**SUPPLEMENTAL PATIENTS AND METHODS**

***Sample Processing and NB5 Assay***

NB5 TLDA assays were performed on mononuclear cells from heparinized blood and BM (pooled bilateral aspirates) isolated by density separation with Ficoll-Hypaque.[23](#_ENREF_23) Pelleted fresh cells were treated with buffer RLT (QIAGEN) containing 1% β-mercaptoethanol to lyse cells, and the lysate was stored at -80oC until RNA was prepared. Viably frozen specimens were defrosted in RNAprotect Cell Reagent (QIAGEN) to protect RNA during thawing, and thawed cells were then pelleted by centrifugation. Total RNA was prepared using the TRIzol® reagent (Invitrogen) and processed with the RNeasy® Mini Kit (QIAGEN). The RNA Integrity Number (RIN) was obtained using the Agilent Bioanalyzer, and only specimens with RIN >5.5 were tested. Two-step RT-PCR was performed using Oligo-dT plus gene specific primers for CHGA, DCX, DDC, PHOX2B, and TH. Reverse transcription of 2,500 ng total RNA in 20 μl was carried out with M-MLV Reverse Transcriptase (Life technologies). The TLDA assay for each specimen quantified the expression of NB-associated genes CHGA, DCX, DDC, PHOX2B, and TH and of housekeeping genes B2M (beta-2 microglobulin), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), HPRT1 (hypoxanthine guanine phosphoribosyltransferase), and SDHA (succinate dehydrogenase complex, subunit A) using pre-designed and pre-optimized probe and primer sets (Supplemental Table A2), 2,500 ng of cDNA, and standard cycling conditions to enable assays for all genes to be run together with a single TLDA card using the 7900HT instrument (Applied Biosystems). The Ct (Cycle Threshold) value for each gene was the cycle number where the amplification signal reached a threshold of 0.5 over baseline. A Ct value of 40 was assigned when this threshold was not reached by the 40th cycle. A summary ΔCt for the five detection genes was computed as the geometric mean (GM) of the Cts for the five detection genes minus GM of the Cts of the four housekeeping genes (TLDA ΔCt). Lower TLDA ΔCt values indicate higher NB-mRNA. Results were classified as “undetectable” when none of the five NB gene Ct values was < 40.

***Disease evaluation***

CT/MRI images were reviewed by one radiologist (FG) and assessed by RECIST 1.0 criteria for presence and size of all target and non-target lesions.[24](#_ENREF_24) The sum of the longest diameter (LD) of all target lesions was evaluated for all CT/MRI scans. MIBG scans were reviewed by one radiologist (HJ), who performed Curie scoring.[25](#_ENREF_25),[26](#_ENREF_26) Histopathology of bilateral BM biopsy specimens was reviewed by one pathologist (HS), and the maximum percentage of tumor cells from either side was assigned as the percent of tumor involvement

Response was graded at each time point for CT/MRI and MIBG lesions and for BM, and these assessments were combined into an overall response per the NANT Response Criteria (v1.0). Overall response was based on central review of the radiological scans and BM biopsies as well as review of institutional reports by one oncologist (AM).

Soft tissue lesions that remained stable in size after protocol therapy were considered stable disease (SD) even if MIBG uptake was no longer present within those lesions. MIBG response was considered complete remission (CR) if all lesions disappeared, partial response (PR) if there was a 50% reduction in the Curie score, SD if the score showed reduction of less than 50%, and progressive disease (PD) for appearance of new lesions or increase in score of >20%. BM response was defined as CR, SD, or PD, with CR defined as no tumor cells on two bilateral BM aspirates and biopsies obtained at least 3 weeks apart. BM PD was defined as 1) no tumor cells at enrollment and then tumor cells observed on two consecutive examinations at least 3 weeks apart (or one examination at the treating physician’s discretion); or 2) tumor cells at enrollment with a subsequent increase to a minimum of 25% tumor cells and a doubling of the amount at enrollment. BM SD was any amount of residual BM involvement not qualifying as CR or PD. Central review of BM biopsies and CT/MRI were not available in 14% and 20% of assessments, respectively, in which case institutional data were used. The correlation between central and institutional review of evaluations was high when both were available (Pearson r = 0.73 for CT/MRI LD and r=0.98 for BM tumor cell percent).

The overall response assigned was CR, PR, mixed response (MR) (one site PR or CR while another stable), SD, or PD when compared to baseline or best response. For patients with multiple disease evaluation time points, each disease progression was considered a new baseline for subsequent response evaluation. Time to progression was calculated from the first baseline as well as after each subsequent progression.

**SUPPLEMENTAL TABLES**

**Table A1.** Therapies received for samples submitted on this study.

|  |  |  |  |
| --- | --- | --- | --- |
| Protocol | Treatment | BM n (%) | Blood n(%) |
| N1999-02 | Phase I BSO + Melphalan + PBSC | 6 (3) | 3 (2) |
| N2004-04 | Phase I Oral Fenretinide | 39 (17) | 16 (11) |
| N2007-02 | Phase I Cyclophosphamide + Bevacizumab + Zoledronic Acid | 5 (2) | 7 (5) |
| N2007-03 | Phase I MIBG + Vorinostat + PBSC | 12 (5) | 5 (4) |
| N2008-02 | Phase I Isotretinoin + Vorinostat | 25 (11) | 19 (13) |
| N2009-03 | Phase I MLN8237 + Irinotecan + Temozolamide | 22 (10) | 11 (8) |
| N2011-04 | Phase I Lenalidomide + ch14.18 + Isotretinoin | 4 (2) | 1 (1) |
| N2012-01 | Phase I DFMO + Cyclophosphamide + Topotecan | 4 (2) | 0 |
| Non NANT therapies\* | immunotherapy (ANBL1021, compassionate ch14.14), I131-MIBG, cyclophosphamide/topotecan, Ifosfamide/carboplatin/etoposide, irirnotecan/temozolamide, local radiation | 106 (48) | 80 (56) |
| All |  | 223 (100) | 142 (100) |

**Table A2.** Genes and Primer and Probe Sets for NB5 assay

|  |  |  |
| --- | --- | --- |
| Gene Symbol | Gene Name | Primer and Probe Set |
| Neuroblastoma |  |  |
| CHGA | chromogranin A (parathyroid secretory protein 1) | CHGA-Hs00154441\_m1 |
| DCX | doublecortin | DCX-Hs00167057\_m1 |
| DDC | dopa decarboxylase (aromatic L-amino acid decarboxylase) | DDC-Hs00168031\_m1 |
| PHOX2B | paired-like homeobox 2b | PHOX2B-Hs00243679\_m1 |
| TH | tyrosine hydroxylase | TH-Hs00165941\_m1 |
|  |  |  |
| Housekeeping |  |  |
| B2M | beta-2-microglobulin | B2M-Hs99999907\_m1 |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase | GAPDH-Hs99999905\_m1 |
| HPRT1 | hypoxanthine phosphoribosyltransferase 1 | HPRT1-Hs99999909\_m1 |
| SDHA | succinate dehydrogenase complex, subunit A, flavoprotein | SDHA-Hs00417200\_m1 |

**Table A3.** Clinical Evaluations and NB5 TLDA Assays Performed

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Clinical Evaluation | TLDA Assay | | | |  |
|  | BM only | BLD only | Both | Neither | Total |
| CT-MRI/MIBG/BM | 106 | 10 | 101 | 15 | 232 |
| CT-MRI/MIBG | 1 | 24 | 0 | 20 | 45 |
| CT-MRI/BM | 1 | 0 | 0 | 0 | 1 |
| MIBG/BM | 7 | 0 | 4 | 1 | 12 |
| CT-MRI | 0 | 0 | 0 | 1 | 1 |
| MIBG | 0 | 2 | 0 | 9 | 11 |
| BM | 2 | 0 | 1 | 0 | 3 |
| Total | 117 | 36 | 106 | 46 | 305 |

**Table A4.** Contribution of Individual Gene to NB5 Signature and AUC

|  |  |  |
| --- | --- | --- |
| Signature | AUC | % Change from NB5 |
| NB5 | 0.622 | NA |
| NB5 minus CHGA | 0.592 | - 4.8% |
| NB5 minus DDC | 0.608 | - 2.3% |
| NB5 minus DCX | 0.627 | + 0.8% |
| NB5 minus PHOX2B | 0.609 | - 2.1% |
| NB5 minus TH | 0.633 | + 1.8% |
|  |  |  |
| TH/DCX | 0.497 | - 20% |
| PHOX2B/TH/DDC | 0.595 | - 4.3% |
| PHOX2B/TH/DCX | 0.565 | - 9.2% |
| PHOX2B/TH | 0.572 | - 8.0% |