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Tobacco Smoke and *Ras* Mutations Among Latino and Non-Latino Children with Acute Lymphoblastic Leukemia

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Abstract

Background and Aims—Childhood acute lymphoblastic leukemia (ALL) is a biologically heterogeneous disease, and mutations in the *KRAS* and *NRAS* oncogenes are present at diagnosis in about one-fifth of cases. *Ras* mutations were previously associated with environmental exposures in leukemias as well as in many other cancer types. This study examined whether *Ras* mutation could define a unique etiologic group of childhood ALL associated with tobacco smoke, a well-established mutagen and carcinogen.

Methods—We included 670 children with ALL enrolled in a case-control study in California (1995–2013), including 50.6% Latinos. Parental and child exposure to tobacco smoke was obtained from interviews. Sanger sequencing was used to detect the common *KRAS* and *NRAS* hotspot mutations in diagnostic bone marrow DNA. ALL cases were also characterized for

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common chromosome abnormalities. In case-case analyses, logistic regression analyses were used to estimate odds ratios to describe the association between tobacco smoke exposure and childhood ALL with *Ras* mutations.

Results—*KRAS* or *NRAS* mutations were detected in ~18% of children diagnosed with ALL. *Ras* mutations were more common among Latino cases compared with non-Latino whites and in high-hyperdiploid ALL. No associations were observed between parental smoking or child's passive exposure to smoke and *Ras* positive ALL.

Conclusions—The apparent lack of association between tobacco smoke and *Ras* mutation in childhood ALL suggests that *Ras* mutations do not specifically define a tobacco-related etiologic pathway. Reasons for racial and ethnic differences in ALL are not well understood and could reflect differences in etiology that warrant further examination.

Keywords

Tobacco smoke; Ras mutation; Childhood acute lymphoblastic leukemia; Latino; Hispanic

Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of leukemia in children <15 years of age, representing 78% of all leukemia cases (1). Whereas 5-year survival for ALL is >90%, children still suffer long-term treatment-related morbidities, and racial and ethnic disparities exist in both incidence and treatment, highlighting the need for etiologic research to direct prevention efforts (2–4). Latino (also referred to as Hispanic) children in the U.S. have the highest incidence of ALL and the poorest survival rates, yet the causes of those disparities, particularly in the biology, are not well understood (4). ALL is a biologically heterogeneous disease with subtypes differing in prognosis and, potentially, in etiology. The latter is supported by the association of heritable genetic variants with particular subtypes of ALL, for example, single nucleotide polymorphisms in *PIP4K2A* and *GATA3* are exclusively associated with the high-hyperdiploid and Philadelphia chromosome-like subtypes of ALL, respectively (5,6). Further, tobacco smoke exposure has been associated with an increased risk of childhood ALL with *TEL-AML1* fusion but not with increased risk of high-hyperdiploid ALL (7). Whether any environmental exposures may be associated with specific acquired tumor genetic abnormalities in childhood ALL has yet to be comprehensively evaluated, although this may have important implications for treatment and indeed prevention.

Mutations in the *Ras* subfamily of proteins are frequent in cancer and have an estimated prevalence of 15–31% at diagnosis in childhood ALL (8–12). The *Ras* oncogenes, including *KRAS*, *NRAS*, and *HRAS*, are part of the mitogen-activated protein kinase (MAPK) signaling pathway and code for GTPases that regulate pathways responsible for cell growth and survival (13). Mutations in *Ras* genes may lead to constitutive activation of these pathways, promoting cell proliferation and tumorigenesis. Identifying mechanisms leading to somatic *Ras* mutations in leukemia may reveal avenues for targeted therapy (8,14,15) and may also point to specific environmental risk factors.

Adult myeloid leukemias have been associated with *Ras* mutations in occupational settings, suggesting a link with environmental chemicals (16,17). Although children are not directly exposed to occupational exposures, they may be associated indirectly via their parents and home environments. Human and animal studies have indicated that exposure to chemical carcinogens such as cigarette smoke, organochlorines, hydrocarbons and mind-altering drugs can induce *Ras* mutations (9,18–20), further suggesting that *Ras* mutations in ALL could define a unique chemically associated etiological group. Moreover, *Ras* mutations in childhood ALL cases enrolled in the California Childhood Leukemia Study (CCLS) were not found to be present at birth, suggesting that *Ras* mutations occur postnatally (21). Further, previous CCLS analyses also found suggestive associations between race/ethnicity and *Ras*, which may result from postnatal differences in environmental or lifestyle factors (12,21).

Tobacco smoke is carcinogenic and an established risk factor for many cancers, including leukemias in adults (22) and possibly in children (23–25). An established human leukemogen-benzene and a recently classified leukemogen-formaldehyde are both present in tobacco smoke. A recent meta-analysis shows that *in utero* and early-life exposure to benzene is associated with the increased risk of childhood leukemia (26). Another study reported that abnormal Ras signaling (by *Nf1* deletion) could enhance the toxicity of hydroquinone, a metabolite of benzene, in murine bone marrow stem/progenitor cells (27).

Carcinogenesis in children may potentially be associated with parental smoking before conception and/or during pregnancy or by the child's exposure to secondhand smoke after birth. The literature on childhood ALL demonstrates little to no evidence for an effect of maternal smoking, but many studies and meta-analyses have shown evidence for an association with paternal smoking (23,28). A previous CCLS report found an increased ALL risk only in children with both a history of paternal smoking during preconception and postnatal passive smoking (7) in support of the two hit (pre- and post-natal) model for leukemogenesis. Here we examine whether tobacco smoke and racial/ethnic characteristics are associated with *Ras* mutations in childhood ALL, using an ethnically diverse study population in California.

Methods

Study Population

The CCLS is a case-control study conducted in 45 counties in California from 1995–2015 (i.e., 17 counties from northern California [1995–1999], expanded to an additional 18 counties in central California [2000–2008], and 10 counties in southern California [2009–2015]) investigating the causes of childhood leukemia. Incident cases of childhood leukemia (age 0–14 years) were identified using the *International Classification of Diseases for Oncology* and rapidly ascertained at pediatric hospitals, usually within 72 h of diagnosis (29,30). Pre-treatment bone marrow and peripheral blood specimens were collected by clinical staff at participating hospitals according to the study protocol and available for molecular characterization. Eligibility criteria for the CCLS included: a) age at diagnosis <15 years of age, b) no prior cancer history, c) residence in the study area, and d) one biological parent able to speak English or Spanish. The study population is described in

more detail elsewhere (7). Additional eligibility for this analysis included complete information on all covariates and availability of diagnostic bone marrow for characterization of *Ras*.

Of the 1058 ALL cases interviewed in the CCLS during the study period, 672 cases had diagnostic bone marrow samples available for *Ras* mutation assessment. Cases with and without *Ras* data were similar with respect to child's age, sex, and race/ethnicity as well as parental age and income. We chose to limit the analysis to cases with complete covariate information, resulting in a final sample size of 670. Table 1 describes the population's sociodemographic characteristics. The study protocol was approved by the Institutional Review Boards at the University of California, Berkeley, California Department of Health Services and participating hospitals. Written informed consent was obtained from parents of all participating study subjects, and assent was obtained for children 7 years and older.

Data Collection

Information on sociodemographic characteristics and tobacco smoking were collected during in-home visits (1995–2008) or by phone (2009–2015) with the child's biological mother (98% of interviews) or father (2%). Interviews were usually conducted within a few months to a year after the child's diagnosis. The interviews covered information on mother's reproductive and health history, parental occupational history, residential history, mother's pregnancy and child's delivery, child's health history, family illness, and other household chemical exposures.

Exposures considered for this analysis were paternal ever smoking during his lifetime; paternal smoking during preconception; maternal ever smoking; maternal smoking before and during pregnancy; and child's passive smoking postnatally. The preconception period encompassed the 3 months prior to conception and the postnatal period encompassed time from birth to child's diagnosis for cases or to his/her third birthday (whichever came first). The prenatal period refers to preconception and pregnancy jointly. Parental active smoking was defined as ever smoking at least 100 cigarettes, pipes, or cigars during their lifetime. "Ever smoking" and "history of smoking" are used interchangeably in this study. Parents who began smoking after the child's diagnosis were not considered as being exposed to tobacco smoke in our analyses. Parental passive smoking was defined as regular smoking by a third party in the individual's presence indoors. Child's passive smoking was defined as indoor presence of a regular smoker postnatally up to the child's diagnosis or third birthday (whichever came first). We also had information on the number of cigarettes, pipes, or cigars smoked per day for fathers during preconception and mothers during preconception and pregnancy (details available elsewhere) (7).

Ras Mutation Sequencing

Ras mutation was defined as either *KRAS* or *NRAS* codon 12 or 13 hotspot mutations in this study. For both *KRAS* and *NRAS*, PCR and Sanger sequencing were carried out across exon 1, which includes codons 12 and 13 that are commonly mutated in childhood ALL, represented as *Ras* positive. For this, genomic DNA was extracted from patients' diagnostic bone marrow samples using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA).

HRAS mutations were not considered for this study because of their low prevalence in leukemias. PCR amplification of exon 1 of *KRAS* and *NRAS* was performed separately using the following sets of oligonucleotide primers: *KRAS*_F: GGTCCTGCACCAGTAATATGC, *KRAS*_R: CTTAAGCGTCGATGGAGGAG; and *NRAS*_F: TCCGACAAGTGAGAGACAGG, *NRAS*_R: TGGAAGGTCACACTAGGGTT. Bidirectional sequencing of PCR products was carried out on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA), with sequence data compared to the human reference genome to confirm presence/absence of *KRAS* or *NRAS* exon 1 mutations.

Cytogenetic Classification

Information on cytogenetic classification was obtained from medical records released by hospitals where cases were treated. The medical records were abstracted for conventional G-banding karyotypes and/or fluorescence *in situ* hybridization (FISH) screening on pretreated bone marrow specimens. Abstracting was reviewed for accuracy by a consulting clinical oncologist. When the clinic medical records in some cases showed missing cytogenetic data or normal karyotypes by G-banding or had no observed high-hyperdiploidy (51+ chromosomes) and/or a common translocation—*t*(12;21), FISH analysis was performed again in house to further screen for the high-hyperdiploidy and t(12;21) in those ALL cases. The karyotypes were classified according to clonal genetic aberration using the International System for Human Cytogenetic Nomenclature 1995 criteria (31). More detailed information on the methods can be found in a previous study (7).

Statistical Analysis

Pearson's χ^2 tests and Student's *t* tests were used to assess the association between sociodemographic characteristics and *Ras* mutation. We also assessed whether cytogenetic abnormalities were associated with *Ras*.

We conducted case-case analyses using logistic regression to estimate the odds ratios (OR) and 95% confidence interval (CI) of having a *Ras* positive ALL (dependent variable) associated exposure to tobacco smoke (independent variable). Potential covariates identified using prior literature were sex, age at diagnosis, race/ethnicity (Latino, non-Latino white, non-Latino other), mother's age, and annual household income. Child's race and ethnicity was determined by the biological parent, and in a few instances ($n = 5$ missing Latino ethnicity and $n = 16$ missing race), missing information was supplemented by birth certificate data on parental race (child defined as "white" if both parents were white and "other" if either parent was not white) and Hispanic ethnicity (child defined as Latino if either one or both parents were Hispanic). For the multivariate model, we retained only race/ethnicity because it was significantly associated with *Ras* mutation in the bivariate analyses (p value cut-off = 0.20), and we tested for interactions with race/ethnicity. Analyses were also carried out separately for high-hyperdiploidy, a subtype of ALL that correlates with higher frequency of *Ras* mutation.

Results

One hundred and twenty-two cases (18.2%) had somatic mutations in exon 1 of *KRAS* or *NRAS*. Of these, 55 cases had *KRAS* and 64 had *NRAS* hotspot mutations, whereas three cases had both *KRAS* and *NRAS* mutations. Race/ethnicity was significantly associated with *Ras* mutation status, with more *Ras* mutations detected in Latinos (20.1%) compared with non-Latino whites (12.7%). The other demographic characteristics did not differ significantly by *Ras* mutation status (Table 1). High-hyperdiploidy was also significantly associated with *Ras* mutation (55.9% of *Ras* positive cases were high-hyperdiploids compared with 24.7% of *Ras* negative cases, $p < 0.001$). In contrast, *TEL-AML1* translocation was less common in *Ras* positive cases (11.9%) than in *Ras* negative cases (25.7%) ($p = 0.002$). High-hyperdiploidy and Latino ethnicity were independent predictors of *Ras* positive childhood ALL, with significant associations observed for Latinos compared with non-Latino whites (OR = 2.04; 95% CI = 1.19, 3.49), for non-Latino others compared with non-Latino whites (OR = 2.34; 95% CI = 1.21, 4.53), and for high-hyperdiploidy (OR = 4.23; 95% CI = 2.62, 6.84) (Table 2).

No statistical evidence was found between history of paternal and maternal smoking (ever and at various periods of exposure) or child's passive smoking and *Ras* in ALL (Table 3), with race/ethnicity included in the logistic model. Similar results were reported for the number of cigarettes, pipes, or cigars per day smoked by the parents before the child's birth. Analyses stratified by child's race/ethnicity or by high-hyperdiploidy led to similar findings (data not shown).

Discussion

This is the largest study to date that confirms a higher proportion of *Ras* positive ALL in Latino children. However, no association was detected between the presence of *Ras* and prior exposures to tobacco smoke. Since discovery in the 1980s, the *Ras* oncogene mutations have attracted much attention for their high prevalence and role in cancer development (13,32). Tobacco smoke is established as a risk factor for many cancers and diseases, many with a high rate of *Ras* mutations such as cancers of the lung, pancreas, and colon (33,34). There is evidence for genotoxic effects from smoking. Paternal smoking causes DNA damage in spermatozoa; fetuses of mothers who smoke have more mutations and chromosomal abnormalities; and children exposed to passive smoking have more DNA damage (35). However, in this current study, we found that *Ras* mutation was likely not a pathway through which tobacco smoke affects leukemogenesis. In contrast, a previous study of 837 childhood ALL cases reported that parental occupational exposure to hydrocarbons such as solvents or plastic materials and specific medications may be associated with *Ras* mutations in ALL (9). That study did not correct analyses for multiple comparisons, which therefore increases the likelihood of false positives. In our study, the postnatal period examined in our analysis was up to 3 years after birth, limiting our ability to assess long-term exposure to passive smoking.

Recent whole genome sequencing studies of multiple cancer types have revealed a mutational signature associated with tobacco smoke exposure, in particular in lung, head and

neck, and liver cancers that are known to be associated with tobacco smoking (36). This signature is characterized by frequent C>A mutations; however, the majority of *KRAS* and *NRAS* codon 12 and 13 mutations are G>A mutations, supporting the notion that tobacco smoke does not induce *Ras* mutations. Although Alexandrov et al. did not report enrichment of the tobacco smoke-associated mutational signature in ALL, whole genome sequencing of large numbers of ALL cases from the CCLS would enable assessment of this signature in tobacco-exposed compared with non-exposed cases.

We did find significant associations between *Ras* mutation and child's race/ethnicity, with a lower frequency of *Ras* mutations in non-Latino white cases as shown previously (12,21). We also confirmed the known higher frequency of *Ras* mutations in high-hyperdiploidy (12) and lower frequency of mutations in *TEL-AML1* fusion cases, the two most common cytogenetic subtypes of ALL. High-hyperdiploidy is more frequent in Latino ALL cases (37); however, we still found a significant association between *Ras* mutation and ethnicity even when including both ethnicity and cytogenetic subtype in the model (Table 2). This suggests a potential role for heritable genetic and/or environmental factors that may explain the inter-ethnic difference in *Ras* mutation frequency.

The strengths of the study include a large sample size with molecular classification of childhood ALL including the use of sequencing and FISH analyses and ethnic diversity as well as detailed information on pre- and postnatal exposures to tobacco smoking. An important limitation of this analysis is the self-reported smoking history. Whereas the use of *Ras* mutation as the outcome in a case-case analysis does limit potential differential recall of the exposure compared with a case-control analysis, misclassification is still a concern. Smoking is commonly underreported, especially when mothers are asked about smoking during pregnancy (29,30). Most respondents in this study were biological mothers so there may be concern about their reporting of paternal smoking; however, a previous study on tobacco smoke in CCLS found agreement in a subset of 107 cases and 108 controls on maternal and paternal self-reported information on paternal smoking. Using the scale proposed by Landis and Koch (40), the study observed a kappa of 0.73 (95% CI: 0.63–0.83) for paternal ever smoking and a kappa of 0.70 (95% CI: 0.56–0.84) for paternal smoking during preconception (7). Some parents had to recall exposures from many years prior, especially if their child was diagnosed with leukemia at an older age, although we did not find that the age at diagnosis was associated with smoking exposures, so issues with recall over time was likely unimportant. Given our findings and this discussion, the self-reported nature of the data likely should not have substantially influenced the results.

The detection of *Ras* mutations was limited to the codon 12 and 13 hotspot mutations in exon 1 of *KRAS* and *NRAS*, which may miss functional mutations at other loci such as codons 61 and 146. Also, we may have missed subclonal *Ras* mutations that would not have been easily detectable by Sanger sequencing. Therefore, we have likely underestimated the full extent of *Ras* mutations in our samples, which could affect the accuracy of our analyses. Furthermore, mutations in other genes in the *Ras*/MAPK signaling pathway such as *FLT3* and *PTPN11* are common in ALL (21). These mutations could have similar environmental risk factors as *KRAS* and *NRAS* mutations. Thus, it is possible that tobacco smoke's overall effects were not discovered due to our lack of complete ascertainment of Ras/MAPK-

pathway positive cases. Another consideration is the fact that the rate-limiting factor in our detection of *Ras* mutations is genetic selection rather than cause of the mutation. Thus, *Ras* mutation may be a common event induced by tobacco carcinogens, but we are only able to detect those mutations that are genetically selected in the context of other complementary mutations in the leukemia cell. It is possible that tobacco affects other pathways instead, which remain unmeasured in this study.

In conclusion, this study suggests that although prior evidence points to tobacco smoke as a possible risk factor for childhood ALL, it may affect the risk of ALL through leukemogenic events other than *Ras* gene mutations. The observation of ethnic differences in the development of *Ras* positive childhood ALL warrants further research on whether the *Ras*/MAPK pathway is involved in the higher rate of leukemia among Latinos.

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Table 1

Sociodemographic characteristics of subjects from the case-case analysis, California Childhood Leukemia Study (1995–2013)

Characteristics	**Total *n* = 670 *n* (%)**	***Ras*+ *n* = 122 *n* (%) [%][a]**	***Ras*− *n* = 548 *n* (%) [%][a]**	**Test statistic**	***p***
Race/ethnicity				$\chi^2 = 6.89$	0.03
Latino	339 (50.6)	68 (55.7) [20.1]	271 (49.5) [79.9]		
Non-Latino white	213 (31.8)	27 (22.1) [12.7]	186 (33.9) [87.3]		
Non-Latino other	118 (17.6)	27 (22.1) [22.9]	91 (16.6) [77.1]		
Sex				$\chi^2 = 0.23$	0.64
Male	377 (56.3)	71 (58.2) [18.8]	306 (55.8) [81.2]		
Female	293 (43.7)	51 (41.8) [17.4]	242 (44.2) [82.6]		
Annual household income (USD)				$\chi^2 = 2.71$	0.74
<15,000	114 (17.0)	19 (15.6) [16.7]	95 (17.3) [83.3]		
15,000–29,999	129 (19.3)	23 (18.9) [17.8]	106 (19.3) [82.2]		
30,000–44,999	100 (14.9)	23 (18.9) [23.0]	77 (14.1) [77.0]		
45,000–59,999	104 (15.5)	21 (17.2) [20.2]	83 (15.1) [79.8]		
60,000–74,999	46 (6.9)	8 (6.6) [17.4]	38 (6.9) [82.6]		
75,000+	177 (26.4)	28 (22.9) [15.8]	149 (27.2) [84.2]		
Child's age at diagnosis, mean ± SD	670	5.3 ± 3.1	5.7 ± 3.5	$t = 1.21$	0.23
Maternal age at birth, mean ± SD[b]	666	28.2 ± 5.6	28.4 ± 6.3	$t = 0.37$	0.71
Paternal age, at birth mean ± SD[b]	642	30.6 ± 6.5	31.0 ± 7.3	$t = 0.55$	0.58

SD, standard deviation, Ras^+, Ras positive, Ras^-, Ras negative.

[a]Numbers within square brackets refer to % of each category relative to total number of *Ras* + or *Ras* − cases.

[b]Maternal and paternal age were missing 14 and 28 observations, respectively.

Table 2

Race/ethnicity, major cytogenetic subtypes, and *Ras* mutation in childhood ALL, California Childhood Leukemia Study, 1995–2013

Variables[a]	*Ras*+	*Ras*−	OR	95% CI	*p*
Race/ethnicity					
Non-Latino white	24/107	173/492	1 (ref)		
Latino	60/107	233/492	2.04	(1.19, 3.49)	0.009
Non-Latino other	23/107	86/492	2.34	(1.21, 4.53)	0.01
High-hyperdiploidy	62/107	121/492	4.25	(2.63, 6.86)	<0.001
TEL-AML1	13/107	124/492	0.82	(0.42, 1.62)	0.57

[a] All variables were adjusted in the same model.

$n = 599$ because of missing cytogenetic data for some cases.

Table 3

Tobacco smoke exposure and *Ras* mutation in childhood ALL, California Childhood Leukemia Study, 1995–2013

Exposures (yes/no)[a]	*n*[b]	*Ras*+	*Ras*−	OR	95% CI
Paternal ever	616	53/114	199/502	1.34	(0.89, 2.03)
Maternal ever	665	23/120	140/545	0.74	(0.45, 1.23)
Child passive	656	24/119	120/537	0.89	(0.54, 1.46)
Paternal preconception	614	25/113	134/501	0.78	(0.48, 1.27)
Maternal preconception	665	15/120	72/545	0.99	(0.54, 1.80)
Maternal pregnancy	665	8/120	52/545	0.72	(0.33, 1.57)
Paternal preconception (CPD)	597			0.94	(0.81, 1.10)
Maternal preconception (CPD)	664			1.02	(0.82, 1.26)
Maternal pregnancy (CPD)	663			0.83	(0.49, 1.40)

CPD, cigarettes per day.

[a] All smoking exposures were analyzed separately and adjusted for race/ethnicity.

[b] Each analysis had different sample sizes due to varying degrees of missing data among smoking exposures.