ORIGINAL ARTICLE

Prognostic value of stage IV neuroblastoma metastatic immunophenotype in the bone marrow: preliminary report

M Nowicki, D Ostalska-Nowicka, B Miskowiak

J Clin Pathol 2006;**59**:150–152. doi: 10.1136/jcp.2004.024687

See end of article for authors' affiliations

Correspondence to: Dr M Nowicki, Department of Histology and Embryology, Poznan University of Medical Sciences, ul. Swiecickiego 6, 60-781 Poznań, Poland; mnowicki@amp.edu.pl

Accepted for publication 14 May 2005

Aim: To correlate the immunophenotype of metastatic cells in the bone marrow of patients with neuroblastoma with early treatment failure.

Methods: The studies were performed on bone marrow material obtained from children treated in the department of paediatric oncology, haematology, and transplantology, Poznan University of Medical Sciences, Poland from 1996 to 2003. Immunocytochemical analysis of nervous tissue markers (using the immunomax technique) was performed on 108 bone marrow preparations obtained from 36 children diagnosed with neuroblastoma (stage IV with bone marrow metastases). The analysis included expression of PGP 9.5 protein, substance P, chromogranin A, bombesin, galanin, neuropeptide Y (NPY), and vasoactive intestinal peptide in neuroblastoma metastatic cells defined by the expression of neurone specific enolase.

Results: Nineteen relapses occurred within 12 months of the end of treatment. Correlation between the various markers studied and early treatment failure, using Fisher's exact test, revealed that chromogranin A and NPY are strong indicators of an unfavourable prognosis in patients with stage IV neuroblastoma ($p < 0.001$ and $p < 0.0002$, respectively).

Conclusion: Determination of metastatic cell immunophenotypes in bone marrow (particularly chromogranin A and NPY) may help establish the short term prognosis in children with neuroblastoma.

Neuroblastoma is one of the most frequently occurring malignant solid tumours in children up to the age of 15 years.[1] The international neuroblastoma staging system distinguishes four stages of the disease. Children in the early stages (I and II) are generally not seen in clinical practice, and unfortunately at presentation the condition is usually advanced (stage III) or present with distant metastases to lymph nodes, bones, bone marrow, or liver (stage IV). The relatively rare condition in which there is metastasis to the liver, skin, or bone marrow, but no primary focus can be found, used to be defined as stage IV-s.[1]

> ''Neuroblastoma is one of the most frequently occurring malignant solid tumours in children up to the age of 15 years''

A new treatment protocol for advanced neuroblastoma (stages III and IV), the Tokyo programme of the Polish paediatric group for solid tumours, was established here in 1991. Despite this intensive programme, which combines surgery, chemotherapy, and radiotherapy, the probability of remission lasting longer than three years in stage IV patients older than 1 year usually does not reach 10%.

The development of oncological genetics and increasing knowledge concerning the biological properties of tumour cells led to the discovery of several factors of prognostic relevance in the course of neuroblastoma. The most important of these are amplification of the *N-myc* proto-oncogene and deletion of the short arm of chromosome 1, which indicate an unfavourable prognosis, and aneuploidy, which indicates a favourable prognosis.[2] However, these factors cannot be used to establish a prognosis in children with stage IV disease because aneuploidy is not seen in these children, and *N-myc* amplification affects most tumour cells.

No studies have been published that aimed to determine the relevance of the numerous peptides present in neuroblastoma cells to the pathogenesis or prognosis of the disease. In a previous study, we showed that metastatic neuroblastoma cells in bone marrow have a different immunophenotype to that found in cells of the primary focus.[3] Some of the markers investigated were only present on metastatic cells.

Assuming that metastases indicate a more pronounced malignant character in neuroblastoma, we attempted to correlate the immunophenotype of metastatic cells in the bone marrow in patients with stage IV disease, recorded before the start of treatment, with early treatment failure.

MATERIAL AND METHODS

The material consisted of bone marrow aspirates obtained by needle biopsy from 36 children (aged 4–18 years with stage IV neuroblastoma with bone marrow metastases) hospitalised between 1996 and 2003 in the department of paediatric oncology, haematology, and transplantology, Poznan University of Medical Sciences, Poland. We obtained local ethics committee approval and informed consent from all of the patients' parents.

Bone marrow was obtained from the posterior superior iliac spine after administration of intravenous pethidine (1 mg/kg body weight), midazolam (0.1 mg/kg body weight), and local anaesthesia (1% lignocaine). The control group consisted of 10 children in whom no neoplastic disease had been diagnosed; the bone marrow of these children was normal and the final diagnosis was reactive lymphadenitis.

Neoplastic cells in the bone marrow aspirates, defined by the expression of neurone specific enolase, were investigated for the presence of PGP 9.5 protein, substance P, chromogranin A, bombesin, galanin, neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP). Commercially available antihuman antibodies (substance P, NPY, and VIP; Sigma,

Abbreviations: NPY, neuropeptide Y; VIP, vasoactive intestinal peptide

Poole, Dorset, UK) and antirat antibodies (NSE, PGP 9.5, chromogranin A, bombesin, galanin; Sigma/ Amersham, Little Chalfont, Buckinghamshire, UK) were used. The immunohistochemical analysis of nervous tissue markers was performed by indirect immunohistochemistry with the use of the biotin–streptavidin system and tyramine signal amplification (the immunomax technique), which was followed by incubation with diaminobenzidine (Dako, Glostrup, Denmark).[4]

Immunohistochemical staining was examined under a light microscope (Eclipse 600, Nikon, Tokyo, Japan) at ×200 magnification. The percentages of positive cells were related to the total number of blast cells in haematological smears by means of Microimage (Olympus, Tokyo, Japan) morphometric software.

Negative and positive controls were used in every case.[5] Doubtful or equivocal cells were excluded from the analysis.

The data obtained were correlated with disease relapse noted up to 12 months after completion of the treatment, using Fisher's exact test in view of the size of the study group, which was less than 100. Statistical analysis involved the use of Statistica '99 software.

RESULTS

Neuroblastoma bone marrow metastases were detected in all 36 children in our study group. The percentage of tumour cells in the bone marrow ranged from 34.8% to 75.9% of nucleated cells (mean, 46.8%).

During the observation period, early relapse of the disease was noted in 19 children (subgroup A). The remaining 17 children (subgroup B) remained in remission for longer than 12 months.

Immunohistochemical expression of substance P and bombesin was confirmed in most of the metastatic cells of all 36 children. The percentage of substance P positive cells ranged from 88.4% to 98.2%, with a mean of 91.8%. The percentage of bombesin positive cells ranged from 72.6% to 96.2%, with a mean of 84.5%. PGP 9.5, chromogranin A, galanin, NPY, and VIP were expressed in some of the children in both subgroups. In subgroup A, the mean percentage of PGP 9.5 positive cells was 60.3%; this figure was 90.2% for chromogranin A, 23.9% for galanin, 53.3% for NPY, and 8.4% for VIP. In children who were in remission for more than a year after treatment (subgroup B) the mean percentages of positive cells were significantly different. The corresponding figures were 35.0% for PGP 9.5 ($p = 0.045$, McNemar's analysis), 26.9% for chromogranin A ($p = 0.034$), 12.1% for galanin ($p = 0.048$), 1.7% for NPY ($p < 0.001$), and 20.5% for VIP ($p = 0.027$). Table 1 summarises these results.

Despite the significantly different expression of these markers, statistical analysis (using Fisher's exact test) revealed that only chromogranin A and NPY were strong markers of an unfavourable prognosis in patients with stage IV neuroblastoma ($p < 0.001$ and $p < 0.002$, respectively).

DISCUSSION

The principal variables affecting the results of neuroblastoma treatment include the stage of the disease, the age of the patient, and the primary location of the tumour.[1] In addition, the histological type of the tumour, the serum ferritin value, and the genetic markers of the disease mentioned above need to be taken into account.[2] In patients with stages I, II, and IV-s disease, three year disease free survival is seen in 75–90% of patients. In children below 1 year of age with stages III and IV disease the survival rates are 80–90% and 60–70%, respectively. In children older than one year of age, three year disease free survival is 50% for patients with stage III disease and from 4% to 15% for those with stage IV disease. Patients with suprarenal involvement have a worse chance of a longterm cure compared with those with tumours at other locations.[1]

> ''A significant correlation was shown between early recurrence of the disease and the presence of chromogranin and neuropeptide Y in the metastatic cells''

The age of the 36 children with neuroblastoma ranged from 4 to 18 years. A suprarenal location of the tumour was found in 12 of the children (six patients in each of the subgroups A and B). In all the patients with stage IV disease, *N-myc* protooncogene amplification was detected in metastatic cells in their bone marrow. Nevertheless, no significant correlations between age, primary location of the tumour, or *N-myc* protooncogene amplification on the one hand, and early relapse of the disease on the other, could be found. However, a significant correlation was shown between early recurrence of the disease and the presence of chromogranin and NPY in the metastatic cells.

Chromogranin A is the index member of the chromogranin/secretogranin (or granin) family of regulated secretory proteins that are ubiquitously distributed in the amine and peptide containing secretory granules of endocrine, neuroendocrine, and neuronal cells.[6] It was initially described in the core of adrenal medullary chromaffin granules, but was subsequently identified in secretory granules throughout the neuroendocrine system, and in a variety of both central and peripheral neurones. Because of their abundance and such widespread occurrence, granins have often been used as prototype proteins to investigate the mechanisms of protein targeting into dense core secretory granules. Neurones and neuroendocrine cells release secretory vesicles (granules) into the ''regulated'' secretory pathway, where the vesicles remain in the cell for an extended period of time, or the ''constitutive'' pathway, in which the secretory cargo leaves the cell promptly after transit through the trans-Golgi network, even in the absence of stimulation.[7 8] Chromogranin A is responsible for the regulated pathway of exocytosis.[6] It is also known as a marker of active cells. In tumour cells it could be considered as a marker of greater malignancy, which is reflected by the worse clinical course seen in neuroblastoma.

Table 1 Immunohistochemical detection of nervous tissue markers in neuroblastoma cells in the bone marrow before treatment

	Subgroup A (n = 19)		Subgroup B (n = 17)	
Neuropeptide	No. of patients (%)	Mean percentage	No. of patients (%)	Mean percentage
Substance P	19 (100)	91.8%	17 (100)	93.4%
Bombesin	19 (100)	84.5%	17 (100)	88.3%
PGP 9.5	9 (47.4)	60.3%	8 (47.1)	35.0%
Chromogranin A	17 (89.5)	90.2%	3 (17.6)	26.9%
Galanin	5 (26.3)	23.9%	5 (29.4)	12.1%
NPY	16 (84.2)	53.3%	5 (29.4)	1.7%
VIP	3 (15.8)	8.4%	4 (21.1)	20.5%

NPY, neuropeptide Y; VIP, vasoactive intestinal peptide.

Take home messages

- Determination of the immunophenotype of metastatic cells in bone marrow (particularly chromogranin A and neuropeptide Y) may be valuable in determining the short term prognosis for children with neuroblastoma
- Neuropeptide Y and chromogranin A may be involved in the modulation of the malignant process by angiogenesis control and regulation of the exocytotic pathway, respectively

NPY, a 36 amino acid neurotransmitter peptide, is widely distributed in sympathetic nerves. Originally, NPY was known for its role as a potent vasoconstrictor and neuromodulator. Released by stress,[9 10] exercise,[11] and myocardial ischaemia,[12] NPY has been implicated in coronary heart disease, congestive heart failure, and hypertension.[9] More recently, because of its ability to stimulate appetite, it has been suggested that it plays a role in obesity and diabetes.[13] It has also been found that NPY is a mitogen for rat aortic vascular smooth muscle cells,[14] and may be an important factor in angiogenesis during tissue development and repair.[15] This may explain why the expression of NPY in neuroblastoma metastatic cells is correlated with a significantly higher risk of early relapse of the disease. It might also indicate that the neuropeptide is potentially important not only in physiological angiogenesis, but also in the pathological course of that angiogenesis.

These observations, together with the finding that chromogranin A and NPY are present in neuroblastoma metastatic cells, not only point to the possible control of the malignant process by neuropeptides but also provide an opportunity to make more accurate prognoses in patients with stage IV disease in the future.

ACKNOWLEDGEMENTS

The authors thank Professor G Shaw for his language editing.

Authors' affiliations

M Nowicki, Department of Histology and Embryology, Poznan University of Medical Sciences, Swiecickiego 6, 60-781 Poznań, Poland
D Ostalska-Nowicka, Department of Cardiology and Nephrology, Poznan University of Medical Sciences
B Miskowiak, Department of Optometry and Biology of the Visual System, Poznan University of Medical Sciences

REFERENCES

1 **Leventhal BG**. Solid tumors. In: *Nelson textbook of pediatrics*, 14th ed. Washington: Behrman, 1992:1481–3.
2 **Brodeur GM**, Maris JM, Yamashiro DJ, *et al*. Biology and genetics of human neuroblastomas. *J Pediatr Hematol Oncol* 1997;**19**:93–101.
3 **Nowicki M**, Miskowiak B. Comparison of the cell immunophenotype of metastatic and primary foci in stage IV-s neuroblastoma. *Folia Histochem Cytobiol* 2002;**40**:297–303.
4 **Shi SR**, Cote RJ, Taylor CR. Antigen retrieval immunocytochemistry: past, present and future. *J Histochem Cytochem* 1997;**45**:327–43.
5 **Elias J**, Gown A, Nakamura R. Special report: quality control in immunohistochemistry. *Am J Clin Pathol* 1989;**92**:836–43.
6 **Taupenot L**, Harper KL, Mahapatra NR, *et al*. Identification of a novel sorting determinant for the regulated pathway in the secretory protein chromogranin A. *J Cell Sci* 2002;**115**:4827–41.
7 **Halban PA**, Irminger JC. Sorting and processing of secretory proteins. *Biochem J* 1994;**299**:1–18.
8 **Kelly RB**. Pathways of protein secretion in eukaryotes. *Science* 1985;**230**:25–32.
9 **Zukowska-Grojec Z**, Wahlestedt C. Origin and actions of neuropeptide Y in the cardiovascular system. In: Colmers WF, Wahlestedt C, eds. *The biology of neuropeptide Y and related peptides*. Totowa, NJ: Humana Press, 1993:315–88.
10 **Zukowska-Grojec Z**, Dayao EK, Karwatowska-Prokopczuk E, *et al*. Stress-induced mesenteric vasoconstriction in rats is mediated by neuropeptide Y Y1 receptors. *Am J Physiol* 1996;**270**:H796–800.
11 **Lundberg JM**, Martinsson A, Hemsen A, *et al*. Co-release of neuropeptide Y and catecholamines during physical exercise in man. *Biochem Biophys Res Commun* 1985;**133**:30–6.
12 **Franco-Cereceda A**, Owall A, Settergren G, *et al*. Release of neuropeptide Y and noradrenaline from the human heart after aortic occlusion during coronary artery surgery. *Cardiovasc Res* 1990;**24**:241–6.
13 **Gerald C**, Walker MW, Criscione L, *et al*. A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* 1996;**382**:168–71.
14 **Zukowska-Grojec Z**, Karwatowska-Prokopczuk E, Fisher TA, *et al*. Mechanisms of vascular growth-promoting effects of neuropeptide Y: role of its inducible receptors. *Regul Pept* 1998;**25**:75–6, 231–8.
15 **Zukowska-Grojec Z**, Karwatowska-Prokopczuk E, Rose W, *et al*. Neuropeptide Y: a novel angiogenic factor from the sympathetic nerves and endothelium. *Circ Res* 1998;**83**:187–95.