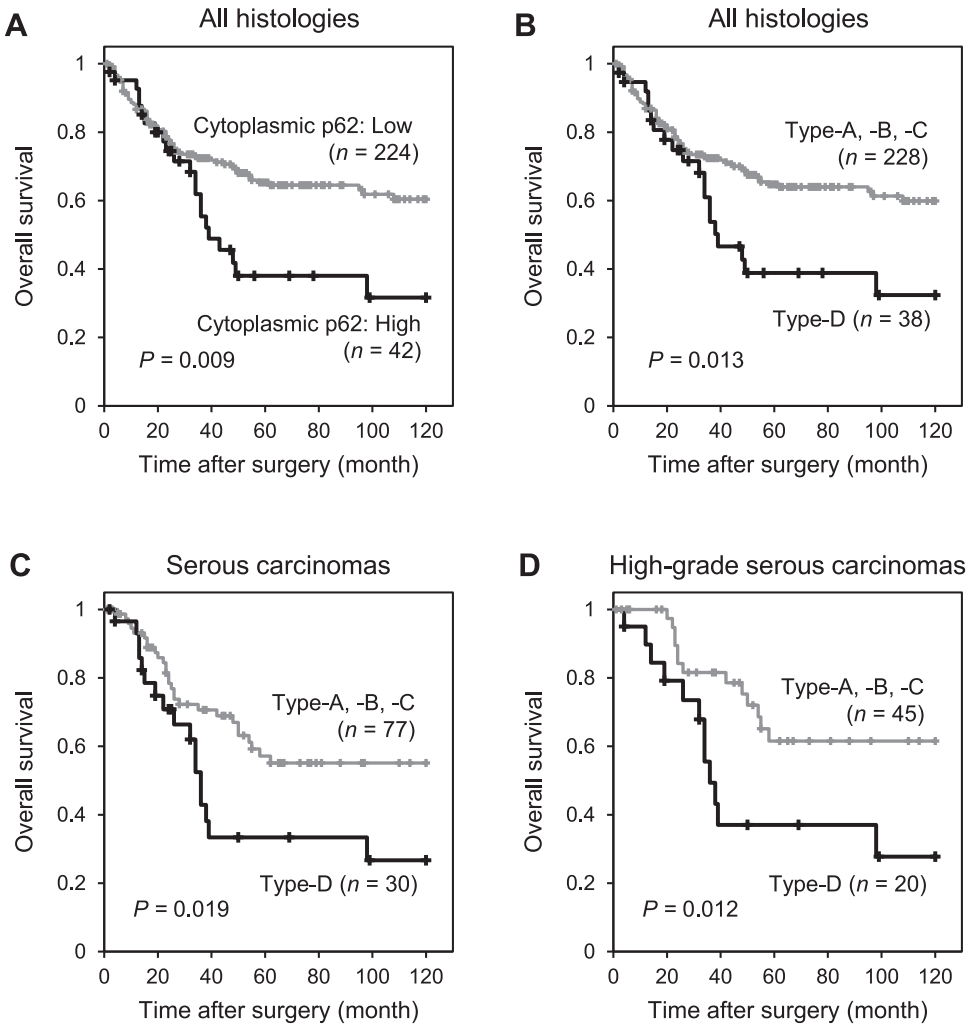


**Fig. 2.** Prognostic implication of the p62 expression status in epithelial ovarian cancer. **A.** Overall survival curves of all 266 patients with epithelial ovarian cancer (EOC) according to the expression level of cytoplasmic p62. This figure shows survival curves for 42 patients with high cytoplasmic p62-expressing tumors and for 224 patients with low cytoplasmic p62-expressing tumors. The two curves differ significantly. **B.** Overall survival curves of all 266 patients with EOC according to the p62 expression subtypes. This figure shows the survival curves for 38 patients with Type-D tumors (high expression of cytoplasmic p62 and low expression of nuclear p62; $Cyto^{High}/Nuc^{Low}$) and for 228 patients with Type-A, -B, or -C tumors ($Cyto^{Low}/Nuc^{Low}$, $Cyto^{Low}/Nuc^{High}$, or $Cyto^{High}/Nuc^{High}$, respectively). The two curves differ significantly. **C.** Overall survival curves of 107 patients with serous carcinoma according to the p62 expression subtypes. This figure shows the survival curves for 30 patients with Type-D tumors and 77 patients with Type-A, -B, or -C tumors. The two curves differ significantly. **D.** Overall survival curves of 65 patients with high-grade serous carcinoma according to the p62 expression subtypes. This figure shows the survival curves for 20 patients with Type-D tumors and 45 patients with Type-A, -B, or -C tumors. The two curves differ significantly.

nosis in patients with EOC, especially those with serous carcinomas.

## IV. Discussions

In the present cohort, we found that the Type-D ($Cyto^{High}/Nuc^{Low}$) p62 expression subtype is correlated with poor prognosis in epithelial ovarian cancers (EOC), especially in serous histology. However, at present the mechanism underlying the change to high expression of p62 protein is unclear. While p62 protein is continuously degraded by the autophagy system, it has been demonstrated that this protein is highly accumulated in liver tumors that develop in mice lacking the *Atg5* or *Atg7* genes [9, 15, 26]. On the other hand, it has been known that p62 expression is regulated in a cellular context-dependent manner at the transcriptional level [12, 19, 25]. The p62 mRNA level is positively correlated with its protein level examined by immunohistochemistry in oral squamous cell carcinomas [18]. Thus, high level of p62 protein may be attributed to dysregulation at the transcription and/or translation levels.

Furthermore, we showed two characteristic distributions of p62 protein expression. We have observed localization of p62 in the nucleus and not only in the cytoplasm. It has been experimentally demonstrated that p62 protein can be shuttled between the nucleus and cytoplasm due to its signal for nuclear localization and export [20]. Furthermore, this distribution has been observed in human prostate

**Table 3** *Cox proportional hazard regression analysis for overall survival*

| | Univariate analyses | | | Multivariate analyses | | |
|---|---|---|---|---|---|---|
| | HR | (95% CI) [a] | *P* [b] | HR | (95% CI) [a] | *P* [b] |
| All histologies (*n*=266) | | | | | | |
| Age >50 years (vs. ≤50 years) | 0.84 | (0.56–1.26) | 0.401 | | | |
| Serous histology (vs. the others) | 1.44 | (0.96–2.16) | 0.082 | | | |
| FIGO stage III–IV (vs. I–II) | 4.23 | (2.58–6.95) | <**0.001** | 2.76 | (1.41–5.41) | **0.003** |
| Residual tumor >0 cm (vs. =0 cm) | 3.68 | (2.28–5.95) | <**0.001** | 1.73 | (0.90–3.33) | 0.100 |
| Type-D p62 expression (vs. Type-A, -B, -C) [c] | 1.85 | (1.13–3.04) | **0.015** | 1.28 | (0.77–2.12) | 0.337 |
| Serous carcinomas (*n*=107) | | | | | | |
| Age >50 years (vs. ≤50 years) | 0.75 | (0.41–1.36) | 0.341 | | | |
| Histological grade 2 or 3 (vs. 1) | 0.90 | (0.31–2.60) | 0.849 | | | |
| FIGO stage III–IV (vs. I–II) | 2.40 | (0.86–6.72) | 0.094 | | | |
| Residual tumor >0 cm (vs. =0 cm) | 3.29 | (1.02–10.65) | **0.047** | 3.00 | (0.92–9.75) | 0.068 |
| Type-D p62 expression (vs. Type-A, -B, -C) [c] | 2.03 | (1.11–3.72) | **0.022** | 1.87 | (1.02–3.44) | **0.044** |

[a] HR, hazard ratio; CI, confidence interval.

[b] Statistically significant values are in boldface type.

[c] Expression status of p62 was assessed by IHC and classified into four subtypes based on expression level (Low or High) and distribution (cytoplasm; Cyto or nucleus; Nuc): Type-A ($Cyto^{Low}/Nuc^{Low}$), Type-B ($Cyto^{Low}/Nuc^{High}$), Type-C ($Cyto^{High}/Nuc^{High}$), and Type-D ($Cyto^{High}/Nuc^{Low}$).

and oral cancers, and has been reported to be correlated with favorable prognosis at least in oral cancers [13, 18]. In the serous carcinomas of the present cohort, the patients with Type-D p62 expression ($Cyto^{High}/Nuc^{Low}$) had poorer prognosis than those with the other expression subtypes. This suggests that the high expression of p62 protein in the cytoplasm, not in the nucleus, may contribute to the malignancy of EOC cells, but the molecular mechanism is largely unknown. Expression of p62 was also stronger in the invasive front area than in the intratumoral area in primary EOC tumors with high expression of cytoplasmic p62. In this invasive front area, EOC tumor cells with p62-positive aggregate-like structures were occasionally observed. Many studies have suggested that excess p62 expression, notably formation of p62-positive aggregates, contributes to tumor growth and tolerance to cellular stress in tumor cells by the activation of oncogenic signals, including the NF-κB, Wnt, mTOR, and NRF2 pathways [4, 6, 9, 15–17]. This suggests that activation of these oncogenic signaling pathways may be involved in the migration and/or invasion of EOC cells within the invasive front area.

A pattern of cytoplasmic p62-expression was extremely infrequent in other histological types than serous carcinoma, such as mucinous, endometrioid, and clear cell, suggesting that p62 expression may be associated with tumor development only in the process of serous carcinoma, although its mechanism is largely unknown. Recently, it has been suggested that sub-classification of serous carcinomas by histological grade is important for proper prognosis; HGSCs are more malignant than low-grade serous carcinomas [23]. In patients with HGSCs from our cohort, patients with the Type-D subtype showed significantly shorter overall survival time than patients with other p62 expression subtypes. This suggests that determination of the expression status of p62, together with histological grade, may be helpful for diagnosis of patients with serous carcinomas. Thus, understanding the significance of the heterogeneous distribution of p62 protein and the mechanisms underlying the involvement of p62 in the malignancy of EOC cells is required to develop a better personalized therapeutic approach for EOC with high expression of cytoplasmic p62.

## V. Disclosure

The authors declare no competing financial interests.

## VI. Acknowledgments

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