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EWS/FLI-1 and *EWS/ERG* fusion genes in Ewing family tumours of children and adolescents

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Introduction. Ewing's family tumours often show characteristic chromosomal abnormalities: t(11;22)(q24;q12) and t(21;22)(q22;q12) with a formation of EWS/FLI-1 and EWS/ERG fusion genes.
Aim of the study. The presence of specific fusion genes in Ewing family tumours: EWS/FLI-1 and EWS/ERG was assessed in order to establish their clinical role in children with these tumours.
Material and methods. 46 patients (28 boys and 18 girls) entered this multicenter study. Tissue samples were collected and PNET or EES diagnosis was retrospectively confirmed by two independent pathologists. Institutional ethical board consent was obtained for all participating institutions. Patient age ranged between 9 and 207 months (mean 114±62 months). The extent of the disease was graded according to the clinical staging system with the following distribution: 5 children with stage II, 30 with stage III and 11 with stage IV disease having distant metastases. RNA was obtained both from fresh-frozen and paraffin embedded tissue biopsy samples. RT-PCR specific primers were used on isolated tumour RNA, followed by electrophoresis for screening of two targeted translocations.
Results. Fusion genes were detected in 31 (67%) tumour samples. The most common fusion gene was EWS/FLI-1 7-6, which was detected in 18 patients. We observed no significant differences between patients with different mutation variants according to age, sex, tumour size and localisation, disease stage, regional lymph node involvement, metastasis event-free and overall survival.
Conclusions. Because of a considerable heterogeneity of genetic changes observed in Ewing's family tumours, results in this group of patients were not fully clear. We were not able to confirm if any mutation is connected with better or poorer prognosis, or with any of the studied clinical parameters.

Obecność genów fuzyjnych *EWS/FLI-1* i *EWS/ERG* w guzach rodziny Ewinga u dzieci i młodzieży

Wprowadzenie. Guzy z rodziny Ewinga często demonstrują charakterystyczne translokacje t(11;22)(q24;q12) oraz t(21;22)(q22;q12), z tworzeniem genów fuzyjnych, odpowiednio EWS/FLI-1 i EWS/ERG.
Celem badania była ocena obecności genów fuzyjnych EWS/FLI-1 i EWS/ERG w guzach z rodziny Ewinga celem określenia ich klinicznego znaczenia wśród dzieci z tymi nowotworami.
Materiał i metody. Do badania zakwalifikowano 46 pacjentów (28 chłopców i 18 dziewczynek). Od każdego pacjenta z rozpoznanym PNET lub EES uzyskano fragment guza do badania. Badanie zostało zaaprobowane przez komisje etyczne wszystkich ośrodków biorących udział w analizie. Wiek pacjentów wahał się od 9 do 207 miesięcy (średnio 114±62 miesięcy). Stopień zaawansowania choroby określono na podstawie kryteriów klinicznych z następującym rozkładem: 5 dzieci w stadium II, 30 w stadium III oraz 11 w stadium IV choroby z obecnością przerzutów odległych. RNA uzyskiwano zarówno z materiału świeżo zamrożonego, jak również z materiału utrwalonego w parafinie. Uzyskane RNA amplifikowano w obecności specyficznych primerów dla poszukiwanych translokacji w reakcji RT-PCR, a uzyskane produkty rozdzielano za pomocą elektroforezy żelowej.

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Wyniki. Obecność genów fuzyjnych stwierdzono w przypadku 31 (67%) nowotworów. Najczęstszym genem fuzyjnym był EWS/FLI-1 7-6, który wykryto u 18 pacjentów. Nie obserwowano istotnych różnic pomiędzy pacjentami z różnymi wariantami mutacji odnośnie wieku, płci, wielkości i lokalizacji guza, stadium choroby, zajęcia regionalnych węzłów chłonnych, obecności przerzutów, przeżyciem wolnym od choroby i przeżyciem całkowitym.
Wnioski. Ze względu na dużą różnorodność zmian genetycznych, stwierdzanych w guzach z rodziny Ewinga, uzyskane wyniki nie są w pełni jednoznaczne. Nie stwierdzono, aby którykolwiek wariant mutacji związany był z gorszym rokowaniem, lub z którymkolwiek analizowanym parametrem klinicznym.

Key words: PNET, Ewing sarcoma, fusion genes, children
Słowa kluczowe: PNET, guz Ewinga, geny fuzyjne, dzieci

Introduction

Primitive peripheral neuroectodermal tumour (PNET) and extraosseous Ewing's sarcoma (EES) account for approximately 13% of soft tissue malignant neoplasms in children and adolescents [1, 2]. Both tumours are predominantly localised within pelvis (45%), thorax wall (including Askin's tumours – 34%) and in the paravertebral region (12%) (2). Metastases are most common to the lungs (38%), the bones (31%), and to the bone marrow (12%) (2). Although for many years both tumours were regarded as separate entities, recent findings of typical cytogenetic abnormalities have allowed to classify both these tumours together with the classic Ewing's sarcoma of bones to the family of Ewing's tumours. The most frequent and characteristic feature of the Ewing's family tumours chromosomal abnormality is translocation t(11;22)(q24;q12) with a formation of the *EWS/FLI-1* fusion gene. As the fusion gene could be formed from different exons, several types of *EWS/FLI-1* exist. Less common are translocations t(21;22)(q22;q12) (*EWS/ERG* fusion gene), t(7;22)(p22;q12) (*EWS/ETV-1* fusion gene), and t(17;22)(q12;q12) (*EWS/EAF-1* fusion gene) [3, 4].

In 1991 a new classification of PNET and EES tumours was suggested [5]. According to this classification, tumours which demonstrate at least two neuronal markers (e.g. NSE, S-100 protein, HNK-1, neurofilaments or GPAP) and/or create Homer-Wright rosettes can be classified as PNET; and tumours which present none or only one neuronal marker can be classified as Ewing's sarcoma [5]. However, until the final consensus is not ready, it is postulated to handle all these tumours together in scientific analyses.

The normal function of the *EWS* gene is unclear, but it is ubiquitously expressed in cells and contains an RNA-binding domain [6]. The *FLI-1* gene is a member of the *ETS* family of proto-oncogenes. The gene contains a DNA-binding domain and functions as a transcriptional activator [7]. The fusion transcript in EES and PNET includes the promoter region of the *EWS* gene and a protein-protein interaction domain [8, 9]. The RNA-binding domain of the *EWS* gene is lost and replaced by the DNA-binding domain of the *FLI-1* gene via a hinge region of variable length, depending on the breakpoints in the two genes [9]. The fusion protein shows increased transcriptional activity *in vitro* compared with the normal *FLI-1* gene and can induce neoplastic transformation in fibroblasts [9]. The other genes, which were proved to form fusion genes with *EWS* gene in Ewing family tumours, belong all to the ETS family of transcription factors. The *ERG* gene is highly homologous to the *FLI-1* gene and is believed to have similar activity [10]. The *EAF-1* gene encodes adenovirus E1A enhancer binding protein, which activates matalloproteinase genes [9].

As the fusion genes seem to play an important role in carcinogenesis of Ewing's family tumours, it seemed reasonable to compare the clinical behaviour of tumours with different variants of translocations.

Material

Tissue samples were retrospectively obtained from 50 patients with PNET or EES diagnosed in different institutions. Four patients were excluded from further analysis as the tissue samples were negative (absence of *GAPDH* gene sequences) suggesting poor quality of the isolated RNA. The final evaluation thus included 46 patients: 33 (71.7%) with PNET and 13 (28.3%) with EES. There were 28 (60.9%) boys and 18 (39.1%) girls, aged between 9 and 207 months (mean: 114±62 months, median value 126 months). The morphologic diagnosis of PNET or EES was confirmed by two independent referent pathologists. Clinical staging on presentation revealed 5 patients (10.9%) having stage II disease, 30 stage III (65.2%) and 11 (23.9%) with stage IV with distant metastases. Regional lymph node involvement was found in 6 children (13%). Nine (19.6%) primary tumours were limited to the organ or tissue of origin (stage T1 according to the TNM classification), while in 37 (80.4%) patients the tumour infiltrated the neighbouring organs (stage T2). Primary tumour size below 5 cm occurred in 9 (19.6%) children, and over 5 cm – in 37 children (80.4%). The primary tumours were localised within the trunk in 20 cases (43.4%), on the extremities in 13 cases (28.3%), on the head/neck in 10 cases (21.7%), and in the pelvis in 3 cases (6.5%) children. In spread tumours, the most common sites of metastases were the lungs – 6 patients, the bone marrow – 2 patients, the liver – 2 patients, the bones – 2 patients, non-regional lymph nodes – 2 patients, the pleura – 2 patients, and the skin – 1 patient. The site of metastases was not precisely indicated in 1 child.

All PNET/EES children underwent multimodal chemotherapy, combination radiotherapy and surgery. Treatment protocols used were: the CWS-91 protocol [11] for 10 children (21.7%), the EICESS-92 protocol for 3 children (6.5%) and the CWS-96 protocol [12] for 32 children (71.7%). The median follow up for all patients was 26.5 months – 39 months for surviving patients (n=25).

Methods

Samples for RNA investigation according to the method modified by Stegmaier et al. [13] were obtained both from fresh-frozen and paraffin embedded tissue biopsies. All tissue samples were checked by two reference pathologists in order to confirm that the evaluated material is representative for RMA.

RNA isolation from paraffin embedded tissue samples

Several 4-5 μm thick sections were prepared from each sample. All paraffin sections put into microtubes underwent xylene deparaffinisation (15 min. at 37°C). The samples were than washed out twice with 100% ethanol. After evaporation at room temperature sediments were diluted in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 20 mM EDTA, 1% SDS (sodium dodecyl sulfate) and 0.08% Proteinase K solution (Roche, No 1 964 364). The total RNA content was obtained after cell lysis (12 hours at 56°C) with Trizol Reagent (Invitrogen No 15596-018) according to the instruction supplied by the manufacturer. The obtained sediment was rinsed out with 75% ethanol, dried out at room temperature and diluted once more in H_2O_{DEPC}. The obtained RNA was treated with 10 U DNase (RNase–free, Roche No 776785) (15 min., 25°C) in the presence of Rnasin (Roche, 75 U). For DNase inactivation the Trizol Reagent was used for a second time. The precipitated RNA was diluted in H_2O_{DEPC} and stored at –70°C until investigation.

RNA isolation from fresh-frozen material

The fresh-frozen samples were homogenized with the Miccra D-8 homogenizator (Art-moderne LaBortechnik). Each sample was than treated with Trizol Reagent in the same way as was described above except for the DNase procedure.

RT-PCR

RNA concentration was measured with the spectrophotometer (λ=260nm – Smartspec™ 3000, BIORAD). For cDNA synthesis we used 1 μg of RNA obtained from each sample. The transcription was performed using a reverse transcriptase provided in a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche No 1483188) in the presence of 5 mM $MgCl_2$ and primers (dT_{15}-primer for fresh-frozen material, and dN_6–primer for RNA obtained from paraffin-embedded samples) according to the manufacturer's instruction. The quality of the isolated RNA was checked by amplification of a 495bp sample for the human beta-actin in a case of fresh-frozen tissues (primers sequence according to Stegmaier, GenBank accession, No M 10277) or a 226bp sample for the human 3-phosphoglyceraldehyde dehydrogenase (*GAPDH* – primers sequence according to PE Biosystem, TaqMan Gold RT-PCR Kit, GenBank J04038) in a case of paraffin-embedded samples. The single strain of cDNA obtained in the RT-PCR was amplified in PCR using specific primers for *EWS/FLI-1 7–6*, *EWS/FLI-1 7-5*, *EWS/FLI-1 7-8*, *EWS/FLI-1 10-5*, *EWS/FLI-1 10-6* and *EWS/ERG 7-6* fusion genes. PCR was performed with an Ampli Taq Gold®Taq DNA polimerase (Applied Biosestem) to a final concentration of 1.5 mM $MgCl_2$ and each primer at 0.5 μM. In the case of fresh-frozen samples 35 cycles of PCR, and in the case of paraffin-embedded tissue samples 60 PCR cycles were performed. The RT-PCR products underwent electrophoresis in a 1.5% agarose gel with TAE buffer (0.04 mM TRIS-acetate, 0.001 M EDTA, 0.5 μg/μl etidine bromide) (Figure 1).

In each case a positive tumour sample (with previously confirmed translocation) and negative control (evaluated tissue sample, however, without primers added) were added. Each step of the procedure (tissue preparation, RNA isolation, PCR amplification and electrophoresis) was performed in a separate room in order to avoid contamination.

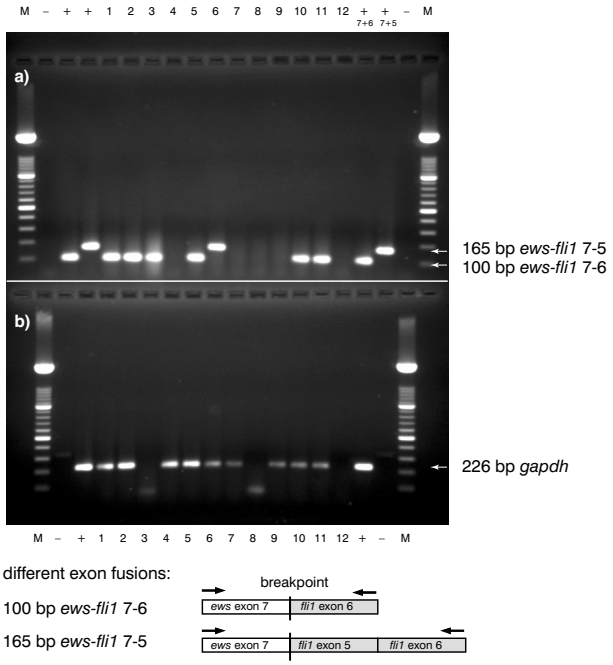

Figure 1. Agarose gel electrophoresis of RT-PCR products from Ewing's sarcoma/PNET patients 1-12. (M = size marker (100 bp fragment ladder). Positive and negative PCR controls as indicated).

a) *EWS/FLI-1* fragments 100 bp (exon fusion *EWS 7 – FLI1* 6) and 165 bp (exon fusion *EWS 7 – FLI-1* 5). Forward primer EWS-794-814, reverse primer FLI-1-854-834: Patients 1-3, 5, 10 and 11 show 100 bp fragments. Patient no 6 shows 165 bp fragment. For patients 4, 7-9 and 12 no *EWS/FLI-1* products were amplified with these primers.

b) *GAPDH* control fragments 226 bp. For patients 3, 8 and 12 no *GAPDH* fragments were amplified, indicating poor quality of RNA. Primers according to Perkin Elmer / Applied Biosystems ('TaqMan® Gold RT-PCR Kit' mannual)

Statistical analysis

The possible differences between the evaluated groups of patients were assessed with the Student's *t*-test in case of parametric values, or with the χ^2 test with appropriate modifications in case on nonparametric values. The Kaplan-Meier overall survival (OS) was calculated from the date of the start of therapy until the latest follow-up or death from any cause, the Kaplan-Meier event-free survival (EFS) – from the date of the start of therapy until the time of treatment failure. Failure was defined as relapse or death from any cause. The differences between the curves were estimated by the log rank test and p-values less than 0.05 were considered as significant.

Results

Fusion genes were detected in 31 (67%) tumour samples (Figure 1 and 2). Eighteen (39%) tumours presented *EWS/FLI-1 7-6* fusion gene, 6 (13%) *EWS/FLI-1 7-5* fusion gene, 3 (7%) *EWS/FLI-1 10-5* fusion gene, 2 (4%) *EWS/FLI-1 10-5* fusion gene, 1 (2%) *EWS/FLI-1 7-8* fusion gene, and 1 (2%) *EWS/ERG* fusion gene (Figure 3). The remaining 15 tissue samples did not show any of the assessed genetic abnormalities.

Table I. Clinical characteristics of tumours with different fusion genes

Characteristics	Fusion genes						
	EWS/FLI-1 7-5 n=6	*EWS/FLI-1 7-6* n=18	*EWS/FLI-1 7-8* n=1	*EWS/FLI-1 10-5* n=2	*EWS/FLI-1 10-6* n=3	*EWS/ERG* n=1	No fusion genes n=15
Gender:							
– male	4 (67%)	8 (44%)	1 (100%)	1 (50%)	2 (67%)	1 (100%)	11 (73%)
– female	2 (33%)	10 (56%)	–	1 (50%)	1 (33%)	–	4 (27%)
Histology:							
– EES	4 (67%)	5 (28%)	–	1 (50%)	–	–	3 (20%)
– PNET	2 (33%)	13 (72%)	1 (100%)	1 (50%)	3 (100%)	1 (100%)	12 (80%)
T-stage:							
– T1 (non-invasive)	1 (17%)	4 (22%)	–	1 (50%)	1 (33%)	–	2 (13%)
– T2 (invasive)	5 (83%)	14 (78%)	1 (100%)	1 (50%)	2 (67%)	1 (100%)	13 (87%)
Tumour size:							
– <5 cm	2 (33%)	3 (17%)	–	–	1 (33%)	–	3 (20%)
– ≥5 cm	4 (67%)	15 (83%)	1 (100%)	2	2 (67%)	1 (100%)	12 (80%)
Localisation							
– Head/neck	1 (17%)	4 (22%)	1 (100%)	–	–	–	4 (27%)
– Trunk	2 (33%)	9 (50%)	–	2 (100%)	1 (33%)	–	6 (40%)
– Pelvis	1 (17%)	–	–	–	–	1 (100%)	1 (6%)
– Extremities	2 (33%)	5 (28%)	–	–	2 (67%)	–	4 (27%)
Regional lymph node involvement							
– Yes	–	2 (11%)	–	–	–	–	4 (27%)
– No	6 (100%)	16 (89%)	1 (100%)	2 (100%)	3 (100%)	1 (100%)	11 (73%)
Metastases:							
– Yes	1 (17%)	2 (11%)	–	1 (50%)	–	1 (100%)	6 (40%)
– No	5 (83%)	16 (89%)	1 (100%)	1 (50%)	3 (100%)	–	9 (60%)
Disease stage							
– II	–	2 (11%)	–	–	–	–	3 (20%)
– III	5 (83%)	14 (78%)	1 (100%)	1 (50%)	3 (100%)	–	6 (40%)
– IV	1 (17%)	2 (11%)	–	1 (50%)	–	1 (100%)	6 (40%)
Metastases localisation:							
– Lungs	–	1	–	–	–	1	4
– Bone marrow	–	1	–	1	–	–	–
– Liver	–	–	–	–	–	–	2
– Non-regional lymph nodes	1	1	–	–	–	–	–
– Bones	–	–	–	1	–	–	1
– Pleura	1	1	–	–	–	–	–
– Skin	–	1	–	–	–	–	–

Table II. Treatment results of patients suffering from peripheral primitive neuroectodermal tumour or extraosseous Ewing's sarcoma according to the presence of different fusion genes

Clinical outcome	Fusion genes						
	EWS/FLI-1 7-5 n=6	*EWS/FLI-1 7-6* n=18	*EWS/FLI-1 7-8* n=1	*EWS/FLI-110-5* n=2	*EWS/FLI-1 10-6* n=3	*EWS/ERG* n=1	No fusion genes n=15
Alive	3 (50%)	11 (61%)	1 (100%)	2 (100%)	2 (33%)	–	6 (40%)
Dead	3 (50%)	7 (39%)	–	–	1 (67%)	1 (100%)	9 (60%)
Response to chemotherapy (only stage III and IV):							
Regression of tumour >2/3	1 (17%)	7 (44%)	1 (100%)	1 (50%)	2 (67%)	–	8 (67%)
Regression of tumour <2/3	5 (83%)	9 (56%)	–	1 (50%)	1 (33%)	1 (100%)	4 (33%)
Disease status:							
Alive in 1st CR	1 (17%)	8 (44%)	1 (100%)	1 (50%)	2 (67%)	–	5 (33%)
Progression	2 (33%)	2 (11%)	–	1 (50%)	–	1 (100%)	4 (27%)
Relapse	3 (50%)	8 (44%)	–	–	1 (33%)	–	6 (40%)
– local	1 (17%)	5 (28%)	–	–	–	–	2 (13%)
– metastatic	1 (17%)	2 (11%)	–	–	–	–	2 (13%)
– mixed	1 (17%)	1 (6%)	–	–	1 (33%)	–	2 (13%)
Second CR	1* (17%)	–	–	–	–	–	–

CR – complete remission

* Second CR was achieved in one patient with local relapse

The patients' clinical characteristics depending on the stated fusion gene are listed in Table I. We did not find any relationships between any of the specific type of translocations and patients' age, gender, tumours' size, stage and localisation. It seemed that in the case of tumours with translocations regional lymph node involvement was less frequent than in the case of tumours without translocations (2/31 and 4/15, respectively; p=0.08) as were distant metastases (5/31 and 6/15, respectively; p=0.08). The results of treatment are shown in Table II. As the group of patients presenting tumours with specific fusion genes was very heterogenous, we did not find any significant differences concerning the outcome among the assessed subgroups. We noticed that patients who presented with translocations, had a slightly better overall survival as compared to patients without translocations (estimated 5-year OS 0.53 and 0.27, respectively), but the difference was not statistically significant (p=0.15; Figure 4). Similar results were achieved for event free survival: estimated 5-year EFS 0.42 and 0.29, respectively; p=0.39.

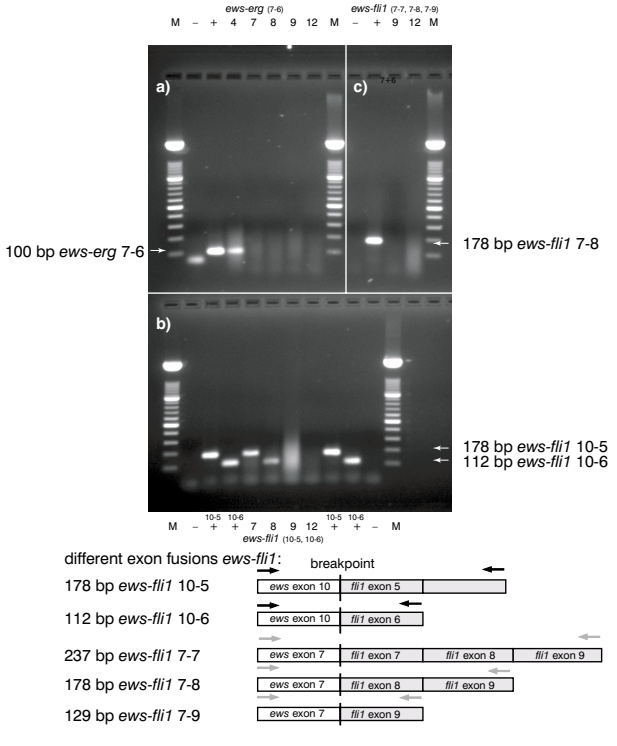

Figure 2. Agarose gel electrophoresis of RT-PCR products from Ewing's sarcoma/PNET patients 4, 7-9, 12. (M = size marker (100 bp fragment ladder). Positive and negative PCR controls as indicated).

a) *EWS/ERG* fragments 100 bp (exon fusion *EWS 7 – ERG* 6). Forward primer EWS-794-814, reverse primer ERG-652-628. Patient 4 positive.

b) *EWS/FLI-1* fragments 178 bp (exon fusion *EWS 10 – FLI-1* 5), patient 7 positive; and 112 bp (exon fusion *EWS 10 – FLI-1* 6) patient 8 positive.

c) *EWS/FLI-1* fragment 178 bp (exon fusion *EWS 7 – FLI-1* 8), 129 bp (exon fusion *EWS 7 – FLI-1* 9; no positive control available) and 237 bp (exon fusion *EWS 7 – FLI1* 7; no positive control available).
For patient 12 no *EWS/FLI-1* or *–ERG* products were amplified with these primer pairs

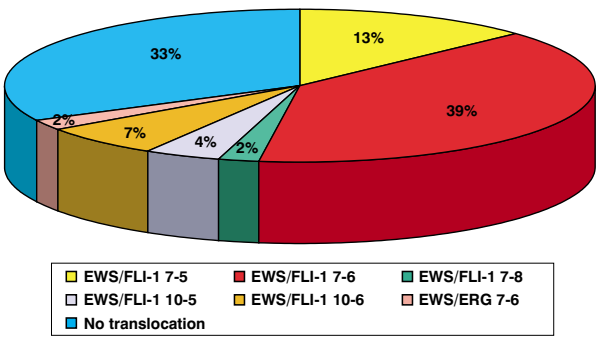

Figure 3. Frequency of fusion genes among analysed tumours

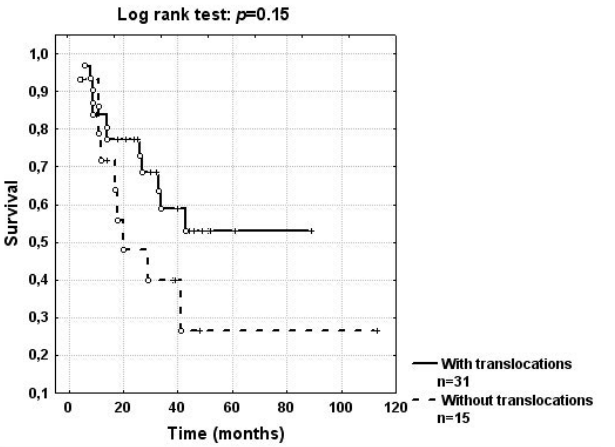

Figure 4. Comparison of overall survival between patients with tumours with and without fusion genes characteristic for tumours of the Ewing's family

Discussion

The precise histological diagnosis of soft tissue tumours is essential for the selection of the most effective anti-tumour therapy. Several immunohistochemical markers, as well as electron microscopy, have proven their efficacy in the differential diagnosis of different malignancies in children and adolescents. However, even with the help of electron microscopy and immunohistochemistry, the precise diagnosis can still be hard to establish in some patients with very low tumour differentiation [14]. Moreover, the increased use of guided-needle biopsy methods has reduced the availability of adequate tissue volume for optimal histological studies. Therefore, it seems that the detection of specific fusion genes, characteristic for different sarcomas, could be very helpful and important in the differential diagnosis of children with soft tissue neoplasms. We have shown that the evaluation of fusion genes is also possible to perform in archival paraffin-embedded tissue samples. We detected the characteristic translocations for Ewing's family tumours in 67% malignancies.

However, still little is known concerning the significant clinical differences between Ewing's sarcomas with different fusion genes. Ginsberg et al., analysing 136 patients with Ewing's sarcoma, did not find any significant

clinical differences between tumours demonstrating *EWS/FLI-1* and *EWS/ERG* fusion genes according to age at diagnosis, sex, metastasis at diagnosis, primary sites, event-free survival and overall survival [15]. As in our patient group only one tumour demonstrated *EWS/ERG* fusion gene, we were not able to confirm these findings. Some authors have demonstrated that the precise exon composition of *EWS/FLI-1* fusion transcripts was found to be a prognostic indicator for Ewing's sarcoma [16, 17]. For localized Ewing's sarcoma, patients with tumours that express the type 1 fusion, where exon 7 of *EWS* is joined to exon 6 of *FLI-1*, have an improved overall survival as compared to patients with tumours that express non-type 1 *EWS/FLI-1* fusions [16, 17]. Because of a considerable heterogeneity of genetic changes observed in Ewing's family tumours, the results in this group of patients were not fully clear. We were not able to confirm whether any mutation related to better or poorer prognosis. We could only state that patients with *EWS/FLI-1 7-6* had slightly better outcome than others (data not shown), however, the observed differences were not significant ($p=0.6$). This discrepancy could be explained by the fact, that the previously mentioned studies included all patients with Ewing's family tumours, with the predominance of Ewing's sarcoma of bones. In our study we have investigated only these tumours, which had primarily originated from soft tissue. It is possible that soft tissue tumours could show different biological properties than bone tumours.

The genetic analysis of paediatric neoplasms has resulted in the identification of new genetic abnormalities and in improved understanding of tumour origin and progression. It is possible that these findings may allow to develop new therapeutic strategies in the future, for example the employment of anti-sense nucleotides sequences, monoclonal antibodies against fusion proteins or specific inhibitors of fusion proteins.

To summarise, proper pre-treatment stratification of patients with soft tissue sarcomas allows to optimise anti-tumour therapy. Despite the significant progress in the therapy of soft tissue sarcomas it is still necessary to search for new prognostic markers, which would lead to a better classifications of patients according to their real chance for successful therapy. Although the presence of different fusion transcripts could have an impact on the clinical course of Ewing's tumours, further investigations are necessary to clarify the exact importance of these genetic abnormalities. However, as we have not observed any significant relationship between the presence of fusion genes and the clinical behaviour of the tumours, it is possible, that molecular changes play only a minor part in the determination of the clinical course of Ewing's sarcoma.

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