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ORIGINAL REPORT

Murine Anti-GD2 Monoclonal Antibody 3F8 Combined With Granulocyte-Macrophage Colony-Stimulating Factor and 13-*Cis*-Retinoic Acid in High-Risk Patients With Stage 4 Neuroblastoma in First Remission

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A B S T R A C T

Purpose
Anti-GD2 monoclonal antibody (MoAb) combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) has shown efficacy against neuroblastoma (NB). Prognostic variables that could influence clinical outcome were explored.

Patients and Methods
One hundred sixty-nine children diagnosed with stage 4 NB (1988 to 2008) were enrolled onto consecutive anti-GD2 murine MoAb 3F8 ± GM-CSF ± 13-*cis*-retinoic acid (CRA) protocols after achieving first remission (complete remission/very good partial remission). Patients enrolled in regimen A (n = 43 high-risk [HR] patients) received 3F8 alone; regimen B (n = 41 HR patients), 3F8 + intravenous GM-CSF + CRA, after stem-cell transplantation (SCT); and regimen C (n = 85), 3F8 + subcutaneous GM-CSF + CRA, 46 of 85 after SCT, whereas 28 of 85 required additional induction therapy and were deemed ultra high risk (UHR). Marrow minimal residual disease (MRD) was measured by quantitative reverse transcription polymerase chain reaction. Survival probability was calculated by the Kaplan-Meier method, and prognostic variables were analyzed by multivariate Cox regression model.

Results
At 5 years from the start of immunotherapy, progression-free survival (PFS) improved from 44% for HR patients receiving regimen A to 56% and 62% for those receiving regimens B and C, respectively. Overall survival (OS) was 49%, 61%, and 81%, respectively. PFS and OS of UHR patients were 36% and 75%, respectively. Relapse was mostly at isolated sites. Independent adverse prognostic factors included UHR (PFS) and post–cycle two MRD (PFS and OS), whereas the prognostic factors for improved outcome were missing killer immunoglobulin-like receptor ligand (PFS and OS), human antimouse antibody response (OS), and regimen C (OS).

Conclusion
Retrospective analysis of consecutive trials from a single center demonstrated that MoAb 3F8 + GM-CSF + CRA is effective against chemotherapy-resistant marrow MRD. Its positive impact on long-term survival can only be confirmed definitively by randomized studies.

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INTRODUCTION

Despite advances in supportive therapy and diagnostic precision in identifying risk groups, curing high-risk patients with stage 4 neuroblastoma (NB) presenting at age ≥ 18 months or with *MYCN* amplification has been daunting.[1] Although multimodality induction can improve remission quality, the maintenance of long-term tumor control remains suboptimal. Myeloablative therapy with autologous stem-cell transplantation (SCT),[2] differentiation therapy using 13-*cis*-retinoic acid (CRA),[2] and immunotherapy using anti-GD2 antibody[3] have shown efficacy in randomized trials. However, given the substantial acute and late toxicities after intensive chemoradiotherapy,[4,5] it is imperative to identify treatments with fewer adverse effects.

From the first phase I study of murine anti-GD2 antibody[6] to the most recent randomized proof of clinical benefit,[3] cytokines have played key roles. In the Children's Oncology Group (COG) trial of anti-GD2 therapy, antibody ch14.18 was combined with two cytokines (ie, granulocyte-macrophage colony-stimulating factor

[GM-CSF] and interleukin-2 [IL-2]).[3] Yet most therapeutic antibodies, including rituximab, trastuzumab, and cetuximab, are effective without cytokines.[7] Because cytokines are potentially toxic,[3] their additional benefits when combined with antibodies need to be examined.

There are strong in vitro rationales for GM-CSF in 3F8-mediated and ch14.18-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) of NB.[8-11] Myeloid activation markers on granulocytes (CD11a, CD63, CD87, and CD11b and its activation epitope CBRM1/5) increased after GM-CSF therapy in patients with metastatic NB.[12] CBRM1/5 activation was associated with favorable patient outcome in a multivariable model. Moreover, CBRM1/5 activation was significantly higher after subcutaneous (SC) GM-CSF when compared with intravenous (IV) GM-CSF. In light of the effectiveness of 3F8 + GM-CSF against histologic and radiographic chemotherapy-resistant bone marrow disease,[13] and the relatively mild toxicity profile of GM-CSF alone or in combination with 3F8,[13] we evaluated survival and pattern of relapse among 169 high-risk patients whose first complete remission (CR)/very good partial remission (VGPR) was consolidated by 3F8 ± GM-CSF ± CRA in consecutive trials. Multivariate models were built to test for prognostic variables that could influence clinical outcome.

PATIENTS AND METHODS

Patient Characteristics

Patients characteristics are listed in Appendix Table A1 (online only). Patients received chemotherapy based on the Memorial Sloan-Kettering Cancer Center or COG3973 induction, hyperfractionated 21-Gy local radiation, and surgical resection of the primary tumor,[14] with or without second-line therapy to achieve remission,[15] and with or without myeloablative therapy with stem-cell rescue.[2,16] Once CR/VGPR was confirmed according to International Neuroblastoma Response Criteria,[17] informed consent was obtained for treatment (Appendix Table A2, online only) according to institutional review board–approved protocols: regimen A (3F8 [1988 to 2000]; n = 43 high-risk [HR] patients),[18] regimen B (addition of IV GM-CSF and oral CRA [1999 to 2003]; n = 41 HR patients),[13] and regimen C (route of GM-CSF changed to SC [2003 to 2008]; n = 85 [57 HR and 28 ultra–high-risk (UHR) patients]). UHR patients had refractory disease, requiring additional cyclophosphamide ± topotecan or irinotecan[15] after standard induction therapy to achieve CR/VGPR before enrollment for immunotherapy. GM-CSF was yeast-derived human recombinant protein (Sargramostim; Immunex, Seattle, WA). Only patients with stage 4 NB age 18 months to 12 years at diagnosis, or infants with stage 4 *MYCN*-amplified NB, were enrolled. Twenty-four of 43 patients receiving regimen A also received ^{131}I-3F8 (20 mCi/kg) on the N7 protocol (Appendix Table A2, online only); their PFS and OS were no different from patients who did not receive ^{131}I-3F8.

Treatment Regimens

Treatment regimens are summarized in Appendix Table A3 (online only). Dosing of GM-CSF for each patient was increased from 250 to 500 ug/m^2 after the second day of 3F8, as long as the absolute neutrophil count was ≤ 20,000/μL. Treatment cycles were separated by 2- to 4-week rest periods through four cycles, then by 6- to 8-week rest periods through 24 months from study entry or until progressive disease (PD), whichever occurred earlier. For patients with elevated human antimouse antibody (HAMA) titer,[19] treatment was deferred until their titers turned negative. After the second cycle of immunotherapy, oral CRA was initiated (used as described × six cycles[2]) between cycles of 3F8 + GM-CSF.

Disease Evaluation

Disease status was evaluated at enrollment and at least every 3 months, through 3 years from study entry. CR/VGPR were defined as the absence of disease by urinary catecholamines, metaiodobenzylguanidine (MIBG) scan, marrow histology, computed tomography (CT)/magnetic resonance imaging (MRI), or bone scan.[17] ^{131}I-MIBG (through November 1999) or ^{123}I-MIBG scans were dosed as 1 mCi (37 MBq)/1.73 m^2 and 10 mCi (370 MBq)/1.73 m^2 body surface, respectively.

Marrow Assessment by Histology

Each examination consisted of two biopsies from bilateral anterior and posterior iliac crests and four aspirates from bilateral anterior and bilateral posterior iliac crests.[20] Heparinized aspirate samples pooled from four sites were used for minimal residual disease (MRD) measurement.

MRD Detection by Quantitative Reverse Transcription Polymerase Chain Reaction

MRD detection was carried out as previously described.[21,22] The MRD marker panel included cyclin D1 (*CCND1*), ISL LIM homeobox 1 (*ISL1*), paired-like homeobox 2b (*PHOX2B*), and GD2 synthase (*B4GALNT1*). β_2-microglobulin (*β2M*) was used as the endogenous control, and NB cell line NMB7 as the positive control. Each sample was quantified using the comparative CT method as fold difference relative to NMB7. All gene expression assays were from Applied Biosystems (Foster City, CA): *CCND1*: Hs00277039_m1; *ISL1*: Hs00158126_m1; *PHOX2B*: Hs00243679_m1; *B4GALNT1*: Hs00155195_m1; *β2M*: 4326319E. For each marker, positivity was defined as greater than upper limit of normal.[22] All samples were run in duplicates. MRD was measured in marrow before treatment (pre-MRD) and after two cycles of 3F8 (post-MRD), at a median of 3.1 months from start of immunotherapy.

FCGR2A Polymorphism, Human Leukocyte Antigen, and Killer Immunoglobulin-Like Receptor Genotyping

These were carried out as previously described.[23-25] Allelic discrimination of *FCGR2A* was identified as [R/R] versus [H/H] versus [H/R]. Killer immunoglobulin-like receptor (KIR) genotyping (2DL1, 2DL2, 2DL3, 3DL1, and 3DL2) was performed on DNA samples.[24] Patients were considered as missing KIR ligand if they lacked any human leukocyte antigen (HLA) class I ligand by HLA genotype for their inhibitory KIR identified by KIR genotype. Patients with all ligands present possessed all HLA class I ligands for their identified inhibitory KIR.[25]

Statistical Analysis

The clinical end points tested were progression-free survival (PFS) and overall survival (OS) from start of 3F8 immunotherapy. Kaplan-Meier method was used to estimate survival probabilities, and log-rank test was used to test the univariate association between variables and PFS/OS. Multivariate Cox regression model was fitted with variables that had a univariate *P* value of less than .1 and the variable missing KIR ligand. Development of HAMA response was included as a time-dependent covariate using the hazard model $\lambda(t|Z(t)) = \lambda_0(t)\exp(\beta Z(t))$, where $Z(t) = 1$ for any time t after patient developed HAMA, and $Z(t) = 0$ otherwise; $\lambda_0(t)$ was the unknown baseline hazard, and $\exp(\beta)$ was the hazard ratio corresponding to the HAMA effect. Logistic regression was used to test the association between binary variables and treatment regimen. Time between diagnosis and start of immunotherapy was correlated with SCT using exact Wilcoxon rank sum test.

RESULTS

Survival After Anti-GD2 Antibody 3F8 Therapy in Children With HR Stage 4 NB

Survival is summarized in Table 1 and Figures 1A and 1B. All progression-free patients had at least 2.9 years of follow-up from the beginning of immunotherapy and at least 3.6 years from diagnosis. Among HR patients, 5-year PFS increased from 44% for those receiving regimen A (n = 43) to 56% and 62% for those receiving regimens B (n = 41) and C (n = 57), respectively. Four patients who died as a result of therapy-related acute myeloid leukemia or infection were scored as having PD. Similarly, 5-year OS improved from 49% to 61% and 81%, respectively. PFS and OS at 5 years for 28 UHR patients

Table 1. Survival Outcome at 5 Years After 3F8 Immunotherapy in Consecutive Regimens Among 169 Patients With HR* Stage 4 NB in First Remission

Treatment Group	SCT	All Patients					Patients Receiving Regimen C				
		No. of Patients	PFS (%)	95% CI	OS (%)	95% CI	No. of Patients	PFS (%)	95% CI	OS (%)	95% CI
Regimen A (3F8 alone)											
HR	—	43	44	32 to 62	49	36 to 66					
Regimen B (3F8 + IV GM-CSF + CRA)											
HR	Yes	41†	56	43 to 74	61	48 to 78					
Regimen C (3F8 + SC GM-CSF + CRA)											
HR	Yes	57	62	50 to 76	81	70 to 92	37	69	55 to 86	78	65 to 95
	No						20	50	32 to 78	84	69 to 100
UHR‡	Yes	28	36	22 to 59	75	60 to 93	9	44	21 to 92	78	55 to 100
	No						19	32	16 to 61	74	56 to 96

Abbreviations: 3F8, anti-GD2 monoclonal antibody 3F8; CRA, 13-*cis*-retinoic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; HR, high risk; IV, intravenous; NB, neuroblastoma; OS, overall survival; PFS, progression-free survival; SC, subcutaneous; SCT, stem-cell transplantation; UHR, ultra high risk.
*HR patients had stage 4 NB diagnosed at age ≥ 18 months or with *MYCN* amplification.
†Among those receiving regimen B, 40 patients underwent SCT before 3F8 immunotherapy.
‡UHR patients received additional cyclophosphamide ± topotecan or irinotecan[15] for refractory disease after standard induction therapy to achieve complete remission/very good partial remission before 3F8 immunotherapy; these salvage therapies were not available until era of regimen C (2003).

receiving regimen C were 36% and 75%, respectively. In univariate analysis, comparison of all four groups (regimens A, B, C [HR], and C [UHR]) found they were significantly different in PFS and OS ($P = .018$ and $P = .003$, respectively). Among those receiving regimen C, OS was similar for patients with or without SCT (Table 1; Appendix Fig A1 [online only]; $P = .64$). Patients undergoing SCT received immunotherapy after a longer median time from diagnosis compared with those who did not undergo SCT (8.8 *v* 5.8 months; $P < .001$). All three regimens were administered as outpatient treatment. Common adverse effects (during or shortly after 3F8 infusions) were grade 2 pain and grades 1 to 2 urticaria; SC GM-CSF occasionally caused local erythema. Toxicity profile was generally milder when compared with that of the published experience when both GM-CSF and IL-2 were used.[3] There were no capillary leak syndromes or deaths resulting from toxicity during immunotherapy (Appendix Table A4, online only).

Frequency and Pattern of Relapse Among Treatment Groups

Relapse is summarized in Table 2. The median times to relapse or death from the start of immunotherapy were 2.7 years (regimen A [HR]), 1.5 years (regimen C [UHR]), and not reached (regimen B [HR] and regimen C [HR]). Most relapses were surprisingly focal or isolated. Isolated marrow/bone recurrences (22% to 29%) were defined as either only marrow or ≤ two MIBG-positive sites. CNS relapse was detected radiologically by CT and MRI and confirmed by biopsy or resection, being mostly isolated (regimen A, 30%; regimen B, 18%; regimen C, 21%). Patients with isolated soft tissue relapses detected by CT/MRI had no skeletal uptake by MIBG and no marrow disease by histology. In contrast to regimens A and B, it was noteworthy that the relapse pattern in regimen C changed to fewer multiple sites and more isolated soft tissues. Twenty-one

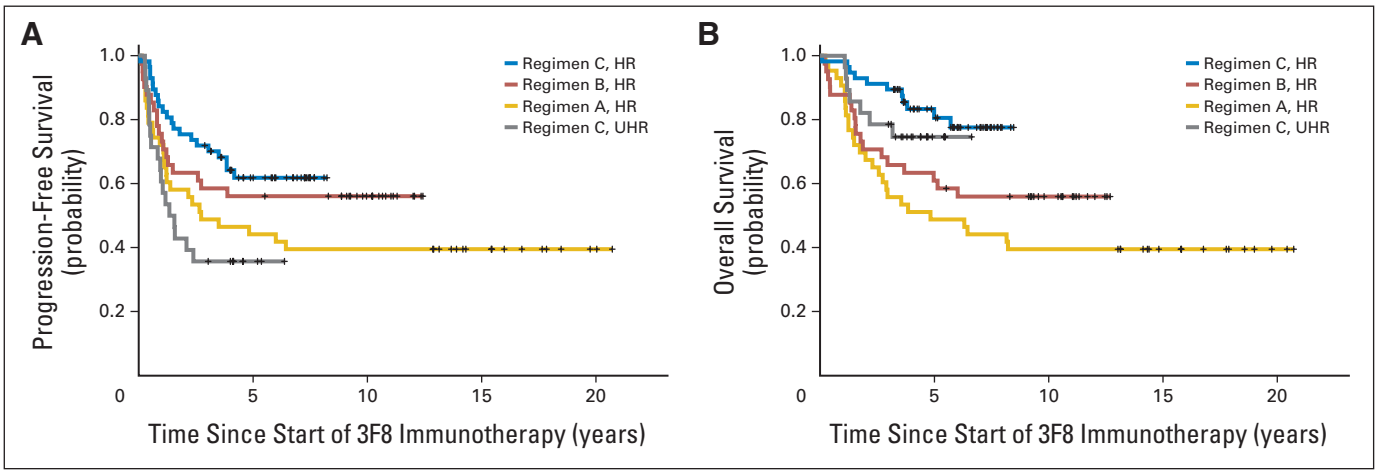

Fig 1. (A) Progression-free survival (PFS) for 169 patients with stage 4 neuroblastoma in first remission after consecutive immunotherapy regimens: 3F8 alone (regimen A–high risk [HR]; n = 43), 3F8 + intravenous granulocyte-macrophage colony-stimulating factor (GM-CSF) + 13-*cis*-retinoic acid (CRA; regimen B-HR; n = 41), and 3F8 + subcutaneous GM-CSF + CRA (regimen C-HR; n = 57 and regimen C–ultra HR [UHR]; n = 28); $P = .018$ (derived from log-rank test to compare PFS among these four groups). (B) Overall survival (OS) among same cohort of patients; $P = .003$ (derived from log-rank test to compare OS among these four groups).

Table 2. Frequency and Pattern of Relapse and Time to Relapse Among Treatment Groups

Treatment Group	Total Patients		PF Patients*		Patients Experiencing Relapse (%)	Pattern of Relapse							
						Multiple Sites		Isolated Marrow/ Bone		Isolated CNS		Isolated Soft Tissue	
	No.	Median Time to Relapse or Death From Start of 3F8 (years)	No.	Median Follow-Up From Start of 3F8 (years)		No.	%	No.	%	No.	%	No.	%
Regimen A (3F8 alone)													
HR	43	2.7	20	15.5	53	9	39	5	22	7	30	2	9
Regimen B (3F8 + IV GM-CSF + CRA)													
HR	41	NR	24	10.2	41	8	47	5	29	3	18	1	6
Regimen C (3F8 + SC GM-CSF + CRA)													
HR	57	NR	36	5.9	37	5	24	6	29	5	24	5	24
UHR	28	1.5	10	4.4	64	5	28	5	28	3	17	5	28

Abbreviations: 3F8, anti-GD2 monoclonal antibody 3F8; CRA, 13-*cis*-retinoic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; HR, high risk; IV, intravenous; NB, neuroblastoma; NR, not reached; PF, progression free; SC, subcutaneous; UHR, ultra high risk.
*Three patients receiving regimen A and one patient receiving regimen B did not experience relapse but died as a result of therapy-related acute myeloid leukemia or infection.

patients (10 HR and 11 UHR) receiving regimen C were back in remission after experiencing relapse after surgery ± focal radiation ± short courses of chemotherapy and then re-treatment with 3F8-based immunotherapy. Eleven patients continued in second CR (range, 1.3 to 6.4 years), five had stable disease, and five had further PD. Of seven patients with isolated CNS relapse, six continued in second CR (median, 3.9 years), and one had both CNS and systemic relapses. All 21 patients remain alive, with median follow-up of 2.7 years since relapse.

Post-MRD As Early Indicator of Treatment Responsiveness and Its Prognostic Significance

Because all patients were treated in CR/VGPR with negative marrow histology, subclinical disease could only be measured by quantitative reverse transcription polymerase chain reaction. Pre-MRD was positive (regimen A, 24%; regimen B, 29%; regimen C, 37% [HR] and 43% [UHR]; Appendix Table A1, online only). Among patients receiving regimen C, pre-MRD was identical (39%) with or without SCT. When marrows were studied after two cycles of immunotherapy (post-MRD), before any exposure to CRA, MRD remission was achieved in 70% of patients with positive pre-MRD among those receiving regimen A, 83% among those receiving regimen B, and 76% [HR] and 67% [UHR] among those receiving regimen C ($P = .79$). Post-MRD was significantly associated with PFS and OS (both $P < .001$), whereas pre-MRD was not ($P = .85$ and $P = .86$, respectively; Appendix Table A1, online only). Kaplan-Meier PFS plots illustrated the strong association with post-MRD (Fig 2; $P < .001$) and lack of association with pre-MRD status (Appendix Fig A2, online only). Irrespective of regimen, both 5-year PFS and OS were markedly different between post-MRD–positive and –negative patients (Table 3).

Univariate Analysis of Prognostic Factors

Risk factors for survival were tested in univariate analyses (Appendix Table A1, online only). Tumor and patient characteristics included sex, age, *MYCN* amplification, lactate dehydrogenase level, bone disease, marrow histology, *FCGR2A* polymorphism, and missing KIR ligand. Treatment variables included induction protocols, immunotherapy regimens, UHR, SCT, and CRA. Immunotherapy regimens (categorized as A [HR], B [HR], C [HR], and C [UHR]), SCT, post-MRD, and UHR were significantly associated with PFS. For OS, the statistically significant variables included immunotherapy regimen, post-MRD, and HAMA response. Induction protocols were found not to be prognostic for PFS or OS. CRA effect was not tested, because it was always administered with GM-CSF.

Multivariate Analysis of Prognostic Risk Factors

Variables with univariate $P < .1$ were included in the multivariate model (Table 4). Although missing KIR ligand was not significant in the univariate analysis, this variable was included, because its effect on PFS and OS was partly masked by the effect of post-MRD (Appendix Fig A3, online only). Regimens A and B were combined into a single category because there was no significant difference between their effectiveness in the multivariate models.

Positive post-MRD (measure of lack of responsiveness to immunotherapy) was an independent adverse prognostic factor for PFS and OS, whereas UHR (measure of refractoriness to induction) was independently prognostic for adverse PFS. In contrast, missing KIR ligand

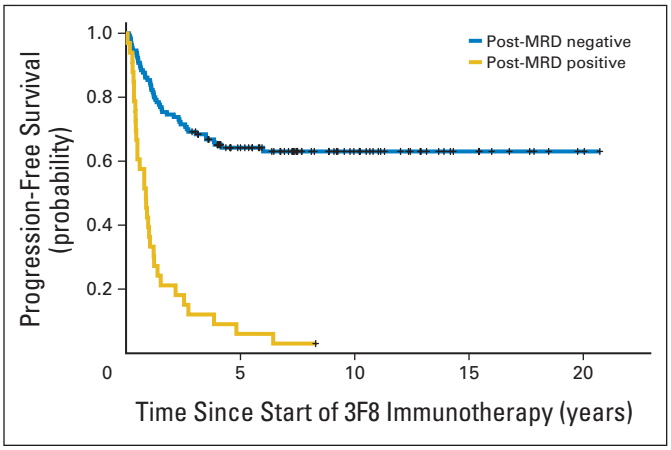

Fig 2. Strong association between minimal residual disease status after two cycles of 3F8 therapy (post-MRD) and progression-free survival for 169 high-risk patients with stage 4 neuroblastoma; $P < .001$.

Table 3. Prognostic Impact of Pre- and Post-MRD on 5-Year Survival Outcome

Treatment Group	MRD Marker Panel*	Pre-MRD PFS %	Pre-MRD PFS 95% CI	Pre-MRD OS %	Pre-MRD OS 95% CI	Post-MRD PFS %	Post-MRD PFS 95% CI	Post-MRD OS %	Post-MRD OS 95% CI
Regimen A (3F8 alone)									
HR	Negative	45	31 to 67	52	37 to 73	62	47 to 83	66	50 to 85
	Positive	50	27 to 93	50	27 to 93	8	1 to 54	17	5 to 59
Regimen B (3F8 + IV GM-CSF + CRA)									
HR	Negative	55	40 to 77	55	40 to 77	66	51 to 84	72	58 to 89
	Positive	58	36 to 94	75	54 to 100	14	2 to 87	14	2 to 88
Regimen C (3F8 + SC GM-CSF + CRA)									
HR	Negative	62	48 to 81	78	65 to 94	72	60 to 86	87	78 to 99
	Positive	62	44 to 87	84	70 to 100	NE†		43	18 to 100
UHR	Negative	38	20 to 71	87	72 to 100	48	30 to 75	90	79 to 100
	Positive	33	15 to 74	57	35 to 94	NE†		29	9 to 92

Abbreviations: 3F8, anti-GD2 monoclonal antibody 3F8; CRA, 13-*cis*-retinoic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; HR, high risk; IV, intravenous; MRD, minimal residual disease; NE, not estimable; OS, overall survival; PFS, progression-free survival; post-MRD, minimal residual disease after two cycles of 3F8 therapy; pre-MRD, minimal residual disease before therapy; SC, subcutaneous; UHR, ultra high risk.

*Marker panel included *CCND1*, *ISL1*, *B4GALNT1*, and *PHOX2b*. MRD positivity was defined as any one of four markers being positive, and negativity as all four markers being negative.

†All patients experienced progression before 5 years.

had a favorable influence on PFS and OS. For OS, having HAMA response and the addition of SC GM-CSF in regimen C versus no GM-CSF (regimen A) or IV GM-CSF (regimen B) had an independent positive impact. However, definitive proof of efficacy contributed by regimen C will require randomized comparisons.

To address the potential confounding factors on survival after relapse, we categorized the postrelapse therapies received as follows: one, high-dose cyclophosphamide-based therapy[14,15,26] ± SCT[27]; two, irinotecan + temozolomide[28]; three, local control by radiation ± intrathecal ^{131}I-8H9[29] ± surgical resection; and four, re-treatment with 3F8-based immunotherapy (Appendix Table A5, online only). When survival was calculated from the time of relapse, each of the four salvage modalities was significant in univariate analysis, with $P \leq .01$. We also included time from diagnosis to relapse (< 18 *v* ≥ 18 months; $P = .02$ for association with postrelapse survival), previously shown to be a highly significant prognostic variable for survival after NB progression[30] in a multivariable model (Appendix Table A6, online only). In addition to the statistical impact of time to relapse on OS, regimen C received before relapse remained significant after adjusting for other variables. Postrelapse therapies, including high-dose cyclophosphamide-based therapy and re-treatment with 3F8, were also found to have a positive impact on survival. However, these findings can only be confirmed by randomized studies.

DISCUSSION

This retrospective analysis of consecutive immunotherapy regimens reflected the clinical experience spanning 20 years by a single institution using anti-GD2 antibody 3F8 in the treatment of patients with high-risk stage 4 NB in their first CR/VGPR. Even though 3F8 + SC GM-CSF + CRA (regimen C) was identified as being independently

Table 4. Multivariate Analysis of Variables on Survival Outcome Among 169 HR Patients With Stage 4 NB in First Remission Treated With 3F8 ± GM-CSF ± CRA

Variable Tested	PFS Hazard Ratio	PFS 95% CI	PFS *P*	OS Hazard Ratio	OS 95% CI	OS *P*
Refractory to induction (UHR)*	**2.2**	**1.2 to 4.4**	**.02**	—	—	—†
SCT consolidation before immunotherapy	0.82	0.51 to 1.3	.41	—	—	—
Positive post-MRD	**6.52**	**3.9 to 10.8**	**< .001**	**7.9**	**4.4 to 14.3**	**< .001**
Missing KIR ligand	**0.62**	**0.38 to 1**	**.05**	**0.55**	**0.31 to 0.99**	**.045**
HAMA response‡	—	—	—	**0.36**	**0.21 to 0.64**	**< .001**
Addition of SC GM-CSF + CRA (regimen C)	**0.85**	**0.49 to 1.6**	**.58**	**0.52**	**0.29 to 0.92**	**.026**

NOTE. Bold font indicates significance.

Abbreviations: 3F8, anti-GD2 monoclonal antibody 3F8; CRA, 13-*cis*-retinoic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; HAMA, human antimouse antibody; HR, high risk; KIR, killer immunoglobulin-like receptor; OS, overall survival; PFS, progression-free survival; post-MRD, minimal residual disease after two cycles of 3F8 therapy; SC, subcutaneous; SCT, stem-cell transplantation; UHR, ultra high risk.

*Refractory: UHR patients requiring second-line high-dose cyclophosphamide/topotecan or cyclophosphamide/irinotecan therapy after standard induction therapy to achieve complete remission/very good partial remission before 3F8 immunotherapy.

†$P \geq .1$ in univariate analysis and therefore not included in multivariable model.

‡HAMA modeled as time-dependent covariate.

prognostic for patient survival, the definitive test of efficacy would require a randomized trial, because there might have been unmeasured confounding factors during preimmunotherapy treatments or postrelapse therapies. Our analysis, nevertheless, did suggest an improvement in OS over time, in part because of the effective anti-NB activity of regimen C. The effects of GM-CSF and CRA cannot be separated, because they were always administered together. However, use of the SC route of administration of GM-CSF instead of the IV route seemed to provide maximal benefit. Nevertheless, by compressing 10 days of treatment (regimen B) into 5 days (regimen C), the advantage of SC GM-CSF could be confounded by higher 3F8 serum levels. Because approximately 80% of de novo patients achieved CR/VGPR and therefore qualified for immunotherapy,[14] overall cure rate is still suboptimal. Although no patient in our cohorts suffered major organ toxicity or died as a result of immunotherapy, toxicity profile of antibody alone, versus its combination with IL-2 or GM-CSF, will require randomized comparisons.

NB recurrence among patients with stage 4 disease reported in the literature has generally involved multiple sites.[31,32] Focal relapses were uncommon (< 10%).[32,33] In contrast, a majority of relapses in the present analysis were isolated (CNS, marrow/bone, or soft tissue). CNS disease is uncommon at diagnosis and was relatively rare before the era of immunotherapy.[34,35] It is striking that CNS relapse after immunotherapy was mostly focal without evidence of systemic disease. Despite effective systemic chemotherapy,[3,14] given the inability of 3F8 to cross the blood-brain barrier, eventual CNS relapse seems unavoidable. Isolated CNS relapse could be indirect evidence for the effectiveness of 3F8 for systemic disease. Such CNS relapses were previously reported among patients with *HER2*-amplified breast cancer after trastuzumab therapy.[36] CNS spread is generally followed by further recurrence along the craniospinal axis, with or without systemic relapse. In our study, six of seven such patients were back in remission after salvage protocol employing intrathecal radiolabeled antibodies.[29]

Responsiveness to induction chemotherapy is well known to be prognostic for patient outcome.[37-39] Necessity of second-line therapy to achieve CR/VGPR to qualify for immunotherapy (UHR) foreshadowed a more aggressive tumor, reflected by its being an independent adverse predictor of PFS. As for SCT, it was found not to be independently prognostic for outcome (Table 4), with comparable survival plot for patients receiving regimen C with or without SCT (Appendix Fig A1, online only). The most common reason for not receiving SCT was parental concern over transplantation toxicity; no known disease-related factors were used in the decision. In using immunotherapy, SCT may not have had the same benefit as that demonstrated in the earlier protocols, where induction therapy was less intensive.[2] Omission of high-dose consolidation therapy might spare many patients unnecessary treatment toxicities.

Curability of high-risk patients with stage 4 NB remained less than 40% despite intensive therapies that included SCT for MRD.[2,40,41] Using a marker panel derived from genome-wide search,[22] abnormal levels of tumor transcripts were detected in 24% to 43% of patients before immunotherapy. The level of pre-MRD was least favorable among patients receiving 3F8 + SC GM-CSF + CRA (regimen C) and best among the 3F8-alone group (regimen A; Appendix Table A1, online only). After two cycles of immunotherapy, 67% to 83% of patients achieved MRD remission. In contrast to pre-MRD, positive post-MRD was an independent adverse predictor of PFS and OS (Table 4). This early indicator of immunotherapy resistance was akin to the observation of early MRD in leukemia as an indicator of induction resistance.[42,43]

Previous studies have implicated high-affinity Fc receptor for human[44,45] and mouse immunoglobulin G[23] as well as the adhesion molecule CD11b in improving patient survival.[12] Unlike in patients with primary refractory NB,[46] *FCGR2A* polymorphism did not reach significance in this cohort of patients treated in first remission. On the other hand, although missing KIR ligand was not significant in the univariate model, when added to the multivariate model, it achieved statistical significance after adjusting for the effect of post-MRD. Missing KIR ligand was previously shown to be prognostic for survival after treatment with 3F8-based immunotherapy.[25,47] Ability of unlicensed natural killer (NK) cells to kill NB efficiently in the presence of 3F8 despite HLA upregulation could be an explanation.[48,49] Similar effects of missing KIR ligand in NB were found in autotransplantation[50] and more recently with hu14.18–IL-2.[51]

The favorable impact of developing HAMA response against mouse antibody, although counterintuitive, confirmed a previous observation of HAMA as a surrogate marker of an anti-idiotype network in prolonging the antitumor effect.[52] However, the potential importance of a host immune response for long-term NB control has not been extensively explored. Furthermore, although marrow MRD is useful for quantifying NB responsiveness to myeloid ADCC, other MRD measurements are likely to be necessary for detecting NB sensitivity to NK-mediated ADCC. Given the potential benefit of myeloid and NK cells in antibody-based treatment of metastatic NB, further optimization of their effector functions should be considered.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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