Non-invasive diagnosis of retinoblastoma using cell-free DNA from aqueous humour

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ABSTRACT
Retinoblastoma is the most common eye malignancy in childhood caused by mutations in the RB1 gene. Both alleles of the RB1 gene must be mutated for tumour development. The initial RB1 mutation may be constitutional germline or somatic (originating in one retinal cell only). Distinguishing between these alternative mechanisms is crucial, with wider implications for management of the patient and family members. Bilateral retinoblastoma is nearly always due to a constitutional mutation; however, approximately 15% of unilateral cases also carry a germline mutation, and identifying these cases is important. This can be achieved by identifying both mutation types in tumour tissue and excluding their presence in blood. Modern eye-saving chemotherapy treatment (systemic, intra-arterial and intravitreal) has resulted in fewer enucleations. As a result, tumour tissue required to identify sporadic RB1 mutation(s) is not always available. Modern intravitreal chemotherapeutic techniques for retinoblastoma involve aspiration of aqueous humour (AH), providing a novel sample source for analysis. By analysing cell-free DNA present in the AH fluid of eyes affected with retinoblastoma, we have developed a screening test capable of detecting somatic RB1 mutations that is comparable to current tests on enucleated tumour tissue. The results obtained with fluid from enucleated eyes were concordant with tumour tissue in all 10 cases analysed. In addition, AH analysis from two patients undergoing intravitreal chemotherapy successfully identified somatic variants in both cases. Our findings suggest that AH fluid is a promising source of tumour-derived DNA in retinoblastoma eyes to monitor somatic variants.

INTRODUCTION
Retinoblastoma (RB) is the most common childhood eye cancer with an incidence of −1:20 000 live births (reviewed in Lohmann and Gallie). Most retinoblastomas are caused by biallelic mutations in the RB1 gene, with non-germline cases being caused by two somatic hits, and inherited cases caused by an initial germline hit and a further somatic hit. Determining the genetic cause of RB is important for planning management of individual cases and prognostic counselling. Patients with RB1 germline mutations are at risk of bilateral disease and non-ocular cancers. There is also the additional risk that other family members may carry the same mutation. Analysis of peripheral blood samples along with tumour DNA (tDNA) accessed through enucleation allows for the complete genetic picture to be uncovered. The possibility that a patient has inherited RB can only be discounted by the detection of two mutations within the tumour sample, which are not present in the blood. Increased treatment success with modalities such as systemic or locally delivered chemotherapy suggests that tDNA is often unavailable rendering it impossible to identify somatic RB1 mutations.

The existence of cell-free DNA (cfDNA) in peripheral blood was noted in 1989 and has since been utilised for many applications including cancer detection (reviewed in Stewart and Tsui) as well as for non-invasive prenatal diagnostics (reviewed in Wong and Lo). We hypothesised that intraocular fluid removed during intravitreal chemotherapy (IVC) treatment would be a source of cell-free tumour DNA and therefore useful for detection of somatic variants in patients with retinoblastoma and other ocular disorders. This publication reports our findings using next-generation sequencing (NGS).

Together with the recently published study of Berry et al., this demonstrates that aqueous humour (AH) can be used as a surrogate biopsy for analysis of tumour-derived DNA in retinoblastoma eyes to monitor somatic variants.

MATERIALS AND METHODS
Sample collection and processing
Following ethical approval, subjects were recruited through the national referral retinoblastoma unit. Approximately 100 µL of AH fluid was taken from chemonaive primary enucleated eyes by anterior chamber tap prior to opening the eye for tumour dissection, or prior to injection during IVC treatment in eyes with recurrent disease following prior systemic chemotherapy and local treatment. Venous blood was collected at the same time for genetic testing.

Targeted massively parallel sequencing (MPS) and bioinformatic analysis
DNA extracted from blood, AH fluid and tumour (enucleated samples only) was sequenced by MPS following targeted capture enrichment for the promoter and exonic regions of the RB1 gene (NM_000321; chr13: 48,877,549–49,056,076, hg19), highly heterozygous single nucleotide polymorphisms and non-polymorphic regions across chromosome 13. Bioinformatic analysis performed for single-nucleotide variant (SNV), copy number
Table 1 MPS results for enucleated (E1–10) and IVC (IVC1–2) samples

| Patient | cfDNA conc (ng/μl) | RB1 mutation | gDNA % mutation | tDNA Result % mutation | cfDNA Result % mutation |
|---------|------------------|--------------|-----------------|-----------------------|------------------------|
| E1      | 2.12             | c.13663C>T p.(Arg455*) |                | 76                    | 91                     |
| E2      | 228              | c.751C>T p.(Arg251*)   |                | 91                    | 99                     |
| E3      | 0.183            | c.1959dupA         |                | 76                    | 87                     |
| E4      | 394              | c.763C>T p.(Arg255*) |                | 99                    | 90                     |
| E5      | 0.169            | c.1251_1252delAA    |                | 99                    | 90                     |
| E6      | 0.141            | Deletion exons 1–17  |                | 91                    | 94                     |
| E7      | 244              | c.1496_97dup p.Arg500Alafs*20 | 8 | 90                    | 94                     |
| E8      | 1.96             | c.1072 C>T p.(Arg358*) | 21†            | 97                    | 100                    |
| E9      | 1.01             | c.958C>T p.(Arg320*) |                | 43                    | 44                     |
| E10     | 1.38             | c.147delT          |                | 38                    | 46                     |
| IVC 1   | <0.100           | c.751C>T p.(Arg251*) |                | N/A                   | 49                     |
| IVC 2   | <0.100           | c.1654C>T p.(Arg552*) |                | N/A                   | 58                     |

Results are shown as percentage of mutation sequencing reads in gDNA, tDNA and cfDNA where appropriate. Complete LOH corresponds to LOH of whole chromosome 13; partial LOH indicates LOH of parts of chromosome 13, encompassing 13q14.

AH, aqueous humour; IVC, intravitreal chemotherapy; LOH, loss of heterozygosity; MPS, massively parallel sequencing; RB, retinoblastoma; cfDNA, cell-free DNA; gDNA, genomic DNA; tDNA, tumour DNA.
that our NGS assay can detect a variety of RB1 mutations at varying levels. We then attempted to detect these mutations in cfDNA extracted from the AH. In all but one case, mutation results from cfDNA were fully concordant with results obtained from tumour tissue (table 1). In the remaining patient, while we were able to detect the two large RB1 exonic deletions present in the tumour, the start point of each deletion was discordant between the two sample types (figure 2). Interestingly, in all cases, the mutant RB1 allele frequency was shown to be >90% in the cfDNA where one allele of RB1 remained (LOH cases) or >45% where two RB1 alleles (one mutant and one wild type) were present, suggesting that >90% of the cfDNA present in AH of enucleated eyes is of tumour origin. This result supports our size profile results.

**DISCUSSION**

The introduction of new techniques in the conservative management of retinoblastoma, including IVC, has dramatically reduced enucleation rates. This study demonstrates the feasibility of testing AH samples in non-enucleated eyes, with AH cfDNA enabling detection of somatic variants in patients undergoing IVC where tumour is not available. Capture-based technology was used to identify previously undetected RB1 gene mutations and LOH alterations in AH cfDNA. This extends the findings of Berry et al and demonstrates the clinical utility of our approach although further development is required to increase the accuracy in detecting larger exon-spanning RB1 mutations. Our data also suggests that the majority of cfDNA in the AH is from the tumour due to the compartmentalised nature of ocular fluids, a factor which facilitates this test.

Among the enucleated eyes tested, higher levels of cfDNA were seen in eyes undergoing primary enucleation. Adequate but lower DNA levels were present in eyes treated with systemic chemotherapy and local treatment such as laser and cryotherapy. This would suggest the cfDNA load varies with the tumour load.

The distinction between germline and somatic mutations is vital, as germline cases need close monitoring with short-term risk of new ocular tumours and long-term risk of second systemic cancers. The risks of both are greatly reduced if there is evidence to indicate a constitutional mutation is highly unlikely. Furthermore, there are important implications for screening examinations in the wider family (siblings and offspring).Confirming the non-heritable nature of the retinoblastoma can avoid screening

**Figure 2** Example of copy number variant (CNV) analysis within the genomic DNA (gDNA), tumour DNA (tDNA) and cell-free (cfDNA) of sample E6 showing the detection of two large RB1 deletions in both the tDNA and cfDNA. CNVs are detected by comparing the relative depth of coverage achieved at each target site within the sample against a reference set of normal controls. Vertical lines indicate the positions of the target sites capturing the RB1 exons.
examinations in many family members, with significant cost savings and avoid potential morbidity in unaffected family members, previously screened unnecessarily.

In view of the extensive safety record of carefully performed intraocular procedures in eyes with retinoblastoma (Munier, personal communication), the results of our study, and that of Berry et al, there appears to be a clear clinical benefit of diagnostic AH taps in apparent non-genetic retinoblastoma where the affected eye is retained.

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Competing interests None declared.

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