

## Exploiting Gene Expression Profiling to Identify Novel Minimal Residual Disease Markers of Neuroblastoma

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**Abstract** **Purpose:** Minimal residual disease (MRD) presents a significant hurdle to curing metastatic neuroblastoma. Biological therapies directed against MRD can improve outcome. Evaluating treatment efficacy requires MRD measurement, which serves as surrogate endpoint. Because of tumor heterogeneity, no single marker will likely be adequate. Genome-wide expression profiling can uncover potential MRD markers differentially expressed in tumors over normal marrow/blood. **Experimental Design:** Gene expression array was carried out on 48 stage 4 tumors and 9 remission marrows using the Affymetrix U95 gene chip. Thirty-four genes with a tumor-to-marrow expression ratio higher than tyrosine hydroxylase were identified. Quantitative reverse transcription-PCR was done on all 34 genes to study the dynamic range of tumor cell detection and the expression of these genes in normal marrow/blood samples and in stage 4 neuroblastoma tumors. Top ranking markers were then tested for prognostic significance in the marrows of stage 4 patients collected from the same treatment protocol after two cycles of immunotherapy. **Results:** Based on sensitivity assays, 8 top-ranking markers were identified: CCND1, CRMP1, DDC, GABRB3, ISL1, KIF1A, PHOX2B, and TACC2. They were abundantly expressed in stage IV neuroblastoma tumors ( $n = 20$ ) and had low to no detection in normal marrow/blood samples ( $n = 20$ ). Moreover, expression of CCND1, DDC, GABRB3, ISL1, KIF1A, and PHOX2B in 116 marrows sampled after two treatment cycles was highly prognostic of progression-free and overall survival ( $P < 0.001$ ). **Conclusions:** Marker discovery based on differential gene expression profiling, stringent sensitivity and specificity assays, and well-annotated patient samples can rapidly prioritize and identify potential MRD markers of neuroblastoma.

A major obstacle to curing metastatic neuroblastoma is the presence of minimal residual disease (MRD) in the bone marrow and peripheral blood even after the patient has achieved clinical remission. Before MRD can be targeted by either immunotherapy or myeloablative therapy, it needs to be detected and quantified. Although there are only a few established MRD markers for neuroblastoma, there is increasing evidence that MRD markers can be clinically useful (1–5). Residual occult tumor cells at the end of each phase of treatment can have an adverse effect on disease relapse and patient survival.

Despite their clinical utility in proof-of-principle studies, single markers are likely to be inadequate because tumor heterogeneity is a hallmark in cancers including neuroblastoma. For example, although GD2 synthase (GalNAcT) is a highly sensitive MRD marker, it is generally believed that some tumors treated with GD2-directed therapy, be it antibody, immunocytokine, or single-chain Fv-modified T cells, can down-regulate the enzyme and the antigen GD2 as an escape mechanism. Similarly, tumors treated with another common modality (<sup>131</sup>I-MIBG) are expected to down-regulate its metabolic pathway, where tyrosine hydroxylase (TH) is a critical step. The rationale for using multiple markers is compelling (6). However, there is a paucity of MRD markers and previous efforts of tumor marker discovery have failed to identify candidates solely intended for MRD measurement because of suboptimal specificity and sensitivity (7).

For an orphan disease like neuroblastoma with few known markers, genome-based expression screening is most likely to be useful. This is particularly true when marker discovery takes into account the context where tumor measurement is most relevant, informative, and feasible, that is, the bone marrow and peripheral blood compartment. Using this approach, our laboratory was able to identify new markers of subclinical disease for Ewing's family of tumors (8). As to neuroblastoma, this gene expression profiling strategy uncovered cyclin D1 as a novel molecular marker of MRD for patients with metastatic neuroblastoma (9). In this report, an array analysis based on

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## Translational Relevance

Despite achieving clinical remission, cancers like metastatic neuroblastoma often recur due in part to the presence of subclinical MRD. Targeted therapy directed against MRD will improve outcome. The ability to measure MRD is critical for gauging the success of these targeting strategies, especially in the key metastatic compartments of marrow and blood. However, no single MRD marker will be adequate because of tumor heterogeneity. Tumor-selective mRNAs are sensitive and specific markers of active disease. In this report, using Affymetrix U95A-E gene expression array analysis on tumors from 48 stage 4 neuroblastoma patients, 34 potential MRD markers were identified. Sensitivity and specificity studies narrowed the list to 8 top-ranking candidates. Using 116 well-annotated bone marrow samples collected from the same phase of an immunotherapy protocol, we further narrowed the list to 6 novel markers based on their prognostic effect on clinical outcome. We conclude that a genome-wide marker discovery approach could identify MRD molecular markers. This is particularly relevant for orphan diseases where known markers are generally scarce, and the ranking of potential markers by clinical significance can reduce false leads. We believe these novel markers will augment the precision in measuring MRD in metastatic neuroblastoma.

tumors from 48 stage 4 neuroblastoma patients was used to rapidly filter 34 potential MRD markers from ~16,000 unique genes. Sensitivity and specificity studies narrowed the list to 8 top-ranking markers. They were further evaluated for prognostic significance in the marrows of 116 stage 4 patients collected from the same treatment protocol after two cycles of immunotherapy.

## Materials and Methods

**Identification of potential MRD markers of neuroblastoma by genome-wide gene expression array analyses.** Affymetrix human U95 oligonucleotide array was carried out on 48 tumors (18 of 48 were *MYCN* amplified tumors) and 9 remission marrows from stage 4 neuroblastoma patients diagnosed after age 18 months and 12 neuroblastoma cell lines [SH-SY5Y, SK-N-BE(1), SK-N-BE(2), SK-N-BE(2)M17, LAI-55N, SK-N-LP, SK-N-ER, SK-N-JD, BE(2)C, LAI-5S, SH-EP1, and SK-N-BE(2)S]. Absolute values of expression were calculated and normalized (scaling factor of 500) using Affymetrix Microarray Suite 5.0 (10).

**Specimens for sensitivity and specificity studies.** Neuroblastoma cell lines LAN1 and NMB7 were used for tumor cell seeding experiments. Buffy coat was obtained from New York Blood Center. Fresh-frozen stage 4 tumors were obtained at diagnosis and relapse at Memorial Sloan-Kettering Cancer Center in accordance to the guidelines of the institutional review board.

**Patient characteristics of marrow samples.** Bone marrows were collected from patients with stage 4 neuroblastoma treated with an immunotherapy protocol using anti-GD2 monoclonal antibody 3F8 plus granulocyte-macrophage colony-stimulating factor following chemotherapy (11). Archived marrows tested by quantitative reverse

transcription-PCR were all collected after two treatment cycles at a median time of 2.5 months from protocol entry. The median age at diagnosis was 3.3 years, with 108 of 116 patients diagnosed at age  $\geq 18$  months, the highest age risk group. Twenty-seven patients had *MYCN* amplified tumors.

**Molecular analysis.** Mononuclear cells were isolated and total RNA was isolated and quality assessed as described previously (12, 13). cDNA was synthesized from 1  $\mu$ g total RNA. cDNA (1  $\mu$ L) was used for real-time quantitative PCR using Applied Biosystems Sequence Detection System 7300. All endogenous controls were purchased from Applied Biosystems. For sensitivity assay,  $\beta_2$ -microglobulin (4326319E) was used for normalization. Tumor samples were normalized using the geometric mean of two endogenous controls: hypoxanthine phosphoribosyltransferase 1 (4326321E) and succinate dehydrogenase complex, subunit A, flavoprotein (Hs00188166\_ml; ref. 14). Each sample was quantified using the comparative  $C_T$  method (Applied Biosystems) as a relative fold difference to the positive control cell line NMB7. All genes selected from expression profiling data were tested using TaqMan Gene Expression Assays with fluorogenic probes from Applied Biosystems; their assay IDs are shown in Table 1.

**Statistical analysis.** Bone marrow was classified as marker positive if the gene transcript level was greater than the upper limit of normal as defined as mean  $\pm 2$  SD of 20 normal marrow and blood samples. The clinical endpoint tested was progression-free survival (PFS) and overall survival (OS) from the beginning of immunotherapy using Kaplan-Meier method and compared by the log-rank test.

## Results

**MRD marker discovery by gene expression profiling.** For each probe in the U95 chip, the gene expression levels of 48 stage 4 neuroblastoma tumors were compared with their levels in 9 remission stage 4 marrow samples and 12 neuroblastoma cell lines. We employed three criteria for marker discovery: (a) statistical significance of gene expression in tumor over marrow, using Bonferroni correction for multiple comparisons; (b) the signal ratio of gene expression in tumor versus marrow for each gene for ranking; and (c) superior ratio of tumor versus marrow when compared with TH, a widely used neuroblastoma marker that served as the gold standard. Using Student's *t* test, only genes with highly significant tumor expression were chosen ( $P < 6 \times 10^{-7}$  using Bonferroni correction for multiple comparisons). TH was found to have a median tumor to marrow expression ratio of 37:1. After excluding genes of ubiquitous nature like collagen and pseudogenes, as well as genes with known expression in marrow, 34 genes with ratios of  $>37$  (superior to TH) and a median expression level of  $\geq 2,500$  units were filtered from ~16,000 unique genes. The 34 genes identified were *CCND1*, *CHGB*, *CNTFR*, *CRMP1*, *CXXC4*, *DDC*, *DPYSL3*, *ELAVL4*, *GABRB3*, *GAP43*, *GRIA2*, *ISL1*, *KIF1A*, *KIF21A*, *KIF5C*, *L1CAM*, *MAB21L1*, *MAOA*, *MAP2*, *MEG3*, *MLLT11*, *NPY*, *PCSK1N*, *PFN2*, *PGP9.5*, *PHOX2B*, *RBP1*, *RGS5*, *RTN1*, *SCG2*, *SOX11*, *STMN2*, *TACC2*, and *TAGLN3*. They were ranked in descending tumor to marrow ratio (Table 1).

**Sensitivity of novel neuroblastoma markers.** Cells from neuroblastoma cell lines LAN1 and NMB7 (defined as 100,000 transcript units) were seeded into  $10^7$  normal peripheral blood mononuclear cells, as well as peripheral blood stem cells, ranging from 1 to 1,000 tumor cells. Sensitivity of all 34 genes was tested by quantitative reverse transcription-PCR. Detection by TH was used as a reference. To prioritize potential MRD markers, the acceptance criteria in

these sensitivity assays were as follows: detection limit must be at least 1 tumor cell in 10<sup>6</sup> normal cells in both cell lines tested. If normal hematopoietic cell alone had detectable expression, the gene expression signal of 10<sup>-6</sup> tumor cells must be at least two times higher than normal cell expression. Moreover, the marker must be superior to the sensitivity of TH. The highest rank was 4, and the lowest was 0 (Table 2). The rank of our reference TH was 2.

Of the 34 genes tested, 11 were superior to TH in these sensitivity assays. Among those that had the highest rank, CRMP1, DDC, GABRB3, ISL1, KIF1A, and PHOX2B also had no detectable expression in the normal cells (Fig. 1). As to CCND1 and TACC2, their sensitivity corrected for expression in normal mononuclear cells remained high (data not shown). In contrast, some genes that had high tumor to marrow ratios in the expression profiling lost their superiority when their sensitivity was tested by tumor cell spiking experiments. For

example, as shown in Table 1, *STMN2*, a gene with the highest tumor to bone marrow ratio (449:1), had a sensitivity rank of only 1. Another example was *DPYSL3*, with a tumor to bone marrow ratio of 240:1, had an unacceptable profile with its high expression in normal mononuclear cells.

**Marker specificity and expression in stage 4 neuroblastoma tumors.** To further narrow down our selection of potential MRD markers of neuroblastoma, only the top 8 genes, all ranked 4 in the sensitivity test, were studied using a panel of 20 normal peripheral blood and marrow samples to evaluate the specificity of these candidate markers in non-tumor-bearing samples. In addition, these markers were tested by quantitative reverse transcription-PCR in 20 stage 4 neuroblastoma tumors. Transcript units in log scale of these 8 novel markers were all highly expressed in all stage 4 tumors tested with low to no detection in normal samples (Fig. 2). They compared favorably with TH.

**Table 1.** Thirty-four genes identified from gene expression array data with tumor to bone marrow ratio higher than TH

U95 chip	Probe	GenBank	Gene symbol	Gene name	Tumor to bone marrow ratio	Applied Biosystems assay ID
A	38800_at	D45352	<i>STMN2</i>	Stathmin-like 2	449	Hs00199796_m1
A	33426_at	Y00064	<i>CHGB</i>	Chromogranin B (secretogranin 1)	287	Hs00174956_m1
A	39297_at	U38810	<i>MAB21L1</i>	Mab-21-like 1 ( <i>Caenorhabditis elegans</i> )	263	Hs00366575_s1
A	36149_at	D78014	<i>DPYSL3</i>	Dihydropyrimidinase-like 3	240	Hs00181665_m1
A	36990_at	X04741	<i>UCHL1</i>	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	222	Hs00188233_m1
A	35778_at	AB011103	<i>KIF5C</i>	Kinesin family member 5C	203	Hs00189672_m1
A	37714_at	M25667	<i>GAP43</i>	Growth-associated protein 43	182	Hs00176645_m1
B	47939_at	AA102788	<i>ELAVL4</i>	Embryonic lethal abnormal vision, <i>Drosophila</i> -like 4 (Hu antigen D)	175	Hs00222634_m1
A	40272_at	D78012	<i>CRMP1</i>	Collapsin response mediator protein 1	140	Hs00609714_m1
A	38551_at	U52112	<i>L1CAM</i>	L1 cell adhesion molecule	110	Hs00544069_m1
A	36924_r_at	M25756	<i>SCG2</i>	Secretogranin II (chromogranin C)	104	Hs00185761_m1
C	64258_f_at	AW016235	<i>MEG3</i>	Maternally expressed 3	100	Hs00292028_m1
C	61320_g_at	AL037611	<i>TACC2</i>	Transforming, acidic coiled-coil containing protein 2	99	Hs00610617_m1
A	39990_at	U07559	<i>ISL1</i>	ISL LIM homeobox 1	93	Hs00158126_m1
A	35020_at	D82344	<i>PHOX2B</i>	Paired-like homeobox 2b	85	Hs00243679_m1
C	63848_s_at	AI199503	<i>PCSK1N</i>	Proprotein convertase subtilisin/kexin type 1 inhibitor	81	Hs00560041_m1
A	39178_at	L10333	<i>RTN1</i>	Reticulon 1	71	Hs00382515_m1
A	38418_at	X59798	<i>CCND1</i>	Cyclin D1	68	Hs00277039_m1
A	32650_at	Z78388	<i>TAGLN3</i>	Transgelin 3	66	Hs00203119_m1
D	73450_at	AI687064	<i>GRIA2</i>	Glutamate receptor, ionotropic, AMPA 2	59	Hs00181331_m1
A	41771_g_at	AA420624	<i>MAOA</i>	Monoamine oxidase A	55	Hs00185140_m1
B	43976_at	AI857856	<i>KIF21A</i>	Kinesin family member 21A	51	Hs00286908_m1
A	36941_at	U16954	<i>MLLT11</i>	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homologue, <i>Drosophila</i> ); translocated to, 11	50	Hs00199111_m1
A	38604_at	AI198311	<i>NPY</i>	Neuropeptide Y	47	Hs00173470_m1
A	38634_at	M11433	<i>RBP1</i>	Retinol binding protein 1, cellular	47	Hs00161252_m1
E	91882_at	AI573279	<i>SOX11</i>	Sex determining region Y-box 11	47	Hs00846583_s1
A	40201_at	M76180	<i>DDC</i>	Dopa decarboxylase (aromatic L-amino acid decarboxylase)	47	Hs00168031_m1
E	73596_at	AI377558	<i>CXXC4</i>	CXXC finger 4	46	Hs00228693_m1
E	88926_at	AA029437	<i>CNTFR</i>	Ciliary neurotrophic factor receptor	45	Hs00181798_m1
B	54897_at	AA167714	<i>MAP2</i>	Microtubule-associated protein 2	44	Hs00159041_m1
A	33890_at	AB008109	<i>RGSS5</i>	Regulator of G-protein signaling 5	43	Hs00186212_m1
B	52176_at	W21875	<i>KIF1A</i>	Kinesin family member 1A	41	Hs00188913_m1
A	38839_at	AL096719	<i>PFN2</i>	Profilin 2	40	Hs00160050_m1
C	63823_at	AL120032	<i>GABRB3</i>	γ-Aminobutyric acid A receptor, β3	39	Hs00241459_m1
A	32300_s_at	M17589	<i>TH</i>	Tyrosine hydroxylase	37	Hs00165941_m1

**Table 2.** Thirty-four novel genes were ranked according to the sensitivity selection criteria

Gene symbol	Tumor to bone marrow ratio	Sensitivity rank*
CCND1	68	4
CRMP1	140	4
DDC	47	4
GABRB3	39	4
ISL1	93	4
KIF1A	41	4
PHOX2B	85	4
TACC2	99	4
CHGB	287	3
GAP43	182	3
SOX11	47	3
CNTFR	45	2
PFN2	40	2
RBP1	47	2
RGS5	43	2
TH	37	2
CXCC4	46	1
ELAVL4	175	1
GRIA2	59	1
MAB21L1	263	1
MAOA	55	1
NPY	47	1
STMN2	449	1
TAGLN3	66	1
UCHL1	222	1
DPYSL3	240	0
KIF21A	51	0
KIF5C	203	0
L1CAM	110	0
MAP2	44	0
MEG3	100	0
MLLT11	50	0
PCSK1N	81	0
RTN1	71	0

NOTE: Selection criteria are discussed in detail in Results.  
\*Highest rank was 4.

**Detection of novel markers in bone marrows from 116 stage 4 neuroblastoma patients treated after two cycles of immunotherapy.** To determine the clinical relevance of these markers, their transcript levels were determined using marrows from 116 stage 4 patients after two cycles of immunotherapy on protocol IRB9418. Because not all patients underwent full extent-of-disease workup until after the fourth cycle of treatment, their remission status at the time of marrow sampling could only be estimated: based on available tests, 50 patients were in complete remission and the remaining 66 patients had clinical evidence of disease (18 very good partial response, 2 partial response, 24 stable disease, and 22 progressive disease). Disease status of patients according to marker positivity was analyzed (Supplementary Table S1). There was a general trend toward a higher percentage of patients with positive markers when they had clinical evidence of disease.

Marrow disease was evaluated histologically. Each marrow study consisted of 2 biopsies at 2 separate sites (usually posterior right iliac crest and posterior left iliac crest) plus 4 aspirates at 4 separate sites (usually posterior and anterior right and left iliac crests). Histology-negative marrows had complete negativity by biopsy (2 of 2 sites) and aspirates (4 of 4 sites).

The frequency of multiple marker positivity according to histology status is summarized in Supplementary Table S2. By Kaplan-Meier analyses, both PFS (Fig. 3) and OS (data not shown) for 6 of 8 markers (CCND1, DDC, GABRB3, ISL1, KIF1A, and PHOX2B) were all highly prognostic ( $P < 0.001$ ). The median follow-up of survivors was 5.9 years.

Among patients with MRD in the bone marrow, the prognostic effect of marker positivity on PFS and OS is summarized in Table 3. Three levels of marrow MRD were analyzed. For level 1, histology-negative MRD (2 of 2 negative biopsy plus 4 of 4 negative aspirates), only CCND1 positivity was found to correlate with PFS and OS with statistical significance. For level 2, aspirate-negative MRD (4 of 4 negative aspirates), both CCND1 and PHOX2B positivity was statistically associated with poorer PFS and OS. It should be noted that marrow samples for our marker study were all derived from pooled aspirates. Level 3 uses a broader definition of marrow MRD with  $\geq 4$  of 6 sampling sites being negative. CCND1, DDC, GABRB3, ISL1, KIF1A, and PHOX2B detection were all predictive of PFS, and all, except GABRB3, were predictive of OS.

## Discussion

Historically, many tumor targets and markers were discovered by serology. With the advent of hybridoma technique, whole tumor cells were used to immunize lymphocytes, and clone selection was based on their differential expression of tumor over marrow/blood or tumor over normal tissues. However, this marker discovery approach has major limitations. Lymphocytes, regardless of its being *in vivo* or *in vitro*, may be anergic or tolerized to certain antigens. Depending on the types of antigens studied, the immune repertoire of these lymphocytes can be limited or skewed by dominant clones.

Tumor marker discovery using genome-wide expression array approach is an attractive alternative. Screening of expressed genes overcomes the issues of clonal frequency and lymphocyte restrictions. Whereas the whole tumor approach identifies antibody before the antigen, the expression profiling approach pinpoints known genes, which are likely to have Web-based tissue expression information available to filter out rapidly false leads. This marker discovery strategy was successfully applied to identify three novel markers of Ewing family tumors (8). The detection of STEAP1, CCND1, and/or NKX2-2 at diagnosis was informative and was predictive of whether patients had a higher likelihood to eventually develop new metastases and to die of this cancer. When applied to other tumor expression arrays, novel gene lists have recently been generated (data not shown). Besides genomics, proteomics and glycomics can take advantage of this approach.

The marker discovery outlined in this report was based on profiling a substantial number of stage 4 neuroblastoma tumors, ensuring a representative spectrum of this metastatic cancer. The differential gene expression of neuroblastoma tumors to remission marrow ratios as well as expression level was taken into account. Selection was based on tumor to marrow expression ratio superior to that of TH, a widely accepted neuroblastoma marker, which serves as the gold standard. Based on these criteria, ~16,000 genes were filtered to a list of 34 candidate genes as potential markers. However, there was no one-to-one corresponding correlation between



gene expression array ranking and the ranking derived from the sensitivity experiments (Table 2). This is likely because the expression level on the array depends on the target sequences of the specific gene chips. For example, although 18 of 48 tumors in the array had genomic *MYCN* amplification, *MYCN* expression level did not meet the criteria we set and was rejected in our final list of candidate genes. Additionally, glycolipid markers including  $\beta$ 1,4-*N*-acetyl-galactosaminyl transferase (GD2 synthase) and sialyltransferase STX (ST8SialII) were not detectable by the Affymetrix U95 Chip. This was most likely due to the substantial difference between the Affymetrix

target sequence and the sequence deposited at GenBank. These findings reinforced our continual effort to discover novel molecular markers using a multipronged approach, be it genome-wide expression screen, or focusing on aberrations (e.g., *MYCN*) and pathways specific for tumor or metastases. We believe that both global screening and pathway-based approaches can complement the MRD marker discovery process.

Besides being highly sensitive with detection in  $10^{-6}$  frequency, the 8 top ranking markers were also specific with high expression in stage 4 neuroblastoma tumors (Fig. 2).

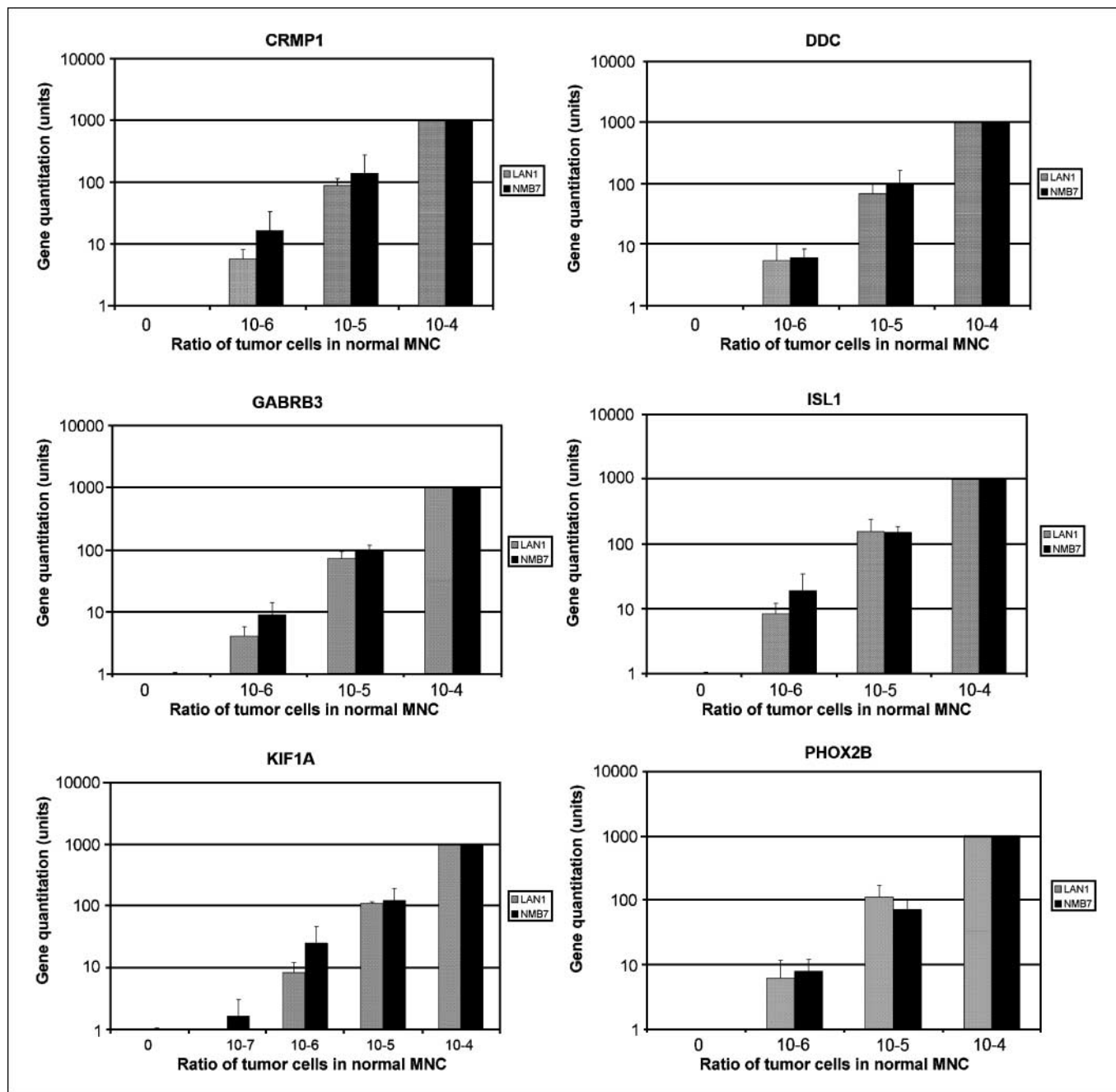
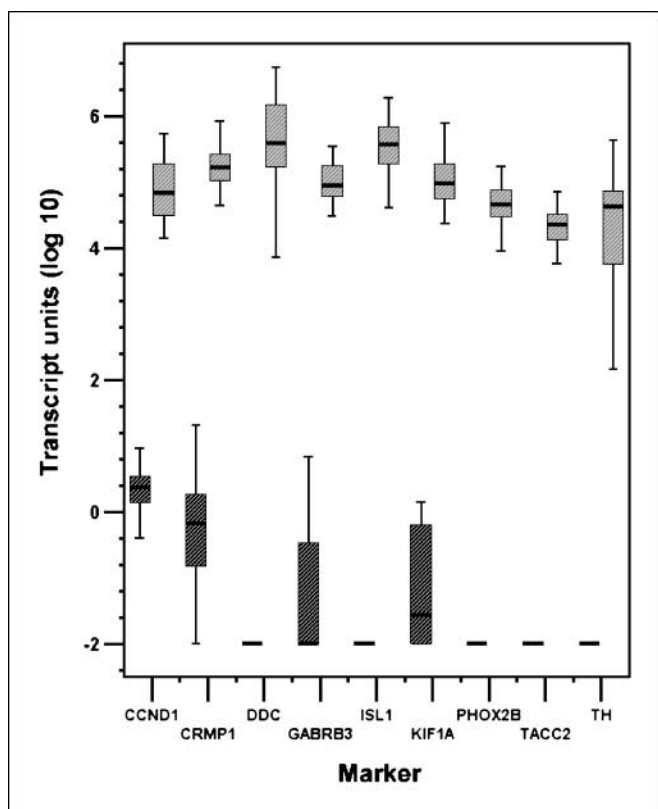


Fig. 1. Sensitivity of novel markers CRMP1, DDC, GABRB3, ISL1, KIF1A, and PHOX2B was evaluated by seeding neuroblastoma cell lines LAN1 and NMB7 ranging from 1 to  $10^4$  cells in  $10^7$  normal mononuclear cells. Gene expression level of  $10^4$  tumor cells in  $10^7$  normal mononuclear cells was defined as 1,000 units. Mean  $\pm$  SD of three experiments.



**Fig. 2.** Differential expression of 8 top ranking novel markers (CCND1, CRMP1, DDC, GABRB3, ISL1, KIF1A, PHOX2B, and TACC2) and TH in 20 stage 4 neuroblastoma tumors and 20 normal mononuclear cells. Gray, tumors; black, normal mononuclear cells. Transcript units in log<sub>10</sub> scale.

CCND1 has a pivotal role in controlling cyclin-dependent kinases during cell cycle progression (15), and it is overexpressed and has adverse prognostic impact in human cancers including neuroblastoma (16). In addition to CCND1, DDC,

and PHOX2B are also associated with neuroblastoma. DDC, an enzyme involved in the pathway of catecholamine synthesis, was shown to have utility as a tumor marker for neuroblastoma (17). PHOX2B is a gene involved in the development of several major noradrenergic neuron populations. It is highly expressed in neuroblastoma, and its germ-line mutation may be linked to hereditary neuroblastoma (18).

The rest of our novel markers, CRMP1, GABRB3, KIF1A, ISL1, and TACC2, have not been reported previously to be associated with neuroblastoma. CRMP1 belonging to a family of cytosolic phosphoproteins expressed exclusively in the nervous system is involved in signal transduction pathway during neural development. GABRB3, which encodes a member of the chloride ionic channel family, serves as the receptor for  $\gamma$ -aminobutyric acid, the major inhibitory transmitter of the nervous system. KIF1A belongs to the microtubule family involved with ATP binding. ISL1 is a zinc finger transcription factor, whereas TACC2 belongs to a conserved family of proteins that are implicated in tumorigenesis by encoding a protein that concentrates at centrosomes throughout the cell cycle.

As part of our discovery algorithm, we tested these 8 candidate markers on the post-cycle 2 bone marrows of a cohort of stage 4 patients treated uniformly with an immunotherapy protocol. In addition to CCND1, a novel MRD response marker of neuroblastoma reported previously (9), 5 additional markers, DDC, GABRB3, ISL1, KIF1A, and PHOX2B, were found to be useful in predicting patient outcome. These markers will need to be tested using an independent set of patient samples collected prospectively to confirm clinical utility. Moreover, a further fine-tuning will be required to determine which markers should be combined with GD2 synthase or TH to optimize the detection of marrow MRD in metastatic neuroblastoma patients.

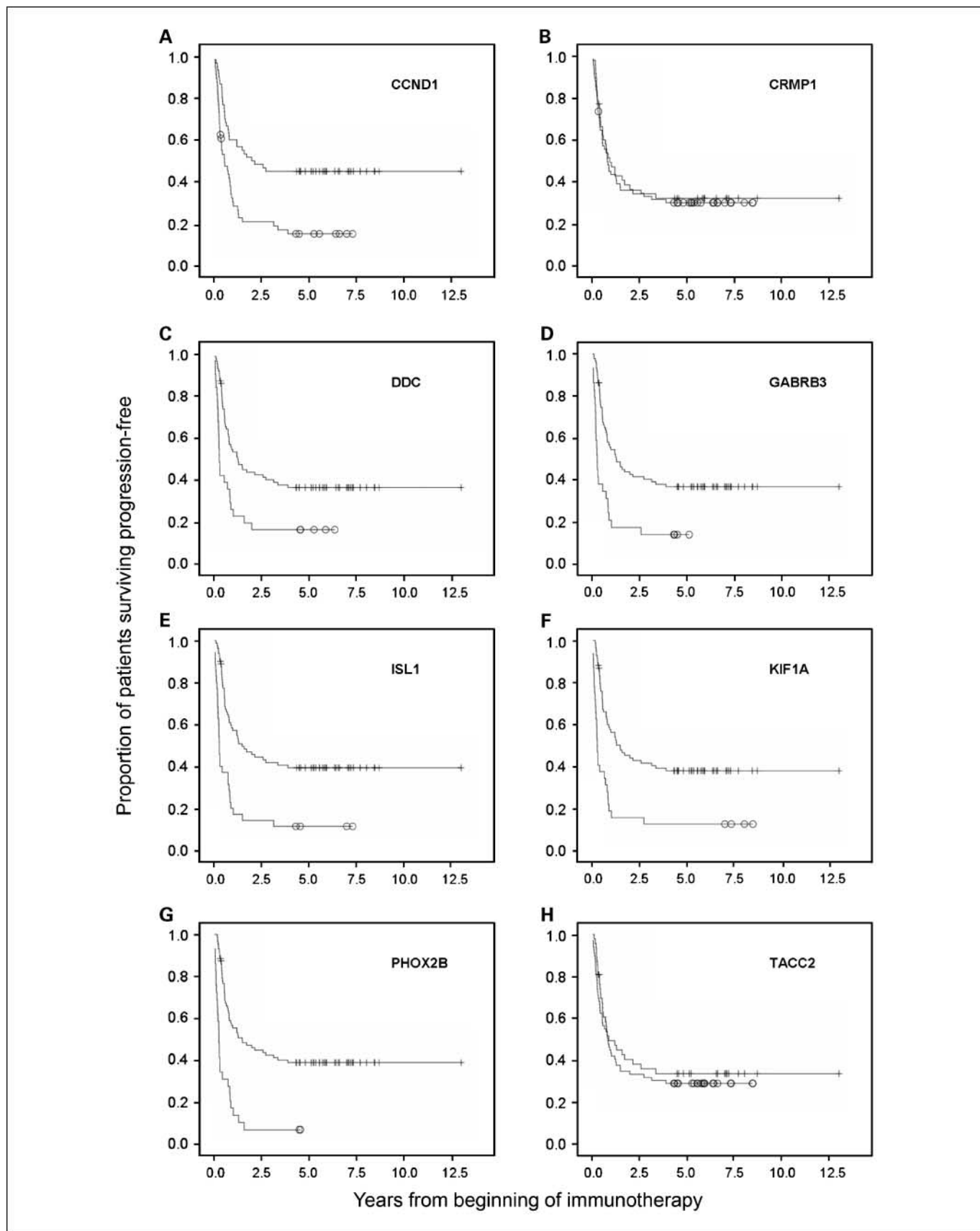
**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Table 3.** Univariate analyses of PFS and OS of stage 4 patients with MRD in the bone marrows after 2 cycles of immunotherapy

Marker	Level 1: histology-negative (n = 90)		Level 2: aspirate-negative (n = 94)		Level 3: isolated infiltration (n = 105)	
	PFS P	OS P	PFS P	OS P	PFS P	OS P
CCND1	0.033	0.015	0.027	0.013	0.002	0.001
DDC	NS	NS	NS	NS	0.014	0.022
GABRB3	NS	NS	NS	NS	0.009	0.067
ISL1	0.08	NS	0.06	0.08	<0.001	<0.001
KIF1A	NS	NS	0.08	0.08	0.001	0.002
PHOX2B	0.07	NS	0.009	0.032	<0.001	<0.001

NOTE: Histology-negative: negative in 2 of 2 biopsy and 4 of 4 aspirate sites. Aspirate-negative: only aspirates were considered, negative in 4 of 4 sites. Isolated infiltration: negative in  $\geq 4$  of 6 sampling sites. NS,  $P > 0.1$ .



**Fig. 3.** Kaplan-Meier plots of PFS for the 8 top ranking novel markers (CCND1, CRMP1, DDC, GABRB3, ISL1, KIF1A, PHOX2B, and TACC2) with respect to marker status. *Open circle*, marker positive; *vertical line*, marker negative. Except for CRMP1 and TACC2, these markers were highly prognostic of outcome ( $P < 0.001$ ).

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