Abstract. Background: Ewing family tumours (EFT) are the second most common bone tumours in children and adolescents. In the majority of EFT, EWSR1-FLI1 (Ewing sarcoma breakpoint region 1-Friend leukaemia virus integration 1) fusion proteins can be detected and EWSR1-FLI1 substantially contributes to the malignant phenotype of EFT. Therefore, inactivation of EWSR1-FLI1 is an interesting strategy for EFT therapy. Materials and Methods: A ribozyme with specificity for EWSR1-FLI1 was developed and the activity in vitro was investigated. Synthetic RNAs corresponding to EWSR1-FLI1 were used as substrates. In addition, the total RNA from EFT cells was used as substrate and the rapid amplification of cDNA ends method for the detection of the cleavage products was used. Results: The ribozyme cleaved the synthetic RNA in a sequence specific manner with high efficiency in vitro. Furthermore, the expected cleavage products were detected after digestion of the total cellular RNA with this ribozyme. A point mutation in the catalytic centre of the ribozyme abolished enzymatic activity. Conclusion: The RNA corresponding to EWSR1-FLI1 is accessible for ribozyme mediated inactivation and ribozymes are able to cleave EWSR1-FLI1 specific RNA in the presence of a high background of normal cellular RNAs.

Ewing family tumours (EFT) are highly aggressive neoplasias with a high metastatic potential, a high rate of early relapse and a bad prognosis especially for patients with more advanced disease (1, 2). The histogenetic origin of EFT has not been clarified, but DNA-microarray analyses indicate a close relationship between EFT and neuroectodermal, as well as mesenchymal stem cells (3-7). At the molecular level, EFT are defined by the expression of EWSR1-ETS (Ewing sarcoma breakpoint region 1-avian erythroblastosis virus E26 oncogene homolog) fusion genes which confer transformation and transactivation ability (8). In about 95% of EFT reciprocal translocations of the EWSR1 gene on 22q12 with FLI1 (Friend leukaemia virus integration 1) or ERG (ETS related gene) located on 11q24 or 21q22, respectively, are detectable. EWSR1 is an ubiquitously expressed gene. FLI1 and ERG belong to the ETS proto-oncogene family and are DNA binding transcription factors. They are involved in the regulation of normal development, including embryonic and blood cell development. All ETS genes are transcriptional activators, repressors have not been found. By mapping of chromosomal translocation breakpoints, different EWSR1-FLI1 fusion transcripts have been identified. The most frequent types are EWSR1-FLI1 type 1 with EWSR1 exon 7 fused to FLI1 exon 6 (about 50%) and type 2, characterized by exons 7 to 5 fusion (about 25%). The products of EWSR1-ETS fusion genes are aberrant transcription factors that are capable of transforming fibroblasts in vitro. These topics have been reviewed elsewhere (9).

EWSR1-FLI1 represents a highly attractive target for therapy of EFT because the expression of the fusion protein is restricted to tumour cells and expression of the chimeric transcription factors seems to be obligatory for tumour growth. Therefore it seems unlikely that escape variants of tumour cells with loss of this target are selected during targeted therapy. The induction of tumour specific immune responses against EWSR1-ETS fusion proteins has been suggested as a possible therapeutic strategy (10-13). However, it remains unclear whether high-avidity T cells with specificity for EWSR1-FLI1 can be stimulated and can kill EFT cells in vivo. In addition, multiple immune escape mechanisms for tumour cells have been described.

Some molecular strategies for the inactivation of oncifusion proteins are based on the functional inhibition of these proteins, e.g. by using inhibitors of enzyme
activity (14). A direct method for the inhibition of onecofusion proteins is the inhibition of expression. Thus the inactivation of EWSR1-FLI1 appears to be another promising approach for developing new therapeutic strategies for EFT. It has been shown that inactivation of EWSR1-FLI1 by antisense oligonucleotides inhibits the growth of tumour cells (15-18). In addition, EWSR1-FLI1 has been successfully targeted by RNA interference (RNAi) (4). Because RNAi has been shown to induce several off-target effects (19-21) and immunological side-effects (22, 23), the cleavage of the corresponding RNA by ribozymes that can destroy high numbers of target molecules per ribozyme molecule might be an interesting alternative to RNAi.

Ribozymes are small catalytic RNA molecules that possess sequence-specific RNA cleavage activity. They occur naturally, but can also be artificially created to target sequences in cis or trans. Ribozymes have been successfully used to target and destroy both viral and cellular RNAs in cell culture systems and in animals. Several successful experiments using ribozymes for suppression of gene expression have been reported (24). Hammerhead ribozymes are the simplest and best characterized ribozymes. They are RNA molecules that hydridise to complementary RNA sequences in which the central part of the sequence forms a characteristic secondary structure where reactive groups are located close to each other and mediate specific cleavage of the target RNA (25-27). The development of ribozymes has opened a new field of therapeutic options against various human diseases (28-37), including the possibility of designing ribozymes for the cleavage of tumour specific fusion transcripts.

Antisense oligonucleotides, as well as hammerhead ribozymes, are powerful instruments for specific inhibition of gene expression. In contrast to antisense oligonucleotides, ribozymes have additional enzymatic activity (25). Thus, the overall activity of ribozymes is contributed by the antisense effect and by the enzymatic activity (38). The multiple turnover (binding and cleaving of the substrate, dissociation and binding of a new target molecule) by using the ribozyme technique promises advantages in comparison to the use of antisense nucleotides. In the present study, this approach was used for the inactivation of EWSR1-FLI1 and a hammerhead ribozyme was designed with specificity for the fusion region of the chimeric transcript.

Materials and Methods

Cell culture. EFT cell lines (39) A-673 and RD-ES were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum (Biochrom). The A-673 cell line was initially established from a patient with rhabdomyosarcoma (40), but recently shown to be an Ewing family tumour (41).

Construction and synthesis of EWSR1-FLI1 RNA substrates. Two EWSR1-FLI1 specific substrate RNAs (fusion type 1 and type II) were synthesized by in vitro transcription. Each in vitro transcription template was generated by cloning the specific cDNA with the EWSR1-FLI1 fusion in the middle of the sequence into the vector pGEM-T Easy (Promega, Mannheim, Germany). The vector was linearised with restriction endonuclease EcoRV and a T added to both 3’-ends. So it was possible to clone the polymerase chain reaction (PCR) fragments without any further modification. The EWSR1-FLI1 type 1 and type 2 cDNAs were generated by reverse transcription (RT)-PCR from the total RNA from A-673 (fusion type 1) and RD-ES (fusion type 2) cells using EWSR1-FLI1 specific primers (sense: 5’-CCT ATG GCC ACA CGA GCA G-3’; reverse: 5’-GGA GGA ATT GCC ACA GCT G-3’). cDNA was synthesized from 2 μg total RNA under standard conditions (Superscript II reverse transcriptase; GibcoBRL, Life Technologies, Karlsruhe, Germany) and amplified in a PCR reaction for 35 cycles (94°C, 30 sec; 60°C, 39 sec; 72°C, 60 sec) using a mastercycler personal (Eppendorf, Hamburg, Germany). The vector was transformed into bacteria and the exact size and orientation of the insert was analysed by restriction analysis with restriction endonucleases BamHI and PstI. The fragment including the T7 promoter and the EWSR1-FLI1 cDNA was generated by digestion of the vector with restriction endonuclease SaII, gel purified, phenol/chloroform extracted, ethanol precipitated and washed with 80% ethanol. The dried pellet was reconstituted in RNase free water and analysed by gel electrophoresis and ethidium bromide staining. The EWSR1-FLI1 specific substrate RNAs were then generated and fluorescein labelled by in vitro transcription for 2 h at 37°C. The reaction conditions for the in vitro transcription of the DNA template were: 1x in vitro transcription buffer (Promega), 10 U Rnasin (Promega), 10 mM DTT, fluorescein labelling mix nucleotides (1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP, 0.35 mM fluorescein-12-UTP (Roche, Mannheim, Germany)) and 15 U of T7 polymerase (Promega) in a volume of 20 μl. One μg of DNA template was used and the reaction was started by the addition of T7 polymerase. After in vitro transcription the DNA template was removed by incubation with DNase for 15 min. Then the in vitro transcript was purified by ammonium acetate (NH4OAc)/ethanol precipitation and washed with 80% ethanol. Air dried pellets were reconstituted in RNase-free water, analysed and quantified by gel electrophoresis and staining with ethidium bromide.

Construction and synthesis of anti-EWSR1-FLI1 hammerhead ribozymes (Rz+, Rz–). Based on a thermodynamic analysis of the predicted three-dimensional structure of the EWSR1-FLI1 mRNA the fusion-region was expected to be accessible to ribozyme-mediated cleavage (data not shown) and an EWSR1-FLI1 type 1 specific hammerhead ribozyme was designed accordingly. The anti-EWSR1-FLI1 hammerhead ribozyme (see Figure 1) was obtained by in vitro transcription of a ribozyme template. This template was generated by the extension of overlapping oligonucleotides (a 36-mer with T7-promoter sequence: 5’-TAA TAC GAC TCA CTA TAG CAT AAG CTG AGT AGT CGG-3’; and a 31-mer: 5’-AGC AGA ACC TCT TCG TCC TTT CGG ACT CAT C-3’) under PCR conditions. In the same way an inactive ribozyme was synthesized by changing one base in the catalytic core of the ribozyme (Figure 1). The PCR was performed under standard conditions for 36 cycles (94°C, 30 sec; 50°C, 60 sec; 72°C, 120 sec) using a mastercycler personal (Eppendorf). The DNA templates for the ribozyme production were purified by
phenol/chloroform extraction and NH₄OAC/ethanol precipitation and washed with 75% ethanol. Air dried pellets were reconstituted in RNase-free water, analysed and quantified by gel electrophoresis and ethidium bromide staining. The anti-EWSR1-FLI1 hammerhead ribozyme was then synthesized by enzymatic T7 \textit{in vitro} transcription for 2 h at 37°C. The reaction conditions for the \textit{in vitro} transcription were: 1x \textit{in vitro} transcription buffer (Promega), 10 U Rnasin (Promega), 10 mM DTT, 1 mM NTP, 15 U of T7 polymerase (Promega) in a volume of 20 μl. One μg of DNA template was used and the reaction was started by the addition of T7 polymerase. After \textit{in vitro} transcription the DNA template was removed by DNase incubation for 15 min. The \textit{in vitro} transcript was purified by LiCl/ethanol precipitation and washed with 80% ethanol. Air dried pellets were reconstituted in RNase-free water, analysed and quantified by gel electrophoresis and staining with ethidium bromide.

\textit{In vitro cleavage assay.} The active and inactive ribozymes were incubated with \textit{in vitro} synthesized EWSR1-FLI1 type 1 RNA substrate that contained the fusion point of EWSRI and FLI1 and was fluorescein labelled to test the catalytic activity. The catalysis was tested in 50 mM Tris-HCl, pH 8.0, and the reaction was started by the addition of 20 mM MgCl₂ in a total volume of 20 μl. The molar ratio of ribozyme to RNA was 10:1. The incubation time was 2 h at 37°C except for the kinetic analysis where the range was from 5 min to overnight incubation. The reaction was then stopped by the addition of an equal volume of formamide buffer (80% formamide, 50 mM Tris-Borat, pH 8.0, 10 mM EDTA, 1 mg/ml xylencyanol, 1 mg/ml bromphenol blue), heat denatured at 95°C for 5 min, and visualized by 8% denatured PAGE and subsequent staining with Gel Star nucleic acid gel stain (FMC Bio Products, Rockland, ME, USA). For quantification, a Fluor-S™ Multimager system (Bio-Rad Laboratories, Munich, Germany) was used.

**Figure 1.** Generation of anti-EWSR1-FLI1 hammerhead ribozymes. A: DNA sequences of the active (Rz+) and inactive (Rz–) anti-EWS-FLI1 ribozymes are shown. Both ribozyme sequences contain a T7 promoter at the 5’ end and a catalytic domain, flanked by complementary, substrate-specific sequences. B: A schematic presentation of the anti-EWSR1-FLI1 hammerhead ribozyme hybridised with the target RNA (EWSR1-FLI1 fusion type 1). The position of the nucleotide exchange in the inactive ribozyme (Rz–) is indicated.
RACE-PCR assay for detection of ribozyme activity against cellular RNAs. In order to detect the ribozyme activity against the EWSR1-FLI1 transcript in the total RNA from EFT cells, the rapid amplification of cDNA ends (RACE) technique was employed. For this purpose the 5′-RACE procedure utilizing an oligo dT-anchor primer and FLI1 specific primer FLI1_1, followed by a second amplification step (nested PCR) with a PCR anchor primer and FLI1 specific primer FLI1_3 was used.

**Results**

Cleavage of synthetic EWSR1-FLI1 substrate RNAs by ribozymes in a cell-free system. Analysis of the in vitro ribozyme transcription products by gel electrophoresis and ethidium bromide staining showed single RNA bands of the expected size (data not shown). As shown in Figure 3, the test of catalytic activity of the ribozymes demonstrated cleavage of EWSR1-FLI1 at the expected position in a time dependent manner. After 2 h over 60% of the substrate was cleaved as indicated by the emergence of a 231 nucleotide fragment and a 224 nucleotide fragment.
The specificity of the ribozyme was tested by incubation of \textit{EWSR1-FLI1} type 1 and \textit{EWSR1-FLI1} type 2 RNA with Rz+ as well as Rz–. As shown in Figure 4, incubation of Rz+ with type 1 substrate resulted in the expected cleavage products of 231 and 224 nucleotides. In contrast, no cleavage products could be detected after incubation of Rz+ with the type 2 substrate. In addition, the inactive form of the ribozyme (Rz–) was unable to cleave the \textit{EWSR1-FLI1} substrates.

\textit{Cleavage of \textit{EWSR1-FLI1} mRNAs in the context of cellular RNAs}. The following PCR products were expected: un-cleaved full-length \textit{EWSR1-FLI1} RNA: 1041 base pairs; cleaved full-length \textit{EWSR1-FLI1} RNA: 196 base pairs; un-cleaved synthetic \textit{EWSR1-FLI1} RNA: 420 base pairs and cleaved synthetic \textit{EWS-FLI1} RNA: 196 base pairs. The results of this analysis are shown in Figure 5. Specific bands corresponding to cleaved \textit{EWSR1-FLI1} RNA could be detected after incubation of RNA from A-673 cells (fusion type 1), but not after incubation of RNA from RD-ES cells (fusion type 2) in the presence of the active ribozyme. In addition, cleavage of synthetic RNA occurred as indicated by presence of the expected PCR products.
Discussion

The cleavage efficiency of ribozymes is dependent on a number of factors, including target site selection as well as length of the complementary arms flanking the catalytic core domain of the ribozyme (25, 42). Computer programs can help to find suitable RNA target sites for the ribozyme attack, but the best ribozyme target sites must often be determined experimentally (43, 44). One common method to determine effective cleavage is based on *in vitro* cleavage assays (45). The ribozyme described in the present paper was able to cleave synthetic EWSR1-FLI1 RNA as well as EWSR1-FLI1 transcripts in total RNA, extracted from Ewing tumour cells. Thus, this anti-EWSR1-FLI1 ribozyme could find the specific target in the presence of an excess of unspecific cellular RNAs and the predicted cleavage site was available for the ribozyme. Ribozyme reaction was time-dependent and highly sequence-specific since the predicted cleavage products were only detected when type 1 EWSR1-FLI1 was used as the substrate.

The activity of ribozymes *in vitro* does not necessarily predict the *in vivo* activity. The reasons for such ineffectiveness *in vivo* could be that: cellular proteins may inhibit the binding of the ribozyme to its target RNA or may disrupt the active conformation of the ribozyme; intracellular metal ions do not have the necessary concentration for sufficient ribozyme-mediated cleavage or ribozymes are easily attacked by RNase (46, 47). Previous studies have shown that the following factors are important for ribozyme activity *in vivo*: a high level of ribozyme expression (48); the intracellular stability of the ribozyme (26); co-localization of the ribozyme and its target RNA in the same cellular compartment (27, 49, 50) and the cleavage activity of the transcribed ribozyme (51).

For applications *in vivo*, systems for highly effective delivery of ribozymes to target cells are required. Delivery of a ribozyme to the cell can be achieved either endogenously, employing a vector-based system to promote ribozyme expression, or by exogenous ribozyme drug delivery. Several viral or non-viral expression constructs have been widely used, but this approach is hindered by cell specificity, efficacy and safety issues. The exogenous delivery on the other hand, offers a direct method of ribozyme application, which requires extensive chemical modifications of the RNA ribozyme molecules due to their high instability and poor cellular uptake. Recently a new method to overcome this problem with the use of polyethylenimines has been described (52). This development offers a new method for the therapeutic inactivation of onc fusion transcripts.

A highly efficient strategy for the inactivation of target RNAs uses small interfering RNAs (53). This strategy uses the phenomenon of RNA interference and utilizes double-stranded RNA (dsRNA) for the activation of an enzymatic pathway that leads finally to the degradation of the complementary target RNA. Anti-sense oligonucleotides as well as ribozymes exert their function via hybridisation to complementary sequences in the target RNA and therefore form dsRNA intermediates. One could speculate whether these intermediates also activate the cellular dsRNA specific RNA degradation machinery. Ribozymes have the great advantage over the other approaches in that they encode by themselves an enzymatic activity. This makes ribozymes more resistant against escape strategies that inactivate distinct components of the cellular dsRNA activated RNA degradation machinery. Such escape mechanisms have been described (54). In addition, dsRNA induces several side-effects (19-23). Major problems of sequence-specific RNA knockdown strategies are off-target effects. One advantage of ribozymes in comparison to other knockdown strategies is the fact that ribozymes have a relatively high sensitivity for sequence variations at the cleavage site. Furthermore, the specificity of ribozymes can be regulated by variation of the length of the hybridization arms (55).

Taken together, our data show that the fusion region of the EFT specific EWSR1-FLI1 oncogenic RNA is accessible to ribozyme mediated cleavage. Thus, the ribozyme approach for the inhibition of EFT cell growth seems to be feasible for the development of new treatment strategies for EFT patients. The *in vivo* activity of our ribozyme and the optimal length of the hybridization arms requires further evaluation.

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References


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