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ARTICLE

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Parental age and retinoblastoma—a retrospective study of demographic data and genetic analysis

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LEARNING OBJECTIVES

Upon completion of this activity, participants will be able to:

1. Assess the proportion of sporadic vs heritable retinoblastoma and the association between parental age gap and risk for retinoblastoma development in offspring, based on a retrospective study in India.
2. Evaluate the associations between maternal age and paternal age with risk for retinoblastoma development in offspring, based on a retrospective study in India.
3. Determine the clinical implications of the associations between parental age gap, maternal age, and paternal age and risk for retinoblastoma development in offspring, based on a retrospective study in India

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ABSTRACT

OBJECTIVE: To determine the association between the parental age gap and the absolute parental age with the risk of retinoblastoma (RB) development in an offspring.

METHODS: RB individuals diagnosed between March 2013 and December 2019 in a single tertiary eye care centre were included. We recorded the demographic data, parental age and *RB1* gene mutation status in the patient's tumour, blood and the parental blood. We categorised *RB1* mutation inheritance as sporadic RB with somatic mutations (only present in tumour), heritable RB with de novo (present in patient's blood) and familial (present in patient and parents' blood) germline mutations. The statistical significance was confirmed by Fisher's exact/Chi-square test.

RESULTS: Out of 259 RB patients, 247 were included in our study. Heritable RB with de novo germline mutations was significantly less common (p value: 0.0387; 95% CI: 0.2676–0.9329) and sporadic RB with somatic mutations was more common (p value: 0.0545; 95% CI: 1.025–3.39), if the parental age gap was <10 years. There were increased odds of a heritable RB with de novo germline mutation with an increase in paternal age and this was more intensified when combined with parental age gap of more than ≥10 years. The heritable RB with de novo germline mutations significantly increased as maternal age progressed, only when it was adjusted to ≥10 years parental age gap (p value: 0.0262; 95% CI: 1.26–17.91).

CONCLUSIONS: An increased parental age gap and increased paternal age are independent risk factors for the development of heritable RB with de novo germline mutation.

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INTRODUCTION

Retinoblastoma (RB), the most common paediatric intraocular malignancy, has been reported to occur 1 in 15,000–20,000 live births [1]. It usually occurs in children before the age of 2 years [2]. The tumour arises as a result of mutation affecting both alleles of *RB1* gene at chromosome 13q14. Depending on the pattern of inheritance, two types of RBs have been defined: Heritable and Sporadic RB. Heritable RB is caused by germline mutation (first hit) that occurs pre-zygotically and mostly present as bilateral disease, while in sporadic cases, both the mutations (first and second hit) are somatic and occur post-zygotically [3].

About 90% of cases, carrying a germline mutation in *RB1* gene, have been known to develop RB during their early childhood [4, 5]. The germline mutation can either be inherited as in familial RB or occur de novo. Familial RB has an autosomal dominant inheritance with one of the parents affected [6]. When it occurs de novo, phenotype is seen only in child but not in parents. It is well known that the number of new mutations in germ cells increases with age [7] and that advanced paternal age is associated with a number of congenital syndromes, including a number of cancers like RB [8–10]. It has also been observed that a paternal transmission will occur in populations in which older men frequently marry much younger women. However, an increasing maternal contribution is to be expected when women conceive at 35 years of age or older [11]. We conducted this study to determine the association of paternal age, maternal age and difference in parental age with an increased risk of RB in offspring. To date this is the first study that has been conducted in the Indian population looking specifically at the age difference between parents and its possible impact in RB.

METHODS

This was a single-centre, retrospective study that adhered to the tenets of the declaration of Helsinki. An ethical clearance was obtained from the Institutional review board (Ethical approval no: IRB2016017BAS) before undertaking a review of medical records. The medical records of all patients who had been diagnosed with RB, between the 1st of March 2013 and the 31st of December 2019 were evaluated.

All the records were critically evaluated for the completeness of data and records with missing entries were excluded. Moreover, if a patient and/or the parent(s) had not undergone genetic analysis from the requisite tissue (at least a blood sample from both the patient and the parent(s) and the tumour tissue from the patient, if available) at our centre or if the *RB1* gene mutations that were tested for (deletions, duplications and promotor methylation of *RB1* gene) were absent, he/she was excluded from the study. Thereafter, all the 'tiered' consent forms for genetic analysis at the time of collection of tissue specimens were evaluated and the medical records of patients, whose legal representatives/guardians had declined the use of this genetic data for future research, were excluded. None of the participants had reached an age of 18 years at the time of data collection for this study and so no attempt was made to re-trace them or obtain a re-consent (Supplementary Fig. 1).

All study subjects and their parents underwent genetic testing for identification of mutations and for the diagnoses of germline or somatic mutations. Blood samples were drawn for all the cases included in the study. Tumour samples, whenever available (following enucleation) were analysed for mutations. The *RB1* gene was analysed for point mutations, indels and frameshift mutations using Sanger sequencing and for large deletions using Multiplex Ligation-dependent Probe Amplification to conclude whether the case was a somatic mutation or a germline mutation. If no mutation was found in the patient's blood, the case was grouped under RB due to somatic mutation and labelled as sporadic RB. If the mutation was present in the patient's blood, with or without tumour tissue being available for analysis, then he/she was defined as a case of heritable RB with germline mutation. If the mutation was present in the parent's blood, then the case was defined as Familial RB. If the mutation was present in the patient's blood but not in the parent's blood, then the case was labelled as a de novo germline RB.

The study population was divided into groups depending upon the maternal age, paternal age and parental age gap at the time of birth of the patient. The groups based on maternal age were of 5-year difference (<20 years, 21–25 years, 26–30 years, >30 years). Similarly, for paternal age the groups were made (<25 years, 26–30 years, 31–35 years, 36–40 years, >40 years). Based on the parental age difference the cases were divided into <10 years or ≥10 years difference in parental ages for statistical analysis.

All the statistical analyses were performed in GraphPad Prism 7.03 (GraphPad Software Inc., USA). Mean age of parents was compared among mutational groups using unpaired student t test or Mann–Whitney U test. Bivariate analysis for independent variables of parental age and mutational group was performed using Fisher's exact test/Chi-square test to calculate p value and Odds ratio. A p value < 0.05 and 95% confidential intervals that exclude 1 were considered as the statistically significant.

RESULTS

A total of 259 patients were diagnosed with RB in the study period. None of the records had to be excluded for missing or incomplete entries. A genetic analysis was not done in five cases

Table 1. Age difference of parents with mutational inheritance pattern.

RB1 mutation inheritance pattern	Age difference between parents		p value	Odds ratio (95% CI)
	<10 years ($n = 194$), *N* (%)	≥10 years ($n = 53$), *N* (%)		
De novo germline ($n = 69$)	48 (25)	21 (40)	0.0387	0.501 (0.2676–0.9329)
Sporadic ($n = 155$)	128 (66)	27 (51)	0.0545	1.868 (1.025–3.39)
Familial ($n = 23$)	18 (9)	5 (9)	>0.9999	0.9818 (0.3459–2.52)
Heritable RB ($n = 92$)	66 (34)	26 (49)	0.0545	0.5355 (0.295–0.9754)

N Frequency, % Percentage.

Table 2. Mean age of parents with mutational inheritance pattern.

	RB1 mutation inheritance pattern				p value			
	De novo germline ($n = 69$)	Sporadic ($n = 155$)	Familial ($n = 23$)	Heritable RB ($n = 92$)	De novo germline vs. sporadic	Sporadic vs. familial	De novo germline vs. familial	Sporadic vs. heritable RB
Mean parental age ± SD	28.8 ± 5.7	28 ± 5.4	28.7 ± 6.7	28.8 ± 5.9	0.1112	0.8	0.8957	0.2641
Mean paternal age ± SD	32.2 ± 4.8	30.9 ± 4.8	31 ± 7.1	31.9 ± 5.4	0.0286	0.963	0.4383	0.0682
Mean maternal age ± SD	25.3 ± 4.2	25.0 ± 4.3	26.3 ± 5.5	25.6 ± 4.5	0.5931	0.0006	0.4525	0.336

SD Standard deviation, *RB* retinoblastoma, Age is in months.

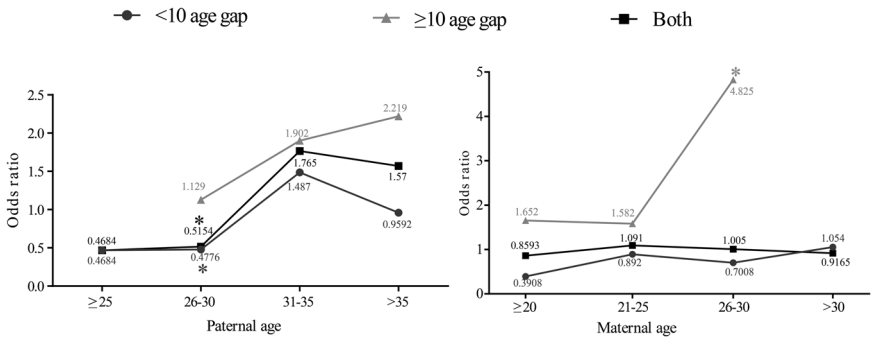

Fig. 1 De novo germline vs. sporadic *RB1* mutation in parental age groups. Increased parental age adjusted to higher age gap between parents had shown more significance as compared to increased parental age alone. Asterisks represent the odds ratio with a significant *p* value and a 95% confidential interval. Missing of few values in ≥10 year group is due to 0 or infinity value by absence of individual in one/two group of comparison.

and in seven cases the tested mutations of the *RB1* gene were absent. In all the remaining cases, the legal representatives agreed to the use of the genetic data for research. Therefore, the records of 247 patients were included in our study and their data analysed (Supplementary Fig. 1).

A total of 155 participants had sporadic RB and 92 had heritable RB. Supplementary Figure 2 shows the distribution of the study population according to the results of the genetic analysis. The study population was divided into two groups depending upon the parental age gap (<10 years, ≥10 years). We found heritable RB with de novo germline mutations (de novo germline RB) were higher in ≥10 years age gap group, whereas sporadic RB were higher in <10 years age gap group (*p* value = 0.0346) (Table 1).

The de novo germline RB group had a, statistically significant, higher mean paternal age than the sporadic RB group (*p* value = 0.0286) (Table 2). Upon dividing the patient's paternal age into 5-year age groups, we found increased odds of having a heritable RB with de novo germline mutation versus the odds of having sporadic RB as the paternal age increased (Fig. 1 and Supplementary Table 1).

The association between the parental age gap and the mutational inheritance pattern is shown in Table 1. We further analysed the mutation inheritance pattern with paternal age adjusted to age difference between parents. We found that the significance of de novo germline mutation rate in increased paternal age group was further intensified when accompanied by higher age difference between parents (≥10 years) [OR 2.219 (0.8749–5.379)] (Fig. 1 and Table 3). Thus, combining the two, we noted that both increased paternal age and an increased parental age gap (≥10 years) have independent impacts on the incidence of de novo germline RB. Thereby we confirmed our hypothesis that parental age gap and an increased paternal age have a significant role to play in the heritable RB with de novo germline mutations.

Analysis of maternal age revealed that the mean maternal age of sporadic RB was lesser than mean maternal age of familial RB with statistical significance (*p* value = 0.0006) but there was no statistical significance in comparison of the mean maternal age of the sporadic versus de novo germline RB (Table 2). We further analysed the mutation inheritance pattern with maternal age

Table 3. Mutation inheritance pattern with paternal age adjusted to age difference between parents.

RB1 mutation Inheritance	<10-year age difference								≥10-year age difference							
	≤25		26–30		31–35		>35		≤25		26–30		31–35		>35	
	p value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)
De novo germline vs. sporadic	0.2275	**0.4684 (0.1665–1.315)**	**0.0301**	**0.4776 (0.254–0.9345)**	0.2574	**1.487 (0.797–2.803)**	>0.9999	0.9592 (0.3581–2.447)	–	–	>0.9999	**1.129 (0.3018–4.279)**	0.1944	**1.902 (0.7176–5.276)**	**0.0875**	**2.219 (0.8749–5.379)**
Sporadic vs. familial	>0.9999	1.38 (0.3344–6.36)	0.2402	**2.094 (0.7328–5.363)**	0.6022	**1.597 (0.5513–4.547)**	**0.0084**	**0.227 (0.081990.6018)**	–	–	0.276	0.4228 (0.097032.182)	– >0.9999	1.517 (0.2306–17.19)	0.6768	0.8021 (0.1993–3.839)
De novo germline vs. familial	0.6375	0.6462 (0.1426–3.604)	>0.9999	1 (0.3459–2.782)	0.1902	2.375 (0.7197–6.995)	0.016	0.2177 **(0.066520.7248)**	–	–	0.5957	0.4773 (0.093232.855)	0.4414	2.885 (0.4579–33.35)	0.7234	1.78 (0.4125–8.615)
Sporadic vs. heritable RB	0.2667	1.883 (0.7432–4.763)	**0.0157**	**2.094 (1.148–3.823)**	0.5521	**0.8094 (0.4647–1.451)**	0.2913	**0.6034 (0.2782–1.34)**	–	–	0.5441	**0.7007 (0.1993–2.088)**	0.4593	**0.636 (0.2421–1.534)**	0.1728	**0.5093 (0.2246–1.182)**

RB1 mutation Inheritance	<10-year age difference				≥10-year age difference			
	36–40		>40		36–40		>40	
	p value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)
De novo germline vs. sporadic	0.7800	1.133 (0.4174–3.2)	>0.9999	0.5551 (0.04477–3.452)	**0.0966**	**2.41(0.8522-6.774)**	>0.9999	1.242 (0.216–6.201)
Sporadic vs. familial	**0.0288**	**0.2483 (0.0762–0.7268)**	0.1735	0.2781 (0.06224–1.549)	>0.9999	1.197 (0.1626–13.85)	0.4279	0.4342 (0.06277–5.876)
De novo germline vs. familial	0.1137	0.2813 (0.07873–1.021)	0.1531	0.1544 (0.01054–1.413)	0.4414	2.885 (0.4579–33.35)	>0.9999	0.6567 (0.074–9.914)
Sporadic vs. heritable RB	0.2348	0.5655 (0.2221–1.444)	0.7137	0.7859 (0.2073–3.179)	0.1966	0.5019 (0.1876–1.267)	0.6736	0.5855 (0.1347–2.556)

The values in bold highlight the increasing or decreasing trend of Odd's Ratio across the different categories.
OR odds ratio, *CI* confidence interval, *RB* retinoblastoma.

Table 4. Mutation inheritance pattern with maternal age adjusted to age difference between parents.

RB1 mutation Inheritance	<10-year age difference								≥10-year age difference							
	≤20		21–25		26–30		>30		≤20		21–25		26–30		>30	
	p value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)	*p* value	OR (CI)
De novo germline vs. sporadic	0.3534	0.3908 (0.08471–1.686)	0.7649	0.892 (0.502–1.588)	0.3281	0.7008 (0.3656–1.343)	>0.9999	1.054 (0.4117–2.743)	0.5208	**1.652 (0.5736–4.954)**	0.3578	**1.582 (0.6729–3.648)**	**0.0262**	**4.825 (1.26–17.91)**	>0.9999	0 (0–4.861)
Sporadic vs. familial	>0.9999	1.681 (0.2648–18.9)	0.1667	**2.153 (0.754–5.511)**	0.8093	**0.906 (0.3442–2.311)**	**0.0348**	**0.3036 (0.1013–0.9142)**	0.1233	0.3153 (0.0759–1.204)	>0.9999	2.357 (0.4048–25.97)	0.4279	0.4342 (0.06277–5.876)	>0.9999	Infinity (0.0678–infinity)
De novo germline vs. familial	>0.9999	0.6567 (0.074–9.914)	0.3059	1.92 (0.6402–5.159)	0.4085	0.6349 (0.2301–1.679)	0.1666	0.4065 (0.117–1.327)	0.4076	0.5208 (0.1198–2.121)	0.28	3.729 (0.5481–42.22)	0.6754	2.095 (0.3023–25.04)	–	–
Sporadic vs. heritable RB	0.2633	**2.266 (0.6713–7.748)**	0.4088	**1.299 (0.7638–2.199)**	0.4616	**1.261 (0.7124–2.264)**	0.4085	**0.7143 (0.3332–1.641)**	0.1597	0.4339 (0.156–1.438)	0.6688	**0.789 (0.3593–1.717)**	**0.0425**	**0.2397 (0.06638–0.8956)**	0.5306	Infinity (0.2746–infinity)

The values in bold highlight the increasing or decreasing trend of Odd's Ratio across the different categories.
OR odds ratio, *CI* confidence interval, *RB* retinoblastoma.

alone (Supplementary Table 2) and adjusted to age difference between parents and found significant association, only when maternal age adjusted to ≥10 parental age gap (Fig. 1 and Table 4), which shows that higher age gap between parents affects more impact in de novo germline RB than increased parental age.

DISCUSSION

RB1 is a tumour suppressor gene involved in cell cycle regulation. Malignant tumours occur in retinal cells having mutations in both copies of the *RB1* gene. Approximately 25–35% RB cases are bilateral, who inherit an *RB1* mutation, either in an autosomal dominant manner from a parent or due to de novo germline mutation. An estimated 10% of all RB cases can be attributed to a positive family history. But family history information and genetic testing has revealed that some unilateral cases (10–15%) involve a germline mutation in the *RB1* gene [12, 13]. In our study population we found 62.75% of cases were sporadic RB while 37.25% were heritable RB. Among the heritable RB, 75% were de novo germline RB.

Epidemiological studies of paediatric cancers done previously have evaluated several birth characteristics as postulated risk factors [14–16]. One of the suggested risk factors of childhood malignancies is parental age [17, 18]. Advanced maternal and paternal age have been associated with a number of congenital syndromes and cancers like RB [19].

There is no published literature on the possible association of parental age gap and RB. In our study population we noted that 21.5% of cases had a large parental age gap (≥10 years). This occurs as a result of increased prevalence of consanguinity in the local community, in the form of young women being married to their maternal uncles. We noted that even without a family history of RB a significant number ($n = 23$; 43.4%) of these cases exhibited clinical characteristics of germline RB (Bilateral RB, multifocal lesions). Hence, in order to explore this possible association between increased parental age gap and RB we collected relevant data (Parental ages and genetic analysis of mutations to assess inheritance pattern). What we found was that the risk of development of de novo germline mutation was more for patients whose parents had an age gap of more than 10 years [p value 0.0346; OR 2.074 (1.089–4.081)]. While age gap of <10 years was present in patients with sporadic RB [p value 0.0346; OR 0.4821 (0.245–0.9185)], implying that the development of sporadic RB occurs irrespective of age difference while de novo germline RB could occur in cases with a large (≥10 year) parental age gap.

In large epidemiological studies conducted for RB, the incidence rate ratio (IRR) was highest in maternal age group of more than 40 years (IRR 5 2.39; 95% CI 5 1.17–4.85), regardless of paternal age [20]. Germline mutations occur less frequently in oocytes (maybe because of the fact that oocytes undergo far fewer cell divisions during gametogenesis) in comparison to sperm. Mechanisms include age related differential gene expression in oocytes like promoter DNA methylation and de novo epimutations in oocyte genes. There are also a number of characteristics of the mother that have been suggested to be associated with childhood cancer risk and potentially confound the effect of parental age. Such factors include smoking in pregnancy, high birth weight, history of spontaneous abortions and poorer diet and alcohol in pregnancy [21–24]. Use of assisted reproductive technology, which increases with maternal age, has been postulated to elevate the cancer risk in offspring conceived by these means [25].

Data we collected showed that the major portion of our cases had younger mothers [$n = 218$ (88.26%) were <30 years of age] which could explain why there was no statistical significance in comparison of the mean maternal age of the sporadic versus de novo germline RB (Table 1) and also why upon studying the mutation inheritance pattern of maternal age groups the chance of somatic mutation decreased as age progress as compared to familial mutation [OR 2.522 (0.9451–6.566)]. Also, the mean maternal age in consanguineous marriages was lesser than non-consanguineous marriages, with p value approaching significance (p value 0.0503). This again consolidated on the evidence that lesser maternal age probably would not contribute to germline mutations, while the paternal age might have an independent role to play.

Frequencies of mutation in germ cells of father as well as frequencies of chromosomal aberrations during the maturation of maternal germ cells increase with age and thus may increase the chance of developing cancer in offspring [20]. Several autosomal dominant genetic conditions are known to be associated with advanced paternal age (generally considered to be 40 years of age or older) [26, 27]. It is a suspected risk factor for several autosomal dominant traits such as multiple endocrine neoplasia type 2B, neurofibromatosis and syndromic associations such as Marfan syndrome, Achondroplasia, and Apert syndrome [28, 29]. Paternal age has been suggested to have an effect with RB, albeit a weaker effect than the conditions mentioned above [8–10].

In published literature, the existence of an association of RB with parental age is controversial. Most of the available studies in this regard are based on a small sample size [25]. Previously conducted larger studies, detected associations of parents' age with RB, but led to different results: Pellié et al. [30] established a paternal age effect, Matsunaga et al. [31] suggested only an age effect for fathers older than 35 years, Moll et al. [32] established both paternal and maternal age effect, while DerKinderen et al. [11] and Johnson et al. [25] found a maternal age effect. Saremi et al. concluded that advanced maternal age can increase the risk of RB in offspring but the paternal age did not have a statistically significant effect on RB risk [22]. A study conducted in India observed that children of fathers who were smokers and had an advanced age (35 years), were more likely to be affected by cancers like RB. Very high levels of sperm 8-hydroxy 2-deoxyguanosine levels and DNA damage was noted in fathers of children who developed RB by the age of 2 years [33].

There are few mechanisms which have been postulated and studied to understand the development of de novo germline RB. Girardet et al. attempted to address the question of whether segregation distortion of mutant *RB1* alleles occurs before fertilisation by performing the sperm-typing technique based on single-cell PCR analysis. The said segregation distortion favouring the mutant *RB1* allele does not seem to occur during spermatogenesis. Thus, meiotic drive may result from various mechanisms, such as a fertilisation advantage or a better mobility in sperm bearing a mutant *RB1* gene or from the existence of a defectively imprinted gene located on the human X chromosome [34].

DNA investigations of some RB patients suggest that new germline mutations principally have paternal origin. It has been estimated that 85% of new *RB1* germline mutations are paternal in origin, therefore, it would be expected that older paternal age might be related to the appearance of heritable RB with de novo germline mutations [35]. We found an increase in odds ratio for cases of de novo germline RB with increase in paternal age although no statistically significant p value was obtained on comparison (Supplementary Table 1).

Upon analysing the effect of paternal age and increased parental age gap we found a statistically significant impact for the de novo germline mutation group (p value = 0.0346). Thus, highlighting the effect of increased paternal age on de novo germline mutations causing RB significantly, as none of our patients had the mother older than the father and majority of the mothers were <30 years of age (88.26%).

What was interesting to note was that data analysed regarding a large parental age gap (≥10 years) and de novo germline RB mutations, which has not been studied previously, showed a statistical significance (p value = 0.0346).

Our study does have a few limitations in terms of the study population having certain cultural characteristics, like consanguineous marriages that are more prevalent in the population studied, which allow us to study the effect of a large parental age gap, without a high maternal age, which might not be so elsewhere in the world. Thus, our findings might not be applicable to populations elsewhere. There are other risk factors, which might act as confounding factors in our study. We have not taken into consideration previously known risk factors like smoking or alcohol intake in antenatal period, high birth weight and history of abortions etc. As the study group having an increased parental age gap has relatively young mothers (<30 years) we plan to prospectively follow up this subset of patients with heritable RB having de novo germline mutations and evaluate their future sibling's antenatal, pre and postnatal environmental characteristics and phenotypical presentation to ascertain whether our hypothesis holds true while excluding as many other known risk factors as possible.

To conclude, an increased paternal age increases the risk of development of de novo germline mutations causing RB. Difference in parental age has a definite role to play in the pathogenesis of heritable RB with de novo germline mutations, which needs further evaluation about possible mechanisms of causation.

SUMMARY

What was known before

- The positive association of advanced paternal and maternal age with RB development was known.
- The association of increased parental age with disease predisposition is controversial as conflicting results reported from different studies.

What this study adds

- The correlation of parental age gap with the risk of RB development is measured in this study.
- This study also revisits the positive association of paternal and maternal age with the risk of tumour development in large set of RB patients from Indian population.

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