COMMENTARY

Retinoblastoma: A Diagnostic Model for India

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Abstract

Purpose: Molecular genetic diagnostics for retinoblastoma are prerequisite for accurate risk prediction and effective management. Developing a retinoblastoma diagnostic model to establish a flow for laboratory tests is thus a necessity for tertiary ophthalmic institutions. An efficient diagnostic model could reduce the overall health care costs, redirect the resources to the high risk group and also avoid unnecessary worry forfamilies. To the best of our knowledge there has hitherto been no comprehensive diagnostic model for retinoblastoma implemented in any institution in India. Methods and Discussion: The diagnostic model demonstrates the logical and practical flow of various genetics tests like karyotyping, loss of heterozygosity analysis, molecular deletion, linkage analysis (familial cases), mutation screening of - CGA exons first and then non-CGA exons, methylation screening of RB1 and essential promoter regions screening in a laboratory. Conclusions: The diagnostic model proposed offers acomprehensive methodology to identify the causative two-hits for retinoblastomas that could be used while genetic counseling families. This model is applicable in tertiary hospitals in India and neighboring countries, which have the highest incidence of retinoblastoma and fertility rates in the world. We suggest that this diagnostic model could also be applied with modification for other cancers.

Key Words: DNA sequencing - genetic testing - loss of heterozygosity - RB1 gene - retinoblastomas

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Introduction

Retinoblastoma is responsible for approximately 1% of all deaths caused by childhood cancer and for about 5% of blindness in children (van der Wal et al., 2003). It is the leading pediatric tumor in India and more so in Chennai (Sunderraj, 1991). Gallie (2004) estimated the burden of retinoblastomas in various countries like India, China, Canada, UK & USA, based on population size, birth and infant mortality rates, and stated that India stands first. The reason could be the large population size combined with high birth rate, which accounts for approximately 1,532 new retinoblastoma cases per annum.

With retinoblastomas, the disadvantage of clinical management of patients and relatives is the necessity for repeated eye examinations, which is often under anesthesia. During this process several relatives who might not be at-risk also might undergo clinical examination and this could lead to huge financial expenditure and threat to life due to general anesthesia. Delayed diagnosis in bilateral retinoblastoma cases would result in loss of the eye (Butros et al., 2002) and life. Accurate and sensitive molecular genetic diagnosis model might avoid unnecessary anesthetic examination for unaffected relatives and help in saving visual morbidity and mortality of probands. Lohmann et al (1996) thererfore suggested that a protocol for routine mutation analysis for RB1 gene should be in place to convert scientific research into clinical practice for the benefit of at-risk individuals or families.

Before automated technology era, sequencing of the RB1 gene was cumbersome, often ending with ambiguous results. Prior to the introduction of MS-PCR, methylation analysis of RB1 gene was difficult and tedious due to the requirement of large amount of tumor DNA for Southern blotting. Even though these issues were addressed, cost effectiveness of the tests prevented implementation of an efficient routine molecular genetic diagnostics.

An efficient diagnostic model for retinoblastoma could save considerable money, reduce the overall health care costs and also avoid unnecessary anxiety and worry for the family. Molecular diagnostic methods are now routinely used in the University of Toronto, Canada and University of Essen, Germany (Lohmann et al., 2003). By comparing the cost of genetic testing and clinical strategy we observed cost saving of 3.5 fold for the proband and 6.1 fold for the family (Joseph et al., 2004b). The cost saving for genetic testing against clinical screening at University of Toronto and our institution is shown in Table 1. Further discovery of BRCA1/2, APC,

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Table 1. Cost Comparison of Clinical and Genetic Screening for Retinoblastomas

Study	Proband / Family	Clinical Screening	Genetic Testing	Saving
Richter et al., (2003)	Proband	3200*	468	2732
	Family	3520	536	2984
Joseph et al., (2004b)	Proband	536	152	384
	Family	1071	175	896

*US$

MSH2, MLH1 and few other inherited cancer predisposing genes emphasized the necessity for having genetic testing both at research and hospital (Cohen et al., 2001).

A diagnostic model for retinoblastoma in India, which has the highest incidence of retinoblastoma, is worth considering and establishing. To the best of our knowledge there is no comprehensive diagnostic model for retinoblastoma implemented in any institution in India. On an average 60 new retinoblastoma patients are seen every year at Sankara Nethralaya, who come from all over India and neighboring countries and nearly half of these are one-child families (Harini et al., 2001). A molecular diagnostic model for retinoblastoma susceptibility gene with reliable results will be useful for these families in taking a decision to have another child. In addition, it will help the ophthalmologist to decide whether a normal child can be spared unnecessary anesthetic examination. The genetic results would also be useful in suggesting the risk for non-ocular tumors in the surviving patient. In an earlier mutation screening report knowing that RB1 mutations were of somatic origin predicted reduced risk for further siblings of retinoblastoma probands (Kumaramanickavel et al., 2003). During the early establishment of genetic testing, investigators were not able to identify a comprehensive genetic screening model. We here suggest a diagnostic model for retinoblastoma in India.

Methods and Discussion

The present study explains a model, which describes an ideal way for collecting genetic material from retino-blastoma patients and their families with analysis in a sequence. Once a child is diagnosed to have retinoblastoma by the ophthalmologist the patient is referred for pre-test counseling. During the non-directive counseling session, the parents are explained about the disease, its probable progression, current treatment and basic genetic information why the disease has affected the child. In this session, the importance of genetic testing and its relevance are also emphasized. We recommend ophthalmic examination for parents to rule out the presence of any regressed tumors. Extreme care is taken not to reveal the inheritance of the disease is from which parent, in case, if one of them has a regressed tumor, more particularly if it is through the mother; in India where culturally, in the majority of situations, the father is employed but not the mother, a divorce could be an additional devastation to both the mother and the child.

In children with the disease complete eye examination is done including visual acuity, external eye and fundus examinations, in uncooperative younger children clinical examination is done under general anesthesia. All first and second degree-children are clinically evaluated if genetic testing showed risk for developing retinoblastoma. Venous blood is collected from the proband and parents (and if necessary extended family members). When the patient undergoes enucleation the tumor tissue is harvested and later DNA isolated from the tissue (Kumaramanickavel et al., 2003). Paraffin embedded mounted sections of enucleated eyeballs with retinoblastoma are obtained from pathology laboratory if fresh tumors are not available. The personnel, equipments and consumables required for retinoblastoma diagnostic model have been described earlier (Kumaramanickavel et al., 2003).

As a first step of investigations peripheral blood collected from proband and parents were used for cytogenetic studies by giemsa banding as described in Harini et al., (2001). Cytogenetic deletions that constitute 6-8% can be identified by this method (Harbour, 1998). Automated karyoanalysis using ikaros karyotyping system (Zeiss-metasystems, Germany) helps to reduce cost and time for karyotyping. On karyotyping if a deletion is identified in the proband then parents are karyotyped to identify the parental origin of the deletion. Translocation though rare could be identified and this is transmitted by one of the parents. Careful counseling methods would help to prevent matrimonial disruption when one of the parents is identified to have transmitted the chromosomal abnormality. In general mosaicism if identified could establish the post-zygotic error and reduce the risk for the next child to insignificant. We at our laboratory confirm cytogenetic deletion by testing fluorescent microsatellite markers across 13q14 cytogenetic region (D13S263, D13S153, RB1.20, D13S1320, D13S1296 and D13S156). Fluorescent in-situ hybridization (FISH) would help to identify subtle deletions that could be missed by conventional cytogenetic methods (Lohmann et al., 2002). In families with clear autosomal dominant inheritance, haplotype or linkage analyses are undertaken. This method would help to track the risk allele that is inherited in a family. This has to be done carefully taking into consideration the recombination events.

Next would be to detect loss of heterozygosity (LOH) to identify whether one allele – the defective one is present in the tumor or not. About 70% of tumors have LOH (Cavenee et al., 1983). This is the most common mechanism for the second hit in retinoblastoma (Cavenee et al., 1983). Next mutational screening is done with tumor DNA (or genomic DNA if it is a bilateral case) for the 11 CpG sites (CGA codons in exons: 8, 10, 11, 14, 15, 17, 18, 23, 27 and two CpGs in conserved splice sites in introns: 5 and 12). Nearly 50% of DNA mutations of retinoblastoma is caused by change of CGA to TGA resulting in arginine to termination codon, hence if we screen these codons the possibility of identifying the mutations in the given specimen is high (Lohmann, 1999). The disadvantage of using tumor DNA is

that it will be of low quantity and therefore precious. If no mutation is identified in these CpG sites then rest of the 15 exons are sequenced for mutations. Nearly 80% of a hit is due to point mutations (missense or nonsense) (Harbour, 1998). If all these are negative for a hit (or two) then essential promoter of the RB gene is screened for nucleotide change but this is extremely rare (0.48%) (Fujita et al., 1999). Then the tumor is subjected for methylation test by PCR of the promoter region (Joseph et al., 2004a); methylation rate of retinoblastoma tumor is about 10% (Zeschnigk et al., 1999). Invariably by this stage 85–90% chances are there to identify both the hits. Only 10% chance is there that only one or no hits might be identified and this could be due to mosaicism (Richter et al., 2003). In post-test counseling, if any close relatives should undergo eye examination, the information is conveyed giving a reassurance rather than an unwarranted alarm. The results are explained and doubts are clarified depending on what socio-economic status they belong. The parents who want to know about risk for next child, the option of prenatal testing counseling is given. The hierarchical structure of the diagnostic model was established based on the experience and inputs from the molecular investigations carried out in our laboratory and literature (Figure 1).

Harbour proposed that a screening approach involving a series of complementary tests might allow rapid screening of majority of RB1 germ line mutations (Harbour, 1998). European Molecular Quality Network has evolved the 'best

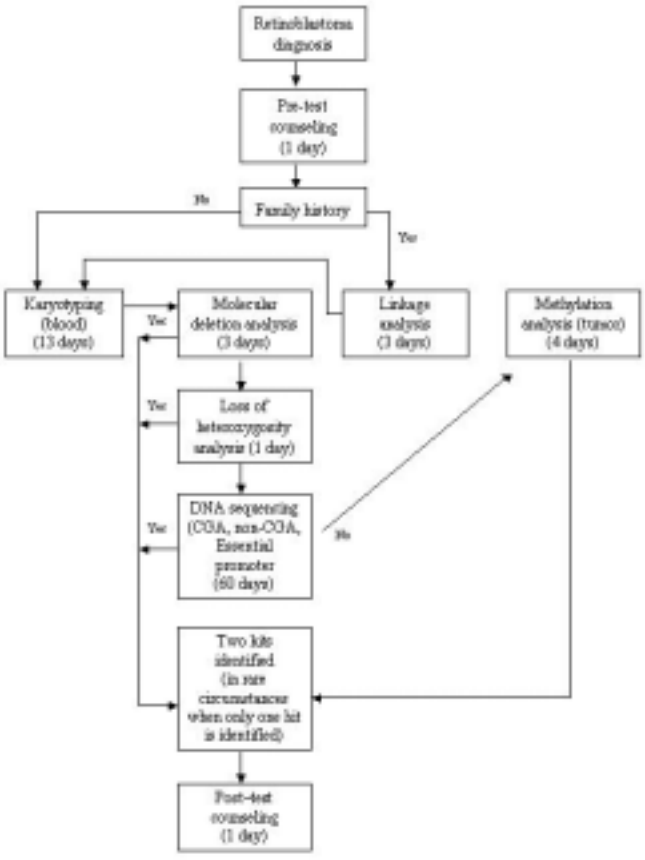

Figure 1. Diagnostic Model for Retinoblastomas

practice guidelines for molecular analysis of retinoblastoma' based on the reports drawn up from the workshops run by EMQN (Lohmann et al., 2002). In the direct testing strategy, mutation analysis was done from peripheral blood of bilateral and from tumor of unilateral cases. In familial retinoblastoma cases genotyping using co-segregating linked markers were used and mutational analysis is carried out from the peripheral blood DNA.

Richter et al., (2003) designed a sensitive and efficient RB1 gene mutation detection strategy using a combination of quantitative multiplex PCR for changes in copy number, allele specific PCR for four recurrent mutations, double-exon sequencing and promoter targeted methylation screening. Assay ordering of QM-PCR multiplexes and AS4-PCR reduced the turnaround time for RB1 screening to 2.7 weeks and detected 89% of the mutations in bilateral probands and both mutant alleles in 84% of the tumors from unilateral probands (Richter et al., 2003).

In the proposed model, LOH analysis costs around US$ 9.72 (5.3%), RB1 gene mutational screening US$ 146.46 (79.88%), methylation analysis US$ 3.67 (2.0%) and cytogenetic analysis US$ 22.37 (12.8%). We prefer to perform the gold standard test of DNA sequencing for all the exons rather than SSCP or any other rapid methods, as this is a critical test that cannot be compromised for the inherent disadvantages of these rapid methods. Assuming we did not exit till the last step in the genetic testing algorithm and did the entire spectrum of the tests with some of them being carried out simultaneously, about 84 days are required to exit from the diagnostic model.

DNA diagnosis in retinoblastoma, either by direct or indirect analysis of the RB1 gene defects, will be helpful for retinoblastoma families in counseling. Retinoblastoma families with several severely affected patients might consider prenatal diagnosis. Newborn infants and young children carrying a predisposing RB1 germinal mutation could be examined every two to four weeks immediately after birth. Children found not to be at risk could be spared unnecessary ophthalmic examinations and general anesthesia. Clinically unaffected sibs and offspring planning to start a family of their own might want to know whether they carry the RB1 mutation. The usefulness of RB1 genetic testing has been proved in follow up studies (Cohen et al., 2001).

In the current study, a diagnostic model for retinoblastoma genetic testing was done under research setting and currently the model is incorporated in our regular clinical service. We also suggest that this model could be extended in any tumor suppressor genetic defect for example in familial adenomatous polyposis coli with some modifications.

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