Chapter 6
General discussion and future perspectives
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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Retinoblastoma is the most common childhood eye cancer, initiated in the developing retina. While significant progress has been made in the treatment and care of retinoblastoma patients, there is still an urgent need to improve diagnosis and care. With the work presented in this thesis, we aimed to improve molecular understanding, and thereby contribute to improved diagnosis, counselling, and ultimately treatment of retinoblastoma.

Discovery of $RB1^{+/+}MYCNA$ a new subtype of retinoblastoma with wildtype $RB1$, improves retinoblastoma diagnosis and care

Retinoblastoma is historically known as the cancer initiated by two mutations or other (epi) genetic inactivations of the retinoblastoma tumour suppressor gene, $RB1$. We found evidence contradicting the current dogma. We discovered a new subtype of retinoblastoma without detectable mutations in $RB1$, but with a strikingly high amplification of the $MYCN$ oncogene, $RB1^{+/+}MYCNA$ retinoblastoma (Chapter 3, and Figure 1). This finding directly improved genetic counselling, diagnosis, and possibly treatment choices in the future.

As we had strong proof that $RB1^{+/+}MYCNA$ retinoblastoma is non-hereditary, siblings and offspring of the affected subjects are not a risk for retinoblastoma, and consequently they do not require the cumbersome screening for retinoblastoma under general anaesthesia. Furthermore, we had no indication that patients with $RB1^{+/+}MYCNA$ retinoblastoma are at risk for second primary tumours. We did find that $RB1^{+/+}MYCNA$ retinoblastomas grew fast and aggressively, with possible risk of invasion and metastasis if left untreated. Therefore, it is important that ophthalmologists are aware that when very young children present with sporadic large unilateral retinoblastoma, the chance is high that they will have $RB1^{+/+}MYCNA$ retinoblastoma. While $RB1^{+/+}MYCNA$ retinoblastoma only accounts for 1.4% of unilateral non-familial retinoblastoma, we predicted that 18% of children diagnosed with unilateral non-familial retinoblastoma at the age of 6 months or younger will have $RB1^{+/+}MYCNA$ retinoblastoma. This percentage will even increase when tumour size is taken into account.

Since $RB1^{+/+}MYCNA$ tumours are unilateral and vision preservation of the affected eye is unachievable due to the large size of the tumours, we believe that enucleation is currently the best treatment regimen for these aggressive tumours. In the future, $MYCN$ targeted therapy might be suitable for these children, particularly in developing countries where patients often present in the clinic with disseminated disease. Currently there are no $MYCN$ targeted therapies in the clinic, since the development of $MYCN$ targeted therapies is challenging for several reasons. Firstly, the structure of the protein complicates small-molecule binding. Secondly, MYC transcription factors are essential proteins that regulate the genome in all proliferating cells. Despite these challenges, several recent studies showed promising results with $MYCN$ inhibition in preclinical models,
associated with reduced MYCN levels, tumour burden, and extended survival in mouse tumour models with notably few side effects.\(^3\) \(^4\)

Retinoblastoma tumours without detectable RB1 mutations: RB1\(^{+/+}\) and RB1\(^{+-}\) tumours

Besides the RB1\(^{+/+}\)/MYCN\(^A\) tumours, there is a group of RB1\(^{+/+}\) retinoblastomas without MYCN amplification, accounting for 1.3% of unilateral retinoblastomas (Chapter 2 and Figure 1). Possibly with current genome diagnostics the RB1 mutations of these tumours are being missed, or these tumours are caused by defects in other genes. In the latter case, one could hypothesize that a tumour suppressor gene is inactivated in these tumours by two sequential mutational events, because the age of onset is similar to the classic RB1\(^{-/-}\) retinoblastoma (Chapter 3, Figure 3). Whole genome sequencing could aid in the discovery of the initiating hits in the tumours, although additional acquired mutations will complicate the identification of the initial mutation.

Furthermore, in several tumours that were scanned for RB1 mutations (4 out of 53, 7.5%) only one RB1 allele was mutated (RB1\(^{+/+}\) retinoblastoma). It seems plausible that in these cases the second mutation has been missed with current screening methods, especially for one of the tumours where LOH of chromosome 13 was detected. Another possibility is that in these tumours only one mutation was sufficient to cause retinoblastoma. Possible other mechanisms that may result in sufficient inhibition of the retinoblastoma pathway are aberrant expression of RB1, epigenetic changes, and mutations, or SNPs in modifier genes.\(^5\) \(^6\). Examples of modifier genes, which might play a role in the onset of retinoblastoma are MDM2, TP53, and CDKN1A. It has been shown that MDM2 functions as a modifier gene in retinoblastoma, with the minor allele of MDM2 SNP rs229744 (MDM2 309T>G) associated with the presence of retinoblastoma among mutation carriers in
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Retinoblastoma families\(^7\). De Oliveira Reis et al. confirmed this and found that the rs229744G allele was associated with lower survival in retinoblastoma patients with a germline \(RB1\) mutation. They also found that the \(MDM4\) rs116197192G allele showed a significantly higher frequency in patients compared to controls\(^8\). In contrast, Epistolato et al. detected a negative association with the minor allele of \(MDM2\), while they did find the p53 SNP (p.Arg72Pro, rs1042522:G4C) to be significantly associated with disease\(^9\). Furthermore, the minor alleles of polymorphisms rs1801270 C>A and rs1059234 C>T in the \(CDKN1A\) (p21) gene were associated with an increased risk of the development of retinoblastoma\(^10\). Modifier SNPs might influence the penetrance of the disease in case of incomplete penetrance \(RB1\) mutations, the age of onset, or the occurrence of second cancers in retinoblastoma.

**Bilateral or familial retinoblastoma patients without detectable \(RB1\) mutations in blood**

In addition to tumours with wildtype \(RB1\) alleles, in a small group of patients with sporadic bilateral retinoblastoma (8% of bilateral cases) or familial retinoblastoma (8% of familial cases) no \(RB1\) mutation could be detected in DNA from blood (Chapter 2, and Figure 1). In chapter 2, we investigated several of these patients with additional methods. We sequenced an extended region of the \(RB1\) promotor and investigated also the \(RB1\) family members \(RBL1\) and \(RBL2\) (p107 and p130). These additional methods did not reveal evidence for mutations. We speculate that for some patients the potential \(RB1\) mutation is missed with current \(RB1\) genome diagnostics. Low-level germline mosaic \(RB1\) mutations could explain the non-familial bilateral retinoblastoma without detectable RB1 mutations in blood.

New sequencing methods have modestly improved the detection frequency of these mutations, Chen et al. described an increased sensitivity from 96% to 97% for bilateral retinoblastoma and 13% to 18% for unilateral retinoblastoma by deep sequencing\(^11\). For the familial patients without \(RB1\) mutations from our cohort, the question remains whether \(RB1\) mutations have been missed as there was no tumour material available from these subjects. Possibly other genes besides \(RB1\) and its family members \(RBL1\) and \(RBL2\) initiate retinoblastoma in these patients. Whole genome sequencing of several of these families might identify possible other genes that cause retinoblastoma. This will be challenging, nevertheless, due to co-segregation within families, and even more challenging if multiple genes are involved.

**Retinoblastoma oncogenomics and the cell of origin**

Besides the initiating mutations in retinoblastoma, the work presented in this thesis focused on secondary genetic and transcriptomic alterations associated with retinoblastoma progression. Zhang et al. claimed, based on a whole genome sequencing study of 4 retinoblastomas, that retinoblastoma tumours have a relatively stable genome\(^12\). Although we also detected retinoblastomas with
a stable genome, we observed a gradual increase in chromosomal instability associated with distinct gene expression profiles and clinical characteristics (Chapter 4 and 5). Retinoblastomas diagnosed early in life, often hereditary patients, were genomically stable and showed a gene expression profile with expression of genes involved in photoreceptor cell development. Retinoblastomas from patients that were diagnosed later, and were often non-hereditary, showed increased copy number changes and a loss of photoreceptor-cell gene expression. The latter tumours have M-phase and mRNA/ribosome synthesis gene expression profiles, and these were more sensitive to chemotherapeutic agents. Furthermore, these tumours were often larger in volume and poorly differentiated compared to tumours from young patients.

The observed differences between retinoblastomas might be simply due to progression. Older age may allow accumulation of chromosomal alterations, and these DNA alterations result in altered gene expression. It seems unlikely, however, that the differences we observed between retinoblastomas are only a result of diagnosis delay. According to the Knudson hypothesis, hereditary retinoblastomas are initiated earlier due to their germline predisposition and the need for only one somatic \textit{RB1} mutation in any retinal cell\textsuperscript{13}. Due to the early initiation, hereditary tumours arise early and are detected early in life. In case of non-hereditary retinoblastoma, two somatic mutations in the same cell (or daughter cell) are required to initiate retinoblastoma and as a result it is believed that these tumours arise later.

Some diagnosis delay might be introduced between hereditary and non-hereditary patients. In retinoblastoma families, offspring is under ophthalmologic surveillance from birth, and consequently these familial patients are diagnosed early. Familial retinoblastoma patients account for approximately 10% of all retinoblastoma patients and due to their young age at diagnosis and the early detection of the tumours, these patients are often treated with local therapies instead of enucleation. So, this was of minor influence on the results we observed in the profiles of the enucleated tumours, of which we had material available for our laboratory studies.

Patients with peripheral tumours present later in the clinic, and therefore the location of the tumour could also introduce a diagnosis delay\textsuperscript{14–16}. However, it has been observed that peripheral tumours also arise later, and so the late detection of peripheral tumours is more likely a result of the timing of initiation of the tumour rather than a delay of diagnosis.

Our results suggested that the differences in gene expression and chromosomal instability may be related to the timing of tumour initiation and the developmental stage of the retinoblastoma cell of origin. Tumours that were diagnosed early showed a stable copy number genome and a photoreceptor-cell gene signature. The latter tumours were often well differentiated and showed the characteristic Flexner-Wintersteiner rosettes. We hypothesize that these tumours arise from early cone photoreceptor precursor cells. These were shown to be
particularly sensitive for proliferation after RB1 loss, and the cells have a tumour favourable (apoptosis resistant) genetic make-up with high expression of genes like MDM2, MYCN, TR62, and RXRγ. Accordingly, RB1 loss in these cells might necessitate few additional chromosomal alterations for tumour progression, while later precursor cells might be less sensitive for RB1 loss due to a less tumour favourable genetic make-up. Other genetic alterations will be required for tumour progression in these later precursors with a loss of photoreceptor cell gene signature as a result. Subsequently it may take longer for these tumours to arise, but when these tumours do progress they might have more proliferative potential and grow to a larger size and outgrow the more differentiated precursor lesions.

In some papers it was suggested that differences in gene expression might be the consequence of different precursor cells or a hybrid cell of origin. The gradual increase in copy number changes and continuous change in gene expression we observed, nevertheless, suggests a common photoreceptor cell precursor in different developmental stages. This is in line with others, who suggested a photoreceptor cell precursor as retinoblastoma cell of origin.

While the differences between retinoblastomas on a genomic and transcriptomic level initially might seem of small importance from a clinical point of view, we observed that the retinoblastomas respond differently to chemotherapeutic agents, possibly due to differences in mitotic capacity. Undifferentiated retinoblastomas with low expression of genes involved in photoreceptor cell development seemed especially sensitive for actinomycin D. Importantly, the genomic profiles could be linked to clinical characteristics that can be determined non-invasively. This is important, since biopsies are avoided in retinoblastoma due to the risk of iatrogenic metastasis. To provide the most optimal treatment for each individual patient, there is a need for non-invasive means to determine the appropriate chemotherapeutic agent for each tumour. The ultimate goal would be that clinical characteristics of the patient can predict the molecular profile and the associated chemosensitivity of the tumour. This would particularly be aided when the standard diagnosis would be supported by imaging techniques such as MRI and optical coherence tomography.

**Retinoblastoma driver genes**

While many studies focused on identifying candidate driver genes for retinoblastoma, this remains challenging due to large chromosomal gains and losses that are common in retinoblastoma. We performed high resolution SNP arrays that can detect smaller gains and losses. Common focal aberrations appeared to occur infrequently, however. The only common focal aberration we detected was gain or amplification of the oncogene MYCN. In one tumour we detected several focal amplifications on chromosome 14q, including an amplification at 14q23.1 that included the gene OTX2 (Chapter 4). This gene is often amplified and overexpressed in the paediatric cancer medulloblastoma.
More recently, McEvoy et al. identified focal amplification of \textit{OTX2} in 3\% of retinoblastoma samples\textsuperscript{30}. \textit{OTX2} is a transcription factor essential for neuronal development, and specifically important for retinal development functioning as a key regulator of photoreceptor genesis and differentiation\textsuperscript{31}.

The only common focal loss in retinoblastoma is deletion of \textit{BCOR}, a gene located on the X-chromosome (unpublished data). This gene was also identified by McEvoy et al. in 4\% of retinoblastoma samples\textsuperscript{30}. \textit{BCOR} was also the most frequently mutated gene (13\%) in a whole genome sequencing study of retinoblastoma\textsuperscript{12}, and is often mutated in medulloblastoma\textsuperscript{32}, Ewing’s sarcoma\textsuperscript{33} and acute myeloid leukaemia\textsuperscript{34,35}. \textit{BCOR} is a transcriptional co-repressor of the oncoprotein \textit{BCL6}\textsuperscript{36}, and is expressed in the embryonic mouse retina\textsuperscript{37}. Germline mutations in \textit{BCOR} lead to congenital disorders that include microphthalmia\textsuperscript{38}. These data together imply that \textit{BCOR} could play an important role in retinoblastoma. Besides determining candidate genes by analyses of tumour DNA profiling, an integrative approach of copy number and expression profiling will aid in pinpointing the important driver genes in retinoblastoma (Kooi et al. Manuscript in preparation).

Besides genomic changes, epigenetic deregulation seems to play an important role in retinoblastoma, too. It has been shown that in many solid paediatric tumours, and especially in retinoblastoma, somatic mutation frequency is low compared to that observed in adult cancers\textsuperscript{12,39–42}. An integrated analysis of genomic and epigenomic data revealed that multiple cancer pathways were epigenetically deregulated in retinoblastoma\textsuperscript{12}. Furthermore, our preliminary analysis of methylation array data showed that there were marked differences between the extent of methylation between retinoblastoma tumours (unpublished data). Moreover, the \textit{RB1} gene is implicated in many epigenetic processes\textsuperscript{43–46}. The candidate tumour suppressor gene \textit{BCOR} plays a role in epigenetic modification of histone methylation. \textit{BCOR} inhibits H3K4 and H3K36 methylation on target gene promoters resulting in repression of gene transcription\textsuperscript{47}. Possibly, mutations or deletions in \textit{RB1} and/or \textit{BCOR} play a role in the epigenetic deregulation observed in retinoblastoma.

**Oncogenomics of retinoblastoma to identify leads for treatment**

Zhang et al. identified the proto-oncogene \textit{SYK} as new drug target for retinoblastoma\textsuperscript{12}. Inhibition of \textit{SYK} with the preclinical small-molecule inhibitor BAY-6306 caused retinoblastoma cell death both in vitro and in vivo. However, local subconjunctival administration of another preclinical \textit{SYK} inhibitor, R406 (further along in clinical testing compared to BAY-6306), failed to accomplish tumour cell death in a human orthotopic xenograft mouse model\textsuperscript{48}. Another targeted treatment that is in retinoblastoma clinical trials is the MDM2-p53 and MDMX-p53 interaction inhibitor Nutlin-3\textsuperscript{49}. MDMX is often overexpressed in retinoblastoma, and thereby prevents p53 induced apoptosis in tumour cells\textsuperscript{50,51}. While these studies showed promising results, currently the majority of patients are still treated with cytotoxic agents such as melphalan, vincristine, etoposide,
and carboplatin which can result in severe side effects. So, there is a requirement for further research that focuses on selecting new or less toxic cancer treatments. In vitro studies of retinoblastoma are challenging, since retinoblastoma cell lines for long term culture are difficult to establish, the cell lines often grow slowly in suspension, and are difficult to transfect. Furthermore, cell lines can acquire additional genetic changes that do not reflect the original tumour. We detected differences in gene expression and the amount of copy number changes between primary retinoblastoma tumours that were reflected in differences in chemosensitivity ex vivo. This indicates that it is important to use oncogenomic data and clinical characteristics in the analysis of drug screens, to be able to identify drugs that convey sensitivity to a subset of tumours.

**Conclusion and future perspectives**

While many improvements have been made in the treatment of retinoblastoma, still there is need for further improvement of diagnosis and care. In a subset of retinoblastoma patients the underlying mutations can still not be identified, complicating risk estimations. Improved genome sequencing methods might aid in the identification of the disease causing gene(s) in these patients. As we have found evidence that there is heterogeneity between tumours, attempts should be made in identifying less toxic treatment agents for retinoblastoma with a focus on the molecular background of the tumour, to select the optimal treatment regimen for each individual patient and tumour.

The further exploration of (epi) genomic data linked to clinical data and a focus on targeted-treatment drug-sensitivity determination will aid in developing new leads for the clinic.

With the identification of a new subtype of retinoblastoma, $RB1^{+/-}MYCN^+$, we improved diagnosis and counselling for retinoblastoma patients. The results from our oncogenomic analyses gave insights in the development and progression of retinoblastoma and leads for further research on (individualized) treatment.
REFERENCES


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