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Scientific and clinical implications of heterogeneity in uveal melanoma
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Summary and Discussion

BACKGROUND

Cellular replication is an ongoing process in which identical copies of progenitor cells are formed. This process is not flawless, and random mutations can be introduced with every cell division[1]. The occurrence of mutations results in natural variation which is the basis of evolution. However, in time the accumulation of mutations can result in aberrant cell behaviour and eventually tumour formation[2]. Due to selection pressure, the process of cellular evolution drives carcinogenesis which makes it an ideal model for studying the biology of these mutations[3]. By genetic analysis of large patient populations, mutation recurrence and mutation rate can be linked to tumour progression and survival[4,5]. Still, it is very difficult to study tumour evolution, since the process is often interrupted by treatment and measurements are usually performed at a single point in time[6]. With the use of deep sequencing, attempts are made to backtrack the order in which mutations occurred[7,8]. Though, adequate analysis is strongly dependent on sequence depth, and moreover, prone to loss of context due to DNA/RNA isolation and analysis. Single cell analysis can help overcome these problems, preserving context, but this is inadequate in understanding the intricate nature of tumour development.

Tumours are not only characterised by the mere presence of a specific set of mutations, but also by the percentage of cells that these mutations occur in. Starting with one transformed cell, many subsequent mutations can follow leading to a variety of subclones. The resulting diversity is referred to as **heterogeneity** and is of vital importance to comprehend the complexity of cancer and ultimately the treatment thereof. In this thesis, we make the translation of the common qualitative approach to unravelling tumour genetics into a quantitative approach by using digital PCR (dPCR) analysis. This is a targeted method that is accurate and sensitive because of the depth of analysis.

Uveal melanoma
Uveal melanoma (UM) is the most common intraocular neoplasm that can affect the choroid, ciliary body and iris. Similar to cutaneous melanoma (CM), UM originate from melanocytes but their biology differs fundamentally [9,10]. UM most commonly originate from the choroid and can extend into the corpus vitreous (CV). Since no contaminating interference of other non-cancer related cells can reasonably be expected in the CV, UM serves as an ideal model to study heterogeneity. As opposed to CM, UM generally do not carry the infamous *BRAF V600E* mutation [11,12]. This mutation is already found in most nevi in the skin and is commonly accepted as the primary hit in CM development [13,14]. Instead, UM carry the *GNAQ/11* mutations in the majority of cases, followed by a wide array of secondary mutational or chromosomal aberrations [15,16]. Solely based on *GNAQ/11* mutations, UM could be considered genetically homogeneous. However, in **chapter 5** we infer that different

mechanisms may be involved in the different driver mutations that can be detected in UM. Functionally homologous point mutations may be caused by very different molecular mechanisms. Whereas specific mutations are detected in the light-exposed part of the choroid, other mutations may be correlated to light-independent mechanisms [17]. It is, however, unsure whether these mechanisms are only playing a role in the development of primary mutations, or in secondary (such as *EIF1AX*, *SF3B1* and *BAP1*) as well [18]. In CM development, the effect of environmental radiation is complicated because exposure is not controlled and nonexposed areas are non-existent. The question remains, however, to what degree UV light exposure contributes to tumorigenesis[19]. This is not only important because of prevention but also because different mutation mechanisms possibly reveal different intrinsic vulnerabilities. These vulnerabilities may play a role in susceptibility to chemotherapy, since this is shown previously by mismatch repair deficient breast cancer cells that respond well to PARP inhibitors while proficient breast cancers do not [20]. In UM, rare examples of repair deficiency due to *MBD4* variants are known to respond well to immune checkpoint inhibitor treatment [21,22].

Moreover, it has been shown that subclones exist within one UM, which have a common progenitor cell, but still carry their own specific set of mutations [7,23]. This genetic heterogeneity can be used for studying tumour biology and optimise personalised treatment. Furthermore, analysis of all these subclones by molecular deconvolution allows for the investigation of another dimension, time [23]. By accurately determining the percentage of cells in which a specific set of mutations is present, a sequence of events can be determined. Thereby, the heterogeneity reflects the point in time where the sample was taken and may allow a personalised prognosis, specifying which steps in tumour development will take place next. Genetic heterogeneity is accompanied by the presence of a wide array of different cells. Next to tumour cells, UM consist of other types of cells which are mostly related to the immune response[24–26]. This heterogeneity is referred to as cellular heterogeneity and is of peak interest in determining prognosis and treatment plan. Previously, the general assumption was that tumours are predominantly monoclonal and can be treated following a one-size-fits-all therapy. We showed that heterogeneity is largely present in UM which can have major repercussions for treatment choice.

Genetic heterogeneity in uveal melanoma
In UM, heterogeneity can be studied in a unique way since it often extends into the acellular part of the corpus vitreous, where there is low stromal contamination and thus a high tumour purity. As a result, this creates an opportunity to discuss the actual contents of the tumour. Heterogeneity can be portrayed as an ancestral tree, in which the branches represent different mutations that a progenitor cell can acquire (**chapter 3**). Given this scenario, a wide variety of mutations can lead to the formation of different subclones that are present

within one tumour (**intratumoral heterogeneity**). Additionally, since every tumour is unique, their genetic make-up also differs between patients (**intertumoral heterogeneity**). Not all mutations will lead to the formation of a detectable subclone, unless they are so-called driver mutations. In UM, *GNAQ* and *GNA11* are the most common driver mutations, since they occur in over 85% of all UM cases worldwide [15,16]. One could assume that this makes UM genetically homogeneous, and they would not be entirely wrong. However, since carcinogenesis is a multi-step process, the presence of *GNAQ/11* mutations alone would not be enough to form a tumour. Additionally, *GNAQ/11* mutations are found in retinal naevi, and they do not necessarily lead to tumour formation [27]. In order to become a successful tumour, so-called secondary hits are necessary. Both chromosomal aberrations, as well as genetic mutations, have been extensively described in UM. Amongst others, Monosomy 3, gain of 8q and mutations in *BAP1*, *SF3B1* and *EIF1AX* have been marked as anomalies that could serve as a second hit [18,28–30]. To properly understand the progression and prognosis of UM, especially chromosome 8q analysis seems to be of vital importance [8].

Cellular heterogeneity in uveal melanoma

The fraction of non-malignant cells can strongly differ between UM[23,31]. The majority of these cells originate from the immune system[24,32,33]. The role of the immune system in cancer development is still a topic of debate, since the interaction between cancer and the immune system is an evolving process[34,35]. For UM, the role of the immune system remains poorly understood. It is unclear whether the immune system can recognise tumour cells as foreign, or if it is actively recruited by tumour cells to aid in tumour progression. Epidemiologically, numerous studies have shown strong correlations between the infiltration of innate and adaptive immune cells on the one hand, and the presence of genetic abnormalities and increased metastatic risk on the other [25,36,37]. However, the mechanism of how the immune system is involved remains unclear and might simply reflect co-occurring events[29]. In **chapter 3** we describe that immune invasion appears to occur alongside progression and increases in a linear fashion, and not in a stepwise fashion that one would expect if immune invasion was correlated to a specific mutation. Genomic profiling of a set of 64 UM showed a clear subdivision in 3 classes (I, IIa and IIb) which correlate to a specific immunological response. The actual presence of T cells is exclusive to class IIa and IIb, but the amount of T cells that is present within one tumour increases gradually when UM show more class IIb-like characteristics. In **chapter 4**, we determined that the main driver behind this process is most likely CXCL10. This shows that the recruitment of immunological cells is an active tumour-driven process that moreover correlates with a bad prognosis for the patient. Most likely, immune infiltrate is a consequence rather than a cause of tumour progression.

From bench to bedside

By means of whole-genome sequencing, differences in chromosomal aberrations, copy-number variations and gene expression signatures were found within one tumour[28]. This makes it extremely difficult to use tumour genetics for prognostication and therapy, since the result of conventional tests simply depend on where in the tumour the biopsy was taken. Subsequently, conventional tests like PCR and karyotyping only show the presence of a specific anomaly within a sample rather than its fractional abundance (FA). However, with the more refined dPCR technique, in-depth analysis of bulk tissue is made possible (**chapter 2**). dPCR is more precise than traditional PCR in the way that a digital measurement quantitatively measures a variable. This makes it possible to investigate the FA of chromosomal aberrations and mutations, even when an anomaly occurs once in every ten thousand cells. Using this technique in UM, a defined sequence of events can be deduced based on this fraction of cells that contains an anomaly. One cell can acquire multiple hits over time and, based on evolution, will grow out to become a clone. Though, this stochastic model for tumour development is not the only theory out there. Other theories may involve the presence of cancer stem cells as main drivers behind the tumour mass (**chapter 1**). A fixed, relatively dormant group of stem cells give rise to daughter cells which, in turn, form the proliferative pool of cells [38–40]. This pool contains the majority of the tumour mass and will primarily be genetically identical to their ancestor cells. Regardless of which ancestral theory of the development of UM, dPCR is capable of accurately determining the tumour composition.

Carcinogenesis is a complicated process which is difficult to map. However, UM provides the ideal model to unravel all anomalies that belong to the tumour, and analysis creates a clear picture of the tumour consistency as well as interactions with non-tumour fractions, including immune cells. In this thesis, we set out to study tumour heterogeneity and showed that events in UM development are not punctuated but a constantly ongoing process. By means of dPCR analysis, we showed which anomalies are most abundant and, more importantly, in which chronological order they occurred. dPCR analysis may not be as powerful as single cell RNA (scRNA) analysis, which gained popularity recently, but showed to be an accurate and valuable tool that can be routinely applied. For a long time it has been presumed that tumours as a whole contain one specific set of mutations, but in this thesis and additional recent research involving scRNA analysis, it was shown that UM development is stochastic[41,42]. The exact role and interplay of the different tumour fractions are yet to be determined, but genetic analysis showed to hold accurate prognostic value. Subsequently, this opens the door to a wide variety of possible personalised treatments. Genetic prognostication offers the possibility to either explore immune therapy or targeted therapy. For instance, in **chapter 6**, we investigated the role of Src kinase in tumour proliferation by using the kinase inhibitor Dasatinib. Dasatinib is a small molecule

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capable of inhibiting a wide array of kinases which potentially results in stalling tumour growth. Subjecting UM with different kinase profiles to Dasatinib resulted in the detection of responders and non-responders. This emphasises that molecular deconvolution of UM and tumours in general is very relevant to determine treatment.

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