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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.

Keywords

RB1 gene; mutations; methylation; Guatemala; ethnicity; health disparity

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Conflicts of Interest

The authors have no conflicts of interest regarding this manuscript to report.

1. 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 to 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.

2. Materials and methods

2.1 Subjects

We examined consecutive medical records from 2000–2012 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 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.

2.2 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.

2.3 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 sub-divisions) from census data was used. Age correction with US population figures was calculated as described (http://seer.cancer.gov/seerstat/tutorials/aarates/step1.html)

2.4 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).

2.5 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).

3. Results

3.1 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 2000–2012 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

3.2 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 were insertion/deletions (p.Ala17ProfsX3, p.Glu19ThrfsX10 and p.Phe198PhefsX4 and four were single base substitutions (p.Ser648X, c.607+1G>C, c.2326-2A>G). The only bilateral mutation carrier harbored a nonsense mutation (p.Ser648X) and was diagnosed at 2.6 years of age and the mutation was confirmed in the patient's parent, who had RB diagnosed at 23 years of age. Because *RB1* can be affected by larger in/dels MLPA was used on a total of 18 patients to identify germline copy number variants. However, no gains and losses were detected (data not shown).

We assessed the somatic methylation of the *RB1* promoter by using methylation-specific PCR in 15 RB tissue samples; and identified two hypermethylation events (2/15; 13%). Both patients with methylated *RB1* were of admixed ethnicity and diagnosed as unilateral RB. Thirteen tumor samples (13/15; 87%) did not exhibit gene hypermethylation (Table 4).

3.3 Disease stage and progression by ethnicity

To support our hypothesis that the lower apparent incidence of rural indigenous retinoblastoma cases is due to late or absent diagnosis, we compared the mortality and stage of diagnosis of indigenous and admixed cases. We found a significantly higher mortality of indigenous cases (65 vs. 40%, P= 0.00011; Table 2). Survival analysis from diagnosis demonstrated a highly significant difference [Hazard Ratio 2.66 (1.86–3.82) P = 2.45×10-8] for indigenous cases (Figure 1). Both indigenous unilateral and bilateral cases show a significantly increased mortality (Table 2). This disparity is reflected in the stage of diagnosis with significantly more indigenous cases being diagnosed at stage II, III, and IV (P=0.00045). Survival of indigenous and admixed cases, adjusted for stage of diagnosis was not significantly different, demonstrating that care after diagnosis is equivalent (Supplemental Figure 1).

4. Discussion

This work presents the first comprehensive study of retinoblastoma in Guatemala. As in several low and middle income countries, such as Mexico (22%), Nigeria (20%) and India (13%); retinoblastoma accounts for a large percentage of pediatric cancer cases. In

Guatemala retinoblastoma is 9.4% of all cancers, more than double that in high income countries (3–5%) [5, 9, 15, 18]. Because only 8 (2.4%) of the 327 cases had a sibling with RB, familial cases do not account for the elevated incidence in Guatemala. We also did not see gross epigenetic changes (only 13% of 15 tumors have a methylated *RB1* gene), and find typical germline mutations, indicating that the molecular basis of the disease is not grossly different. But we do find that a significantly larger percentage of retinoblastoma cases have a father employed in agriculture, suggesting that there may be environmental/dietary factors for the disease. There is evidence for a larger percentage of undiagnosed ALL cases than RB, contributing to the higher relative fraction of patients diagnosed with RB.

Access to healthcare screening may confound our case ascertainment. Moreover, low income level, education status and limited accessibility to medical services in Guatemala likely influence our findings. While we believe that the majority of diagnosed retinoblastoma cases in the Guatemala capital region are included in the hospital's cancer registry, there is a need for a nationwide cancer registry to improve studies of cancer incidence.

Economic disadvantage, a rural location and membership in minority and/or indigenous populations are all associated with under-diagnosis, and poor treatment outcomes for pediatric cancers [13]; and our data from Guatemala support this. There is a clear trend of earlier ascertainment of urban and non-indigenous cases as compared to rural and isolated regions. This disparity is supported by a highly significant increase in death as an outcome for indigenous RB cases, that holds for both unilateral and bilateral disease. In addition, indigenous children with RB present at a much higher rate with stage II or greater disease, and more often require palliative care. Undoubtedly language barriers, access to health care, transportation and communication barriers contribute to this disparity. However, once diagnosed and treated at UNOP, indigenous children have similar stage-corrected survival (Supplemental Figure 1). To begin to address late diagnosis, a pilot program with posters in Spanish and three major Mayan languages has been initiated and resulted in earlier diagnosis of at least two cases (data not shown, Supplemental Figure 2).

The pattern and the nature of mutations are very similar to those reported in other populations. The main mutation type are frameshifts (43%), followed by splicing and non-sense (29%) alterations, and 67% of all of them were newly described mutations [1, 12]. Typically, nonsense and frameshift mutations lead to bilateral tumors because of the complete absence of the RB protein, some splicing mutations are associated with incomplete penetrance and milder expressivity due to residual function of RB1.

By using the SIFT program, the rare exonic variants we described were not predicted to be pathogenic. However, the role of the synonymous variants in the etiology of RB require further evaluation because there is evidence showing that certain synonymous SNPs involving frequent-to-rare codon substitutions may result in ribosome stalling, due to either a lower concentration of cognate tRNAs or an alteration of the RNA structure [19].

Taken together, the high incidence, late onset, high percentage of sporadic retinoblastoma and low mutation detection rate in the *RB1* gene of Guatemalan retinoblastoma patients

supports the hypothesis that other genes or environmental factors may contribute to the disease. Although amplification of *MYCN* gene has been described in some tumors, at least in North American and European patients non-*RB1* mechanisms account for less than 3% of retinoblastoma [17].

In conclusion, we document an elevated incidence of retinoblastoma in Guatemala, but provide evidence of under-diagnosis in rural and indigenous groups suggesting that the actual incidence may be higher. We identified sequence alterations, but no gross insertions or deletions of the *RB1* gene, as mechanisms of germline inactivation. Because of the apparent elevated RB incidence, the involvement of other molecular mechanisms, viruses or lifestyle in retinoblastoma etiology in Guatemala needs to be further evaluated in larger cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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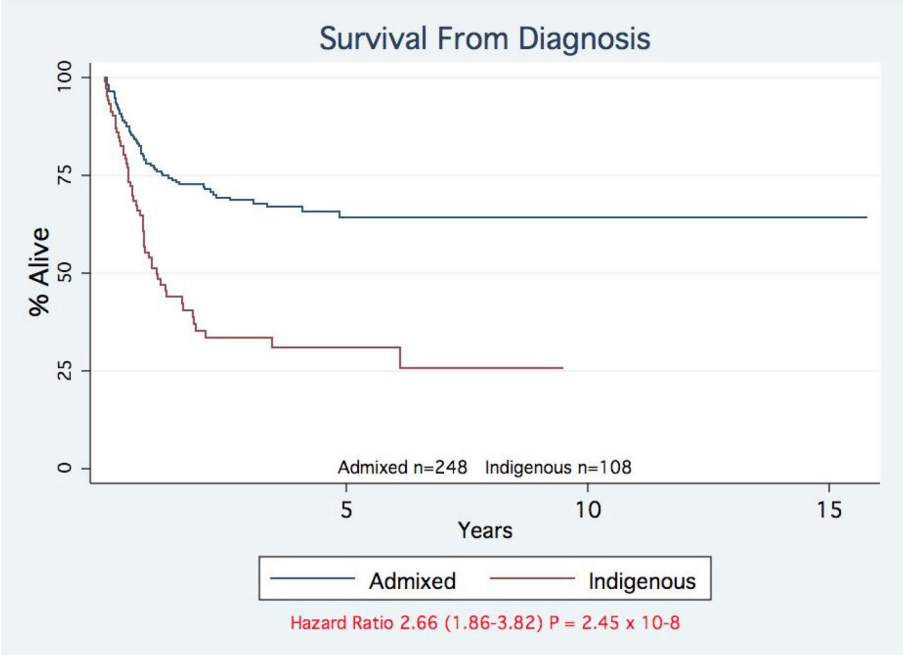

Figure 1. Survival of Indigenous and admixed patients with retinoblastoma

The survival of patients with retinoblastoma is shown by ethnicity. Admixed children (blue line); indigenous children (red line). Hazard Ratio 2.66 (1.86–3.82) P = 2.45×10^{-8}.

Table 1

Estimated Incidence of retinoblastoma and ALL in Guatemala from 2000–2012

Department	Cases	Admixed	Indigenous	Population <14	Estimated Incidence	Adm. Inc.	Ind. Inc.
ALTA VERAPAZ	14	2	12	496,634	2.4	-	2.3
BAJA VERAPAZ	10	6	4	118,154	7.1	9.6	-
CHIMALTENANGO	19	8	11	263,098	6.0	11.2	4.5
CHIQUIMULA	7	6	1	157,963	3.7	4.5	-
EL PROGRESO	4	4	0	60,615	-	-	-
El QUICHE	41	13	28	443,546	7.7	14.4	6.3
ESCUINTLA	6	5	1	254,221	2.0	1.8	-
GUATEMALA	85	75	10	1,016,936	**7.0**	7.0	6.8
HUEHUETENANGO	26	18	8	493,222	4.4	8.5	2.1
IZABAL	9	7	2	170,073	4.4	4.5	-
JALAPA	10	10	0	141,725	5.3	10.8	-
JUTIAPA	11	11	0	184,761	4.5	4.8	-
PETEN	9	9	0	273,323	2.7	3.7	-
QUETZALTENANGO	18	10	8	311,118	4.8	6.7	3.6
RETALHULEU	7	4	3	118,895	4.9	-	-
SACATEPEQUEZ	6	4	2	117,327	4.3	-	-
SAN MARCOS	14	12	2	422,176	2.8	4.2	-
SANTA ROSA	8	8	0	139,814	4.8	4.9	-
SOLOLA	6	4	2	187,357	2.7	-	-
SUCHITEPEQUEZ	10	7	3	210,668	4.0	6.4	-
TOTONICAPAN	8	3	5	208,296	3.2	-	2.1
ZACAPA	1	1	0	86,737	-	-	-
Total RB	**327**	**225**	**102**	**5,876,659**	**4.6**	**5.7**	**3.3**
Total ALL	**1264**	**914**	**350**	**5,876,659**	**17.9**	**23.1**	**11.3**

Adm. Inc. = Estimated admixed incidence; Ind. Inc.=Estimated indigenous incidence, Values only calculated for cells with 5 or more entries.

Table 2

Demographics and survival data of Study Subjects

		All subjects N (%)	Indigenous N (%)	Admixed N (%)	*P* value
Total		356 (100)	102 (31)	225 (69)	
Laterality	Unilateral	239 (76)	74 (73)	165 (78)	0.54
	Bilateral	75 (24)	28 (27)	47 (22)	
Gender	Feminine	168 (51)	57 (56)	111 (49)	0.27
	Masculine	159 (49)	45 (44)	114 (51)	
Age of Diagnosis (yrs)		2.8	3.2	2.3	
	Unilateral	3.1	3.5	3.1	0.85
	Bilateral	1.8	2.3	1.6	
		RB cases	**ALL cases**		
Occupation of Father	Agriculture	110 (50)	335 (37)		0.02
	Data on occupation	219	912		
Survival Data[a]	**Alive**	**Deceased**	**% Deceased**		
Indigenous	29	55	65%		0.00011
Admixed	103	68	40%		
Unilateral	101	84	45%		
Bilateral	29	29	50%		
Indigenous Unilateral	25	36	59%		0.0091
Admixed Unilateral	76	48	39%		
Indigenous Bilateral	4	18	82%		0.00015
Admixed Bilateral	25	11	31%		
Stage Global SJ	Admixed	Indigenous			
I	66	17			
II	16	15			
III	14	13			
IV	4	3			
	I	II, III, IV			
Admixed	66	34			0.00045
Indigenous	17	31			

[a] Last known status, after elimination of cases that have abandoned therapy

Table 3

Mutations identified in RB patients

Patient	Exon	DNA position (GRCh37)	Mutation	Consequence	Reference#
AP701348	Exon 1	g. 48878093	c.45_79 del TGCCGCCGCGGAACCCCC GGCACCGCCGCCGC/-	p.A15X p.A16fs*3	COSM29200
AP700763	Exon 1	g. 48878103	C.55_57delGA	p.E19X*	
AP700069	Exon 6	g. 48923151	c.600_601insT TTA >TTT	p.L200fs	
AP700815	Intron 6	g. 48923159	c.607+1 G>T	Splicing	
AP700815	Exon 15	g. 48954199	c.1420 A>G AGC>GGC	p.S474G	
AP700826	Exon 17	g.48955529	c.1646 A>T CAT>CTT	p. H549L	
AP700603	Exon 19	g. 49030468*	c.1942 C>A TCA>TAA	p. S648X	
AP700759	Exon 19	g. 49030457	C.1931 C>A TCT>TAT	P.S644Y	
AP700480	Exon 25	g.49050889	c.2272 G/A GTG> GAG	p.V858M	

*de novo mutation;

#Catalogue of somatic mutations in cancer (COSMIC)

Cancer Lett. Author manuscript; available in PMC 2015 August 28.

Table 4

Methylation changes in RB Tumors.

Samples Id	Ethnicity	Phenotype	% Methylation
EO-07-2584	Admixed	Unilateral	0.04
EO-10-2778	Admixed	Unilateral	0.43
2475	Admixed	Bilateral	0.05
3304	Admixed	Bilateral	0.06
EO-07-1624	Admixed	Unilateral	0.01
EO-07-3700	Indigenous	Unilateral	0.03
EO-08-2510	Admixed	Unilateral	0.02
EO-10-2108	**Admixed**	**Unilateral**	**81.51**
EO-08-447	Admixed	Unilateral	0.3
1042	Admixed	Unilateral	0.4
EO-07-1518	Admixed	Bilateral	0.2
EO-08-576	Admixed	Unilateral	Undetermined
EO-08-1413	**Admixed**	**Unilateral**	**37.7**
EO-07-2550	Indigenous	Unilateral	0.5
3340	Admixed	Bilateral	Undetermined
EO-07-2769	Admixed	Unilateral	0.3
EO-07-3639	Admixed	Unilateral	0.1
EO-10-2917	Admixed	Unilateral	Undetermined

Bold character: Hypermethylation > 25%