Polymorphism G80A in the Reduced Folate Carrier Gene and its Relationship to Survival and Risk of Relapse in Acute Lymphoblastic Leukemia

Marco Antonio Leyva-Vázquez, PhD, Jorge Organista-Nava, MSc,† Yazmín Gómez-Gómez, MSc,† Adriana Contreras-Quiroz, MSc,* Eugenia Flores-Alfaro, PhD,‡ and Berenice Illades-Aguiar, PhD**

Background: The reduced folate carrier (*RFC1*) is a major methotrexate transporter whose impaired function was recognized as a frequent mechanism of antifolate resistance. Recently, a G80A polymorphism has been described in the RFC1. This study evaluated the effect of the G80A polymorphism in the *RFC1* gene on survival and risk of relapse of acute lymphoblastic leukemia.

Methods and Results: Seventy patients with acute lymphoblastic leukemia were genotyped by polymerase chain reaction restriction fragment length polymorphism method. An association between the polymorphism and risk of relapse was found ($P < 0.05$). Patients with the G/A genotype have 3.97 (95% confidence interval, 1.12–14.06) and carriers of the A/A genotype have 7.84 (95% confidence interval, 1.66–37.10) higher chance of a relapse. Other variables such as age and leukocyte count were associated ($P < 0.05$) with the risk of relapse of disease. Individuals with G/A or A/A genotypes had poorer survival (log-rank test, $P = < 0.05$).

Conclusions: These data suggest a role of the polymorphism G80A in the risk of relapse and the mortality risk in patients with acute lymphoblastic leukemia from the State of Guerrero, Mexico.

Key Words: acute lymphoblastic leukemia, reduced folate carrier, risk of relapse, G80A polymorphism

(*J Investig Med* 2012;60: 1064–1067)

In Mexico, acute leukemia is considered a problem of public health; it represents the fourth position of mortality of all neoplastic malignancies in children younger than 15 years. Date from 1996 to 2000 show a mortality rate of 63.7 per million children, one of the highest rates reported in the world.[1] In 2005, leukemia was the second cause of mortality in the State of Guerrero in children younger than 15 years.[2] An antineoplastic agent used as treatment for patients with acute lymphoblastic leukemia (ALL) is the methotrexate (MTX), which was introduced 5 decades ago to clinical oncology. The reduced folate carrier gene (*RFC1*) is a major MTX transporter whose impaired function was recognized as a frequent mechanism of antifolate resistance.[3] Recently, the G80A polymorphism, which replaces His by Arg at position 27 of the RFC1 protein, was identified. Children with ALL with the A variant had worse outcome than patients with the G/G genotype.[4] In the present study, we have retrospectively analyzed the association between the genetic polymorphisms G80A and both survival and risk of relapse in childhood ALL in a Mexican population.

Study Population

The patients (n = 70) with ALL were included in this study of the pediatric oncology service of the State Cancer Institute from the south of Mexico (Acapulco, Guerrero) between August 2005 and August 2010, diagnosed with ALL through bone marrow aspirate based on French-American-British morphological criteria, cytochemical staining properties, and immunophenotyping of blast cells. The diagnosis of ALL was further subclassified into T-lineage ($CD3^+$, $CD7^+$, plus $CD2^+$ or $CD5^+$, or both) or B-lineage ($CD22^+$, $CD19^+$, HLA-DR^+, and $CD10^+$). Multiagent chemotherapeutic protocols used were 96091, 96092, or CIE-10:C9.1.0 of the Cancer Institute from Guerrero State and previously described.[5,6]

Children with low-risk ALL received a triple intrathecal therapy (MTX, cytarabine, hydrocortisone [HDC]) during the induction phase: MTX, 8 mg/m^2 for 1 to 2 years, 10 mg/m^2 for 2 to 3 years, 12 mg/m^2 for 3 to 8 years, and 15 mg/m^2 after 8 years; cytarabine, 16 mg/m^2 for 1 to 2 years, 20 mg/m^2 for 2 to 3 years, 24 mg/m^2 for 3 to 8 years, and 30 mg/m^2 after 8 years; HDC, 8 mg/m^2 for 1 to 2 years, 10 mg/m^2 for 2 to 3 years, 12 mg/m^2 for 3 to 8 years, and 15 mg/m^2 after 8 years at days 1, 22, 29, and 36. During the consolidation phase, the patients received 2 g/m^2 per 24 hours of MTX at weeks 7, 10, 13, 16, 19, and 22; and 48 hours after, they received folinic acid rescue, 15 mg/m^2 every 3 hour for 9 times; and during maintenance therapy, they received 50 mg/m^2 of MTX weekly until week 130. Children with high-risk ALL received a triple intrathecal therapy (MTX, cytarabine, HDC) during the induction phase: MTX (8–15 mg), cytarabine (16–30 mg), HDC (8–15 mg), depending on age, at days 1, 15, 29, 45, and 59. During the consolidation phase, they received 5 g/m^2 of MTX at days 8, 22, 36, and 50 and triple intrathecal therapy at days 8, 22, 33, and 50, with leucovorin rescue, 15 mg/m^2, after 48 hours. During the maintenance phase, they received 25 mg/m^2 MTX weekly[5,6] until week 130.

The bone marrow samples of patients or blood samples used in this study were part of the samples taken for clinical diagnostic tests in the hospital; informed consent was obtained. The study and the informed consent procedure were approved by the Institutional Review Board of the Cancer Institute. Complete remission was defined by less than 5% blast cells in the bone marrow and normalization of peripheral blood counts at 4 weeks after starting

From the *Laboratory of Molecular Biomedicine, School of Biological Sciences, Guerrero State University, Chilpancingo, Guerrero; †Institute of Cellular Physiology, National Autonomous University of Mexico (UNAM), University City, DF; and ‡Laboratory of Chronic Degenerative Diseases and Infections, School of Biological Sciences, Guerrero State University, Chilpancingo, Guerrero, Mexico.

Received February 7, 2012, and in revised form June 20, 2012.

Reprints: Marco Antonio Leyva-Vázquez, PhD, Laboratory of Molecular Biomedicine, School of Biological Sciences, Guerrero State University, Av Lázaro Cárdenas S/N, Ciudad Universitaria, Chilpancingo, Guerrero, México 39090. E-mail: leyvamarco13@gmail.com.

MALV, JON, and YGG contributed equally to this study; MALV, JON, YGG, and BIA designed the study, analyzed and interpreted data, and drafted the manuscript; JON, YGG, EFA, and ACQ contributed to data collection; MALV and BIA revised and approved the final version of the paper.

All authors have no conflicts of interest to declare.

Accepted for publication June 23, 2012.

ISSN: 1081-5589

DOI: 10.231/JIM.0b013e31826803c1

TABLE 1. General Characteristics of Population and Clinical Data of Patients With ALL

Variable	Patients With ALL N = 70
Age, yrs	7.65 ± 4.67
Number of leukocytes, mm^3	13,000 (5400–39,000)*
Sex	
Male	45 (64.29)
Female	25 (35.71)
Status of participants	
Alive	30 (42.86)
Deceased	40 (57.14)
Immunophenotype	
B-lineage	66 (94.28)
T-lineage	4 (5.72)
Risk by age	
Standard (1–9 years)	18 (25.71)
High (<1 and >9 years)	52 (74.29)
Relapse during treatment	
Yes	48 (68.57)
No	22 (31.43)

Data are presented as n (%) and mean ± SD.

*Median (percentiles 25–75).

induction therapy. Relapse was defined as the reappearance of more than 20% blast cells in the marrow or the presence of localized leukemic infiltrates at any site after completion of induction chemotherapy. Worse outcome was defined as a lack of response to induction therapy, a relapse after achieving complete remission, or death due to any cause. Risk classification (standard risk: 1–9 years of age and presenting white blood cell [WBC] count of <50,000/mm^3; high risk: <1 and >9 years of age and WBC count >50,000/mm^3). Patients' characteristics and clinical prognostic factors at diagnosis (sex, age, WBC count, and risk group) are given in Table 1.

Genotyping

The G80A polymorphism was detected using the reported polymerase chain reaction primers by Dervieux et al.[7] in 2004: forward primer (5′-AGTGTCACCTTCGTCCCCTC-3′) and reverse primer (5′-CTCCCGCGTGAAGTTCTT-3′) and using previously established protocols.[8] The polymerase chain reaction products (230 bp) were digested with 3 units of the *HhaI* enzyme (New England Biolabs, Berverly, MA). Individuals with the A/A genotype presented 2 fragments (162 and 68 bp), individuals with the G/A genotype presented 4 fragments (162, 125, 68, and 37 bp), and those with the G/G genotype 3 fragments (126, 68, and 37 bp).

Statistical Analysis

Univariate logistic regression analysis for the association with the risk of relapse of ALL was tested first for G80A genetic polymorphism, sex, and other clinical characteristics, and those factors were included in a second analysis, the multivariate logistic analysis. The log-rank test and Kaplan-Meier curves were used to analyze the effect of the G80A genetic polymorphism, and relapse of ALL on overall survival. Overall survival (OS) was defined as the time between surgery and either death or the time of the last follow-up. $P < 0.05$ was considered statistically significant. All statistical analyses were done using SPSS software, version 15.0 (SPSS, Chicago, IL) and STATA software, version 9.2 (StataCorp, College Station, TX).

RESULTS AND DISCUSSION

The 70 patients with ALL had ages ranging from 1.0 to 18 years (mean ± SD age, 7.65 ± 4.67 years). There were 45 males (64.29%) and 25 females (35.71%). Eighteen patients (25.71%) were in the

TABLE 2. Association of the G80A Polymorphism in the *RFC* Gene and Other Clinical Features With the Risk of Relapse of ALL

			Univariate Analysis			Multivariate Analysis		
	n	%	OR	95% CI	*P**	OR	95% CI	*P*‡
Sex								
Female	25	35.71	1.00					
Male	45	64.29	1.38	0.49–3.92	0.540			
Risk by age								
2–9 years (low-risk)	18	25.71	1.00					
<1 and >10 years (high-risk)	52	74.29	4.54	1.14–18.09	0.032†	0.86	0.25–2.96	0.814
Leukocytes at diagnosis								
<50,000/mm^3	18	25.71	1.00					
>50,000 /mm^3	52	74.29	7.64	1.90–30.73	0.004†	0.52	0.11–2.36	0.395
G80A genotype								
G/G	20	28.57	1.00					
G/A	20	28.57	3.36	1.02–11.12	0.047†	3.97	1.12–14.06	0.033†
A/A	30	42.86	6.92	1.58–31.38	0.012†	7.84	1.66–37.10	0.009†

**P* was obtained by logistic regression analysis in reference to AA and CC genotypes, female, 2–9 years (low-risk), and <50000 leukocytes per cubic millimeter.

†Significant: $P < 0.05$.

‡*P* was obtained by multivariate logistic regression analysis.

95% CI indicates 95% confidence interval.

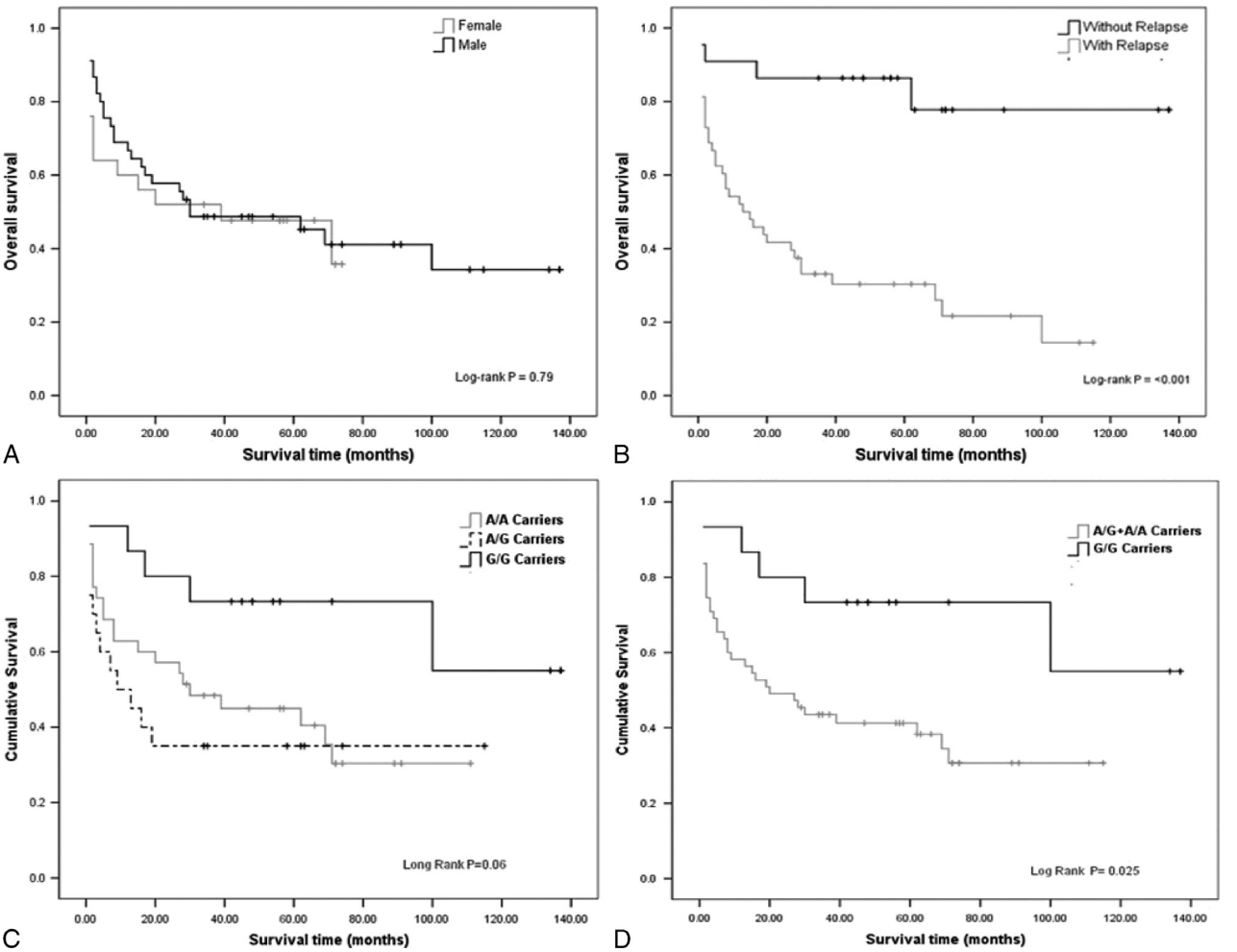

FIGURE 1. Kaplan-Meier curves considering the influence of the sex, relapse, *RFC1* G80A polymorphism on overall survival of patients with ALL. A, Overall survival in female and male with ALL. B, Overall survival between patients with and without relapse. C, Association between overall survival and G80A polymorphism. D, Combined genotypes A/G + A/A vs G/G in 70 pediatric patients with ALL.

age group of 1 to 9 years (standard risk). Fifty-two patients (72.29%) were younger than 1 year old and older than 9 years (high risk) at the time of initial diagnosis; 68.57% had a relapse of ALL. White blood cell count and characteristics of immunophenotype are described in Table 1.

This report is the first to describe the contribution of polymorphism in RFC1, to the effects of both survival and risk of relapse in childhood ALL. A significant difference in the frequency of RFC1 genotypes was observed between children with and without relapse (Tables 1, 2). Carriers of the RFC1 A variant had a higher risk for events than those with the G/G genotype (odds ratio [OR], = 6.92; 95% confidence interval, 1.58–31.38; P = 0.012). Similarly, the Kaplan-Meier analysis showed that carriers of the A variant had the worst ALL outcomes (P = 0.026; Fig. 1D). In the multivariate analysis, OR estimates for patients with RFC1 A/A genotype retained their significance (OR, 7.84; 95% confidence interval, 1.66–37.10; P = 0.009) in the presence of other prognostic factors, which also influenced ALL outcome (age, WBC, and risk classes; Table 2). The Kaplan-Meier analysis of overall survival curves showed significant results of relapse of ALL and G80A genetic polymorphism. We found no significant associations between the sex and overall survival (log-rank test, P = 0.79; Fig. 1A). The different overall survival was evident between the individuals with and without relapse of ALL (log-rank test, P = <0.001; Fig. 1B).

We found patients who did not respond to chemotherapy with MTX of which 9 (45.00%) of 20 patients were carriers of the G/G genotype, 17 (85.00%) of 20 patients were carriers of the G/A genotype, and 22 (73.00%) of 30 patients were carriers of the A/A genotype (P = 0.007, Fisher exact test). Moreover, the patients were evaluable for toxicity. The incidence of renal, hepatic, and mucous membrane toxicities during induction or consolidation phase was mainly found in the patients with the risk genotype (A/A), a result similar to that reported by Laverdiere et al., in children carriers of the A/A genotype, who had worse response than patients with the G/G genotype (P = 0.04). Evidence is shown here of the correlation between the genotypes, the polymorphism G80A in the *RFC1* gene, and lack of response to MTX. Most of the A/A patients experienced lack of response to MTX more frequently.

The association between RFC1 polymorphism and ALL outcome suggests that this variant might contribute to the estimation of ALL prognosis. Our results are in line with those previously reported by Laverdièr et al., showing an association between the A/A genotype and a reduced survival in the patients with ALL.[4] However, the genotypic frequencies reported in this

study differ ($P < 0.001$) from those reported by Laverdièr et al. in French Canadian origin population with ALL, where the G/A genotype (48.0%) was the most frequently reported, followed by the G/G genotype (29.9%) and the A/A genotype (22.1%). The differences found in the distribution of the risk genotype (A/A) in the French Canadian population compared to the Mexican population, can be due to differences in genetic background between populations. The population from the State of Guerrero in Mexico has 22.0% African genetic background according to the report by the INMEGEN in 2007.[9,10] Morin et al.[11] suggested that patients with A/A genotype of RFC1 tended to have higher folate polyglutamate levels in blood cells. Recent evidence suggests that patients with the RFC1 A/A genotype responded to therapy better than those with the G/G and G/A genotypes.[7] Other studies have explored variations in the RFC1 expression in leukemic cells and demonstrated that low expression of RFC1 is related to a poor outcome in childhood leukemia.[12] Jansen et al.,[13] suggested that higher messenger RNA levels was related to the RFC1 A variant.

Therefore, additional studies are needed to explain the underlying mechanism linking RFC1 polymorphism and ALL outcome. A prospective study assessing intracellular MTX levels and RFC1 substrate binding affinities in patients with and without RFC1 A variant is under way in our laboratory. It would also be important to assess the relative impact of RFC1 polymorphism on ALL outcome with regard to other variants relevant to MTX response.

ACKNOWLEDGMENTS

The authors thank their patients and the patients' parents for their collaboration; and Ana Bertha Rivera Ramírez and Marco A. Terán Porcayo of the State Cancer Institute for their contributions of biologic material and facilitating access to clinical data. The authors also thank the National Council of Science and Technology (CONACYT, Mexico) for a fellowship awarded to Adriana Contreras Quiroz (August 2007 to July 2009).

REFERENCES

1. Mejia-Arangure J, Bonilla M, Lorenzana R, et al. Incidence of leukemias in children from El Salvador and Mexico City between 1996 and 2000: population-based data. *BMC Cancer*. 2005;5:33–38.
2. Salud/INEGI, S.d., Fuente: base de datos de defunciones INEGI/ Secretaria de Salud, Dirección General de Información en Salud, México. Febrary, 2006. Available at: http://www.inegi.org.mx/. Accessed April 29, 2008.
3. Sierra EE, Goldman ID. Recent advances in the understanding of the mechanism of membrane transport of folates and antifolates. *Semin Oncol*. 1999;26:11–23.
4. Laverdiere C, Chiasson S, Costea I,et al. Polymorphism G80A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia. *Blood*. 2002;100:3832–3834.
5. Seguro-popular: Secretaria de Salud/ Seguro Popular. August 03, 2007. Available at: http://seguropopular.col.gob.mx:81/repss/protocolos/6LLAEH.pdf. Accessed May 4, 2009.
6. Gómez-Gómez Y, Organista-Nava J, Saavedra-Herrera MV, et al. Survival and risk of relapse of acute lymphoblastic leukemia in Mexican population is affected by dihydrofolate reductase gene polymorphisms. *Experimental and Therapeutic Medicine*. 2012;3:665–672.
7. Dervieux T, Furst D, Lein DO, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. *Arthritis Rheum*. 2004;50:2766–2774.
8. Organista-Nava J, Gómez-Gómez Y, Saavedra-Herrera MV, et al. Polymorphisms of the γ-glutamyl hydrolase gene and risk of relapse to acute lymphoblastic leukemia in Mexico. *Leukemia Res*. 2010;34:728–732.
9. INMEGEN. Mapa genético de los mexicanos. May, 2009 Available at: http://www.inmegen.gob.mx/. Accessed June 18, 2009.
10. Silva-Zolezzi I, Hidalgo-Miranda A, Estrada-Gil J, et al. Analysis of genomic diversity in Mexican Mestizo populations to develop genomic medicine in Mexico. *Proc Natl Acad Sci U S A*. 2009;106:8611.
11. Morin I, Devlin AM, Leclerc D, et al. Evaluation of genetic variants in the reduced folate carrier and in glutamate carboxypeptidase II for spina bifida risk. *Mol Genet Metab*. 2003;79:197–200.
12. Levy AS, Sather HN, Steinherz PG, et al. Reduced folate carrier and dihydrofolate reductase expression in acute lymphocytic leukemia may predict outcome: a Children's Cancer Group study. *J Pediatr Hematol Oncol*. 2003;25:688–695.
13. Jansen G, Mauritz R, Drori S, et al. A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance. *J Biol Chem*. 1998;273:30189–30198.