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We report the first use in Hong Kong of molecular techniques to screen prenatally for retinoblastoma and review 17 cases of retinoblastoma seen at the Hong Kong Eye Hospital from 2001 to 2006. A pregnant couple whose first child had retinoblastoma requested prenatal screening for retinoblastoma during their second pregnancy in 2000. Whole *RB1* coding gene sequencing was performed on peripheral blood cells taken from family members and cultured amniocytes collected from the foetus during the 14th week of gestation. No *RB1* gene mutations were found in the amniocyte samples and at birth the baby had no evidence of ocular tumours. During 5 years of follow-up the child remained healthy with intact visual function. Prenatal diagnosis of retinoblastoma alleviates parental stress and improves the perinatal care of affected family members.

Introduction

Retinoblastoma (*RB1*; OMIM accession number: 180200) affects 1:15000-28000 live births in most populations.¹ It is the most common primary paediatric intra-ocular malignancy² accounting for about 3% of all childhood cancers,³ and has no predilection for sex,^{3,4} ethnicity,^{5,6} or geographical location.⁵ About 60% of cases are sporadic and these account for most unilateral cases. The rest are hereditary and, in 85% of these, both eyes are affected. Both sporadic and hereditary retinoblastoma involve the inactivation of the *RB1* gene.^{7,8}

Although retinoblastoma is treatable and the 5-year survival rate in Chinese populations is quite high (reported as 80.9% in Taiwan⁹), delayed diagnosis leads to severe impairment of visual acuity and hence a poor quality of life. Moreover, the costs of managing retinoblastoma diagnosed late, such as chemotherapy drugs and the special aids needed by low-vision patients, are a burden for both the health care system and the family. In the United States and Canada, it has been reported that molecular *RB1* mutation identification has saved at least US\$4200 per family,¹⁰ and has removed the need for expensive and time-consuming clinical examinations. Therefore, we believe early retinoblastoma screening should be made available for high-risk foetuses and describe Hong Kong's first successful case of prenatal screening for retinoblastoma.

Genetics of retinoblastoma

Retinoblastoma is inherited as an autosomal dominant trait¹¹ and is an archetypal model of Knudson's "Two Hit Hypothesis" of tumour genesis.¹² The causative Retinoblastoma 1 (*RB1*) gene, located in chromosome 13q14.1-q14.2, was the first tumour suppressor gene identified⁸ in cancer development. It consists of 27 exons of different sizes ranging from 31 to 1889 base pairs.¹³ Several possible forms of genetic and epigenetic change to the *RB1* gene have been reported in retinoblastoma, including small deletions, point mutations, and DNA hypermethylation at the promoter region.¹⁴⁻¹⁶ The *RB1* mutations scatter in most coding exons (*RB1* gene mutation database, <http://www.verandi.de/joomla/>). Although no 'hotspot' mutation has been identified, two recurrent nonsense mutations, R320X and E54X have been identified in our local Hong Kong population.¹⁷ Nonsense mutations that abrogate the two globular (A and B) domains required for the binding of Rb1-associated proteins account for around 80% of *RB1* gene mutations,¹ and together with splice mutations are the most frequently identified (50% and 26% respectively) mutations in familial hereditary retinoblastoma.¹⁸ In Chinese populations, *RB1* inactivation by loss of heterozygosity at chromosome 13 is associated with 60% of sporadic retinoblastoma cases.¹⁷ Apart from the *RB1* gene, epigenetic silencing of DNA repairing genes (*MGMT* 35%, and *MLH1* 67%) and another tumour suppressor gene (*RASSF1A* 82%) are also associated with retinoblastoma pathogenesis.¹⁹⁻²¹ Such epigenetic events where promoter hypermethylation occurs in these three genes are not mutually exclusive but frequently occur together (86%) in sporadic retinoblastoma.

Key words

Eye neoplasms; Mutation; Prenatal diagnosis; Retinoblastoma; Tumor cells, cultured

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香港視網膜母細胞瘤的產前篩查

我們報告香港首宗應用分子標記作視網膜母細胞瘤產前檢查的案例，並回顧2001到2006年香港眼科醫院處理過的17宗視網膜母細胞瘤病例。一對夫婦的長女患有視網膜母細胞瘤，2000年他們要求為其第二胎作視網膜母細胞瘤的產前檢查。醫院為他們及其長女的外周血細胞作*RB1*基因全序列測定，並於妊娠14週時從胎兒抽取羊水細胞檢驗。羊水細胞內並未發現有*RB1*基因突變，這名小孩出生時也沒有眼部腫瘤的徵象，五年後視覺仍然良好。視網膜母細胞瘤的產前檢查可減低父母的壓力，並且對受影響的家庭成員可以有更妥善的圍產期護理。

and Scheffler⁶). Therefore the investigation of the *RB1* mutation pattern in an at-risk foetus offers not only an early diagnosis of retinoblastoma, but also evaluates the likelihood of other non-ocular tumours developing.

Successful prenatal diagnoses using mutation screening have been demonstrated in the United States and Canada¹⁰ where nine foetuses carrying germline *RB1* mutations were identified. In the only four families that opted to continue the pregnancy, all the infants, who were delivered at 35 weeks of gestation, developed bilateral retinoblastoma (1/4) or macular tumours (3/4). Fortunately, early focal treatment enabled retention of 6/6 vision in all eight eyes. All the infants with negative mutation screening results remained free of retinoblastoma.

Incidence of retinoblastoma in the Hong Kong Eye Hospital

A total of 17 new retinoblastoma cases were diagnosed and treated in the Hong Kong Eye Hospital (not including referred cases) from January 2001 to December 2006 (Fig 1). The male-to-female ratio was 2.4:1 (12:5). All patients had unremarkable family histories. Ten had retinoblastoma in one eye only, while the other seven had bilateral involvement, a ratio consistent with findings reported by other centres.⁹ Of the seven patients with bilateral involvement, one individual had neoplastic changes in the pineal gland (trilateral retinoblastoma with germline 744X truncated *RB1* mutation), and died 12 months after the diagnosis. No secondary tumours were found in the other 16 patients who were all followed up at the Hong Kong Eye Hospital.

First case of prenatal screening for retinoblastoma in Hong Kong

Background

The first attempt to screen for retinoblastoma prenatally was successfully performed in the Hong Kong Eye Hospital. A pregnant couple (father aged 45, mother aged 30 years) approached the Hong Kong Eye Hospital in 2000 for genetic counselling about the likelihood of their second child developing retinoblastoma. The couple had no history of retinoblastoma, or other relevant eye diseases, but their first child, a daughter, had developed bilateral retinoblastoma at 4 months of age. Her left eye was enucleated and the other was treated with a combination of cryotherapy and radiotherapy. She had been reviewed at the Hong Kong Eye Hospital every 4 months since then. A genetic analysis identified a truncated germline *RB1* mutation (g64348C/T; R320X). The parents were worried that their second child may have inherited the same mutated *RB1* gene and be likely to develop retinoblastoma, so they requested

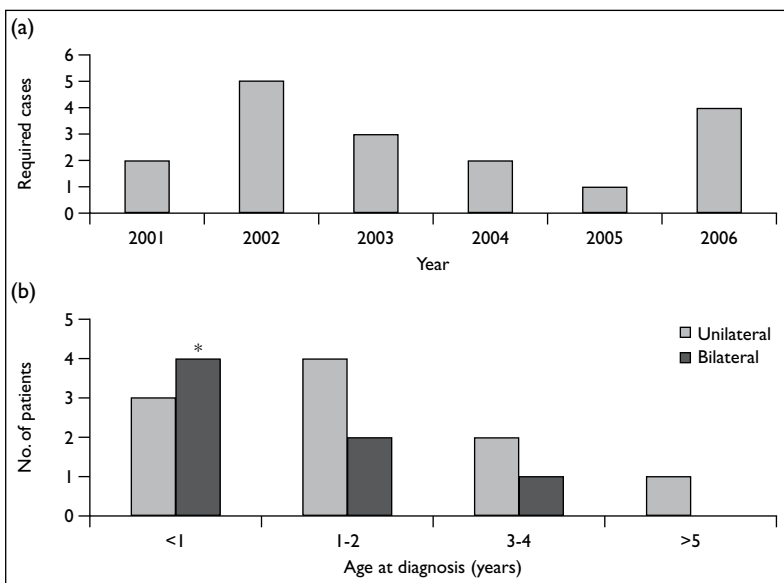


FIG 1. (a) Distribution of retinoblastoma cases at the Hong Kong Eye Hospital from 2001 to 2006. (b) Relationship of age at diagnosis with number of eyes involved
* Trilateral retinoblastoma case

Significance of prenatal *RB1* mutation screening

Penetrance and disease expression are associated with the specific mutation pattern (genotype), ie the genotype-phenotype correlation.¹⁸ It has been found that retinoblastoma patients carrying premature stop codons and large rearrangements in the *RB1* have the highest penetrance whereas missense mutations have the lowest penetrance of all types of mutation.¹⁸ In Hong Kong, 19% of retinoblastoma cases carry a germline *RB1* mutation, and 47% (7/15) of all *RB1* mutations are nonsense mutations.¹⁷ Patients with germline (hereditary) mutations are more likely to develop second tumours (30% after 40 years) and have a 22% higher chance of developing a third tumour.²² Second non-ocular cancers observed in retinoblastoma survivors with germline *RB1* mutations include osteosarcoma, lung cancer, pineoblastoma and other paediatric cancers (reviewed by Abramson

prenatal screening for retinoblastoma.

Methods using DNA extraction and polymerase chain reaction amplification

Cultured amniocytes taken from the mother at 14 weeks of gestation and peripheral blood cells taken from the couple and the sister with retinoblastoma were used for DNA extraction for *RB1* mutation analysis. Genomic DNA from all family members was extracted using the QIAmp Blood Kit (Qiagen Inc, CA, US) according to the manufacturer's protocol. The promoter and all 27 coding exons of the *RB1* gene were amplified using specific forward and reverse primers for each exon.²³ Subsequently, the amplified *RB1* gene products were sequenced and analysed using the dRhodamine Dye Terminator Cycle sequencing kit (Perkin Elmer, CA, US) on an automated ABI PRISM 377 Sequencer (Applied Biosystem). Sequence alterations were further confirmed by both forward and reverse sequencing.

Results

Whole *RB1* gene screening detected three nucleotide alterations in the *RB1* genes (g.2302G/A, g.39606T/C and g.174438T/C) of the parents, the affected child, and the foetus (Fig 2). In addition, an extra nucleotide alteration (g.64348 C/T) was found in the affected child. The hereditary de-novo mutation, g.64348 C/T, leads to a truncated mutation at codon 320 (R320X), which is a recurrent mutation that has been reported previously.^{17,24} This was not detected in the foetus, nor was any other known disease-associated *RB1* mutation found on whole gene sequencing. In the absence of any germline *RB1* mutations, the foetus' chances of getting sporadic retinoblastoma were similar to those of normal children: approximately 1 in 15000-28000 live births. The parents were satisfied with the result, and their anxiety was alleviated. A few months later a healthy infant with no evidence of retinoblastoma was born. The child is now 5 years old and has passed the peak age for retinoblastoma (0-4 years; 10.6 per million) and has perfectly normal ocular function. The two children will be followed up by paediatric ophthalmologists from the Hong Kong Eye Hospital.

Discussion

Prenatal screening is indicated for couples at risk of giving birth to babies with serious genetic disorders to relieve their anxiety, particularly that of the mother. Although retinoblastoma is a rare neoplasm, it is the most common intra-ocular tumour in children. More importantly, retinoblastoma is curable and the prognosis is highly dependent on the timing of diagnosis. In addition, the genetic features of the pathogenesis of retinoblastoma are now well

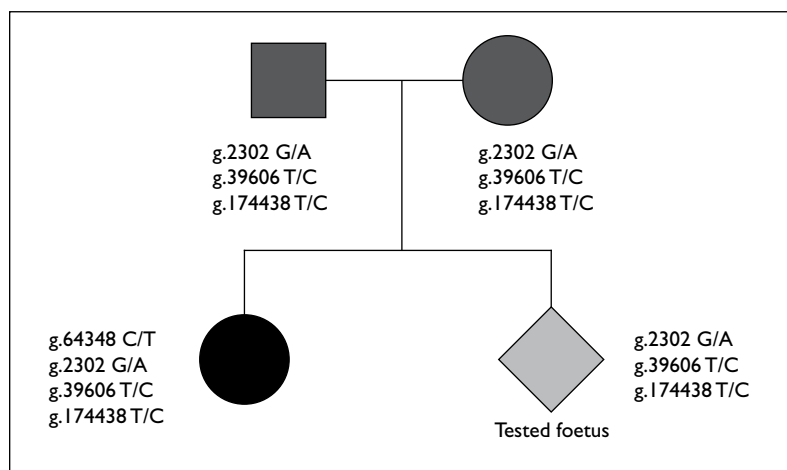


FIG 2. Pedigree diagram showing nucleotide alterations in family members
Circle in black denotes bilateral retinoblastoma

understood, further improving the sensitivity of molecular diagnosis. For these reasons, genetic screening should be included in the management plan for patients with retinoblastoma and those suspected to have it. Prenatal amniocyte *RB1* screening as described in this report provides a means of early antenatal management for the foetus at risk. We have demonstrated that the development of sensitive genetic tests for the detection of *RB1* mutations has improved the identification of children at risk. This genetic screening method provides an indication for detailed neonatal ophthalmic examinations and potentially diminishes the economic and psychological impact of the disease.

The reasons for amniocentesis and the risk of miscarriage are important issues to be discussed with the parents. A meta-analysis reported 99.32% sensitivity and 99.86% sensitivity for using amniocytes for prenatal diagnosis of cytogenetic alteration-related diseases; the excess miscarriage risk of amniocentesis was reported as 0.75% in the same study.²⁵ Similar results (0.6% excess miscarriage risk) were also found by a meta-analysis of over 1000 amniocentesis cases.²⁶ These results show that prenatal diagnosis using amniocentesis carries low risks for both the mother and foetus. Amniocentesis and chorionic villus sampling have been performed for prenatal diagnosis of genetic diseases such as thalassaemia, haemophilia A, and Huntington's disease for about 20 years in Hong Kong, so we have skilled obstetricians and mature techniques able to ensure safe clinical procedures during sample collection.

There have been extensive molecular studies on the *RB1* gene alterations associated with retinoblastoma. Since no 'hotspot' mutations have been identified in retinoblastoma, whole *RB1* gene screening remains the best option for prenatal

diagnosis, with a reported high sensitivity (89%).¹⁰ Given the accuracy and safety of prenatal genetic analysis, the benefits patients gain from early management of the disease and the costs saved by

avoiding unnecessary ophthalmic follow-up, prenatal diagnosis using *RB1* mutation screening is a good choice for early diagnosis of retinoblastoma in Hong Kong.

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