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Original contribution

MDM2 copy number increase: a poor prognostic, molecular event in esophageal squamous cell carcinoma☆

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Summary The present study aimed to elucidate the clinicopathological significance of molecular alterations in *MDM2* in esophageal squamous cell carcinoma (ESCC). A total of 399 resected cases of ESCC were examined by dual-color in situ hybridization for *MDM2* and immunohistochemistry for p53 using tissue microarrays. Clinicopathological features were correlated with the *MDM2* status. Among 362 cases with a successful dual-color in situ hybridization analysis, 19 (5%) and 13 (4%) had *MDM2* amplification and chromosome 12 polysomy, respectively, and these were examined as an MDM2-positive group. A comparison between amplified and polysomic cases revealed that the latter were more strongly associated with preoperative chemotherapy than the former. Sixteen (50%) of 32 MDM2-positive cases had positive results in all tissue cores examined, indicating diffuse *MDM2* alterations. Cases with the diffuse alteration of *MDM2* were characterized by an advanced pT stage and extensive vascular infiltration. The relationship between *MDM2* copy number increases and p53 mutations was weak, with the overexpression of p53 being similarly detected in MDM2-positive and MDM2-negative cases (59% versus 49%; $P = .267$). Overall survival was shorter in patients with MDM2-positive ESCC than in those without *MDM2* alterations ($P = .033$). The poor prognostic value of *MDM2* alterations became more obvious when only diffusely altered cases were counted ($P = .005$). In conclusion, the present study revealed that *MDM2* copy number increases occurred in 9% of ESCC cases, and *MDM2* alterations, particularly diffuse abnormalities, were associated with a poor prognosis. *MDM2*-altered ESCC may achieve beneficial effects from MDM2-targeted therapy.

Abbreviations DISH, dual-color in situ hybridization; ESCC, esophageal squamous cell carcinoma; FISH, fluorescence in situ hybridization; MDM2, murine double minute-2; PCR, polymerase chain reaction

☆ Disclosures: None to declare.

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1. Introduction

Esophageal cancer is the eighth most common cancer worldwide and causes the death of an estimated 400,000 individuals annually [1]. Esophageal cancer is classified by histology as esophageal adenocarcinoma or ESCC, with the latter being the most frequent subtype, particularly in eastern/southeast Asia and some parts of Africa [2,3]. More than 50% of patients with ESCC are diagnosed at an advanced stage because of the lack of sensitive methods for the early detection of ESCC [4], and the 5-year survival rate of patients with unresectable disease is 14% to 25% [5]. Furthermore, genetic alterations that may be targeted therapeutically similar to HER2 in breast cancers or EGFR in lung cancers have been elusive in ESCC [6].

Recent sequencing studies on ESCC identified copy number alterations and structural rearrangements, including high-level amplification in 11q13.2-q13.3, homozygous deletions in 9p21.3, and in-frame fusion between *TRAPPC9* and *CLVS1* [7-9]. Sequence analyses also detected 22 significantly mutated genes, such as *TP53*, *NOTCH1*, *NFE2L2*, *MLL2*, *ZNF750*, and *FAT1* [6,7,10,11]. Many genetic alterations detected in ESCC are involved in several signaling pathways. Mutations in *TP53* and *CDKN2A* and the amplification of *CCDN1* and *CDK6* dysregulate the cell cycle, whereas the amplification of *EGFR* and *FGFR1* affects receptor tyrosine kinase signaling pathways [6]. Some of these pathways and their downstream molecules are potentially targeted by drugs that have already been approved for other cancers.

The human homologue of the MDM2, a ubiquitin-protein ligase, is a known negative regulator of p53. MDM2 interferes with p53 tumor suppressor function by directly binding to its transactivation domain and promoting proteasome-mediated degradation [12,13]. The overexpression of MDM2 also confers p53-independent activity linked to the dysregulation of cell cycle progression, DNA replication, and DNA repair [14-16]. Therefore, MDM2 is considered to be a promising target for the treatment of human cancers, regardless of the p53 status in tumor cells [17]. The overexpression of MDM2 may be caused by gene amplification. The overall frequency of *MDM2* amplification in human tumors is 7% [18]. A higher frequency has been reported in soft tissue tumors (20%) [18], particularly in well-differentiated/dedifferentiated liposarcoma (almost 100%) [19]. In contrast to nonepithelial tumors, a small number of studies have examined the *MDM2* copy number status in epithelial malignancies, and *MDM2* amplification has been identified as a poor prognostic factor for salivary gland and breast cancers [20,21].

Previous studies identified *MDM2* amplification in 0 to 18% of ESCC cases by Southern blot hybridization, FISH, array comparative genomic hybridization, and quantitative PCR; however, its exact frequency and clinical significance remain unclear [22-25]. In the present study, we examined the clinicopathological features of ESCC with *MDM2* copy number increases using DISH, which is a highly reproducible method for the analysis of gene amplification and copy number variations.

2. Materials and methods

2.1. Case selection and tissue samples

This study was approved by the Ethics Committee of Kobe University Graduate School of Medicine. Informed consents were obtained from all patients. The study cohort consisted of 337 consecutive patients with ESCC who underwent surgical resection between 2007 and 2016, and 62 patients with ESCC who underwent endoscopic submucosal resection in 2009 at Kobe University Hospital. Among the former surgical series, 208 patients with stage II/III disease received preoperative chemotherapy with cisplatin and 5-fluorouracil according to the result of the Japan Clinical Oncology Group 9907 trial [26]. Patients who underwent palliative resection or salvage esophagectomy were excluded. Uncommon histologic variants, such as adenosquamous carcinoma or sarcomatoid carcinoma (so-called carcinosarcomas), were also excluded. According to the protocol in our institute, the resected esophagus was entirely submitted for histologic examinations. Formalin-fixed, paraffin-embedded tissue was used for pathological analyses.

2.2. Evaluation of clinicopathological features

Clinical data, including age, sex, the use of preoperative chemotherapy, and outcomes, were retrieved from electronically stored medical records. The median follow-up period after surgery was 32 months (range, 1-138 months). Histology slides and pathology reports were also reviewed in terms of the depth of invasion, degree of tumor differentiation, lymphovascular invasion, lymph node involvement, resection margin status, and the presence or absence of multiple lesions. Lymphatic invasion was evaluated on D2-40–stained sections. Tissue microarray blocks were constructed using Tissue Microarrayer JF-3 (Pathology Institute, Toyama, Japan). Two or 3 representative tissue cores (2 mm in diameter) were obtained from each case depending on the tumor size. In patients with multiple cancers, the most advanced lesion was selected for study. When there were multiple tumors of the same stage, the largest was selected.

2.3. Dual-color in situ hybridization

DISH for *MDM2* was performed on tissue microarray sections using an automated staining platform (Ventana BenchMark XT system; Ventana Medical Systems, Tucson, AZ). The DISH DNA Probe Cocktail (Ventana Medical Systems) consisting of MDM2 DNP probes and chromosome 12 (CHR12) DIG probes was obtained from Ventana Medical Systems. The hybridized DNP-labeled MDM2 probe was visualized in dark brown by the ultraView SISH DNP Detection Kit (Ventana Medical Systems), whereas the DIG-labeled CHR12 probe was colored in red using the ultraView Red ISH DIG Detection Kit (Ventana Medical Systems). Staining

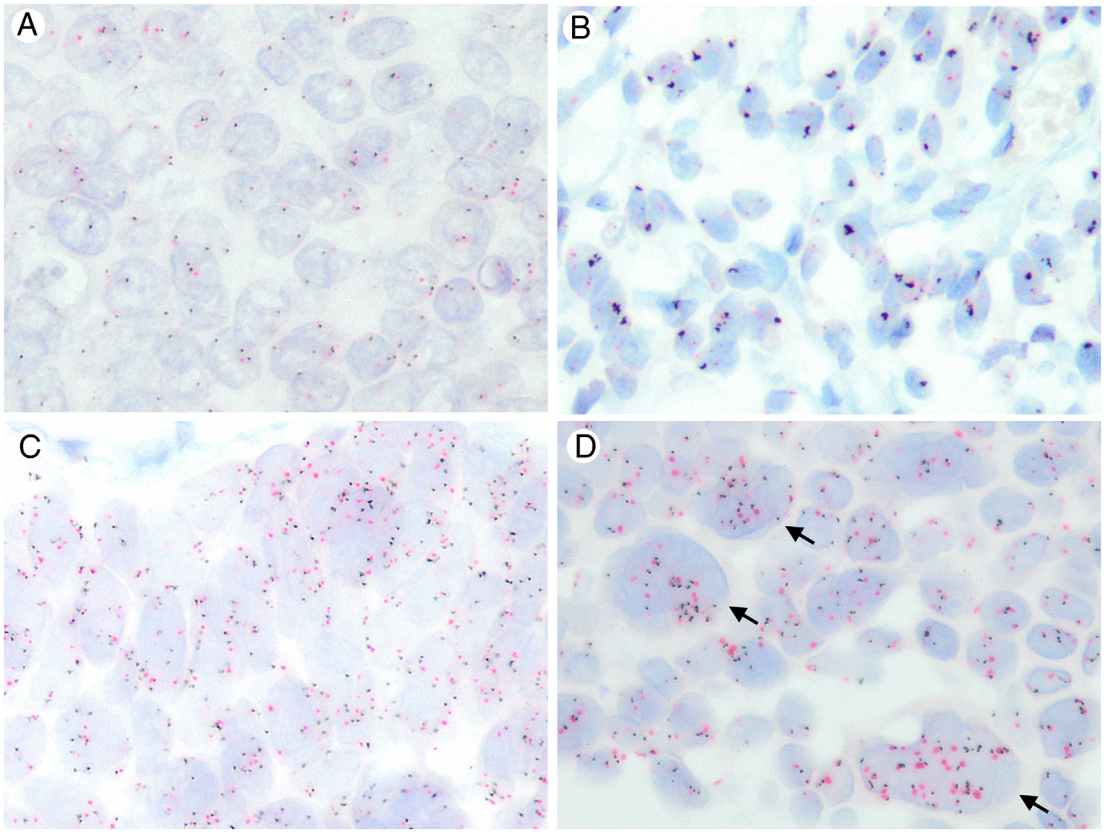

Fig. 1 *MDM2* analysis by DISH. A, No *MDM2* amplification is observed. Each cell has only 1 or 2 *MDM2* signals. *MDM2* is colored in brown and CHR12 in red. B, Clustered signals for *MDM2* are identified in the nuclei of cancer cells, consistent with amplification. C, The presence of many signals of both *MDM2* and CHR12 indicates CHR12 polysomy. D, The polysomic amplification of *MDM2*/CHR12 is observed in occasional large cells (arrowheads). Original magnification ×800 (A-D).

was performed according to an Food and Drug Administration–cleared protocol. Sections of dedifferentiated liposarcoma were used as positive controls. The MDM2 to CHR12 ratio was counted in 40 tumor cells in each case, and an average ratio >2.0 was considered to be amplification based on previous findings on soft tissue sarcomas [27,28]. Cases with >4 MDM2 signals and a ratio of ≤2 were considered to have chromosome polysomy. ESCC cases with *MDM2* copy number increases (either amplification or CHR12 polysomy) were examined as an MDM2-positive group. Small clusters of multiple signals were counted as 6 signals, whereas large clusters were counted as 12 signals based on the manufacturer's instructions and previous studies [29]. Cases in which amplification or polysomy was observed in all tissue cores examined

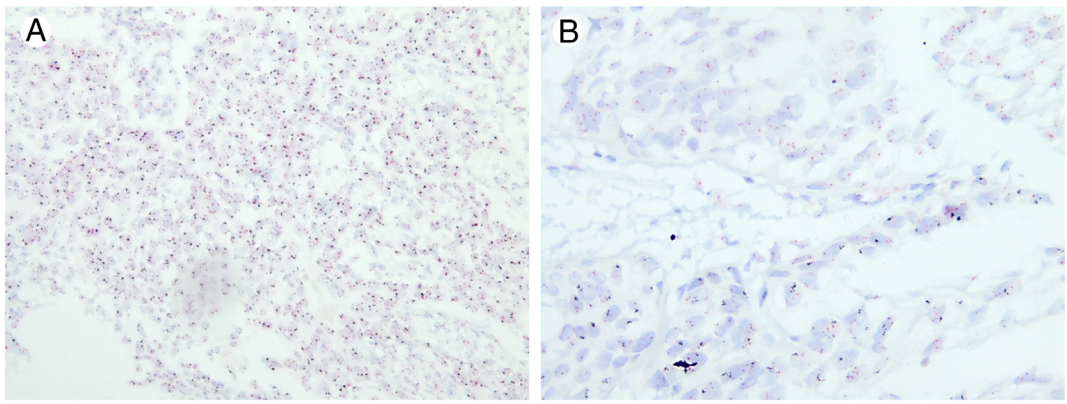

Fig. 2 Heterogeneity of *MDM2* alterations. A, *MDM2* amplification with clustered *MDM2* signals is observed in most cancer cells in this field. B, The lower part shows features consistent with *MDM2* amplification, whereas the upper part is negative for amplification. Original magnifications ×200 (A) and ×400 (B).

were considered to have diffuse *MDM2* alterations. A whole section of cancer tissue was also stained in 11 representative cases of ESCC with diffuse copy number increases in *MDM2* to examine the heterogeneity of the genetic event. In cases where DISH interpretation was difficult, 2 investigators (R.S. and Y.Z.) reviewed the slides together using a multi-headed microscope and evaluated the results with consensus.

2.4. p53 immunostaining

Immunostaining for p53 was performed on tissue microarray sections using an automated immunostainer (Ventana Benchmark XT, Ventana Medical Systems). The antibody used was a monoclonal antibody against human p53 (clone DO-7; dilution 1:300; Leica Microsystems, Wetzlar, Germany). Immunohistochemical results of p53 are known to correlate well with the mutational status of the gene. The diffuse nuclear staining of p53 in almost all tumor cells was regarded as a positive result, indicating a *TP53* mutation.

2.5. Statistical analysis

Continuous variables were compared between 2 groups using the Student *t* test. Categorical variables were compared between 2 groups using the χ^2 or Fisher exact test. Disease-free survival curves were constructed using the Kaplan-Meier method, with the log-rank test being applied for the comparison of survival curves. Prognostic significance was assessed by a multivariate analysis using Cox proportional hazard model. Statistical analyses were performed using JMP 13 (SAS Institute Inc., Cary, NC). All tests were 2-sided, and a probability of $P < .05$ was considered significant.

3. Results

3.1. *MDM2* amplification in ESCC

Among the 399 cases examined, 362 (91%) had interpretable results with countable signals of both MDM2 and CHR12. As described above, *MDM2* alterations were evaluated by the MDM2/CHR12 ratio and number of MDM2 signals. MDM2-negative cases showed 1 or 2 copies of MDM2 and CHR12 supposedly depending on how each cell was cut within a tissue section (Fig. 1A), whereas cases with *MDM2* amplification had multiple signals or clusters of MDM2 with fewer signals of CHR12 (Fig. 1B). In addition, some cases had multiple copies of both CHR12 and MDM2, indicating CHR12 polysomy, particularly in scattered large cancer cells (Fig. 1C and D). Therefore, *MDM2* amplification was detected in 19 (5%) of 362 cases of ESCC, whereas increased copy numbers of MDM2 and CHR12 were noted in 13 (4%) of 362 cases. These *MDM2*-altered cases were hereafter examined as MDM2-positive ESCC.

Diffuse *MDM2* alterations (abnormalities in all tissue cores examined) were observed in 10 (53%) of 19 *MDM2*-amplified and 6 (46%) of 13 CHR12 polysomy cases (Fig. 2). Additional examinations using whole sections were also conducted in representative MDM2-positive cases to elucidate heterogeneity. Whole-section hybridization revealed that 6 of 11 invasive cancers examined had diffuse amplification. One case with diffuse amplification in the invasive area was associated with high-grade dysplasia, and the intraepithelial lesion was negative for *MDM2* amplification.

Table 1 Comparison of clinicopathological features between MDM2-positive and MDM2-negative ESCCs

	MDM2-positive (n = 32)	MDM2-negative (n = 330)	*P*
Sex			
Male	28 (88%)	283 (86%)	.787
Female	4 (13%)	47 (14%)	
Mean age (y)	69	66	.070
Preoperative chemotherapy	17 (53%)	168 (51%)	.811
Resection procedure			
Esophagectomy	25 (78%)	277 (84%)	.398
Endoscopic dissection	7 (22%)	53 (16%)	
Tumor location			
Cervical	3 (10%)	19 (6%)	.597
Upper	2 (6%)	37 (11%)	
Middle	16 (50%)	167 (50%)	
Lower	11 (34%)	95 (29%)	
Abdominal	0	12 (4%)	
Degree of differentiation			
Well/moderately	27 (84%)	282 (85%)	.869
Poorly [a]	5 (16%)	48 (15%)	
Vascular invasion	12 (38%)	128 (39%)	.886
Lymphatic invasion	15 (47%)	142 (43%)	.675
pT classification			
pT1	15 (47%)	179 (54%)	.518
pT2	2 (6%)	31 (10%)	
pT3	14 (44%)	103 (31%)	
pT4	1 (3%)	17 (5%)	
Lymph node metastasis			
Present	19 (59%)	145 (44%)	.094
Absent	13 (41%)	185 (46%)	
Resectability			.593
R0	26 (81%)	279 (85%)	
R1	5 (16%)	34 (10%)	
R2	1 (3%)	17 (5%)	
Multiple tumors	2 (6%)	39 (12%)	.343
Synchronous SCC in other organs	6 (19%)	30 (9%)	.081
Recurrence			
Present	13 (41%)	113 (34%)	.469
Absent	19 (59%)	217 (66%)	
p53 expression	19 (59%)	161 (49%)	.267

Abbreviation: SCC, squamous cell carcinoma,
[a] Including 6 cases of basaloid squamous cell carcinoma.

Table 2 Comparison between cases with diffuse *MDM2* alterations and those with partial abnormalities

	Diffuse alterations (n = 16)	Partial alterations (n = 16)	*P*
Sex			
Male	12 (75%)	16 (100%)	.033
Female	4 (25%)	0	
Mean age (y)	70	68	.467
Preoperative chemotherapy	12 (75%)	5 (31%)	.013
Resection procedure			
Esophagectomy	14 (88%)	11 (69%)	.200
Endoscopic dissection	2 (13%)	5 (31%)	
Tumor location			
Cervical	1 (6%)	2 (13%)	.935
Upper	1 (6%)	1 (6%)	
Middle	8 (50%)	8 (50%)	
Lower	6 (38%)	5 (31%)	
Degree of differentiation			
Well/moderately	12 (75%)	15 (94%)	.144
Poorly [a]	4 (25%)	1 (6%)	
Vascular invasion	10 (63%)	2 (13%)	.004
Lymphatic invasion	10 (63%)	5 (31%)	.077
pT classification			
pT1	4 (25%)	11 (69%)	.013
pT2-4	12 (75%)	5 (31%)	
Lymph node metastasis			
Present	11 (69%)	8 (50%)	.280
Absent	5 (31%)	8 (50%)	
Resectability			
R0	13 (81%)	13 (81%)	.549
R1	2 (13%)	3 (19%)	
R2	1 (6%)	0	
Multiple tumors	1 (6%)	1 (6%)	1.000
Synchronous SCC in other organs	1 (6%)	5 (31%)	.070
Recurrence			
Present	9 (56%)	4 (25%)	.072
Absent	7 (44%)	12 (75%)	
MDM2 status			
Amplification	10 (63%)	9 (56%)	.719
Polysomy	6 (37%)	7 (44%)	
p53 expression	9 (56%)	10 (63%)	.719

Abbreviation: SCC, squamous cell carcinoma.
[a] Including 1 case of basaloid squamous cell carcinoma.

Table 3 Comparison of features between *MDM2* amplification and polysomic amplified *MDM2*

	MDM2 amplification (n = 19)	CHR12 polysomy (n = 13)	*P*
Sex			
Male	16 (84%)	12 (92%)	.496
Female	3 (16%)	1 (8%)	
Mean age (y)	68	70	.536
Preoperative chemotherapy	7 (37%)	10 (77%)	.026
Resection procedure			
Esophagectomy	13 (68%)	12 (92%)	.108
Endoscopic dissection	6 (32%)	1 (8%)	
Tumor location			
Cervical	3 (16%)	0	.513
Upper	1 (5%)	1 (8%)	
Middle	9 (47%)	7 (54%)	
Lower	6 (32%)	5 (38%)	
Degree of differentiation			
Well/moderately	18 (95%)	9 (69%)	.051
Poorly [a]	1 (5%)	4 (31%)	
Vascular invasion	7 (37%)	5 (38%)	.926
Lymphatic invasion	6 (32%)	9 (69%)	.036
pT classification			
pT1	10 (53%)	5 (38%)	.430
pT2-4	9 (47%)	8 (62%)	
Lymph node metastasis			
Present	9 (47%)	10 (77%)	.095
Absent	10 (53%)	3 (23%)	
Resectability			
R0	15 (79%)	11 (85%)	.700
R1	3 (16%)	2 (15%)	
R2	1 (5%)	0	
Multiple tumor	2 (11%)	0	.227
Synchronous SCC in other organs	4 (21%)	2 (15%)	.687
Recurrence			
Present	6 (32%)	7 (54%)	.208
Absent	13 (68%)	6 (46%)	
MDM2 localization			
Diffuse	10 (53%)	6 (46%)	.719
Partial	9 (47%)	7 (54%)	
p53 expression	12 (63%)	7 (54%)	.598

Abbreviation: SCC, squamous cell carcinoma.
[a] Including 1 case of basaloid squamous cell carcinoma.

3.2. Clinicopathological findings

Table 1 compares clinicopathological features between MDM2-positive and MDM2-negative ESCC. There were no significant differences in age, sex, pT stage, tumor location, or history of preoperative chemotherapy between the 2 groups. Microscopic parameters, such as the degree of differentiation and lymphovascular invasion, also did not significantly differ. Lymph node metastasis and synchronous squamous cell carcinoma in other organs were slightly more common in MDM2-positive cases than in MDM2-negative cases (P = .094 and P = .081, respectively). No significant differences were observed in p53 expression between MDM2-positive (59%) and MDM2-negative (49%) cancers (P = .267).

3.3. Clinicopathological features associated with diffuse copy number increases in *MDM2*

Table 2 compares features between cases with diffuse *MDM2* alterations and those with partial abnormalities. Partial abnormalities were associated with male sex (P = .033), whereas a larger number of patients with diffuse *MDM2*

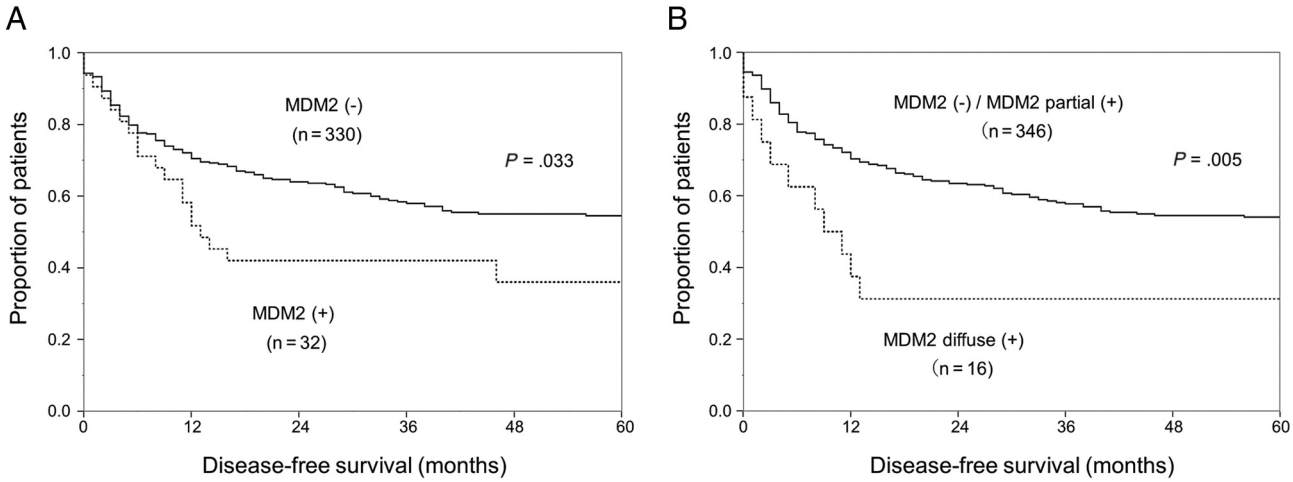

Fig. 3 Survival analysis. A, Patients with MDM2-positive ESCC have significantly poorer disease-free survival than do those with MDM2-negative cancer. B, When MDM2-positive cases are classified into partly and diffusely altered cases, patients with diffuse *MDM2* abnormalities show a significantly worse prognosis compared with combined MDM2-negative and partially altered cases.

alterations had preoperative chemotherapy (P = .013). Vascular invasion was more common in ESCC with the diffuse abnormality of *MDM2* than in those with partial alterations (P = .004). Moreover, advanced pT stage was associated with diffuse genetic alterations (P = .013). No significant differences were observed in p53 expression between the 2 groups (P = .719).

As shown in Table 3, ESCC with CHR12 polysomy more commonly had preoperative neoadjuvant chemotherapy and lymphatic infiltration than cases with *MDM2* amplification. The remainder of the parameters examined were similar between cases with *MDM2* amplification and those with CHR12 polysomy.

3.4. Postoperative survival and prognostic factors

As shown in Fig. 3, MDM2-positive cases showed significantly shorter postoperative survival than did MDM2-negative cases (P = .033, Fig. 3A). The median disease-free survival period was 13 months for MDM2-positive cases and 98 months for MDM2-negative cases. Additional analyses comparing cases with the diffuse alteration of *MDM2* (n = 16) and the remaining cases (n = 346) revealed a more obvious difference (P = .005, Fig. 3B). A multivariate analysis with the application of 3 other prognostic parameters (pT classification, nodal metastasis, and the surgical margin status) did not identify diffuse alterations as an independent prognostic factor for disease-free survival (P = .129; hazard ratio, 1.7; 95% confidence interval, 0.8-3.0), whereas the other 3 independently correlated with patient prognosis.

4. Discussion

The results of the present study are summarized as follows: (1) 32 (9%) cases of ESCC had MDM2 copy number increases. (2) Clinicopathological features were similar between MDM2-positive and MDM2-negative cases. However, cases with the diffuse alteration of *MDM2* were more advanced with more frequent vascular invasion than the others. Cases with *MDM2* copy number increases due to CHR12 polysomy were more strongly associated with preoperative chemotherapy than those with *MDM2* amplification. (3) Genetic alterations, particularly diffuse abnormalities, in *MDM2* seemed to be a poor prognostic factor for patients with ESCC.

MDM2 is a primary negative regulator of p53 and has recently emerged as a key player in many cellular functions associated with cancer progression. MDM2 has also been identified as a drug target in various cancers, particularly those with wild-type p53 [30]. Two types of MDM2 inhibitors have been developed. One is a small MDM2 antagonist, whereas the other is an α-helical p53-stapled peptide capable of inhibiting both MDM2 and MDMX [31,32]. Clinical trials using these inhibitors (eg, RG7112 and ALRN-6924) have provided promising findings [33,34], suggesting that the present results may be of immediate translational value. In the present study, 13 of 32 cases with *MDM2* amplification lacked the diffuse expression of p53. These cases will be a good candidate for MDM2-targeted therapy. Furthermore, recent studies using immunotherapy demonstrated that *MDM2* amplification is a potential biomarker for hyperprogression cases against immune checkpoint inhibitors [35,36].

Our recent study on *MDM2* amplification in cholangiocarcinomas revealed that *MDM2* amplification was identified in ~15% of large duct cholangiocarcinoma, and the molecular alteration was diffusely observed in invasive areas in most cases [37]. Therefore, the proportion of partial abnormalities was higher in ESCC than in cholangiocarcinoma, suggesting that *MDM2* alterations occurred at a late stage in some cases of ESCC. In addition, a single case was found to have *MDM2* amplification restricted to the invasive area in whole section staining, indicating that this genetic disruption developed just

before invasive growth started in that particular case. Therefore, the timing of *MDM2* genetic alterations seems to vary among cases. Another interesting result is that a history of neoadjuvant chemotherapy was positively associated with CHR12 polysomy, suggesting that preoperative chemotherapy causes *MDM2* alterations.

Two earlier studies using Southern blotting suggested *MDM2* amplification in 13 (18%) of 72 and 2 (11%) of 19 ESCC cases [22,38]. However, subsequent experiments using a more sensitive method (quantitative or differential PCR) identified *MDM2* amplification in only 3 (8%) of 40, 2 (4%) of 48, and 0 (0%) of 27, questioning the original data [24,25,39]. In the present study, a more reliable method (DISH) was applied, and the frequency of *MDM2* amplification in ESCC was demonstrated to be 19 (5%) of 362. An advantage of DISH is that only cancer cells are analyzed, whereas Southern blotting and differential PCR examine cancer cells and stromal or lymphoid cells together. It is also important to note that this is the first study to systematically analyze CHR12 polysomy in ESCC because Southern blotting and differential PCR generally fail to assess polysomic amplification. CHR12 polysomy was suggested to occur in ESCC at a similar frequency to *MDM2* amplification.

Intratumor genetic heterogeneity has been confirmed in many types of cancers, and it is speculated to contribute to treatment failure and drug resistance [40-42]. A previous study revealed 2 main distribution patterns of heterogeneity: a clustered form characterized by pockets of highly amplified cells and a mosaic form in which distinct subpopulations comingle [43]. Consistent with the argument that the mosaic form may be an ISH artifact, all cases with partial alterations in *MDM2* in the present study showed the clustered pattern, indicating the true mixed existence of distinct subclones with or without MDM2 alterations. In terms of the clinical significance of partial alterations in *MDM2*, ESCCs with partially altered *MDM2* less frequently had vascular invasion, were less advanced, and had a slightly better prognosis than those with globally altered *MDM2*. In a large cohort study on the *HER2* status in breast cancers, no significant differences were observed in 5-year disease-free survival between patients with ~30% of amplified cells and those without amplified cells. However, disease-free survival was significantly reduced in those with 30% to 100% of amplified cells [44]. Therefore, the heterogeneity of *MDM2* alterations warrants further study, and a low proportion of amplified cells may not confer a clinical outcome or the efficacy of targeted drugs.

Gene amplification is a copy number increase in a restricted region of a chromosome arm and is associated with the overexpression of the amplified gene [45]. In contrast, polysomy is defined as the presence of extra copies of one or more whole chromosomes. In breast cancers, it remains unclear how to interpret increased signal counts of chromosome enumeration probe 17 (CEP17) in the HER2 examination [43]. Some groups argue that chromosome 17 polysomy is not related to HER2 overexpression [46,47]. A previous study showed that CEP17 increases in FISH were simply due to a gain in the centromeric region and were not associated with true chromosome 17 polysomy [48]. In contrast, other groups suggested the combined use of the mean HER2 copy number and HER2/CEP17 ratio to exclude false-positive results [43,49]. The ASCO/CAP 2013 guidelines currently endorse the latter approach [50]. In the present study, we used stringent criteria for polysomic amplification; however, further studies are needed to elucidate the cutoff points that are the most relevant to clinical outcomes.

This study has some limitations. Some cases (9%) without countable signals of either MDM2 or CER12 were excluded from the analysis. Moreover, it was occasionally difficult to precisely evaluate MDM2 positivity in cases with borderline amplification or polysomy. Approximately 20 cases (~5%) fell into that category. Although DISH is a highly useful approach, additional examinations using Southern blot hybridization or PCR may need to be considered for borderline cases. Immunostaining for MDM2 was not conducted because of our experience that MDM2 immunostaining is less reproducible and more difficult to interpret than DISH. Therefore, how MDM2 amplification and CHR12 polysomy correlate with protein expression values remains to be examined. Finally, because partial and diffuse *MDM2* alterations were determined on tissue arrays, it may not represent abnormalities in whole tumors.

In conclusion, the present study revealed that *MDM2* amplification or CHR12 polysomy occurs in 9% of ESCC cases, and *MDM2* alterations, particularly diffuse abnormalities, were associated with a poor prognosis. *MDM2*-altered cases may achieve benefits from *MDM2*-targeted therapy.

CRediT authorship contribution statement

Ryuichiro Sawada: Data curation, Formal analysis, Investigation, Writing - original draft. **Ritsuko Maehara:** Data curation, Formal analysis, Investigation, Writing - original draft. **Taro Oshikiri:** Investigation, Writing - review & editing. **Tetsu Nakamura:** Investigation, Writing - review & editing. **Tomoo Itoh:** Investigation, Writing - review & editing. **Yuzo Kodama:** Investigation, Writing - review & editing. **Yoshihiro Kakeji:** Investigation, Writing - review & editing. **Yoh Zen:** Conceptualization, Methodology, Supervision, Writing - original draft.

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