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Research Article

Germline Deletions in *RB1* Gene and in 13q14 Chromosomal Region in Retinoblastoma

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Abstract

Carriers of germline constitutional pathogenic mutations tend to develop bilateral retinoblastoma. Among carriers of germline mutations in *RB1* gene, about 14% of the probands carry gene or chromosomal deletions that include partial or complete loss of one allele of *RB1* gene. We have used MLPA technique to search for germline deletions in *RB1* and in five adjacent genes (*SUCLA2*, *MED4*, *ITM2B*, *RCBTB2*, and *DLEU1*), in 13q14 chromosomal region in 9 carriers of retinoblastoma without germline point mutation in *RB1*. The deletions were confirmed by array-CGH and quantitative relative real-time PCR techniques. Partial *RB1* deletions were found in four probands, and five probands carried complete *RB1* deletion that varied in size from 12 to 42 Mb. All four cases with at least one breakpoint within *RB1* gene developed bilateral disease. Among five carriers of complete *RB1* deletion, two were unilateral. In order to investigate the hypothesis that complete *RB1* deletions are associated with incomplete penetrance, we have identified 14 studies where germline *RB1* deletions could be correlated with disease laterality. Fisher's test showed that carriers of partial *RB1* deletion more probably develop bilateral/trilateral disease, whereas carriers of complete *RB1* deletions have a higher probability of incomplete penetrance (unilateral disease) (p=0.000017). In addition, is demonstrated difference statistically significant for partial deletions that affect female patients.

ABBREVIATIONS

Mb: Megabases; MLPA: Multiplex Ligation-probe amplification

INTRODUCTION

Retinoblastoma constitutes the most common childhood intraocular malignancy [1], compromising immature retinal cells. Most cases are diagnosed between birth and six years of age [2]. Tumor development is mostly dependent on the occurrence of two loss-of-function mutational events in both alleles of the tumor suppressor gene *RB1* in the same retinal cell, in accordance with the Knudson two hit model for cancer development [3]. In the absence of the normal activity of *RB1*, there is an accumulation of genomic instability and chromosomal aberrations that promote tumor initiation and development [4].

Most germline mutations found in the *RB1* gene are cytosine to thymine transitions (C>T) in CGA codons, generating stop codons [5]. About 10-15% of the probands with hereditary disease carry gene or chromosomal deletions that include partial or complete loss of one allele of *RB1* [6]. Large chromosomal deletions are usually associated with growth retardation, microcephaly, developmental delay, and dysmorphic features [7]. Matsunaga (1980), observed that unilateral cases are more frequent among carriers of 13q deletion when compared to non-carriers [28]. Subsequently, Mitter et al. (2011), suggested that deletions larger than 1 Mb are associated with incomplete penetrance, with greater proportion of unilateral retinoblastoma among carriers of these deletions [7]. The phenotypic findings in our study accordance with these findings.

MATERIALS AND METHODS

Genomic DNA was isolated from peripheral blood samples from 64 probands with retinoblastoma (42 unilateral, 22 bilateral) according to standard protocols [8]. Sanger sequencing of the coding region of *RB1* did not disclose disease-causing mutations in *RB1*. However, *RB1* copy number analysis was performed from 9 probands (2 unilateral, 7 bilateral) with SALSA MLPA P047 *RB1* kit (MRC Holland®) according to the manufacturer's recommendations.

In order, to confirm the findings of the MLPA technique, we performed the analysis for array-CGH (7 probands), and quantitative real-time PCR (5 probands) techniques.

The literature review for *RB1* deletions and disease laterality was performed with the keywords "13q14 deletion" and/or "retinoblastoma" and/or "*RB1* deletion". Cases with identified somatic or germline mosaicism where excluded from the analyses, as well as cases associated with chromosomal translocations involving 13q14. We found 31 studies between 2003 and 2020 where germline *RB1* deletions were searched for in retinoblastoma cohorts. Seventeen studies were excluded because the genomic data could not be correlated with laterality in each proband.

RESULTS AND DISCUSSION

The genomic and clinical data of nine unrelated probands with retinoblastoma are summarized in Table 1. Four probands carried partial *RB1* deletions, with at least one breakpoint within *RB1* and 5 probands with complete deletion in *RB1* gene and 13q14 region. Array-CGH and Real-time PCR for validations of the deletions found by MLPA was performed in five probands (data not show). Relative estimation of *RB1* copy number of probands 1 and 3, carriers of partial *RB1* deletion, showed copy number compatible with the deletion, and normal copy number for *MED4* and *SUCLA2* genes. Probands 5, 8 and 9, carriers of complete *RB1* deletion, presented copy number estimation compatible with deletion of adjacent genes *MED4* and *SUCLA2*.

For the analysis of type of germline *RB1* deletion and disease laterality we identified 14 published studies of retinoblastoma cohorts where the *RB1* data could be correlated with disease laterality in each proband. These cohorts are form 11 different countries, and where published between 2003 and 2018. A total of 194 probands could be included in the analysis, as well as the nine probands identified in our cohort (n=203). Gender information was available in 8 of the 15 studies. Among 203 probands there were 52 unilateral, 150 bilateral, and one trilateral case (Table 2).

A contingency 2x2 table was built comparing type of *RB1* germline deletion (complete or partial), with disease laterality (unilateral x bilateral/trilateral), and sex (male x female). Fisher's test showed that carriers of partial *RB1* deletion more probably develop bilateral/trilateral disease, whereas carriers of complete *RB1* deletions have a higher probability of incomplete penetrance (unilateral disease) (p=0.00017). When the data are stratified by sex, the difference is not statistically significant. However, when stratified the laterality of the disease by sex, the difference is statistically significant for patients with partial deletion and female. These results are shown in Tables 3.

In the present study we have performed MLPA analysis in 9 probands with retinoblastoma carrying partial or complete deletion of *RB1* gene and no point mutations in *RB1* coding and introinc flanking region [26]. These findings were subsequently confirmed by array-CGH (7 probands) and quantitative real-time PCR (5 probands) [27]. In all cases these methodologies confirmed the partial or complete deletions observed by MLPA. The data are shown in Table 1.

Matsunaga (1980), reviewed laterality and age at diagnosis in 27 previously published cases of retinoblastoma associated with 13q chromosomal deletion. He observed a larger proportion of unilateral retinoblastoma among carriers of 13q deletion when compared to patients with hereditary retinoblastoma (p=0.0008) [28]. He did not observe statistical difference in age at diagnosis and sex ratio, although there were more females than males [28].

Mitter et al. (2011), reviewed clinical and genotypic findings in 63 retinoblastoma patients carriers of 13q deletion. They observed higher probability of unilateral disease in carriers of deletions larger than 1 Mb, and including the adjacent *MED4* gene [7]. They have also established a relationship between haploinsufficiency of the *ITM2B* gene, also located in the chromosomal region 13q14, with the occurrence of bilateral disease. Also, they associated the loss of the *ITM2B*, *RB1* and *RCBTB2* genes to above-standard stature, a factor that was not evaluated in our study [8]. Subsequently, Dehainault et al. (2014), have demonstrated *in vitro* that cells with double inactivation of the *RB1* gene cannot survive in the homozygous absence of the *MED4* gene [9].

It is not yet known exactly whether specific genes in this 13q14 region affect specific phenotype characteristics seen in patients with retinoblastoma and such deletions [7; 10]. Germline mutations in *SUCLA2* gene, adjacent to *RB1* in 13q14, were associated with encephalomyopathy and association in bilateral retinoblastoma. According to Matinailen et al. (2015), the *SUCLA2* gene can be considered a cause for neurological deficits in 13q14 deletion retinoblastoma patients [11]. Caselli et al. (2007), reported a retinoblastoma patient with normal clinical features and a small deletion (1.7 Mb), and two other patients with large deletions (19 to 45 Mb) that showed varied clinical features including cranio facial dysmorphism, psychomotor delay, hypotonia, short stature and anomalies in phalanges and brain [10].

Brennan et al. (2016), compared chemotherapy-related toxicities between 11 patients with retinoblastoma and 13q deletion and 57 patients without 13q deletion. They state the there were 7 females and 4 males, and that there was no statistically significant difference in the proportion of unilateral to bilateral cases, but the data are not shown [12].

Table 1: Summary of clinical and molecular findings of nine probands with partial or complete *RB1* deletion in the germline.

case	fh	age	lat	MLPA / Array	size	*SUCLA2*	*MED4*	*ITM2B*	*RB1*	*RCBTB2*	*DLEU1*
1	-	8	B	48,941,725-48,945,175	1.1 Kb	N	N	N	**E10-11**	N	N
2	-	8	B	49,027,069-49,107,316	>80kb	N	N	N	**E18-27**	del	N
3	-	5	B	48,807,274-48,934,489	276 kb	N	N	del	**E1-7**	N	N
4	Y	4	B	48,807,274-48,878,185	>70kb	N	N	del	**pro-E1**	N	N
5	-	8	B	44,619,278-60,527,495	15.4 Mb	del	del	del	**complete**	del	del
6	-	NS	U	43,140,102-58,050,846	15 Mb	del	del	del	**complete**	del	del
7	-	20	U	38,048,439-80,896,580	42 Mb	del	del	del	**complete**	del	del
8	-	12	B	38,999,215-49,055,894	12 Mb	del	del	del	**complete**	N	N
9	-	17	B	38,999,215-66,611,427	27 Mb	del	del	del	**complete**	del	del

Abbreviations: fh: family history; age: age at diagnosis (months); y: yes; NS: not specified; lat: laterality. Ref. Seq.: NG_009009.1 Ref. Seq.: NC_000013.10.

Table 2: References for 13q14 deletion in retinoblastoma patients. The total deletions identified and those corresponding to the complete deletion of *RB1* gene were observed.

Country	N* Total	Deletion *RB1*			Sex		Laterality			Ref.
		n (%)	Complete (%)	Partial (%)	M	F	U	B	T	
Argentina	144	12 (5%)	9	3	NS	NS	5	7	-	Ottaviani et al., 2013 [13]
Brazil	64	9 (14%)	5	4	4	5	2	7	-	This study
China	85	8 (9%)	5	3	3	5	-	8	-	He et al., 2014 [14]
France	192	18 (9%)	6	12	NS	NS	1	17	-	Houdayer et al., 2014 [15]
Germany	63	62 (98.4%)	53	9	26	27	20	42	-	Mitter et al., 2011 [7]
India	50	5 (10%)	1	4	NS	NS	1	4	-	Singh et al., 2016 [16]
	33	4 (12%)	1	3	-	4	-	4	-	Devarajan et al., 2015 [17]
	74	11 (15%)	2	9	NS	NS	2	9	-	Parsam et al., 2009 [18]
Iran	121	14 (12%)	8	6	NS	NS	5	9	-	Ahani et al., 2013 [19]
Italy	65	10 (17%)	6	4	7	4	5	4	1	Grotta et al., 2015 [20]
Malaysia	19	3 (16%)	2	1	-	3	1	2	-	Khalid et al., 2015 [21]
Netherlands	529	12 (2%)	12	-	7	5	5	7	-	Dommering et al., 2014 [22]
USA	30	4 (13%)	3	1	NS	NS	-	4	-	Li et al., 2016 [23]
	358	25 (7%)	16	9	NS	NS	5	20	-	Richter et al., 2003 [24]
Vietnam	34	6 (18%)	5	1	2	4	-	6	-	Nguyen et al., 2018 [25]
Total	**1.861**	**203 (11%)**	**134 (66%)**	**69 (34%)**	**49 (47%)**	**56 (53%)**	**52 (25,6%)**	**150 (73,9%)**	**1 (0,5%)**	

Abbreviations: *N= total sample size; NS: not specified; REF: reference.

Table 3: Contingency table 2x2: Fisher Exact Test applied to meta-analysis.

Laterality x deletion type			
	Complete	Partial	Totals
Unilateral	47	5	52
Bi-Trilateral	87	64	151
Totals	134	69	203
Pearson's goodness-of-fit chi-square test: X^2: 18.5115; p = 0.000017 Fisher's exact test: p < 0.05			
Laterality x sex			
	Male	Female	Totals
Unilateral	16	15	31
Bi-Trilateral	33	41	74
Totals	49	56	105*
Pearson's goodness-of-fit chi-square test: X^2: 0.43; p = 0.5108 *NS cases for sex were excluded from the analysis, totaling 60 cases.			
Type of deletion x sex			
	Male	Female	Totals
Complete	44	41	85
Partial	5	15	20
Totals	49	56	105
Pearson's goodness-of-fit chi-square test: X^2: 4.66; p = 0.0308 Fisher's exact test: p < 0.05			

The meta-analysis of 14 papers published between 2003 and 2018, we observed cases of deletion involving the *RB1* gene, with complete or partial deletion, with sex, laterality and age at the diagnosis of each proband (Table 2), and the cases of deletion involving the *RB1* gene is 5% - 10% of the cases of retinoblastoma and we also observed that the prevalence of cases of the bilateral form of the disease in cases of complete and partial deletion, emphasizing the greater frequency of bilateral cases to the detriment of unilateral form in cases of partial deletion of the *RB1* gene, as showed by Fisher's statistical test applied to the data (Tables 3).

One potential caveat of our study is that we could not identify multifocal disease cases among carriers of unilateral disease. We also have not correlated the phenotype with the size of the deletion, only with the total or partial compromise of *RB1* gene. The absence of statistical significance when the data are stratified by sex may be accounted for by the smaller sample size available for analysis.

CONCLUSION

In the present study, we present the search for deletions involving the *RB1* gene and other adjacent genes in the 13q14 region, carried out on 9 probands with retinoblastoma. In addition to the MLPA technique, the techniques of array-CGH and quantitative real-time PCR were used, which estimated the size of

the deletion (1.1 Kb - 42 Mb). With the meta-analysis performed, it was possible to determine the prevalence of deletions involving the *RB1* gene and its correlation with tumor laterality. The frequency of deletions in different cohorts ranged from 5% -10%. Due to the small number of studies available for analysis, it was not possible to determine the existence of a possible association between laterality and the size of the deletion, as well as it was not possible to evaluate the cases with the deletion involving the *RB1* gene concomitant with the adjacent genes located in the 13q14 region (*SUCLA2* and *MED4*, for example).

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CONFLICT OF INTEREST

The author does not have conflict of interest. The project "Cytomolecular studies in retinoblastoma" was approved by the Nacional Cancer Institute (INCA) from Rio de Janeiro (protocol 40/00).

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