



ORIGINAL ARTICLE

TP53 p.Arg337His geographic distribution correlates with adrenocortical tumor occurrence

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Abstract

Background: The p.Arg337His mutation of the *TP53* is the most frequent germline missense variant associated with cancer described so far in this gene. It is mainly found in the South and Southeastern regions of Brazil, where it has been associated with a high incidence of pediatric adrenocortical (ACT) and choroid plexus tumors. The frequency and geographic distribution of this mutation is largely unknown, except for the Parana State, where a mean prevalence of 0.27% was reported. In the present study, we developed a high-throughput method for p.Arg337His genotyping, what allowed us to determine the frequency and geographic distribution of this mutation in a cohort from the most populous state in Brazil.

Methods: Consecutive samples from 31,612 newborns from São Paulo State were screened for p.Arg337His. The allelic discrimination was done by real-time polymerase chain reaction (PCR) and the presence of haplotype A3 in carriers was examined by using allele-specific oligonucleotide PCR, followed by nested-PCR to detect the SNP rs9894946.

Results: We found 67 (0.21%) samples positive for this mutation. The highest p.Arg337His frequencies were found in the cities close to the boundary between São Paulo and Minas Gerais State. No association could be found between p.Arg337His and gender, ethnicity, premature birth or twinning. Remarkably, a trend was found between the geographic distribution of p.Arg337His carriers and occurrence of ACT.

Conclusion: We presented for the first time the p.Arg337His frequency among individuals unselected for any disease from a subset of the São Paulo State, the most populous in Brazil. The allele discrimination assay we presented here has proven to be a reliable and efficient method for high-throughput genotyping. ACT was found to be a good sentinel cancer to suppose p.Arg337His presence in our region.

KEYWORDS

genetic counseling, high-throughput genotyping, p.Arg337His, pediatric oncology, *TP53*

Ana Luiza Seidinger and Isabel Pereira Caminha should be considered joint first author.

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1 | INTRODUCTION

The tumor suppressor gene *TP53* (tumor protein p53, OMIM 191170) has a fundamental role in cell cycle regulation, apoptosis, and DNA repair (Lane, 1992). Germline mutations in this gene are usually associated with Li-Fraumeni Syndrome (LFS), an inherited disorder characterized by predisposition to a broad spectrum of tumors. Individuals affected by LFS are usually diagnosed with cancer during childhood and early adulthood and present a very high cumulative risk for developing cancer throughout their life (Guha & Malkin, 2017).

The p.Arg337His mutation of the *TP53* (NG_017013.2:g.21852G>A) is known to contribute to the development of several types of cancer, such as adrenocortical tumors (ACT), choroid plexus carcinoma, osteosarcoma, breast cancer, and neuroblastoma (Assumpção et al., 2008; Seidinger et al., 2010; Seidinger, et al., 2015). Brazil is especially affected by this mutation. Prevalence studies performed in the Southern region have found p.Arg337His in ~0.3% of the healthy population (Custódio et al., 2013; Palmero et al., 2008). This frequency is 300 times higher than any single *TP53* mutation associated with LFS (Garritano et al., 2010). Genotype analysis based on a series of SNPs loci near in or around the *TP53* revealed that p.Arg337His carriers share the same relatively rare haplotype, raising the hypothesis of a founder effect (Garritano et al., 2010).

Although different tumors have been linked to the p.Arg337His, ACT presents the most striking association with the mutation. More than 90% of Brazilian patients diagnosed with ACT are carriers of this missense variant, a finding that explained the reason why Brazil has the highest incidence in the world for this type of tumor (Ribeiro et al., 2001).

The geographic distribution of the p.Arg337His mutation in Brazil is not known. Cities/regions with the highest incidence of the mutation would be first candidates for a cancer surveillance program. The recent p.Arg337His screening performed in the state of Paraná revealed an uneven geographic distribution of the mutation, and the observed prevalence ranged from 0.075% to 0.51% (Custódio et al., 2013). There is currently no similar study in the four other states of Brazil where the mutation is thought to occur. Here we report on the frequency and geographic distribution of the p.Arg337His in a subset of São Paulo State, the most populous state of Brazil. Findings from this region may have an impact over millions of people at risk. In addition, first evidence on the geographic superposition of this mutation and pediatric adrenocortical cancer is presented.

2 | METHODS

2.1 | Ethical compliance

The study was approved by the Ethics Research Committee of the Faculty of Medical Sciences at the State University

of Campinas (CAAE: 1026.0.146.000-11), which waived the signature of Informed Consent, given the research was designed to be retrospective and conducted anonymously.

2.2 | Cohort sampling

The samples studied in this work consisted of surplus blood samples directly spotted on the "Guthrie card," collected for routine testing of hemoglobinopathies within the National Program for Newborn Screening, conducted by our institution. We selected samples from the Regional Health Board VII (RHB-VII), which comprises 42 cities and represents a subset of São Paulo State.

In the year 2005 (year selected for the sample collection), the population of RHB-VII was 3,707,627 individuals. The calculated sample size for an estimated frequency of 0.2%, absolute precision of 0.05%, and confidence level of 95% would be 30,671.

Demographic data (gender, twinning, ethnicity, and city of residence) were collected anonymously from the Guthrie cards.

2.3 | High-throughput genotyping method development

After the routine testing for hemoglobinopathies, 31,612 samples consecutively collected from January to September of 2005 were analyzed for the p.Arg337His mutation.

By using an automatic puncher, a disk of 3 mm in diameter was removed from each Guthrie card. The DNA extraction method was adapted from Sepp, Szabo, Uda, & Sakamoto, 1994, by using the resin Chelex[®] 100 (Bio-Rad Laboratories, Inc). Initially, the blood disks were rehydrated with 200 µL of deionized water and maintained at constant shaking. After that, the supernatant was discarded and 200 µL of 5% (in water) Chelex[®] 100 was added. The plates were sealed and incubated at 56°C for 90 min followed by 10 min of incubation at 99°C. The supernatant obtained was used as a template for real-time PCR. Both, DNA extraction and PCR were performed as a pool, with three different samples per well.

DNA extracted with Chelex[®] 100 was quantified by using the Qubit[®] 2.0 Fluorometer (Invitrogen, Life Technologies, Thermo Fisher Scientific Inc) and revealed concentrations ranging from 0.1 to 2.5 ng/µl DNA. Serial dilutions of DNA from p.Arg337His carriers were performed in water to assess the sensitivity of the method. By doing this, we confirmed that even samples with DNA at low concentrations were amplified successfully (Figure 1a).

The allelic discrimination by real-time polymerase chain reaction (PCR) consisted of amplification of exon 10 from the *TP53*(NG_017013.2), using primers flanking the region of the p.Arg337His mutation: (CCTCCTCTGTTGCTGCAGATC)

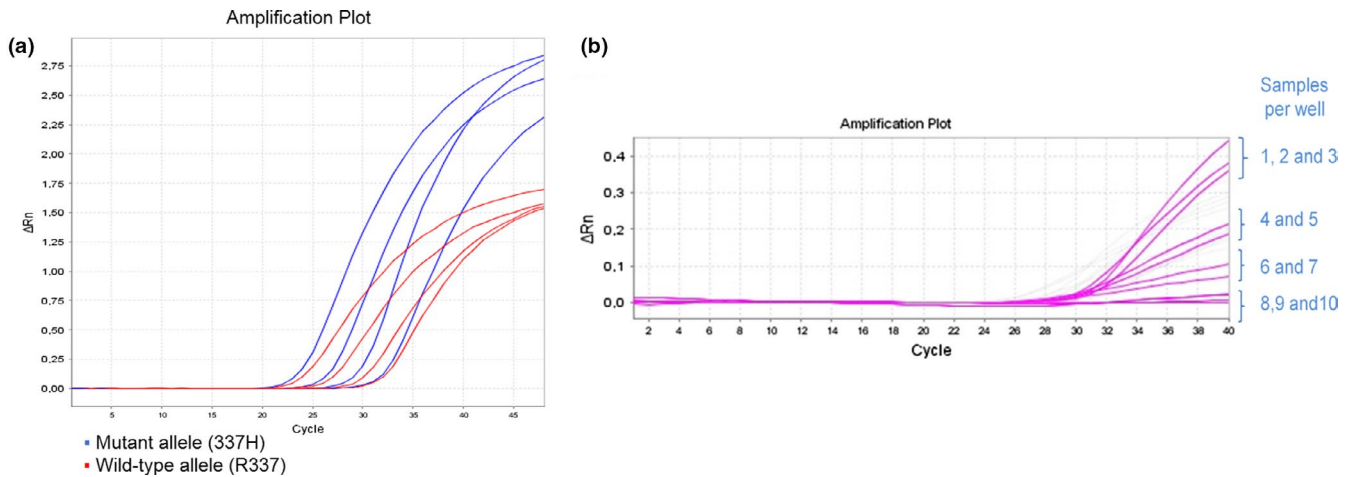


FIGURE 1 Standardization of a high-throughput method for p.Arg337His genotyping. (a) Allelic discrimination of DNA serial dilutions from a heterozygous p.Arg337His carrier. Allelic discrimination of p.Arg337His was conducted with DNA concentrations ranging from 50 to 0.19 ng/ul in water. The blue curve refers to the mutant allele, while the red curve refers to the wild-type allele. From left to right the curves represent the amplification of both alleles in a sample with 50, 6.25, 0.78, and 0.19 ng of DNA, respectively. Even at low DNA concentrations the amplification of both alleles succeeded. (b) RQ-PCR reaction containing different numbers of pooled samples. The wells contain from 1 to 10 samples, and each well always contain one sample heterozygous for p.Arg337His mutation. The chart suppresses the amplification of the wild-type allele for better visualization of results. The best results were obtained in wells with up three samples. From four samples on, the sensitivity drops considerably, and with 8–10 samples the reaction does not occur, probably due to excess inhibitors. PCR, polymerase chain reaction

and (CCTCATTGAGATCTCTCGGAAC). In the same reaction, we included two Minor Groove Binder TaqMan probes: one of them to identify the wild-type allele (CGTGAGCGCTTCGAG) and the other to recognize the mutated allele (CGTGAGCACTTCGAG). Reactions were carried out in a 12- μ L reaction volume, containing 2 μ L of DNA extracted from Guthrie card. The remainder of the reaction consisted of 6 μ L of 2x TaqMan[®] Universal PCR Master Mix (Life Technologies, Thermo Fisher Scientific Inc), 0.2 μ M of probes, 0.9 μ M of primers, and 3.85 μ L of deionized H₂O. Preheating of the mixture at 95°C for 10 min was followed by 40 cycles of a two-step cycling (15 s at 95°C and 1 min at 60°C) using an Applied Biosystems 7500 fast Real-Time PCR System. The identification of a mutation was achieved by the amplification of the mutant allele. Since a probe for both alleles was included in the reaction, we expect to find at least one amplification curve from each reaction.

In order to verify the reliability of testing pooled samples, reactions containing 1–10 disks per well were performed. Analyses indicated that the best results were obtained in reactions with three pooled samples. The sensitivity in detecting the p.Arg337His allele decreased, but was still possible, when attempting to find a mutant among four or five samples per well. At six samples per well the result was untrustworthy and, with eight or more samples, the amplification did not occur, most likely due to excess inhibitors (Figure 1b).

Once a mutation was found, the pool was split, so the newborn carrying p.Arg337His could be identified. To perform

quality control and to calculate the failure percentage of this approach, 10% of all pooled samples were also run in separate replicate reactions, as singlets; a total of 3,325 samples were reanalyzed. Of these, six were positive for the p.Arg337His allele both by pooled and individual analysis. However, 0.42% of samples in the individual analysis render no amplification product. In our experience, PCR failures were likely due Chelex[®] 100 contamination when pipetting DNA from the extraction to the PCR reaction plate.

2.4 | Founder haplotype

The presence of haplotype A3 was examined in p.Arg337His positive samples by using allele-specific oligonucleotide PCR, followed by nested-PCR to detect the SNP rs9894946, as previously described by Garritano et al., 2010. Furthermore, all cases were genotyped by direct sequencing of SNPs rs1642785 and rs1800370 to distinguish between the haplotypes A1 and A3, as described by Giacomazzi, Selistre, Duarte, et al., 2013.

2.5 | Geographic distribution of p.Arg337His and ACT cases

The plotting of p.Arg337His carriers on Brazilian map was done by using the software ArcGis v10.1. After observe that the distribution of p.Arg337His carriers was uneven and to better explore the distribution pattern of carriers along our region, we conducted statistical analysis to detect potential deviations from

the expected random occurrence of p.Arg337His. For this purpose and considering that cities with small population presented a nonrepresentative sample size, we estimated 7,668 samples as the minimum number of samples to be compared in each group, considering a frequency of 0.2% and absolute precision of 0.1%, at the confidence level of 95%. For that reason, we merged cities into four distinct groups, according to proximity, in order to sum at least 7,668 samples analyzed in each group.

Since ACT is strongly associated with p.Arg337His in our region (Seidinger et al., 2010), we matched the geographic occurrence of p.Arg337His mutation within the RHB-VII with that of ACT. The data from ACT patients were obtained from an updated series previously published by Seidinger et al., 2010 ($n = 36$). Fisher's exact test was conducted to detect differences in both p.Arg337His frequency and ACT cases between the regions.

3 | RESULTS

3.1 | Prevalence of p.Arg337His in RHB-VII

In the year 2005, 53,936 births were recorded in RHB-VII. Among these, 31,612 samples were collected and analyzed, representing almost 60% of newborns (Table S1). Among the 31,612 samples analyzed, 67 (0.21%, 95% CI: 0.16, 0.26%) were heterozygous for p.Arg337His, corresponding to the estimated prevalence of this mutation in the region analyzed. No association could be found between p.Arg337His and gender, ethnicity, premature birth or twinning (Table 1). Haplotyping of mutation carriers with available DNA sample revealed the presence of haplotype A3 in all p.Arg337His carriers tested ($n = 63$).

The mutation was found in 26 of 42 cities analyzed (Table S1). After analyzing 31,612 samples, we noticed that some cities had a p.Arg337His frequency much higher than the overall average. However, these cities presented small populations and, therefore, few births per month, resulting in skewed results because a positive newborn found among so few samples leads to a high mutation frequency.

Therefore, we conducted a survey of additional samples in cities with p.Arg337His frequency higher than $\geq 0.64\%$, in order to double-check the frequency of the mutation in these cities. Originally, newborn samples were collected between January and September of 2005; this additional survey included samples from the remainder of the year 2005, in addition to births recorded in the year 2006. The cities reanalyzed were the following: Águas de Lindóia (p.Arg337His frequency: 0.64%); Cabreúva (0.75%); Joanópolis (0.89%); Nazaré Paulista (0.68%); Socorro (1.03%); Tuiuti (2.43%); and Vargem (4.76%).

After collecting a larger set of samples, we obtained approximately 90% of live births in the 2005–2006 period,

TABLE 1 Frequency distribution of the birth variables according to p.Arg337His status

	p.Arg337His(-)		p.Arg337His(+)		p-value
	N	%	N	%	
Gender					
Male	15,972	51%	36	54%	.71 ^a
Female	15,404	49%	31	46%	
Without information	169	1%	—	—	
Ethnicity					
White	23,710	75%	53	79%	.63 ^b
Black	756	2%	1	1%	
Brown	5,491	17%	11	16%	
Yellow	98	0%	0	0%	
Without information	1,490	5%	2	3%	
Blood transfusion					
Yes	76	0.2%	0	0.0%	.99 ^a
No	31,158	98.8%	66	98.5%	
Without information	311	1.0%	1	1.5%	
Premature birth					
<37 weeks	1821	6%	3	4.5%	.99 ^a
≥ 37 weeks	29,362	93%	63	94%	
Without information	362	1%	1	1.5%	
Twinning					
Yes	608	2%	0	0%	.64 ^a
No	30,937	98%	67	100%	

^aFisher's exact test.

^bChi-square for trend.

considerably increasing the confidence of the results (Table S1). At the end of this additional survey, 2,214 samples were collected from the six cities of interest, among which seven were carriers of the mutation. The updated results were the following: Águas de Lindóia (p.Arg337His frequency: 0.43%); Cabreúva (0.34%); Joanópolis (0.33%); Nazaré Paulista (0.56%); Socorro (0.7%); Tuiuti (1.92%); and Vargem (1.47%). Although the frequency of p.Arg337His has decreased for all of the cities reanalyzed, the concluding frequency remained higher than the overall average for the region (Table S1). The data obtained after this second survey were intended to be used only for adjustment of local frequency and were not incorporated in the final frequency of p.Arg337His. Once the second survey was not random, the inclusion of data enriched from regions with high p.Arg337His frequency could certainly imply in mutation frequency bias.

3.2 | Geographic distribution of p.Arg337His carriers correlates with ACT occurrence in Brazil

The place of residence at the time-of-collection from the 74 p.Arg337His carriers was plotted onto a map of São Paulo State. Remarkably, the geographic distribution of p.Arg337His was not even, and cities close to the border state of Minas Gerais (northeast region in the map) presented two to three times higher incidence of the mutation than the mean (Figure 2).

The cities analyzed were then merged into four subgroups by proximity (Figure 3a), in order to sum a minimum of 7,668 samples analyzed per sub region, which corresponds to the estimated sample size for a frequency of $0.2 \pm 0.1\%$ at 95% confidence level (Table S2). Occurrence of p.Arg337His carriers was significantly higher in subgroup 4 (0.39%, 95% CI: 0.25, 0.53%) corresponding to cities on the eastern side of the map (Figure 3a,b). Nine of the 10 top cities regarding p.Arg337His frequency belong to subgroup 4. It is worth mentioning that the frequency of p.Arg337His was higher than 0.5% in most of the top 10 cities and higher than 1% in two of them (Table 2).

We then analyzed to what extent the place of residence of ACT patients positive for the p.Arg337His treated at our institution overlapped with the geographic distribution found among p.Arg337His newborns. Remarkably, positive

p.Arg337His children with ACT (updated series previously published by Seidinger et al., 2010) were more frequently found in the subgroup 4 of cities (Figure 3c), which is the subgroup with the highest frequency of newborns positive for the p.Arg337His mutation.

4 | DISCUSSION

We presented for the first time the p.Arg337His frequency among individuals unselected for any disease from a subset of the São Paulo State, the most populous in Brazil. The observed frequency after studying 31,612 individuals (0.21%, 95% CI: 0.16, 0.26%) is in accordance (Fisher's exact test $p = .07$) with previous studies conducted in the Southern region states of Parana (0.3%) and Santa Catarina (0.25%) (Costa et al., 2019). The identification of haplotype A3 in 63 unrelated carriers corroborates the hypothesis of a common ancestor for p.Arg337His in our country (Garritano et al., 2010; Giacomazzi, Correia, et al., 2014; Paskulin et al., 2015; Pinto et al., 2004).

The allele discrimination assay we presented here has proven to be a reliable and efficient method for high-throughput genotyping. The method currently employed to analyze the p.Arg337His mutation has been PCR followed by restriction fragment length polymorphism

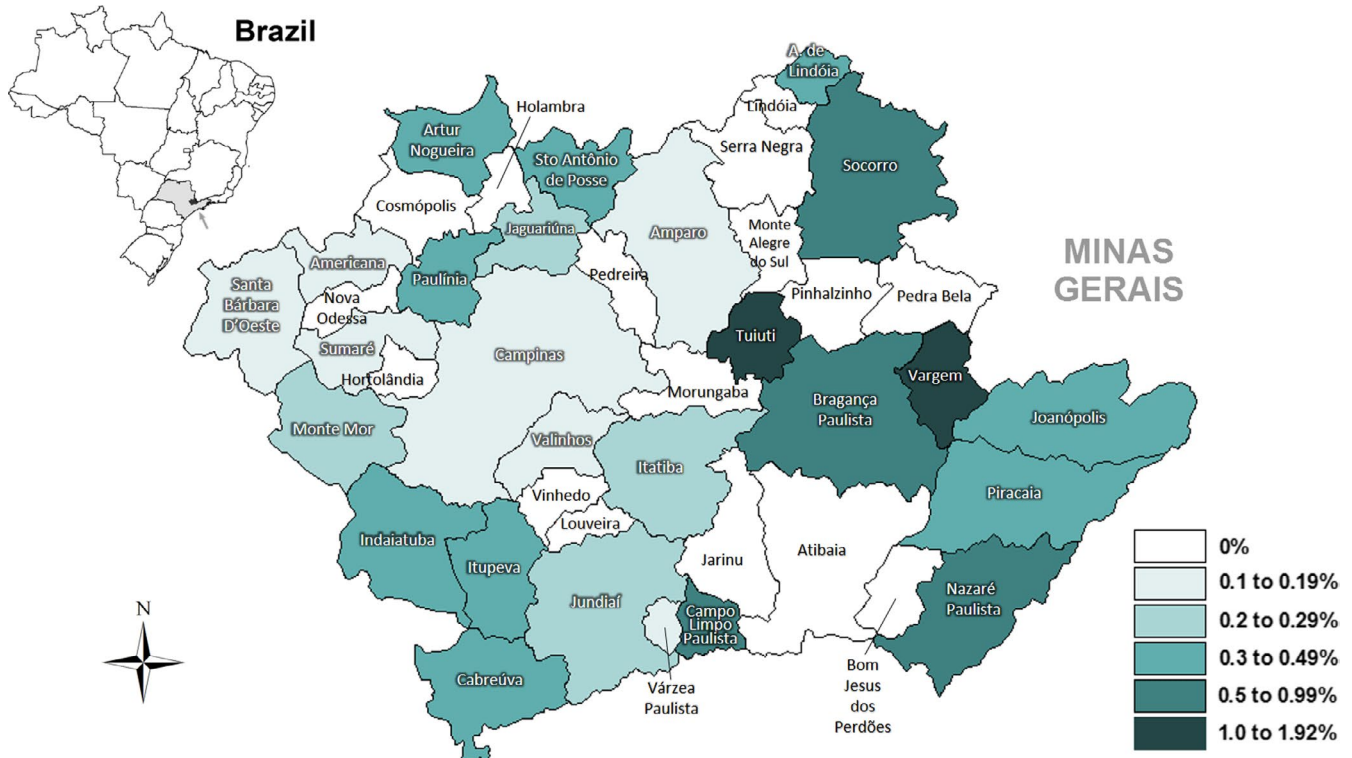


FIGURE 2 Frequency (%) of newborn carriers of the *TP53* p.Arg337His germline mutation in 42 cities from the São Paulo State's VII Regional Health Board (RHB VII). The São Paulo State is represented in gray color and RHB-VII region is indicated by an arrow in Brazilian map at the left. The zoomed RHB-VII region was depicted in gradient colors representing the range of estimated mutation frequency in each city. The absolute frequency for each city is detailed in Table S1

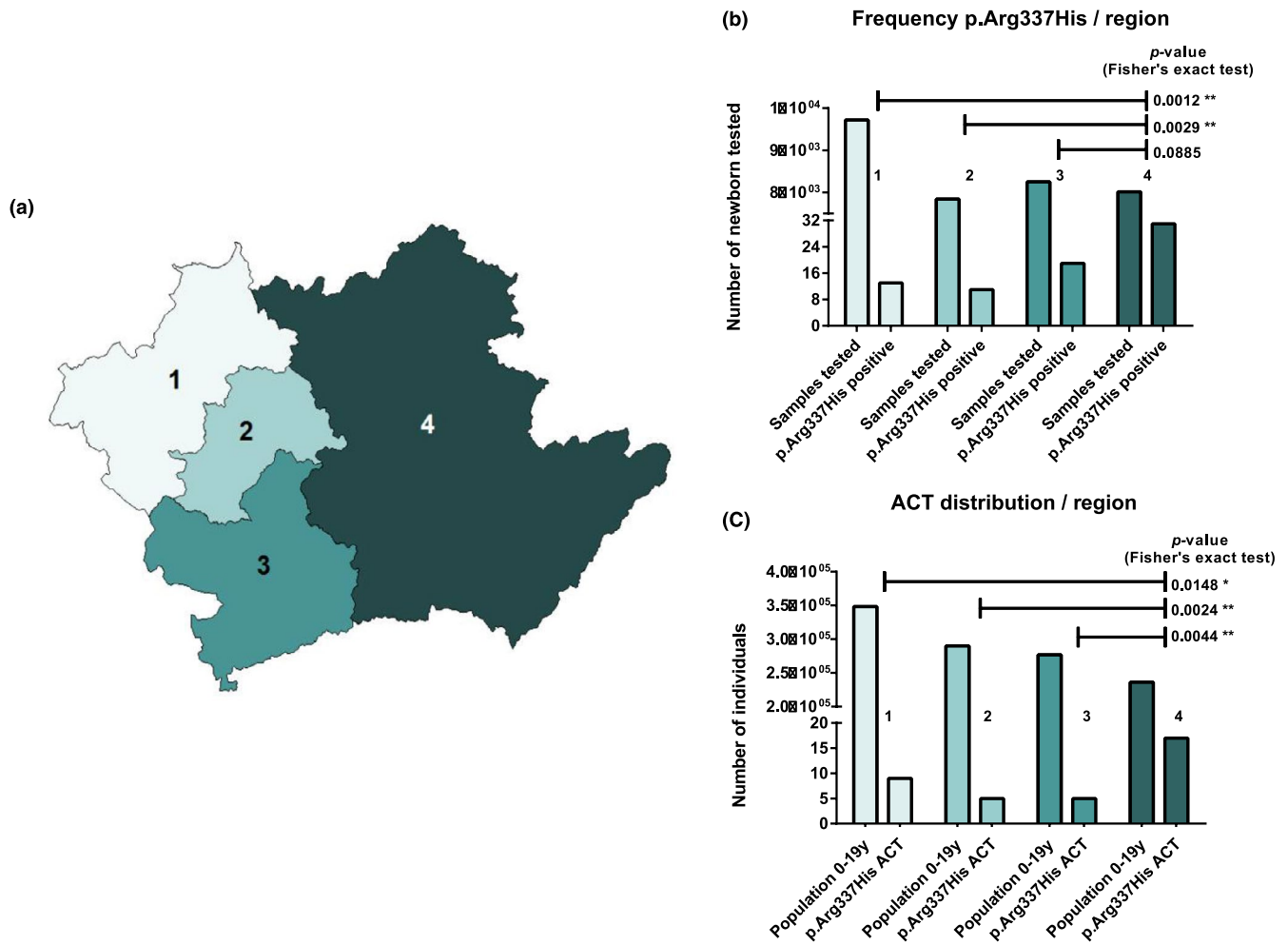


FIGURE 3 Uneven geographic distribution of the p.Arg337His mutation and its coincidence with pediatric cancer. (a) Cities were merged considering proximity in order to sum at least 7,668 samples analyzed. Four subgroups were generated. (b) Distribution of p.Arg337His carriers along the four subgroups. Left bars represent the total number of samples tested, whereas the right one refers to the number of p.Arg337His carriers. Chi-square statistical analysis shows a higher mutation frequency in region 4. (c) Distribution of pediatric ACT patients carriers of p.Arg337His treated at our institution along the four subgroups. As a reference, left bars represent the population 0–19 years inside each subgroup, accordingly to the 2010 Brazilian census, while the right ones refer to the number of ACT patients. Chi-square test showed higher incidence of cancer cases in region 4. ACT, adrenocortical tumors

analysis (PCR-RFLP) of an *Hha*I site abolished by the p.Arg337His mutation (Ribeiro et al., 2001). However, analysis by restriction enzyme requires several post-PCR reaction steps, including digestion and electrophoresis of the digested amplicon. This technique is efficient and relatively simple to use in laboratory routine, but for large-scale analysis, the cost and the numerous steps make it unfeasible. The method presented here exempts post-PCR reaction steps, requiring less time and at least four times lower cost per sample (USD 0.29 in reagents and plastics) than the PCR-RFLP method commonly employed in p.Arg337His genotyping (USD 1.20 per sample).

Geographic distribution of the p.Arg337His mutation was found to be uneven in the tested region. The cities close to the border of the Minas Gerais State were found to have exceptionally high p.Arg337His frequency among the analyzed

newborns. We have no explanation for this uneven distribution, though it may be related to lower population migration rates or a potential diffusion center for p.Arg337His. Importantly, considering the uneven distribution of the mutation, the 0.21% prevalence of this variant cannot be extrapolated to the whole Sao Paulo State.

Adrenocortical tumors was found to be a good sentinel cancer to suppose p.Arg337His presence in our region. We observed a pronounced correlation between the occurrence of this tumor and p.Arg337His mutation, a finding that reinforces the predominant role of genetic predisposition over environmental risk factors on ACT tumorigenesis. In addition, this correlation gave us a clue to the investigation on the putative diffusion center of p.Arg337His. The spread of an allele, in a relatively homogeneous environment, results in a concentric distribution, with the highest gene frequency at the diffusion center, which is

TABLE 2 Top 10 cities with high frequency of the *TP53* p.Arg337His germline mutation within the São Paulo State's VII Regional Health Board (RHB VII)

City	Cluster region ^a	Analyzed samples	Live	p.Arg337His carriers	
			births analyzed	N	%
Tuiuti	4	104	90%	2	1.92
Vargem	4	136	89%	2	1.47
Socorro	4	853	94%	6	0.70
Campo Limpo Paulista	4	634	54%	4	0.63
Bragança Paulista	4	1,171	55%	7	0.60
Nazaré Paulista	4	359	89%	2	0.56
Santo Antônio de Posse	4	204	74%	1	0.49
Águas de Lindóia	4	465	81%	2	0.43
Paulínia	1	694	60%	3	0.43
Piracaia	4	232	61%	1	0.43

Abbreviation: N, number.

^aAs shown in Figure 2.

presumably the geographic origin of the mutation. The same is true for an allele brought in by a single immigrant or by a small group (Cavalli-Sforza & Bodmer, 1971). Among the newborns included in the present study, we observed a high p.Arg337His frequency near the border of Minas Gerais State, which may suggest a proximity with the p.Arg337His diffusion center. ACT clusterization in that region corroborates this hypothesis. Therefore, we propose the analysis of nationwide ACT distribution might definitely elucidate the diffusion center of p.Arg337His mutation in Brazil.

The diagnosis of childhood ACT is frequently delayed. ACT is a rare disease and, moreover, the patient usually does not seem to be sick because hormonal changes due tumor expansion lead to an accelerated growth rate and development (Michalkiewicz et al., 2004). Tumor size at diagnosis is highly correlated with prognosis and given that ACT has a poor response to cytotoxic treatment, total surgical resection is essential for the ultimate control of the disease (Bugg et al., 1994; Cagle et al., 1986; Fassnacht et al., 2012; Michalkiewicz et al., 1997; Ribeiro et al., 1990; Weiss, Medeiros, & Vickery, 1989; Wieneke, Thompson, & Heffess, 2003; Zancanella et al., 2006). A prospective study conducted in the Brazilian state of Paraná found that p.Arg337His children enrolled in an ACT surveillance program had earlier diagnosis and better prognosis than those who did not adhere to the cancer surveillance program (Custódio et al., 2013). One cost-effective option for improve ACT rate cures is the education of pediatricians and families to early diagnosis of ACT in p.Arg337His carriers, as demonstrated recently by Costa et al., 2019. Besides education, is important to consider offering genetic testing as well genetic and psychological counseling to relatives from the carrier side. Periodic exams should be done according to physician management of symptoms

related to cancer manifestations. Additionally, considering the increased susceptibility of p.Arg337His carriers to multiple cancers, both during childhood (Custódio et al., 2013; Giacomazzi, Selistre, Rossi, et al., 2013; Seidinger et al., 2010; Seidinger, et al., 2015) and adulthood (Achatz et al., 2007; Assumpção et al., 2008; Cury, Ferraz, & Silva, 2014; Giacomazzi, Graudenz, et al., 2014; Mastellaro et al., 2017), there would be a further increase in the number of people who could benefit from early detection of the p.Arg337His mutation.

Newborn screening of the p.Arg337His mutation has the potential for improving early diagnosis and prognosis of ACT patients (Custódio et al., 2013). Analysis of the geographic distribution of the mutation, as presented here, will indicate the cities deserve more attention for an eventual screening program. Although such a screening program may contribute to alerting family members of an increased susceptibility to cancers, it would also expose the majority of the carriers to an unnecessary psychological burden. For genetic counseling purposes, it remains to be determined the full spectrum of cancer associated with p.Arg337His mutation, both in childhood and adulthood and, perhaps most importantly, the psychological consequences of knowing it beforehand.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors have a patent application related to this study to declare: JAY, IPC, ALS and CSG are inventors of the application BR 10 2012 031182 8 A2, submitted on December 7th, 2012 to Brazilian National Institute of Industrial Property (Instituto Nacional da Propriedade Industrial - INPI), covering the high-throughput method for p.Arg337His genotyping, which is fully described in the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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