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RESEARCH

The Evaluation of CD99 Immunoreactivity and EWS/FLI1 Translocation by Fluorescence in situ Hybridization in Central PNETs and Ewing's Sarcoma Family of Tumors

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Abstract Ewing's sarcoma family of tumors (ESFTs) are indicated by malignant, small, round and blue cell tumors of the bone and soft tissue. Gene rearrangements between EWS gene on chromosome 22q12 and members of the ETS gene family are common in and specific to ESFTs. Another defining characteristic of ESFTs is their membranous expression of the CD99. In contrast, such translocations and immunoreactivity are not found in central primitive neuroectodermal tumors (cPNETs). The aim of this study was to investigate the detection of EWS/FLI1 translocations and CD99 immunoreactivity in order to evaluate their clinicopathological features and their roles in the differential diagnosis of these tumors. In this study, we investigated CD99 immunoreactivity using immunohistochemistry and Ewing's sarcoma / Friend leukaemia virus integration 1 (EWS/FLI1) translocation using the fluorescence in situ hybridization (FISH) method in 23 cases. CD99 expression was detected in 10/11 (90%) ESFT cases and 2/7 cPNET cases. In 18 cases EWS/FLI1 translocation was examined using the FISH method. The EWS/FLI1 translocations were detected in 7/8 (87.5%) ESFTs cases, whereas non of 8 cPNET cases were detected with this translocation. One case could not be classified as either central or peripheral, showed EWS/FLI1 translocation. There was a statistically significant difference in CD99 expression (p=0.0013) and EWS/FLI1 translocation (p=0,002) between cPNETs and ESFTs cases. In conclusion, CD99 expression and EWS/FLI1 translocation are specific and sensitive markers in the diagnosis of ESFTs. However, these were often not found in cases of cPNET. Therefore, in the diagnosis of ESFTs, clinical, radiological, histopathological and immunohistochemical parameters should always be evaluated together.

Keywords Ewing's sarcoma family of tumors · Peripheral primitive neuroectodermal tumor · CD99 · EWS-FLI1 translocation · Fluorescence in situ hybridization

Abbreviations

ESFTs	Ewing's sarcoma family of tumors
EWS	Ewing's sarcoma
cPNETs	Central primitive neuroectodermal tumors PNET
EWS/FLI1	Ewing's sarcoma / Friend leukaemia virus integration 1
FISH	Fluorescence in situ hybridization
PNET	Primitive neuroectodermal tumor
pPNET	Peripheral primitive neuroectodermal tumors PNET
EWS/FLI1	Ewing Sarcoma (EWS) / Friend leukaemia virus integration 1 (FLI1)
RT-PCR	Reverse transcriptase-polymerase chain reaction

Introduction

Primitive neuroectodermal tumor (PNET) is a malignant, embryonal neoplasm consisting of poorly differentiated

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neuroepithelial cells occurring in the children and young adults. Recently, these tumors were divided into central PNET (cPNET) such as medulloblastoma and supratentorial PNET, originating in the central or sympathetic nervous system, and peripheral PNET (pPNET), originating in the soft tissue or bone [1, 2]. Although Ewing's sarcoma and pPNET were orginally regarded as totally separate entities, it has subsequently been established that both Ewing's sarcoma and pPNET share a balanced translocation in over 90% of cases [t (11;22)(q12;q24)] and they are now almost universally regarded as ends of a common histologic spectrum, known as the "Ewing's Sarcoma Family of Tumors (ESFTs)" [3, 4]. These groups of tumors include Ewing's sarcoma (EWS: in the osseous and extraosseous forms), pPNET, and malignant small round blue cell tumors of the thoracopulmonary region (Askin tumor) [5, 6].

ESFTs are relatively uncommon, account for only 6–8% of primary malignant bone tumors [5, 7–9]. The mean age at diagnosis is around 14 years [5, 10, 11]. It generally tends to arise in the diaphysis and metaphyseal-diaphyseal portion of long bones (45%, most commonly in the femur and tibia), pelvic bones (27%) and the chest wall (19%). It may occasionally affect the soft tissue, skin and visceral organs [9, 10].

The histologic features of ESFTs and cPNETs include a solidly packed, lobular pattern of strikingly uniform round cells which have a scant cytoplasm and hyperchromatic nuclei with inapparent or small nucleoli [3]. Approximately 90% of ESFT cases are CD99 positive, while CD99 immunoreactivity is not a feature of cPNETs [12]. However ESFTs and cPNETs share similar immunoprofile, as they are reactive for neural markers, including neural spesific enolase, neurophilament, CD-56, Leu-7, S-100 protein, synaptophysin, chromogranin, and PGP9.5 [3, 13, 14].

ESFTs are characterized by a chromosomal translocation resulting in the production of the Ewing Sarcoma (EWS) / Friend leukaemia virus integration 1 (FLI1)(EWS/FLI1) fusion gene [15]. Almost all ESFTs (more than 95% of cases) express non-random chromosomal translocations mainly t(11;22)(q24;q12) or t(21;22)(q22;q12). These translocations result in a fusion with 5′ portion EWS gene located on 22q12 region and either the 3′ portion of FLI1 gene located on 11q24 region (90–95% of cases) or ERG (ets-related gene) gene located on 21q22 region (5–10% of cases) [16]. In addition to t(21;22)(q22;q12), other minor changes like t (7;22), t(17;22) and t(2;22) and inv(22) may also confirmed in these lesion spectrum. EWS/FLI1 fusion gene is rarely reported in other tumors, and it is extremely specific to the diagnosis of ESFTs [13,17-19].This nonrandom translocation and CD99 immunoreactivity are not found in cPNETs.

The aim of this study was to investigate the detection of EWS/FLI1 translocations and CD99 immunoreactivity in a total of 23 patients, diagnosed with ESFTs and cPNETs at the Department of Pathology at Gazi University Faculty of Medicine, by using the FISH method and immunohistochemistry respectively, in order to evaluate their clinicopathological features and their roles in the differential diagnosis of these tumors.

Materials and Methods

Patients

In this study a total of 23 patients, diagnosed as ESFTs and cPNETs between the years 1999–2007 at the Department of Pathology at Gazi University Faculty of Medicine, were examined. Hematoxylin-eosin and immunohistochemical stained sections of all cases were re-evaluated retrospectively. Patient details (age, tumor size, radiological information, treatment and prognosis) were recorded from the follow-up files (Table 1).

Immunohistochemistry

Only 18 of 23 cases were available for immunohistochemical analysis for CD99. Tissue sections of 4 μm were cut from representative formalin-fixed and paraffin-embedded tissue blocks. Sections were deparafinized in xylene and rehydrated. Immunoperoxidase staining was performed using the streptavidin-biotin peroxidase method. The sections were treated with 0.3% H_2O_2 to suppress endogenous peroxidase activity. Antigen retrieval was performed with microwave processing for CD99 (DakoCytomation, Denmark) using 0.01 M citrate buffer (pH 6.0). Diaminobenzidine substrate (DAB, LabVision, NeoMarkers) was used as a chromogen for color development. The slides were counterstained with hematoxylin, dehydrated, and mounted. In the negative control, the primary antibody was replaced by PBS. Membranous staining was considered positive immunoreactions for CD99. The extent and intensity of expression was semiquantitatively evaluated.

Fluorescence In Situ Hybridization (FISH)

Sections of 4 μm thickness were cut from representative blocks onto positively charged slides. After dewaxing the slides, they were immersed in 0.2 N HCL for 20 min and pretreatment solution (Paraffin Pretreatment Kit, Vysis) at 80°C for 30 min. They were then digested with protease for 60 min at 37°C, washed in 1 x phosphate buffer saline for 5 min at room temperature, fixed in 10% formaldehyde for 10 min at room temperature, washed in 1 x phosphate buffer saline for 5 min at room temperature and dehydrated by immersing 70%, 85% and 100% ethanol for 1 min each at room temperature.

Table 1 ESFT and cPNET cases types, clinical features, CD99 immunoreactivity and EWS/FLI-1 translocations using the FISH method

Case	Age	Sex	Location	Tumor Size	Stage	Treatment[a]	Follow-up Time (month)	Outcome	FISH	CD99	ESFTs Type[b]
1	N/A	M	Epigastric region	15	N/A	N/A	N/A	N/A	N/P	Positive	EES
2	22	M	Proximal part of left humerus	N/A	N/A	N/A	N/A	N/A	N/P	Positive	EWS/pPNET
3	13	F	Left femur	13	IV	S+C	17	Exitus (progressive disease, metastasis)	Positive	Positive	EWS/ pPNET
4	9	F	Left frontoparietal region	6	N/A	S+C+R	33	Exitus (herpesencephalitis)	Negative	Negative	Central PNET
5	16	M	Around left lateral ventricle	N/A	I	S+C+R	54	Remission	Negative	Negative	Central PNET
6	19	M	Left temporal region	N/A	N/A	S+C+R	N/A	N/A	Negative	Negative	Central PNET
7	9	M	Right tibia	14	N/A	S+C	N/A	N/A	N/D[c]	Negative	EWS/ pPNET
8	19	F	Left frontoparietal region	8	N/A	S	37	Remission	Negative	Negative	Central PNET
9	15	F	Left frontoparietal region	7	N/A	S	N/A	Recurrence	Negative	Positive	Central PNET
10	8	M	Right mandibula, corpus	N/A	N/A	N/A	N/A	N/A	N/P	Positive	EWS/ pPNET
11	15	M	Right chest wall	6,5	IV	S+C+R	21	Exitus	Negative	N/P	Askin tumor
12	10	F	Left parietooccipital region	N/A	N/A	S+C+R	81	Recurrence	Positive	N/P	Central/peripheral PNET?
13	2	F	Suprasellar region	5,5	N/A	S+C+R	19	Remission	Negative	N/P	Central PNET
14	16	F	Kidney with brain metastasis	4	IV	C+BMT	17	Progressive disease with metastasis	Positive	Positive	EES
15	17	M	Right infrahiatal region	N/A	N/A	N/A	N/A	N/A	Positive	Positive	EES
16	18	F	Intranasal region	N/A	N/A	S+C	N/A	N/A	Positive	Positive	EWS/pPNET
17	29	F	Right tibia	N/A	IV	C	14	Progressive disease with metastasis	Positive	Positive	EWS/pPNET
18	17	F	Amputation stump, left lower extremity	N/A	N/A	N/A	N/A	N/A	Positive	Positive	EWS/pPNET
19	12	F	Left pontocerebellar angle	5	N/A	S+C+R	8	Residual tumor	Negative	Positive	Central PNET
20	28	M	Thoracal-extradural 4–6	N/A	N/A	N/A	N/A	N/A	Positive	Positive	EES
21	12	F	Supratentorial region	8	N/A	S	4	Refracter	Negative	N/P	Central PNET
22	12	M	Left temporobasal region	6	N/A	S+C+R	75	Remission	N/P	Negative	Central PNET
23	26	F	Left frontal region	4	N/A	S	1	Postoperative	N/P		Central PNET

N/A Not available, *N/P* Not performed

[a] Treatment; S:surgery, C: chemotherapy, R: radiotherapy, BMT: Bone marrow transplantation

[b] ESFT type; EES: Extraosseous Ewing sarcoma, EWS/PNET: Ewing sarcoma/primitive neuroectodermal tumor in bone

[c] N/D: Not determined; We have not evaluated EWS/FLI1 translocation after decalcification process with formic acid

For interfase FISH, the slides were subjected to hybridisation with an LSI EWSR1 (22q12) dual color, breakapart rearrangement probe (Vysis). The probe consisted of a mixture of 2 FISH DNA probes. The first was a 500-kb probe, labelled orange in the spectrum, flanking the 5′side of the EWSR1 gene extending inward to intron 4. The second probe was a 1,100 kb probe, labelled green in the spectrum, and flanking the 3′ side of the EWSR1 gene (22q12). The FISH probe mix (10 μl) was added to the sample area of the slides. The slides were coverslipped, sealed with rubber cement, and incubated at 73°C for 5 min and 37°C for 16 h in a humidified chamber. The slides were then washed in posthybridisation wash buffer at 73°C. Subsequently, 10 μl of DAPI counterstain was placed on the slide, which was then coverslipped. After hybridisation, all slides were maintained in complete darkness at −20°C. Hybridisation signals were visualised with an epifluorescence microscope. In normal cells with intact 22q12 region, each of EWSR1gene alleles reflect itself with dual orange and green signal pattern next to each other. In the presence of translocations related to the 22q12 region of the gene allele, one orange (denoting the 5 primed site of the gene) and one green (denoting the 3 primed site of the gene) signals apart from each other is expected For each sample, a minimal of 200 non-overlapping tumor cells were evaluated for the presence of both normal (two fused orange and green signal) and abnormal (at least one apart green and red signal) signals. A positive result was defined as >20% of cells having apart orange and green signals.

Statistical Analysis

SPSS 15.0 was used for the statistical analysis of the data and the comparisons were made using the chi-square test with significance defined as $p<0.05$. The results were expressed as median values.

Results

The clinical features, immunohistochemical CD99 positivity and EWS/FLI-1 translocations performed with the FISH method on the series of 23 cases are shown in Table 1. The cases were classified into 7 cases of EWS/pPNET (30,4%) , 4 cases of EES (17,4%), 10 cases of central PNET (43,5%), 1 case of PNET (not classifiable as either central or peripheral) (4,35%), and 1 case of Askin tumor (4,35%). Thirteen (57%) of the patients were female and 10 (43%) were male. The ages of the patients ranged between 2 years and 29 years, (median, 16 years). The size of the tumors ranged between 4–15 cm (median, 7 cm). The median value of patients' follow-up time was 19 months (range 1–81 months). The most common location of the ESFTs was the extremities (5 cases, 42%), and in descending order, abdomen (3 cases, 25%; in 1 case, the tumor originated in the kidney and metastasized to the brain), head and neck (2 cases, 17%), thorax (1 case, 8%) and vertebra (1 case, 8%).

Histologically, either ESFTs or cPNETs were composed of sheets of uniform primitive neoplastic cells with scant cytoplasm, hyperchromatic nuclei with irregular nuclear contours and inconspicuous nucleoli (Fig. 1). In some cases extensive areas of necrosis and viable tumor cells around the blood vessels were observed. In addition, Homer-Wright rosettes and Flexner-Wintersteiner rosettes were seen in some cases (Fig. 2).

CD99 expression was immunohistochemically evaluated in 18 patients that 11 of them was ESFT cases and 7 of them was cPNET cases. The CD99 antibody stained the tumor cells diffusely in 10 of 11 ESFT cases (90%) and 2 of 7 cPNET cases. There was a statistically significant difference with CD99 expression between cPNETs and ESFTs ($p=0.0013$).

In 18 cases EWS/FLI1 translocation was examined using the FISH method. 8 of these patients was ESFTs, 8 of them was cPNETs, and 1 of them was unclassifiable as either central or peripheral PNET. Due to nonoptimal staining, 1 patient could not be evaluated. The EWS/FLI1 translocations were detected in 7 of 8 ESFT cases (87.5%) [one patient had brain metastases originating from the kidney (EES)] (Fig. 3), whereas non of 8 cPNET cases were detected with this translocation. The remaining 1 case could not be classified as either central or peripheral, showed EWS/FLI1 translocation. In EWS/FLI1 translocations, there was a statistically significant difference between cPNETs and ESFTs ($p=0,002$).

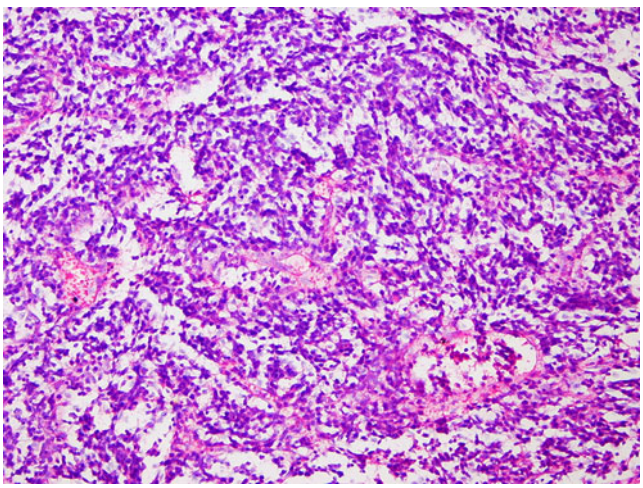

Fig. 1 EWS/PNET: the tumor contains small, round cells with hyperchromatic nuclei, and scant cytoplasm (Hematoxylin-eosin x 200)

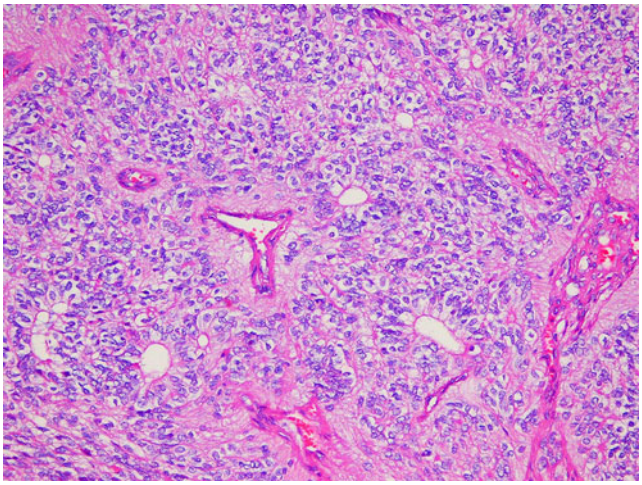

Fig. 2 EWS/PNET: small, round, blue cell tumor consisting of a large number of cells in rosette formation (Hematoxylin-eosin x 200)

Discussion

ESFTs are the second most common malignant neoplasms of the bone among children and adolescents after osteosarcoma [3, 7, 8, 10]. ESFTs may arise virtually anywhere in the body, including the bones, soft tissue, skin and visceral organs, but it is rarely found in the intracranial region. Similar to the literature, in our study; the tumors were mostly located in extremities (5 cases, 42%), followed by the abdomen (3 cases, 25%), head and neck (2 cases, 17%), thorax (1 case, 8%), and vertebra (1 case, 8%), respectively [10, 21].

PNETs which are located in intracranial region are divided into 2 groups called central PNET (cPNET) and peripheral PNET (pNET; extraosseous Ewing's sarcoma of the central nervous system). cPNET arises from the central or sympathetic nervous system, whereas there could also be cases of pPNET arising from cranial soft tissue and bone [1–3, 9, 21–23]. Although both groups seem to have the same histological pattern, a distinction should be made due to the differences in their biological behavior and treatment protocols [24, 25]. The absence of t(11; 22) and CD99 positivity, and the lack of invasion of the tumor in the brain or skull with the dura mater and neighbouring structures may help to distinguish cPNET from pPNET [14, 24]. In our study, 10 cases had primary intracranial locations (cPNET). They were located intraparenchymal and were unrelated to the skull, as such, these cases were accepted as cPNETs. One case, which presented with intracranial-intraparenchymal located recurrence, could not be distinguished as central or peripheral PNET, as the radiological findings regarding connection between localization and bone structure were not known.

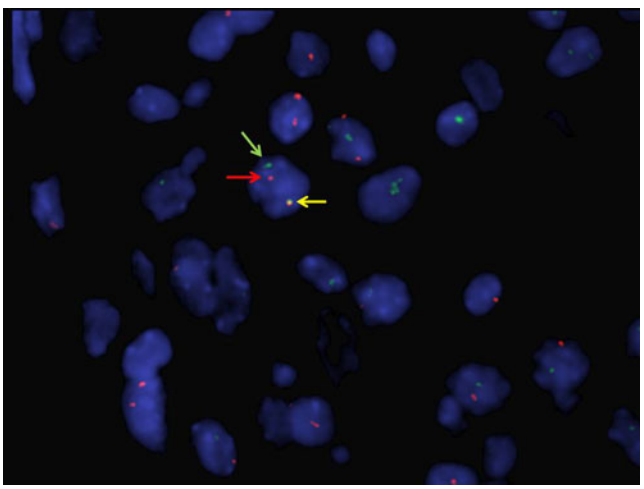

Fig. 3 Result of EWSR1 (22q12) dual colour break apart rearrangement probe by FISH method. Tumour cells of a EWS/PNET show one fusion (yellow arrow), one orange (red arrow), and one green (green arrow) signal pattern, indicative of a rearrangement of one copy of the EWSR1 region. (x 400 and 450 nm. wave lenght)

ESFTs constitute a family of neoplasms characterized by a continuum of neural differantiation, where EWS is at the most undifferantiated end of the spectrum and PNET is at the other extreme. The histologic evidence of rosette formation, the demonstration of immunohistochemical evidence of neural differentiation in PNET, more diffuse and uniform immunoreactivity with CD99, stronger vimentin positivity than PNET, and presence of intracytoplasmic glycogen in EWS may help to separate these two tumors [3, 13]. Schmidt et al stated in their study, that the cases diagnosed as PNET showed a more aggressive clinical course than those diagnosed as EWS. However, other prospective studies found that there were no clinical differences between these two types of tumors. It may be suitable to classify these tumors as members of ESFTs, in addition to ascertaining presence or absence of the neural differantiation of light microscopic, immunohistochemical, or ultrastructural features [3].

The functional role of CD99 is not well known. CD99 (also known as HBA-71 antigen, O-13, glycoprotein p30/32, or MIC2 gene product), a 32-kDa transmembrane protein, is encoded by the MIC2 gene located in the end of the short arm of the X and Y chromosomes [9]. CD99 engagement, using monoclonal anti-CD99 antibodies, has been found to induce apoptosis, inhibit growth and increase sensitivity to chemotherapeutic agents in ESFTs cell lines [3, 10, 14, 19, 26–28]. Although CD99 is usually positive (90%) in ESFTs, this marker is not specific for these tumors, as CD99 can also be expressed in other small, round, and blue cell tumors, as well as many neoplastic and normal tissues. (Table 2) [26]. Immunohistochemistry for CD99 is usually detected in ESFTs but is not detected in cPNETs [1, 16, 21–25]. In our study we found CD99 positivity in 10 of 11ESFT cases (90%), and 2 of 7 cPNET cases were detected CD99 immunoreactivity, similar to the literature. There was a

Table 2 Normal and Neoplastic tissues that can express immunoreactivity for CD99 [5]

Normal tissue	Neoplastic tissue
Some lymphocytes	ES/PNET
Some columnar epithelia	T-cell lymphoblastic lymphoma
Pancreatic islets	Poorly differentiated synovial sarcoma
Renal collecting ducts and distal convulated tubules	Small cell osteosarcoma
Urothelium	Rhabdomyosarcoma
Vaginal squamous epitelium	Desmoplastic small round cell tumor
Sertoli cells	Small cell carcinoma
Granulosa cells	Merkel cell carcinoma
Fibroblasts (variable)	Neuroblastoma
Endothelium (variable)	Leiomyosarcoma
	Malignant fibrous histiocytoma
	Chondrosarcoma
	Fibrosarcoma
	Thymoma
	Schwannoma
	Astrocytoma
	Neuroendocrin tumors
	Bladder carcinoma
	Ependymoma
	Wilms tumor
	Glioblastoma
	Uterine stromal sarcoma

statistically significant difference in CD99 expression between intracranial located cPNETs and other locations of ESFTs (p=0.0013). It has been considered that in the differential diagnosis of cPNETs, which has a different treatment protocol and worse prognosis, CD99 negativity may be used in addition to radiological findings.

Only two cases of cPNETs showed CD99 positivity without EWS/FLI1 translocation and although we repeated the immunohistochemical procedures several times, the positive membranous staining for CD99 retained in those cases. Since the number of the cPNETs were very limited, we could not make a statistically significant conclusion. In our opinion, CD99 immunohistochemistry should not be the only criterion for differentiation cPNETs from pPNETs.

The reciprocal translocation of the EWS gene in 22q12 is characteristic of ESFTs. t(11;22)(q24;q12) (EWS/FLI1 translocation) is detected in ESFTs with extremely high frequencies (up to 95%) [26]. These translocations can be detected with conventional cytogenetic studies, Southern blot and Northern blot analyses, FISH and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques [29–31]. Although extremely rare cases of olfactory neuroblastoma, small cell osteosarcoma, mesenchymal chondrosarcoma, polyphenotypic round cell tumor, desmoplastic small round cell tumor, neuroblastoma and rhabdomyosarcoma have been reported to contain either EWS/FLI1 or EWS/ERG fusion genes, the identification of these translocations is for the most part regarded as highly specific to ESFTs and is increasingly recognized as the "gold standard" for diagnosis [3, 4, 13, 17–20]. In contrast, such translocations are not found in cPNETs [1, 2, 16, 21–25]. Thorner et al reported that identification of the t(l1;22) (q24; q12) by cytogenetics should not be taken as absolute proof of a diagnosis of ESFTs in the absence of confirmatory histology to include positive staining for MIC2 [28]. In our study, the rate of EWS/FLI1 translocation in ESFTs was 87.5% , whereas none of 8 cPNET cases were detected with this translocation. Other than from central PNETs, we found only 1 negative EWS/FLI1 translocation located in the chest wall, which is supported by the literature showing EWS/FLI1 translocation to be fairly sensitive in the diagnosis of ESFTs. In addition, it is known that decalcificated preparations may not show this translocation [29]. In our study, we could not evaluate EWS/FLI1 translocation using the FISH method in a tumor located in right tibia after performing the decalcification process using formic acid.

In our series, two cases located intracranially showed EWS/FLI1 translocation and CD99 positivity. One of them was a metastatic brain mass originated from a kidney tumor (case 14). The other case (case 12) was a recurrent mass which we have learned this feature from the clinical history. Although this tumor was unrelated to bone and soft tissue of the cranium, due to presence of EWS/FLI1 translocation and CD99 positivity, we thought that this case may indicate pPNET (extraosseous Ewing Sarcoma of central nervous system). However, we could not reach a clear conclusion, due to the fact that the radiologic findings of this case, prior to the first operation, could not be accessed.

It has been concluded that detection of EWS/FLI1 translocation could be used for distinguishing central and peripheral PNETs. It is important to separate these tumors from each other, due to comparatively worse prognosis of cPNETs. In our study, there was no statistically significant difference between central and peripheral PNET in the clinical course (p=0.536).

Conclusion

CD99 immunoreactivity and EWS/FLI1 translocation were fairly specific and sensitive in the diagnosis of ESFT group tumors. However, in cPNET cases, these analyses were negative. Due to the fact that translocations and CD99 immunoreactivity can rarely be detected in other kinds of tumors, in such cases clinical, radiological, histopathological and immunohistochemical parameters should be always evaluated together.

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