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Increased incidence and disparity of diagnosis of retinoblastoma patients in Guatemala

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ABSTRACT

Analysis of 327 consecutive cases at a pediatric referral hospital of Guatemala reveals that retinoblastoma accounts for 9.4% of all cancers and the estimated incidence is 7.0 cases/million children, higher than the United States or Europe. The number of familial cases is low, and there is a striking disparity in indigenous children due to late diagnosis, advanced disease, rapid progression and elevated mortality. Nine germline mutations in 18 patients were found; two known and five new mutations. Hypermethylation of *RB1* was identified in 13% of the tumors. An early diagnosis program could identify cases at an earlier age and improve outcome of retinoblastoma in this diverse population.

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Introduction

Retinoblastoma (RB: OMIM + 180200) is the most common pediatric ophthalmological cancer, and represents a significant proportion of pediatric cancers in several developing countries [5,11,13]. Retinoblastoma, is typically diagnosed before age five, and exists in inherited and sporadic forms. Inherited RB accounts for 40% of cases and results from dominantly inherited germline mutations in *RB1*, is associated with bilateral disease, and early onset. Sporadic disease presents with unilateral tumors, with somatic alterations in both *RB1* gene alleles, and no family history [8]. Nevertheless, 10–15% of hereditary cases exhibit a unilateral pattern and cannot be distinguished from the sporadic form without molecular studies.

The high heterogeneity underlying *RB1* inactivation (over 2750 known mutations) makes molecular testing of RB a challenge (http://rb1-lovd.d-lohmann.de/). And in fact, gross deletion or duplication, promoter methylation of the *RB1* gene and *MYCN* amplification without *RB1* mutation have been identified in RB tumors [2,4,7,10,14,16,17]. The aim of the current study was to understand the incidence of retinoblastoma in Guatemala and the nature of the *RB1* mutations in patients with this intraocular tumor.

Materials and methods

Subjects

We examined consecutive medical records from 2000 to 2012 in the cancer registry of the major pediatric oncology hospital, Unidad Nacional de Oncología Pediátrica (UNOP) in Guatemala City. UNOP is the only dedicated pediatric oncology hospital in the country, care is free-of-charge, and transportation, housing and nutritional assistance are also provided. All retinoblastoma cases diagnosed in ophthalmology clinic and hospitals refer to UNOP. UNOP specialists have access to laser and cryotherapy, localized radiotherapy, imaging (RetCam) and telemedicine contact with an international team of experts (Orbis and cure4Kids.com). All

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patients are documented in an electronic registry supported in part by the International Outreach Program of St. Jude Children's Research Hospital (Memphis, USA). Therefore, we estimate that over 90% of diagnosed cases of retinoblastoma in the country are entered in the registry. The Guatemala City region encompassed 20% of the pediatric population of the country, is expected to have a very low rate of undiagnosed retinoblastoma, and was therefore used for incidence estimation.

The study was conducted with the approval of the ethic and research committee of UNOP, the NIH Office of Human Research Studies and Stanford University. Patients (with parental consent for minors) were consented and enrolled by trained investigators in small groups. Nearly all indigenous parents of patients speak and understand Spanish, and Spanish–Mayan interpreters are available when required. We have documented approximately 5% of adults (including indigenous adults) refusing to participate, indicating that comprehension of the voluntary nature of the study is achieved. All identifying information remains in the cancer registry and all samples are coded to maintain privacy. Clinical and genetic counseling is provided by staff oncologists, as needed.

Patients and families used for genetic analysis

To identify the spectrum of germline *RB1* mutations in patients from Guatemala we included blood or saliva DNA from 18 cases and their parents. The germline DNAs were identified as part of an ongoing collection of cases and family members initiated in 2009 [6]. For epigenetic analysis 18 formalin-fixed, paraffin-embedded (FFPE) tumor specimens were available stored samples from patients that have undergone enucleation surgery. There is no overlap between the germline and tumor DNA samples. Families were self-identified as being either indigenous or admixed and checked against cancer registry data on languages spoken by parents and grandparents and in the household.

Incidence estimation

Incidence of retinoblastoma and acute lymphocytic leukemia (ALL), as a comparison group, were estimated by calculating observed cases/million children under the age of 14. There were 327 RB and 1264 ALL cases from the same time period for comparison. In total, 409 of the ALL cases were from the Guatemala capital region (estimated ALL incidence = 33.5 (34.2 in admixed and 28.7 in indigenous)). Guatemalan census data (http://www.ine.gob.gt/np/poblacion/index.htm) was used for the numerator and for age-correction. To determine estimated incidences for admixed and indigenous populations by region of Guatemala, the estimated percentage of the indigenous population for each department (22 political subdivisions) from census data was used. Age correction with US population figures was calculated as described (http://seer.cancer.gov/seerstat/tutorials/aarates/step1.html).

Occupational exposures and outcome

Father's occupation was available in the registry for 219 of the 327 retinoblastoma cases and 912 ALL cases. Agriculture was the only occupation frequent enough for analysis and the data for the two groups were compared by Chi square statistics. Outcome was assessed by last known status of the patient, stage of disease at presentation, as well as by survival statistics. Last-known status of indigenous and admixed cases was tabulated both with and without elimination of cases lost to follow-up, abandonment of therapy or transfer to another hospital. Both comparisons showed significantly higher mortality in indigenous patients. Stage at presentation (St. Jude's Staging) was also tabulated for both groups. For survival analysis, date of diagnosis was used as a baseline and death as the outcome. Analysis was performed in STATA (StataCorp, College Station, TX).

DNA extraction and sequencing

Genomic DNA from saliva (DNA Genotek Inc., Ontario, CA.) and tumor DNA (QIAamp DNA FFPE Tissue Kit, Qiagen, CA, USA) was extracted according the manufacturer's instructions. The entire *RB1* gene was sequenced using an ABI PRISM 3130XL (Applied Biosystems, Foster City, CA, USA) and sequence data was analyzed using Mutation Surveyor V9.1 software (Soft Genetics, State College, PA). Exon 15 was analyzed by using TOPO TA cloning® and sequencing of 10 clones from each patient. Mutations were named according to the genomic *RB1* sequence with GenBank reference (L11910) and the *RB1* mutation database LOVD2 (http://rb1-lovd.d-lohmann.de/home.php).

Molecular analysis included 18 genomic DNA and 18 tumors samples from 36 unrelated Guatemalan patients with retinoblastoma, 6 (17%) with bilateral and 32 (83%) with unilateral tumors. No bilateral and only one unilateral case had a family history of retinoblastoma. Mean age of RB onset was 33 months among all cases (bilateral: 32 and unilateral: 33 months). Notably, only one (17%) bilateral patient was diagnosed at <1 year old. To assess the DNA methylation status of the *RB1* promoter, tumor DNA was analyzed and bisulfite analysis was performed [Zymo EZ ADN Methylation-Gold™ (Zymo Research; Irvine, CA)] with gene-specific primers (Supplementary Table 1). Three tumors did not yield adequate DNA quantity for analysis. Mutations were compared with data in the *RB1* mutation database, and newly described missense variants analyzed by SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html).

Results

Retinoblastoma in Guatemala: clinical characteristics and estimated incidence

To determine the frequency of retinoblastoma in Guatemala, 327 consecutive RB patients diagnosed from the UNOP cancer registry from 2000 to 2012 were examined. Retinoblastoma accounted for 9.4% of all cancer cases during this period; and is the most common solid tumor. The estimated incidence of RB in the Guatemalan capital department is 7.0 cases/million children under the age of 14 (6.7 cases/million, age-adjusted) (Table 1).

Approximately 40% of Guatemalans are indigenous from one of 22 different, mostly Mayan, ethnic groups [3], while the remainder of the population is admixed (European, Amerindian and to a lesser extent, African). In the Department of Guatemala, the crude incidence of retinoblastoma in admixed and indigenous populations is similar, 7.0 and 6.8, respectively. However, in departments where there were enough cases of each ethnicity to compare, the apparent incidence in indigenous children was consistently lower (Table 1), particularly in regions far from the capital, probably reflecting lack of diagnosis or referral. We performed the same analysis with 741 consecutive cases of acute lymphoblastic leukemia (ALL), the most common cancer. The incidence of ALL under age 14 is 34/million in the Guatemala capital region, similar to Caucasian children in the US and UK (36–38/million) [http://info.cancerresearchuk.org/cancerstats/types/leukaemia/incidence/#trends] [9]. However, both RB and ALL show an apparent incidence much lower in the rest of the country, consistent with a significant under-ascertainment of rural and/or indigenous cases.

The cases studied were 51% female, 31% indigenous, and 24% had bilateral disease (Table 2). As expected, bilateral retinoblastoma cases are diagnosed at an earlier age and unilateral cases show an older age distribution. Indigenous unilateral cases show the oldest age of onset, and only 17% of indigenous unilateral cases are diagnosed by the age of 2 compared to 35% for admixed children and 63% in the US (Table 2, data not shown).

An elevated incidence of retinoblastoma could be due to (1) an increased number of familial cases due to a common founder mutation(s); (2) a unique environmental factor(s) contributing to disease; or (3) an epigenetic or unique molecular mechanism of disease such as *MYCN* amplification reported in some non-*RB1* mutated tumors [17]. We determined that only 8 of the cases derived from familial retinoblastoma out of the 327 cases, a frequency lower than typically reported, ruling out explanation 1 above.

To begin to understand environmental factors that may play a role in retinoblastoma we examined the father's occupation (Table 2). There is an association with agriculture in the children with retinoblastoma; 50% of retinoblastoma cases have a father engaged in farming compared to 37% of the ALL cases ($p = 0.019$). Detailed analysis of the environment of cases and controls would be needed to further explore an environmental etiology.

Molecular characteristics of selected tumors

To determine if the molecular etiology of the disease is similar to other countries, the *RB1* gene was sequenced and nine germline oncogenic variations were detected in eight out of 18 patients (44%; Table 3). Bilateral patients (1/2, 50%) and unilateral patients (7/16, 44%) both displayed mutations, and one of the unilateral cases with an identified mutation has familial disease. Three mutations