

## 'Insight' into Molecular Genetic Testing in Retinoblastoma

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**ABSTRACT** Retinoblastoma is an intraocular tumor with hereditary and sporadic forms. Tumor develops when both alleles of the tumor suppressor gene, RB1 is inactivated in the embryonal retinal cells. Clinical screening for retinoblastoma is most often done on examination under anesthesia for proband and closely related infants. Genetic testing in retinoblastoma could avoid unwanted ophthalmic surveillance for infants who do not have mutations in the gene. However establishing a single genetic test is not possible due to the multiple RB1 inactivation mechanisms like gene mutations, deletions and also hypermethylation of the gene promoter. The review focuses on the application of molecular screening methods- multiplex PCR, sequencing of 27 exons of RB1 gene, methylation analysis of promoter region and peripheral blood karyotyping in retinoblastoma. The cost benefit analysis of genetic testing compared to conventional clinical management of retinoblastoma is also discussed. Clinical management of retinoblastoma could be aided by coordinated application of the molecular genetic testing.

### INTRODUCTION

Retinoblastoma (RB) is an intraocular tumor affecting 1 in 17,000 live births (Moll et al. 1997). Age at detection of more than 90% of retinoblastoma is less than three to five years (Brantley Jr and Harbour 2000); but rarely detected as retinomas in adults. Retinomas are tumors that undergo spontaneous growth arrest after a period of proliferation (Gallie et al. 1982). Tumor develops when both alleles of the retinoblastoma susceptibility gene (RB1 gene) are inactivated in the embryonal retinal cells widely referred to as the "two hits" (Knudson 1971). Stallard (1962) suggested that RB1 gene is located on chromosome 13. The ubiquitously expressed RB1 gene with 27 exons spanning over the 180 Kbp at the 13q14 band (Lee et al. 1987) codes for a 110-kDa-tumor suppressor protein - pRB. Susceptibility to retinoblastoma is transmitted as an autosomal dominant trait, if an RB1 mutation is present in the constitutional cells (Knudson 1986). In this situation, the tumors arise at an earlier age, in both eyes and are multifocal. In non-hereditary cases where only one eye is affected, the mutations are of somatic origin and the tumor is unifocal. Majority of the

sporadic cases are accounted for by de novo RB1 mutations.

The genetic alterations that could result in RB1 gene inactivation are chromosomal rearrangements involving the 13q14 region (deletion, translocation), nucleotide changes (substitutions, deletions, insertions and duplications), exonic deletions (single or multiple); loss of heterozygosity (LOH) or CpG island hypermethylation in the RB1 promoter region (de Andrade et al. 2006). Mutational inactivation of RB1 is also associated with small-cell lung cancer, sarcomas and carcinomas (Simpson et al. 2000). Osteogenic sarcoma in childhood and breast or bladder cancer has been reported in retinoblastoma survivors (Hurwitz 2003).

Clinically retinoblastoma screening is done by examination under anesthesia at regular intervals for proband and closely related infants (Musarella and Gallie 1987). Anesthesia related risks, financial burden due to repeated examination and the psychological disadvantage for the proband are the shortcomings. Genetic testing could save money and time by eliminating unnecessary ophthalmic surveillance for proband and infants lacking germline RB1 mutations (Richter et al. 2003).

Despite the use of molecular techniques, establishing a single genetic test in retinoblastoma is not possible because of the wide distribution of mutations throughout RB1 gene (except exons 26-27) and inactivation due to hypermethylation of CpG island in the promoter region (Lohmann 1999; Richter et al. 2003).

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Complementary tests like sequencing, karyotyping and comparative genotyping allow rapid RB1 mutation screening (Harbour 1998). Bamne et al. (2005) suggested a multi-technique approach involving karyotyping, *Fluorescent In Situ Hybridization* (FISH) and PCR-SSCP-DNA sequencing for genetic testing in retinoblastoma. Protein truncation testing is suggested as an effective, rapid single-modality screen for RB1 mutations in retinoblastoma (Tsai et al. 2005).

The review focuses on the molecular screening methods in retinoblastoma - multiplex PCR and sequencing of 27 exons of RB1 gene, methylation analysis of RB1 promoter region and peripheral blood karyotyping. In addition, the cost benefit analysis of genetic testing compared to clinical management is discussed.

### Mutation Screening

Conclusive identification of RB1 mutations in retinoblastoma improves the clinical management of affected children and relatives. An infant having highly penetrant RB1 germline mutations could be delivered earlier and treated to save the vision (Richter et al. 2003). RB1 mutations could be classified into single base substitutions (nonsense and missense mutations), splice site mutations, small deletions or insertions and complex mutations (generally involve deletions and insertions). Review of RB1 mutations by Harbour (1998) reported 78% of nonsense and frameshift mutations while Lohmann (1999) reported 62% of single base substitutions.

RB1 mutation testing in clinical practice is done by analysis of sequencing of entire coding region, selected exons or RNA and by scanning methods like heteroduplex assay or denaturing gradient gel electrophoresis (<http://www.genetests.org>). Solutions by Sequence Inc (Canada), Universidad de Buenos Aires (Argentina) and University of Pennsylvania School of Medicine offer genetic testing for retinoblastoma at research level (<http://www.genetests.org>).

Comparative multiplex PCR has been developed to detect RB1 deletions of one or few exons and exonic size changes due to deletions or insertions (Du and Gallie 1999). Quantitative multiplex PCR (QM-PCR) accurately measured deletions and insertions for 23 RB1 exons and promoter region (Richter et al. 2003). Mutation screening by denaturing high-performance liquid

chromatography (Houdayer et al. 2004) was aided by multiplex PCR of RB1 exons. Dehainault et al. (2004) suggested the combination of multiplex PCR and liquid chromatography using DHPLC system as a cost-effective method for RB1 copy number analysis. According to Tsai et al. (2005), identification of the truncated fragment, using the Protein Truncation Test (PTT), the efficiency of RB1 mutation detection by sequencing could be increased. Direct sequencing of the RB1 exons (Ata-ur-Rasheed et al. 2002; Kumaramanickavel et al. 2003; Joseph et al. 2005) or combined with SSCP analysis (Kiran et al. 2003; Bamne et al. 2005) have been used in mutational screening of Indian retinoblastoma patients. Sequencing has been suggested to detect 75% of the predisposing RB1 mutations in "best practice guidelines for screening for retinoblastoma".

### Methylation Analysis

Hypermethylation of CpG islands in promoter region and resultant inactivation of the RB1 gene could be a potential mechanism for retinoblastoma. The RB1 promoter region hypermethylation frequency in retinoblastoma varies from 4-10 % (Sakai et al. 1991; Ohtani-Fujita et al. 1997; Stirzaker et al. 1997; Zeschnigk et al. 1999). RB1 promoter hypermethylation was not reported in samples from Chinese retinoblastoma patients. But, hypermethylation was reported in the Mut L homologue 1 (MLH1)-as well as the O<sup>6</sup>-Methylguanine-DNA Methyltransferase (MGMT) genes in retinoblastoma (Choy et al. 2002; Choy et al. 2004).

The differentiation of hypermethylation being associated with tumorigenesis from its involvement as a causative factor is crucial in understanding the molecular mechanism of retinoblastoma. During the molecular screening of retinoblastoma, methylation analysis of RB1 promoter is done, if only one or no RB1 mutations were identified, and hence hypermethylation could be a possible mechanism. Determination of methylation status of a gene by PCR amplification, followed by restriction digestion and Southern hybridization has the disadvantages of requirements of large amount of DNA and multiple restriction enzymes. Sodium bisulfite conversion and MS-PCR simplified the methylation screening by the requirement of less DNA, no restriction digestion and determination of individual CpG site methylation.

Hypermethylation results are useful in genetic counseling of retinoblastoma. A unilateral retinoblastoma patient with hypermethylation of RB1 gene is unlikely to have a germline mutation (Vogel 1979). Identifying the CpG sites preferentially hypermethylated could lead to 'CpG island methylation phenotype' in retinoblastoma and 'tailor-made' demethylation therapy could be instituted. Jones (1997) stated that complete hypermethylation abolishes entire RB1 gene activity.

### Peripheral Blood Karyotyping

The most frequent chromosomal abnormality in retinoblastoma is interstitial deletion of 13q14 band (Yunis and Ramsay 1978). Chromosomal abnormalities involving 13q14 region have been reported in 7.5–8.0 % of bilateral and 1.0–4.9 % of unilateral retinoblastoma (Ejima et al. 1988). Retinoblastoma susceptibility is transmitted to 50% of offspring of patients with 13q14 deletion in blood.

The first report of constitutional D group deletion in retinoblastoma was by Lele et al. (1963). Howard et al. (1978) established the deletion to be in chromosome 13. Ophthalmic evaluation after interstitial 13q14 deletion detection by karyotyping led to earlier detection of retinoblastoma (Kennerknecht et al. 1994). Potluri et al. (1986) reported monosomies and deletions of chromosome 13, extra chromosome 6 and additional copies of 1q and 6p in retinoblastoma. Balanced translocations in retinoblastoma are t(1:13)(p22;q12)(Davison et al. 1979); t(13:18)(q14.1;q12.2) (Motegi 1982) and t(2:13)(p24.3;q14.2) (Turleau et al. 1985). In a study, chromosomal deletion was not demonstrable in Indian retinoblastoma patients. Gandhewar et al. (2004) demonstrated a higher frequency of q14 fragile site expression in retinoblastoma patients having 13q14 deletion (Amare Kadam et al. 2004).

Mosaic and non-mosaic chromosomal deletions of 13q14 region did not differ regarding the age at diagnosis, laterality of tumor, and presence of family history (Kivela et al. 2003). Amare Kadam et al. (2004) suggested that mosaicism for 13q14 deletion should be considered in genetic counseling of unilateral retinoblastoma. FISH is sensitive in detecting monoallelic RB1 deletion/deletion mosaicism in blood in 20% of Indian retinoblastoma cases (Bamne et al. 2005). Tharapel and Kadandale (2002) indicated that primed in situ labeling

(PRINS) could serve as an alternative to FISH for defining deletions involving 13q14 region in hematological malignancies.

### Cost Analysis of Genetic Testing

Cost analysis of genetic testing is becoming more important with the possibility of being used in the decisions varying from employability to health insurance coverage. Efficiency plays an important role in deciding the wider implementation of retinoblastoma genetic testing in clinical practice (Richter et al. 2003; Joseph et al. 2005b). This is very important in India with the highest projected incidence and also the low economic status of these patients (Gallie 2004). Noorani et al. (1996) showed a 3.6 fold savings by comparing the cost of RB1 gene and clinical screening. In a questionnaire-based follow-up of retinoblastoma families, most respondents expressed usefulness from genetic testing (Cohen et al. 2001). Children without germline RB1 mutations by genetic screening could avoid cumbersome screening procedures and in children established to have RB1 mutations, earlier delivery could result in saving the vision (Smith and O'Brien 1996; Raizis et al. 2002; Richter et al. 2003).

Karyotype analysis by 72-hour peripheral leucocyte culture has been ongoing in the SN ONGC Genetics and Molecular Biology department from 1993 (Harini et al. 2001). We reported 8.33% of 13q14 deletion in our patients (Harini et al. 2001). Further, the advantages of statistical analysis in deriving meaningful 13q14 deletion results were established in another study (Joseph et al. 2005a). mPCR for RB1 exons resulted in significant cost, time and reagent saving in RB1 mutation screening (Joseph et al. 2005b). The benefits due to RB1 mutation screening, conventional karyotyping and methylation analysis in Indian patients were established (Kumaramanickavel et al., 2003). The first methylation report from Indian retinoblastoma patients showed 6.6% of hypermethylation of RB1 promoter region (Joseph et al. 2004a). At our hospital, genetic screening showed a financial savings of 3.5 fold for the proband and 6.1 fold for a nuclear family compared to examination under anesthesia (Joseph et al. 2004b).

### Best Practice Guidelines for Retinoblastoma Screening

With the advent of improved and newer molecular testing techniques, establishing

screening guidelines are important to ensure their optimal use in cancer genetics. European Molecular Quality Network (EMQN) is a not-for-profit organization promoting quality in molecular genetic testing through the organization of best practice meetings and publication of guidelines. Guidelines have been developed for hereditary breast and ovarian cancer, haemoglobinopathies and Angelman syndrome from the meetings held at various places over the years. Table 1 summarizes the genetic tests included in 'best practice guidelines for retinoblastoma screening' developed after the Manchester (2002) meeting. Tests like karyotyping, sequencing, methylation-specific PCR and comparative genotyping could be done in a basic molecular genetic laboratory with minimum infrastructure while FISH requires a workstation and RB1 probes which would add to the costs. Southern hybridization is not used currently due to the disadvantages described elsewhere in this article. Quantitative multiplex PCR is not used routinely due to requirement of specialized equipments.

With improvements in molecular techniques coupled with miniaturization of instruments, RB1 screening is expected to be widely used in clinical practice with potential benefits for the society.

**Table 1: Genetic tests in retinoblastoma**

| S. No. | Technique                   | Mutation detected                                           |
|--------|-----------------------------|-------------------------------------------------------------|
| 1      | Karyotyping                 | Large deletions, translocations                             |
| 2      | Sequencing                  | Small length mutations, base substitutions                  |
| 3      | FISH                        | Large deletions, translocations                             |
| 4      | Methylation-specific PCR    | Promoter hypermethylation                                   |
| 5      | Comparative genotyping      | Large deletions                                             |
| 6      | Southern blot hybridization | Deletions, insertions, rearrangements; promoter methylation |
| 7      | Quantitative multiplex PCR  | Deletions, insertions                                       |

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