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M. Brinkhuis · L.C.D. Wijnaendts · J.C. van der Linden
A.J.M. van Unnik · P.A. Voûte · J.P.A. Baak
C.J.L.M. Meijer

Peripheral primitive neuroectodermal tumour and extra-osseous Ewing's sarcoma; a histological, immunohistochemical and DNA flow cytometric study

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Abstract Although peripheral primitive neuroectodermal tumour (pPNET) and extra-osseous Ewing's sarcoma (EES) are thought to be closely related neoplasms, their clinical behaviour differs considerably. To determine the clinical relevance of the Schmidt classification scheme for differentiating pPNET and EES, 20 tumour specimens of poorly differentiated round cell tumours were evaluated. In addition, the diagnostic value of several neural markers and the prognostic value of quantitative morphological variables (DNA ploidy, S-phase fraction, and the mitotic activity) were assessed. Homer-Wright rosettes were present in 9 tumours. Neuron specific enolase (NSE) was expressed in 11 tumours, 8 of which expressed a second neural marker (CD57, S100, or neurofilament). According to the Schmidt classification, 11 pPNET and 5 EES were distinguished. HBA-71 was exclusively expressed in pPNET and EES. The remaining tumours were classified as sarcoma not otherwise specified (n = 2), rhabdomyosarcoma (n = 1), and desmoplastic tumour with divergent differentiation (n = 1). EES611 patients fared significantly better than the pPNET patients (100% versus 42% 5-year survival). Neither DNA ploidy nor S-phase fraction assessed in 12 evaluative histograms (9 pPNET and 3 EES), nor mitotic activity yielded information of additional prognostic value. On the basis of this study and the Schmidt classification scheme, it can be concluded that if the diagnosis of EES and pPNET is based on light microscopy (Homer-Wright rosettes) and/or immunohistochemistry (at least two neural markers, i.e. NSE, S-100, CD57, and neurofilament), the classification provides important clinical information. Furthermore, positivity for HBA-71 is helpful in differentiating pPNET and EES from all other small round cell tumours.

Key words Peripheral primitive neuroectodermal tumour · Extra-osseous Ewing's sarcoma
Schmidt classification scheme · DNA flow cytometry
Mitotic avtivity index

M. Brinkhuis · L.C.D. Wijnaendts (✉) · J.C. van der Linden
J.P.A. Baak · C.J.L.M. Meijer
Department of Pathology, Free University Hospital,
de Boelelaan 1117, NL-1007 MB Amsterdam, The Netherlands

A.J.M. van Unnik
Department of Pathology, Groot Ziekengasthuis,
's-Hertogenbosch, The Netherlands

P.A. Voûte
Department of Paediatric Oncology,
Emmakinderziekenhuis/Academic Medical Centre,
Amsterdam, The Netherlands

Introduction

Peripheral primitive neuroectodermal tumour (pPNET) and extra-osseous Ewing's sarcoma (EES) belong to the group of small round cell tumours and are thought to be related neoplasms. Histologically, they are characterized by the presence of small poorly differentiated cells with uniform nuclei and scanty-cytoplasm. Cytogenetically, they share the same reciprocal translocation (11;22) (q24;q12) [6, 34] and express the same oncogenes, i.e. c-*myc*, c-*myb*, c-*ets*-1 and N-*myc* [24]. Additionally, the neoplastic cells of both tumour types share reactivity with HBA-71, an antibody raised against the cell surface antigen MIC2 [1, 15]. The *MIC2* gene is a pseudoautosomal gene, located on the short arms of the sex chromosomes [13]. In the haematopoietic system the MIC2 gene product appears to be involved in cell adhesion processes like rosette phenomenon [12]. Although the distinction between EES and pPNET is often difficult, the need to make this distinction has become apparent from the finding of several authors that pPNET has a worse prognosis than EES [2, 16, 20, 21, 27]. Recently, Schmidt and coworkers [27] presented a new classification scheme for the differential diagnosis of pPNET and EES. This classification scheme was based on the recognition of neural differentiation in pPNET. This neural differentiation was characterized histologically by the presence of Homer-Wright rosettes and/or immunohistochemically by the expression of at least two different neural markers, such as neuron specific enolase (NSE), protein S-100, CD57,

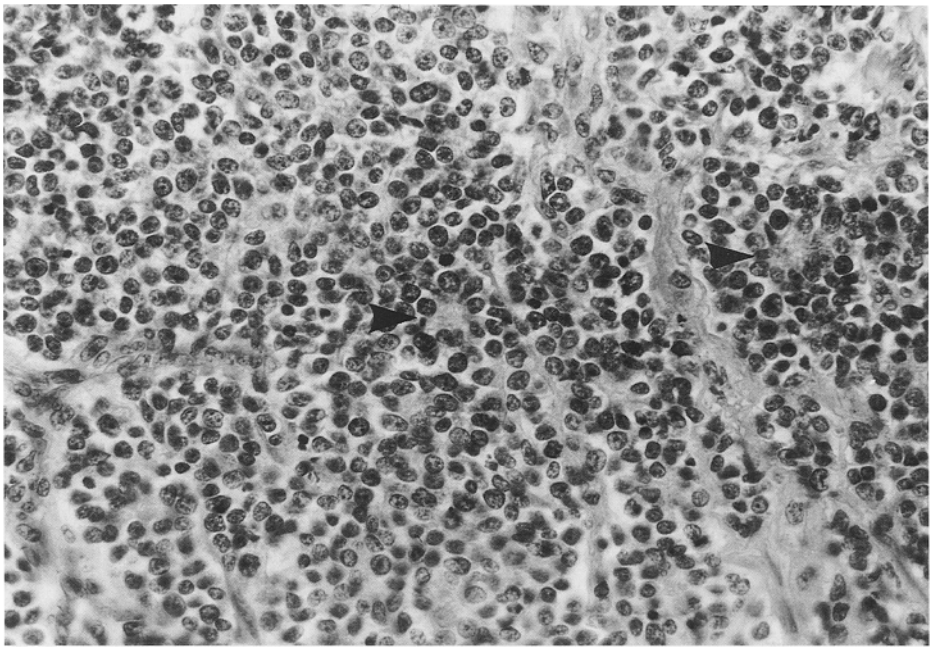

Fig. 1 Histological illustration of a peripheral primitive neuroectodermal tumour in which several pseudorosettes of the Homer-Wright type are present (*arrowhead*). Haematoxylin-eosin stain, ×400

neurofilament (NF), and glial fibrillary acidic protein (GFAP). The presence of ganglion cells was also regarded as sufficient evidence for neural differentiation to warrant the diagnosis of pPNET, but this finding was noticed in only one case. EES was diagnosed in cases lacking Homer-Wright rosettes, ganglion cells, and expressing a maximum of one neural marker in immunohistochemistry.

Alteration in the cellular DNA content, as can be reflected by change in DNA ploidy, increase of the S-phase fraction, and increase of the mitotic activity, was previously shown to be related to the clinical outcome of a number of patients with paediatric tumours. Patients with DNA aneuploid neuroblastoma [11], rhabdomyosarcoma (RMS) [35], and acute lymphoblastic leukaemia [33] fared significantly better than those with DNA diploid tumours.

The aim of the present study was to evaluate the clinical value of the Schmidt classification scheme and to determine whether the immunohistochemical panel could be optimized by the addition of other immunohistochemical markers. Furthermore, the diagnostic and prognostic value of cellular DNA content, S-phase fraction, and mitotic activity was investigated.

Materials and methods

Patients

A total of 20 cases diagnosed between 1969 and 1989 as EES (n = 10), Askin's tumour (n = 2) or RMS (n = 8) were retrieved from the files of the Free University Hospital (Amsterdam, The Netherlands), the Dutch Study group on Paediatric Tumours (WKT) and from the consultation file of one of the authors (A.v.U.). The 8 patients initially diagnosed as having RMS were excluded from a study on RMS, because they showed an undifferentiated morphology on histological re-evaluation. Relevant clinical data were retrieved from patient registration files. All patients were staged at diagnosis according to the TNM classification for soft tissue tumours [28]. There were 13 male and 7 female patients. Their ages ranged from 1 month to 32 years (mean 10.6 years) at the time of diagnosis. All patients were treated with surgery and chemotherapy and/or radiotherapy. Follow-up information was available in all cases; follow-up ranged from 19 months to 15.5 years (median 9 years) in surviving patients. Eleven patients died of their disease.

Conventional light microscopy

The paraffin-embedded formalin-fixed specimens of the primary tumour were available in all cases. Sections were stained with haematoxylin-eosin (H&E), Gomori's silver stain for reticulin and the periodic acid-Schiff (PAS) reagent, with and without diastase for glycogen detection. According to Schmidt and co-workers [27], a diagnosis of pPNET was made only in cases that had Homer-Wright rosettes (Fig. 1) and/or expressed at least two different neural markers (see Table 1). Although rarely present, ganglion cells are also regarded as sufficient evidence for neural differentiation to warrant the diagnosis of pPNET. EES was diagnosed in cases lacking Homer-Wright rosettes, ganglion cells, and expressing a maximum of one neural marker in immunohistochemistry. The nuclei in EES are uniform, with a fine chromatin network, whereas in pPNET clumping of chromatin is a frequent feature [9].

The mitotic activity index (MAI) was assessed according to Baak [3]. Mitoses were counted by two observers in ten consecutive fields at 400× magnification (40× objective, field diameter 450 μm) starting at the spot within the counting area with the highest number of mitotic figures. Fields with necrosis or inflammation and fields containing a low number of tumour cells were avoided.

Immunohistochemistry

Immunohistochemical investigations were performed in all cases on 4-μm-thick paraffin sections using the indirect peroxidase (IP), the peroxidase-antiperoxidase (PAP) and the avidin-biotin complex (ABC) methods [17, 29, 31] (Table 1). The immunohisto-

Table 1 Antibodies, pretreatment procedure, and immunohistochemical methods used in this study (*D* Dakopatts, *B* Becton Dickinson, *M* Monosan, *ABC* avidin-biotin complex, *IP* indirect peroxidase, *PAP* peroxidase-antiperoxidase, *GFAP* glial fibrillary acidic protein, *LCA* leucocyte common antigen)

Antibodies	Source	Pretreatment	Method
Neuron specific enolase	Mouse (D)	–	ABC
CD57 (Leu-7, HNK-1)	Mouse (B)	–	ABC
Protein S-100	Rabbit (D)	trypsin	PAP
Neurofilament protein	Mouse (M)	–	ABC
Chromogranin	Mouse (D)	–	ABC
GFAP	Rabbit (D)	–	IP
HBA-71 (O13)	Mouse (ref)	–	ABC
LCA	Mouse (D)	trypsin	ABC
Muscle-actin (HHF35)	Mouse (D)	–	ABC
Desmin (D33)	Mouse (M)	–	ABC
Cytokeratin	Rabbit (D)	trypsin	ABC
Vimentin (V9)	Mouse (D)	–	ABC

chemical panel included antibodies against NSE, CD57 (Leu-7, HNK-1), protein S-100, NF, chromogranin, GFAP, and HBA-71, a monoclonal antibody directed against the MIC2 cell surface antigen [15]. For differential diagnosis the following antibodies were used: leucocyte common antigen (LCA), desmin, muscle-specific actin (HHF35), pan-cytokeratin, and vimentin.

DNA flow cytometry

Preparation of cell suspensions for flow cytometry from the formalin-fixed, paraffin-embedded tumour specimens was performed according to a modified Hedley technique [32], applying an extra digestive step on the material remaining on the mesh after the first enzymatic treatment. DNA flow cytometry on the cell suspensions was performed after DAPI (4', 6'-diaminido-2-phenyl-indole dihydrochloride) staining with a mercury lamp based Pas II Analyser (Partec, Münster, Germany). Tumour cells were always present in H&E sections taken before and after the tumour material used to prepare the cell suspension (sandwich technique).

DNA histograms

The first modal cell peak was regarded as the diploid peak. If, in addition to the diploid (G0/G1) and G2/M peak, one or more additional peaks were detected, the tumour was classified as DNA aneuploid. DNA tetraploidy was defined by the presence of more than 10% of cells in the G2/M phase of the diploid cell cycle and had a clear tail at the right of that G2/M as well, with a second G2/M peak at a double distance. The coefficient of variation of the first G1 peak ranged from 3.3 to 11.3 (mean 6.38) in 12 tumour samples. The proportion of cells in the S-phase in each tumour cell population was determined by cell cycle analysis using the semi-automated program Multicycle (Phoenix Flow Systems, Philadelphia, Pa., USA), which offers after background fit an adequate correction for debris and sliced nuclei.

Statistical analysis

Survival rates were calculated according to the method of Kaplan and Meier and Mantel-Cox (MC) test statistics were assessed using the BMDP statistical software package (BMDP, Los Angeles, Calif., USA).

Results

Classification based on histology and immunohistochemistry

By light microscopy, all 20 tumours were composed of fields with monotonous small dark cells, with scanty pale eosinophilic cytoplasm. These fields were separated by collagenous or reticular fibres. The nuclei of the tumour cells were monotonous dark and round, but showed polymorphism, differences in size, irregular shape and diverge chromatin contents in some areas of the tumour.

Homer-Wright rosettes were present in 9 tumours (Table 2). NSE was expressed in 11 tumours, 8 of which expressed a second marker (CD57, S100, or NF) (Table 3). According to the classification scheme of Schmidt and co-workers [27], 11 tumours were classified as pPNET (Fig. 1), 5 as EES (Fig. 2). HBA-71 was exclusively present in pPNET ($n = 6$) and in EES ($n = 4$).

The remaining tumours were classified, on histological and immunohistochemical grounds, as sarcoma not otherwise specified ($n = 2$), RMS ($n = 1$), and desmoplastic tumour with divergent differentiation ($n = 1$).

None of the pPNET expressed GFAP or chromogranin. Patient survival was not correlated with the expression pattern of the neural markers. Three pPNET expressed pan-cytokeratin. In both pPNET and EES negative reactions were obtained for LCA, desmin, and muscle-specific actin. Glycogen was present in all EES and to a lesser extent in 3 pPNET. The Gomori silver stains showed similar patterns in both tumours.

Quantitative morphological parameters

Evaluative DNA histograms were obtained in 12 cases (Table 2). Eight pPNET were DNA diploid, and 1 DNA tetraploid. All 3 EES were DNA diploid. The S-phase fraction in pPNET ranged from 2.1% to 21.2% (mean 6.5%); in only one case was the S-phase fraction high (>15%). The percentage of cells in the S-phase in EES ranged from 4.6% to 13.2% (mean 9.1%). No significance for these variables in relation to prognosis was present. Similarly, MAI was low (<10) in 8 pPNET and in all EES.

Clinical findings

Males predominate in pPNET (7/11) and EES (4/5) (Table 2). Age at the time of diagnosis was higher in pPNET than in EES patients (mean: 14 and 9 years respectively).

Table 2 Clinical, histological, and quantitative morphological variables in 11 patients with peripheral primitive neuroectodermal tumours and 5 with extra-osseous Ewing's sarcomas (*ThP* thoracopulmonary, *MAI* mitotic activity index, *NE* not evaluated, *NED* no evidence of disease, *DOD* died of disease)

Patient no.	Age (months) sex	Location	TNM stage	Diagnosis	Homer-Wright rosettes	MAI	% S-Phase	DNA ploidy	Follow up (month)
1.	163 M	Axilla	II	pPNET	+	12	4.5	Diploid	108 NED
2.	164 M	ThP	II	pPNET	+	62	21.2	Diploid	21 DOD
3.	89 M	Shoulder	II	pPNET	+	0	3.5	Diploid	41 DOD
4.	133 M	ThP	IV	pPNET	+	7	4.0	Diploid	8 DOD
5.	305 M	Lower leg	II	pPNET	+	5	7.4	Diploid	56 DOD
6.	169 F	Paravertebral	I	pPNET	–	4	6.4	Diploid	10 DOD
7.	94 M	Paravertebral	I	pPNET	+	3	3.7	Diploid	38 NED
8.	385 M	ThP	II	pPNET	+	24	2.1	Diploid	9 DOD
9.	3 F	Head and neck	I	pPNET	–	0	6.4	tetraploid	186 NED
10.	162 F	Paravertebral	I	pPNET	+	0	NE	NE	61 NED
11.	168 F	Abdominal	I	pPNET	+	8	NE	NE	113 NED
12.	142 F	Paravertebral	II	EES	–	1	4.6	Diploid	123 NED
13.	115 M	Paravertebral	II	EES	–	0	NE	NE	82 DOD
14.	63 M	Paravertebral	I	EES	–	0	NE	NE	164 DOD
15.	48 M	Abdominal	IV	EES	–	0	9.5	Diploid	186 NED
16.	192 M	ThP	III	EES	–	8	13.2	Diploid	100 NED

Table 3 Immunohistochemical staining results of 11 peripheral primitive neuroectodermal tumours and 5 extra-osseous Ewing's sarcomas (● antigen-positive; ○ antigen-negative; *NA* not available, *NSE* neuron specific enolase, *NF* neurofilament, *V9* vimentin). Chromogranin and glial fibrillary acidic protein were negative in all cases

Patient no.	NSE	CD57	S-100	NF	HBA-71	Pan-cytokeratin	V9
1.	●	○	●	○	●	●	●
2.	●	●	○	○	●	○	○
3.	●	○	○	○	●	○	●
4.	●	●	○	○	●	●	●
5.	●	●	○	●	●	○	●
6.	●	○	○	●	○	○	●
7.	●	○	○	○	○	○	●
8.	●	○	●	○	●	○	○
9.	●	●	○	○	○	●	●
10.	●	○	○	○	NA	○	●
11.	●	○	●	○	○	○	●
12.	○	○	○	○	●	○	●
13.	○	○	○	○	●	○	●
14.	○	○	○	○	●	○	●
15.	○	○	○	○	●	○	●
16.	○	○	○	○	○	○	●

Localization, TNM stage, and treatment modalities were comparable in both pPNET and EES patients. EES patients fared significantly better than the pPNET patients with a 5-year survival of 100% compared to 42% ($P = 0.05$ MC = 3.9). No improvement in survival was observed for patients diagnosed before and after 1980; during both periods of time patients with pPNET fared less well than those with EES.

Two EES patients died from a local recurrence and successive metastatic relapses 7 and 14 years after diagnosis. A more favourable prognosis was observed for patients with paravertebrally located tumours than those with thoracopulmonary located tumours.

Discussion

pPNET and EES seem to form part of a single disease spectrum, with the more differentiated pPNET, characterized by the presence of Homer-Wright rosettes and/or at least the expression of two neural markers and the less differentiated EES, in which almost no histological or immunohistochemical neural differentiation is present. The commitment of EES to the neurogenic pathway is, however, suggested by the detection in EES cell lines of high levels of choline acetyltransferase [24], the expression of neural growth factor receptor [19], and the development of a morphological and immunohistochemical neural phenotype [5, 22, 26]. Moreover, the expression of HBA-71 in the majority of pPNET and EES provides further evidence of a close relationship of these neoplasms. Because of its high specificity [1, 10], HBA-71 is of great value in differentiating pPNET and EES from all other small round cell tumours.

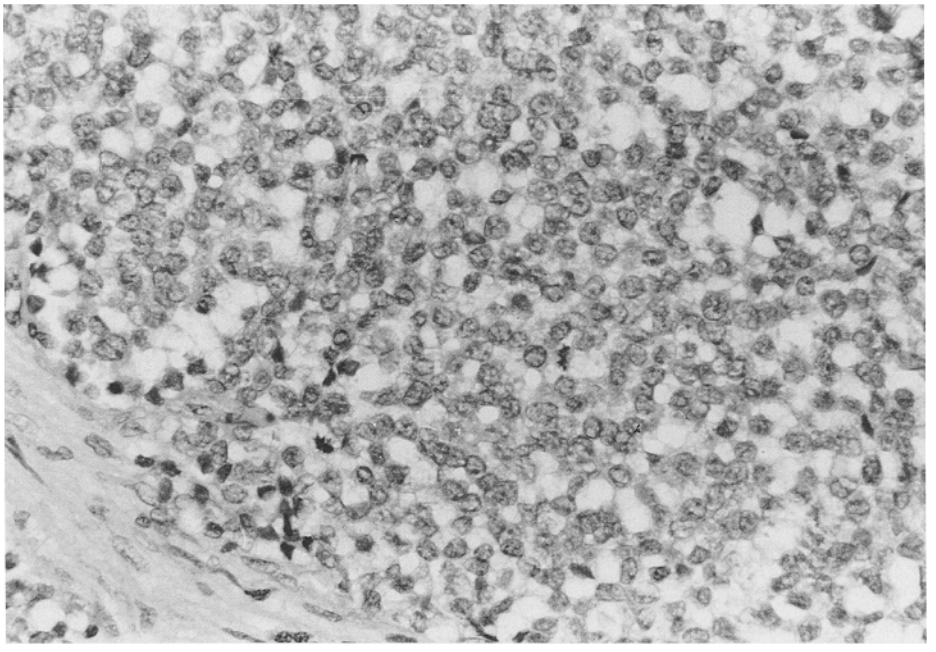

Fig. 2 Histological illustration of an extra-osseous Ewing's sarcoma. Haematoxylin-eosin stain, ×400

In all pPNET, neural differentiation as evidenced by the presence of Homer-Wright rosettes was supported by the expression of at least one neural marker (NSE, S100, NF, or CD57). None of the pPNET expressed GFAP or chromogranin, both of which are of a more mature neural differentiation [8]. In the study of Schmidt and coworkers [27], the only 3 pPNET which expressed GFAP also showed immunoreactivity for at least two other neural markers (NSE and S-100 in 2 cases and NSE, S-100, CD57, and NF in 1 case). These results, and those of the present study, suggest that the diagnostic value of GFAP and chromogranin is limited.

Neither the MAI nor the cellular DNA content, which proved to be prognostic in other blastemal tumours [4, 7, 11, 14, 23, 35], were related to the clinical outcome in our cases of pPNET and EES. The evaluative DNA histograms showed a DNA diploid stemline in 11 cases. Although other investigators have reported a higher incidence of DNA aneuploidy [18, 25, 30], no prognostic information could be obtained from DNA flow cytometry in their studies either. The lower incidence of DNA aneuploidy in the present study is possibly due to tumour heterogeneity or to the small number of patients included. Concordantly, no prognostic information was derived from the S-phase fraction. Therefore we advise the omission of quantitative morphological variables such as DNA ploidy, S-phase fraction, and MAI in the diagnostic and prognostic evaluation of these tumours.

The lower 5-year survival rate for pPNET patients compared with EES patients suggests a more aggressive clinical behaviour at onset of the more differentiated pPNET. However, localization, clinical stage, and age may have additional influence on patient outcome. When considering a longer follow-up period, it appeared that some EES patients developed late relapses. This difference in clinical behaviour substantiates the clinical importance of distinguishing pPNET from EES.

In conclusion, the present study confirmed that the Schmidt classification [27] for pPNET and EES provides important clinical information. In addition, based on our results, we advocate the use of HBA-71, since positivity for this marker is helpful in differentiating pPNET and EES from other small round cell tumours, and the exclusion of GFAP and chromogranin from the immunohistochemical panel, since they do not add relevant information. We advise the use of the following stainings for the identification of pPNET and EES from the group of small round cell tumours: HBA-71, NSE, protein S-100, CD57, and NF. Quantitative morphological variables such as DNA ploidy, S-phase fraction and MAI do not add diagnostic or prognostic information and should be omitted when evaluating these tumours.

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