Aus dem Universitätsklinikum Münster

Gerhard-Domagk-Institut für Pathologie

- Institutsleitung (kommissarisch): Univ.-Prof. Dr. med. Gabriele Köhler -

Molecular and immunohistochemical characterisation of matched primary and metastatic Ewing sarcomas

INAUGURAL – DISSERTATION

zur

Erlangung des doctor medicinae

der Medizinischen Fakultät

der Westfälischen Wilhelms-Universität Münster

vorgelegt von Wentker, Dennis

aus Münster

2012

Gedruckt mit Genehmigung der Medizinischen Fakultät der Westfälischen

Wilhelms-Universität Münster

Dekan: Prof. Dr. med. Wilhelm Schmitz

1. Berichterstatter: Univ.-Prof. Dr. med. Gabriele Köhler

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Tag der mündlichen Prüfung: 21.05.2012

Aus dem Universitätsklinikum Münster
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ZUSAMMENFASSUNG

Molecular and immunohistochemical characterisation of matched primary and metastatic Ewing sarcomas

Dennis Wentker

Die Diagnostik von Ewing-Sarkomen (ES) basiert auf molekularpathologischen und immunhistochemischen Analyseverfahren. In einer früheren Arbeit wurde ein Patient mit zwei separaten ES beschrieben, in denen unterschiedliche EWSR1-Translokationen nachgewiesen wurden. Zur Differenzierung, ob es sich bei manchen vermeintlichen Metastasen um eigenständige Tumoren handelt, wurden Primärtumoren und Metastasen auf die ES-spezifischen EWSR1-Translokationen untersucht. Zudem wurden immunhistochemische Färbungen durchgeführt, um Unterschiede in der Markerexpression von Primärtumoren und korrespondierenden Metastasen zu evaluieren.

Formalin-fixiertes, Paraffin-eingebettetes (FFPE) bzw. gefrorenes Material von 39 Primärtumoren und den korrespondierenden Metastasen wurde auf ES-spezifische Fusionstranskripte molekularpathologisch untersucht. Des Weiteren wurden tissue microarrays erstellt, um die immunhistochemischen Expressionsprofile von Primärtumoren und Metastasen miteinander zu vergleichen. Die Primärtumoren und die korrespondierenden Metastasen wiesen alle den jeweils identischen Translokationstyp auf. AK16-, CD99- und FLI-1-Antikörper zeigten eine hohe Sensitivität sowie eine gute Konkordanz in der Markerexpression zwischen Primärtumoren und Metastasen.

AK16-, CD99- und FLI-1-Antikörper können zur Diagnose von ES-Metastasen verwendet werden, falls der Nachweis eines ES-spezifischen Fusionstranskriptes an FFPE-Tumormaterial nicht gelingt. Primärtumoren und Metastasen weisen in der Regel identische Fusionstranskripte auf, und das Vorhandensein zweier separater ES mit divergierenden Translokationstypen bei einem Patienten stellt ein seltenes Ereignis dar.

Tag der mündlichen Prüfung: 21.05.2012

ERKLÄRUNG

Ich gebe hiermit die Erklärung ab, dass ich die Dissertation mit dem Titel:

Molecular and immunohistochemical characterisation of matched primary and metastatic Ewing sarcomas

in der/im (Klinik, Institut, Krankenanstalt):

Universitätsklinikum Münster, Gerhard-Domagk-Institut für Pathologie

unter der Anleitung von:

Frau Univ.-Prof. Dr. med. Gabriele Köhler

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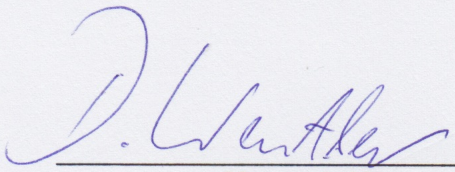

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Abbreviations

A	Alive (outcome)
D	Dead (outcome)
EFS	Event free survival
ES	Ewing Sarcomas
FFPE	Formalin-fixed, paraffin-embedded
FISH	Fluorescence in situ hybridization
FT	Frozen tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
LR	Local relapse
Mono	Monoclonal (antibodies)
Mths.	Months
N/A	Not available
N/E	Not evaluable
PanCK	Pancytokeratin
PAS	Periodic acid-schiff reaction
PCR	Polymerase chain reaction
Poly	Polyclonal (antibodies)
RT-PCR	Reverse-transcription-polymerase chain reaction
TMA	Tissue microarray
Yrs.	Years

List of tables and figures

1 Introduction

Ewing sarcoma (ES) is a malignant small round-cell tumor of the bone and was described for the first time by James Ewing in 1921. [10] It is the second most common malignant bone tumor in childhood and young adults after osteosarcoma. The incidence of ES in children and adolescents is approximately 3 per million annually. The progress in local therapy and systemic chemotherapy in the last decades improved the 5-year survival from 44% to 68% for patients with localised disease. [9, 14]

About 25% of the patients have detectable metastases at diagnosis, which is a major adverse prognostic factor. The most common metastatic sites are the skeletal system, the lungs and the bone marrow. Patients with pulmonary metastases have a better prognosis than patients with bone or bone marrow metastases at primary diagnosis. The 5-year survival for patients presenting with metastatic disease at primary diagnosis increased from 16% to 39% in the last decades. [1, 9] Local relapse within 2 years after the primary diagnosis is another prognosis-deteriorating factor. However, the local relapse rate could be decreased to 15% for axial tumors and 4% for all other sites since 1986. [5]

In nearly all cases of ES the EWSR1 gene is rearranged to one ETS family transcription factor. The most common fusion partner of EWSR1 is FLI-1 [t(11;22)(q24;q12)]. About 85% of ES tumors carry this translocation and the EWSR1/FLI-1 fusion protein functions as a aberrant transcription factor which causes the dysregulated expression of several genes. [18] In 10-15% of cases the ETS DNA-binding-domain is contributed from ERG [t(21;22)(q22;q12)] and in rare cases from ETV-1 [t(7;22)(q22;q12)], E1AF [t(17;22)(q12;q12)], or

FEV [t(2;22)(q33;q12)]. [6] For EWSR1/FLI-1 and EWSR1/ERG translocations, variants with breakpoints in different introns of EWSR1, FLI-1 and ERG can be found. In the most common translocations EWSR1 exon 7 is juxtaposed to exon 6 (type 1, 55.5%) or exon 5 (type 2, 18.5%) of FLI-1 or exons 6 (3.5%), 7 (2.0%) or 9 (2.0%) of ERG. EWRS1 exon 10 and FLI-1 exon 6 combination (type 3) account for another 11% of translocations. [15]

Due to the specificity of EWSR1 translocations for ES and the presence in nearly all ES cases, the detection of these rearrangements is an important support in the diagnosis of ES. Here, the use of reverse-transcription-polymerase chain reaction (RT-PCR) has become the most preferred method. Another important diagnostic tool is the immunohistochemical analysis of ES tumor samples. Although no immunohistochemical stain can exclusively be used for diagnosis of ES, anti-CD99 and -FLI-1 antibodies are useful markers. [6, 12, 16, 20, 24]

We once presented a patient, in whom two separate ES with different EWSR1 translocations were observed and speculated that EWSR1 translocations might not be the first step in evolution of these ES, but could be preceded by so far undetected genomic aberrations. [2]

To further investigate whether the appearance of separate ES with differing EWRS1 translocations are frequent events in ES patients, we analysed samples taken from another 39 ES patients, who either presented with metastases or multifocal disease at diagnosis (n = 5) or developed metastases/ relapses during the period under review (n = 35), for t(11;22) or t(21;22)

translocations by RT-PCR. In addition, we performed immunohistochemistry on suitable samples to analyse whether corresponding primary and metastatic tumor samples differ in marker expression.

2 Materials & Methods

2.1 Case selection

The files of the Department of Pathology of the University Hospital Muenster, Germany, were searched for ES patients presenting with metastatic, multifocal and/or local relapsing disease and of whom specimens of primary tumors and metastases were available for molecular and immunohistochemical analysis. In addition to the then described patient (no. 26 of Table 1 [2]), 39 patients were identified to fulfil these criteria. Ninety-one tumor samples of these 39 patients were available for analysis by RT-PCR (20 fresh frozen (FT = frozen tissue) and 71 formalin-fixed, paraffin-embedded (FFPE) specimens). Fifty-four FFPE samples taken from 34 of all 40 patients were suitable for immunohistochemical research (patient no. 26 included). In 2 cases, specimens of the first metastasis but not of the primary tumor were available. Hence, further material of elsewhere localised and temporally differing metastases was analysed. The patients were either treated according to the EURO-E.W.I.N.G. 99 (n = 20), the EICESS 92 (n = 15) or the CESS 86 protocol (n = 1). [21]

Four patients were consult patients and not treated according to these protocols. Table 1 gives an overview of all patients and their courses of disease.

Patient No.	Primary tumor site	Age at diagnosis [yrs.]	EFS [mths.]	1. metastasis	EFS [mths.]	2. metastasis	Period under review [mths.]	Outcome
1	humerus	29	9	multifocal (lung)	15	pelvis	26	D
2	spine	16	47	spine (LR)	23	pleura	97	A
3	pelvis	10	5	lung			24	A
4	pelvis	16	43	lung			75	A
5	femur	17	0	lung			39	A
6	pelvis	35	45	lung				N/A
7	femur	16	24	lung			65	D
8	tibia	12	11	lung			17	A
9	tibia	15	97	lung	7	pleura	119	D
10	pelvis	18	50	bronchus	10	lung	62	D
11	humerus	39	96	pleura	9	lung	112	A
12	tibia	19	59	multifocal (lung)			83	A
13	rib	16	20	pericardium			41	D
14	pelvis	41	11	lung			23	D
15	pelvis	21	6	lung			44	A
16	fibula	24	6	multifocal (lung / pericardium)			25	A
17	spine	14	67	lung			98	A
18	vulva	13	4	multifocal (groin / perianal)				N/A
19	tibia	17	0	pelvis			14	A
20	femur	9	64	skull	4	ribs	203	A
21	scapula	24	6	scapula (LR)			29	D
22	fibula	15	20	skull			31	D
23	neck	42	11	neck				N/A
24	femur	35	27	mamma			76	A
25	pelvis	18	13	pelvis				N/A
26	Spine [2]	14	58	humerus			101	A
27	hallux	14	1	tibia			15	D
28	fermur	10	13	pelvis			23	D
29	pelvis	11	12	pelvis (LR)			12	D
30	tibia	13	48	pelvis			62	D
31	pelvis	17	19	multifocal (pleura)	9	Pleura	37	D
32	pelvis	13	0	humerus			118	A
33	spine	9	0	femur	39	spine	52	A
34	left femur	12	13	right femur			22	D
35	pelvis	20	14	lung			19	D
36	spine	20	19	multifocal (spine)				N/A
37	right tibia	16	38	multifocal (heart / soft tissue mediastinal)			40	D
38	spine	10	14	mandible			25	D
39	femur	17	19	humerus	8	rib	40	D
40	pelvis	10	19	soft tissue (skull temporal)			32	D

Table 1: Course of disease. EFS = event free survival; outcome: A = alive, D = dead; LR = local relapse; N/A = not available; yrs. = years; mths. = months.

2.2 RNA extraction

Three to five 10 µm thick FFPE tissue sections were deparaffinized two times with 500 µl xylene (incubation at 36°C for 15 min), washed twice with 100% ethanol, dried for 2 min using a vacuum concentrator (Bachofer, Reutlingen, Germany), and lysed for 16-24 h in 230 µl of RNA digestion-buffer (20 mM Tris pH 7,5, 20 mM EDTA, 1% SDS in DEPC treated water) with 20 µl of 2% proteinase K solution (Merck, Germany) at 56°C. If lysis was incomplete, another 20 µl of the proteinase K solution were added and incubated for 2-3 h until complete disappearance of tissue fragments. In order to extract RNA from the lysate, 2 ml of Trifast® (PeqLab, Erlangen, Germany, contains guanidine isothiocyanate and phenol) were added and further steps were done according to the manufacturer's protocol. The precipitated RNA was resuspended in 90 µl of DEPC treated water, DNA digested by adding 10 µl of RNase-free DNase (final concentration: 0.1 U/µl, Eurogentec Germany, Cologne, Germany) supplemented by 0,5 µl of RNAguard™ (final concentration: 0,162 U/µl, GE Healthcare, Munich, Germany) and again Trifast® extracted.

RNA concentration and purity was measured at 280 nm in a photometer. The RNA was afterwards stored at -80°C.

2.3 Nested PCR

Reverse transcription and first round PCRs were performed in one tube using the Titan One tube RT-PCR System (Roche, Mannheim, Germany) with 1 µg RNA (in a final reaction volume of 25 µl). Reverse transcription was carried out for 30 min at 50°C followed by first denaturation and inactivation of the Reverse

Transcriptase at 94°C for 2 min. Afterwards, amplification was performed by 35 cycles of 30 sec 94°C denaturation, 30 sec annealing at optimum temperature (Table 2), 60 sec 68°C polymerisation, with extension of the polymerisation-time in the last 25 cycles by additional 5 seconds for each cycle. A final step of 7 min at 68°C was added. To evaluate whether the extracted RNA was appropriate for further analysis, RT-PCR was first performed for a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) fragment and products were analysed by gel electrophoresis on 12% polyacrylamide gels. Only samples in which a GAPDH fragment could be amplified were further analysed. For detection of EWSR1-translocation, first round PCR was followed by nested PCR using 2 µl of first round PCR as template (30 sec 94°C denaturation, 30 sec annealing at optimum temperature, 45 sec 72°C polymerisation). First, all samples were tested for fusion products like EWSR1 7/FLI-1 6 or EWSR1 7/FLI-1 5 (primer pair A) and for EWSR1/ERG fusion products (primer pair B). In case of negative results, primers for longer transcripts (like EWSR1 10/FLI-1 6 or EWSR1 10/ FLI-1 5) were used (primer pair C). To exclude false negative results caused by too much amplification-product, samples were additionally used in a 1:50 dilution within the nested PCR reaction. Each trial was accompanied by positive and negative controls. PCR reactions were performed on a MJ Research PTC 200 Thermal Cycler and products were subsequently analysed by gel electrophoresis. After purification using the Qiagen PCR purification Kit, PCR products were directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the same primers as used in the nested PCR. Sequencing-reaction conditions were: 4 min at 96°C followed by

35 cycles of 30 sec 96°C, 20 sec 50°C and 2 min 60°C. To remove unincorporated dyes from the sequencing reaction, the Qiagen DyeEx 2.0 Spin Kit was used. Capillary electrophoresis was performed on a 3730 capillar sequencer (Applied Biosystems) and the results were evaluated using the Seqscanner programme of Applied Biosystems. The exact exon breakpoints were determined by comparing the sequences with the NCBI database using the Blast 2 software (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?0).

In one case (no. 14 of Table 1), 2 bands were seen after electrophoresis on the polyacrylamide gel. This sample was unsewed on a 1% agarose gel and the bands were separately cut out of the gel and extracted using the Qiaquick gel extraction kit (Qiagen) and afterwards analysed as described above.

PCR	**Primer-pairs**	**Sequence [5' -> 3']**	**Annealing temperature**
First round PCR	A: EWS	TCCTACAGCCAAGCTCCAAGTC	65 °C
	A: FLI-1	GAATTGCCACAGCTGGATCTGC	65 °C
	B: EWS	TCCTACAGCCAAGCTCCAAGTC	65 °C
	B: ERG	GAGTTGGAGCTGTCCGACAGG	65 °C
	C: EWS	GAGAGCGAGGTGGCTTCA	55 °C
	C: FLI-1	GGATCTGATACGGATCTGGC	55 °C
Nested PCR	A: EWS	CAGAGCAGCAGCTACGGGCA	60 °C
	A: FLI-1	AGGGTTGGCTAGGCGACTGCT	60 °C
	B: EWS	CAGAGCAGCAGCTACGGGCA	60 °C
	B: ERG	AGSAGCTCCAGGAGGAATTGCCA	60 °C
	C: EWS	GTGGCTTCAATAAGCCTGGT	55 °C
	C: FLI-1	GGCCGTTGCTCTGTATTCTT	55 °C
GAPDH-PCR	GAPDH	GCATTGCTGATGATCTTGAGGCT	63 °C
	GAPDH	CACCCATGGCAAATTCCATGGC	63 °C

Table 2: Primers used in first round and nested PCR.

2.4 Fluorescence in situ hybridization (FISH)

Three-µm sections were cut from FFPE samples and processed for FISH using the EWSR1 (22q12) dual color, break apart rearrangement probe (Vysis, Abbott diagnostics, Wiesbaden, Germany). This probe consists of a mixture of two FISH DNA probes. The first probe is labelled in spectrum orange and flanks the 5‘ side of the EWSR1 gene and extends inward to intron 4. The second probe is labelled in spectrum green and flanks the 3‘ side of the EWSR1 gene. There is a 7 kb between the two probes. The known breakpoints within the EWSR1 gene are restricted to introns 7 through 10. FISH was performed according to the manufacturer‘s instructions. Cells lacking a t(22q12) rearrangement are expected to show two intact copies of EWSR1. Abnormal cells with t(22q12) show one fusion, one green and one orange signal pattern. For each sample, 20 tumor cells were reviewed for the presence of fused or split green and orange signals. We defined a positive result as > 20% of cells having split signals.

2.5 Tissue Microarray (TMA) and Immunohistochemistry

The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD). Hematoxylin and eosin (H&E) slides were reviewed, representative areas with ES tumor cells were marked on the slides and the corresponding areas on the tissue block were transferred into the recipients blocks, using a 1.5 mm diameter stylet. After construction, 3 µm sections were cut and afterwards dried on slides at 56°C over night. After drying, slides were placed into a Lab vison PT module and treated with Citrate

buffer (Thermo Scientific, pH 6,0) for dewaxing and epitope recovery for 35 min at 95°C. Only slides for pancytokeratin staining were exposed to xylol, a series of alcohol concentrations and washed in aqua dest. for dewaxing and additionally pretreated with Proteinase K (Dako). After dewaxing, S100 staining was performed without pretreatment. Afterwards, slides were incubated with primary antibodies for 25 min:

CD99 (1:500, clone 12E7, Dako), FLI-1 poly (polyclonal antibody, 1:1000, SC-356, Santa Cruz), FLI-1 mono (monoclonal antibody, 1:1000, clone G146-222, BD Pharmingen), pancytokeratin (1:300, MNF-116, Dako), ETAA16 (monoclonal antibody, AK16, 1:400, clone SN 58 1857.15.30, Alexis Switzerland), caspase 8 (polyclonal antibody, 1:50, SC7890, Santa Cruz), neuron-specific enolase (1:3000, BBS/NC/VI-H14, Dako), CD57 (monoclonal antibody, 1:300, clone TB01, Dako), neurofilaments (monoclonal antibody, 1:5000, clone 2F11, Dako) and S100 (1:5000, Z 0311, Dako). This step was followed by incubation with Dako REAL Link, Biotinylated secondary antibodies (20 min), incubation with Dako REAL Streptavidin Alkaline Phosphatase (20 min) and visualisation by RED chromogen (2 x 8 min) using the Dako REAL Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (this Kit is based on the labeled streptavidin-biotin method). Primary antibodies were diluted in Dako Real antibody diluent. All staining-steps were performed using a Dako autostaining system.

3 Results

3.1 Patient data

Twenty-seven patients were male (67.5%) and 13 female (32.5%). Five of 40 patients had metastases or presented with multifocal disease at diagnosis (time from diagnosis until detection of metastases or multifocal disease within ≤ 3 months), 35 patients developed metastases or relapses at least 3 months after initial diagnosis (median time from diagnosis until first metastasis: osseous metastases (n = 12): 28.3 mths., pulmonary metastases (n = 16): 35.1 mths., local relapse (n = 3): 21.7 mths.). Nineteen patients died of disease (median time from diagnosis until death for patients harbouring: pulmonary metastases (n = 7): 4.18 yrs., osseous metastases (n = 7): 2.60 yrs., local relapse (n = 2): 1.71 yrs.). Survival data of 5 patients were not available. Two primary tumor samples were not available. Hence, temporally differing metastases were researched. Seven patients presented 2 contemporaneous first metastases (multifocal) while 9 patients developed secondary metastases.

3.2 Molecular pathology

To analyse whether metastases of ES might in some cases actually represent different tumors, we analysed the EWSR1 translocations of primary tumors and the corresponding metastases of 39 patients by RT-PCR for EWSR1/FLI-1 and EWSR1/ERG fusion transcripts. RNA was extracted from 36 primary, 46 first and 9 secondary metastatic FFPE (n = 71) or FT (n = 20) tumor samples. RT-PCR for a GAPDH-fragment was used to evaluate whether the samples contained suitable RNA for further analysis, and from 80 samples (87.9%) a

GAPDH-fragment could be amplified. All 11 GAPDH negative samples (12.1%) were FFPE samples. As GAPDH negative samples were not further analysed, 32 matched samples (24 x primary tumor / first metastasis; 5 x primary tumor / first metastasis / secondary metastasis; 3 x first metastasis / secondary metastasis) remained for analysis of EWSR1 translocations by RT-PCR using the primers listed in Table 2. In 7 cases no amplificates were detected by RT-PCR in 2 independent RT-PCR attempts. Four of these samples were available and additionally analysed by FISH with break-apart probes for EWSR1. Two of these samples had a break in EWSR1 while 2 samples were not evaluable.

Thus, 27 matched samples (19 x primary tumor / first metastasis; 1 x primary tumor / secondary metastasis; 4 x primary tumor / first metastasis / secondary metastasis; 3 x first metastasis / secondary metastasis) remained for comparison of ES specific fusion transcripts and were sequenced. In all but one cases, one fusion transcript was obtained per sample.

The sample taken from the primary tumor site of patient no. 14 harboured two different fusion transcripts [1 x t(11;22) EWSR1 7/FLI-1 6 and 1 x t(11;22) EWSR1 7/FLI-1 7)]. FISH of this sample revealed that only one t(22q12) rearrangement per cell was present. Thus, the two different EWSR1/FLI-1 fusion transcripts are most likely due to differential splicing and not to the presence of 2 different translocations.

Comparison of the matched samples revealed that all matched pairs carried the same translocation types. Table 3 shows the frequency of the obtained translocation variants revealed by molecular-pathological analysis.

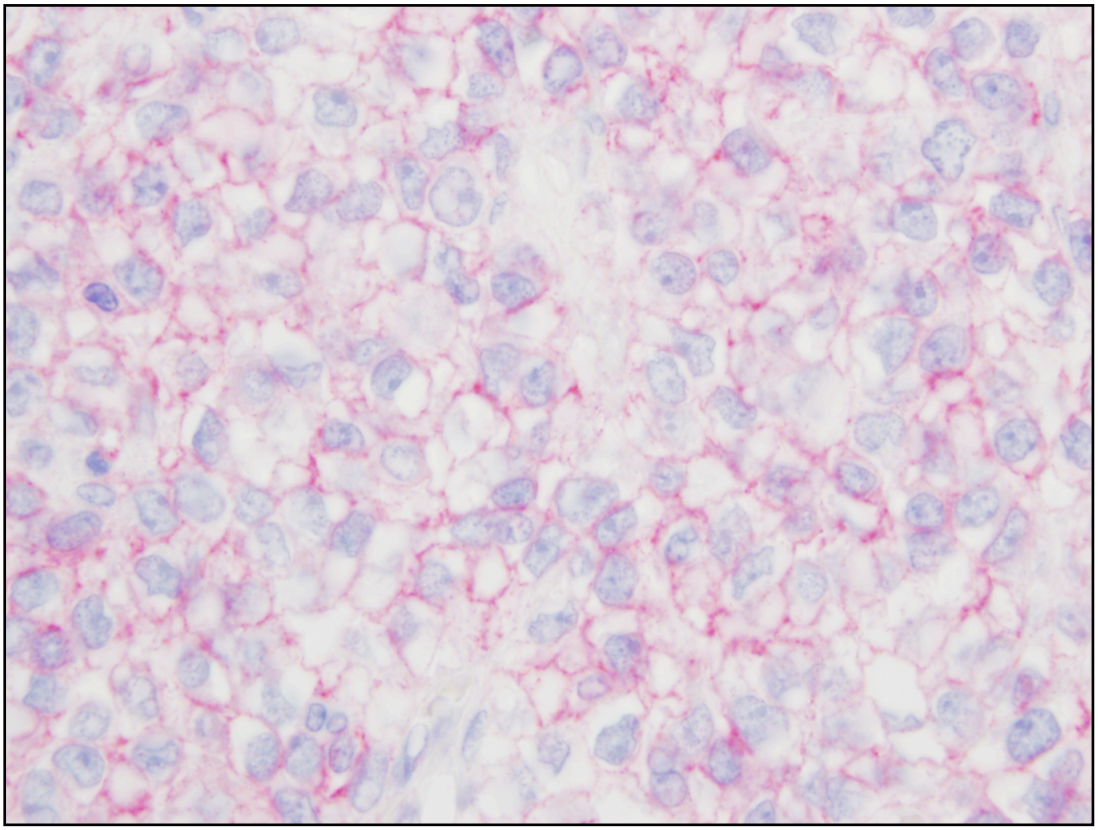
Translocation type	Total n = 74
t(11;22) EWSR1 7/FLI-1 6	45 (60.8%)
t(11;22) EWSR1 7/FLI-1 5	17 (23%)
t(11;22) EWSR1 10 /FLI-1 5	4 (5.4%)
t(11;22) EWSR1 10/FLI-1 8	2 (2.7%)
t(11;22) EWSR1 10/FLI-1 6	2 (2.7%)
t(11;22) EWSR1 7/FLI-1 8	1 (1.4%)
t(11;22) with unknown exon breakpoints	3 (4.1%)

Table 3: Frequency of the obtained translocation types.

3.3 Immunohistochemistry

Fifty-four FFPE specimens taken from 34 of all 40 patients were available for TMA production. The markers listed in Table 4 were previously described in several studies as possible diagnostic tools for ES. The yet published reference data in this Table (second column) give an overview of the normal distribution of these markers in ES. Therefore, immunohistochemical staining of the assembled TMAs were performed in order to evaluate whether corresponding primary and metastatic tumor samples differ in expression of these markers. We scored the results as (+) = positive or (-) = negative. CD99, FLI-1 poly and Ak16 expression in ES tumor cells is shown in Figure 1. In 18 cases, we had the possibility to assemble several tumor samples from each individual patient, so that we were able to observe a chronological sequence of immunohistochemical and PAS staining (14 x primary tumor / first metastasis; 2 x primary tumor / first

metastasis / secondary metastasis; 2 x first metastasis / secondary metastasis). The concordance between the primary tumor site and all of its corresponding metastases or between first and secondary metastases is shown in Table 4. In case of patient no. 1 and no. 13 immunohistochemical staining for AK16 was constantly detectable while CD99 and FLI-1 poly differed in marker expression (Table 5; FLI-1 poly was also negative in a FFPE sample of patient no. 34. Here, only a few tumor cells were seen, CD99 and AK16 were weakly positive in a few of those cells).



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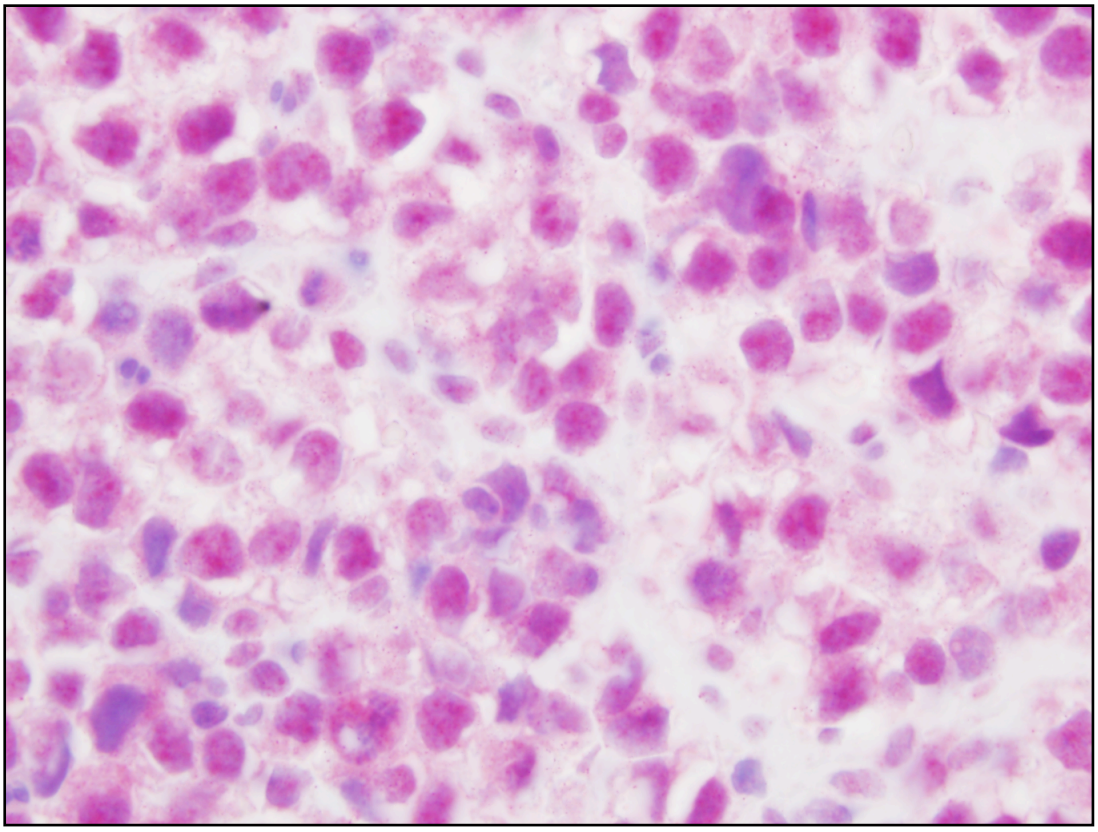

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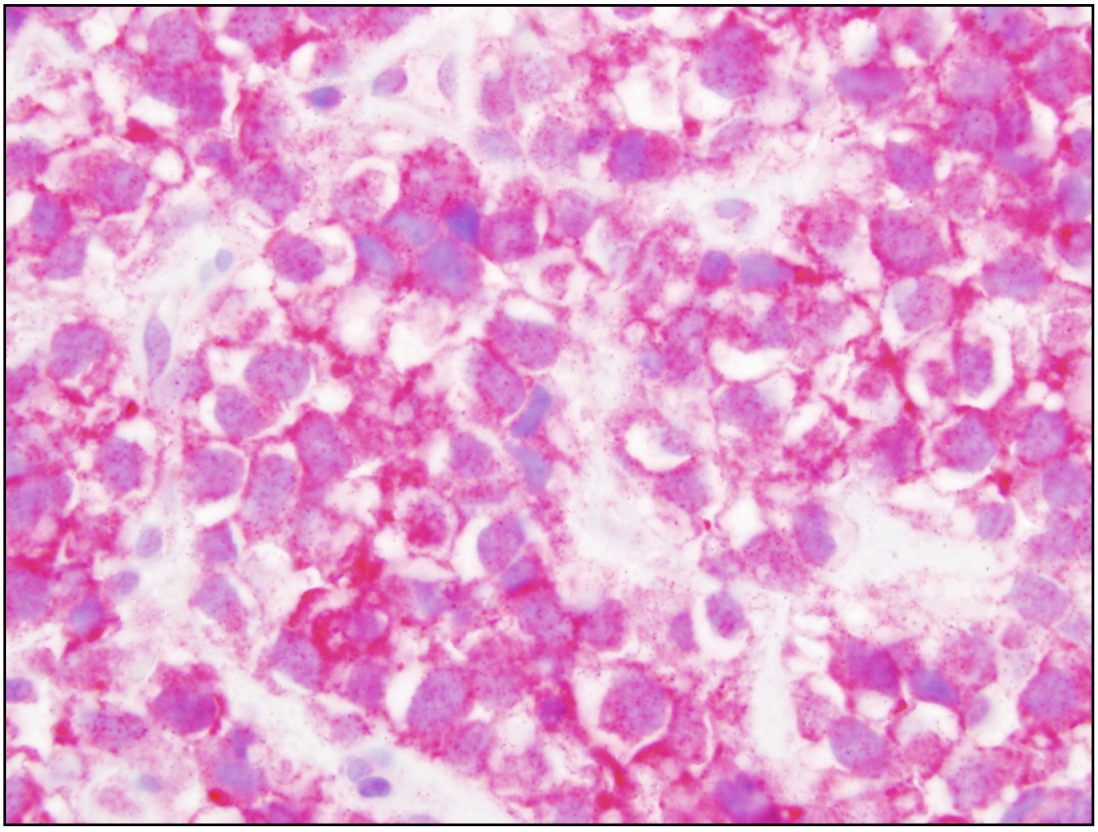

C

Figure 1: CD99, FLI-1 poly and AK16 marker expression. Exemplarily, the tumor cells express CD99 (membranous pattern) (A), FLI-1 poly (nuclear pattern) (B) and AK16 (membranous pattern) (C) (original magnification, x100 for A, B and C).

Antibody	Reference	Primary tumor site	1. metastasis	2. metastasis	Total	Concordance
CD99	93% - 100% [16, 17, 24]	20 / 20 (100%)	25 / 27 (92.6%)	5 / 6 (83.3%)	94.3% (50 / 53)	17 / 18 (94.4%)
panCK	20 - 25 % [11, 13]	4 / 21 (19%)	7 / 27 (26%)	1 / 6 (16.7%)	22.2% (12 / 54)	15 / 18 (83.3%)
NSE	47.5% [19]	10 / 21 (47.6%)	15 / 26 (57.7%)	2 / 6 (33.3%)	50.9% (27 / 53)	9 / 17 (52.9%)
S100	44% [17]	5 / 21 (23.8%)	10 / 27 (37%)	3 / 6 (50%)	33.3% (18 / 54)	10 / 18 (55.6%)
NF	12.5% [4]	5 / 21 (23.8%)	7 / 26 (26.9%)	3 / 6 (50%)	28.3% (15 / 53)	10 / 17 (58.8%)
FLI-1 poly	71 - 81% [12, 16]	21 / 21 (100%)	25 / 27 (92.6%)	5 / 6 (83.3%)	94.4% (51 / 54)	15 / 18 (83.3%)
FLI-1 mono	97 - 100 % [16, 20]	18 / 21 (85.7%)	22 / 27 (81.5%)	4 / 6 (66.7%)	81.5% (44 / 54)	12 / 18 (66.6%)
AK 16	not available	21 / 21 (100%)	27 / 27 (100%)	6 / 6 (100%)	100 % (54 / 54)	18 / 18 (100%)
Caspase 8	92.6% [7]	20 / 21 (95.2%)	22 / 27 (81.5%)	5 / 6 (83.3%)	87% (47 / 54)	13 / 18 (72.2%)
CD57	0% - 25% [4, 17]	7 / 21 (33.3%)	12 / 27 (44.4%)	4 / 6 (66.7%)	42.6% (23 / 54)	8 / 18 (44.4%)
PAS	84 % [23]	20 / 21 (95.2%)	27 / 27 (100%)	6 / 6 (100%)	98.1% (53 / 54)	17 / 18 (94.4%)

Table 4: Staining data for immunohistochemical markers used in this study and PAS staining. Concordance includes all available samples of each patient. PanCK = pancytokeratin, FLI-1 poly / mono = polyclonal / monoclonal antibody. PAS = Periodic acid-schiff reaction.

Patient no.:	Primary tumor site				
	CD99	FLI-1 poly	AK16	Fusion transcript	FISH t(22q12)
1	N/A	N/A	N/A	t(11;22) 7/6	N/A
13	+	+	+	t(11;22) 7/6	+
	1. metastasis				
	CD99	FLI-1 poly	AK16	Fusion transcript	FISH t(22q12)
1	-	+	+	-	+
13	-	-	+	-	N/E
	2. metastasis				
	CD99	FLI-1 poly	AK16	Fusion transcript	FISH t(22q12)
1	-	-	+	N/E	N/E
13	N/A	N/A	N/A	N/A	N/A

Table 5: Data of immunohistochemical staining, RT-PCR and FISH of patients no. 1 and no. 13 (+ = positive, - = negative; N/A = not available; N/E = not evaluable).

4 Discussion

The diagnosis of ES is based on molecular-pathological techniques and immunohistochemical examination of tumor samples. We once found 2 separate ES with distinct EWSR1 translocations in one patient and this disposed us to analyse primary tumors and corresponding metastases for differing translocation types. [2] We also analysed whether immuno-histochemical marker expression varies during the course of disease.

Thirty-nine patients were analysed for ES specific fusion transcripts in primary and metastatic tumor samples. All thereby received 27 matched tumor samples carried the same fusion transcripts. We additionally analysed 4 GAPDH positive FFPE samples lacking ES specific fusion transcripts by FISH. Two samples showed a t(22q12) rearrangement, while 2 samples were not evaluable. This indicates that FISH can improve and supplement the diagnostic specificity in ES. The appearance of 2 different ES in one patient with distinct EWS translocations is - according to this - a rare event.

The immunohistochemical markers were previously described as important tools for diagnostic differentiation of ES. The best immunohistochemical staining data in this study were provided by the monoclonal antibody AK16. It was positive in all FFPE samples. AK16 recognises a surface antigen named ETAA16. Borowski et al. described that surface expression of ETAA16 seems to be restricted to ES cells – other small, blue, round cell tumors did not show an ETAA16 surface expression - and represents a highly selective and unique feature of ES cells. [3, 22] We are not aware of any published data of AK16

expression in FFPE samples. Therefore, further analyses should be made to confirm the sensitivity and especially the specificity of AK16.

CD99, a cell-surface glycoprotein, is known to be a highly sensitive immunohistochemical marker for ES. [6, 24] In this study, it was negative in 3 samples and we were not able to detect a ES specific fusion transcript by RT-PCR in these tumor samples.

FLI-1 poly is a relatively sensitive and highly specific marker, while FLI-1 mono is more sensitive but lacks specificity. [12, 20] Mhawech-Fauceglia proposed, that the most sensitive and specific test panel for the diagnosis of ES / PNET is the combination of CD99 and FLI-1 poly. [16] Here, the FLI-1 poly antibody was – in comparison to the FLI-1 mono antibody - a highly sensitive immunohistochemical marker for ES. It was negative in 3 samples as well. Due to the fact that all CD99 and FLI-1 poly negative samples were taken from metastases, chemotherapeutical treatment might have affected the tumor cells. Both, CD99 and FLI-1 poly showed a high sensitivity and a comparable high concordance. Pancytokeratin was expressed in about 22% of samples and the concordance was comparatively high. This indicates that pancytokeratin - if expressed - is a relatively stable marker. PAS was as well a stable and sensitive marker, but its diagnostic benefit remains inferior. Caspase 8 was described as a sensitive marker for ES, but its expression showed intertumoral and intratumoral variation in intensity and heterogeneity of staining. [7] In our study, concordance data were inferior to those of AK16, CD99 and FLI-1 poly. Neuron-specific enolase (NSE), S100, neurofilaments (NF) and CD57 also revealed inappropriate concordance data. NSE, S100, CD57 and NF can be helpful to

identify the neuronal differentiation of ES cells [8], but the concordance data show that there is a high variation rate for each marker in between the primary tumor and its metastases.

In concordance to the literature, our collective showed comparable clinical data. [5] Patients who developed pulmonary metastases showed a longer event-free-survival than patients who developed osseous metastases. Furthermore, patients harbouring osseous metastases died earlier than patients that had pulmonary metastases. Ancillary, patients with local relapses died earlier than patients with pulmonary or osseous metastases, indicating that local relapsing is intended by a worst prognosis.

Taken together, metastases of ES usually carry the same translocations as the corresponding primary tumors and the presence of two different ES with distinct translocations in one patient is a rare event. FISH can improve and supplement the diagnostic specificity in ES. AK16, CD99 and FLI-1 poly should be used for immunohistochemical diagnostics of ES if RT-PCR does not detect ES specific fusion transcripts in FFPE samples. AK16 seems to be the most sensitive marker in metastases.

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Danksagung

An dieser Stelle möchte ich all denen Dank aussprechen, die zur Entstehung dieser Arbeit beigetragen haben.

Frau Prof. Dr. Gabriele Köhler danke ich für die freundliche Überlassung dieses Themas, die intensive Betreuung und ihr Engagement bei der Erstellung dieser Arbeit.

Weiterhin gilt mein besonderer Dank den (teils ehemaligen) wissenschaftlichen und technischen Mitarbeitern des Gerhard-Domagk-Instituts für Pathologie für die vielen wertvollen Ratschläge und Hilfeleistungen. In diesem Zusammenhang sind insbesondere Herr Prof. Dr. Bräuninger, Herr Dr. Minin, Herr Dr. Schmidt, Frau Fischer und Frau Brune zu nennen. Frau Kloss möchte ich für die Unterstützung bei der Zusammenstellung der klinischen Daten danken.

Nicht zuletzt gilt mein Dank meiner Familie und meiner Freundin Marga, die durch die große Unterstützung während meines Studiums und ihre unermüdliche Motivation die Fertigstellung dieser Arbeit ermöglicht haben.

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