Molecular genetic analysis of paediatric small, round cell tumours (Ewing's sarcoma, pPNET)

PhD Thesis

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Introduction

The Ewing family of tumors (EFT) is an undifferentiated small, round cell tumor of the bone and soft tissue, including Ewing sarcoma (ES), extraosseus Ewing tumor and peripheral primitive neuroectodermal tumor (pPNET). These tumours share common specific reciprocal chromosomal translocations which generally involve the TET family member EWSR1 gene (22q12) and one of the five transcription factors of the ETS family, namely the FLI1 gene (11q24), the ERG gene (21q22) and in sporadic cases the ETV1 (7p22), ETV4 (17q12) or FEV genes (2q33). In recent years the heterogenity of the variant translocations in EFT have increased by the discovery that both fusion partners of EFT-associated translocations are interchangeable and another member of TET family, the FUS (16p11) gene, can participate in translocations. Furthermore, novel sporadic C-terminal partners of the fusion have been identified in EFT.

The diversity of EFT-related translocations is enhanced by the significant translocation breakpoint variability as well. For instance, in the case of the most common EWSR1-FLI1 translocation, the position of the breakpoints has been shown to be localized in introns 7, 8, 9 or 10 of the EWSR1 gene and in introns 3, 4, 5, 6, 7 and 8 of the FLI1 gene. Although only 12 types of EWSR1-FLI1 fusion transcripts were considered to be the most prevalent, there are 24 possible translocation combinations between four introns of the EWSR1 gene and six introns of the FLI1 gene, and theoretically all types of EWSR1-FLI1 fusion transcripts can occur. Of these types, EWSR1(ex 7)-FLI1(ex 6) (51%) and EWSR1(ex 7)-FLI1(ex 5) translocations (27%), called type I and type II, are the most frequent. Fusion proteins translated from fusion transcripts act as an aberrant transcription factor which disrupts transcriptional control and transforms cells, leading to the development of the Ewing family of tumors.

Because of the relatively large region of the fusion partner genes, which may involved in the translocation, the application of molecular diagnostics is limited to RNA-based methods. Besides the diagnostic difficulties of EFT due to the diversity of the translocation partner genes and the breakpoint heterogenity in each translocation type, in the case of formalin-fixed paraffin-embedded (FFPE) EFT-tissue the degradation of RNA also presents a significant difficulty. In spite of the many methods

for the diagnosis of EFT, these difficulties in archived tissue specimens have not been solved adequately.

Aims

The original aim of the work was:

- to apply RNA based molecular genetics method in Hungarian diagnostics of the Ewing family of tumors first time in Hungary.
- to establish a new method based on the multiplex fluorescent PCR and laser-induced fluorescent capillary electrophoresis in molecular diagnostics of fresh frozen EFT samples.
- to establish a new method based on the multiplex fluorescent PCR and laser-induced fluorescent capillary electrophoresis which is able to identify all possible EWSR1-FLI1 and EWSR1-ERG translocation transcripts in the formalin-fixed paraffin-embedded EFT samples
- after the processing of EFT samples received from the Tumor Bank at the Department of Orthopaedics, Semmelweis University, to determine the frequency of various EWSR1-FLI1 and EWSR1-ERG fusion types in Hungarian tumor cohort.
- to compare the distribution of the various EWSR1-FLI1 and EWSR1-ERG translocations with international data
- to determine the relationship of EWSR1-FLI1 and EWSR1-ERG genotype to phenotype in clinical manifestation of EFT.
- to determine the sequence and the frequency of the multiple fusion transcripts in the EFT tumor sample.
- to characterize the phenomenon of multiple fusion transcripts and the role in the pathomechanism of Ewing family of tumor on the basis of results of the present study and the review of similar studies.

Methods

This retrospective project was based on the processing of 23 fresh frozen and 60 formalin-fixed paraffin-embedded tissue samples from 48 patients, resected between 1996 and 2006. 25 patients had multiple samples, but only one paraffin-embedded and/or fresh frozen tissue was made from each sampling. All samples were received from the Tumor Bank at the Department of Orthopaedics, Semmelweis University.

The histopathologic diagnosis of Ewing sarcoma (ES)/primitive neuroectodermal tumours (PNET) was based on the current histological criteria defined by WHO. Of all processed tumour samples, 22 FFT and 53 FFPE tissue samples had ES/PNET histopathological diagnosis. We added a further eight samples with the diagnoses of small round cell tumours, but whose identification as ES/PNET was questionable.

To identify all possible fusion transcripts of the most prevalent EFT translocations, five primers sets (EWS A, EWS B, FLI1 A, FLI1 B, ERG) were designed according to the breakpoint regions of EWSR1, FLI1 and ERG genes reported previously. One of the most important criteria of primer selection was the size of the amplicons amplified by outer primer sets which had to be less than 200 bp. Inner primer sets were designed to produce PCR products of different sizes: a minimal size difference of 2-3 base pairs was required between amplicons of every possible fusion transcript to identify the amplicons reliably by capillary electrophoresis. For the control of RNA quality, one outer (ACTB A) and one inner (ACTB B) primer sets were designed for the housekeeping gene β-actin.

The EWS and FLI1 primer sets consist of four forward or reverse primers. The ERG primer set had two reverse primers. Each of the four inner forward primers of the EWS B primer set was labeled by one of the 6FAM, VIC, NED or PET fluorescent dyes.

Total RNA was extracted from fresh frozen tissues with TRIZOL reagent and from FFPE tissue samples using the High Pure RNA Paraffin Kit or PureLink™ FFPE RNA Isolation Kit. Isolated RNAs were stored at –80°C until use.

In case of the fresh frozen samples monoplex RT-PCR and nested fluorescent multiplex PCR were used to amplify EWSR1-FLI1 and EWSR1-ERG transcripts, using the designed gene specific primers and GeneAmp RNA PCR Kit or AmpliTaq Gold DNA Polymerase. In case of the paraffin-embedded, formalin-fixed tissues the combination of multiplex RT-PCR and the fluorescent multiplex PCR was used to amplify fusion transcripts. Fusion transcripts were identified by laser-induced fluorescent capillary electrophoresis on the basis of amplicon size and forward primers labelled by distinct fluorophores. Sequence of the detected fusion transcript was always confirmed by DNA sequence analysis.

As a control for RT-PCR and sample quality, each FFPE sample was reverse transcribed and amplified for the housekeeping gene β-actin using the outer and inner primer sets.

Results

EWSR1-ETS fusion variants were identified in 21 (91%) of the 23 fresh frozen tissue samples. In the analyzed samples, 9 distinct EWSR1-FLI1 fusion variants, one type of EWSR1-ERG translocation transcript and one alternative splicing variant of the type 2 transcript were detected. EWSR1-FLI1 type 1 fusion [EWSR1(ex 7)-FLI1(ex 6)] occurred in 11 samples from 10 patients, type 2 [EWSR1(ex 7)-FLI1(ex 5)] and EWSR1(ex 7)-ERG(ex 6) translocation transcripts were detected in four cases each and the following seven EWSR1-FLI1 fusion variants occurred only in single cases: EWSR1(ex 7)-FLI1(ex 7), EWSR1(ex 7)-FLI1(ex 8), EWSR1(ex 8)-FLI1(ex 5), EWSR1(ex 8)-FLI1(ex 6), EWSR1(ex 8)-FLI1(ex 8), EWSR1(ex 9)-FLI1(ex 7), EWSR1(ex 10)-FLI1(ex 6). In one sample, a splicing variant of EWSR1-FLI1 translocation type 2 was identified [EWSR1(ex 7)-FLI1(ex 5,8)].

Of the 23 fresh frozen tumor samples from 22 patients who had EFT confirmed by molecular genetic diagnosis, we identified multiple fusion transcript isoforms of EWSR1-FLI1 translocations in 5 samples. In one case, three variant fusion transcripts were found in the same tumor sample [EWSR1(ex 7)-FLI1(ex 6), EWSR1(ex 8)-FLI1(ex 6), EWSR1(ex 8)-FLI1(ex 8)] and in three cases two different fusion

transcripts were identified [EWSR1(ex 7)-FLI1(ex 8), EWSR1(ex 7)-FLI1(ex 6) in the first, EWSR1(ex 7)-FLI1(ex 5), EWSR1(ex 8)-FLI1(ex 5) in the second and EWSR1(ex 7)-FLI1(ex 7), EWSR1(ex 9)-FLI1(ex 7) in the third case]. Finally, in one case we identified one fusion transcript and its alternative splicing variant [(EWSR1(ex 7)-FLI1(ex 5), EWSR1(ex 7)-FLI1(ex 5,8)] in the same sample.

To optimize our method, we extracted RNA from SK-ES-1 cell lines. Interestingly, the RNA extracted from an early passage was identified as type 2, but in extracted RNA from a later passage transcripts of EWSR1(ex 8)-FLI1(ex 5) and EWSR1(ex 7)-FLI1(ex 5) were also detected.

To analyze EWSR1 gene on the other alleles in our 23 samples, we amplified and analyzed EWSR1 gene from exon 7 to exon 11, but transcript variants of these exons were not detected.

The RT-PCR analysis was performed on 45 FFPE samples from 39 patients, because of the poor quality of the RNA in the remaining 15 tumor samples. Six patients had duplicate FFPE samples, but each tissue section has been made from different samplings. EWSR1-FLI1 or EWSR1-ERG fusion transcripts were detected in 27 samples from 25 patients (60%), of which 22 patients had EWSR1-FLI1 and 3 had EWSR1-ERG fusion transcripts in at least one of their samples, as identified by capillary electrophoresis and confirmed by DNA sequencing. In the analyzed samples, the following six distinct types of EWSR1-FLI1 and one type of EWSR1-ERG fusion transcripts were detected: EWSR1 (ex 7)-FLI1(ex 6), EWSR1(ex 7)-FLI1(ex 5), EWSR1(ex 7)-FLI1(ex 7), EWSR1(ex 10)-FLI1(ex 6), EWSR1(ex 7)-ERG(ex 6), and finally EWSR1(ex 8)-FLI1(ex 7) and EWSR1(ex 9)-FLI1(ex 7) transcripts which occurred surprisingly in the same sample, together with EWSR1(ex 7)-FLI1(ex 7) fusion transcript.

Conclusions

In the present study a new molecular diagnostic method was established to identify the EWSR1-FLI1 and EWSR1-ERG chimeric transcripts and to confirm the

results of the histopathologic diagnosis of Ewing sarcoma/primitive neuroectodermal tumours by molecular genetic analysis.

Within the frame of the new method monoplex or multiplex RT-PCR and nested fluorescent multiplex PCR were used to amplify EWSR1-FLI1 and EWSR1-ERG fusion transcripts. Fusion transcripts were identified by laser-induced fluorescent capillary electrophoresis and were subjected to direct sequence analysis. This method is adapted for the molecular analysis of fresh frozen and formalin-fixed, parafin-embedded EFT samples, as well, and is suitable for identifying all EWSR1-FLI1 and EWSR1-ERG chimeric transcripts.

By this new method 23 fresh frozen and 60 formalin-fixed paraffin-embedded tissues of which the majority is histologically confirmed as EFT were processed from the Tumor Bank at the Department of Orthopaedics, Semmelweis University. Present study with this tumor cohort is the largest published molecular genetic analysis of EFT tumor samples in Hungary, and also considerable at the international field/range.

EWSR1-ETS fusion variants were identified by the molecular genetic method in 91% of the fresh frozen and in 60% of the formalin-fixed, paraffin-embedded EFT samples. After the summary of difficulties of the present method connected with the diagnosis of EFT in FFPE tissue samples, modifications of diagnostic method were proposed. After these modifications, present method can offer an effective alternative answer to diagnostic challenges connected with heterogenity of the EFT-related translocations as well as the RNA-degradation of archival tissues and requires significantly less RNA and reagents than the previously used methods.

EWSR1-FLI1 or EWSR1-ERG transcripts were confirmed by the present method in all 68 EFT samples from 43 patients and ten different EWSR1-FLI1 translocation transcripts and one type of EWSR1-ERG transcript were identified, including two fusion transcript types (EWSR1(ex 8)-FLI1(ex 5), EWSR1(ex 8)-FLI1(ex 8)) that, to our knowledge, have not been previously described. Occurrence of different EWSR1-FLI1 and EWSR1-ERG transcripts of Hungarian EFT samples shows similarity to the international results.

In EFT samples of five patients multiple fusion transcripts of EWSR1-ETS fusion gene were identified. Alternative mRNA splicing offers the most probable explanation for the phenomenon of these multiple fusion transcripts.

Our results and a review of similar studies led us to the conclusion that the alternative splicing of EWSR1-ETS transcipts is a frequent mechanism. While the occurence of the multiple fusion transcripts in previously reported EFT-cases with multiple fusion transcripts was 4%, our study showed a 22% occurence. However the frequency of the alternative splicing can be over 30% according to studies of molecular characterization of EWSR1-FLI1 chromosomal breakpoints.

Alternative splicing can play an important role in the pathogenesis of the Ewing family of tumours. In cases of out-of-frame gene fusions encoding a truncated inactive EWS protein without the DNA binding domain of FLI1, alternative splicing contributes to the production of active fusion proteins by creating in-frame transcripts, thus leading to the development of EFT. However the numerous cases with multiple in-frame transcripts suggest an unknown role of the alternative splicing as well.

Due to the small number of EFT patients with complete clinical documentation we were not able to study the possible relationship of EWSR1-FLI1 and EWSR1-ERG translocations or cases with the multiplex fusion transcripts to the survival data of EFT patients.

Publications

Candidate's publications Related to the Thesis:

Patócs B, Németh K, Garami M, Arató G, Kovalszky I, Szendrői M, Fekete G. Multiple splice variants of EWSR1-ETS fusion transcripts co-existing in the Ewing sarcoma family of tumors. Cell Oncol. 2013;3:191-200. **IF (2011): 3,105**

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Candidate's lectures Related to the Thesis:

Patócs B, Németh K, Garami M, Arató G, Kovalszky I, Szendrői M, Fekete G. Ewing-sarcoma / pPNET molekuláris genetikai diagnosztikája Magyarországon. VII. Magyar Genetikai Kongresszus és XIV. Sejt- és Fejlõdésbiológiai Napok, Balatonfüred, 2007.04.15-17.

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