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Original Article

Semi-comprehensive analysis of gene amplification in thymic malignant tumors using multiplex ligation-dependent probe amplification and fluorescence in situ hybridization

Seiichi Kakegawa[1], Isao Matsumoto[1], Masaya Tamura[1], Munehisa Takata[1], Shuhei Yoshida[1], Daisuke Saito[1], Yusuke Tanaka[1], Hirofumi Takemura[1], Akishi Ooi[2]

[1]*Department of Thoracic, Cardiovascular and General Surgery, Graduate School of Medical Science, Kanazawa University, Ishikawa, Japan;* [2]*Department of Molecular and Cellular Pathology, Graduate School of Medical Science, Kanazawa University, Ishikawa, Japan*

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Abstract: Research on the amplification of oncogenes in thymic malignant tumor is limited. In this study, we aimed to determine the gene amplification status of receptor tyrosine kinases and other cell regulator genes in thymic malignant tumors, with a view toward the future introduction of molecular targeted therapy. In addition, we examined the usefulness of multiplex, ligation-dependent probe amplification (MLPA) in the semi-comprehensive detection of these gene amplifications. The participants of this study were nine patients with thymic carcinoma and one patient with atypical carcinoid who underwent resection at our department from 1999 to 2016. Twenty-four oncogenes (*MDM4*, *MYCN*, *ALK*, *PDGFRA*, *KIT*, *KDR*, *DHFR, EGFR*, *MET*, *SMO*, *BRAF*, *FGFR1*, *MYC*, *ABL1*, *RET*, *CCND1*, *CCND2*, *CDK4*, *MDM2*, *AURKB*, *ERBB2*, *TOP2A*, *AURKA*, *AR*) were analyzed for amplification by MLPA. In cases where amplification by MLPA was suspected, confirmation was performed by fluorescence in situ hybridization (FISH). Immunostaining for detected oncoproteins and p53 were performed in cases with confirmed oncogene amplification. *MYC* (2/10, 20%) and *MDM2* (1/10, 10%) amplifications were detected using MLPA and FISH. Immunostaining in both cases was positive. The *MDM2*-amplified tumor relapsed and spread rapidly after operation despite the use of post-operative chemo-radiotherapy. *MYC* amplification may be involved in the carcinogenesis of thymic malignant tumors. In addition, *MDM2* amplification may be a concern in the increased malignancy.

Keywords: Thymic carcinoma, gene amplification, multiplex ligation-dependent probe amplification, fluorescence in situ hybridization, *MYC*, *MDM2*

Introduction

Thymic epithelial tumors, including thymoma, thymic carcinoma, and thymic neuroendocrine tumors, are rare primary tumors of the mediastinum and are derived from the thymic epithelium. Thymoma is the most common primary anterior mediastinal mass, while thymic carcinomas or thymic neuroendocrine tumors are far rarer, but are more likely to spread [1]. Although the treatment of thymic epithelial tumors requires a multidisciplinary approach, surgery is the mainstay of treatment in the management of thymic tumors for those patients where complete resection is possible. Systemic chemotherapy represents the standard of care for metastatic or inoperable refractory/recurrent disease; however, there remains a lack of standard treatment after first-line failure [1]. The five-year survival rate of thymic carcinoma patients treated with multimodal therapy, including surgical resection, chemotherapy, and radiotherapy, is 40-65.7%, with poorer prognoses reported for incompletely resected patients [2-5]. To improve the prognoses of patients with metastatic or inoperable refractory/recurrent disease, an examination of the efficacy of molecular target therapies should be undertaken [6].

In various cancers, including non-small cell lung cancer, molecular target therapies have been

Table 1. Patient characteristics

	Patients (n=10)
Age, years	
Median	66
Range	55-76
Gender	
Male	8
Female	2
Histology	
Squamous cell carcinoma	7
Adenocarcinoma	1
Basaloid carcinoma	1
Atypical carcinoid	1
Surgical Procedure	
Complete resection	9
Biopsy	1
Masaoka Stage	
I	1
II	1
III	3
IVb	5

shown to be effective in cases with target gene mutation or amplification [7, 8]. While many thymic carcinomas are immunohistochemically positive for EGFR, HER2, and c-KIT, actual mutations in these oncogenes are detected only in a limited number of cases [9]. For this reason, molecular target therapies have not been extensively evaluated in thymic carcinoma patients [6], and studies of the amplification of oncogenes in thymic carcinoma are limited [10-12].

Therefore, the aim of the present study was to determine the amplification status of oncogenes semi-comprehensively in thymic malignant tumors using the multiplex ligation-dependent probe amplification (MLPA) method, with a view toward the future introduction of molecular targeted therapy.

Patients and methods

Patients

A total of 10 patients with thymic malignant tumors who underwent surgery at the Department of Thoracic, Cardiovascular and General Surgery in Kanazawa University Hospital between 1999 and 2017 were examined. The Medical Ethics Committee of Kanazawa University approved this laboratory study. Serial sections cut from representative formalin-fixed and paraffin-embedded cancer tissues were used for hematoxylin-eosin staining, MLPA, fluorescence in situ hybridization (FISH), and immunohistochemistry.

Relevant patient characteristics are listed in **Table 1**. Patient age ranged from 55 to 76 years, with a median age of 66 years. According to the World Health Organization Classification of Tumors [13], the histological subtype of the thymic malignant tumors was squamous cell carcinoma in seven cases, and one case each of adenocarcinoma, basaloid carcinoma, and atypical carcinoid. Complete resection was performed in nine patients, with only one case of pulmonary metastasectomy used for diagnosis. According to the system described by Masaoka et al. [14], the pathological stage was stage I in one patient, stage II in one patient, stage III in three patients, and stage IVb in five patients. Four patients are still alive without recurrence while three patients are alive with recurrence, two patients died from thymic malignant tumor, and one patient died from another disease. Post-operative survival time ranged from 6 to 221 months, with a median time of 36 months.

MLPA

A cancer area was selected on a 6 μm-thick representative tumor section, with reference to the adjacent hematoxylin-eosin staining section, taking care that non-neoplastic cells were excluded as much as possible. DNA was manually extracted from each section using proteinase K (Rosch Diagnostics, Manheim, Germany) according to the manufacturer's protocol (MRC-Holland, Amsterdam, The Netherlands). By using this protocol, > 1 μg of sample DNA with an OD260:OD280 ratio within 1.1-1.7 was obtained from each tumor. DNA from the cell lines MKN7, A431, HSC39, and MKN45, previously shown to display amplified *ERBB2*, *EGFR*, *MYC*, and *MET*, respectively, were used as positive controls [15, 16]. MLPA was performed by using the kit from MRC-Holland. The SALSA MLPA P175-A2 Tumor-Gain kit contains two or three probes for each of 24 genes including *MDM4*, *MYCN*, *ALK*, *PDGFRA*, *KIT*, *KDR*, *DHFR*, *EGFR*, *MET*, *SMO*, *BRAF*, *FGFR1*, *MYC*, *ABL1*, *RET*, *CCND1*, *CCND2*, *CDK4*, *MDM2*, *AURKB*, *ERBB2*, *TOP2A*, *AURKA,* and *AR*. PCR products

Table 2. Genes with increased copy number detected by multiplex ligation-dependent probe amplification in thymic malignant tumors

Name of gene	MYC	MDM2	CCND1
Chromosomal locus of gene	8q24.13	12q15	11q13.3
FISH probe	RP11-440N18	RP11-775J10	RP11-300I6
No. of amplified cases detected by MLPA	3	1	1
No. of amplified cases confirmed by FISH	2	1	0

Abbreviation: MLPA, multiplex ligation-dependent probe amplification. FISH, fluorescence in situ hybridization.

Table 3. Antibodies and dilutions applied for immunohistochemistry

Antibody	Clone	Dilution	Antigen retrieval	Manufacturer
MYC	Y69	1:50	PC pH9, EDTA	Pharmingen, San Diego, CA, USA
MDM2	IF2	1:40	PC pH6, citrate	Calbiochem, La Jolla, CA, USA
p53	Do-7	prediluted	PC pH6, citrate	DAKO IR616

Abbreviation: PC, pressure cooking.

were separated on an ABI-310 capillary sequencer (Applied Biosystems, Foster City, CA, USA) and interpreted with Genemapper software (Applied Biosystems). Data analysis was performed with Coffalyser MLPA-DAT software (version 9.4, MRC-Holland) in order to generate normalized peak values. Average peak values below 0.7 were defined as "lost", between 0.7 and 1.3 as "normal", between 1.3 and 2.0 as "gain", and > 2.0 as "amplified", using a previously established system [17, 18].

FISH

Tumors with "amplified" genes as determined by MLPA were further examined using FISH to validate the accuracy of the MLPA results for respective gene amplification. The FISH probes acquired from BACPAC Resources (Oakland, CA, USA) are summarized in **Table 2**. Probes were labeled with SpectrumOrange or SpectrumGreen using a nick translation kit (Abbott Laboratories, Abbott Park, IL, USA). For the detection of gene amplification, a SpectrumGreen-labeled pericentromeric probe (Abbott), specific to each chromosome on which the particular gene was located, was co-hybridized to standardize the chromosome number. The removal of proteins from the tissue sections, denaturation, hybridization, and post-hybridization washing were performed as described previously [18]. Tissue sections were counterstained with DAPI II (Abbott) and examined using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Triple Bandpass Filter set (Abbott) for DAPI II, SpectrumOrange, and SpectrumGreen, using a filter set specific for SpectrumOrange or SpectrumGreen. FISH images were recorded through a cooled, charge-coupled device camera (DP-70, Olympus) linked to a computer software program (DP Manager, Olympus). The scoring and evaluation of the FISH slides was performed manually by counting the gene signals and the centromere signals on which the gene was located, with 20 tumor nuclei per case. The average copy number of the gene and the centromere, along with their ratio, was determined for each case. According to the criteria of ERBB2 amplification approved by the American Society of Clinical Oncology/College of American Pathologists, gene amplification was determined when the quantity of positive cells was defined as more than six gene copies per nucleus or gene signal/centromere signal > 2.2 [19].

Immunohistochemistry

Immunohistochemistry for MYC, MDM2, and p53 was performed on tumors with FISH-confirmed *MYC* or *MDM2* amplification. Antibody clones, manufacturers, dilutions, and the antigen retrieval methods are summarized in **Table 3**. Antibody binding was visualized using the LSAB system (Dako, Glostrup, Denmark). For the evaluation of the positive staining of p53, each tumor was scored using a four-tier system (0, 1+, 2+, 3+) according to the criteria recommended by Dako for the HercepTest. For the evaluation of MYC and MDM2 staining, only nuclear immunostaining, which was significantly higher than that of the control cells of normal thymus, was considered as positive.

Table 4. Thymic malignant tumor cases with gene amplification

Case No.	Histological classification	FISH		Immunohistchemistry		
		MYC	*MDM2*	MYC	MDM2	p53
1	Basaloid carcinoma	amplified		positive		positive
2	Squamous cell carcinoma	amplified	amplified	positive	positive	positive

Abbreviation: FISH, fluorescence in situ hybridization.

Results

MLPA

MLPA was successfully performed on all 10 of the formalin-fixed and paraffin-embedded tumor tissue samples. The gene status of the 24 oncogenes analyzed in the 10 tumors were categorized as "amplified", "gain", or "normal" based on mean MLPA peak values. There were no cases with "lost" genes. The number of tumors designated as "amplified" for specific genes by MLPA is summarized in **Table 2**. All tumors displayed a "normal" or "gain" copy number for *MDM4*, *MYCN*, *ALK*, *PDGFRA*, *KIT*, *KDR*, *DHFR*, *EGFR*, *MET*, *SMO*, *BRAF*, *FGFR1*, *ABL1*, *RET*, *CCND1*, *CCND2*, *CDK4*, *AURKB*, *ERBB2*, *TOP2A*, *AURKA,* and *AR*.

FISH

Out of the 10 total thymic malignant tumors, four tumors (40%) displayed gene amplification of *MYC, MDM2*, or *CCND1* by MLPA. FISH analyses were performed in these "amplified" tumors to confirm gene amplification. From the FISH analysis, two tumors had cancer cells with gene amplifications of *MYC* or *MDM2* that could be detected by FISH; these tumors are shown in **Table 4**.

Immunohistochemistry

Immunohistochemistry for MYC was positive in both 2 tumors with *MYC* amplification. Immunohistochemistry for MDM2 was positive in one tumor with *MDM2* amplification. We examined p53 immunohistochemistry because MDM2 is an antagonist for p53. Eight cases out of the 10 cases showed a positive reaction to p53 immunohistochemistry. Cases with *MDM2* or *MYC* amplifications were both positive for p53 immunohistochemistry as shown in **Table 4**.

Clinical courses and pathological findings of the two cases with oncogenic amplification

Case 1, with *MYC* amplification, was a 74-year-old man, diagnosed with stage I basaloid carcinoma who underwent total thymectomy (**Figure 1**). Macroscopic examination revealed the presence of a multicystic lesion that was approximately 6 cm in size, with relatively well-defined borders and scattered white nodules on the cystic walls. Thymic tissue was found around the cyst. Furthermore, microscopic examination revealed that the cyst wall was a relatively thick fibrous tissue, with a multilayer of tumor cells with high N/C ratio and proliferated papillary in the mural node. Basaloid cells with palisaded nuclear arrangement proliferated at the margin of the tumor. The nuclei of the tumor cells were irregular in size and shape, and mitotic figures were scattered. A small fraction of the tumor cells invaded beyond the cyst wall. There was an image in which vein invasion was suspected; however, lymphatic invasion was unclear. Immunostaining revealed that the tumor cells were CD5(-), CK5/6(+), CAM5. 2(+), TTF-1(-), chromogranin(-), and synaptophysin(-) and had MIB-1 indexes of 30%-40%. Basaloid carcinoma was the diagnosis on morphologic findings. He is alive without recurrence at 74 months after the operation.

Case 2, with both *MYC* and *MDM2* amplifications, was a 63-year-old man, diagnosed with stage IVb squamous cell carcinoma invading the right upper lung, pericardium, and mediastinal lymph nodes (**Figure 2**). He underwent a total thymectomy with right pulmonary and pericardial partial resection and mediastinal lymph node dissections. Macroscopic examination revealed that the tumor was 6.5×4.2×3.0 cm in diameter, and the tumor was mainly located in the thymus and invaded into the right lung. A part of the tumor was necrotic, with slight adhesion to the pericardium. Microscopic examination revealed that the histology of the tumor was that of squamous cell carcinoma and mainly comprised well-differentiated elements with keratinization, which proliferated in an invasive manner. The tumor directly invaded the right lung, particularly with severe lymphatic invasion. Not only the lymph vessels in the mediastinum but also the lymph vessels in the

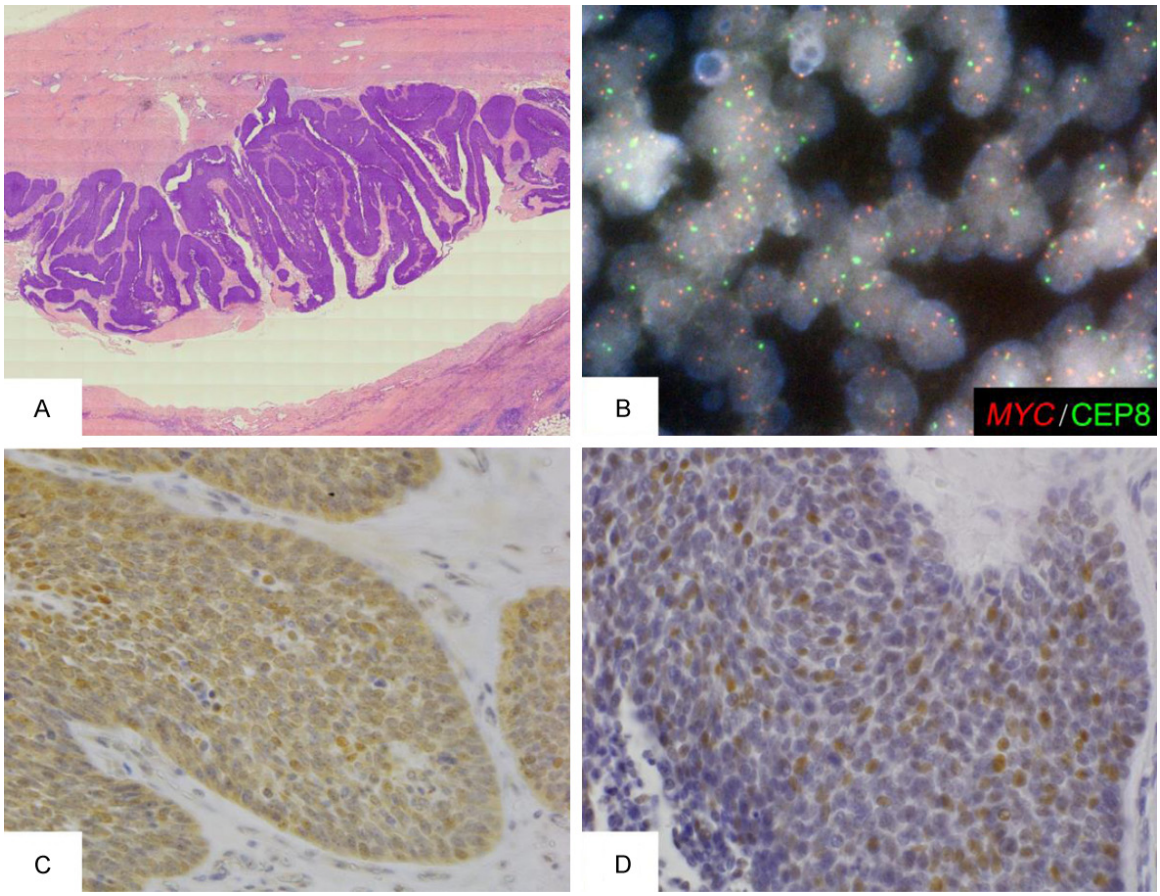

Figure 1. Case 1 with *MYC* amplification was diagnosed histopathologically as basaloid carcinoma (A: hematoxylin and eosin stain, original magnification 10×). *MYC* amplification was confirmed by FISH (B: orange fluorescence, *MYC*; green fluorescence, centromere probe for chromosome 8). And the protein overexpression of *MYC* and p53(2+) were detected by immunohistochemistry (C: MYC, original magnification 400×; D: p53, original magnification 400×).

lungs and lymph vessels just beneath the pleura were extensively infiltrated. Immunostaining (CD5 and c-KIT) was negative. The macroscopic shape of the tumor suggested that the tumor invaded to the lung from the thymus side. The sarcomatous element, which was found in the liposarcoma cases with *MDM2* amplification, was not detected. Chemo-radiotherapy was performed after the operation. However, the tumor relapsed 4 months after the operation and spread rapidly. The patient died of cancer at 6 months from the operation.

Discussion

In this study, we investigated the amplification of oncogenes in thymic malignant tumors using both MLPA and FISH methods. Examining multiple genetic amplifications from multiple samples using FISH is time-consuming, labor-intensive, and unrealistic. Thus, the use of the MLPA method in this study allowed for a relatively accurate assessment of the presence or absence of the amplification of multiple genes for a number of samples in a single experimental procedure. FISH was then used on the samples that were expected to be amplified by this method. In this way, the time, labor, and costs required for the experiment can be dramatically reduced. In a previous study of gastric cancer, FISH was performed to evaluate the amplification in samples categorized as “gain” and “amplified”; however, no significant amplification was observed in cases categorized as “gain” [20, 21]. Therefore, in this study, FISH was performed only on samples evaluated as ‘amplified’.

Genetic mutations associated with receptor tyrosine kinases are relatively common in lung adenocarcinoma and have been associated with the therapeutic effects of molecular targeted therapies [22]. On the other hand, thymic tumors have been researched for genetic

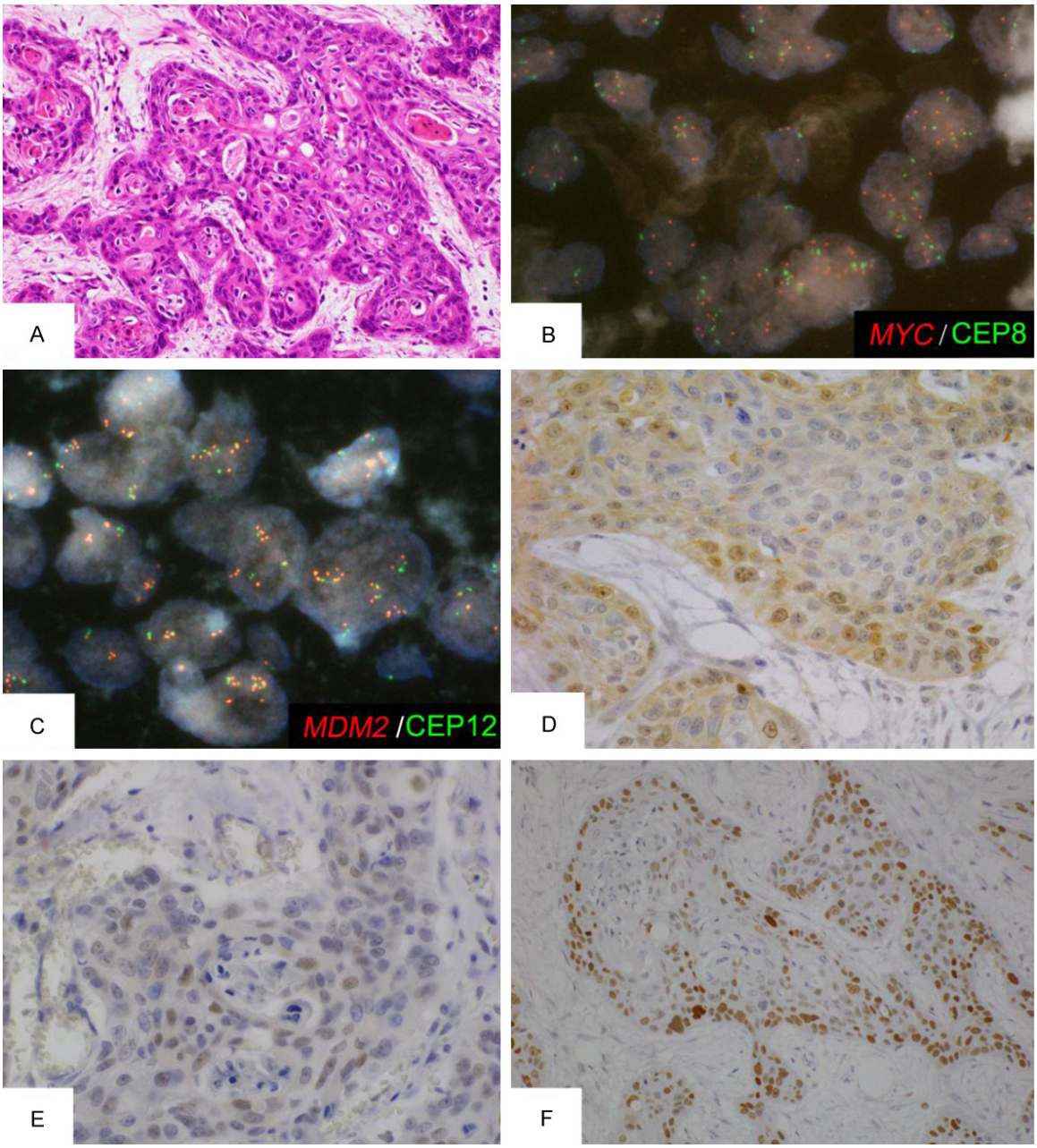

Figure 2. Case 2 with *MYC* and *MDM2* amplifications was diagnosed histopathologically as keratinizing squamous cell carcinoma (A: hematoxylin and eosin stain, original magnification 200×). *MYC* and *MDM2* amplifications were confirmed by FISH (B: orange fluorescence, *MYC*; green fluorescence, centromere probe for chromosome 8, C: orange fluorescence, *MDM2*; green fluorescence centromere probe for chromosome 12). And the protein overexpression of MYC, MDM2, and p53(3+) were detected by immunohistochemistry (D: MYC, original magnification 400×; E: MDM2, original magnification 400×; F: p53, original magnification 200×).

abnormalities, but no results have been found to be relevant to treatment [6]. Thymomas and thymic carcinomas appear to be genetically quite different tumors, thus both should be considered separately when investigating genetic abnormalities [23]. The opportunity to examine thymic malignant tumors is limited because their frequency is rare in comparison with thymomas. The evaluation of the oncogenes related to thymic carcinoma has been primarily performed for receptor tyrosine kinases, including *EGFR*, *HER2* (*ERBB2*), and *KIT*. Though positive results were comparatively recognized at a high frequency by immunos-

Table 5. Reports on amplification of oncogenes in thymic malignant tumor detected by FISH

year	Author	*EGFR*		*HER2 (ERBB2)*		*MYC*		*MDM2*	
2003	Pan CC			0/12	(0%)				
2005	Ionescu DN	1/4	(25%)						
2012	Weissferdt	0/24	(0%)	1/24	(4.2%)				
2019	our report	0/10	(0%)	0/10	(0%)	2/10	(20%)	1/10	(10%)

Abbreviation: FISH, fluorescence in situ hybridization.

taining, the frequency of recognized gene mutations was rare, and these mutations were not associated with treatment [9]. Eighteen cases of poorly differentiated nonkeratinizing squamous cell carcinomas of the thymus were analyzed using next-generation sequencing; no "actionable" genes amenable to currently available targeted therapies were identified [24].

Relevant studies on gene amplification that report on the oncogenes of thymic carcinoma are shown in **Table 5** [10-12]. As for gene amplification, only *EGFR* and *HER2 (ERBB2)* have been reported to date. Therefore, to our knowledge, this is the first report to semi-comprehensively examine the amplification of thymic malignant tumors, including oncogenes other than *EGFR* and *HER2 (ERBB2)*. In the present examination, the genetic amplification of *EGFR* or *HER2 (ERBB2)* was not observed. Thus, the effects of EGFR tyrosine kinase inhibitors on thymic malignant tumors may be limited. Although KIT is overexpressed in many thymic carcinomas (46-86%) as detected by immunostaining, mutations in this gene are only identified in as few as 9% of cases. In addition, there are several types of mutations, and it has been reported that molecular target drugs such as imatinib show effects only in cases with mutations [6, 9]. No studies on gene amplification for *KIT* have been reported. In the present study, no amplification of *KIT* was observed, thus we can presume that the frequency of *KIT* amplification is not high.

In this study, amplifications of *MYC* and *MDM2* were observed, and p53 immunostaining was positive in both cases. *MYC* is the most frequently amplified oncogene in human cancers and its alteration is observed in a wide range of tumors, including breast, lung, and prostate cancers [25]. *MYC* encodes a transcription factor that triggers selective gene expression amplification in order to promote cellular growth and proliferation [26]. *MYC* amplification has been pointed out in a detailed report of a thymic adenocarcinoma case, for which whole exome sequencing and whole transcriptome sequencing were performed [27]. It was discussed that *MYC* amplification might contribute to the high malignancy of thymic adenocarcinoma because such a focal *MYC* amplification had been unprecedented in type A and type AB thymomas [23, 28, 29]. Since the amplification of *MYC* was recognized in 2 (20%) of the 10 cases examined in this study, the amplification of *MYC* may be a concern in carcinogenesis and the progress of thymic malignant tumor at a constant rate. Currently, a drug specifically for MYC is under development and it may be associated with a specific treatment strategy in the future [26, 30].

MDM2 is an oncogene that suppresses the actions of p53; the amplification of *MDM2* is a frequent abnormality observed in liposarcomas [31]. The overexpression of MDM2 is often seen in various human cancers, and correlates with high-grade, late-stage, and more treatment-resistant tumors [32]. *MDM2* amplification has not been detected in thymic carcinoma cases, and this is the first report that pointed out a case with *MDM2* amplification. This tumor with *MDM2* amplification was a high-grade malignant tumor like the other organ tumors with *MDM2* amplification. *MDM2* amplification may be also a concern in the increased malignancy of thymic carcinoma.

The high frequency of p53 protein overexpression in thymic carcinoma has been reported previously [33]. The authors suggest that a certain oncoprotein may interact with the p53 protein in thymic carcinomas, leading to the stabilization of the wild-type p53 protein. It is interesting to note that the presence of p53 overexpression is associated with an increase in carcinogenicity due to *MYC* [30], and that both cases in which *MYC* amplification was positive in this study also had p53 protein overexpression.

There are some limitations to this study. As thymic malignant tumor is a very rare disease and has a lot of subtypes; therefore, further cases may need to be accumulated, for example, by collecting cases in a multicenter study. Thymic carcinoma is defined as a primary thymic epithelial neoplasm displaying overt cytologic evidence of malignancy with loss of organotypical features of thymic differentiation. No reliable histopathologic features have yet been identified that may help distinguish these tumors from metastases from other organs. In most cases, thymic carcinoma remains a diagnosis of exclusion [13]. This study also concerns relatively old specimens and the possibility of false negatives by FISH or immunostaining cannot be denied due to storage problems. However, the MLPA method was performed without issues in all cases.

In conclusion, we detected *MYC* (20%) and *MDM2* (10%) amplifications through the semi-comprehensive examination of oncogenic amplifications in thymic malignant tumors by using both MLPA and FISH methods. *MYC* amplification may be involved in the carcinogenesis of thymic malignant tumors. In addition, *MDM2* amplification may be a concern in the increased malignancy of thymic carcinoma.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Seiichi Kakegawa, Department of Thoracic, Cardiovascular and General Surgery, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi Kanazawa, Ishikawa 920-8641, Japan. Tel: +81-76-265-2354; Fax: +81-76-222-6833; E-mail: seiichi.kakegawa@gmail.com

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