Virchows Arch (1998) 432:505–513

ORIGINAL ARTICLE

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Differentiation in paediatric peripheral primitive neuroectodermal tumours of bone

A critical contribution to its assessment

Received: 15 September 1997 / Accepted: 15 December 1997

Abstract Differentiation was studied in 73 paediatric peripheral primitive neurorectodermal tumours (pPNETs) of bone observed during 1974 through 1992. The presence of rosettes, pseudorosettes, and/or a rosette-like arrangement of tumour cells (the morphological neural marker, MNM) occurred in 29% of these cases. NSE and N-CAM were expressed by nearly all tumours; synaptophysin was present in 30% of cases, not significantly associated with the MNM status. Neuroendocrine (NE) markers were present in 25% (chromogranin B, secretogranin II) to 40% (chromogranin A, 7B2 protein) of cases. Focal expression of cytokeratins, S100 protein and/or desmin was also noted in a minority of cases. In univariate statistical analysis, only the presence of MNM conferred a significantly higher (about twofold) risk of death than its absence. This study demonstrates the occurrence of at least one immunocytochemical N and/or NE differentiation marker in all pPNETs of bone and a focal expression of cytokeratins, S100 protein and/or desmin in a minority of cases. Synaptophysin and MNM were present each in less than 1/3 of the cases, and no association was noted between them. Statistical analyses highlighted the prognostic role of MNM per se and discourage the sole use of immunocytochemistry in the assessment of neuroectodermal differentiation for prognostic purposes in paediatric pPNETs of bone.

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Key words Peripheral primitive neuroectodermal tumour · Immunocytochemistry · Differentiation · Survival · Statistical analysis

Introduction

Peripheral primitive neuroectodermal tumours (pPNETs) [10], also known as the "Ewing's sarcoma family of tumours" [23], encompass small round cell neoplasms arising in bone or in soft tissues, having an alleged common origin from the multipotential neuroectoderm [8, 10] and displaying peculiar cytogenetic abnormalities [2, 8, 23]. At the histoimmunocytochemical level, pPNETs are characterized by diastase-sensitive periodic acid–Schiff (PAS) postivity, and immunoreactivity for vimentin (VIM) and CD99 (p30/32 protein^{MIC2}) [1, 2, 8, 14, 20, 21, 30]. Their neuroectodermal differentiation is characterized by variable expression of neural (N) and/or neuroendocrine (NE) immunocytochemical markers [2, 3, 5–13, 16, 18, 22, 23, 25, 26, 30–33, 35, 36, 42, 43, 48, 50, 52, 56, 59, 60], as well as by the occurrence of pseudorosettes, rosette-like arrangement of tumour cells, fibrillary background, and – in exceptional cases – true rosettes [3, 9, 13, 18, 22, 25, 26, 29, 34–37, 45, 48–50, 52, 56, 59].

According to the grade of neuroectodermal differentiation and to the site of origin, pPNETs arising in bone may be classified as neuroectodermal tumour (NET) of bone [25], Askin's tumour (AT) [3, 7, 13, 36, 37] or Ewing's sarcoma (ES), this last reflecting the least differentiated form [2, 3, 5–8, 10–13, 16, 18, 20, 22, 23, 25, 30, 32, 35, 36, 56]. This presence of neuroectodermal differentiation in pPNETs has been reported to entail a poor prognosis and resistance to chemotherapy [2, 5, 13, 22, 30, 36, 50]. However, little consensus has been obtained on its assessment, which is variably based on immunophenotype (using both N and NE markers) and/or morphology [1, 3, 5–7, 9, 11–14, 16, 18, 20, 22, 23, 25, 26, 29, 31–37, 41, 42, 45, 48–50, 52, 56, 59, 60]. Furthermore, most of the series previously studied included extraosseous pPNETs [5, 22, 26, 31, 32, 41, 50, 52, 60].

While studies are found on the immunocytochemical expression of cytokeratins (CKs) and/or S100 protein in pPNETs of soft tissues and bone [5–8, 16, 18, 32, 36–38, 41, 47, 50, 52, 60], expression of desmin (DES) has been reported only in pPNETs of soft tissues [46, 47, 53].

Accordingly, we decided to evaluate the N and NE status separately in a series of 73 paediatric pPNETs of bone, exploring their mutual relationships and their separate and combined influence on overall patient survival. In addition, the expression of CKs, S100 protein and/or DES, their association with the other variables and their influence on overall patient survival were also investigated.

Materials and methods

A search was made in the files of the Division of Paediatrics of the Istituto Nazionale Tumori of Milan, Italy for the period 1974–1992. In all, 220 consecutive cases of pPNETs with radiologically proven bone involvement were retrieved. Only those cases for which sufficient formalin- and/or Bouin-fixed, paraffin-embedded material from the primary tumour was available (n=73) were selected for this study. The case material had been originally diagnosed as NET of bone in 2 cases, AT in 4, and ES in the remaining 67. For each case, patient's sex and age at diagnosis, and site of origin of the primary tumour were recorded. Follow-up was collected up to May 1995 or until the death of the patient.

All the original slides were reviewed. The morphological N marker (MNM) was defined as the presence in the primary tumour of at least one of the following features: (a) rosettes with an empty core, (b) well-developed Homer Wright pseudorosettes, (c) a rosette-like arrangement of tumour cells around a fibrillary core. The occurrence of a fibrillary background was also noted [13, 37, 52]. The aggregation of cells around necrotic foci or vascular structures was not taken into account.

One representative specimen from each primary tumour was selected for histochemical and immunocytochemical staining. The former included PAS, before and after diastase digestion, and Grimelius' argyrophilic procedures. For immunocytochemistry, paraffin sections were incubated with primary monoclonal or polyclonal antibodies (Table 1) and an avidin–biotin (ABC) peroxidase method (Vector Labs., Burlingame, Calif.) with development in 3-3' diaminobenzidine (DAB), plain or silver-intensified [15]. In some instances, prior to incubation with the primary antibody, the sections were heated in a microwave oven (6 min at 95° C, in 0.005 M citrate buffer, potency at 450 W) or digested with trypsin (20 min at 37° C, in 0.05 M Tris buffer+0.1% $CaCl_2$). To rule out neuroblastoma, NB84-monoclonal antibody (NB84a, anti-human-neuroblastoma, Dakopatts, Glostrup, DK) was applied to CD99-unreactive cases after trypsin pretreatment [57].

Adequate positive and negative controls were performed. Negative controls included preabsorption of the polyclonal (serum) antibodies with saturating amounts of the peptides used as immunogens. The reactivity of the monoclonal antibodies was checked by comparison with unrelated, species- and subtype-matched immunoglobulins at the same concentration.

The results of histo- and immunocytochemical stains were scored as "absent" when rare scattered or no reactive cells were seen, "low" when up to 10% reactive cells were seen, "moderate" when between 11% and 50% reactive cells were seen, and "strong" when >50% reactive cells were seen. For statistical analyses, the results were grouped into "absent" or "present", the latter class including the scores "low" through "strong".

Applying updated criteria, a diagnosis of NET or AT was considered when MNM and/or immunoreactivity for synaptophysin (SYN) were found to be present [13, 22, 35, 50, 56]. This last marker was recently suggested as a reliable alternative to neurofilaments in the assessment of N differentiation in pPNETs of bone [13].

Statistical analyses were performed by means of the Statistical Analyses System package (SAS Institute, Cary, N.C.). The variables studied included sex and age at diagnosis, site of origin of the primary tumour, MNM, and histo- and immunocytochemical stains. The independence between the variables was tested by the Chi-square or (if necessary) Fisher's exact tests. Overall survival (OS) was calculated as the time elapsed from the date of diagnosis to the date of the last control or to the death (for any cause). OS pattern was estimated by means of the product-limit method [28]. Since the proportional hazard assumption was tenable, the Cox regression model was adopted both for univariate and for multivariate analyses. In this model, each of the regression coefficients is the logarithm of the hazard ratio and is constant over the time. For two groups of patients under the null hypothesis of the same OS experience, the hazard ratio is expected to be 1.00. This hypothesis was tested by the Wald statistic. In univariate analysis, for each variable a regression model containing only that variable was interpolated and the unadjusted hazard ratio (UHR) was estimated. The effect of the separate and combined presence of MNM and the immunocytochemical N and NE markers (SYN and

Table 1 Characteristics of the primary antibodies used in the immunocytochemical studies (*KLH* Keyhole limpet haemocyanin)

Code/clone	Directed against	Immunogen	Raised in	Source
CRA-2-3-7[a]	Chromogranin A (CgA)	Human $CgA^{Cys307–320}$ coupled to KLH	Rabbit	Own
B11[a]	Chromogranin B	Chromaffin granules from human phaeochromocytoma	Mouse	Biogenesis, Bournemouth, England
5A7[a]	Secretogranin II	Bovine anterior pituitary homogenate	Mouse	M. Pelagi, H.S. Raffaele, Milan, Italy
7B2-2-3-7[a]	7B2 protein	Human $7B2^{1–14Cys}$ coupled to KLH	Rabbit	Own
AO10[b,c]	Synaptophysin (SYN)	Synthetic human SYN peptide coupled to KLH	Rabbit	Dakopatts, Glostrup, Denmark
BBS/NC/VI-H14	Neuron-specific enolase	γ–γ Enolase purified from human brain	Mouse	Dakopatts
OB11[a]	Neural cell adhesion molecule	Enriched rat forebrain extract	Mouse	Sigma, St. Louis, Mo.
V9	Vimentin	Purified vimentin from porcine eye lens	Mouse	Signet Labs., Mass., USA
[a, d]	Cytokeratins	See specification sheets	Mouse	[d]
D33[a]	Desmin	Human muscle purified desmin	Mouse	Dakopatts, Glostrup
Z311[a, c]	S100	S100 purified from cow brain	Rabbit	Dakopatts
O13[a]	CD99 ($p30/32^{MIC2}$)	MeWo human melanoma cells	Mouse	Signet Labs. USA

[a] Microwave oven, [b] trypsin, treatment of the sections prior to application of primary antibody
[c] IgG fraction of the serum
[d] Pool of clones 35βH11, 34βE12 (Dakopatts, Glostrup, Denmark), CAM 5.2 (Becton Dickinson, Calif.), KL1 (Immunotech, Marseille, France), AE1 and AE3 (Hybritech, San Diego, Calif.)

secretogranin II (SgII), respectively) was tested in a multivariate analysis. SYN and SgII were chosen on the basis of their UHR.

Results

There were 48 male and 25 female patients (male/female ratio, 1.9/1). Their median age was 12 years. Sixteen tumours arose in the thoracopulmonary region, 6 in the axis, 15 in the pelvic bones, 25 in the extremities, and 11 from other sites. The follow-up period ranged from 1 to 231 months (median, 38 months). At the last control, 53 patients (73%) had died and 20 (27%) were still alive. Death was due to the disease in 50 cases, and 19 of the surviving patients were disease-free.

As shown in Table 3, MNM was present in 21 cases (29%). Pseudorosettes and/or a rosette-like arrangement of tumour cells occurred, in most cases focally, in 17 (81%) and 11 (52%) of the cases with MNM present, respectively (Fig. 1). A fibrillary background was found in 18 (86%) of the cases with MNM present, and occasional rosettes with an empty core were seen in 3 cases. The MNM status was not significantly associated with the site of origin of the primary tumour.

Table 2 shows also the histoimmunocytochemical results. Both fixatives gave similar staining patterns. A moderate to strong diastase-sensitive PAS positivity was recorded in 44 cases (60%). Strong CD99 and VIM immunoreactivity was detected in nearly all cases. Cytoplasmic VIM immunoreactivity was diffuse in 52 cases (74%) and dot-like in 18 cases (26%).

In the 6 CD99-unreactive cases (5 of which were strongly reactive for VIM), absence of NB84 immunoreactivity and several clinical evidences supported the di-

Table 2 Histo-immunocytochemical variables, their classes and relative frequencies

	Absent		Present	
	(*n*)	%	(*n*)	%
Common markers				
Diastase-sensitive PAS	29	40	44	60
Vimentin	3	4	70	96
CD99 (p30/32 protein^{MIC2})	6	8	67	92
Other markers				
Desmin	68	93	5	7
Cytokeratins	62	85	11	15
S100 protein	54	74	19	26
Morphological neural marker (MNM)	52	71	21	29
Immunocytochemical neural markers				
Neuron-specific enolase	5	7	68	93
Neural cell-adhesion molecule	7	10	66	90
Synaptophysin	51	70	22	30
Histoimmunocytochemical neuroendocrine markers				
Argyrophilia	42	58	31	42
Chromogranin A	40	55	33	45
Chromogranin B	56	77	17	23
Secretogranin II	55	75	18	25
7B2 protein	41	56	32	44

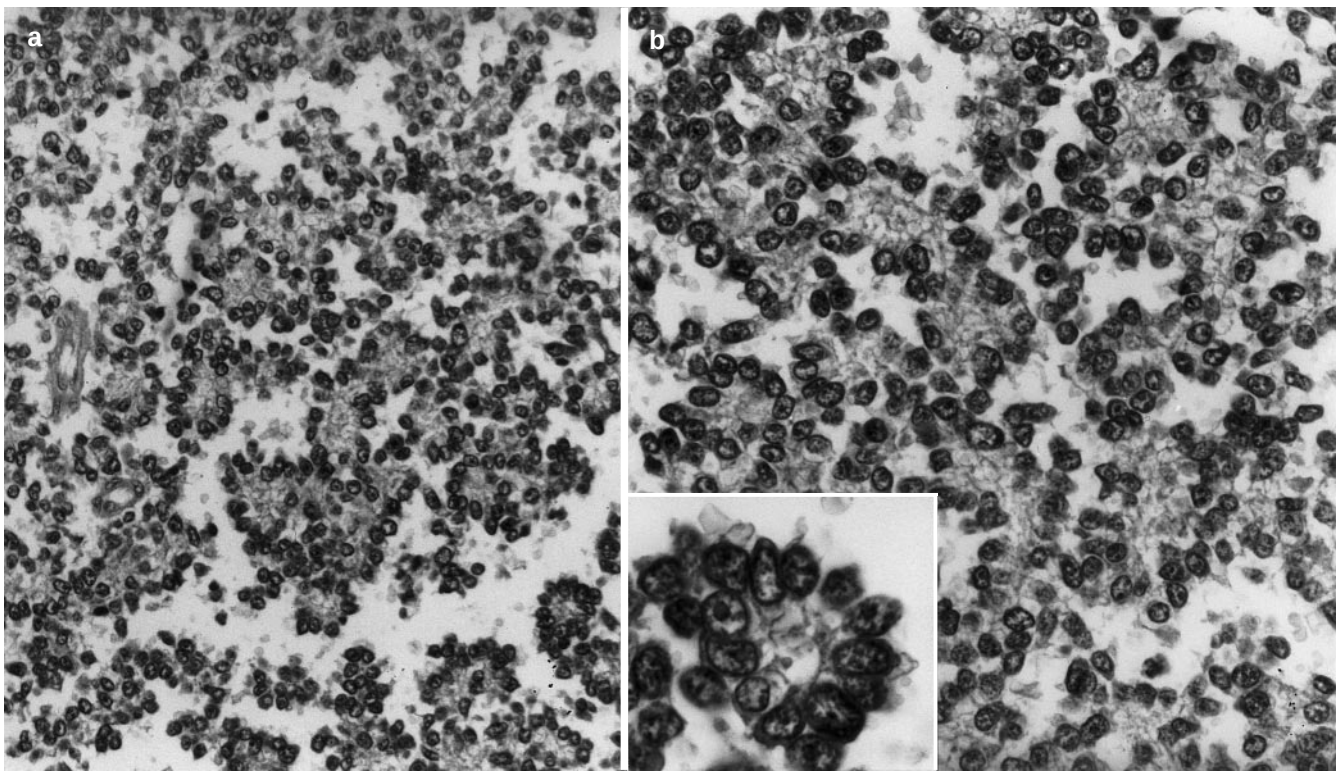

Fig. 1 **a** Presence of the morphological neural marker in a case of pPNET of bone, characterized by a rosette-like arrangement of tumour cells (**b**) and focal evidence of well-developed Homer Wright pseudorosettes (*inset*). Haematoxylin-eosin, **a** ×200, **b** ×320, *inset* ×624

agnosis of pPNET of bone and excluded that of a metastatic neuroblastoma. In fact, the ages at onset were 2, 9, 13, 15, 18, and 19 years, respectively, which – save for the first patient – are unusual for a neuroblastoma. The work-up at the time of the diagnosis involved physical examination, complete blood biochemistry, plain X-ray, tomography or computed axial tomography (CAT) of the chest, CAT and/or ultrasonography of the abdomen and pelvis, ^{99}Tc-diphosphonate bone scan, and iliac crest bone marrow biopsy and aspirate. None of these 6 patients presented neoplastic lesions at the sites where primary neuroblastomas are usually located: 5 of them had tumours in a monostotic localization (3 in the diaphysis of the long bones, and 2 in flat bones), which was predominantly lytic, and had no other clinical or radiological sign of neoplastic localization. Such a presentation – a monostotic lesion and occult primary tumour – is extremely unusual for neuroblastoma. The remaining patient was a 13-year-old girl with a lytic lesion of the left femur and synchronous bone metastases, normal levels of urinary cathecolamine excretion and normal serum neuron-specific enolase (NSE).

At least one immunocytochemical N marker was expressed by all cases. At least one immunocytochemical NE marker was present in 62/73 cases (85%).

Nearly all tumours were moderately to strongly immunoreactive for NSE and N cell-adhesion molecule (N-CAM). N-CAM was observed along the cell membrane as well as in the cytoplasm. A diffuse cytoplasmic SYN immunoreactivity was detected in 22 cases (30%); in 14 of these cases the reaction was moderate to strong. The expression of SYN was not significantly associated with the MNM status. No significant association was found between diastase-sensitive PAS positivity and the presence of N markers. Since NSE and N-CAM were detected in over 90% of the cases (Table 2), in statistical analyses only SYN was considered as the immunocytochemical N marker.

A faint argyrophilic reaction was present in 31 cases (42%); in all but 3 of them it co-occurred with immunoreactivity for at least one granin. Chromogranin A (CgA) and 7B2 protein (7B2) were each present in over 40% of cases, with only a partial overlap between them. A strong expression, however, was detected in only 15 (CgA) and 2 (7B2) cases. Neither of the two granins was significantly associated with the presence of SYN. The prevalence of SgII and chromogranin B (CgB) expression was lower: each of these proteins was detected at a low to moderate reactivity level in about one quarter of the tumours and with almost no overlap. Their expression was significantly associated with that of SYN and with the tumour site. In fact, SYN immunoreactivity was detected in 12 of the 18 tumours with SgII present and in 10 of the 55 with SgII absent (χ^2=15.142, P<0.001), in 9 of the 17 with CgB present and in 13 of the 56 tumours lacking CgB (χ^2=5.474, P=0.019). Likewise, SgII was detected in 7 of the 16 tumours from the thoracopulmonary region, and was absent from those taken from the axis (P=0.05, Fisher's exact test), whereas CgB was almost always absent from the former tumours (P=0.06, Fisher's exact test). Argyrophilia or reactivity for at least one granin was found in 61 cases (84%). No association was found between granin expression or argyrophilia and MNM.

On the basis of the combined presence of morphological and immunocytochemical (MNM and SYN) N features, four subsets could be identified:

1. MNM present/SYN present (n=9, 12%). These tumours were characterized by a rich content in granins, present in all cases, with a prevalent expression of SgII and near-total absence of CgB.
2. MNM absent/SYN present (n=13, 18%). These tumours expressed SgII or CgB in all but 3 cases, with a moderate to marked content in granins present in all but 1 cases.
3. MNM present/SYN absent (n=12, 16%). These tumours were characterized by the almost complete absence of SgII and CgB in the presence of a moderate expression of CgA and 7B2. No granin immunoreactivity was found in 3 cases.
4. MNM absent/SYN absent (n=39; 54%). There were 28 cases showing a moderate expression of granins, with a poor reactivity for SgII and CgB. Eleven cases lacked any granin immunoreactivity.

No correlation was found between the number of NE and N markers expressed, and the MNM status.

A diagnosis of AT was made in 8 cases (11%); of these, 2 were MNM present/SYN present, 3 were MNM present/SYN absent, and 3 were MNM absent/SYN present. A diagnosis of NET of bone was made in 26 cases (36%); of these, 7 were MNM present/SYN present, 9 were MNM present/SYN absent, and 10 were MNM absent/SYN present. The remaining 39 cases (53%) were considered to be ESs.

CK, S100 protein or DES immunoreactivities were present focally in 11 (15%), 19 (26%) and 5 cases (7%), respectively (Tables 2, 3); these three immunocytochemical variables were not significantly associated with any of the other variables considered. Immunoreactivity for CKs and DES was dot-like in all cases. No distinctive

Table 3 Presence of cytokeratins, S100 protein and desmin in 29 pPNETs of bone (*VIM* vimentin, *MNM* morphological neural marker, *SYN* synaptophysin, *CKs* cytokeratins, *S100* S100 protein; *DES* desmin)

CD99	VIM	MNM	SYN	CKs	S100	DES	CKs +S100	DES +S100	CKs+DES +S100	CKs+DES
+	+	+	+		2		1			
+	+	+	–	1	3	1				
+	+	–	+	1	2	1	2		1	
+	+	–	–	5	7	1		1		

Table 4 Clinicopathological characteristics of the five DES-reactive cases of pPNETs of bone (*CgA* chromogranin A, *CgB* chromogranin B, *SgII* secretogranin II, *7B2* 7B2 protein, *DOD* dead of disease)

Case no.	Site	Sex	Age (years)	Follow-up (months)	MNM	CD99	VIM	DES	CKs	S100	SYN	NSE-N-CAM	CgA	CgB	SgII	7B2
1	Axis	M	8	DOD 23	+	+	+	+	–	–	–	+	+	–	–	–
2	Pelvis	M	15	DOD 8	–	+	+	+	–	–	+	+	+	–	+	–
3	Scapula	M	12	DOD 26	–	+	+	+	+	+	+	+	+	+	–	+
4	Pelvis	M	17	DOD 23	–	+	+	+	–	+	–	+	–	–	+	+
5	Femur	F	16	DOD 45	–	+	+	+	–	–	–	+	+	+	+	–

Table 5 Univariate analysis for overall survival (all χ^2 have 1 *df*; *UHR* unadjusted hazard ratio, *CI* confidence interval)

Variables	UHR	95% CI	χ^2	*P*
Morphological neural marker (MNM)				
absent vs present	1.93	1.08–3.44	4.931	0.0264
Immunocytochemical neural markers				
Neuron-specific enolase, absent vs present	1.52	0.47–4.89	0.4968	0.4809
Neural cell-adhesion molecule, present vs absent	1.44	0.61–3.39	0.7091	0.3997
Synaptophysin, absent vs present	1.48	0.83–2.65	1.7686	0.1836
Histoimmunocytochemical neuroendocrine markers				
Argyrophilia, absent vs present	1.15	0.67–1.99	0.2596	0.6104
Chromogranin A, absent vs present	1.02	0.59–1.76	0.0059	0.9386
7B2 protein, absent vs present	1.23	0.71–2.11	0.5394	0.4627
Secretogranin II, absent vs present	1.36	0.74–2.53	0.9722	0.3241
Chromogranin B, present vs absent	1.22	0.64–2.34	0.3762	0.5397
Other markers				
Desmin, absent vs present	1.41	0.32–3.55	0.9654	0.3334
Cytokeratins, absent vs present	1.39	0.67–2.87	0.7917	0.3736
S100 protein, absent vs present	1.17	0.64–2.14	0.2743	0.6004

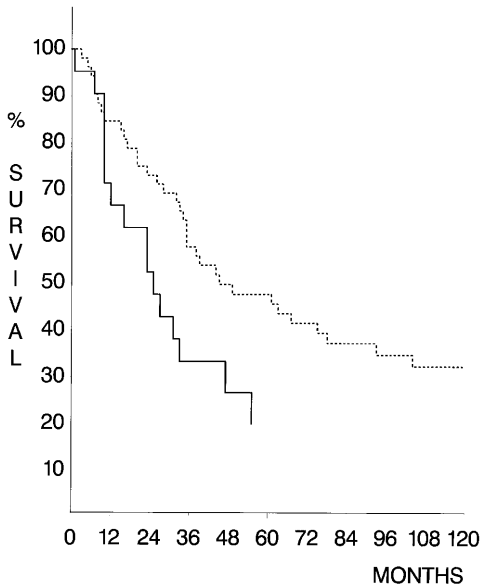

Fig. 2 Overall survival curves by the presence (——) or absence (- - - -) of the morphological neural marker in the primary tumour

morphological features characterized the 5 DES-reactive tumours. CD99, VIM, NSE, N-CAM, and at least one granin were present in all 5 cases, 1 of which displayed the simultaneous expression of VIM, CKs, S100 protein, DES, NSE, N-CAM, SYN, CgA, CgB and 7B2 protein in the absence of MNM. The patient concerned was a 12-year-old boy with a 4-cm lytic lesion in the scapula that was infiltrating the surrounding soft tissues, without any other clinical or radiological sign of neoplastic localization. Bilateral lung metastases appeared 10 months later and he died of the disease 26 months after surgery.

The clinicopathological characteristics of the DES-reactive cases are given in Table 4.

In the univariate analysis for OS, a hazard ratio significantly greater than 1 was detected only for MNM (Fig. 2, Table 5). The occurrence of S100 protein together with MNM and/or SYN immunoreactivity did not influence the OS. In the multivariate analysis, the interactions between MNM, SYN, and SgII were not statistically significant. In fact, the prognostic impact of MNM did not differ significantly between patients with SYN-absent tumours and those with SYN-present tumours. Similarly, the prognostic impact of SYN did not differ significantly between the two MNM modalities. A similar result was observed for MNM and SgII and for SYN and SgII.

Discussion

Our results confirmed the strong and widespread expression of VIM and CD99 in pPNETs of bone previously reported by others [37]. In keeping with previous reports, we did not find lower diastase-sensitive PAS positivity in the presence of N markers [33, 60].

The presence of variable grades of neuroectodermal (N or NE) differentiation in all pPNETs of bone was confirmed by immunocytochemistry and morphology.

The analysis of the immunoreactivity patterns for classical N markers confirmed the widespread presence of NSE and N-CAM [12, 14, 16, 18, 23, 25, 26, 33, 35, 36, 42, 48, 52, 59, 60]. However, our results with the latter are at odds with a previous study [17], where N-CAM expression was inversely related to that of CD99, currently considered a distinctive though nonspecific marker of pPNETs within small round cell tumours of childhood [54]. This discrepancy could be due either to the use of different primary antibodies or to different immunocytochemical techniques, including the application of microwave irradiation and silver enhancing procedures in our cases. Furthermore, we also found N-CAM reactivity in the cytoplasm, a singular event for a cell membrane-associated molecule, but in keeping with previous observations made in non-N tumours [39, 40]. This unusual immunoreactivity pattern calls for caution in the use of antibodies to N-epitopes [2, 10]. In addition, the widespread presence of NSE and N-CAM in pPNET of bone precludes their use in the characterization of subsets.

The only N immunocytochemical marker restricted to a consistent number of pPNETs was SYN. However, in these tumours SYN reactivity was diffusely cytoplasmic, as described in NE cells and tumours and at variance with that observed in neurons, where it is oriented along the cell periphery [19]. This fact, together with its detection in secretory granules of normal NE cells [27] (L. Scopsi, unpublished work), suggested that in pPNETs of bone SYN plays a role also as a NE marker.

Some authors failed to find reactivity for CgA in pPNETs [5, 32]. Pagani et al. [43] detected the presence of SgII mRNA, but did not find any immunoreactivity for CgA, CgB, or SgII. At odds with these results, we found immunoreactivity for at least one granin in 80% of pPNETs. CgA immunoreactivity was found in a higher proportion of tumours than reported previously [7]. CgB and SgII showed lower expression, but, unlike CgA, showed a correlation with the expression of SYN immunoreactivity. Our study also demonstrates, for the first time, the frequent occurrence of 7B2 in pPNETs. This protein belongs to the granin family [24], is widely present in NE cells and tumours [55] (L. Scopsi, unpublished work), and is thought to represent an NE chaperone interacting with proprotein convertase 2, an NE-specific precursor-processing enzyme [4]. This high prevalence of granin-reactive cases in our series could well be due to the use of discrete primary antibodies along with the application of a microwave pretreatment. The widespread expression of CgA, routinely used as immunocytochemical pan-NE marker, suggests caution in its use for the assessment of the grade of neuroectodermal differentiation in pPNETs of bone.

MNM also characterized a group of tumours. The presence of MNM in only less than one-third of the cases is in keeping with the reported poor morphological differentiation in pPNETs of bone [13]. In addition, it was predominantly characterized by focal expression of a rosette-like arrangement of the tumour cells, considered to be poorly developed Homer Wright pseudorosettes.

The occurrence of MNM was not significantly associated with SYN or granin expression. The discrepancy between neuroectodermal immunophenotype and morphological differentiation in pPNETs has been already pointed out by Navarro and co-workers [42], but, at variance with this group, we found qualitative differences in the expression of immunocytochemical markers. Our results suggest the existence of a spectrum of neuroectodermal differentiation in pPNETs of bone, with expression of NE and N markers resembling that found in neuronal development. In fact, the first evidence of neuronal development is the expression of CgA in primitive cells of the neural tube and crest. The next marker to appear is NSE; the last is SYN, which labels the presynaptic vesicles of well-differentiated neurons [38].

Taking account of the poor degree of differentiation of pPNETs of bone, we consider that SYN could well mark pPNETs with a more N-differentiated phenotype, such as NETs of bone and ATs. A weak, and possibly immature, sign of neuroectodermal differentiation in ESs is characterized by the expression of granins. Recently, the induction of neuronal differentiation in neuroblastoma cells in vitro was related to the switch of granin expression from CgA to SgII [43]. These data would be in keeping with our finding of SgII associated with the expression of SYN in the more differentiated forms of pPNETs of bone. An ascending grade of differentiation can also be identified for MNM, from a rosette-like arrangement of tumour cells to well-developed Homer-Wright pseudorosettes and true rosettes. Conceivably, MNM is associated with the expression of a still unidentified immunocytochemical marker of differentiation. It is not clear whether the combined presence of MNM and SYN marks a higher grade of differentiation than their separate occurrence.

The univariate analysis for OS showed that the presence of MNM conferred a risk of death about two fold that with its absence, while none of the other N and NE markers, whether alone or in combination with MNM, seemed to have any impact on survival. This is in keeping with most earlier observations [22, 50–52, 56, 59] and once more discourages the sole use of immunocytochemistry in the prognostic assessment of differentiation in pPNETs.

As reported in the literature on osseous pPNETs [36, 41, 50, 52, 60], focal expression of CKs and S100 protein was noted in a small percentage of our cases. The aberrant expression of CKs and S100 protein – also called divergent differentiation [8, 47] – has been related to the multipotential neuroectodermal differentiation, which includes the expression of epithelial and schwannian markers [10]. We are at variance with others [50] in that we found the expression of S100 protein in the presence of N markers not related to a more favourable outcome.

Sporadically, reports on the expression of DES by pPNETs of soft tissues have also appeared [46, 47, 53]. Recently, the expression of the *EWS/FLI1* fusion gene was reported in sarcomas of soft tissues with myogenic and neural differentiation, suggesting their close relation to pPNETs, of which they might be a subset with biphenotypic differentiation [8, 53]. We report here for the first time the focal occurrence of DES immunoreactivity in 5 otherwise typical pPNETs of bone. At variance with the hypothesis of Pagani et al. [44], we did not find an inverse relationship between the expression of mesenchimal markers (e.g. DES) and SgII. In fact, 3 of the 5 DES-reactive cases also displayed SgII immunoreactivity and 1 case even expressed mesenchimal, epithelial, schwannian, N and NE differentiation markers, simultaneously. In addition, neither these 5 cases displayed peculiar morphological characteristics compared with the DES-unreactivity cases, and the occurrence of DES seemed to have no influence on patient survival. All these data, together with the presence of CD99, VIM, N and NE markers in all the 5 DES-reactive cases, speaks in favour of their inclusion in a subset of pPNETs of bone with divergent differentiation rather than in the poorly defined "primitive sarcomas of bone" [37]. Studies on larger case series are now needed, to test whether such cases with divergent differentiation represent more than an immunophenotypic variant.

This study confirms the presence of at least one immunocytochemical neuroectodermal differentiation marker in all pPNETs of bone, and of MNM in 29% of the cases. Despite this, no statistically significant association was detected between the expression of SYN, often considered to be a marker for high N differentiation, and MNN. The patterns of expression of MNM and of immunocytochemical markers seemed to point to a spectrum of N and NE differentiation similar to that found in neuronal development, with early expression of NSE and CgA and expression of SYN and SgII (and probably MNM) in the more advanced stages. The presence of MNM per se seemed to imply a worse outcome, while immunocytochemical markers of N or NE differentiation did not have any impact on survival. The focal occurrence of DES in 5 pPNETs of bone is also reported for the first time. The presence of CKs, S100 protein and/or DES immunoreactivity in a small percentage of cases was not associated with any of the other variables studied and had no influence on the OS.

Further studies examining morphological characteristics such as necrosis, mitoses, filigree and large cell patterns [9, 22] as well as clinical variables with a known impact on survival, such as local extension of the disease, the presence of distant metastases at diagnosis, and therapeutic protocols [20, 29, 31, 56, 58], are now warranted to test the prognostic strength of MNM.

Acknowledgements This work was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC). Giuseppe Sampietro was a "Piero Casati" Fellow of the Istituto Nazionale Tumori of Milan. We thank Morena Gobbo and Barbara Vergani for their technical assistance, A. Parma (Ospedali Riuniti, Bergamo, Italy) and A. Parafioriti (Istituto Ortopedico G. Pini, Milan, Italy) for making some of the histological material available to us and M. Pelagi (DIBIT, San Raffaele Hospital, Milan, Italy) for the generous gift of antibodies.

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