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Original Artice

Molecular detection of SYT-SSX fusion gene transcription in monophasic type synovial sarcoma with the use of fomalin-fixed paraffin-embedded specimens -Case report guaranteed by the chromosomal analysis of incubated cells and an establishment of the most suitable condition in Reverse Transcription - Polymerase Chain Reaction (RT-PCR) of SYT-SSX gene -

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Summary

In synovial sarcoma the rearrangement of t (X; 18) (p11.2; q11.2) and its new chimeral gene "SYT-SSX" were reported.(1,3, 4) We studied its application to a histopathological diagnosis of synovial sarcoma with both the use of RNA (ribonucleic acid), extracted from formalin-fixed paraffin-embedded material, and the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) by PCR primers, consisted of SYT-SSX, SYT-SSX1, and SYT-SSX2.

We had a chance to investigate the RT-PCR of SYT-SSX through one case of monophasic type synovial sarcoma(so-called poorly differentiated type synovical sarcoma), which had been reconfirmed by chromosomal analysis. As a result of our examination in this case, an optimal RT-PCR condition for formalin-fixed paraffin-embedded tissues was as follows: 1. whole RNA extracts digested with proteinase K were for use, 2. RT with downstream primers (reverse primers, anti-sense primers) at 42℃, 3. PCR of 50 cycles at 55℃, 4. 12.5% Polyacrylamide Gel Electrophoresis (PAGE), and 5. viewed under ultraviolet light followed by ethidium bromide reaction. In this RT-PCR condition we could confirm chimeral gene SYT-SSX2 in our case of monophasic synovial sarcoma of thigh.

Key words : Synovial sarcoma, SYT-SSX, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), formalin-fixed paraffin-embedded tissue

Introduction

Biphasic synovial sarcoma was easily diagnosed histologically by their two-cell pattern. But there were many troubles in monophasic type synovial sarcoma, so-called poorly differentiated type synovial sarcoma, because of many indistinguishable pathologies consisting of spindle cells. It is the reason that the new diagnostic technique is requested. The translocation between the 18th chromosome and the X chromosome was reported in synovial sarcoma, and resulted in a chimeral gene "SYT-SSX" at the breakpoint in t (X; 18) (p11.2; q11.2). (1, 3, 4) Proving this rearranged SYT-SSX gene by RT-PCR could become reliable diagnostic basis in these laborious subtypes of synovial sarcomas.

When restricted to formalin-fixed paraffin-embedded specimens the tissue chromosomes were severely damaged by their fixation, and then their collected genes were finely fragmented into about 100 (100-300) bps in basal length. (2, 4) We had to select primers for a gene amplification of very short basal length because our routinely usable tissues had to be limited to formalin-fixed specimens. A chimeral RNA "SYT-SSX" was originally a very long basal chain, but our recognizable basal arrangement had to limit it to about 100 bps just around breakpoint, where SYT RNA of 18 th chromosome glued to SSX RNA of X chromosome. Several primers for this purpose reported, and then we discussed the availability of previously reported RT-PCR primer pairs as follows: SYT-SSX, SYT-SSX1, and SYT-SSX2 (1, 3, 4). SYT and SSX was a common upstream primer and a common downstream primer, respectively. SSX1 and SSX2 were specific downstream primers for each synovial sarcoma, and then only one primer subtype was amplified in each case. Their final DNA (deoxyribonucleic acid) products were 98, 118, and 118 bps at the usage of SYT-SSX, SYT-SSX1, and SYT-SSX2, respectively.

Case report

A fifty-years-old man presented with a thigh tumor. Dur-

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ing the autumn of 1999 several times of aspiration therapies had been done and shown blood-natured contents. On June 9, 2000, the curettage had removed coagula, most of which was blood but a very small part of solid tumor (H98-4345-1). Its pathology was undifferentiated malignancy. On June 22, 2000, the complete resection of tumor was done in Niigata Prefectural Cancer Center and the pathological diagnosis was synovial sarcoma, poorly differentiated type, which was reconfirmed by the chromosomal dislocation in cultured tumor cells.

RT-PCR analysis

1. RNA extraction: Several 4 μm-thick sections sliced from paraffin block were deparaffinized, and proteinase K (5mg/ml) was added, and then incubated overnight at 55℃. The supernatant was collected for analysis. Negative control RNA was obtained from normal gastric specimens.

2. Primers: Basal arrangement as follows:

i. Upstream (forward, sense) primer:
SYT: 5'-ccA gcA gAg-gcc ttA tgg AtA-3'

ii. Downstream (reverse, anti-sense) primers:
SSX: 5'-ttt gtg ggc cAg Atg ctt c-3', product=98bps
SSX1: 5'-gtg cAg ttg ttt ccc Atc gt-3', product=118bps
SSX2: 5'-gcA cAg ctc ttt ccc Atc A-3' , product=118 bps

SYT primer was common basal arrangement of upstream. SSX primer was common basal arrangement of downstream for both SSX1 primer and SSX2 one. The downstream basal arrangement of each tumor had either SSX 1 or SSX2 inevitably.

3. RT procedure : RNA PCR kit (AMV) Ver 2.1 (Takara, Kyoto) was used. Reactant consisted of MgCl 2 4 μl, 10xRNA PCR buffer 2 μl, RNase free dH2O 8.5 μl, dNTP Mixture 2 μl, RNase inhibitor 0.5 μl, Reverse transcriptase 1 μl, RT primer 1 μl, and RNA sample 1 μl. RT primer was Random 9 mers or Oligo dT-adaptor primer or specific downstream DNA primers. The positive control RNA was used instead of experimental sample for positive control test. This mixture of 20 μl in 0.5ml tube was placed in a thermal-cycler and set under a single cycle of reaction program as follows: 95℃x5', 25℃x10', 42-50℃x45', 99℃x5', and 5℃x5'.

4. PCR procedure: Same RNA PCR kit (AMV) Ver 2.1 (Takara, Kyoto) was used. Reactant consisted of MgCl2 6 μl, 10xRNA PCR buffer 8 μl, H2O 63.5 μl, Takara Taq 0.5 μl, upstream primer 1 μl, and downstream primer 1 μl. F-1 primer was used instead of upstream primer in positive control. Instead of downstream primer, R-1 primer, M13 primer M4, or pure H2O was used for positive control, dT-adapter primer, or specific downstream primer, respectively. 16 μl of this PCR reactant was mixed with 4 μl RT product (complementary DNA, cDNA) product just before PCR procedure. Each 20 μl of final mixture in 0.5ml tube was placed in thermal-cycler. PCR program was 94℃x2'x1 cycle, 50 cycles of amplification (each cycle consisted of 94℃x30", 55-65℃x30", and 72℃x90"), and 72℃ x7'.

5. Semi-nested PCR procedure: Additional PCR with inner downstream SSX was done in same PCR program of either 25 or 50 cycles of amplification.

Results

There consisted of three examination groups depending on their primers for RT as follows:(1) Random 9 mers, (2) Oligo dT-adaptor primer, and (3) specific downstream primers.

Firstly, in the usage of cDNA extracted by Ramdom 9 mers, specific PCR products were obtained by PCR of 50 cycles at 55℃ (Fig. 1). There were also several non-specific RT-PCR products. These non-specific products were resistant to both high annealing temperature (Fig.1) and additional semi-nested PCR's after the dilution of original PCR products (Fig.2). The RT-PCR procedure suggested that this synovial sarcoma had the rearrangement of SYT-SSX2 type.

		Temperature during RT						
		42℃			45℃	50℃	55℃	60℃
PCR primer		SSX	SSX1	SSX2	SSX	SSX	SSX	SSX
Temperature during PCR, x50 α	55℃	△	×	○	△	△	△	△
	58℃	▲						
	60℃	▲	×	×				
	62℃	▲	×	×				
	63℃						×	
	64℃						×	
	65℃	×						

RT: Reverse Transcription, PCR: Polymerase Chain Reaction, α: cycles
× negative, ▲ weak positivity with non-specific bands,
△ moderate positivity with non-specific bands

Fig 1. RT with Random 9 sarcoma

		Temperature at RT 42℃									
PCR primer		SSX1					SSX2				
Temperature during PCR	55℃	×					○				
Dilution of PCR product		x10	x100	x1000	x10000	x100000	x10	x100	x1000	x10000	x100000
Semi-nested PCR with SSX, x25 α		×	×	×	×	×	×	×	×	×	×
Semi-nested PCR with SSX, x50 α		×	×		×		×	×		×	

RT: Reverse Transcription, PCR: Polymerase Chain Reaction, α: cycles
× negative, ▲ weak positivity with non-specific bands,
△ moderate positivity with non-specific bands,
○ definite positivity with weak non-specific bands

Fig 2. RT with Random 9 mers

Secondly, in the usage of cDNA extracted by Oligo dT-adaptor primer, no RT-PCR product was obtained by SYT-SSX primers (Fig. 3). Additional analysis of PCR by SSX1 or SSX2 primers was omitted because common basal arrangement of SSX could not be amplified.

Thirdly, in the usage of cDNA extracted by specific downstream primers, specific RT-PCR products were obtained (Fig.4). There was no RT-PCR products in normal gastric mucosal tissues as negative control.

No additional semi-nested PCR after the dilution of original PCR product could enhance its positivity and dimin-

		Temperature during RT
		42°C
PCR primer		SSX
Temperature during PCR	55°C	×
	60°C	×
Semi-nested PCR with SSX1, x50 α	Dilution of PCR product, x10	×
	x100	×
	x10000	×
Semi-nested PCR with SSX2, x50 α	x10	×
	x100	×
	x10000	×

RT: Reverse Transcription, PCR: Polymerase Chain Reaction, α: cycles
× negative

Fig 3. RT with Oligo dT-adaptor primer

RT primer	Tempera-ture during PCR	Dilution of PCR product: nested PCR with SSX	Temperature during RT			
			42°C		50°C	60°C
			Nested PCR x25 α	Nested PCR x50 α		
SSX	55°C		△			×
	60°C		△		×	×
SSX1	55°C	—	×			
		x10	×	×		
		x100	×	×		
		x1000	×			
		x10000	×	×		
SSX2	55°C		◎			
		x10	×	×		
		x100	×	×		
		x1000	×			
		x10000	×	×		

RT: Reverse Transcription, PCR: Polymerase Chain Reaction, α: cycles
× negative, ▲ weak positivity with non-specific bands,
△ moderate positivity with non-specific bands,
◎ definite positivity without non-specific bands

Fig 4. RT with downstream (reverse) Primer

ish its non-specific RT-PCR product (Fig. 2, 3, 4).

Discussion

Our RNA's withdrawed from formalin-fixed paraffin-embedded specimens were very short to about 100 bps. (2, 4) This was one of causes of non-specific RT-PCR products (Fig. 1, 4). (4)

In RT with Random 9 mers, every RNA was reversed in spite of their three-dimensional differences. cDNA's against both ribosomal RNA (rRNA) and transfer RNA (tRNA) were also reversed as well as messenger RNA (mRNA). This was the reason that the Random 9 mers was screeningly used for the RT-PCR analysis. We also performed RT-PCR with both Random 9 mers firstly and obtained SYT-SSX products (Fig. 1). But this random amplification in RT accompanied many non-specific bands in PAGE (Fig. 1). These non-specific products could not be removed by semi-nested PCR following any dilution of PCR products (Fig. 1, 2). RT-PCR with Random 9 mers should be given up at last.

In RT-PCR with Oligo dT-adaptor primer, amplifying only mRNA, there was no positive bands in PAGE, which suggested very few complete poly-A-mRNA could be extracted in usual RNA extration methods (Fig. 3). RT-PCR with Oligo dT-adaptor primer was disappointed for this purpose.

In RT-PCR with specific downstream primers, appropriate SYT-SSX2 (or SYT-SSX1 as in case for other synovial sarcoma) product could be obtained without any non-specific ones (Fig. 4). To decrease non-specific products and preserve mRNA's we recommended that the specimens should be shortly fixed with neutral formalin instead of routinely-unsealed strongly-acid formalin. (2, 4)

There is still some uncertainty concerning the RT-PCR in formalin-fixed paraffin-embedded tissues. We must genetically re-examine other synovial sarcomas, reconfirmed by chromosomal analysis, by RT-PCR study. Synovial sarcoma was regarded as a wrong diagnosis, so-called "misnomer", because it was not always related to synovium. Synovial sarcoma should be categorized into the group of SYX-SSX-related sarcomas. So we must genetically re-examine spindle cell sarcomas, which had been diagnosed as ibromatosis, fibrosarcoma, spindle cell habdomyosarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor (MPNST), malignant mesenchymoma, malignant fibrous histiocytoma, mesothelioma, solitary fibrous tumor, gastrointestinal stromal tumor, and so on.

This article provided one case of synovial sarcoma of thigh with SYT-SSX2 fusion gene, verified by RT-PCR analysis using archival formalin-fixed paraffin-embedded tissues. This leads us that this RT-PCR approach is easible and reliable to reconfirm the monophasic type synovial sarcoma or so-called poorly differentiated type synovial sarcoma, which should be distinguished from other spindle cell sarcomas.

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原　著

滑膜肉腫のRT－PCR診断に関する検討

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二相性滑膜肉腫の病理組織学的診断は容易であるが、紡錘形腫瘍細胞より構成される単相性未分化型滑膜肉腫の診断は困難である。ここに、新しい補助診断手技が求められる理由である。滑膜肉腫症例においては、第１８番染色体とＸ染色体転座における組みかえt(X；18)(p11.2；q11.2)が確認され、その結果としてキメラ遺伝子SYT-SSXが形成される。このSYT-SSXキメラ遺伝子の発現をパラフィン切片より抽出されたribonucleic acid（RNA）によるReverse Transcription-Polymerase Chain Reaction（RT-PCR）で同定できれば、診断困難な単相性滑膜肉腫の客観的診断根拠となりうると判断される。しかしながら、ホルマリン固定・パラフィン包埋切片よりのRNA鎖の回収においては、標本作製の途上における染色体障害により、その遺伝子長は約100（100～300）bpsに制限される。

今回、われわれは、染色体転座の確認された大腿部の単相性（未分化型）滑膜肉腫のホルマリン固定・パラフィン包埋切片より抽出されたRNAを材料として、既報告のPCR primer（SYT-SSX, SYT-SSX1, SYT-SSX2）によるRT-PCR検査により、この症例のSYT-SSX遺伝子の検索を行い、同時に、通常業務病理組織材料へのRT-PCR応用の条件設定を検討した。

本症例を検討した結果、最適な条件は、１．検体はproteinase K使用による全量回収RNA溶液を使用し、２．逆転写反応（Reverse transcription, RT）はdownstream primerを使用して42℃とし、３．PCR（Polymerase Chain Reaction）は55℃、50サイクルで、４．12.5%ポリアクリアミドゲル電気泳動、５．エチジウムブロマイド後の紫外線下での観察であった。また、その結果、本滑膜肉腫症例のSYT-SSX geneはSYT-SSX2であることが判明した。

キーワード：滑膜肉腫、SYT-SSX、RT-PCR、ホルマリン固定パラフィン包埋組織

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