Yonago Acta Medica 2019;62:115–123 *Original Article*

Detection of Disease-specific Fusion Genes of Soft Tissue Tumors Using Formalin-fixed Paraffin-embedded Tissues; Its Diagnostic Usefulness and Factors Affecting the Detection Rates

Takahiro Matsushige,* Satoshi Kuwamoto,†‡ Michiko Matsushita,* Lusi Oka Wardhani,‡ Yasushi Horie,† Kazuhiko Hayashi‡ and Yukisato Kitamura*

**Department of Pathobiological Science and Technology, Major in Clinical Laboratory Science, School of Health Science, Tottori University Faculty of Medicine, Yonago 683-8503, Japan, †Department of Pathology, Tottori University Hospital, Yonago 683-8504, Japan, and ‡Division of Molecular Pathology, Department of Pathology, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8503, Japan*

ABSTRACT

Background Recent rapid advances in molecular biology have led the discovery of disease-specific novel fusion genes in a variety of soft tissue tumors. In this study, we attempted to detect these fusion genes using formalin-fixed paraffin-embedded (FFPE) tumor tissues and investigated their clinical utility and factors that affect the results of examination.

Methods Reverse transcription polymerase chain reaction for the detection of tumor-specific fusion genes was performed using 41 FFPE tumor samples obtained from 37 patients representing nine histological types of soft tissue tumors that were diagnosed from 2006 to 2017 in our laboratory.

Results Fusion genes in 19 (51.3%) out of 37 cases were detected successfully. Relatively high detection rates were observed in synovial sarcomas (100%, 4/4) and alveolar rhabdomyosarcomas (75%, 3/4). The detection rates of fusion genes were inversely correlated with the storage period of FFPE blocks. Decalcification by Plank-Rychlo solution significantly affected detection rates of the internal control gene (P = 0.0038). In contrast, there was no significant difference in detection rates between primary and metastatic lesion, or biopsy and resection material, or presence and absence of treatment history.

Conclusion In certain histological types, detection of disease-specific fusion genes of soft tissue tumors using FFPE tissues showed high sensitivity and thus had diagnostic utility. However, due to the diversity of fusion patterns and the low-quality of nucleic acid, the detection rate as a whole was sluggish and required further improvement. For factors affecting the detection results, our results suggested that it was impossible to detect fusion genes by decalcified FFPE tissues, but it may be not necessary to consider factors such as the type of specimen (biopsy or resection) and treatment history of the patients when selecting the FFPE tissues.

Key words fusion gene; reverse transcription polymerase chain reaction; soft tissue tumor

Soft tissue tumors are a heterogeneous group of mesenchymal neoplasms which its histological assessment is challenging, as the morphology and immunoprofile of different tumor types frequently overlap, and can mimic other tumor types. So far, in the diagnosis of several sarcomas such as Ewing sarcoma or Synovial sarcoma, disease-specific fusion gene detection is useful.[1–4] Currently rapid molecular biological progress discovers novel fusion genes. Clinicopathological disease units of some sarcomas have been classified based on their genetic abnormalities.[5] However, regarding the detection of translocation in such sarcomas, there are many types of translocation patterns, and optimization of standard methods for primer designs and reverse transcription polymerase chain reaction (RT-PCR) conditions have not been established, so it is not sufficiently clarified how much fusion gene detection contributes to improvement of diagnostic accuracy in the hospitals. In this study, we have searched fusion genes from soft tissue tumors, examined the diagnostic usefulness and evaluated how much it contributed to improvement of pathological diagnostic accuracy in general hospitals. At the same time, very important factors influencing detection of the fusion genes were also investigated from the standpoint of clinical laboratory technologist.

MATERIALS AND METHODS

Tumor Samples

We identified 37 cases of soft tissue tumors diagnosed between 1 April 2006 and 30 October 2017 with formalin-fixed paraffin-embedded (FFPE) tissue available in

Corresponding author: Takahiro Matsushige, MT
takahiro_610425@yahoo.co.jp
Received 2018 December 4
Accepted 2019 January 28
Abbreviations: bp, base pair; FFPE, formalin-fixed paraffin-embedded; RT-PCR, reverse transcription polymerase chain reaction

Table 1. Tumor tissue types and fusion gene detection results of all samples

Sample no.	Age of block (in years)	Histological diagnosis	Patient age (in years)	Gender	Specimen	Decalcification	Translocation	Fusion point (exon–exon)	*ACTB*
1	5	SS	63	M	excision	–	*SYT-SSX2*	9–7	Positive
2	2	SS	51	F	excision	–	*SYT-SSX1*	9–7	Positive
3	0	SS	38	F	excision	–	*SYT-SSX2*	9–7	Positive
4	0	SS	60	M	biopsy	–	*SYT-SSX1*	9–7	Positive
5	11	ES	36	F	excision	+	Negative		Failed
6	8	ES	52	F	biopsy	–	Negative		Positive
7	4	ES	10	F	biopsy	–	Negative		Positive
8*	2	ES	47	F	excision	–	Negative		Positive
9*	0	ES	48	F	excision	–	*EWSR1-FLI1*	7–5	Positive
10*	0	ES	6	F	excision	–	*EWSR1-FLI1*	7–5	Positive
11*	0	ES	6	F	excision	+	Negative		Failed
12	0	DSRCT	43	M	excision	–	*EWSR1-WT1*	9–8	Positive
13	4	MCS	35	F	biopsy	–	Negative		Positive
14*	1	MCS	67	F	biopsy	–	*HEY1-NCOA2*	4–13	Positive
15*	1	MCS	67	F	excision	–	*HEY1-NCOA2*	4–13	Positive
16	3	AFH	43	M	excision	–	Negative		Positive
17	8	EHE	28	F	excision	–	Negative		Positive
18	2	EHE	77	M	excision	–	Negative		Positive
19	3	MLPS	58	F	excision	–	*FUS-DDIT3*	5–2	Positive
20	3	MLPS	63	M	excision	–	*FUS-DDIT3*	5–2	Positive
21	7	MLPS	59	F	excision	–	Negative		Positive
22	8	MLPS	82	F	biopsy	–	Negative		Positive
23*	0	MLPS	49	M	excision	–	*FUS-DDIT3*	5–2	Positive
24*	0	MLPS	49	M	excision	+	Negative		Failed
25	10	SFT	63	M	excision	–	Negative		Positive
26	5	SFT	79	F	excision	–	Negative		Positive
27	3	SFT	52	F	excision	–	Negative		Failed
28	3	SFT	54	F	excision	–	Negative		Positive
29	3	SFT	83	F	excision	–	*NAB2-STAT6*	4–2	Positive
30	3	SFT	78	M	excision	–	*NAB2-STAT6*	6–17	Positive
31	2	SFT	56	F	excision	–	Negative		Positive
32	2	SFT	60	F	excision	–	*NAB2-STAT6*	4–2	Positive
33	1	SFT	71	F	excision	–	*NAB2-STAT6*	6–17	Positive
34	0	SFT	70	M	excision	–	Negative		Positive
35	0	SFT	48	F	excision	–	Negative		Positive
36	0	SFT	63	M	excision	–	*NAB2-STAT6*	4–2	Positive
37	0	SFT	58	F	excision	–	Negative		Positive
38	11	ARMS	16	F	excision	–	*PAX3/7-FOXO1*	7–2	Positive
39	6	ARMS	74	F	excision	–	Negative		Failed
40	5	ARMS	7	M	biopsy	–	*PAX3/7-FOXO1*	7–2	Positive
41	0	ARMS	9	M	biopsy	–	*PAX3/7-FOXO1*	7–2	Positive

*8 and 9, or 10 and 11, or 14 and 15, or 23 and 24 are the same patients.

AFH, Angiomatoid fibrous histiocytoma; ARMS, Alveolar rhabdomyosarcoma; DSRCT, Desmoplastic small round cell tumor; EHE, Epithelioid hemangioendothelioma; ES, Ewing sarcoma; F, Female; M, Male; MCS, Mesenchymal chondrosarcoma; MLPS, Myxoid liposarcoma; SFT, Solitary fibrous tumor; SS, Synovial sarcoma.

Tottori University Hospital (Table 1). All of 37 cases were reassessed by a pathologist (S.K.) and the diagnoses were confirmed based on histological and immunohistochemical evaluation. In some cases, there were both biopsy and resection specimens, with some specimens being decalcified by Plank-Rychlo solution, and thus a total of 41 FFPE samples were available. Sample 8 and 9, or 10 and 11, or 14 and 15, or 23 and 24 were those from the same patients, respectively (Table 1). The diagnosis of the 37 cases (41 samples) was as follows; 4 (4 samples) synovial sarcoma, 5 (7 samples) Ewing sarcoma, 1 (1 sample) desmoplastic small round cell tumor, 2 (3 samples) mesenchymal chondrosarcoma, 1 (1 sample) angiomatoid fibrous histiocytoma, 2 (2 samples) epithelioid hemangioendothelioma, 5 (6 samples) myxoid liposarcoma, 13 (13 samples) solitary fibrous tumor and 4 (4 samples) alveolar rhabdomyosarcoma (Table 1). The characteristics of all samples as follows: 8 biopsy and 33 resection samples, 34 primary lesion and 7 metastatic lesion samples, and 33 samples without chemoradiation treatment and 11 samples with chemoradiation treatment. There were 2 Ewing sarcoma samples and 1 myxoid liposarcoma sample that were decalcified by Plank-Rychlo solution. All samples were fixed with 10% neutral buffer formalin. Conditions of formalin fixation are 24 hours in biopsy specimens and about 1 week in resection specimens without incision.

Primers and Reverse Transcription-PCR

The primers which were designed with amplicon length of 75–160 bp and amplicon T_m of 60 ± 3 °C were made using the NCBI Primer-BLAST tool (Table 2). RNA was extracted from FFPE tissues by PureLink FFPE Total RNA Isolation Kit (Invitrogen, Waltham, MA). Total RNA from each FFPE sample was used to synthesize the first-strand cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). The subsequent PCR was performed in a final reaction volume of 20 µL containing 10 U TaKaRa Ex Taq Hot Start Version (TaKaRa, Shiga, Japan), 2 µL cDNA, 1 µL gene specific forward primer and reverse primer. The following PCR conditions were used an initial denaturation at 94 °C for 4 minutes 30 seconds, 40 cycles at 94 °C (30 seconds), 55 °C (30 seconds), 72 °C (30 seconds), and one final extension at 72 °C for 4 minutes 30 seconds. Because the amplified products could not be obtained in the preliminary experiment standard RT-PCR in the myxoid liposarcoma cases, semi-nested PCR method which was represented by two types forward primers (external and internal) and one type reverse primer was adopted. The amplification profile of the first-round PCR consisted of 40 cycles of denaturation at 94 °C for 4 minutes 3 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 4 minutes 30 seconds. The second-round PCR was 2 µL of 5-fold diluted solution of the first-round PCR product as a template with the following cycling condition: denaturation at 94 °C for 4 minutes 3 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 4 minutes 30 seconds. PCR products were visualized on a 2% agarose gel using ethidium bromide staining. Several cases which the amplification product could not be detected were used as designed primers for another translocation pattern. In some cases, we tried to improve the detection rate as much as possible. PCR products of several cases were sequenced with forward and reverse primers in the Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). As a control for cDNA synthesis and sample quality, each sample was reverse transcribed and amplified for the housekeeper gene *ACTB* (98 bp).[6]

Statistical Analyses

Fisher's exact test (two-sided) was performed to compare categorical variables. Results were considered significant when *P*-values were less than 0.05. All statistical analyses were conducted using the R ver. 3.3.2.

Ethical Approval

This study has been implemented since it was approved by the Institutional Review Board of Tottori University Hospital (research management number, G179).

RESULTS

Detection Results

Of the all 37 extracted cases (Table 1), fusion gene amplicons were obtained in 19 (51.3%) cases, and *ACTB* amplicons were obtained 34 (91.9%) cases, it indicated adequate RNA quality, except 3 decalcified samples. Of these 34 cases, fusion gene amplicons were obtained in 19 (55.9%) cases. Of these 34 cases, the detection rate for each tissue type was 100% (4/4 cases) in synovial sarcoma, 50% (2/4) in Ewing sarcoma, 100% (1/1) in desmoplastic small round cell tumor, 50% (1/2) in mesenchymal chondrosarcoma, 0% (0/1) in angiomatoid fibrous histiocytoma, 0% (0/2) in epithelioid hemangioendothelioma, 60% (3/5) in myxoid liposarcoma, 42% (5/12) in solitary fibrous tumor and 100% (3/3) in alveolar rhabdomyosarcoma (Fig. 1).

EWSR1ex7-WT1ex8 that detected in more than 80% of cases, the most common fusion gene in one desmoplastic small round cell tumor case,[7,8] was not detected, however we detected *EWSR1ex9-WT1ex8* which was a rare translocation pattern (Fig. 2). In an angiomatoid

Table 2. The retrieved fusion gene and primer sequence

Histological diagnosis	Gene fusion	Forward primer (5' –3')	Reverse primer (5' –3')	Fusion point (exon–exon)	Size (bp)
SS	*SYT-SSX1*	CCAGCAGAGGCCTTATGGATA	ACACTCCCTTCGAATCATTTTCG	9–7	77
	SYT-SSX2	(same as above)	GCACTTCCTCCGAATCATTTCCT	9–7	77
ES	*EWSR1-FLI1*	CCAAGTCAATATAGCCAACAGAGC	CATGTTATTGCCCCAAGCTCCTC	7–5	156
	EWSR1-FLI1	(same as above)	(same as above)	7–6	90
	EWSR1-ERG	(same as above)	TCCAGGAGGAACTGCCAAAG	7–9	154
DSRCT	*EWSR1-WT1*	CCAAGTCAATATAGCCAACAGAGC	GTCTGAACGAGAAAACCTTCG	7–8	102
	EWSR1-WT1	GAGGACGCGGTGGAATGG	(same as above)	8–8	78
	EWSR1-WT1	(same as above)	(same as above)	8–9	116
	EWSR1-WT1	(same as above)	(same as above)	8–10	149
MCS	*HEY1-NCOA2*	ATCCTGCAGATGACCGTGGA	TGGTTTGGCAATAACCTGCC	4–13	104
AFH	*EWSR1-CREB1*	CCAAGTCAATATAGCCAACAGAGC	ACCCCATCGGTACCATTGTTAG	7–7	98
	EWSR1-ATF1	(same as above)	CTCCATCTGTGCCTGGACTTG	7–5	97
	FUS-ATF1	CAGCAGAACCAGTACAACAGC	(same as above)	5–5	109
EHE	*WWTR1-CAMTA1*	CTCCACCCTGCCGTCAGTTC	TGCAGGTCCACTTGATGCCA	4–8	91
	WWTR1-CAMTA1	(same as above)	GCGAGATGATGCGGTGTTTG	4–9	124
MLPS	*FUS-DDIT3*	External: TAATCCCCCTCAGGGCTATGG Internal: CAGCAGAACCAGTACAACAGC	TTCAGGTGTGGTGATGTATGAA	5–2	98
	FUS-DDIT3	External: TATGAACCCAGAGGTCGTGGA Internal: AAGTGACCGTGGTGGCTTC	(same as above)	7–2	75
	FUS-DDIT3	(same as above)	(same as above)	8–2	108
	EWSR1-DDIT3	External: ACTGGATCCTACAGCCAAGC Internal: CCAAGTCAATATAGCCAACAGAGC	(same as above)	7–2	86
SFT	*NAB2-STAT6*	CTTGTCCTCCTTGAAGGGCTC	TTTTTCTGGGGGCATCTTGGA	4–2	139
	NAB2-STAT6	CAGCAGACACTGATGGACGAGG	AGCTGGGACATAACCCCTGC	6–17	144
	NAB2-STAT6	CAGCAGACACTGATGGACGAGG	CTTTGGCAGAGAATGGCTGGATG	6–16	145
ARMS	*PAX3-FOXO1*	CCCAGCACCAGGCATGGATTT	GCTGTGTAGGGACAGATTATGACGA	7–2	138
	PAX7-FOXO1	CTACGGAGCCCGCCACA	(same as above)	7–2	136

AFH, Angiomatoid fibrous histiocytoma; ARMS, Alveolar rhabdomyosarcoma; DSRCT, Desmoplastic small round cell tumor; EHE, Epithelioid hemangioendothelioma; ES, Ewing sarcoma; MCS, Mesenchymal chondrosarcoma; MLPS, Myxoid liposarcoma; SFT, Solitary fibrous tumor; SS, Synovial sarcoma.

fibrous histiocytoma case, neither *EWSR1ex7-CREB1ex7*,[9, 10] *EWSR1ex7-ATF1ex5*[9–12] nor *FUSex5-ATF1ex5*[13, 14] was detected. In two epithelioid hemangioendothelioma cases, no amplified products were obtained for *WWTR1ex4-CAMTA1ex8* (type1) and *WWTR1ex4-CAMTA1ex9* (type2).[15, 16]

Affectors of Detection

Factors affecting detection of fusion genes were examined. To investigate the effects of decalcification, we selected 2 samples of Ewing sarcoma and 1 sample of myxoid liposarcoma that were decalcified FFPE samples. No amplified products either transcription or *ACTB* were detected in these samples (Fig. 3). Therefore, in the subsequent examination, affectors other than decalcification were analyzed in the FFPE samples excluding the decalcified samples. FFPE tissues stored at room temperature have been used as archival resources in many molecular studies. The characteristic of FFPE samples based on storage period as follows: less than one year (11 samples), one to two years (8 samples), three to four years (9 samples), five to seven years (5 samples), and eight to eleven years (5 samples). The detection rates for fusion genes were as follows; 73% (8/11) for samples stored less than one year was positive, 63% (5/8) for samples stored one to two years was positive, 44% (4/9) for samples stored three to four years was positive, 40% (2/5) for samples stored five to seven years was positive, and 20% (1/5) for samples stored eight to eleven years. As described above, the longer the storage period, the

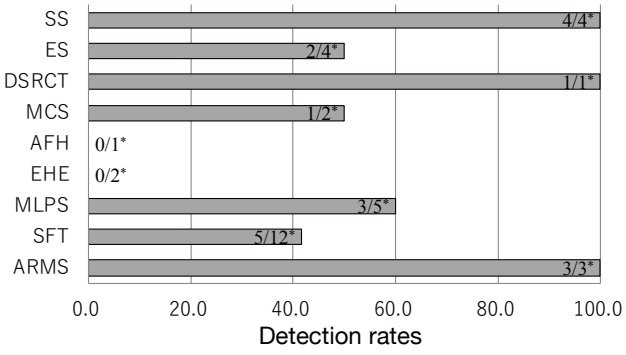

Fig. 1. The detection rates of fusion genes for each tissue types with *ACTB* negative cases exclusion. *Number of fusion gene detection cases and total cases by tissue types. AFH, Angiomatoid fibrous histiocytoma; ARMS, Alveolar rhabdomyosarcoma; DSRCT, Desmoplastic small round cell tumor; EHE, Epithelioid hemangioendothelioma; ES, Ewing sarcoma; MCS, Mesenchymal chondrosarcoma; MLPS, Myxoid liposarcoma; SFT, Solitary fibrous tumor; SS, Synovial sarcoma.

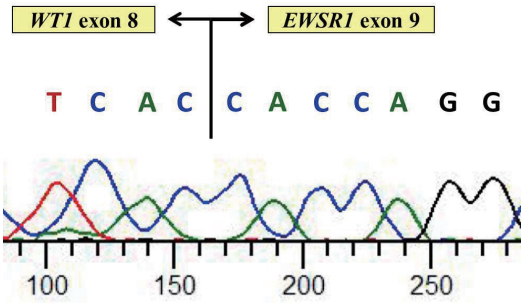

Fig. 2. DNA sequence analysis showing *EWSR1ex9-WT1ex8* gene fusion of Desmoplastic small round cell tumor.

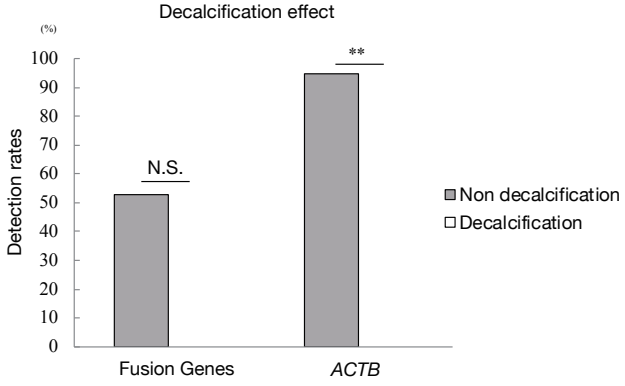

Fig. 3. The effect of decalcification for fusion genes and *ACTB* detection rates. $^{**}P < 0.01$. N.S., not significant

lower the detection rates of fusion transcripts will be. Whereas the detection rates for *ACTB* were as follows; 100% (11/11) for samples stored less than one year was positive, 100% (8/8) for samples stored one to two years was positive, 89% (8/9) for samples stored three to four years was positive, 80% (4/5) for samples stored five to seven years was positive, and 100% (5/5) for samples stored eight to eleven years was positive. As described above, the detection rates were almost constant regardless of the storage period (Fig. 4). There was no significant difference in the detection rates of the fusion genes and *ACTB*, between biopsy and resection samples (Fig. 5A), between with and without chemoradiation treatment samples (Fig. 5B), and between primary lesion and metastatic lesion samples (Fig. 5C).

DISCUSSION

Even though the frequency of each histological types of soft tissue tumors are rare, there are over 100 types of histological type.[5] In addition, we recognize general histopathological diagnosis limitations, as the morphology and immunoprofile of different tumor types frequently overlap and can mimic other tumor types. Furthermore, in recent years, with advances in treatments such as chemotherapy or radiation therapy, opportunities are increasingly demanded for definite diagnosis with a sample volume in minute amounts by preoperative needle biopsy. The detection of tumor-specific fusion genes is becoming an integral part of the diagnostic investigation of soft tissue tumors. In more detail, synovial sarcoma showing from epithelial to sarcoid lesion may cause diagnostic challenges. However, detecting disease-specific fusion genes such as *SYT-SSX1* or *SYT-SSX2* helps diagnosis of this tumor. Furthermore, in round cell sarcomas such as Ewing sarcoma or rhabdomyosarcoma, detecting the fusion genes are important for deciding course of treatment. One reason is that the patients suffering from tumors of specific subtypes of round cell sarcoma, like Ewing sarcoma or rhabdomyosarcoma, respond to well-defined therapeutic regimens. Proper classification of soft tissue tumors is crucial for appropriate patient management. In this study, we have searched fusion genes from soft tissue tumors, examined the diagnostic usefulness and evaluated how much it contributed to improvement of pathological diagnostic accuracy in general foundation hospitals. At the same time, from the standpoint of clinical laboratory technologist, we also analyzed factors influencing the detection of fusion genes.

In the cases of *ACTB* positive, the comprehensive detection rate was not as high as 55% (19/34 cases). It has been suggested that one of the causes could be that primers of our own design may not cover all of the fusion genes that can be expressed in our soft tissue tumor cases because there are multiple types of fusion

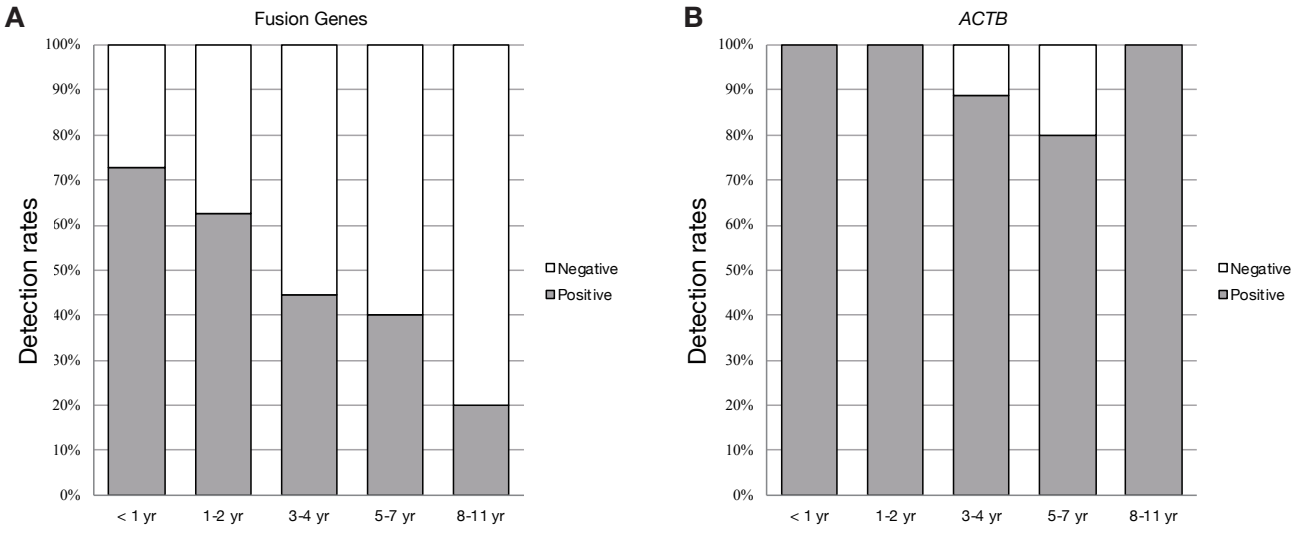

Fig. 4. Comparison of fusion genes and *ACTB* detection rates based on FFPE storage period. FFPE, formalin-fixed paraffin-embedded; yr, year(s).

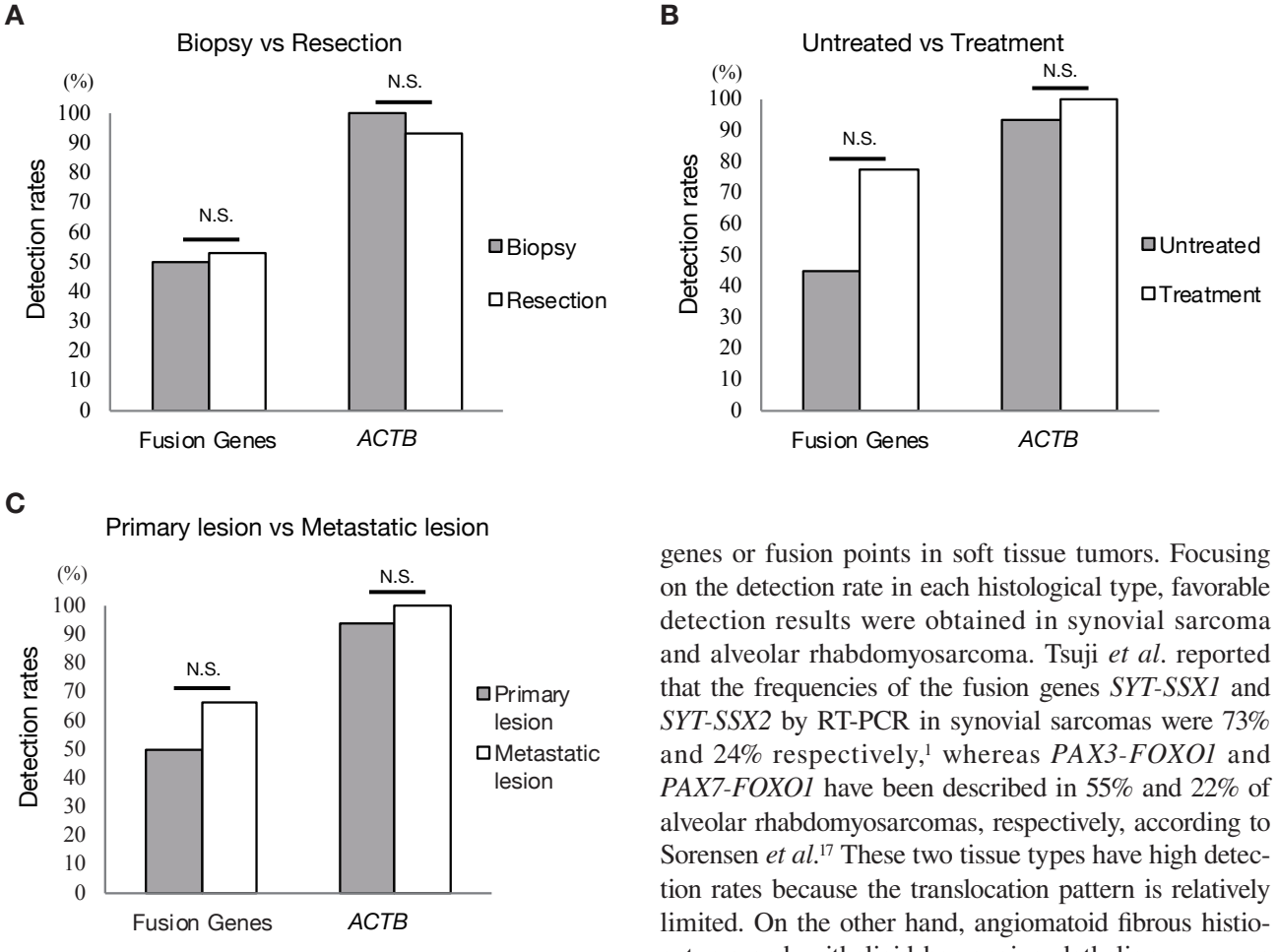

Fig. 5. Comparison of the detection rates of fusion genes and *ACTB* in each factor. N.S., not significant

genes or fusion points in soft tissue tumors. Focusing on the detection rate in each histological type, favorable detection results were obtained in synovial sarcoma and alveolar rhabdomyosarcoma. Tsuji *et al*. reported that the frequencies of the fusion genes *SYT-SSX1* and *SYT-SSX2* by RT-PCR in synovial sarcomas were 73% and 24% respectively,[1] whereas *PAX3-FOXO1* and *PAX7-FOXO1* have been described in 55% and 22% of alveolar rhabdomyosarcomas, respectively, according to Sorensen *et al*.[17] These two tissue types have high detection rates because the translocation pattern is relatively limited. On the other hand, angiomatoid fibrous histiocytoma and epithelioid hemangioendothelioma prove a challenge to detect translocations from FFPE samples by our designed primers and PCR condition. The reason why a sufficient detection rate could not be obtained in these tumors may be attributable to various translocation

pattern, in addition, to the RNA ravages due to storage time in the FFPE samples.

The fusion genes in 5 myxoid liposarcoma cases were not detected by standard RT-PCR in preliminary experiment. It may be affected by the low concentration of the tumor cells. However, the fusion genes in 3 out of 5 cases in myxoid liposarcoma were successfully detected by semi-nested RT-PCR. Powers *et al.* reported that fusion genes of myxoid liposarcoma could be efficiently detected by standard RT-PCR.[18] On the other hand, Hisaoka *et al.* showed that the nested RT-PCR assay could specifically detect multiple consistent fusion gene transcripts of myxoid liposarcoma generated after splicing of introns in which most of the variable breakpoints are located.[19] As described above, the fusion gene retrieval from FFPE samples of this tumor is recommended to use nested RT-PCR or semi-nested RT-PCR. In this study, fusion genes of myxoid liposarcoma could be detected sufficiently even with semi-nested RT-PCR.
We searched for *EWSR1-FLI1*, the most common fusion variant in Ewing sarcoma cases which has two type variants: type 1 (55%) and type 2 (25%), and the second common fusion variant was *EWSR1-ERG* (10%).[2–4] The detection rate was 50% (2/4 cases). Next we evaluated *NAB2-STAT6*, the most common fusion gene variant in solitary fibrous tumor cases which had three type variants as follows: type 1 (45%), type 2 (21%), and type 3 (21%).[20] We found the detection rate for *NAB2-STAT6* was 42% (5/12 cases), which was not high. The detection rate in mesenchymal chondrosarcoma cases was 50% (1/2 cases), we could not obtain adequate result. The results described above seemed also being caused by the quality of FFPE samples or the variation of the fusion genes which influenced the detection rate.

In this experiment, we adopted RT-PCR assay because Patel *et al.* state that RT-PCR targeting the specific fusion transcripts associated with soft tissue tumors are a valuable ancillary tool for diagnosis,[16] particularly as more soft tissue tumors are discovered to harbor recurrent translocations involving overlapping genes. They also made the cautionary comment that fluorescence in situ hybridization alone may be unsuitable for evaluating different types of tumors sharing the same gene rearrangement.[16]

We examined factors that affect in the search for fusion gene using FFPE samples, and as a result, the decalcification was the most influential factor. Primary and metastatic bone tumors decalcify during tissue pathology specimen preparation process. Nam *et al.* showed that the PCR results were significantly affected by the decalcification time and the yield of RNA or DNA from decalcified tissues decreased gradually with increasing decalcification time.[21] Therefore, for examining fusion gene from primary and metastatic bone tumor tissues, it is necessary to selectively cut out portions which not requiring decalcification process. In addition, when selecting FFPE blocks for genetic diagnosis, it is recommended to use nondecalcified blocks.

The detection rates of fusion genes also were greatly affected by the storage period of FFPE samples.[21] In our study, even FFPE samples stored for one to two years showed lower detection rates than FFPE samples stored for less than one year, suggesting that deterioration of RNA is caused even for a storage period about one year depending on the case. Therefore, since an acceptable range was not established for the storage period of FFPE samples in which detection of the fusion gene is possible, it was considered to perform the detection as soon as possible.

We compared the detection rates of fusion genes between biopsy and resection samples, but there was no significant difference. Detection using biopsy samples could have a false negative due to lack of sample volume. However, in this study, even taken from small quantities of specimens it is suggested that detection is possible as long as tumor components are sufficiently contained. Furthermore, it is considered that RNA is not easily influenced by RNase because the biopsy materials undergo formalin fixation completely in a short time. Therefore, it is possible that excellent detection results could be obtained despite a small amount of biopsy materials. In the approximately 1-week formalin-fixed resection specimens, the detection rates were high in *ACTB* despite being fixed in formalin without incision. This means that the influence that the fixed condition had on fusion gene detection was minimal. According to Nam *et al.*, when conducting a gene search study from RNA, it is recommended using 20% formalin for tissue fixation and the tissues should be immersed in formalin solution for 3 to 7 days.[21] In other words, to obtain a highly accurate result, it is important to quickly inactivate RNase in the tissue. However, large resection specimens require considerable time for completing formalin fixation step. In the fusion gene search using RT-PCR, it is recommended that a part of the tumor is collected and fixed from the surgically resected specimen before RNA is affected by RNase to ensure constant detection sensitivity.

We also examined other factors that affect detection results. When comparing the detection rates between with and without chemoradiation treatment samples, or primary lesion and metastatic lesion samples, no significant difference was observed. Metastasis and chemoradiation treatment could cause gene mutations in tumors,

these factors were thought affecting the detection rate, but in this study there were no differences among these factors. This may indicate that the fusion genes are likely to be retained even in metastasis or after treatment. Therefore, it suggests that considering these factors as confounding factors in the fusion gene search might not be necessary. Our results may suggest that the soft tissue tumors are cancer stem cell derived tumors. Zhou *et al*. reported that the development of synovial sarcoma may arise from cancer stem cells.[22] Furthermore, cancer stem cells show resistance to chemotherapy and radiotherapy which known as the cause of recurrence and metastasis.[23] In the light of above, our findings may suggest that soft tissue tumors used in this study are tumors that originated from cancer stem cells. To the best of our knowledge, there was no literature comparing the detection rates of fusion genes which collected from various conditions samples as in our study condition. However, since we analyzed only 41 samples, we consider that further examination is necessary to get more accurate results.

The results obtained in this study, the storage periods or decalcification, were similar to the data reported by Kanai *et al*.[24] However, these published data do not indicate how much they can respond to the routine work in general hospitals or data comparing the detection rates between primary and metastatic lesions, biopsy and resected material, or the presence or absence of treatment history. In our study, it was indicated that the fusion genes of tumors showing relatively simple chromosomal abnormalities can be detected by selecting FFPE blocks with short-storage periods and nondecalcification even at general hospitals. Furthermore, it was indicated that factors such as biopsy or resected material, primary or metastatic lesions, or the presence or absence of treatment history may not be necessary while considering selection of FFPE blocks.

In recent years, rapidly developing genomic medicine adds consideration how to handle and manage the pathological tissue samples storage properly. The quality control of FFPE samples is important in examinations using molecular pathological methods, since numerous factors could affect the detection of fusion genes. The quality control of FFPE samples, formalin fixation and decalcification conditions are becoming the clinical laboratory technologist's responsibility who is involved in pathological examination. These parts will greatly affect gene search, therefore we need to ensure these accuracies. Even more, we consider the same importance of how to handle pathological tissue samples focusing on genetic diagnosis as well as how to handle samples for histopathological and immunohistological diagnosis. At the same time, establishing optimal fixation conditions and standardizing methods for fusion gene search are essential for improving the accuracy of molecular pathological diagnosis in pathology laboratory.

Acknowledgments: The authors thank Chieko Ohno for providing formalin-fixed paraffin-embedded tissues of soft tissue tumors

The authors declare no conflict of interest.

REFERENCES

1 Tsuji S, Hisaoka M, Morimitsu Y, Hashimoto H, Shimajiri S, Komiya S, et al. Detection of *SYT-SSX* fusion transcripts in synovial sarcoma by reverse transcription-polymerase chain reaction using archival paraffin-embedded tissues. Am J Pathol. 1998;153:1807-12. PMID: 9846971.
2 Mariño-Enríquez A, Fletcher CD. Round cell sarcomas - biologically important refinements in subclassification. Int J Biochem Cell Biol.2014;53:493-504. PMID: 24801613.
3 Machado I, Navarro L, Pellin A, Navarro S, Agaimy A, Tardío JC, et al. Defining Ewing and Ewing-like small round cell tumors (SRCT): The need for molecular techniques in their categorization and differential diagnosis. A study of 200 cases. Ann Diagn Pathol. 2016;22:25-32. PMID: 27180056.
4 Gamberi G, Cocchi S, Benini S, Magagnoli G, Morandi L, Kreshak J, et al. Molecular diagnosis in Ewing family tumors: the Rizzoli experience-222 consecutive cases in four years. J Mol Diagn. 2011;13:313-24. PMID: 21458383.
5 Christopher DM, Fletcher Julia A, Pancras CW, Hogendoorn Fredrik Mertens. WHO Classification of Tumours of Soft Tissue and Bone. International Agency for Research on Cancer. Geneva: World Health Organization; 2013. p. 10-11.
6 Puls F, Niblett A, Marland G, Gaston CL, Douis H, Mangham DC, et al. *BCOR-CCNB3* (Ewing-like) sarcoma: a clinicopathologic analysis of 10 cases, in comparison with conventional Ewing sarcoma. Am J Surg Pathol. 2014;38:1307-18. PMID: 24805859.
7 de Alava E, Ladanyi M, Rosai J, Gerald WL. Detection of chimeric transcripts in desmoplastic small round cell tumor and related developmental tumors by reverse transcriptase polymerase chain reaction. A specific diagnostic assay. Am J Pathol. 1995;147:1584-91. PMID: 7495283.
8 Liu J, Nau MM, Yeh JC, Allegra CJ, Chu E, Wright JJ. Molecular heterogeneity and function of *EWS-WT1* fusion transcripts in desmoplastic small round cell tumors. Clin Cancer Res. 2000;6:3522-9. PMID: 10999739.
9 Antonescu CR, Dal Cin P, Nafa K, Teot LA, Surti U, Fletcher CD, et al. *EWSR1-CREB1* is the predominant gene fusion in angiomatoid fibrous histiocytoma. Genes Chromosomes Cancer. 2007;46:1051-60. PMID: 17724745.
10 Kao YC, Lan J, Tai HC, Li CF, Liu KW, Tsai JW, et al. Angiomatoid fibrous histiocytoma: clinicopathological and molecular characterisation with emphasis on variant histomorphology. J Clin Pathol. 2014;67:210-5. PMID: 24043718.
11 Somers GR, Viero S, Nathan PC, Teshima I, Pereira C, Zielenska M. Association of the t(12;22)(q13;q12) *EWS/ATF1* rearrangement with polyphenotypic round cell sarcoma of bone: a case report. Am J Surg Pathol. 2005;29:1673-9. PMID: 16327442.
12 Hallor KH, Micci F, Meis-Kindblom JM, Kindblom LG, Bacchini P, Mandahl N, et al. Fusion genes in angiomatoid

fibrous histiocytoma. Cancer Lett. 2007;251:158-63. PMID: 17188428.
13 Waters BL, Panagopoulos I, Allen EF. Genetic characterization of angiomatoid fibrous histiocytoma identifies fusion of the *FUS* and *ATF-1* genes induced by a chromosomal translocation involving bands 12q13 and 16p11. Cancer Genet Cytogenet. 2000;121:109-16. PMID: 11063792.
14 Raddaoui E, Donner LR, Panagopoulos I. Fusion of the *FUS* and *ATF1* genes in a large, deep-seated angiomatoid fibrous histiocytoma. Diagn Mol Pathol. 2002;11:157-62. PMID: 12218455.
15 Flucke U, Vogels RJ, de Saint Aubain Somerhausen N, Creytens DH, Riedl RG, van Gorp JM, et al. Epithelioid Hemangioendothelioma: clinicopathologic, immunhistochemical, and molecular genetic analysis of 39 cases. Diagn Pathol. 2014;9:131. PMID: 24986479.
16 Patel NR, Salim AA, Sayeed H, Sarabia SF, Hollingsworth F, Warren M, et al. Molecular characterization of epithelioid haemangioendotheliomas identifies novel *WWTR1-CAMTA1* fusion variants. Histopathology. 2015;67:699-708. PMID: 25817592.
17 Sorensen PH, Lynch JC, Qualman SJ, Tirabosco R, Lim JF, Maurer HM, et al. *PAX3-FKHR* and *PAX7-FKHR* gene fusions are prognostic indicators in alveolar rhabdomyosarcoma: a report from the children's oncology group. J Clin Oncol. 2002;20:2672-9. PMID: 12039929.
18 Powers MP, Wang WL, Hernandez VS, Patel KS, Lev DC, Lazar AJ, et al. Detection of myxoid liposarcoma-associated *FUS-DDIT3* rearrangement variants including a newly identified breakpoint using an optimized RT-PCR assay. Mod Pathol. 2010;23:1307-15. PMID: 20581806.
19 Hisaoka M, Tsuji S, Morimitsu Y, Hashimoto H, Shimajiri S, Komiya S, et al. Detection of *TLS/FUS-CHOP* fusion transcripts in myxoid and round cell liposarcomas by nested reverse transcription-polymerase chain reaction using archival paraffin-embedded tissues. Diagn Mol Pathol. 1998;7:96-101. PMID: 9785008.
20 Tai HC, Chuang IC, Chen TC, Li CF, Huang SC, Kao YC, et al. NAB2-STAT6 fusion types account for clinicopathological variations in solitary fibrous tumors. Mod Pathol. 2015;28:1324-35. PMID: 26226844.
21 Nam SK, Im J, Kwak Y, Han N, Nam KH, Seo AN, et al. Effects of fixation and storage of human tissue samples on nucleic Acid preservation. Korean J Pathol. 2014;48:36-42. PMID: 24627693.
22 Zhou Y, Chen D, Qi Y, Liu R, Li S, Zou H, et al. Evaluation of expression of cancer stem cell markers and fusion gene in synovial sarcoma: Insights into histogenesis and pathogenesis. Oncol Rep. 2017;37:3351-60. PMID: 28498445.
23 Radpour R. Tracing and targeting cancer stem cells: New venture for personalized molecular cancer therapy. World J Stem Cells. 2017;9:169-78. PMID: 29104735.
24 Kanai Y, Nishihara H, Miyagi Y, Tsuruyama T, Taguchi K, Katoh H, et al. The Japanese Society of Pathology Guidelines on the handling of pathological tissue samples for genomic research: Standard operating procedures based on empirical analyses. Pathol Int. 2018;68:63-90. PMID: 29431262.