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A Multiplex Real-Time PCR Assay for the Detection of Gene Fusions Observed in Solid Tumors

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SUMMARY: Specific gene fusions observed in solid tumors are extremely useful diagnostic markers. We report the development of a method based on real-time PCR which enables the detection upon identical PCR conditions of the different fusions specifically observed in Ewing tumors (ET), alveolar rhabdomyosarcoma (ARMS), synovial sarcoma (SS), small round cell desmoplastic tumors (SRCDT), extraskeletal myxoid chondrosarcoma, malignant melanoma of soft parts, congenital fibrosarcoma, and anaplastic large cell lymphoma. A simple assay, based on multiplexing of primers and probes, is described for the routine genetic diagnosis of small round cell tumors of children. It enables the detection of the five *EWS-ETS*, the two *PAX-FKHR*, the three *SYT-SSX*, and the *EWS-WT1* fusions of ET, ARMS, SS, and SRCDT, respectively. The sensitivity of this test is high enough to detect all fusions, including the large *EWS-FLI-1* transcripts, with the equivalent of 100 tumor cells as a starting material. This multiplex fluorescent analysis of chromosome translocations (MFACT) was validated in comparison with conventional RT-PCR on a series of 79 tumors. A major advantage of this method is that it completely abolishes the manipulation of PCR-products. It, therefore, considerably lowers the risk of cross-contamination linked to carry-over of RT-PCR products. It also constitutes an important step toward the complete automation of the detection of cancer-specific gene fusions. (*Lab Invest 2001, 81:905–912*).

Some solid tumors are characterized by specific translocations that result in gene fusions. These genetic lesions, which are at the basis of the tumorigenic process, now constitute very powerful diagnostic criteria (Barr, 1998; Bennicelli and Barr,1999; Ladanyi and Bridge, 2000). Most of these gene fusions are listed in Table 1. These tumor markers are particularly useful for the precise diagnosis of sarcomas and small round cell tumors of children and young adults, which can harbor atypical clinical or pathological presentations. Different techniques, including conventional cytogenetics, Southern blotting, fluorescent in situ hybridization, reverse transcription-polymerase chain reaction (RT-PCR), or, more rarely, immunohistochemistry, have been developed to identify these lesions, with RT-PCR being the most widely used approach. Indeed, RT-PCR is a simple, specific, and sensitive technique for analyzing small tumor fragments. However, as with all PCR-based approaches, it is particularly sensitive to the risk of cross-contamination linked to the carry-over of PCR products.

We have developed real-time PCR detections of the different fusions listed in Table 1. Moreover, taking advantage of multiplexing primers and probes, we set up a test that detects the most frequent fusions observed in sarcomas and small round cell tumors of children that raise difficult diagnostic challenges.

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Results

Detection of Single Gene Fusions Using Real-Time PCR

For each gene fusion listed in Table 1, internal probes and primers were designed using Primer Express software (Applied Biosystems, Foster City, California). Our aims were (a) to detect every type of gene fusion associated with a given malignancy, (b) to reach a high sensitivity of detection, and (c) to standardize PCR conditions to facilitate routine analysis.

We first focused on the detection of the various *EWS-ETS* fusions observed in Ewing tumor (Table 1). Concerning *EWS-FLI-1*, the diversity of the position of the breakpoints with respect to the exons of *EWS* and *FLI-1* leads to an important variability of the types of fusion transcripts observed in tumors (Zucman et al, 1993b). The most proximal breakpoint observed within the *EWS* gene lies at codon 205 (Peter et al, 1996). Therefore, we used the primer EWS 3 tqm and the probe EWS S2 tqm, which correspond to sequences of *EWS* proximal to this codon. For *FLI-1*, fusions always contain the exon 9, which encodes the DNA binding domain. Primer FLI 3 tqm, localized within this exon, was therefore used. This set of primers and the probe would be sufficient to amplify all types of *EWS-FLI-1* fusion transcripts of Ewing tumors. Initial experiments were performed using RNAs from the POE cell line, which expresses the most frequent type 1 *EWS-FLI-1* fusion joining *EWS* exon 7 to *FLI-1* exon 6. Optimal conditions for amplifying the 574 bp fragment were as follow: 3 mM $MgCl_2$ and 50 cycles of PCR consisting of denaturation 95° C for 15 seconds, annealing at 66° C for 1 minute, and elongation at 72° C for 1 minute 30 seconds. We then determined

that these primers, the probe, and the PCR conditions enabled the detection of the presently reported *EWS-FLI-1* fusions. Similarly, for the detection of other *EWS-ETS* fusions observed in Ewing tumors, the same EWS 3 tqm primer was used together with 3' oligos corresponding to either *ERG*, *ETV1*, *E1AF*, or *FEV* genes. We verified that each individual fusion, except *EWS-E1AF*, for which no tumor material was available, could be reliably detected with these primers and that a mix of the five 3' primers could be used for a multiplex analysis of these fusions (Fig. 1, Multiplex PCR II). The sensitivity of this multiplex detection will be described below.

For other gene fusions listed in Table 1, primers and Taqman probes were designed to be compatible with PCR conditions determined for *EWS-ETS* fusions of Ewing tumor apart from the $MgCl_2$ concentration, which was adapted for an optimal detection of each fusion (Tables 1, 2, and 3). Primer pairs and probes were tested on tumor RNA previously demonstrated to exhibit the fusion of interest, except for *SYT-SSX4*, *TFG-ALK*, and *ATIC-ALK*, for which no control RNAs were available. Concerning synovial sarcoma (SS), the SSXc.3 tqm primer matches perfectly with the *SSX1* sequence and exhibits the same mismatch with *SSX2* and *SSX4* sequences at position 4. We checked that the presence of this mismatch did not impair the detection of an *SYT-SSX2* fusion. Although it was not tested, we anticipate that an efficient detection of *SYT-SSX4* fusion would also be achieved with these conditions. For ARMS, multiplex analysis with primers Pax3.1 tqm, Pax7.1 tqm, and FKHR1.2 tqm was shown to be as efficient as single PCR in detecting *PAX3-FKHR* and *PAX7-FKHR*. Similarly, multiplex analyses of the fusions of extraskeletal myxoid chondrosarcoma, SS, and anaplastic lymphoma were validated.

Multiplex Fluorescent Analysis of Chromosome Translocations

To set up a diagnostic assay that could detect the most frequent gene fusions observed in small round cell tumors and sarcomas, we took advantage of the use of both multiplex analyses and different dyes for Taqman probes. The following assay consisting of three parallel PCRs was designed: the first PCR consisted of a control amplification of the ubiquitously expressed *EWS* gene and therefore evaluates the quality of the RNA; the second PCR is a multiplex analysis of the different Ewing-specific fusions as described above; the third PCR is a mix of primers and probes for detecting alveolar rhabdomyosarcoma (ARMS), SS, and small round cell desmoplastic tumors (SRCDT). For this last PCR, a specific labeling was used for each probe (Table 1).

The sensitivity of the multiplex detection of *EWS-ETS* fusions of Ewing tumors was determined using serial dilutions of control RNAs. As expected, a linear variation of the Ct (number of the cycle at the threshold) depending on the log of the amount of RNA was observed. The results observed for the control *EWS* amplification and three different *EWS-FLI-1* fusions are shown in Figure 2. For the *EWS* transcript, a Ct lower than 40 was consistently observed for 10 pg of RNA (the estimated amount of RNA of a single cell). The sensitivities of detection for *EWS-FLI-1* type 1 and type 2 (junction between *EWS* exon 7 and *FLI-1* exon 5) were similar and slightly lower than that of the EWS control (Fig. 2). For the larger 892 bp fragment corresponding to a junction between *EWS* exon 10 and *FLI-1* exon 5, a consistent detection of the PCR product with a Ct lower than 40 was observed for 1 ng of RNA. For amounts of RNA lower than 100 pg, this fusion could not be detected (Fig. 2). This result indicated that, as expected, the efficiency of the PCR decreases with the size of the amplification product.

For the multiplex III (*EWS-WT1*, *SYT-SSX1* and *2*, *PAX3*, or *7-FKHR)* and for all other fusions listed in Table 1, the sensitivities of detection were similar to that of the *EWS* control, ie, the fusions were always detected for 10 pg of RNA with a Ct lower than 40. Therefore, the detection of large *EWS-FLI-1* transcripts represents the limit of application of this technique: at least 1 ng of RNAs (around 100 cells) should be used to avoid false-negative results for *EWS-FLI-1* fusions. This amount corresponds to a Ct for the *EWS* control lower than 30 (Fig. 2).

Validation of This Assay on a Series of 79 Tumors

Seventy-nine tumors, referred to the laboratory for the study of gene fusions, were analyzed in parallel by multiplex fluorescent analysis of chromosome translocations (MFACT) and conventional PCR. Results are shown in Table 4. Six cases were assumed noninterpretable (NI) by conventional RT-PCR because the *EWS* control fragment could not be observed by ethidium bromide staining of agarose gel (Delattre et al, 1994). These six cases demonstrated a Ct for *EWS* higher than 30 by MFACT. One additional case exhibited a Ct of 30.7 by MFACT and was thus considered as NI by this approach. Thirty-nine cases were positive for *EWS-ETS* fusions by MFACT, compared with 38 by conventional RT-PCR. The discordant case involved an Ewing tumor confirmed by pathological examination; however, the fragment of tumor received by the laboratory contained only a few tumor cells. Accordingly, the Ct for *EWS-ETS* fusion was 42, whereas that of the *EWS* control was 28. Nine tumors were positive for *PAX 3* or *7-FKHR* by MFACT, compared with 8 by conventional RT-PCR. Interestingly, the discordant case here was a bone marrow aspirate containing a small number of tumor cells from a pathologically confirmed ARMS. Three cases were positive by both approaches for SS and SRCDT. Altogether, 21 cases were negative by conventional RT-PCR and 18 by MFACT. These results indicate that both approaches yield highly consistent results. However, MFACT appears slightly more efficient because two fusions ignored by conventional RT-PCR could be detected by MFACT. Both cases involved samples containing a small number of tumor cells.

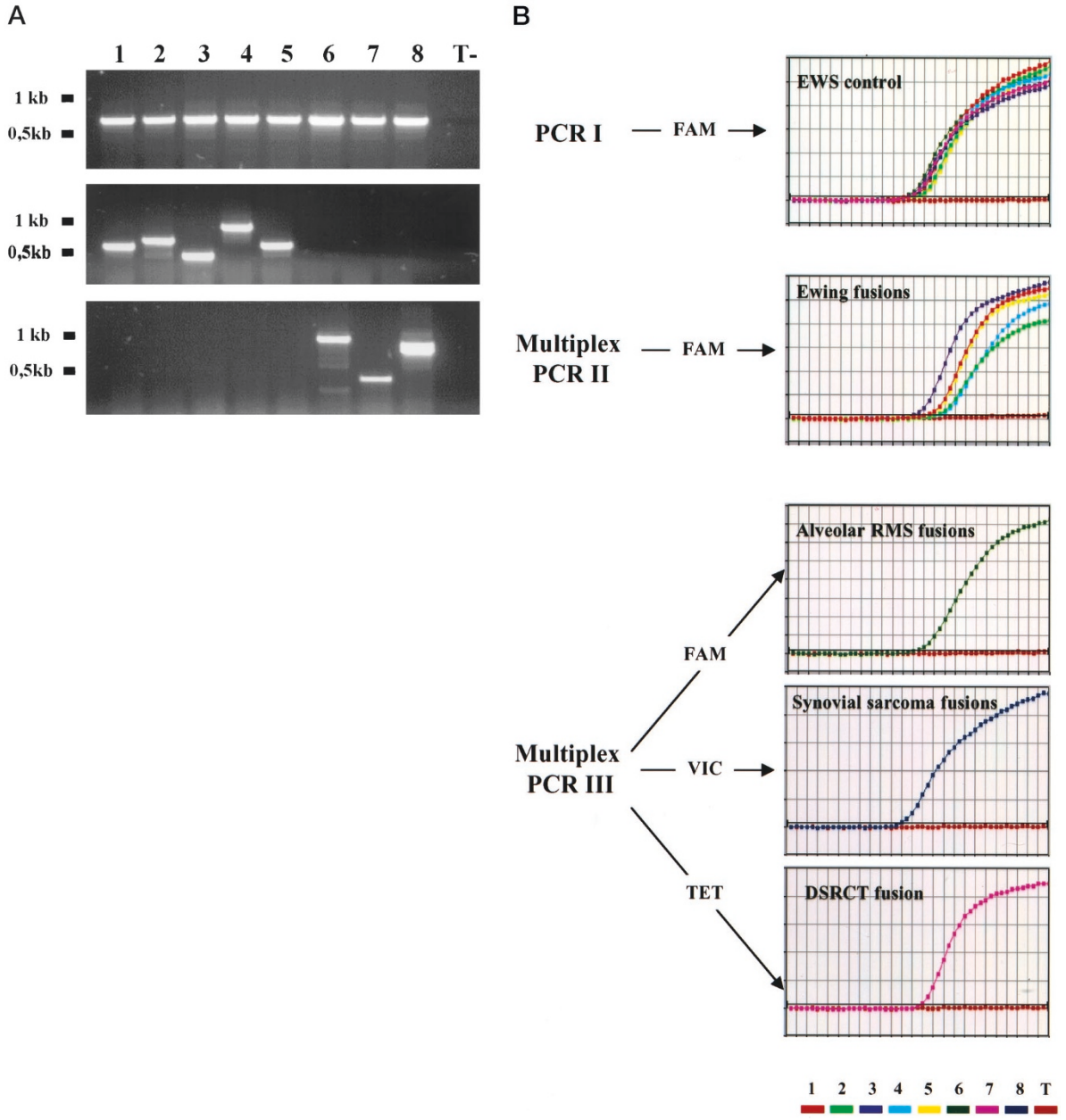

Figure 1.

Detection of specific gene fusions using multiplex fluorescent analysis of chromosome translocations (MFACT). Tumors harboring eight different fusions (1, *EWS*[ex7]-*ERG*[ex6]; 2, *EWS*[ex7]-*ETV1*[ex9]; 3, *EWS*[ex7]-*FLI-1*[ex8]; 4, *EWS*[ex10]-*FLI-1*[ex5]; 5, *EWS*[ex7]-*FLI-1*[ex6]; 6, *PAX3-FKHR*; 7, *EWS-WT1*; 8, *SYT-SSX1*]) were tested with both a conventional reverse transcription polymerase chain reaction (RT-PCR) approach (A) and a multiplex fluorescent analysis of chromosome translocations (MFACT) (B). A, The eight aforementioned tumors were analyzed by RT-PCR with specific primers. The top panel shows the *EWS* control amplification. The middle panel corresponds to the detection of the specific *EWS-ETS* fusions. The bottom panel shows the detection of specific *PAX3-FKHR*, *EWS-WT1*, and *SYT-SSX*. B, The RNAs used for the experiment shown in A were used for MFACT. PCR I is the control amplification with *EWS*-specific primers and an *EWS*-specific probe labeled with FAM. PCR II is aimed at the detection of Ewing-specific fusions with the same *EWS* probe. PCR III is a multiplex PCR used to detect the three indicated fusions with specific probes labeled with three different dyes.

Discussion

We describe primers, probes, and PCR conditions that enable one to detect efficiently most gene fusions observed in solid tumors. The standardization of the reverse transcriptase and PCR cycling conditions enable one to search for these different fusions on the same plate during a single round of PCR. Furthermore, the use of different dyes for the labeling of probes permit multiplex PCR and detection. We propose an assay, termed MFACT, that detects the most frequent fusions observed in sarcomas and small round cell tumors, including those of Ewing tumor, ARMS, SS, and SRCDT. As an internal control, the amplification of the ubiquitously expressed *EWS* gene is used. This control appears particularly appropriate because its level of expression is similar to those of the tested gene fusions.

Table 1. Primers and Probes Used for the Detection of Gene Fusions with Real-Time PCR

Malignancy	Translocation	Genes	References[a]	5′ Primers	3′ Primers	Probes (dye)[b]	$MgCl_2$[c]
Ewing tumor	t(11;22)(q24;q12)	EWS-FLI	1	EWS 3 tqm	FLI.3 tqm	EWS S2 tqm (Fam)	3mM
	t(21;22)(q22;q12)	EWS-ERG	2		ERG 3 tqm		
	t(7;22)(p22;q12)	EWS-ETV1	3		ETV1.1 tqm		
	t(17;22)(q12;q12)	EWS-E1AF	4		E1AF.1 tqm		
	t(2;22)(q33;q12)	EWS-FEV	5		FEV 2 tqm		
ARMS	t(2;13)(q35;q14)	PAX3-FKHR	6, 7	Pax3.1 tqm	FKHR1.2 tqm	RMS S1 tqm (Fam)	4mM
	t(1;13)(P36;q14)	PAX7-FKHR	8	Pax7.1 tqm			
SS	t(X;18)(p11.2;q11.2)	SYT-SSX1	9	SYT.2 tqm	SSXc.3tqm	SYNO S1 tqm (Vic)	6mM
	t(X;18)(p11.2;q11.2)	SYT-SSX2	10, 11				
	t(X;18)(p11.2;q11.2)	SYT-SSX4	12				
SRCDT	t(11;22)(p13;q12)	EWS-WT1	13	EWS 12 tqm	WT1.3 tqm	WT1 S1 tqm (Tet)	6mM
Extrakeletal myxoide chondrosarcoma	t(9;22)(q22;q12)	EWS-TEC	14, 15	EWS.15 tqm	TEC.3 tqm	TEC S1 tqm (Fam)	6mM
	t(9;17)(q22;q11.2)	TAF68-TEC	16, 17, 18	TAF68.1 tqm			
MMSP	t(12;22)(q13;q12)	EWS-ATF1	19	EWS 3 tqm	ATF1.1 tqm	EWS S2 tqm (Fam)	3mM
Congenital fibrosarcoma	t(12;15)(p13;q25)	ETV6-NTRK3	20	ETV6.1 tqm	NTRK3.1 tqm	FCI S1 tqm (Fam)	6mM
Anaplastic large cell lymphoma	t(2;5)(p23;q35)	NPM-ALK	21	NPM.1 tqm	ALK.1 tqm	ALK S1 tqm (Vic)	7mM
	t(1;2)(q25;p23)	TPM3-ALK	23	TM30.1 tqm			
	t(2;3)(p23;q21)	TFG-ALK	22, 24	TFG.1 tqm			
	inv(2)(p23q35)	ATIC-ALK	25	ATIC.1 tqm			

ARMS, alveolar rhabdomyosarcoma; SRCDT, small round cell desmoplastic tumor; MMSP, malignant melanoma of soft parts; SS, synovial sarcoma.

[a] References cited in Table 1: 1, Delattre et al, 1992; 2, Sorensen et al, 1994; 3, Jeon et al, 1995; 4, Kaneko et al, 1996; 5, Peter et al, 1997; 6, Galili et al, 1993; 7, Shapiro et al, 1993; 8, Davis et al, 1994; 9, Clark et al, 1994; 10, Crew et al, 1995; 11, De Leeuw, 1995; 12, Skytting et al, 1999; 13, Ladanyi and Gerald, 1994; 14, Labelle et al, 1995; 15, Clark et al, 1996; 16, Sjogren et al, 1999; 17, Panagopoulos et al, 1999; 18, Attwooll et al, 1999; 19, Zucman et al, 1993a; 20, Knezevich et al, 1998; 21, Morris et al, 1994; 22, Rosenwald et al, 1999; 23, Lamant, 1999; 24, Hernandez et al, 1999; 25, Trinei et al, 2000.

[b] Fam, 6 carboxy fluorescein; Tet, tetrachloro 6 carboxyfluoresceine; Vic, structure not reported.

[b] Optimal concentration.

Table 2. Sequences of Primers Used in This Study

Primer	Sequence
EWS 6f tqm	CTCAGCCTGCTTATCCAGCC
EWS 7r tqm	GCTATATTGACTTGGAGCTTGGC
EWS 3 tqm	GTCAACCTCAATCTAGCACAGGG
FLI 3 tqm	CTGTCGGAGAGCAGCTCCAG
ERG 3 tqm	CTGTCCGACAGGAGCTCCAG
FEV 2 tqm	GAAACTGCCACAGCTGGATC
ETV1.1 tqm	TAAATTCCATGCCTCGACCAG
E1AF.1 tqm	AACTCCATTCCCCGGCC
Pax3.1 tqm	TCCAACCCCATGAACCCC
Pax7.1 tqm	CAACCACATGAACCCGGTC
FKHR1.2 tqm	GCCATTTGGAAAACTGTGATCC
EWS 12 tqm	AGCCAACAGAGCAGCAGCTAC
WT1.3 tqm	TGAGTCCTGGTGTGGGTCTTC
SYT.2 tqm	TACCCAGGGCAGCAAGGTT
SSXc.3 tqm	ATCGTTTTGTGGGCCAGATG
ETV6.1 tqm	CCCATCAACCTCTCTCATCGG
NTRK3.1 tqm	GGCTCCCTCACCCAGTTCTC
ALK.1 tqm	AGGTCACTGATGGAGGAGGTCTT
NPM.1 tqm	CTTGGGGGCTTTGAAATAACAC
TM30.1 tqm	CCGTGCTGAGTTTGCTGAGAG
TFG.1 tqm	AGAACCAGGACCTTCCACCAATA
ATIC.1 tqm	AGGCATTCACTCATACGGCAC
EWS.15 tqm	CCCACTAGTTACCCACCCCAAA
TAF68.1 tqm	AGCAAAACATGGAATCATCAGGA
TEC.3 tqm	TACACGCAGGAAGGCTTGAGTT
ATF1.1 tqm	TGTAAGGCTCCATTTGGGGC

This highly specific method considerably simplifies and reduces the bench work because all the post-PCR steps are suppressed. Consequently, no PCR products are manipulated, which considerably reduces the risk of cross-contamination. In addition, apart from the isolation of RNA, all other steps can follow a fully automated process.

We show that, except for large fusion transcripts of Ewing, the PCR conditions enable the systematic detection of all fusions when 10 pg of tumor RNA, the equivalent of one cell, are used. Taking into account the lower sensitivity for the detection of Ewing transcripts, we propose that a Ct for the *EWS* control less than 30 should be observed for a fully reliable interpretation of results.

Since the set up of this approach in the lab, 799 tumors have been analyzed by MFACT. One hundred one cases (13%) were considered noninterpretable, given a Ct for *EWS* greater than 30. The usual causes were the very small size of the tumor fragments, the necrosis of the fragment, or the poor condition of the sampling. Three hundred fourteen tumors presented specific transcripts as follows: *EWS-ETS* in 215, *SYT-SSX* in 43, *PAX-FKHR* in 35, and *EWS-WT1* in 21 cases. A subset of the 799 tumors was analyzed for specific fusions not included in the MFACT assay and revealed 4 *EWS-ATF1*, 2 *EWS/TAFII68-TEC*, 5 anaplastic lymphoma fusions, and 2 *ETV6-NTRK3* fusions. Finally, 371 tumors were negative for the tested fusions. Interestingly, this analysis shows that no tumor exhibits two different fusions, which confirms the association of these fusions with specific tumor types and which strongly suggests the absence of false-positive cases, thus reinforcing the reliability of the test.

In SS (Inagaki et al, 2000; Kawai et al, 1998; Nilsson et al, 1999), ARMS (Kelly et al, 1997), and Ewing tumor (de Alava et al, 1998; Zoubek et al, 1994), the types of fusion transcripts have been associated with prognostic information suggesting that a precise typing of fusion transcripts might be of clinical interest. By itself, the presently described method does not enable a precise typing of these transcripts. Currently, to type fusion transcripts, we perform a standard PCR with gel electrophoresis and Southern hybridization with specific probes. The primers used for typing are internal to those used for the MFACT. Therefore, the typing step cannot lead to a contamination of the diagnostic step. Alternatively, the real-time PCR could be used for a precise typing of fusion transcripts using pairs of primers and probes specific for certain types of fusion.

In conclusion, the real-time PCR constitutes a simple and efficient method for the detection of the gene fusions observed in human solid tumors. Although this was not tested in the present study, the sensitivity of this approach should enable its use for the detection of residual and minimal disease.

Materials and Methods

RNA Isolation, Reverse Transcriptase

Tumor samples were snap-frozen in liquid nitrogen. RNA was isolated using the Trizole extraction kit (Gibco BRL, Gaithersburg, Maryland). A total of 1 μg of total RNA was reverse transcribed using random hexamers in a final volume of 20 μl using the Gene-

Table 3. Sequences and Labeling of Probes Used in This Study

Probe	Sequence	Fluorescence	Gene
EWS S2 tqm	CTCCTACCAGCTATTCCTCTACACAGCCGACT	Fam	*EWS*
RMS S1 tqm	ATGCTCAATCCAGAGGGTGGCAAGAG	Fam	*FKHR1*
WT1 S1 tqm	TCTCGTTCAGACCAGCTCAAAAGACACCA	Tet	*WT1*
SYNO S1 tqm	ATCATGCCCAAGAAGCCAGCAGAGG	Vic	*SSX1/2/4*
FCI S1 tqm	CTCCCCGCCTGAAGAGCACGC	Fam	*ETV6*
ALK S1 tqm	CAAGCTCCGCACCTCGACCATCA	Vic	*ALK*
TEC S1 tqm	ACCTTGGCAGCACTGAGATCACGGC	Fam	*TEC*

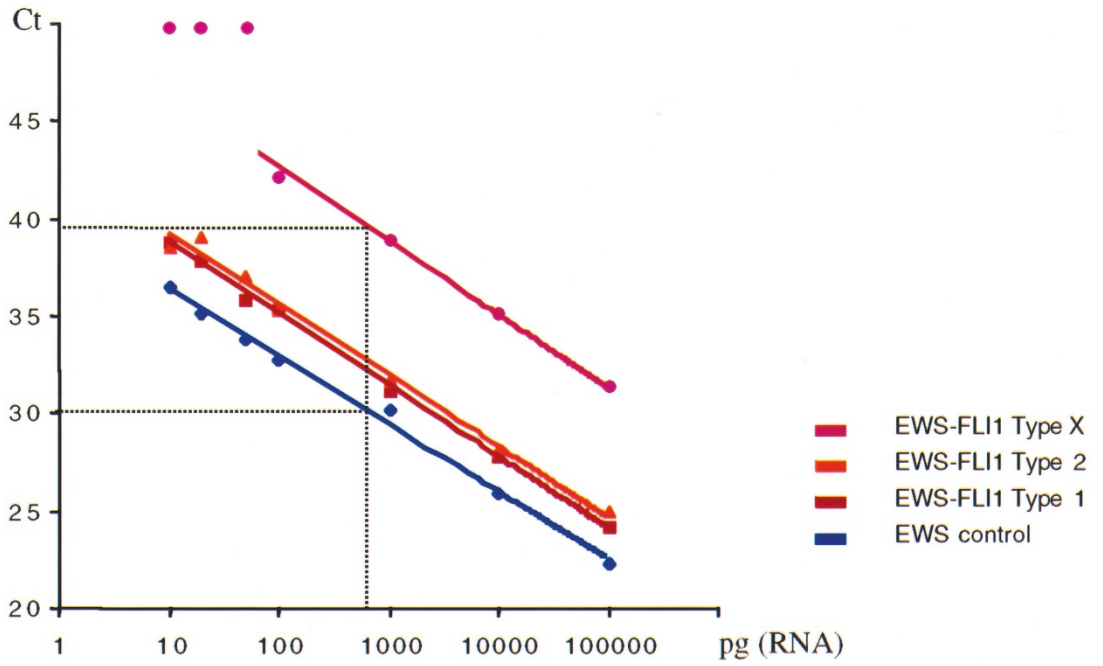

Figure 2.

Sensitivity of the detection of *EWS-FLI-1* fusions using MFACT. The MFACT approach was used to analyze both *EWS* and *EWS-FLI-1* fusion transcripts on serial dilutions of tumor RNA with a type 1, a type 2, or a type X fusion (between *EWS* ex 10 and *FLI-1* ex 5). The amount of RNA (in picograms) is indicated on the x axis and the cycle threshold (Ct) on the y axis. For each fusion, three independent measures of Ct were performed for 100 ng, 10 ng, 1 ng, 100pg, 50pg, 20pg, and 10pg of cellular RNA. Curves are drawn using the mean of these three values. From this diagram it can be concluded that a Ct of 30 for the *EWS* control corresponds to an analysis of less than 1 ng of cellular RNA (around 100 cells). For this Ct, all types of *EWS-FLI-1* transcripts can be detected. If the Ct for *EWS* is higher than 30, large *EWS-FLI-1* transcripts might escape detection. Therefore, a Ct for *EWS* less than 30 is requested to avoid potential false-negative results for large fusion transcripts.

Table 4. Validation of the Fluorescence Detection

Malignancy	Type of fusion transcript	Detection by standard RT-PCR	Detection by MFACT	Range of Ct (fusion)	Mean Ct (fusion)
Ewing tumor	EWS-FLI-1 type 1	19	20	26–42	30
	EWS-FLI-1 type 2	5	5	30–35	32
	EWS-FLI-1 type X	7	7	31–41	36
	EWS-ERG	5	5	28–33	30
	EWS-ETV1	1	1	30	
	EWS-FEV	1	1	39	
ARMS	PAX3-FKHR	7	8	21–30	27
	PAX7-FKHR	1	1	23	
SS	SYT-SSX1	3	3	22–24	23
	SYT-SSX2				
	SYT-SSX4				
SRCDT	EWS-WT1	3	3	25–36	28
	Negative	21	18		
	Noninterpretable	6[a]	7[b]		

RT-PCR, reverse transcription polymerase chain reaction; MFACT, multiplex fluorescent analysis of chromosome translocations; Ct, number of the cycle at the threshold.
[a] Absence of detection of the EWS PCR products by ethidium bromide staining.
[b] The Ct for the control EWS amplification is > 30.

Amp RNA PCR Kit (PE Biosystems, Foster City, California).

Real-Time PCR

Real-time PCR experiments were performed in a final volume of 50 μl containing 2 μl of cDNA, with 200μM each of dATP, dCTP, and dGTP; 400μM of dUTP; 200 nM of each primer; 100 nM of the Taqman probe; 1.5 U AmpliTaq Gold (PE Biosystems); and 0.5 U AmpErase UNG (Uracile N Glycosylase, PE Biosystems). After initial steps of UNG reaction for 2 minutes at 50° C and TaqGold activation for 15 minutes at 95° C, 50 cycles of PCR were performed according to standardized procedures (denaturation at 95° C for 15 seconds, annealing at 66° C for 1 minute, and elongation at

72° C for 1.5 minutes). The primers and probes are described in Tables 2 and 3, respectively. The only variable parameter was the $MgCl_2$ concentration. Optimal concentrations of $MgCl_2$ for the detection of individual fusions are indicated in Table 1. For the MFACT test, the multiplex detection of *PAX-FKHR*, *SYT-SSX*, and *EWS-WT1* was performed at a concentration of 4 mM of $MgCl_2$.

The real-time PCR was carried out using the ABI/PRISM 7700 (PE Applied Biosystems). The fluorescence data were collected during the annealing and extension phases of every cycle.

Conventional PCR Conditions

Standard PCR was performed using the GeneAmp PCR Core Reagents kit N808-0009 (PE Biosystems) in a reaction mixture of 25 μl containing 2 μl of cDNA, 200μM of each dNTP, 500 nM of each primer, 1.5 mM of $MgCl_2$, and 0.7 U TaqDNA polymerase. The sequence of primers used for conventional PCR can be obtained upon request.

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