RESEARCH COMMUNICATION

Genotype-phenotype Correlation Analysis in Retinoblastoma Patients from India

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Abstract

Background: Genetic analysis has a beneficial impact on retinoblastoma management enabling definite risk assessment. However, information regarding genotype-phenotype correlation in retinoblastoma is limited. Aim: To analyze the retinoblastoma susceptibility gene for mutations in retinoblastoma patients and correlate the genotypes the phenotypes. Methodology: Eleven retinoblastoma patients, who underwent molecular genetic studies were classified into high, moderate or low disease severity groups based on phenotype. Results: Seven patients had high disease severity and four moderate disease severity. Eleven truncating mutations were detected; six were in the N-terminus region of the retinoblastoma protein and two in the A/B pocket (p=0.03). Conclusions: No significant association between mutation type and disease severity could be established in the present study. However a positive correlation between location of the mutations in certain domains of the retinoblastoma protein and disease severity was observed. To the best of our knowledge this is the first genotype-phenotype correlation study in retinoblastoma patients from India.

Key Words: Genotype, phenotype, RB1 mutations, Retinoblastoma

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Introduction

Retinoblastoma (RB1; MIM# 180200) is a malignant tumor of the eye that arises from un-differentiated retinal precursor cells. It occurs in children below the age of five years (Brantley and Harbour, 2001). Retinoblastoma manifests in its earliest clinical stage as a small (less than 2 mm in basal diameter), slightly translucent lesion in the sensory retina. Larger tumors present with leukocoria, strabismus and/or neovascular glaucoma. The disease has been categorized as low, moderate or high severity according to disease stage, age at presentation and subsequent clinical course (Qi et al., 2005). Treatment depends on the tumor stage with smaller lesions treated by local modalities such as cryotherapy, photocoagulation, and brachytherapy and larger tumors necessitating external beam radiation therapy (EBRT), chemotherapy, and/or enucleation.

The RB1 gene is a large 180 kilobase, 928 – aminoacid protein at chromosome 13q14, that plays a role in cell growth and development. Mutations in both alleles of RB1 gene are required for development of the tumor (Cavenee et al., 1985). While a *de novo* or inherited germline mutation gives rise to bilateral retinoblastoma, somatic mutations in both the alleles account for 85% of unilateral tumors (Richter et al., 2003). Most RB1 mutations are point mutations (single-base substitutions, short length-alterations, and complex mutations). These can be broadly classified as truncating, non-truncating and promoter mutations (Qi et al., 2005). The RB1 gene codes for the retinoblastoma protein pRB, that has 4 domains (Figure 1); the N terminus (aminoacids 1-392), the A/B domain (aminoacids 393-772), the C pocket (aminoacids 773-869), and the C terminus (aminoacids 870-928).

Genotype-phenotype associations are of great significance since they might help prognosticate the effect of an individual mutation in a carrier (Lohmann and Gallie, 2004). To date conflicting reports exist about correlation between the genotype and phenotype in retinoblastoma. A retrospective analysis of 88 germline RB1 mutation carriers from Canada and the United States (Qi et al., 2005) revealed a significant association of high disease severity with truncating mutations. However, mean residual protein length and location of mutations in domains did not show any association to clinical severity (Qi et al., 2005). Albrecht et

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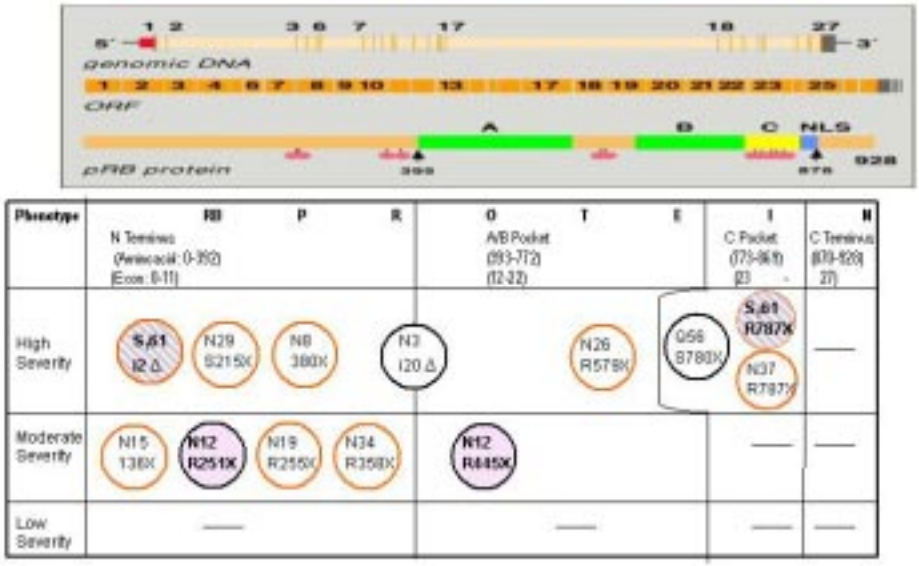

Figure 1. Matrix of genotype- phenotype (clinical and protein) Correlations in Retinoblastoma Patients. Similar hatched pattern circles denote same patient. Black circles represent unilateral retinoblastoma patients. Orange circles represent bilateral retinoblastoma patients. We thank Dr. DRLohmann, University of Essen, Germany, for his permission to use the figure of the RB protein.

al observed a positive correlation between presence of gross deletion with one breakpoint in RB1 gene and occurrence of bilateral retinoblastoma (Albrecht et al., 2005). Alonso et al reported an association between mutation type and time of onset of retinoblastoma (Alonso et al., 2001). Mutations affecting splice junctions resulted in a delayed onset of tumors while nonsense and frameshift mutations were associated with an early age at diagnosis (Alonso et al., 2001). However, studies on Chinese and Indian retinoblastoma patients did not demonstrate any association between the type of mutations and the phenotype (Ata-ur-Rasheed et al., 2002; Choy et al., 2002). Further, families with the same mutation have been shown to demonstrate phenotypic differences viz. the development of lipomas in only one of two retinoblastoma families having an identical splice mutation (resulting in skipping of exon 13) (Lohmann and Gallie, 2004).

We report the results of mutational analyses of the RB1 gene, and the correlation between the genotype and phenotype in 11 retinoblastoma patients from our center.

Materials and Methods

The study was carried out at Vision Research Foundation, Sankara Nethralaya, Chennai, India, a tertiary eye care center for retinoblastoma in India. The study was approved by the institutional ethics review board and adhered to the tenets of the declaration of Helsinki.

The study population comprised of 11 retinoblastoma patients who presented to our hospital during the period 1999-2000. All the cases were staged according to the Reese-Ellsworth classification (Shields and Shields, 2004). Enucleation had to be performed in all the patients due to advanced disease. Diagnosis of retinoblastoma was confirmed by current histopatholgical criteria. Histopathological examination included determination of tumor differentiation, and presence or absence of choroidal and/or optic nerve invasion, tumor cells at the cut end of the optic nerve, and any extra ocular extension. Patients were classified into 3 disease severity groups viz. high, moderate and low, according to criteria adopted by Qi et al (2005) with some modifications (Table 1).

RB1 mutation screening was performed on DNA from fresh tumor and peripheral blood following methods described in a previous study (Kumaramanickavel et al., 2003). Mutations were classified based on the type and aminoacid location in the retinoblastoma protein. They were divided into 3 groups viz. (i) large deletions that included mutations which resulted in loss of multiple exons, (ii) truncating mutations that included nonsense, frameshift or splice site mutations resulting in premature creation of stop codon and (iii) non-truncating mutations, which resulted in change of aminoacids. Predicted residual protein length and domains disrupted by the mutations were calculated for patients with truncating mutations.

Statistical Analysis- The correlation of disease severity with the type of mutations was analyzed using 'Z' test for proportions with SPSS version 13. The exact test based on Montecarlo assumption was used to test for correlation between mean residual protein length and disease severity.

Results

All 11 patients (3 unilateral retinoblastomas / 8 bilateral retinoblastomas) were identified to have moderate to high disease severity, with 7 patients categorized as having highly severe disease and 4 patients identified as having moderately-severe disease. No patient could be placed in the low severity group (Table 2). A total of 13 mutations were detected in the study of which eight were found in patients with a highly

Table 1. Comparison Between the Phenotype Classification Criteria of Qi et al, (2005) and the Present Study

Qi et al, (2005)	Present Study
High Severity (obeying any one or more criteria)	
1. Bilateral patients with age less than 1 year or unilateral disease with less than 2 years and high stage (early presentation with high stage)	1. Bilateral patients with age less than 1 year or unilateral disease with less than 2 years stage IV-V of Reese-Ellsworth classification,
2. High risk features on histopathological exam	2. High risk features on histopathological exam- Infiltration of optic nerve/choroids
3. Clinically advanced disease (neovascular glaucoma, anterior segment involvement, buphthalmos) with average age of presentation	3. and 4. Same as Qi et al, (2005).
4. Bilateral enucleation.	
Low severity (obeying any one or more criteria)	
1. Unilateral disease and positive family history	1. Same as Qi et al, (2005),
2. Bilateral patients with age greater than 1 year and low stage (late presentation with low stage).	2. Bilateral patients with age greater than 1 year and stage I- II of Reese-Ellsworth classification.
Moderate severity (obeying any one or more criteria)	
1. Absence of low or high severity criteria at diagnosis, and on review of disease course	1. Absence of low or high severity criteria at diagnosis, and on review of disease course
	2. Stage III of Reese-Ellsworth classification

severe phenotype and five were found in patients with moderately-severe disease These mutations included nine nonsense mutations, one frameshift mutation (eight bp deletion), and one splice site mutation (all resulting in truncation) and two large deletions (Table 3). Both large deletions were present in the high disease severity group. Of 11 truncating mutations, six were found in the high disease severity group and five were found in the moderate disease severity group (Figure 1). The mean residual protein length was 289 aminoacids for the moderate disease severity group and 588 aminoacids for the high disease severity group (Table 3).

When considering the location of the mutations in the RB1 gene, six truncating mutations (five nonsense, one frameshift and one large deletion) were present in the N terminus region, in moderate and high severity groups, two nonsense mutations were seen in the A/B pocket and three nonsense mutaions were in the C pockets (Figure 1). Of these, one patient with a mutation in A/B pocket (N12) and another patient with a mutation in C pocket (S161) had a second mutation in the N terminus. These patients had moderate and high severe retinoblastoma respectively. In the patient who had an intron 20 deletion, the intron 2 was intact; however the location of the deletion cannot be stated with absolute certainity as PCR was performed using microsatellites in RB1 introns 2 and 20. This deletion was therefore placed between the N terminus and the A/B pocket.

Table 2. Correlation of RB1 Mutations versus Disease Severity

Severity	Patients	RB1 mutations	Truncating	Mean residual protein length
High	7	8	6	588
Moderate	4	5	5	289
Total	11	13	11	-

No statistically significant association was found between disease severity (phenotype) and mutation type ($p>0.05$). The mean predicted protein length also did not show any statistically significant difference between moderate and high disease severity groups. However the number of truncating mutations in the N terminus region were significantly more (6/10) when compared to the A/B pocket region (2/10; $p=0.03$) and C pocket region (3/10; $p=0.08$).

Discussion

Genotype-phenotype information is important for genetic counseling of retinoblastoma families, enabling risk assessment and prognostication of the disease (Lohmann and Gallie, 2004). While no genotype-phenotype correlation could be drawn in Chinese (Choy et al., 2002) and Indian (Ata-ur-Rasheed et al., 2002) populations, gross deletions (with one breakpoint in RB1 gene) were significantly associated with laterality in another study (Albrecht et al., 2005). In the present study, no significant association was observed between truncating mutations and disease severity. This could be because of the small sample size. Additionally, we did not find any correlation between mean predicted residual protein length and disease status for the truncating mutations. While Qi et al (2005) could not detect a relationship between truncating mutations and their location, we found that majority of the truncating mutations (6/10) were in the N terminus of the protein ($p=0.03$).

Nonsense or frameshift mutations occurring in exons 2-25 have been previously associated with bilateral retinoblastoma (Lohmann and Gallie, 2004). Seven patients (S161, N29, N15, N19, N34, N26 and N37) in this study showed a similar association. However, occasionally such mutations have also been found in isolated unilateral patients

Table 3. Phenotype and Genotype Data

S No.	ID	Clinical features	Severity	Mutation/pRB domain (exon)	Mutation type	Truncation	Effect on Protein
1	S161	Optic nerve invasion	High	Intron 2 del. /NA	Large deletion	-	Loss of expression
2				C>T / C(E23)	Nonsense	+	R787X
3	N15	Bilateral, age >1 yr	Moderate	8 bp del./ N (E04)	Frameshift	+	136X
4	N29	Advanced disease	High	C>G /N(E07)	Nonsense	+	S215X
5	N12	Unilateral, no family history, age >2 yrs	Moderate	C>T /N(E08)	Nonsense	+	R251X
6				R445X/N(E14)			R445X
7	N19	Bilateral, age >2 yrs	Moderate	C>T /N (E08)	Nonsense	+	R255X
8	N34	Bilateral, age >2 yrs	Moderate	C>T /A(E11)	Nonsense	+	R358X
9	N8	Bilateral, age < 1 yr	High	Intron 11 splice /NA	Splice site	+	aa 378-405 del; aa 378-379 mutation; 380+ truncation
10	N26	Bilateral, age < 1 yr	High	C>T, /Spacer (E18)	Nonsense	+	R579X
11	N3	Unilateral, age <2 yrs, Choroidal infiltration	High	Intron 20 del./ NA	Large deletion	-	Loss of expression
12	Q56	Unilateral, age <2 yrs	High	C >A / C(E23)	Nonsense	+	S780X
13	N37	Bilateral,age < 1 yr	High	C>T/ C(E23)	Nonsense	+	R787X

(Lohmann et al., 1997) and in the current study patients N12 (two nonsense mutations) and Q56 (one nonsense mutation) had unilateral retinoblastomsa. On the other hand, intron 11 splice mutation in patient N8 resulted in bilateral retinoblastoma. This could be caused by protein truncation and complete penetrance of mutation at invariable splice sites (Lohmann and Gallie, 2004).

Qi et al concluded that large deletions were associated with high disease severity (2005). In the present study, two large deletions, one at intron 2 and another at intron 20 were found in a bilateral (S161) and unilateral retinoblastoma patient (N3), respectively. Though both are large deletions, intron 2 deletion could be expected to result in definite loss of major domains of the retinoblastoma protein critical for most of its function. Moreover, this patient also had a nonsense mutation R787X in the C pocket, which could have disrupted the interaction of the A/B domain with E2F and resulted in the bilateral phenotype. The i20 deletion could have occurred anywhere between introns 2 and 20 resulting in some protein expression and function causing unilateral phenotype. Six out of 9 nonsense mutations detected here occurred in the N terminus region and were associated with moderate and high disease severity. This association is much favorable to the expected outcome of loss of protein beyond the N terminus and thereby loss of A/B pocket and its crucial function in E2F binding. However, patients in low severity group and more number of mutations are required to clearly understand these correlations.

In summary, though no significant association between type of mutations and disease severity could be established in the present study, we found a positive correlation between location of the mutations in certain domains of the retinoblastoma gene and disease severity.

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