Genetic testing of retinoblastoma

Investigators : Dr. A. Vanniarajan, Dr. D. Bharanidharan, Dr. Usha Kim Prof. VR. Muthukkaruppan
Project Fellow : A. Aloysius Abraham
Funding agency : Aravind Eye Foundation, USA

Introduction

Retinoblastoma (RB) is an intraocular childhood malignancy with highest incidence in India among all developing countries. Biallelic inactivation of *RB1* gene is known to be the first step in an avalanche of genetic events. Genetic testing plays a major role in risk prediction of siblings and offspring, thereby enhancing the management of the disease. A stepwise strategy has been adopted for the genetic testing of retinoblastoma using multiple methods such as Sanger sequencing, Multiplex Ligation dependent Probe Amplification (MLPA), and Methylation specific MLPA (MS-MLPA). Recently, Next Generation Sequencing methods using the Illumina Miseq platform were also utilized to understand the additional genetic changes other than *RB1*.

Results

Sanger Sequencing and MLPA

During the year, a total of 34 RB patients were screened for genetic alterations in *RB1* gene. Mutations were identified in 12 out of 14 bilateral patients in their blood samples and hence an increased chance of inheriting the mutation in their offspring was predicted. Among the 20 unilateral patients, tumor was accessible in 3 patients and two heterozygous mutations were identified in tumor but not in blood of 2 patients. In other 17 patients, blood samples were analysed but mutations were not identified implying that they may not have germline mutations and hence their siblings or offspring had very low or no risk of inheriting the pathogenic *RB1* mutation. Based on the test results, genetic counselling was also provided for two older unilateral patients who wanted to know the risk of the secondary malignancies and inheritance of RB to their offspring.

Next Generation Sequencing

Agilent Sureselect XT custom panel was designed and developed for 70 genes that included exonic regions of 24 RB related genes and hotspots of 46 cancer related genes. Libraries were prepared for 28 samples (16 tumor and 12 blood) and 2x150bp paired-end sequencing was performed in Illumina Miseq platform using Miseq V2 kit. A sequencing depth of 250X was achieved with Q30 phred score of 96%. The in-house bioinformatics pipeline used for data analysis could detect both the single nucleotide variants (SNVs) and copy number variants (CNVs).

Some of the key findings of the analysis include:

i. Mosaicism

In the NGS run, the samples with known status of *RB1* mutations were included. All the *RB1* mutations detected by Sanger were concordant in NGS as well. Along with that, a mosaic mutation in a patient as shown in figure 1, which was present at 15 % level (56 out of 378) was detected, which was not detected by Sanger sequencing.

ii. Loss of Heterozygosity (LoH)

The identification of compound heterozygous mutations or a homozygous mutation of *RB1* gene in tumors would be sufficient to find out the causative mutations. However, it may not be possible to detect whether the homozygous nature is due to copy neutral LoH or hemizygosity unless both Sanger sequencing and MLPA are done (Figure 2).

iii. Genetic alterations in other RB and cancer related genes

With the custom panel and bioinformatics pipeline, it was possible to detect both the copy number changes and pathogenic variations in cancer related genes. Out of 16 tumors analysed with CNVkit,

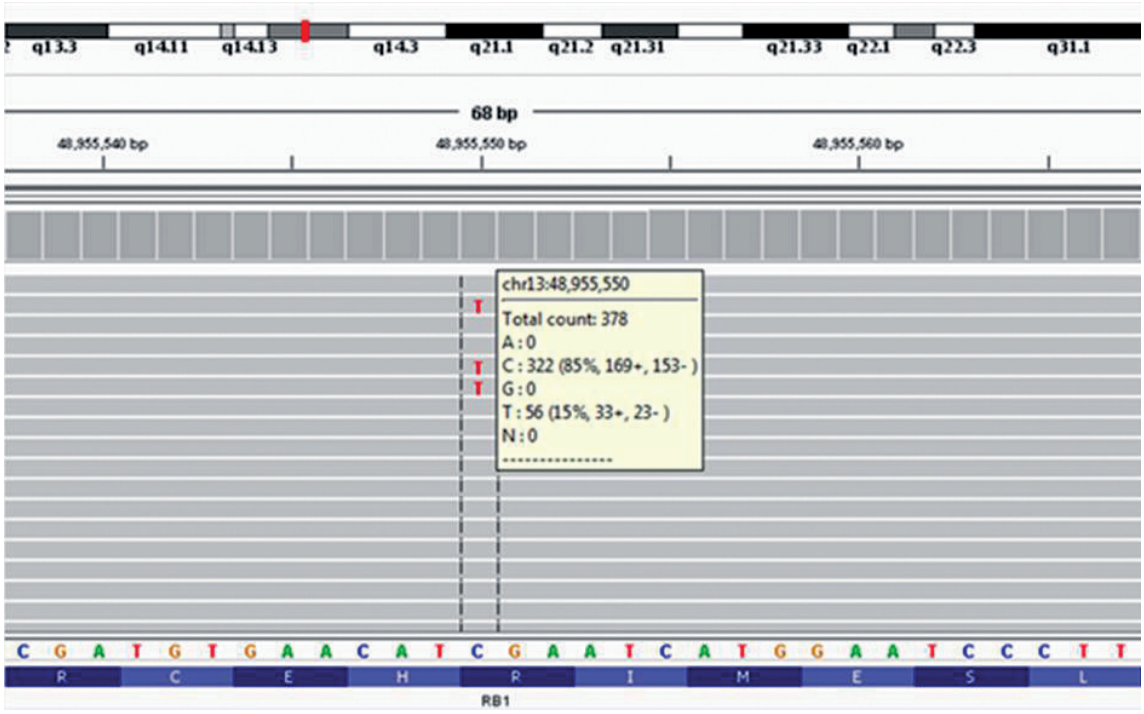

Fig 1: Snapshot of NGS reads by Integrated Genome Viewer (IGV) tool showing the mosaic mutation c.C1666T in exon 17 identified in a patient's blood

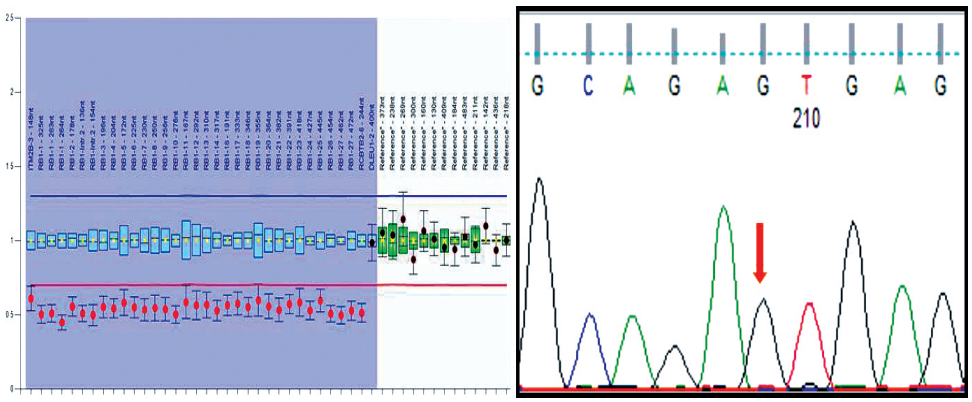

Fig 2: LoH in a patient identified by deletion of one copy of RB1 detected by MLPA leading to homozygous mutation (c.A2069G: p.N690S) detected by Sanger sequencing. Using NGS, it is possible to detect both the loss of RB1 and mutation in a single run.

frequent copy number gains were detected in KIF14 (9), MDM4 (10), MYCN (6), DDX1 (4), DEK (6), E2F3 (8), GATA5 (8) and recurrent loss were identified in TP53 (4), *RB1* (6), CDH11 (8) and CDH13 (8).

SNVs were found in WT1, ATM, FANCF, and BRCA2 that were predicted to have deleterious effect and have involvement in pathogenesis. In few patients without RB1 mutations, variants in tumor suppressor genes TP53, STK11 and cell cycle gene CHEK2 were detected which need further evaluation to elucidate their role in RB tumor formation.

Conclusion

Genetic testing of *RB1* gene has helped our adult RB patients to know the risk of getting secondary malignancies and inheriting the retinoblastoma

to the offspring along with the management in paediatric patients. NGS methods had increased the sensitivity of the genetic testing through detection of mosaic mutations and improved the understanding on the mechanism of LoH in a single run. NGS had also paved the way for understanding the process of tumorigenesis though the analysis of other RB and cancer related genes.

Understanding the molecular basis of chemoresistance in retinoblastoma

Investigators	:	Dr. A. Vanniarajan, Dr. Usha Kim, Dr. R. Shanthi, Prof. VR. Muthukkaruppan
Project Fellow	:	T.S. Balaji
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Introduction

Chemoresistance is clinically defined as lack of reduction in tumor size or relapse after initial response upon chemotherapy. Though different mechanisms have been reported in chemoresistance of cancer, ABC transporter mediated chemotherapeutic drug efflux is widely suggested mechanism. PGP/ABCB1 and MRP1/ABCC1 are widely studied ABC transporters in many cancers including retinoblastoma. One of the objectives of the study is to analyse the role of ABC transporters in chemoresistance of retinoblastoma.

Results

Gene expression analysis of ABC Transporters

Out of 48 ABC transporters, 31 were reported to be expressed in Y79 cell line. Hence gene expression analysis was carried out using semiquantitative reverse transcriptase PCR with Y79 and neural retina samples. The primers were optimised for 17 transporters and real time PCR will give the relative quantity of these transporters in the cell line versus neural retina control.

Selection of patients for studying chemoresistance

The treatment regimens for retinoblastoma include 6 cycles of Vincristine, Etoposide & Carboplatin (VEC) at a suitable concentration. If there is no response or relapse after initial response, Cyclosporine A (modulator of PGP/ABCB1) is added to the normal regimen. For this study, 27 were selected out of 86 retinoblastoma patients, who underwent minimum 6 cycles of VEC and 2 cycles of Cyclosporine A. RNA was isolated from the tumors of 6 eyes enucleated after treatment failure with chemotherapy.

Gene expression of ABC Transporters in tumors with treatment failure

Real-time PCR analysis was done to study the gene expression of 3 ABC transporters (PGP/ABCB1, MRP1/ABCC1 & BCRP/ABCG2) in 6 tumor samples with treatment failure. One of these tumors showed upregulation of 2 ABC transporters (MRP1/ABCC1 & BCRP/ABCG2) and other tumor had increased expression of PGP/ABCB1 compared to neural retina with B2M as normalizing gene.

Conclusion

The preliminary results show that some of the ABC transporters have a potential role in the chemoresistance of retinoblastoma. Further analysis of these transporters and other genes would enhance knowledge on chemoresistance and might help to develop newer drugs.