**British Journal of Cancer (2008) 98,** 263 – 269

**www.bjcancer.com**



# Detection of GD2-positive cells in bone marrow samples and survival of patients with localised neuroblastoma

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The impact of bone marrow (BM) GD2-positive cells on survival has been evaluated in 145 Italian children with localised neuroblastoma (NB) evaluated at diagnosis by anti-GD2 immunocytochemistry. Nineteen of these (13.1%) were found to be BM GD2-positive, with the number of positive cells ranging between 1 and 155 out of $1 \times 10^6$ total cells analysed. Seven/19 (38.8%) GD2-positive *vs* 12/126 (9.5%) GD2-negative patients relapsed. The 5-year event-free survival (EFS) and overall survival of the GD2-positive patients was significantly worse than that of the GD2-negative ones (62.2 *vs* 89.9%, $P<0.001$; and 74.9 *vs* 95.9%, $P=0.005$, respectively). GD2 positivity was not associated to other known risk factors, and in particular to *Myc-N* amplification and 1p deletion. Among *Myc-N*-negative patients, the EFS of those negative for both GD2 and 1p deletion was significantly better than in children positive for either one of these two markers (EFS = 96.9 *vs* 66.0%, $P<0.001$). In conclusion, GD2 positivity may represent a prognostic marker for patients with non-metastatic NB without *Myc-N* amplification, and its combination with genetic alterations might help identifying patients that require a more careful follow-up.
*British Journal of Cancer* (2008) **98,** 263–269. doi:10.1038/sj.bjc.6604179 www.bjcancer.com
Published online 8 January 2008


**Keywords:** neuroblastoma; detection; GD2; immunocytochemistry; survival

Neuroblastoma (NB) is the most common extracranial solid malignancy of childhood (Henry *et al*, 2005). Staging, clinical management and prognosis mainly depend on the presence/absence of bone marrow (BM) and skeletal involvement (Brodeur *et al*, 1993). Almost 50% of NB patients present at diagnosis with localised disease, that is, they do not have evidence of BM metastases, as assessed by morphological examination of both marrow smears and trephine biopsies, nor other distant localisations investigated by $^{123}$I-MIBG scintigraphy.

Usually patients with localised disease are treated by surgery alone (stage 1 and 2) or by standard-dose chemotherapy followed by surgery (stage 3), unless amplification of the *Myc-N* proto-oncogene is detected in their tumour cells (Rubie *et al*, 1997b; Haase *et al*, 1999; Simon *et al*, 2004; Henry *et al*, 2005; Maris, 2005), which requires a more aggressive chemotherapeutic regimen. Event-free (EFS) and overall (OS) survival of the patients with localised disease without *Myc-N* amplification are good (95% for stage 1, 86% stage 2 and 65% for stage 3 patients; Cotterill *et al*, 2000), but a small percentage of them relapse and may die of disease.

Genetic abnormalities at chromosome 1p (Rubie *et al*, 1997a), 3p, 11q (Spitz *et al*, 2003; Attiyeh *et al*, 2005; Simon *et al*, 2006) and 17q (Brinkschmidt *et al*, 2001), as well as biochemical (Simon *et al*, 2003), histological (Perez *et al*, 2000; Navarro *et al*, 2006; Sano *et al*, 2006), and biological factors (Christiansen *et al*, 1995; Cheung *et al*, 1997; Kramer *et al*, 1997; Perez *et al*, 2000; Ladenstein *et al*, 2001; Mora *et al*, 2001; Krams *et al*, 2003; Riley *et al*, 2004; Haber *et al*, 2006; Spitz *et al*, 2006), do not seem to have the same relevance in patients with localised NB as they do in patients with metastatic disease. Gene expression profiling and GCH studies have suggested specific favourable and unfavourable NB signatures (Takita *et al*, 2004; Ohira *et al*, 2005; Vandesompele *et al*, 2005), but presently a widespread identification of patients at risk of relapse by these techniques cannot be envisaged. Therefore, an independent, easily applicable, prognostic marker able to identify patients that would benefit from a more careful follow-up is currently lacking.

In a previous study, aimed to assess the diagnostic and prognostic role of different techniques detection of NB tumour cells in peripheral blood and BM, we observed that in patients with localised NB the GD2 positivity in BM was negatively associated with survival (Corrias *et al*, 2004). However, this finding was based

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Received 23 August 2007; revised 28 November 2007; accepted 5 December 2007; published online 8 January 2008

on a small sample size with a relatively short follow-up. The aim of the present investigation was to evaluate the impact of BM GD2 positivity, evaluated at diagnosis by anti-GD2 immunocytochemistry (IC), and its combined effect with other known risk factors on survival of a larger cohort of patients with localised NB.

## MATERIALS AND METHODS

### Patients

One hundred and forty-five consecutive NB patients, diagnosed with localised disease (stages 1–3) according to INSS criteria (Brodeur *et al*, 1993) at 20 Italian paediatric oncology centres between January 1997 and June 2003, with available information on BM GD2 status at diagnosis, were included in the study. Disease staging (Brodeur *et al*, 1993) at diagnosis, including appropriate imaging, $^{123}$I MIBG scintigraphy and BM evaluation, was made at the referring oncology centre and centrally reviewed at the Gaslini Institute.

Therapeutic approach for stage 1–2 patients included only surgery followed by a complete re-evaluation with appropriate imaging 1 month later. Stage 3 patients also received chemotherapy according to national or international protocols. Stage 3 and 2 *Myc-N*-amplified patients received high-dose chemotherapy, myeloablative therapy with haematopoietic stem cell rescue and local radiotherapy. In case of relapse, a complete restaging, including imaging and BM evaluation, was performed.

For each patient, demographic, clinical and follow-up data, together with information on biological characteristics and other prognostic risk factors as serum LDH, NSE and ferritin, tumour *Myc-N* amplification and 1p status (Ambros *et al*, 2003), are summarised in Table 1. Data were retrieved from the Italian Neuroblastoma Registry (INBR) that collects information on clinical and biological characteristics of patients at diagnosis as treatment; follow-up is sought during protocol administration and then at least yearly after treatment discontinuation (Conte *et al*, 2006). Pathology data regarding the primary tumour were not considered for this study, since information according to the criteria proposed by the International Neuroblastoma Pathology Committee (INPC) was not available for patients diagnosed before 2003.

### BM analysis

In general, BM aspirations and bone trephine biopsies at both iliac crests were performed under general anaesthesia during surgical procedure on the primary tumour or during '*ad hoc*' sedation. For morphological analysis, three May Grünwald–Giemsa-stained slides from each site were examined by an experienced cytomorphologist and centrally reviewed. Trephine biopsies were obtained by a Jamshidi needle, and only biopsies containing at least 5 mm$^3$ of tissue were considered adequate for evaluation. At least 30 high-resolution fields of haematoxylin–eosin-stained sections were evaluated by an experienced pathologist and centrally reviewed.

For the purpose of this study, GD2-IC was centrally performed at the Italian NB reference laboratory, as previously described (Corrias *et al*, 2004). Briefly, six cytospins, each containing $5 \times 10^5$ mononuclear cells, were fixed in cold acetone and incubated with the 3F8 anti-GD$_2$ mAb (kindly donated by Dr Nai-Kong Cheung, Memorial Sloan Kettering Cancer Center, New York, NY, USA). After washing, slides were incubated with a biotinylated anti-mouse antibody and developed with an avidin–alkaline phosphatase conjugate (DAKO, Copenhagen, Denmark). Enumeration of GD2-positive cells was based on both morphological and immunological criteria, according to standardised conditions (Swerts *et al*, 2005). Namely, the presence of round nuclei larger than that of small lymphocytes, granular chromatic structure and scarce amount of cytoplasm were considered positive morphological criteria; strong red staining localised to the entire cell membrane and cytoplasm was the positive immunological criterion. Bone marrow samples were considered positive if at least three positive tumour cells were detected out of the $3 \times 10^6$ cells analysed.

The study was approved by the Institutions' Ethical Committees. All analyses were performed after informed consent was given from the patients themselves or their legal guardians, according to the Helsinki declaration.

**Table 1** Demographic, clinical, biochemical and genetic features of the 145 patients with localised NB stratified by GD2 status

| | GD2 negative ($n=126$) | | GD2 positive ($n=19$) | | | Total ($n=145$) | |
|---|---|---|---|---|---|---|---|
| | ***n*** | **%** | ***n*** | **%** | ***P*** | ***n*** | **%** |
| *Gender* | | | | | 0.059 | | |
| Male | 69 | 54.8 | 6 | 31.6 | | 75 | 51.7 |
| Female | 57 | 45.2 | 13 | 68.4 | | 70 | 48.3 |
| *Age* | | | | | 0.932 | | |
| $<12$ months | 61 | 48.4 | 9 | 47.4 | | 70 | 48.3 |
| $\geqslant 12$ months | 65 | 51.6 | 10 | 52.6 | | 75 | 51.7 |
| *Primary site* | | | | | 0.923 | | |
| Neck | 7 | 5.6 | 0 | 0.0 | | 7 | 4.8 |
| Thorax | 29 | 23.0 | 4 | 21.1 | | 33 | 22.8 |
| Abdomen, adrenal | 44 | 34.9 | 7 | 36.8 | | 51 | 35.2 |
| Abdomen, other sites | 46 | 36.5 | 8 | 42.1 | | 54 | 37.2 |
| *INSS stage* | | | | | 0.315 | | |
| Stage 1 | 59 | 46.8 | 7 | 36.8 | | 66 | 45.5 |
| Stage 2 | 35 | 27.8 | 4 | 21.1 | | 39 | 26.9 |
| Stage 3 | 32 | 25.4 | 8 | 42.1 | | 40 | 27.6 |
| *NSE (78 tested)* | | | | | 0.660 | | |
| $<100$ ng ml$^{-1}$ | 57 | 89.1 | 12 | 85.7 | | 69 | 88.5 |
| $\geqslant 100$ ng ml$^{-1}$ | 7 | 10.9 | 2 | 14.3 | | 9 | 11.5 |
| *LDH (123 tested)* | | | | | 0.999 | | |
| $<1000$ IU ml$^{-1}$ | 86 | 81.9 | 15 | 83.3 | | 101 | 82.1 |
| $\geqslant 1000$ IU ml$^{-1}$ | 19 | 18.1 | 3 | 16.7 | | 22 | 17.9 |
| *Ferritin (95 tested)* | | | | | 0.999 | | |
| $<150$ ng ml$^{-1}$ | 63 | 77.8 | 11 | 78.6 | | 74 | 77.9 |
| $\geqslant 150$ ng ml$^{-1}$ | 18 | 22.2 | 3 | 21.4 | | 21 | 22.1 |
| *Myc-N (124 tested)* | | | | | 0.999 | | |
| Not amplified | 95 | 88.8 | 15 | 88.2 | | 110 | 88.7 |
| Amplified | 12 | 11.2 | 2 | 11.8 | | 14 | 11.3 |
| *1p36 (105 tested)* | | | | | 0.999 | | |
| Not deleted | 60 | 67.4 | 11 | 68.8 | | 71 | 67.6 |
| Deleted | 14 | 15.7 | 2 | 12.5 | | 16 | 15.2 |
| Imbalance | 15 | 16.9 | 3 | 18.8 | | 18 | 17.1 |
| 5-year EFS | 126 | 89.9 | 19 | 62.2 | $<0.001$ | | |
| 5-year OS | 126 | 95.9 | 19 | 74.9 | 0.005 | | |

EFS = event-free survival; NB = neuroblastoma; OS = overall survival.

### Statistical analysis

Descriptive statistics were reported as percentages for categorical variables. For continuous and counting data, medians with interquartile range (IQR) were used due to the non-normal distribution of the observations and to reduce the effect of outliers.

Patients were stratified according to their BM GD2 status and comparisons of frequency data were performed by means of the $\chi^2$-test or the Fisher's exact test, when appropriate. The Wilcoxon Mann – Whitney test was used to compare median values, while the Spearman $\rho$ coefficient was used to assess correlation between variables. Event-free survival and OS analyses were performed according to the Kaplan – Meier method and compared by the log-rank test. A *P*-value $<0.05$ was considered as statistically significant. Analyses were performed using Stata for Windows statistical package (release 7.0; Stata Corporation, College Station, TX, USA).

## RESULTS

During the study period, 145 patients diagnosed with localised NB and registered in the INBR had their BM aspirates analysed at diagnosis by anti-GD2-IC. Patients included in the study were similar for age, sex, stage, *Myc-N* status and survival to the children with localised NB without information on GD2 status at diagnosis (see Supplementary data).

Demographic, clinical, biochemical and genetic features of the 145 study patients stratified according to BM GD2-IC status are reported in Table 1. In more detail, 126 patients (86.9%) were GD2 negative and 19 (13.1%) were GD2 positive. Among the 19 GD2-positive patients (Table 2), the number of positive cells ranged between 1 and 155 (median = 3; IQR 2-20) out of $10^6$ total cells examined. It is to be noted that of the 11 patients with less than five GD2-positive cells/$10^6$ total cells, seven were also evaluated by RT-PCR and all but one were found to be positive for at least one NB molecular marker (data not shown). Three examples of GD2-positive samples are shown in Figure 1. As reported in Table 1, no association was found between GD2 status and each of the other risk factors considered.

Seven (36.8%) of the 19 GD2-positive patients relapsed, all locally, and four of them died due to local disease progression (Table 2). The 5-year EFS and OS of this group were 62.2 and 74.9%, respectively (Table 1; Figure 2A and B). In these patients, an inverse correlation between the number of GD2-positive cells and the time to relapse was observed ($\rho = -0.786$, $P = 0.036$). Conversely, among the 126 BM GD2-negative patients, only 12 (9.5%) relapsed, 10 locally and two with metastatic disease, and five subsequently died (Table 2) with a 5-year EFS of 89.9% and an OS of 95.9% (Table 1; Figure 2A and B). Differences in EFS and OS between the two groups were significant ($P<0.001$ and $P = 0.005$, respectively; Table 1). If, as in our previous study, the five cells/$10^6$ cut off was used to discriminate positive BM samples, differences in survival remained highly significant (data not shown). Table 3 further reports on the 5-year EFS analyses on the entire cohort also considering other clinical and biological risk factors. A statistically significant worse effect was observed for unresectable disease (i.e., stage 3) (EFS = 76.7%) *vs* resectable disease (i.e., stage 1 – 2) (EFS = 89.8%; $P = 0.033$), high LDH levels (72.1 *vs* 89.5%, $P = 0.010$), *Myc-N* amplification (EFS = 57.1 *vs* 90.4%, $P<0.001$)

**Table 2** Features of the GD2-positive patients and of the GD2-negative patients who relapsed

| Stage | Sex/age (months) | GD2+ cells/$10^6$ total | Tumour site | LDH (IU ml$^{-1}$) | *Myc-N* | 1p | Relapse (months) | Type of relapse | Follow-up[a] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M/45 | 1.3 | Thorax | 650 | Normal | Normal | — | — | CR |
| 1 | F/2 | 1.0 | Abdomen | 475 | Normal | Normal | — | — | CR |
| 1 | M/25 | 2.2 | Pelvis | 523 | Normal | Normal | — | — | CR |
| 1 | F/93 | 1.6 | Thorax | 601 | Normal | Normal | — | — | CR |
| 1 | F/2 | 3.0 | Abdomen | 411 | ND | ND | — | — | CR |
| 1 | M/2 | 10.0 | Abdomen | 494 | Normal | Normal | — | — | CR |
| 1 | M/6 | 39.3 | Abdomen | 1961 | Amplified | Deleted | 1.8 | Local | DPD |
| 1 | F/10 | Negative | Abdomen | 1693 | Amplified | Deleted | 1.4 | Local+Met | DPD |
| 1 | F/25 | Negative | Thorax | 617 | ND | ND | 18.0 | Local | AWD |
| 1 | F/1 | Negative | Abdomen | NE | Normal | Normal | 10.0 | Local+Met | AWD |
| 2 | F/59 | 8.0 | Abdomen | 339 | Normal | ND | — | — | CR |
| 2 | F/3 | 2.0 | Abdomen | 336 | Normal | Imbalance | — | — | CR |
| 2 | F/10 | 2.0 | Abdomen | 484 | Gain | Normal | 24.0 | Local | DPD |
| 2 | F/6 | 3.3 | Abdomen | 949 | Normal | Normal | 15.0 | Local | CR |
| 2 | F/43 | Negative | Abdomen | NE | Normal | Deleted | 30.0 | Local | CR |
| 2 | M/7 | Negative | Abdomen | 1481 | Normal | ND | 3.8 | Local | CR |
| 2 | F/11 | Negative | Neck | 444 | Normal | Imbalance | 25.1 | Local | AWD |
| 2 | M/1 | Negative | Abdomen | 409 | Normal | Deleted | 5.0 | Local | DPD |
| 3 | M/15 | 2.0 | Thorax | 477 | Normal | Imbalance | — | — | CR |
| 3 | F/50 | 4.5 | Abdomen | 735 | Normal | Deleted | — | — | CR |
| 3 | F/15 | 5.0 | Abdomen | 1051 | Normal | Normal | — | — | CR |
| 3 | F/6 | 20.0 | Abdomen | 791 | Normal | Normal | — | — | CR |
| 3 | F/8 | 34.6 | Abdomen | 437 | Normal | Normal | 8.1 | Local | CR |
| 3 | F/31 | 29.1 | Abdomen | 2445 | Amplified | ND | 13.3 | Local | DPD |
| 3 | F/18 | 155.0 | Abdomen | ND | Normal | Normal | 1 | Local | DPD |
| 3 | M/73 | 1.0 | Thor/Abd | 800 | ND | Imbalance | 13.2 | Local | CR |
| 3 | F/11 | Negative | Abdomen | 6247 | Amplified | Deleted | 7.0 | Local | DPD |
| 3 | F/118 | Negative | Abdomen | 127 | ND | Imbalance | 7.0 | Local | AWD |
| 3 | M/17 | Negative | Abdomen | 4672 | Amplified | Deleted | 1.0 | Local | DPD |
| 3 | M/13 | Negative | Neck | 536 | Amplified | Deleted | 14.0 | Local | DPD |
| 3 | M/7 | Negative | Abdomen | 401 | Normal | Deleted | 17.0 | Local | CR |

[a]AWD = alive with disease; CR = complete remission; DPD = dead of progressive disease; F = female; M = male; ND = not determined.

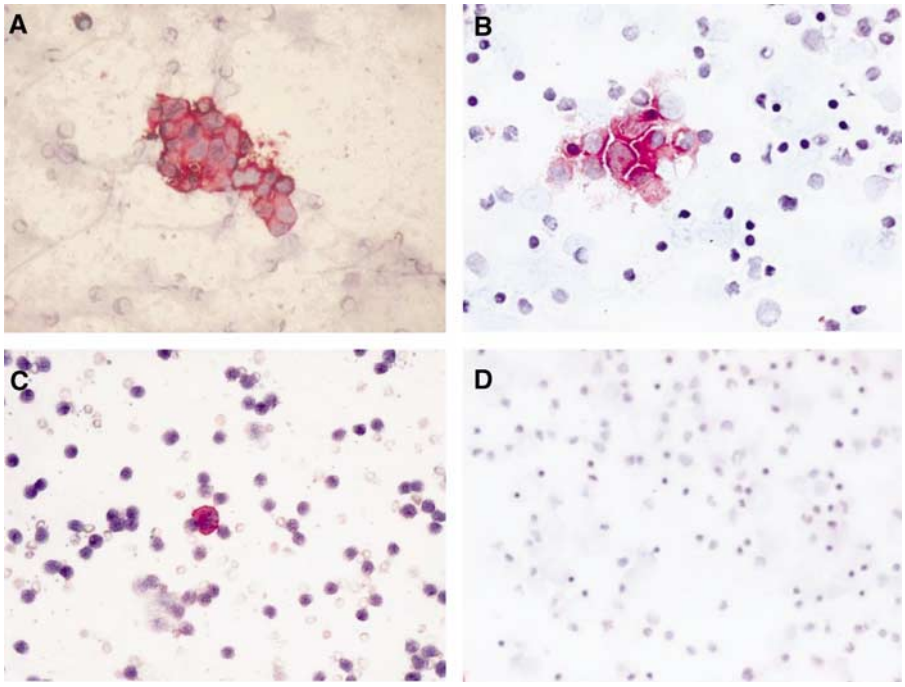


**Figure 1** Cytospin of BM aspirates fixed in acetone and immunologically stained with anti-GD2 antibody. (**A**, **B**) Rosettes of NB cells stained in red, from patients F/18 and F/8, respectively; (**C**) a single NB cell stained in red from patient M/73; (**D**) a completely negative aspirate. Magnification is × 40.

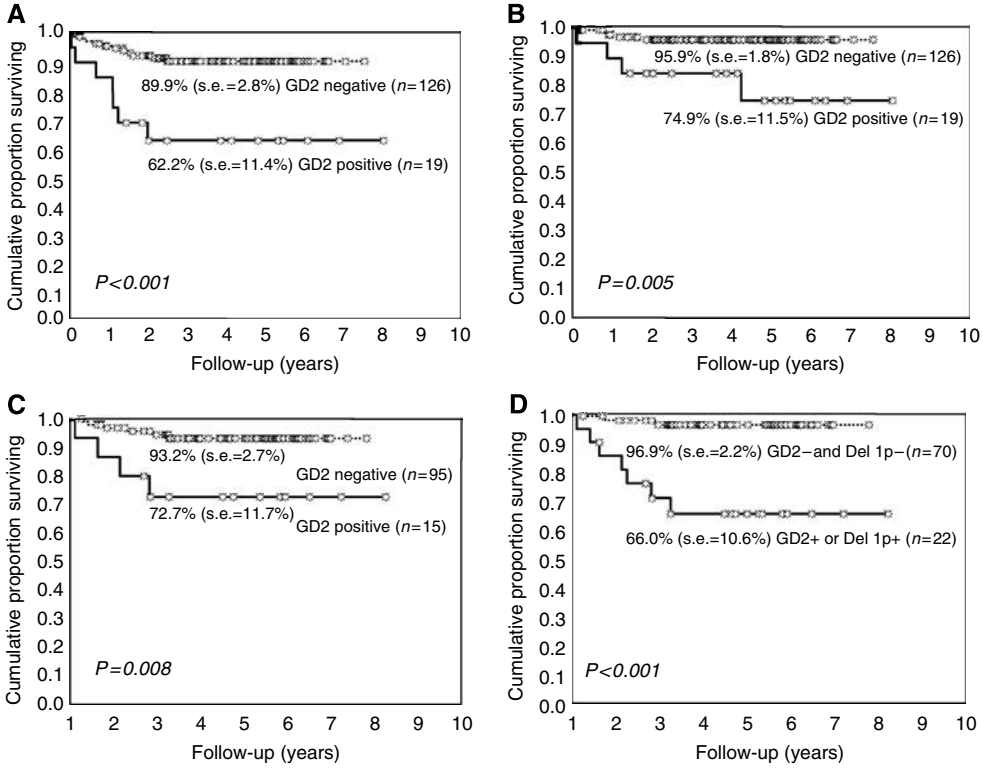


**Figure 2** (**A**) Event-free survival of patients with localised NB stratified by BM GD2-IC status. (**B**) Overall survival of patients with localised NB stratified by GD2-IC status. (**C**) Event-free survival of patients with normal *Myc-N* status stratified by GD2-IC status. (**D**) Event-free survival of patients with normal *Myc-N* status stratified by GD2-IC and chromosome 1p status.

and chromosome 1p status (EFS = 45.8% for deletion, 83% for imbalance and 92.7% for normal, $P < 0.001$). The negative effect of 1p deletion remained even if 1p imbalance was pooled with the not deleted cases (data not shown); for this reason, in subsequent analyses patients with 1p imbalance were grouped with the not deleted ones.

Since *Myc-N* status directs patients towards either standard or high-risk treatment, survival analysis was repeated in patients with

**Table 3** EFS of patients with localised NB stratified according to the indicated variables

| Variables | *n* | 5-year EFS % | s.e.[a] | *P* |
|---|---|---|---|---|
| *GD2* | | | | <0.001 |
| Negative | 126 | 89.9 | 0.03 | |
| Positive | 19 | 62.2 | 0.36 | |
| *Gender* | | | | 0.166 |
| Male | 70 | 90.5 | 0.03 | |
| Female | 75 | 81.2 | 0.05 | |
| *Age* | | | | 0.352 |
| <12 months | 70 | 83.6 | 0.05 | |
| ⩾12 months | 75 | 88.7 | 0.04 | |
| *Primary site*[b] | | | | 0.211 |
| Abdomen | 105 | 84.1 | 0.04 | |
| Thorax | 40 | 91.6 | 0.05 | |
| *Tumour stage (INSS)* | | | | 0.033 |
| Stages 1–2 | 105 | 89.8 | 0.03 | |
| Stage 3 | 40 | 76.7 | 0.07 | |
| *NSE (78 tested)* | | | | 0.445 |
| <100 ng ml$^{-1}$ | 69 | 86.1 | 0.04 | |
| ⩾100 ng ml$^{-1}$ | 9 | 77.8 | 0.14 | |
| *LDH (123 tested)* | | | | 0.010 |
| <1000 IU ml$^{-1}$ | 101 | 89.5 | 0.03 | |
| ⩾1000 IU ml$^{-1}$ | 22 | 72.1 | 0.10 | |
| *Ferritin (95 tested)* | | | | 0.604 |
| <150 ng ml$^{-1}$ | 74 | 84.4 | 0.04 | |
| ⩾150 ng ml$^{-1}$ | 21 | 80.0 | 0.09 | |
| *Myc-N (124 tested)* | | | | <0.001 |
| Not amplified | 110 | 90.4 | 0.03 | |
| Amplified | 14 | 57.1 | 0.13 | |
| *1p36 (105 tested)* | | | | <0.001 |
| Not deleted | 71 | 92.7 | 0.03 | |
| Deleted | 16 | 45.8 | 0.14 | |
| Imbalance | 18 | 83.0 | 0.09 | |

EFS = event-free survival; NB = neuroblastoma. [a]Standard error. [b]Abdomen = adrenal+other abdominal site; thorax = thorax+neck.

and without *Myc-N* amplification. In *Myc-N*-amplified patients, GD2 negativity was associated with a better survival (EFS 66 *vs* 0%), but the difference was not significant ($P = 0.073$), likely due to the small number of cases. However, in *Myc-N* non-amplified patients, the BM GD2 negativity was associated with a significantly better outcome (EFS = 93.2 *vs* 72.7%, $P = 0.008$; Figure 2C). In the same *Myc-N*-negative patients, the combined prognostic role of GD2 and 1p status was also assessed. Patients negative for both markers had a 96.9% EFS, which was significantly better than that observed among children positive for at least one marker (EFS = 66.0%; $P < 0.001$; Figure 2D). In the same group of *Myc-N*-negative patients, the OS showed a similar pattern (98% for the GD2-negative subjects *vs* 83% for the GD2-positive subjects). Finally the OS of patients positive for either GD2 or *Myc-N* was worse than that of patients negative for both the markers (83.1 *vs* 100%), but the very low number of observed deaths prevents one to draw definitive conclusions.

Finally, among BM GD2-IC-positive patients, a worse survival was found in children with ⩾20 GD2-positive cells/$10^6$ total cells, corresponding to the fourth quartile (EFS = 77.1 *vs* 20.0%, $P = 0.002$). A similar result was also observed after excluding the two patients with *Myc-N* amplification (data not shown).

Because of the small sample size (19 BM GD2-positive patients) and low number of events, results from a multivariate analysis should be taken with caution and are herewith reported only as Supplementary data. In this analysis, the GD2-IC status seemed to maintain its prognostic role independently from both *Myc-N* amplification and 1p deletion. It is of note that conversely *Myc-N* amplification and 1p deletion were indeed correlated, being 72.7% of *Myc-N* amplified patients also deleted of 1P.

## DISCUSSION

This study has confirmed our previous observation (Corrias *et al*, 2004) of a negative impact of BM GD2-positive cell infiltration on survival of patients with localised NB. In particular, in this larger cohort of patients with a longer follow-up, we have shown that this effect is not due to the association with other risk factors as *Myc-N* amplification and 1p deletion.

Other reports have previously documented that tumour cells may be detected by IC and/or RT-PCR in BM of children with localised NB (Moss *et al*, 1991; Cheung *et al*, 1998; Trager *et al*, 2003; Corrias *et al*, 2004; Simon *et al*, 2004; Ifversen *et al*, 2005; Russell *et al*, 2005; Swerts *et al*, 2006). However, the limited number of patients analysed and/or the short follow-up of those studies did not allow one to draw definitive conclusions on the prognostic impact of this detection. In addition, a large multicentre European study (Navarro *et al*, 2006) and several other national studies (Christiansen *et al*, 1995; Cheung *et al*, 1997; Ladenstein *et al*, 2001; Simon *et al*, 2003; Sano *et al*, 2006; Spitz *et al*, 2006) have indicated that stage and *Myc-N* status were the only independent risk factors for children with localised NB. However, *Myc-N* amplification is a relatively rare event, occurring, as in our series, in about 10% of localised NB patients (Haase *et al*, 1999; Perez *et al*, 2000; Henry *et al*, 2005; Maris, 2005) and is thus inadequate to identify all the patients who will eventually relapse.

A negative prognostic role of unfavourable histology (Perez *et al*, 2000), of 11q LOH (Attiyeh *et al*, 2005; Simon *et al*, 2006), and of a specific GCH profile (Schleiermacher *et al*, 2007), was also previously reported in patients with localised disease. This effect was independent from *Myc-N* status. Unfortunately, homogeneous information on tumour histology (i.e., INPC classification) was not available in our registry because of the long recruitment period covered by this study. Moreover, our patients were not screened for genetic abnormalities other than *Myc-N* amplification and 1p deletion. Even if genetic screening could have been more precise, our observation of a negative prognostic role of BM GD2-IC positivity suggests that also this technique, which has become accurate, thanks to recent standardisation (Swerts *et al*, 2005), might be of interest in future studies.

The low relapse rate observed in our cohort is similar to that expected in patients with localised NB, and indicates that staging was correct. The fact that presence of GD2-positive cells in the BM correlated with a higher tendency of relapse at the primary site, instead that in the BM, is surprising. However, also Perez *et al* (2000) did not observe further BM involvement in eight stage 1–2 patients found positive by BM immunocytology. Thus, it is conceivable that the very few GD2-positive cells, detected by IC at diagnosis, were unable to survive and actively proliferate in the BM. Whether this inability to invade the marrow compartment was related to the NB cells themselves or to the presence/absence of factors in the BM microenvironment (Fidler, 2003) remains to be determined in future studies.

Our observation that higher number of GD2-positive cells correlated with poorest survival and with a shorter time to relapse is similar to that previously reported by Moss *et al* (1991). Even if this finding was based on a very small sample size (i.e., seven relapses in GD2-positive patients), it suggests that quantification

by IC might add useful clinical information and that BM GD2-IC positivity reflects a biological feature of the neuroblasts that can affect the disease course.

In our study, survival analysis on other prognostic factors confirmed the negative role of *Myc-N* amplification, high LDH serum levels and 1p deletion. Only a multivariable analysis based on a larger series will be able to assess the combined effect of BM GD2 positivity and the other known major risk factors. However, our data suggest that in patients with localised disease without *Myc-N* amplification, the combination of BM GD2-IC and 1p status might help individuating those at risk of relapse.

In conclusion, we believe that BM GD2-IC analysis might have the potential to discriminate different risk groups within localised NB patients. In fact, GD2-IC seems to provide additional information on biological features and has the advantage of generating quantitative data. We neither recommend that BM GD2-IC status be used for staging purposes, nor the patient be shifted to a more intensive treatment because not all the GD2 positive patients relapsed. However, in future multi-national studies, patients with localised disease should be evaluated by GD2-IC. In fact, in the presence of BM GD2-positive cells, especially if combined with chromosome 1p deletion, patients should be closely followed, and in case of relapse, they should be treated more aggressively.

## ACKNOWLEDGEMENTS

This study was supported in part by grants from Fondazione Italiana per la Lotta al Neuroblastoma, Fondazione CA.RI.GE, and Ministry of Health, Italy. SP, FN and BC are recipients of Fondazione Italiana per la Lotta al Neuroblastoma fellowships. The precious INBR data management of Mr Filippo Papio and Ms Barbara Galleni is deeply acknowledged. We thank Veronica Tintori and Cinzia Marchi for providing images of GD2-IC, Bruno De Bernardi for his criticism in reviewing the manuscript and all the physicians who have centralized bone marrow samples for GD2-IC analysis.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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