
CHAPTER

1

GENERAL INTRODUCTION

INTRODUCTION

Retinoblastoma (Rb) is a rare malignant tumour of the developing retina, originating in an embryonic retina cell precursor. It is a disease of early childhood and tumour formation may start before birth. If left untreated, Rb is fatal. With timely screening and diagnosis and adequate therapy, Rb patients have an excellent prognosis: more than 97% of patients survive their eye cancer, many with useful vision.[1-4] Identification of the *RB1* mutations, in the germline and in the Rb tumour, can be important in the clinical management of patients with Rb. Furthermore, this information can aid families in reproductive decision making. As most Rb patients survive into adulthood and childbearing age, they may face an increased risk for a child with Rb. Not much is known about the influence of this increased risk on reproductive decisions of couples with an increased risk for a child with Rb.

Second primary tumours (SPT) are now the leading cause of death in patients with heritable Rb.[5] If genotype-phenotype associations for SPTs exist, this could be of influence on surveillance recommendations and choice of therapy.

Throughout the medical literature and within this dissertation both the words 'hereditary' and 'heritable' are used. The preferred word nowadays is 'heritable', as many retinoblastoma patients have no family history of retinoblastoma.[6]

This thesis addresses the molecular genetic and clinical genetic aspects of Rb. This introductory chapter provides background information on Rb and is followed by a description of the aims and outline of the thesis.

HISTORY OF RB DIAGNOSIS

The first description of Rb has been attributed to a Dutch physician, Pieter Pauw (or Pawius in Latin) in 1597. He described a malignant tumour in a 3-year old child, invading the orbit, the temporal region and the cranium.[7] Some authors, however, claim Pauw's description may have been another tumour.[8] In 1805 William Hey used the term Fungus Haematodes to describe tumours in different parts of the body, including the eye.[9] In 1809 James Wardrop was the first to describe Rb as a clinical entity and noted that the retina seemed to be the origin of the tumour.[10] He also described enucleation as a therapy and observed the tumour was mainly confined to children. In 1851 Helmholz introduced the ophthalmoscope, making earlier diagnosis possible.[11] In 1864, Virchow described the tumour as a retinal glioma.[12] In the 1920s it was the ophthalmologist Verhoeff who proposed the term "retinoblastoma", and in 1926 the American Ophthalmological Society adopted this term.[13]

RB REGISTER

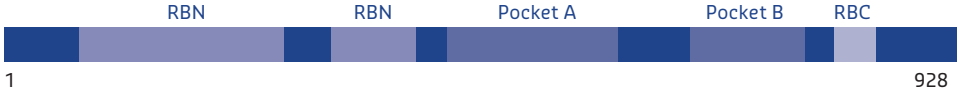
In the Netherlands, ten to twelve children are diagnosed with retinoblastoma each year.[14] In 1930 G.D. Hemmes started to collect information about all Rb patients treated in the Dutch Hospital for Eye Diseased in Utrecht, the Netherlands and patients treated elsewhere, whose enucleated eye had been sent to this hospital for pathological confirmation. A total of 48 Rb patients were collected.[15] In 1957, this information was updated and completed at the request of the Netherlands General Association of Prevention of Blindness.[16,17] This registry has since then been updated regularly. It now contains patients from as early as 1862 and is virtually complete from 1945 onwards.[18-20] Information has been collected on demography, family history of Rb, tumour laterality, treatment for Rb and information on other cancers occurring in Rb patients. The registry forms a unique opportunity for research on Rb and has been used for data collection as part of this thesis.

Since 1991, all Rb patients in the Netherlands are treated in the National Retinoblastoma Treatment Center in the VU University Medical Center (VUMC) in Amsterdam by a multidisciplinary team.

RB1 GENE AND RB PROTEIN

In 1976 it was demonstrated that some patients with Rb carried a deletion on chromosome 13.[21] Dryja and co-workers cloned DNA from chromosome 13 containing the Rb locus and two years later cDNA with the properties of a gene was isolated.[22,23] In 1987 the tumour suppressor retinoblastoma gene *RB1*, located on chromosome 13q14.2, was identified (GenBank accession number L11910, MIM#180200).[24] The *RB1* gene has 27 exons and encodes a ubiquitously expressed nuclear protein of 110 kDa, consisting of 928 amino acids. The Rb protein is a multifunctional protein that is involved in cell cycle regulation (G1 to S transition), maintenance of genome stability and apoptosis.[25] The protein has several domains (**Figure 1**):

- The central pocket domain, consisting of two subdomains, A and B, each resembling a cyclin fold
- The amino-terminal domain (RBN), containing two additional cyclin folds and resembling the pocket domain
- The carboxy-terminal domain (RBC), which is formed by approximately the last 150 residues of the Rb protein
- Linker sequences that contain CDK (cyclin-dependent kinase)-dependent phosphorylation sites, that have a critical role in inactivation of the Rb protein



RBN: Rb amino-terminal domain

RBC: Rb carboxy-terminal domain

Figure 1. Schematic representation of the Rb protein. (Adapted by permission from Macmillan Publishers Ltd: Nature Reviews, Molecular Cell Biology25, copyright 2013)

The Rb protein interacts at least in two pathways involved in cell cycle control:
1) Rb-E2F (E2-binding factor) pathway
2) Rb-SKP2 (S phase kinase associated protein)-p27 pathway

At this moment there are several unresolved issues about these pathways, e.g. it is unclear whether one pathway has a more important role in some tissues than in others, or whether one pathway is crucial under distinct physiological circumstances. Nor is known whether the Rb protein can function in both pathways simultaneously or that they are mutually exclusive.[25]

Knudson's two hit hypothesis

In 1971, Knudson used Rb as a model to formulate his "two-hit" hypothesis for the initiation of cancer.[26] Based upon observations of age of diagnosis on 48 Rb cases and published reports he developed the idea that Rb is caused by two mutational events. In the heritable form one of the mutations is present in the germline and can be transmitted to offspring in an autosomal dominant way. In the non-heritable form, the two mutational events occur in somatic cells. Today this hypothesis still holds true in virtually all cases of Rb, although it is now known that more genomic changes are needed for tumour formation to develop.[27-29] Furthermore, recently it was shown that the paradigm of the two hit-hypothesis does not hold true for all Rb tumours, because in 1% of Rb cases the *RB1* gene is not mutated and MYC-N amplification initiates tumour formation.[30]

MUTATIONS IN THE *RB1* GENE

Over 1000 different mutations have been described in the *RB1* gene. Information about mutations can be accessed through the online Leiden Open Variation Database (LOVD).[31] Mutations have been found scattered throughout exon 1 to exon 27 and in the promoter region. Most mutations result in a premature termination codon. In the *RB1* gene are several methylated CGA codons known to lead to 11 recurrent nonsense mutations by C>T transitions.[32-34] An important

factor in the high recurrence of mutations at these sites was shown to be deamination of 5-methylcytosine.[35]
Low penetrance mutations of the *RB1* gene are defined on the basis of a diseased eye ratio (DER) of ≤1.5. The DER is calculated by dividing the total number of affected eyes per family by the number of mutation carriers in the family.[36] Low penetrance mutations are thought to lead to a protein with retention of partial activity or a reduction in the amount of normal Rb protein that is produced.[37] Missense mutations located in part of the gene coding for the pocket domain and mutations located near the 3'-end of the gene fit the former low penetrance explanation.[36,38] Some splice mutations, promoter mutations and exon 1 mutations are examples of low penetrance mutations due to reduced expression of Rb.[39,40]
In about 8% of cases the cause of Rb is a chromosomal deletion encompassing band 13q14.2. These patients have a developmental delay and often have congenital malformations and dysmorphic features, depending on the extent of the deletion.[41-43]
It is important to test all Rb patients for *RB1* mutations and obtain knowledge about genotype-phenotype relations. This information can aid in risk management and reproductive decisions.

MEDICAL ASPECTS OF RB

Epidemiology

The prevalence of Rb is between one in 16,000 to 18,000 live births.[19,44] Rb is usually diagnosed in the first few years of life. About 60% of individuals are unilaterally affected (mean age of diagnosis 24 months) and 40% are bilaterally affected (mean age of diagnosis 15 months).[45] In 10% of cases the disease is familial, whereas 90% of cases the Rb patient is the first in the family with Rb. Bilateral Rb is always caused by a germline mutation in the *RB1* gene (either inherited or de novo), whereas unilateral Rb can be both heritable or non-heritable. Around 15% of unilateral, non-familial Rb cases are heritable, based on a de novo germline *RB1* mutation.

Presenting signs and diagnosis

The most common presenting sign is leukocoria ("cat's eye") in a child, but strabismus, poor vision, nystagmus, red eye, and such atypical presentations as glaucoma, orbital cellulitis, uveitis can also lead to the diagnosis of Rb.[46]
The diagnosis can be established by examination of the fundus by indirect ophthalmoscopy and is supported by ultrasonography of the eye and MRI (Magnetic Resonance Imaging).

Staging

Staging is important to guide risk stratification and choice of therapy. In 1963 the Reese-Ellsworth classification of intraocular Rb was presented. This classification defined 5 groups based on size, location and number of tumours. It predicted the outcome of external beam radiotherapy. When in the 1980s chemotherapy was introduced, a new classification was needed. In 2003 the International Intraocular Retinoblastoma Classification was therefore introduced. The TNM (Tumour Node Metastasis) classification of malignant tumours is used to evaluate the extend of extraocular disease.

Treatment

In the Western world, Rb can be cured in more than 97% of cases. Choice of treatment depends on: tumour stage, number of tumour foci, location and size of the tumour in the eye, presence of vitreous seeding, laterality, age of diagnosis and familiarity.

Treatment options can include one or more of the following: enucleation of the eye, photocoagulation, cryotherapy, chemotherapy and radiotherapy. Chemotherapy can be administered systemically or locally: as intravitreous chemotherapy or as selective intraocular arterial chemotherapy (SIAC). Radiotherapy can be applied by brachytherapy (episcleral plaques) or by external beam radiation therapy.

When postlaminar involvement of the optic nerve or massive invasion in the choroid is shown on pathological examination after enucleation, systemic chemotherapy is added to the treatment to eradicate possible micrometastases.

Screening for Rb from birth

Children at increased risk for Rb are offered ophthalmological screening from birth, so adequate therapy can be started as soon as tumours arise, thus minimizing the sequellae of therapy. Starting at the age of 3 or 6 months these exams are performed under anaesthesia, depending on the magnitude of the risk of developing Rb. When no retinoblastoma has developed, screening is discontinued at the age of 4 years.[47]

Subsequent primary cancers

Carriers of a germline mutation are at increased risk for new primary tumours. The overall life time risk for subsequent cancers is 28% (CI 21-35%) after 40 years of follow-up.[48] Treatment with radiotherapy is associated with a further Increased risk, increasing the cumulative incidence to 33.2% (CI 24.6% to 41.8%).[48] Irradiation given below the age of 12 months may place the patient at greater risk of a subsequent cancer within the field of radiation, than when irradiation is delayed until 1 year of age.[49,50] The most common second cancers include: osteosarcoma, soft tissue sarcoma (e.g. rhabdomyosarcoma and leiomyosarcoma) and melanoma. Later in life common epithelial cancers are

seen more often, such as bladder cancer, lung cancer and breast cancer. Besides radiation as a risk increasing factor, *RB1* mutation carriers may have an increased susceptibility to carcinogenic effects of tobacco and UV light. The effect of chemotherapy on SPT risk is not well known.
Despite longstanding knowledge about an increased SPT risk for *RB1* mutation carriers, it is not known whether specific mutations might be associated with a greater risk for an SPT. This information may impact surveillance protocols for patients with heritable Rb and eventually may lead to targeted therapy for SPTs.

CLINICAL GENETIC ASPECTS OF RB

As mentioned above, the Dutch National Retinoblastoma Treatment Center is located at the VUMC. All patients are treated by a multidisciplinary team with a central role for two specialized ophthalmologists. All parents of newly diagnosed Rb patients and all adult Rb patients seeking information on the inheritance of Rb are referred to the clinical geneticist of the multidisciplinary team for genetic counselling.

Genetic counselling

In 1947 Reed was the first to use the term genetic counselling.[51] In 1975, the American Society of Human Genetics agreed on a definition of genetic counselling.[52] In 2006 the definition was adapted to better capture modern views on genetic counselling and to "meet health care demands imposed by the emerging era of genomic medicine".[53]
Genetic counselling is the process of helping people understand and adapt to the medical, psychological and familial implications of genetic contributions to disease. This process integrates the following:

— Interpretation of family and medical histories to assess the chance of disease occurrence or recurrence.
— Education about inheritance, testing, management, prevention, resources and research.
— Counselling to promote informed choices and adaptation to the risk or condition.

Since 1991, more than 350 patients with Rb and/or their parents have visited the Department of Clinical Genetics of the VUMC for genetic counselling on Rb.

Increased risk for a child with Rb

Healthy parents of a child with Rb may have an increased risk of having another child with Rb, as do Rb patients. This increased risk varies between less than 1 and 50%, and depends on the results of DNA testing and family history. Four possible situations for a counselee with an increased risk are displayed in **Table 1**.
When a child is affected by unilateral non-familial Rb and the eye is enucleated,

it is possible to perform *RB1* mutation testing on tumour material. When DNA scanning of the tumour detects two *RB1* mutations and when these two mutations are not detected in DNA from lymphocytes of the child, there is no increased recurrence risk for the parents.[45] However, there is a small chance (1–2%) of a (not detected) low-grade mosaicism in the child for one of the *RB1* tumour mutations. Therefore there will be a small recurrence risk for future children of the affected child (0.6%).[45]

Table 1. Possible family situations leading to an increased risk of developing retinoblastoma in offspring of counselees

Family situation	Rb risk for (future) offspring*
Counselee is a carrier of a germline *RB1* mutation	50%
Counselee has had unilateral non-familial Rb, without a detectable germline *RB1* mutation	0.5 - 1%
Counselees are healthy parents of a child with a de novo *RB1* mutation	2 - 3%
Counselees are healthy parents of a child with unilateral non-familial Rb without a detectable germline *RB1* mutation	< 1%

Rb = retinoblastoma

*Based on Genereviews[45] and an *RB1* mutation detection rate of 90%.

Reproductive options

Couples with an increased risk have several reproductive options. They may decide to remain childless, or, if they already have a(n affected) child, have no more children. They may adopt a child or choose gamete donation. If a germline *RB1* mutation is detected, couples can choose prenatal diagnosis (PND). If the child is a carrier of the mutation, they could opt for termination of the pregnancy, although some may find termination of a pregnancy of a potentially treatable disease debatable. If one of the parents is a *RB1* mutation carrier, preimplantation genetic diagnosis (PGD) may also be an option to avoid the birth of an affected child. PGD involves DNA testing of the parental *RB1* mutation in two cells of a 3-day old embryo during in vitro fertilization. Only embryos without the mutation are transferred to the uterus. Alternatively, couples can decide to accept the risk and have biological children without using the above mentioned options. Genetic counselling may facilitate informed reproductive decision- making. A Dutch interview study of 156 Rb survivors found that 12% of all Rb survivors in their study decided not to have children due to the increased risk of having a child with Rb.[54] Little is known, however, about reproductive behaviour of couples at increased risk for a child with Rb. To accommodate the potential needs with regard to genetic counselling of couples at increased risk of a child with Rb it is important to study the reproductive decision-making process of these couples.

IVF AND RB

In the Netherlands, around 2,5% of infants are born after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment, i.e. 1 in every 40 children.[55,56] Since 2001 there has been concern that IVF and ICSI might lead to an increased incidence of imprinting disorders in children conceived by IVF/ICSI. Anteby et al. reported on retinoblastoma in a child conceived by IVF.[57] Since then more cases have been reported.[58-61] Two IVF-register based studies did not find an increased incidence of retinoblastoma after IVF, although these studies may have lacked enough power to detect an increased risk.[62,63] Reports have suggested an association between IVF/ICSI and imprinting disorders like Beckwith-Wiedemann syndrome and Angelman syndrome.[64-66] Since the *RB1* gene contains imprinted regions[67], imprinting has been postulated as the possible link between Rb and IVF/ICSI.[60,68] It is important to get more insight in the possible association between IVF/ICSI and Rb.

AIMS OF THIS THESIS

The focus of this thesis is on clinical genetic aspects of retinoblastoma. In order to optimize genetic counselling for retinoblastoma, three main aims were formulated. The first aim was to extend genotype-phenotype knowledge on the *RB1* mutations of Dutch retinoblastoma patients and investigate cancer risks conferred by germline *RB1* mutations. The second aim was to explore the reproductive behaviour of individuals at risk for a child with retinoblastoma. And lastly, the third aim was to examine the role of in vitro fertilization (IVF) in the aetiology of retinoblastoma.

The research questions were:

- What is the spectrum of *RB1* mutations in the germline and tumour of retinoblastoma cases from the Dutch National Retinoblastoma Register?
  a) Is there a genotype-phenotype relation between germline *RB1* mutations and the risk of second primary tumours in retinoblastoma survivors?
- What is the reproductive behaviour of individuals with an increased risk for a child with retinoblastoma?
  a) What factors are of influence on reproductive decision-making for couples with an increased risk for a child with retinoblastoma?
  b) How does the uptake for prenatal diagnosis for retinoblastoma compare to other hereditary cancer syndromes?
- Is there an association between IVF and retinoblastoma?
  a) What is the incidence of retinoblastoma in children conceived by IVF?
  b) Is there a difference in the type of *RB1* mutations in retinoblastoma tumours occurring in children conceived by IVF when compared to children conceived without assisted interventions?

OUTLINE OF THE THESIS

Chapter 2 describes the complete results of *RB1* mutation screening in the Netherlands, from the start in 1992 up until January 2013. In Chapter 3 the risk of second primary tumours is investigated in patients with hereditary Rb according to type of *RB1* mutation. Chapter 4, 5 and 6 present the results of studies concerning reproductive behaviour of individuals at increased risk of having Wcouples at increased risk of a child with retinoblastoma and explores the impact of prospective risk on reproductive decisions, factors influencing these decisions, and the needs of couples with regard to reproductive counselling. Chapter 5 is a cross-sectional questionnaire survey investigating the reproductive behaviour of individuals at increased risk of having a child with Rb. And in Chapter 6 the uptake of prenatal diagnosis for Rb is compared to prenatal diagnosis for other hereditary tumour syndromes.

In Chapter 7 and 8 the association between IVF and retinoblastoma is examined. In Chapter 7 the incidence of retinoblastoma in Dutch children conceived by IVF between 1995 and 2007 is evaluated. Chapter 8 evaluates the association of IVF, retinoblastoma and tumour characteristics.

Chapter 9 summarizes and discusses the principal findings of the thesis. Ideas for future research are suggested and implications and recommendations for clinical practice are given.

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