Multimerin-1 (MMRN1) as Novel Adverse Marker in Pediatric Acute Myeloid Leukemia: A Report from the Children's Oncology Group

George S. Laszlo1, Todd A. Alonzo2,3, Chelsea J. Gudgeon1, Kimberly H. Harrington1, Robert B. Gerbing5, Yi-Cheng Wang5, Rhonda E. Ries1, Susana C. Raimondi3,4, Betsy A. Hirsch3,5, Alan S. Gamis3,6, Soheil Meshinchi1,3,7, and Roland B. Walter1,8

Abstract

Purpose: Exploratory gene expression array analyses suggested multimerin-1 (MMRN1) to be a predictive biomarker in acute myelogenous leukemia (AML). Following up on these studies, we evaluated the role of MMRN1 expression as outcome predictor in two recent Children's Oncology Group trials.

Experimental Design: We retrospectively quantified MMRN1 expression in 183 participants of AAML03P1 and 750 participants of AAML0531 by reverse-transcriptase PCR and correlated expression levels with disease characteristics and clinical outcome.

Results: In AAML03P1, the highest quartile of MMRN1 expression (expression ≥0.5 relative to β-glucuronidase; n = 45) was associated with inferior event-free survival (EFS; P < 0.002) and higher relapse risk (P < 0.004). In AAML0531, in which we quantified MMRN1 mRNA for validation, patients with relative MMRN1 expression ≥0.5 (n = 160) less likely achieved remission (67% vs. 77%, P = 0.006), and more frequently had minimal residual disease (43% vs. 24%, P = 0.001) after one induction course. They had inferior overall survival (OS; 44% ± 6% vs. 69% ± 4% at 5 years; P < 0.001) and EFS (32% ± 8% vs. 54% ± 4% at 5 years; P < 0.001) and higher relapse risk (57% ± 10% vs. 35% ± 5% at 5 years; P < 0.001). These differences were partly attributable to the fact that patients with high MMRN1 expression less likely had cytogenetic/molecular low-risk disease (P < 0.001) than those with low MMRN1 expression. Nevertheless, after multivariable adjustment, high MMRN1 expression remained statistically significantly associated with shorter OS (HR, 1.57; 95% confidence interval, 1.17–2.12; P = 0.003) and EFS (HR, 1.34; 1.04–1.73; P = 0.025), and higher relapse risk (HR, 1.40; 1.01–1.94; P = 0.044).

Conclusions: Together, our studies identify MMRN1 expression as a novel biomarker that may refine AML risk stratification.

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Introduction

Acute myelogenous leukemia (AML) is a challenging disease with outcomes that vary widely between individual patients (1). Numerous disease-related risk factors have so far been recognized; among those, cytogenetic abnormalities and somatic mutations are the most important ones and provide the framework for diagnostic classification and risk stratification schemes (1). Although such schemes are increasingly used for risk-tailored treatment assignment, there are only a small number of informative predictive markers, and it is a recurrent clinical observation that this limited battery fails to accurately predict outcome for many patients. Thus, there remains a need for refined tools to characterize disease risk in AML. Our recent studies indicate that multimerin-1 (MMRN1) may be such a biomarker.

MMRN1, a member of the elastin microfibrillar interface protein (EMILIN)/multimerin family, has so far primarily been described as a component of secretory granules found in platelets and endothelial cells that may mediate cellular adhesion via integrin receptors (2). During recent discovery studies using gene expression array data from diagnostic specimens of 211 recently treated pediatric AML patients, we identified MMRN1 as a SOCS2 (3) cosegregating gene whose expression varied widely (Supplementary Fig. S1) and appeared to be related to patient outcomes (Supplementary Fig. S2). To follow-up on these studies, we retrospectively quantified MMRN1 expression in pretreatment bone marrow specimens from participants of the Children’s Oncology Group (COG) AML protocol, AAML03P1, and then validated findings in participants of AAML0531 to investigate the potential role of MMRN1 as a predictive biomarker in pediatric AML.

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Personalized Medicine and Imaging

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Conclusions: Together, our studies identify MMRN1 expression as a novel biomarker that may refine AML risk stratification.

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Patients and Methods

Patient samples

Cryopreserved pretreatment (*diagnostic*) specimens from patients enrolled on AAML03P1 or AAML0531 who consented to the biology studies and had bone marrow samples available were included in this study. AAML03P1 (registered at Clinical-Trials.gov as NCT00070174) was a multicenter phase III pilot study that determined the safety and feasibility of adding gemtuzumab ozogamicin (GO) to intensive chemotherapy among 339 eligible children and adolescents (ages 1 month to 21 years) with newly diagnosed de novo AML, excluding those with acute promyelocytic leukemia (APL), bone marrow failure syndromes, juvenile myelomonocytic leukemia, or Down syndrome between 2003 and 2005 (4). AAML0531 (NCT00372593) was the subsequent multicenter phase III study that determined the addition of GO to intensive chemotherapy among 1,070 eligible patients ages <30 years with newly diagnosed de novo non-APL AML, excluding those with bone marrow failure syndromes, juvenile myelomonocytic leukemia, or Down syndrome (if <3 years of age) between 2006 and 2010 (5). The patient and disease (cytogenetic/molecular) characteristics of the subsets of AAML03P1 and AAML0531 patients studied in this analysis were relatively comparable with patients not studied in this analysis. Specifically, for AAML03P1, there were differences with regard to some disease characteristics [i.e., higher white blood cell (WBC) counts (P < 0.001) and higher proportion of patients with NPM1 mutation (P = 0.011) and low-risk disease (P < 0.001)], but short-term outcomes were similar [i.e., complete remission (CR) rate after 1 course of therapy (P = 0.08) and rates of minimal residual disease (MRD; P = 0.55)], as were overall survival (OS; P = 0.22) and event-free survival (EFS; P = 0.93). For AAML0531, there were also some differences in disease characteristics [i.e., higher proportion of patients with inv(16)/(t(16;16) (P = 0.011) and lower risk disease (P < 0.001)] as well as better short-term outcomes [i.e., CR rate after 1 course of therapy (P = 0.001) albeit not rate of MRD (P = 0.95)], but OS was similar (P = 0.52) and EFS was only slightly better (P = 0.04). Informed consent was obtained from all study subjects in accordance with the Declaration of Helsinki, and the Institutional Review Boards (IRB) of all participating institutions approved the clinical protocol. IRB approval was obtained from Fred Hutchinson Cancer Research Center (Seattle, WA) before conduct of this biologic study, which was also approved by the COG Myeloid Disease Biology Committee and the National Cancer Institute Cancer Therapy Evaluation Program.

Detection and quantification of MRD

Residual AML was quantified in bone marrow aspirates collected at the end of the first induction course by multiparameter flow cytometry using a ‘different-from-normal’ approach as previously described (7).

Quantification of MMRN1 expression in unsorted AML specimens and FACS-isolated CD34+/CD33- and CD34+/CD33+ cells

Total RNA from unsorted diagnostic AML specimens was extracted with the AllPrep DNA/RNA Mini Kit using the QIAcube automated system (Qiagen). After quantification with a micro-volume spectrophotometer (NanoDrop; Thermo Scientific), 10 ng of total RNA was subjected to qRT-PCR for MMRN1 Detection and quantification (GO to intensive chemotherapy among 339 eligible children and adolescents (ages 1 month to 21 years) with newly diagnosed de novo AML, excluding those with acute promyelocytic leukemia (APL), bone marrow failure syndromes, juvenile myelomonocytic leukemia, or Down syndrome between 2003 and 2005 (4). AAML0531 (NCT00372593) was the subsequent multicenter phase III study that determined the addition of GO to intensive chemotherapy among 1,070 eligible patients ages <30 years with newly diagnosed de novo non-APL AML, excluding those with bone marrow failure syndromes, juvenile myelomonocytic leukemia, or Down syndrome (if <3 years of age) between 2006 and 2010 (5). The patient and disease (cytogenetic/molecular) characteristics of the subsets of AAML03P1 and AAML0531 patients studied in this analysis were relatively comparable with patients not studied in this analysis. Specifically, for AAML03P1, there were differences with regard to some disease characteristics [i.e., higher white blood cell (WBC) counts (P < 0.001) and higher proportion of patients with NPM1 mutation (P = 0.011) and low-risk disease (P < 0.001)], but short-term outcomes were similar [i.e., complete remission (CR) rate after 1 course of therapy (P = 0.08) and rates of minimal residual disease (MRD; P = 0.55)], as were overall survival (OS; P = 0.22) and event-free survival (EFS; P = 0.93). For AAML0531, there were also some differences in disease characteristics [i.e., higher proportion of patients with inv(16)/(t(16;16) (P = 0.011) and lower risk disease (P < 0.001)] as well as better short-term outcomes [i.e., CR rate after 1 course of therapy (P = 0.001) albeit not rate of MRD (P = 0.95)], but OS was similar (P = 0.52) and EFS was only slightly better (P = 0.04). Informed consent was obtained from all study subjects in accordance with the Declaration of Helsinki, and the Institutional Review Boards (IRB) of all participating institutions approved the clinical protocol. IRB approval was obtained from Fred Hutchinson Cancer Research Center (Seattle, WA) before conduct of this biologic study, which was also approved by the COG Myeloid Disease Biology Committee and the National Cancer Institute Cancer Therapy Evaluation Program.

Risk stratification

A combination of cytogenetic and molecular abnormalities was used to stratify patients into risk groups. A patient was considered low-risk if a chromosomal abnormality/mutation was present in core-binding factors [CBF; t(8;21) or inv(16)/(t(16;16)], nucleophosmin [NPM1; unless a FLT3-ITD mutation with high allelic ratio (>0.4) was also present], or CCAAT/enhancer-binding protein (C/EBP), alpha [CEBPA], for CEBPA, both single and double mutations were considered favorable (6). Patients were classified as high-risk if they had monosomy 5 or deletion of 5q (~5/5q~), monosomy 7 (~7), or FLT3-ITD with high allelic ratio (0.4 or higher). All other patients with data sufficient for classification were considered standard-risk.

Translational Relevance

Although several predictive biomarkers have been described in acute myelogenous leukaemia (AML), current models are unable to accurately forecast therapeutic response and survival. Exploratory gene expression array analyses suggested that multimerin-1 (MMRN1), a hitherto very poorly described gene that may be involved in cellular adhesion via integrin receptors, could be a novel predictive biomarker in AML. Following up on these studies, we investigated the MMRN1 expression in two recent Children’s Oncology Group trials, AAML03P1 and AAML0531. While associated with adverse disease-risk features, we found that high MMRN1 expression was independently associated with shorter overall survival and event-free survival as well as a higher relapse risk in a large set of homogenously treated pediatric patients with AML. Together, these studies identify MMRN1 expression as a novel biomarker that may refine AML risk stratification.
MMRN1 as Adverse Marker in AML

Statistical analysis

Data from AAML03P1 and AAML0531 were current as of December 31, 2013. The median (range) of follow-up for patients alive at last contact was 7.8 (6–9.3) years for AAML03P1 and 4.3 (0.02–7.1) years for AAML0531. The Kaplan–Meier method (10) was used to estimate OS (defined as time from study entry to death) and EFS (time from study entry until failure to achieve CR during induction, relapse, or death). Relapse risk (RR) was calculated by cumulative incidence methods defined as time from the end of induction I for patients in CR to relapse or death where deaths without a relapse were considered competing events (11). Patients who withdrew from therapy due to relapse, persistent central nervous system (CNS) disease, or refractory disease with >20% bone marrow blasts by the end of induction I were defined as induction I failures. The significance of predictor variables was tested with the log-rank statistic for OS, EFS, and with Gray's statistic for RR. All estimates are reported with two times the Greenwood standard errors. Children lost to follow-up were censored at their date of last known contact. Cox proportional hazards models (12) were used to estimate the HR for defined groups of patients in univariate and multivariate analyses of OS and EFS. Analyses of OS for AAML0531 patients across all risk groups and for standard risk patients violated the proportional hazards assumption, and therefore a parametric Weibull regression model was used to estimate the HR. Competing risk regression models were used to estimate HRs for univariate and multivariate analyses of RR. The χ² test was used to test the significance of observed differences in proportions, and the Fisher exact test was used when data were sparse. Differences in medians were compared by the Mann–Whitney or Wilcoxon signed-rank test, as appropriate. A P value <0.05 was considered statistically significant.

Results

Identification of MMRN1 as predictive biomarker in participants of AAML03P1

Among 339 eligible patients enrolled on AAML03P1, 309 (91%) consented to contribute to the biologic aims of the study and provided diagnostic bone marrow specimens. At the time this study was conducted, RNA from 188 of these 309 patients (61%) was available for quantitation of MMRN1 expression levels by qRT-PCR. Five samples were excluded because of inadequate RNA as determined by low GUSB expression (97.5th percentile cut-off for low GUSB in AAML03P1: Ct >33.09). Within the remaining 183 specimens, MMRN1 mRNA was detected in all samples and its abundance varied >80,000-fold relative to GUSB mRNA (0.0002–15.14; median: 0.1793; Fig. 1A). Of note, the median expression of MMRN1 in the AML specimens was similar to the median expression found in a small subset of normal whole bone marrows obtained from volunteers ages 22, 26, 31, and 44 years (median relative expression 0.170; range, 0.094–0.232, n = 4).

Studying the relationship between MMRN1 expression and clinical outcome, we initially analyzed patient outcomes per quartile of MMRN1 expression and noticed that the 45 patients with the highest relative MMRN1 expression (4th quartile, corresponding to an expression of ≥0.5 relative to GUSB) fared worse than patients in the first, second, or third quartile of MMRN1 expression, respectively, with little difference between the first three quartiles. We therefore subsequently compared patients with the highest relative MMRN1 expression (i.e., relative expression ≥0.5) to patients with lower expression (i.e., relative expression <0.5); their baseline characteristics are summarized in Supplementary Table S1. We found that patients with the highest MMRN1 expression had an inferior EFS (P = 0.002; at 5 years: 33 ± 15% vs. 58% ± 9%) and higher RR (P = 0.004; at 5 years: 52% ± 20% vs. 24% ± 8%) than the 138 patients within the lower 3 quartiles of MMRN1 expression, whereas OS was not statistically significantly different (P = 0.135; at 5 years: 57% ± 15% vs. 71% ± 8%; Fig. 2A–C). Of note, exploratory multiple cutpoint analyses for EFS indicated that the most statistically significant results were centered around the Q4 cutpoint region, supporting our approach of comparing patients with the highest quartile of relative MMRN1 expression with those having lower relative MMRN1 expression (data not shown).

Validation of MMRN1 as predictive biomarker in participants of AAML0531

To further validate the role of MMRN1 expression as predictive biomarker in pediatric AML, we quantified MMRN1 expression among 1,070 eligible patients enrolled on AAML0531, and correlated expression levels with clinical outcome and disease characteristics. Among these patients enrolled on AAML0531, 980 (92%) consented to the use of biospecimens for correlative research, with RNA available from 765 patients, 15 of which had inadequate GUSB levels (Ct > 33.09). The remaining 750 patients (77%) were used for quantitation of MMRN1 expression levels. In 740 of the 750 patient specimens, MMRN1 mRNA was detectable, varied >130,000-fold relative to GUSB mRNA (0.0001–18.21; median: 0.1263; Fig. 1B), and was distributed across quartiles similarly to MMRN1 expression in AAML03P1.

Association between MMRN1 expression and characteristics of study population

To investigate associations between relative MMRN1 expression and demographics, baseline laboratory findings, and pre-treatment characteristics of the study cohort, we used the same cut-off as identified in the AAML03P1 training cohort and compared patients with high MMRN1 expression (i.e., relative expression of ≥0.5; n = 160) with those having low MMRN1 expression (i.e., relative expression of <0.5; n = 590). As summarized in Table 1, patients with high MMRN1 expression were younger (P < 0.001), whereas there was no statistically significant difference in gender distribution, WBC count, hemoglobin, platelet count, and proportion of patients with hepatomegaly, splenomegaly, or extramedullary disease (chloroma and/or CNS involvement). There was also no significant correlation between MMRN1 mRNA levels and the percentage of bone marrow blasts (Supplementary Fig. S3). Importantly, patients with high MMRN1 expression less likely had CBF translocations ([t(8;21)] 0% vs. 19%, P < 0.001; inv(16): 2% vs. 15%, P < 0.001) and NPM1 mutations (3% vs. 9%, P = 0.016); conversely, they were more likely to have leukemia with monosomy 7 (7% vs. 1%, P < 0.001) and abnormalities involving 11q23 (33% vs. 17%, P < 0.001). Consistently, patients with high MMRN1 expression less likely had low-risk disease (5% vs. 48%, P < 0.001) and more likely had standard-risk disease (73% vs. 41%, P < 0.001) and high-risk disease (23% vs. 11%, P < 0.001) than those with lower MMRN1 expression.
Association between \(\text{MMRN1}\) expression and clinical outcome

To investigate the relationship between \(\text{MMRN1}\) expression and clinical outcome in the AAML0531 cohort, we first studied responses to initial chemotherapy. We found that the 160 patients with high relative \(\text{MMRN1}\) expression were statistically significantly less likely to have achieved CR after one course of chemotherapy than the 590 patients with lower \(\text{MMRN1}\) expression (67% vs. 77%, \(P = 0.006\)) and more likely had MRD at the end of the first induction course (43% vs. 24%, \(P = 0.001\)). Some patients with high \(\text{MMRN1}\) expression were able to achieve remission with reinduction therapy, and the proportion of patients with high \(\text{MMRN1}\) expression in CR after two courses of induction chemotherapy approached that of patients with low \(\text{MMRN1}\) expression (83% vs. 88%, \(P = 0.153\)). We subsequently evaluated how \(\text{MMRN1}\) expression related to parameters of long-term outcome and found that high \(\text{MMRN1}\) expression was associated with inferior OS (\(P < 0.001\); at 5 years: 44% ± 9% vs. 69% ± 4%) and EFS (\(P < 0.001\); at 5 years: 32% ± 8% vs. 54% ± 4%), and higher RR (\(P < 0.001\); at 5 years: 57% ± 10% vs. 35% ± 5%; Fig. 3A–C). The 5-year survival and relapse estimates,
stratified by disease-risk and relative MMRN1 expression, are summarized in Table 2.

**MMRN1 as independent predictive factor**

We next evaluated the potential role of MMRN1 expression as independent predictor of OS, EFS, and RR in regression models (Table 3). Given the association between disease risk and MMRN1 expression, one might attribute the worse outcome for patients with high MMRN1 expression to the lower prevalence of leukemias with more favorable prognoses in this subgroup. However, after adjustment for disease risk, age, bone marrow blast percentage, and treatment arm, high MMRN1 expression remained statistically significantly associated with inferior OS (HR, 1.57; 1.17–2.12; \( P = 0.003 \)), inferior EFS (HR, 1.34; 1.04–1.73; \( P = 0.025 \)), and higher RR (HR, 1.40; 1.01–1.94; \( P = 0.044 \); Table 3).

**Correlation of MMRN1 expression with outcome in individual risk groups**

Finally, we performed subgroup analyses to investigate the potential role of MMRN1 expression as a predictor for outcome in specific risk groups; these studies were of exploratory nature because our ability to perform these analyses was relatively limited because of the sample size of the individual risk groups. As summarized in Table 2, patients with high MMRN1 expression had generally worse outcomes than those with low MMRN1 expression across all three disease risk categories, although these differences approached statistical significance only in the subset of standard-risk-patients (e.g., RR: \( P = 0.045 \)). Of note, fewer patients with high MMRN1 expression underwent HCT as consolidation therapy relative to those with lower MMRN1 expression [low-risk: 0/7 (0%) vs. 17/276 (6%); standard-risk: 20/111 (18%) vs. 42/238 (18%); and high-risk: 12/35 (34%) vs. 27/63 (43%)]. This difference was at least partly explained by primary failures to achieve remission on study, or early relapse after short remission duration. Nevertheless, analyses in which patients were censored at the time of HCT indicated that the predictive significance of MMRN1 expression was retained (Supplementary Table S2).

**Relationship between MMRN1 expression and differentiation stage of AML cell**

In the analyses presented thus far, we used unsorted bone marrow specimens to quantify MMRN1 expression. To investigate whether levels of MMRN1 mRNA were related to the differentiation stage of the AML cell, we used bone marrow specimens from 10 patients and isolated less mature CD34⁻/CD33⁻ and more mature CD34⁺/CD33⁺ cell subsets by FACS. In these 10
specimens, the relative MMRN1 expression was slightly higher in CD34+/CD33− cells [median: 0.33 (range, 0.01–4.82)] than corresponding CD34+/CD33+ cells [median: 0.14 (range, 0.02–1.44); P < 0.05; Supplementary Table S3].

**Discussion**

Multimerins encompass an elusive family of secreted glycoproteins that are characterized by an N-terminal cysteine-rich EMI domain.
domain thought to be involved in multimerization, a long central region predicted to form coiled-coil structures, and a C-terminal globular C1q domain that mediates binding to integrins (2). The biologic function of MMRN1 is poorly understood. Originally identified as a multimeric glycoprotein released by, and associated with, the surface of platelets following platelet activation (13), MMRN1 expression is highly enriched populations than in leukemic progenitor cells. Data on MMRN1 expression, which is highly associated with lower CR rates, shorter survival estimates, and a higher risk of relapse and, consequently, inferior survival expectations. 

Previous studies have established that proteins involved in cellular adhesion such as L-selectin, β-integrin, and VLA-4 constitute biologic features that can serve as predictive markers in AML (18–21). Ultimately, mechanistic studies will be necessary to fully understand this association between high MMRN1 expression and adverse outcome in AML and to investigate whether secreted levels of MMRN1 also bear predictive information. Still, given the emerging recognition of the importance of cellular adhesion for resistance to chemotherapy in hematologic malignancies, including AML (22), and the ability of multimers to bind to ECM and integrins, it is interesting to speculate that elevated MMRN1 levels could alter AML cell function and adhesion-dependent resistance to chemotherapy. Future studies will...
be required to test whether elevated expression of MMRN1 in AML cells provides further support for the concept of cellular adhesion being a pivotal biologic factor in AML. In addition, MMRN1 has been identified as a TGFβ1-interacting protein (23), and the multimerin family member, EMILIN-3, acts as a TGFβ1 antagonist in vitro (24), raising the possibility that MMRN1 could exert an effect on AML cells via modulation of cytokine signaling (e.g., attenuation of TGFβ1 signaling).

Our study has several strengths, most notably the inclusion of a large number of the diagnostic specimens, thereby increasing the precision of outcome estimates, the use of data from patients treated homogeneously on recent cooperative group trials, and the use of data from two independent trials that allow validation of our findings across independent patient cohorts. On the other hand, several limitations have to be acknowledged. First, despite the use of a large number of specimens, our ability to perform subset analyses was relatively limited because of the sample size of the individual risk groups, for example, for risk-stratified analyses. Likewise, our study was not sufficiently powered to allow for extensive multivariate adjustments. Second, because unsorted bone marrow specimens were used for our studies, differences in MMRN1 abundance between specimens may not necessarily reflect differences in AML blasts but, rather, other cell types such as megakaryocytes or vascular cells. Gene expression studies in humans and mice indicate that higher MMRN1 mRNA levels are found in less mature hematopoietic cells, including LSC populations (17, 25). Consistent with the latter, we found in a small set of AML specimens that MMRN1 levels were higher in less mature less mature CD34+/CD33− cells than the more mature CD34+/CD33+ cell counterparts. Identifying the exact cellular origins of the greatly variable amounts of MMRN1, and more detailed analyses of relative expression levels along the cellular differentiation path of AML cells, may be a subject for future work. And third, only cryopreserved specimens were available for our analyses, and additional studies will be required to determine to what degree, if any, MMRN1 expression changes in the cryopreservation process. Nevertheless, our data indicate that MMRN1 expression is a novel independent adverse predictive marker in pediatric and adolescent AML. For outcome prediction, high MMRN1 expression characterizes patients at significantly increased risk for primary treatment failure, relapse, and poor leukemia-free survival. Thus, MMRN1 may be of use for the refinement of risk-stratification, for example, via assignment of intensified chemotherapy or use of allogeneic hematopoietic cell transplantation in future cooperative study group trials or routine off-protocol care, and improve our abilities to individualize treatment decisions in AML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: G.S. Laszlo, A.S. Gamis, S. Meshinchi, R.B. Walter
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Gudgeon, K.H. Harrington, B.A. Hirsch, A.S. Gamis
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.B. Walter
Study supervision: R.B. Walter

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References

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