

A COMPARISON OF RT-PCR AND FISH TECHNIQUES IN MOLECULAR DIAGNOSIS OF EWING'S SARCOMA IN PARAFFIN-EMBEDDED TISSUE

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Summary

Ewing's sarcoma is relatively uncommon tumor representing 6-8 percent of malignant bone tumors with variable morphology. Cytogenetically, Ewing's sarcomas are characterized by a specific reciprocal chromosomal translocation t(11;22)(q24;q12). The presence of this chromosomal translocation has been detected in approximately 85 percent of the cases. The translocation results in the fusion of EWS gene from chromosome 22 to FLI1 gene at 11q24 which is a member of ETS family of transcription factors. In this study we performed a comparison of two molecular diagnostic strategies, namely RT-PCR and FISH, in fresh, frozen and formalin-fixed paraffin-embedded tissues. We conclude that FISH is a more sensitive technique than RT-PCR for the diagnosis of Ewing's tumors in formalin-fixed paraffin-embedded tissue. In conclusion, molecular pathology techniques, using reverse transcription-polymerase chain reaction (RT-PCR) and/or fluorescence in situ hybridization (FISH) are valuable diagnostic tools for evaluation of undifferentiated small round-cell tumors like Ewing's sarcoma.

Key words: Ewing's sarcoma – chromosomal translocation – RT-PCR – FISH

Souhrn

Molekulární diagnostika Ewingova sarkomu: porovnání RT-PCR a FISH metod pro tkáň zalité do parafinu

Ewingův sarkom je relativně vzácný nádor reprezentující 6–8 procent nádorů kostí. Cytogeneticky je Ewingův sarkom v 85 procentech případů charakterizován specifickou reciproční chromozomální translokací t(11;22)(q24;q12), která má za následek fúzi genu EWS na chromozomu 22 a genu FLI1 na chromozomu 11. V této studii jsme se zaměřili na porovnání dvou molekulárně diagnostických metod – reverzně transkripční polymerázové řetězové reakce (RT-PCR) a fluorescenční in situ hybridizace (FISH). Z našich výsledků vyplývá, že v případě formalínem fixované, do parafinu zalité tkáně je patrně vlivem degradace RNA, FISH senzitivnější než RT-PCR. Závěrem: molekulárně patologické metody RT-PCR a FISH jsou účinným diagnostickým nástrojem pro diagnostiku nádorů Ewingova typu.

Klíčová slova: Ewingův sarkom – chromozomální translokace – RT-PCR – FISH

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Ewing's sarcoma is a relatively uncommon tumor representing 6-8 percent of malignant bone tumors. However, it is the second most common sarcoma in bone and soft tissue in children (22). Ewing's sarcoma and primitive neuroectodermal tumors (PNET) are defined as round cell sarcomas that show varying degrees of neuroectodermal differentiation (14).

In the Ewing's sarcoma, in contrast to PNET, features of neuroectodermal differentiation are lacking as assessed by light microscopy and immunohistochemistry. The primitive round to oval Ewing's sarcoma cells contain in their cytoplasm glycogen aggregates and produce fine cytoplasmic processes with primitive intercellular function (13). No specific immunohistochemical marker of this tumor exists till now.

Cytogenetically, Ewing's sarcomas are characterized by a specific reciprocal chromosomal translocation t(11;22)(q24;q12). The presence of this chromosomal translocation has been detected in approximately 85 percent of the cases (15, 17). Subsequent cloning of the translocation breakpoint showed (24), that chromosomal translocation t(11;22)(q24;q12) results in the fusion of Ewing sarcoma breakpoint region 1 gene (EWS) from chromosome 22 to Friend leukemia virus integration 1 gene (FLI1) at 11q24 which is a member of ETS (v-ets erythroblastosis virus E26 oncogene homolog) family of transcription factors (4, 18). Moreover, another chromosomal translocation t(11;22)(q22;q12)

was found in 10-15 percent of cases, which results in the expression of EWS-ERG fusion transcript. In 1 % or less cases t(7;22), t(17;22), and t(2;22) translocations and inv(22) have been described (4, 9, 12, 19, 21). The mentioned secondary chromosomal aberration resulted in fusion between EWS gene and one of the ETS superfamily: Ets variant gene 1 (ETV1), Ets variant gene 4 (E1AF), fifth Ewing variant gene (FEV), and zinc finger sarcoma gene (ZSG), respectively.

Little is known about the function of the genes involved in this translocation. EWS gene encodes an ubiquitously expressed RNA binding protein of an unknown function. EWS was found to be uniformly expressed in two splicing variants of similar abundance, EWS a and EWS b, which differ in a single amino acid (11). The EWS protein, primarily localized in the nucleus, has been found to associate with components of the basal transcriptional machinery (2,16, 23) and RNA splicing factors (10, 23), as well as with partition into the ribosome-dense fraction of the cytoplasm, in particular, upon G protein coupled receptor signaling (5).

All ETS members are defined by the 87 amino acid domain that is both necessary and sufficient for the site-specific DNA-binding in vitro (6). ETS factors are thought to act by binding to promoter and/or enhancer elements of the target genes and result in the transcriptional activation or repression.

In this study we performed a comparison of two molecular

diagnostic strategies, RT-PCR and FISH, in fresh, frozen and formalin-fixed paraffin-embedded tissues.

MATERIAL AND METHODS

Patient and tumor samples

This study included 5 patients surgically treated for soft tissue sarcoma at the General Faculty Hospital and Faculty Hospital Bulovka between 1975 and 2005. Immediately after the surgical removal, tumor specimens were frozen in liquid nitrogen and used directly for molecular analysis or they were formalin-fixed, paraffin-embedded for later use. Diagnosis was based on the standard histopathological criteria according to WHO classification (22) with additional immunohistochemistry (anti-CD99, 1:10, Dako).

Deparaffinization of tissue section

Block of formalin-fixed, paraffin-embedded tissue was cut using a microtome, and 9 paraffin slides (20 µm thick) were placed directly into a sterile microfuge tube. Then 0.5 ml of xylen was added, after which the specimens were mixed for 5 minutes and centrifuged for 5 minutes in a microfuge tube. The xylen was removed and added again to remove residual paraffin. After centrifugation, the tissue was twice washed in 0.6 ml of 100% ethanol and centrifuged again. The tissue was dried by heating at 45°C for 3 minutes.

RNA isolation, cDNA preparation and RT-PCR analysis

Isolation of total RNA, synthesis of cDNA and RT-PCR analysis were performed by standard procedures described in our previous work (20).

Briefly: total RNA was extracted using an RNeasy Mini Kit (Qiagen), reverse transcription was performed by a RevertAid – First Strand cDNA Synthesis Kit (Fermentas), which employs random hexamer primer and Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase.

The PCR conditions were: initial denaturation at 95°C for 3 minutes, followed by 45 cycles with the program of denaturation at 95°C for 1 minute, annealing at 58-60°C (see Table I.) for 1 minute, and extension at 72 °C for 1 minute. The reaction was accomplished with a final extension at 72°C for 10 minutes.

The complete list of primer sets including their sequences, annealing temperatures, and PCR product sizes are presented in Table I.

Fluorescence in-situ hybridization

Five µm thick sections from paraffin-embedded tissue were processed for FISH using the LSI EWSR1 (22q12) Dual Color, Break Apart Rearrangement Probe from Abbott Vysis (Downers Grove, IL, USA). The assay procedure was carried out according to the manufacturer's recommendations. In brief, the slides were at first deparaffinized in xylene, then pretreated in 0.2N HCl and subsequently in NaSCN solution at 80 °C; the next step was proteolytic treatment. The protease digestion plays a crucial role in terms of obtaining readable and conclusive FISH results. We used Protease II from Abbott

Vysis, 25mg in 50 ml saline solution pH 2, digestion time 60 minutes, since we had therewith supreme experiences. Afterwards the sections were fixed in buffered formalin. Then we denatured the specimen DNA in formamide at 73 °C, applied FISH probe, sealed with liquid rubber cement and let hybridize in a humid chamber overnight. After hybridization, the unbound probe was removed in 0.4xSSC/0.3%NP-40 wash solution at 74 °C, the slides were dehydrated and counterstained with 4,6-diamidino-2-phenylindol (DAPI).

For each sample, a minimum of 200 non-overlapping cells were evaluated on OLYMPUS AX70 True Research Microscope for presence of fused or split signals. A positive result was defined as > 20 % of cells having split signals.

RESULTS

RT-PCR amplification of EWS-FLI in frozen tissue

We chose frozen tumor tissue as positive control of Ewing's sarcoma, whereas synovial sarcoma was selected as a negative control. Specific oligonucleotide primers that span introns were chosen for the amplification of EWS-FLI fusion transcript (see Table I). In each case, the phosphoglycerate kinase (PGK) was used as an internal control for RT-PCR. Negative controls included reactions lacking RNA and reactions lacking reverse transcriptase (not shown).

In order to determine correct quality of PCR amplification, we briefly analysed the presence of phosphoglycerate kinase in each sample. We found the presence of the 247 base pairs PCR product corresponding to spliced mRNA of PGK in both samples of Ewing's sarcoma as well as in synovial sarcoma (Figure 1 a, c).

Then we analysed the presence of EWS-FLI fusion transcript in the tumors. We found that all Ewing's sarcoma tested were EWS-FLI positive (Figure 1d). In contrast, synovial sarcoma was EWS-FLI negative (Figure 1 b).

Faintish nonspecific PCR products were seen in case the EWS and FLI primers were used. This product was absent (not shown) in case the samples were treated with DNase I prior to RT-PCR, suggesting contamination by genomic DNA.

Detection of EWS-FLI fusion transcripts in paraffin-embedded tissue

We studied 5 paraffin blocks of Ewing's sarcoma. These blocks were between 1 and 31 years old. We can easily detect EWS-FLI fusion transcript when paraffin-embedded tissues were not older than 5 years (Figure 1 f). In contrast, we have less success detecting fusion transcripts when older materials were studied (not shown), suggesting that RNAs have been degraded in aged paraffin-embedded tissues. Due to this reason, we decided to perform the analysis on the DNA level.

Detection of EWS rearrangements by using FISH

FISH analysis was performed in 5 patients along with specimens from a PCR-positive Ewing's sarcoma and synovial sarcoma, which constituted appropriate positive and negative control respectively. This probe consists of a mixture of two FISH DNA probes: the first labeled in SpectrumOrange, flanking

Table I. List of primers used for RT-PCR analysis

Each primer was chosen to span introns. Specific annealing temperature (Ta) of each primer and the size of expected PCR products are listed below.

Gene	Primer	Sequence (5' to 3')	Ta(°C)	PCR product (in base pairs)
EWS-FLI	sense	TCC TAC AGC CAA GCT CCA AGT C	58	327
EWS-FLI	antisense	ACT CCC CGT TGG TCC CCT CC	58	327
PGK	sense	CAG TTT GGA GCT CCT GGA AG	60	247
PGK	antisense	TGC AAA TCC AGG GTG CAG TG	60	247

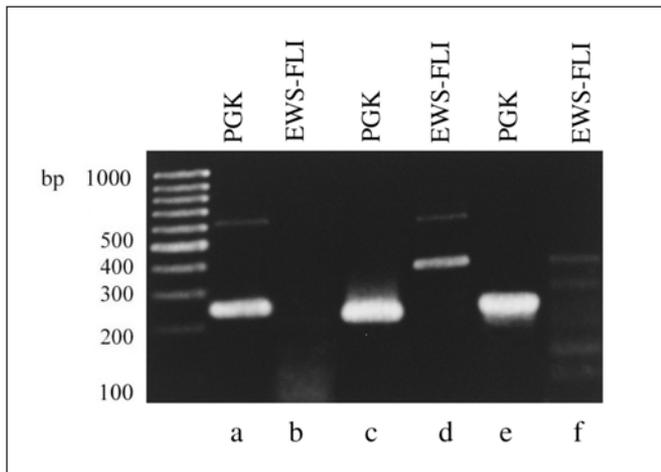


Figure 1. Analysis of the presence of EWS-FLI fusion transcripts. Total RNA was isolated from patients with Ewing's sarcoma (c, d – frozen tissue; e, f – paraffin-embedded tissue). The presence of EWS-FLI fusion transcripts was analysed by RT-PCR. The PCR products were then separated by electrophoresis on an agarose gel and visualized by using ethidium bromide. Phosphoglycerate kinase (PGK) was used as an internal control of PCR reaction. Synovial sarcoma was used as a negative control (a, b).

the 5' side of the EWSR1 gene and extending inward into intron 4, and the second labeled in SpectrumGreen and flanking the 3' side of the EWSR1 gene. The known breakpoints within the EWSR1 gene are restricted to introns 7 through 10. Concerning the results of hybridization, in a cell lacking a t(22q12) in the EWSR1 gene region, two yellow (co-localization of red and green) fusion signal pattern is observed, reflecting the two intact copies of EWSR1. By contrast, in a cell harboring a t(22q12) in the EWSR1 gene region, one green and one orange/red signal pattern is expected.

All tested specimens of the patients with Ewing's sarcoma as well as the positive control displayed 1F1O1G signal pattern in more than 20 % of cells and were therefore evaluated as having the EWSR1 gene rearrangement (Figure 2A). As we anticipated, the specimen from a patient with synovial sarcoma (negative control) showed 2F signal pattern (Figure 2B) in total extent of the tissue section characterizing unmutated EWSR1 region.

DISCUSSION

Many types of sarcomas are characterized by specific chromosomal translocations that result in production of novel chimeric genes. Detection of these fusion genes could be a sensitive molecular diagnostic assay.

Until the first discovery of the t(11;22) chromosomal rearrangement 23 years ago, the unambiguous diagnosis of the family of Ewing's tumors was difficult, especially in cases of unusual locations, recurrences, or poorly differentiated cases (17).

Ewing's sarcoma is characterized by a relatively simple karyotype with only a few numerical and structural aberrations. A reciprocal chromosomal translocation between chromosomes 11 and 22, the t(11;22)(q24;q12), is present in about 85% of these tumors (4) and is therefore considered pathognomic for the disease. This chromosomal translocation results in a juxtaposition of the EWS gene on chromosome 22 with FLI1 gene (friend leukemia virus integration site 1) on chromosome 11. As a consequence chimeric transcripts and proteins are produced that consist of the N-terminus of EWS fused to the C-terminal portion of FLI1 (4). In most of the remaining cases, variant translocations are always observed involving

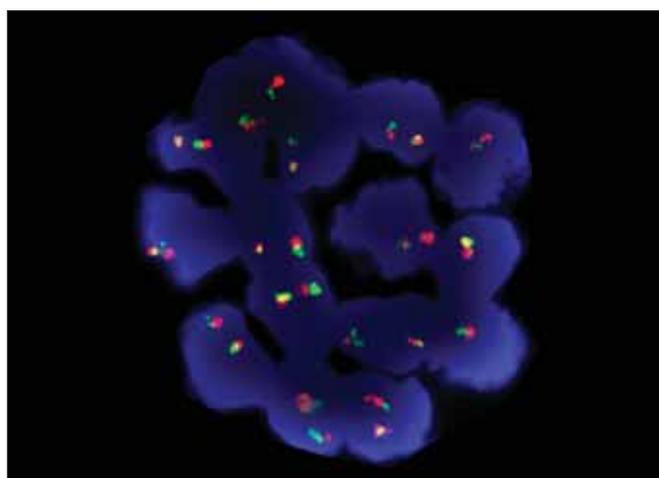
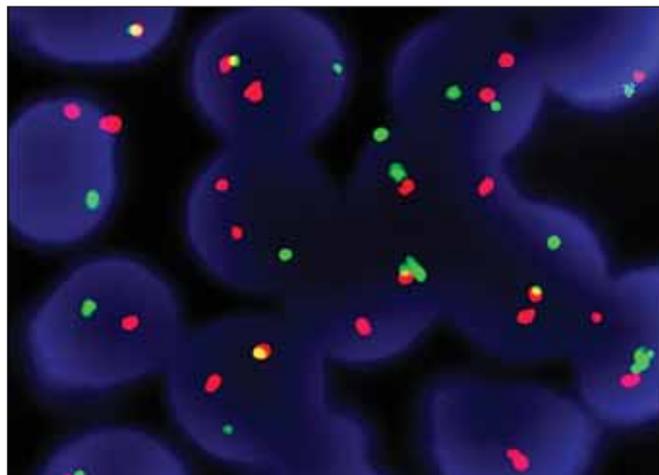


Figure 2. Analysis of EWS rearrangements. Two-color fluorescent in situ hybridization analysis using locus-specific (locus 22q11) EWSR1 dual-color break-apart probe. The hybridization signal was visualized on fluorescence microscope using appropriate filters. Cells with one yellow fusion (co-localization of red and green), one green and one red signal (1F1O1G signal pattern) indicated EWSR1 gene rearrangement (A) while the cells lacking the t(22q12) in the EWSR1 gene region (negative control), two yellow fusion signal (2F signal pattern) is observed reflecting the two intact copies of EWSR1 (B). Magnification x1000.

chromosomes 22q12 and either 21q22 (10% of Ewing's sarcomas) or 7p22, 17q12, and 2q36 (<1% of Ewing's sarcomas each). Variant Ewing's sarcoma family translocations have been also described that join EWS to one of four additional ETS family transcription factors (4, 9, 12, 19, 21). These variant translocations frequently occur as either complex or interstitial chromosomal rearrangements and are therefore difficult to diagnose by conventional cytogenetics. Additional structural changes affect chromosomes 1 and 16 in about 20% of tumors, most frequently leading to a gain of 1q and a loss of 16q and the formation of a derivative chromosome der(1;16) (7).

Several reports have described the RT-PCR detection of the resulting EWS-LFI1 fusion transcript as a valuable diagnostic tool to identify Ewing's sarcoma (1, 3, 8) within other small round-cell tumors. The EWS-FL1 fusion transcript can be identified in up to 95% of Ewing's sarcoma family. In the remaining percentage of these tumors another translocation, namely t(21;22)(q22;q12) is found, resulting in an EWS-ERG fusion transcript which can also be detected by RT-PCR (3).

Our results correlated well with the results of the above mentioned authors, who have reported that RT-PCR is a valuable diagnostic aid for Ewing's sarcoma in frozen tissue. In contrast to other authors (1, 8), our data show that RT-PCR

is less sensitive in formalin-fixed paraffin-embedded tissue. We assumed that the probable cause of this is the fact that RNAs have been degraded in paraffin-embedded tissue, although RT-PCR detects only translocation t(11;22) while FISH detects all breaks of EWS gene including t(11;22) and minor translocations. Due to this reason, we performed the analysis on the DNA level by using FISH with commercial EWS break apart probe set. However, the sensitivity of RT-PCR analysis in paraffin-embedded tissue could be increased in case a smaller-sized amplicon (approximately 100 bp) was used for PCR because of degradation of RNAs in paraffin-embedded tissue (20).

We found that all PCR-negative Ewing's sarcoma cases which we tested gave positive FISH results. We found that the storage time of paraffin block, even for as long as 10 years, did not affect the quality of FISH results. We detected chromosomal aberrations in paraffin-embedded tissues stored as long as 31 years. We conclude that FISH is a more sensitive technique than RT-PCR for the diagnosis of Ewing's sarcoma family in formalin-fixed paraffin-embedded tissue, although RT-PCR analysis provides additional information regarding fusion transcript subtype, which may play a role in prediction of prognosis (7, 22). We assume that diagnosis and prediction of prognosis of Ewing's sarcoma family benefit from molecular testing with both techniques.

In conclusion, molecular pathology techniques, using reverse transcription-polymerase chain reaction (RT-PCR) and/or fluorescence in situ hybridization (FISH) are valuable diagnostic tools for evaluation of the undifferentiated small round-cell tumors. Detection of characteristic translocations by these methods may allow a definitive diagnosis of Ewing's sarcoma, as well as of synovial sarcoma (20).

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Abbreviations

RT-PCR – reverse transcriptase-polymerase chain reaction, PGK – phosphoglycerate kinase, bp – base pairs, FISH – fluorescence in-situ hybridization, EWS – Ewing sarcoma breakpoint region 1 gene, FLI1 – Friend leukemia virus integration 1 gene, ETS – v-ets erythroblastosis virus E26 oncogene homolog, ERG – v-ets erythroblastosis virus E26 oncogene, ETV1 – Ets variant gene 1, E1AF – Ets variant gene 4, FEV – fifth Ewing variant gene, ZSG – zinc finger sarcoma gene, PNET – primitive neuroectodermal tumors

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