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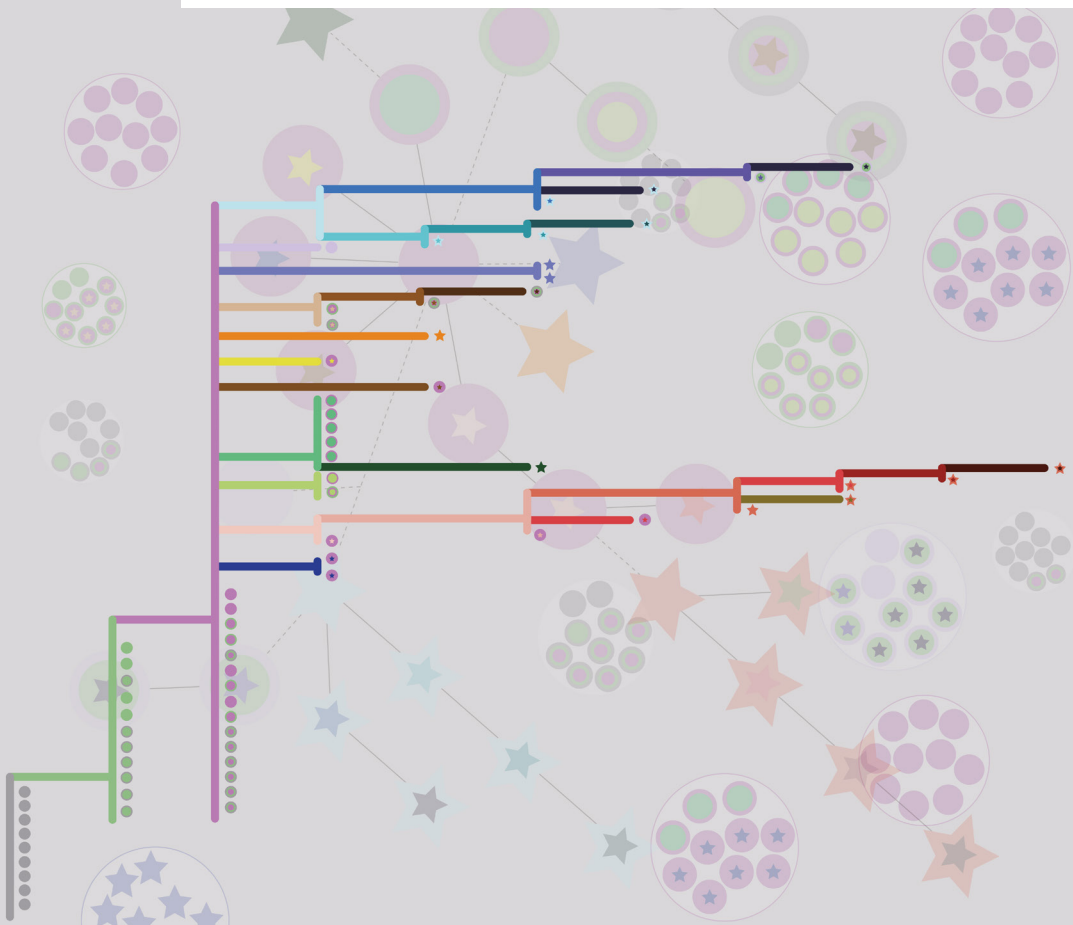

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Abstract

This thesis explores how the tumour microenvironment affects the phenotype and shapes the evolution of cancer cells. It encompasses four separate studies:

First, we explored the effect of chemokines on the peritotumoral microenvironment of ovarian cancer. We found that C-C Motif Chemokine Ligand 2 (CCL2) secreted from mesothelial cells is a dominant chemokine promoting the peritoneal dissemination of ovarian cancer cells.

To evaluate the role of tissue differentiation in tumorigenesis, we then focused on Iroquois Homebox B proteins in the childhood kidney cancer Wilms tumour. We showed that these proteins had key roles in normal embryonic kidney development in humans and also had an impact on the differentiation of Wilms tumour cells.

In the third and fourth studies, we used multiregional genetic analysis of tumour cells to assess evolutionary trajectories in an environment affected by chemotherapy. In patients with the aggressive childhood cancer malignant rhabdoid tumour we found a pattern of branching evolution across metastatic sites, followed by linear evolution regionally. This resulted in a heterogeneous neoantigen profile and a diverse immune checkpoint status within patients.

The fourth study focussed on the childhood cancer neuroblastoma. Here we found two distinct patterns, linear and collateral evolution, coupled to progression and response to chemotherapy, respectively. These patterns were reproduced in a neuroblastoma PDX model and in vitro.

Thus, tumour microenvironment has a significant role in shaping tumour cell phenotype and genotype. Finding patterns in these complex interactions may provide future routes to therapy.

Key words: Cancer genotype, cancer phenotype, malignant rhabdoid tumour, neuroblastoma, ovarian cancer, tumour evolution, Wilms tumour

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MADE IN SWEDEN

To my family, Misano, Kyoka and Karen

"Thank you very much for supporting my PhD life."

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Preface

Cancer was the most common cause of death in Japan already when I was born in 1986. Whenever news-reporters mentioned the most common cause of annual death in Japan, it was cancer. This fact never changed. I finally decided to be a medical doctor for my future occupation, and therefore I (18 years old) entered a medical university, Nagoya University, ***in order to be a clinician***.

There, I learned what cancer is through my medical education. Cancer cells generally emerge from our normal cells because of genetic abnormalities. Although most cells are loyal to our health and help us to maintain our daily life, some become selfish and do not obey the social system of our bodies. Obviously, cancer affects the patient's body and life, but interestingly, I learnt that cancer also is affected by the environment created in the interplay between host and cancer. This environment is now commonly termed ***tumour microenvironment***.

As a clinical doctor whose expertise is obstetrics and gynaecology, I have experienced lots of death from gynaecological cancer. Death by cancer was very close. I was followed with a question through my daily clinical life. The question was why patients died of treatment-refractory metastatic relapse, while an initial treatment was successfully conducted and there seemed to be no tumour cells in the patient's body afterwards. However, I did not have any answer to this question at that time. Thus, I decided to apply for this ***double degree of Doctor of Philosophy (PhD) program*** between Nagoya University and Lund University four years ago, in order to learn more about cancer. The overall aim of the present PhD thesis is to clarify how tumours are organized as systems of phenotype and genotype.

Now, when I'm ready to defend my thesis, I can answer the question why tumour cells become treatment resistant and form treatment-refractory metastasis, better than before starting this PhD course. The following descriptions are my footprints of a four years long journey to seek out ***tumour evolution***. This dissertation is briefly divided into two main parts, "Introduction" and "The present study". A brief summary of essential background knowledge to the present study is summarized in the Introduction. The present study is further subdivided into Aims, Materials and Methods, Results, Discussion and Conclusions.

I (34 years old now) will soon be back as ***a clinician*** as intended and will work with clinical oncology with a little bit matured understanding about cancer. I hope that you will enjoy reading my book and that my four years of contribution will be a tiny step in the scientific progress towards the bright future for which every patient hopes.

Original Articles

Article I **Yasui H**, Kajiyama H, Tamauchi S, Suzuki S, Peng Y, Yoshikawa N, Sugiyama M, Nakamura K, Kikkawa F. CCL2 secreted from cancer-associated mesothelial cells promotes peritoneal metastasis of ovarian cancer cells through the P38-MAPK pathway. Clin Exp Metastasis 2020; 37: 145-158.

Article II Holmquist Mengelbier L, Lindell-Munther S, **Yasui H**, Jansson C, Esfandyari J, Karlsson J, Lau K, Hui CC, Bexell D, Hopyan S, Gisselsson D. The Iroquois homeobox proteins IRX3 and IRX5 have distinct roles in Wilms tumour development and human nephrogenesis. J Pathol 2019; 247: 86-98.

Article III **Yasui H**, Valind A, Karlsson J, Pietras C, Jansson C, Wille J, Romerius P, Backman T, Gisselsson D. A dynamic mutational landscape associated to an inter-regionally diverse immune response in malignant rhabdoid tumour. J Pathol 2020 Jun 16. doi: 10.1002/path.5490. Online ahead of print.
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Article IV Karlsson J*, **Yasui H***, Mañas A, Andersson N, Hansson K, Aaltonen K, Jansson C, Durand G, Chattopadhyay S, Valind A, Bexell D, Gisselsson D. Collateral clonal replacement - a major route to treatment resistance from a substrate of branched evolution in neuroblastoma. *Unsubmitted manuscript draft.* *Equal contribution.

Abbreviations

ALK = Anaplastic Lymphoma Kinase
AT/RT = Atypical teratoid/RT
BRCA = Breast Cancer Susceptibility Gene
CCL = C-C Motif Chemokine Ligand
CCR = C-C Motif Chemokine Receptor
CD = Cluster of Differentiation
CNNI = Copy Number-neutral Imbalance
DNA = Deoxyribonucleic Acid
ELISA = Enzyme-Linked Immunol Sorbent Assay
INRG = International Neuroblastoma Risk Group
INSS = International Neuroblastoma Staging System
IRXB = Iroquois Homeobox B
MYCN = MYCN Proto-oncogene, bHLH Transcription Factor
PD-1 = Programmed Cell Death 1
PD-L = Programmed Death-Ligand
PDX = Patient-derived Tumour Xenograft
PhD = Doctor of Philosophy
RNA = Ribonucleic Acid
RT-PCR = Reverse Transcription-Polymerase Chain Reaction
SIOP = International Society of Paediatric Oncology
SMARCA4 = SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4
SMARCB1 = SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily B, Member 1
SNP = Single Nucleotide Polymorphism
TGF-β = Transforming Growth Factor-β
TP53 = Tumor Protein P53
WHO =World Health Organization

Introduction

General tumour biology

Cancer is growing into one of the most serious healthcare concerns, especially, in my home-country, Japan. Here cancer has been the leading cause of death for more than 30 years and still the number of deaths by cancer is increasing [1]. In addition, cancer is the second most common cause of death annually in Sweden [2]. When we broaden our view to all over the world, it is reported that cancer is the second most frequent cause of death according to the World Health Organization (WHO) [3].

The current treatment strategy of cancer is early diagnosis by screening examination preferably followed by a combination of therapies, including surgery, chemotherapy, and radiotherapy, according to the WHO [3]. However, tumour types that are available for early detection by screening [4] are limited and so far only breast cancer [5], human papilloma virus related cervical cancer [6], lung cancer [7, 8] and colorectal cancer [9-11] have highly-established evidence of early detection and screening showed reduction of mortality in some tumour types [7, 8].

Screening examinations are thus not available for most tumour types. Epithelial ovarian cancer except hereditary breast and ovarian cancer [12, 13], Wilms tumour except Beckwith-Wiedemann spectrum [14, 15], malignant rhabdoid tumour [16] and neuroblastoma [17] that I have focused on for my PhD projects do not generally have effective screening examinations. Lack of effectiveness of early detection means that we cannot find cancer at an early stage for all patients. Thus, whether a patient is diagnosed at an early stage or at an advanced stage with the presence of metastasis depends extensively on chance.

Cancer cells are considered to emerge from the normal cells composing our body [3, 18]. There are various factors behind why cancer cells arise, including germline mutations [19, 20], exposure of carcinogenic substances such as tobacco smoke [21] and ultraviolet radiation [22], and virus infection such as hepatitis virus [23] and human papilloma virus [24]. When the normal cells in our body are exposed to these factors, cells acquire gene alterations such as somatic mutations, chromosomal rearrangements and copy number alterations. When the cells which contain genetic alterations survive, some are going to be life threating cells, i.e. cancer cells, as a consequence [25].

Genetic alterations can lead to structural changes of protein either by exchange of individual base pairs or by chromosome rearrangements that induce fusion genes [26]. Cells are supposed to have responsibility for specific functions, for instance, intestinal epithelial cells work with absorbance of nutrients by covering the surface of organs and stratified epithelial cells should be durable to external impact. However, once these cells become cancer cells, they abandon their responsibilities. For example epithelial cells which are the most frequent cells of origin for adult cancer can show epithelial-mesenchymal transition [27]. Epithelial-mesenchymal transition allows epithelial cancer cells to obtain the phenotypic aspects of mesenchymal cells, such as migration and dissemination to other organs [28].

The phenotypic cancer cell behaviour is highly affected by the tumour microenvironment. The microenvironment, created by cancer cells interacting with benign cells, such as endothelial cells, fibroblasts, and immune cells [29], is regarded as an important biological system that affects not only the cancer phenotype but can also shape the cancer genotype [30]. This is because cancer cells are exposed to a selective pressure from the tumour microenvironment, and tumorigenesis is an ongoing evolutionary process [31]. To understand cancer evolution by phenotypic and genomic analyses became my PhD research project.

To address cancer evolution, I first focused on cell to cell interactions between cancer-associated mesothelial cells and epithelial ovarian cancer cells. Then I investigated how a specific set of transcription factors of the *Iroquois Homeobox B (IRXB)* gene cluster, including *IRX3* and *IRX5*, impact the phenotype of cells in Wilms tumour. Finally, I analysed evolutionary histories in two paediatric cancers, malignant rhabdoid tumour and neuroblastoma.

Tumour microenvironment

The tumour microenvironment is a local milieu containing tumour cells and non-tumour cells such as, fibroblasts, immune cells, extracellular matrix, and local molecular interactions including cytokines, growth factors, and hormones. This microenvironment has a significant impact on tumour initiation, progression and formation of metastasis, i.e. cancer phenotype but also the genome of both primary and metastatic tumours [32].

The cancer-associated fibroblast is a well-studied and well-known component of the tumour microenvironment. Fibroblasts are considered to inhibit early progression of tumour cells. Cancer cells activate fibroblasts to become cancer-associated fibroblasts by increased expression of α-smooth muscle actin during later progression, and these secrete high levels of extracellular matrix-degrading proteases, allowing extracellular matrix turnover [33]. Cancer-associated fibroblasts help tumour growth by secreting growth factors, chemokines and proteases, which promote carcinogenesis [34]. As a result, cancer-associated fibroblasts have been found to have a correlation to poor prognosis [35, 36].

In the tumour microenvironment, cancer cells also interact with immune cells via chemokines. Chemokines are chemotactic cytokines and are small proteins which can be subdivided into four main classes, including CC-chemokines, CXC-chemokines, C-chemokines and CX3C-chemokines. Chemokines work by binding to the receptors that immune cells mainly express and have a function in migration of monocytes, natural-killer cells and T cells in normal immune response [37].

As a significant component of the tumour microenvironment, chemokines are also known to be relevant directly to tumour progression via receptors expressed on the tumour cells. The C-C Motif Chemokine Ligand (CCL) 2 promotes tumour vascularization, cancer extravasation and metastasis, CCL3 promotes cancer extravasation and CCL5 promotes cancer invasion. CXCL8, CXCL12 and CXCL17 promote angiogenesis [38]. The C-C Motif Chemokine Receptor (CCR) 2 is the main receptor of CCL2 and recent reports have suggested that CCL2/CCR2 promotes breast cancer growth and invasion [39].

Discontinuation of anti CCL2 treatment induces breast cancer metastasis in mice [40]. Therefore, chemokines and their receptors are expected to be direct therapeutic targets for future treatment strategies.

Cancer cell phenotype

The most important features of cancer phenotype can be summarized as adhesion, migration, invasion and proliferation. Especially, invasion which is a prerequisite for cancer cells to form metastasis, is considered to be significant because metastasis is the main cause of cancer death [41]. Epithelial mesenchymal transition, demonstrated by loss of tight junction between cells, i.e. an epithelial feature, and activation of actin stress fibres, i.e. a mesenchymal feature, is often found in the tumour cells. This transition is induced by the tumour microenvironmental components, such as transforming growth factor-β (TGF-β), and other cytokines [28].

Genomic alterations

All sufficiently studied cancer cells show somatic genetic alterations, including sequence mutations, chromosomal rearrangements, and copy number variants [42]. Mutations are considered to occur from deoxyribonucleic acid (DNA) damage, which is induced by environmental exogeneous factors or replication errors repaired incorrectly or left unrepaired. Complex somatic mutations are commonly found in cancer genomes [43, 44], together with multiple chromosomal copy number alterations that may be further complicated by shifts in ploidy that arise from whole-genome duplication and other gross mitotic segregation errors [45, 46]. While genetic events in childhood tumours have been reported as less compared to adult cancers, mutational signatures of childhood cancers can sometimes still be very complex [47].

Chromosomal instability

Tumour cells divide more frequently than normal cells, as the former often lose the checkpoint systems that normally stop cell division after the accumulation of mutations. The best known loss of function of a checkpoint protein is that of p53, caused by loss and/or mutations of the corresponding gene, *Tumor Protein P53 (TP53)* [48]. Cell division following loss of checkpoint status leads to mitotic defects and an emergence of aberrant chromosomal segregation, resulting in chromosomal instability that brings on an ongoing acquisition of copy number alterations as gains, losses, and copy number-neutral imbalances (CNNI) of whole chromosomes or chromosomal segments [49]. Chromosomal instability also promotes phenotypic adaptation under selective pressures by the tumour microenvironment during cancer evolution and treatment. Conversely, it has been reported that TGF-β-induced epithelial-mesenchymal transition promotes chromosomal instability of tumour cells [50].

Somatic and germline mutations

All cancers emerge by obtaining somatic mutations. As previously mentioned, the somatic mutations can show several distinct patterns even in the same tumour type, making the cancer genome complex [51]. In addition, the genetic abnormalities of tumour cells are affected by hereditary factors, i.e. germline mutations on top of which somatic mutations are added. The best known cancer predisposition syndromes affecting adults and children are caused by germline mutations in tumour suppressor genes, such as *TP53*,

the *retinoblastoma* (*RB1*) gene, the *breast cancer susceptibility genes BRCA1*/*BRCA2*, the *adenomatous polyposis coli (APC)* gene, and the mismatch repair genes *MutL Homolog1 (MLH1)* and *MutS Homolog2 (MSH2)* [52]. Germline mutations can also contribute to cancer in infants. For example, mutations in the gene *SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily B, member 1 (SMARCB1)* is strongly linked to the emergence of malignant rhabdoid tumour [53]. Around 10%-20% of childhood neoplasms encompass germline predisposition [54, 55].

Tumour heterogeneity and cancer cell evolution

Cancer cells are often undergoing evolution following Darwinian principles [42]. A prerequisite for evolution is hereditary genetic variation. As tumours grow from an ancestral cell, they typically form genetically distinct subclones, that can be distinguished by each having specific somatic mutations and copy number profiles. The temporal and spatial diversity of subclones makes tumour architecture complex and renders interactions between genotype and phenotype even harder to understand. For the understanding of tumour evolution, validation of subclones becomes an important step. Selection of the fittest subclone is the major driver in tumour evolution, but this process can be hard to distinguish from genetic drift. Both processes can result in evolution branching out into different cell lineages or linear evolution as a stepwise accumulation of mutations [31].

Clinical features of the studied cancer types

Most ovarian cancer cells emerge from an epithelial origin [56], while for most paediatric cancers, the cells of origin are not known, although many are assumed to emerge from embryonal cell populations and even to be initiated *in utero*. Further, paediatric malignancies are very rare compared to adult cancers [57]. It was reported that the annual prevalence of ovarian cancer was 11.5 case per 100,000 in the United States of America [58]. On the other hand, the prevalence of neuroblastoma which is the most common solid extracranial paediatric neoplasm was reported to be only 10.5 cases per 1,000,000 [59]. Although the global population of childhood cancer is tiny compared to cancer of adults, death from paediatric cancer still costs many expected future years of life. A big issue for childhood cancer survivors is late adverse effects such as cardiomyopathy, renal failure and risk of secondary malignancy due to the intensive multimodal chemotherapy [60]. Thus, an important future perspective for childhood neoplasms is to explore alternative strategies instead of the current intensive chemotherapy protocols.

Epithelial ovarian cancer

Ovarian cancer is associated with one of the highest mortalities among gynaecological tumours. It is a highly heterogeneous disease, based on its genomic profiles [61], and the many different histological types according to the latest WHO classification [62]. Epithelial ovarian cancer briefly can be categorized into five major types according to their histological appearance, including high grade serous carcinoma, endometrioid carcinoma, clear cell carcinoma, mucinous carcinoma, and low grade serous carcinoma [63]. It should be noted that the distribution of each type highly depends on region/country.

The most frequent epithelial ovarian cancer is serous carcinoma, and the second most frequent tumour type is clear cell carcinoma in Japan [64]. High grade serous carcinomas

have a propensity for early peritoneal dissemination, leading to an advanced stage at the time of diagnosis [63]. On the other hand, more than half of clear cell carcinoma patients are diagnosed with stage I, and 5-year survival rate of stage I clear cell carcinoma reaches approximately 90%. However, prognosis of clear cell carcinoma with advanced stage is poor because clear cell carcinoma is often chemotherapy resistant [65].

Regarding the genomic features of ovarian cancer, *TP53* mutation is a common genetic abnormality in high grade serous carcinoma, being confirmed in 96.7% of pelvic-high grade serous carcinoma [66]. Further, *TP53* mutations are typically found in the trunk of tumour phylogenies and allow ovarian cancer cells to be highly heterogeneous already at an early stage [61]. Over 40% of clear cell carcinomas encompass specific gene mutations, such as *ARID1A* and *PI3CA* [67] and the presence of these two gene mutations correlates to poor prognosis [68].

The main clinical problem in serous carcinoma is thus early peritoneal dissemination [69]. The prognosis of clear cell carcinoma with peritoneal dissemination is poor due to its chemotherapy resistant feature. Importantly, ovarian cancer cells interact with mesothelial cells by cell to cell communication through a molecular signalling network in the malignant ascites [70]. We recently found that epithelial ovarian cancer cells change human mesothelial cells to cancer-associated mesothelial cells in order to promote peritoneal dissemination by TGF-β secreted from cancer cells [71]. Further, we confirmed that plasminogen activator inhibitor-1 from tumour cells increased tumour malignancy [72]. Deeper understanding of the relationship between epithelial ovarian cancer cells and cancer-associated mesothelial cells might have the possibility to suggest a novel treatment strategy, something we address in this thesis.

Wilms tumour

Wilms tumour is the most common paediatric renal tumour. The prevalence of Wilms tumour is highly different among countries: the age-standardized incidence of renal tumour per 1,000,000 in children aged 0-14 years is estimated to be 9.3 in Northern Europe and 5.4 in Southern Asia, from 2001 to 2010 [73].

According to the International Society of Paediatric Oncology (SIOP) approach [74], patients diagnosed with Wilms tumour undergo an initial chemotherapy treatment before surgical resection. Wilms tumour histology is highly heterogeneous with differential structures, and the histological risk assessment is based on chemotherapy-induced histological changes, as well as the proportions of blastemal, epithelial and stromal elements, and the presence or absence of anaplasia. This histological classification defines three major risk groups, i.e. a low risk group (completely necrotic Wilms tumour), a high risk group (blastemal type and diffuse anaplasia) and an intermediate risk group (the others) to which most tumours belong [75].

The 5-years overall survival of patients under the SIOP 2001 protocol was reported as 97.2% in the low risk group, 94.8% in the intermediate risk group, and 74.8% in the high risk group [76]. While the prognosis of Wilms tumour seems better than the other tumour types discussed in this thesis, it should be noted that chemotherapy treatment of Wilms tumour is quite intensive. According to the SIOP 2001treatment protocol, a combination

of dactinomycin, vincristine and doxorubicin is used for 6 weeks and additional post-operative chemotherapy treatment by doxorubicin, carboplatin, cyclophosphamide and etoposide is performed over 34 weeks for high risk patients diagnosed over stage II [76]. These intensive chemotherapy treatments have improved Wilms tumour prognosis significantly from 30% to 90% overall survival [60], but the adverse effects of chemotherapy agents and radiotherapy, including cardiac dysfunction [77], hypertension, renal failure [78], and secondary malignancies [79] have remained a huge clinical problems.

The major genetic abnormalities of Wilms tumour are 1q gain, 1p loss, 16q loss, *MYCN* gain or mutation and 17p loss encompassing the *TP53* locus. Especially *TP53* alterations are strongly associated to Wilms tumour with anaplasia. Tumours with *TP53* mutations and/or 17p loss are associated with a significantly increased risk of death [80]. Regarding the mechanism linking 16q loss to inferior prognosis, a small overlapping copy number alterations of 16q, harbouring the *IRXB* gene cluster, was previously reported in Wilms tumours by us [81]. In the present study, we focused on IRX3 and IRX5 since the role of these proteins has remained unknown. Signalling pathways associated to IRX3 and IRX5 might be novel treatment targets.

Malignant rhabdoid tumour

Rhabdoid tumour is an uncommon, but highly aggressive malignant neoplasm usually emerging in infants and young children. Rhabdoid tumour is briefly categorized into atypical teratoid/RT (AT/RT) which emerges in the central nervous system and extracranial rhabdoid tumour, often termed malignant rhabdoid tumour. Malignant rhabdoid tumour usually arises from the kidney or from soft tissues, including the orbit, thymus, uterus, bladder and neck [53]. The median age of diagnosis is 16 months [82]. Prognosis of malignant rhabdoid tumour is poor with a three-year overall survival of 38.4% and two-year overall survival of 13% for metastatic disease [83].

Although combination of surgery, chemotherapy and radiotherapy is used for the current treatment, whether there is an effective treatment strategy has been unclear because of the poor overall survival. Further, due to a highly intensive treatment protocol, only less than half of the patients complete the protocol [83]. There is in fact no strong evidence to support highly intensive chemotherapy treatment for this tumour type [84]. Thus, a new treatment strategy is necessary to improve the poor prognosis.

Inactivation of the SWI/SNF complex triggers the emergence of malignant rhabdoid tumour. The essential components of the SWI/SNF complex are *SMARCB1* and *SMARCA4*. Complete inactivation of *SMARCB1* in the q arm of chromosome 22 is the most frequent driver of malignant rhabdoid tumour [85]. Around 95% of malignant rhabdoid tumours contains abnormalities of *SMARCB1,* with a significant proportion has having one germ-line and one somatic mutation [86]. Germline nonsense mutations and somatic inactivation of *SMARCA4* are also reported in AT/RT patients and renal malignant rhabdoid tumour patients [87].

Inter-tumour heterogeneity of malignant rhabdoid tumours from different patients has been identified by whole-genome sequencing and this has enabled the subdivision of

malignant rhabdoid tumour into two subgroups [88]. However, there is no systematic study so far of the intra-tumour heterogeneity and tumour evolution of malignant rhabdoid tumour in the same patient.

Because malignant rhabdoid tumour is a highly refractory tumour, the effectiveness of immune checkpoint blockade has been discussed. One paper indicated that 90% of cluster of differentiation (CD) 3 + T cells in malignant rhabdoid tumour also expressed CD8, identifying them as cytotoxic T cells [89]. Another paper confirmed that CD8+ T cells infiltrate malignant rhabdoid tumours and that AT/RT expressed high levels of checkpoints receptors, including programmed cell death-1 (PD-1), and also that PD-1 blockade suppressed tumour volume in an AT/RT mouse model, resulting in improved survival of the mice [90]. These recent reports suggested future treatments by immune checkpoint blockade in malignant rhabdoid tumour, an issue I address in the present thesis.

Neuroblastoma

Neuroblastoma is the most common extracranial solid childhood malignant neoplasm. Neuroblastoma emerges from neural crest cells of the sympathetic nervous system with most cases occurring in the adrenal medulla. The prevalence of neuroblastoma varies depending on geographical regions as is the case of Wilms tumour. Age-standardized incidence of tumours from the sympathetic nervous system including neuroblastoma among children aged 0-14 years is estimated to 10.1 per 1,000,000 children in Northern Europe but only 4.4 in South Asia [73].

Diagnosis of neuroblastoma is based on a histopathological examination of tumour tissue or the presence of tumour cells in a bone marrow aspirate or biopsy with raised concentration of catecholamines in urine [91]. Staging is done according to the International Neuroblastoma Staging System (INSS) [92] and pathological classification could be done by the International Neuroblastoma Pathology Classification (INPC) [93]. However, this INSS staging is based on tumour progression assessed by surgical procedures. To circumvent such procedures prior to treatment, the more recent, imaging based International Neuroblastoma Risk Group (INRG) classification system is now applied for the risk assessment before the treatment. This INRG risk group is determined using multiple variables, including stage by the INRG Staging System [94], age, histologic category, grade of tumour differentiation, presence or absence of amplification of *MYCN*, presence or absence of 11q aberration and status of ploidy (hyper-diploid or diploid), which enables us to define pre-treatment risk groups of very low, low, intermediate and high risk [95].

Overall, treatment strategy for high risk neuroblastoma patients is extremely intense. Combination of pre-induction chemotherapy, post-operative myeloablative therapy with bone marrow transplantation followed by 13-cis-retinoic acid resulted in the best prognosis of 3-year event-free survival for high risk neuroblastoma patients [96]. However, this protocol employs total-body irradiation, which has severe late side effects. Another study assessed the efficacy of total-body irradiation by multivariate analysis and showed that there was no convincing treatment benefit to improve overall survival by total-body irradiation. Therefore, it should not be used for all neuroblastoma patients because of the late adverse effects [97]. According to a randomized control trial to assess

the efficacy of high-dose rapid induction chemotherapy known as rapid COJEC (C; cisplatin, O; vincristine, J; carboplatin, E; etoposide and C; cyclophosphamide), this protocol improved 5-year event-free survival of high risk neuroblastoma patients, but there was no improvement of overall survival [98]. While the efficacy is controversial, the rapid COJEC regimen is employed as induction chemotherapy treatment for the high risk group in Sweden. However, even if we conduct this intensive treatment, prognosis for neuroblastoma high risk patients remains poor, with a survival rate still around only 50% [99].

The accumulated number of gene mutations is not high in neuroblastoma compared to adult cancers. Instead of gene mutations, chromosomal instability highly contributes to the tumorigenesis of neuroblastoma, giving rise to a high number of numerical and structural copy number alterations [100]. The patients with the presence of *MYCN* amplification have the lowest overall survival rate. After them, the patients with the presence of segmental copy number aberrations in the absence of *MYCN* amplification have the poorest outcome. In contrast, the presence of numerical aberrations only is linked to a more favourable outcome [101]. It has been reported that high risk neuroblastomas encompass high intra-tumour heterogeneity of genomic profiles during cancer development and also harbour more complex genomic landscapes than low risk paediatric tumours [102]. Furthermore, a previous study from our group showed that clones carrying *MYCN* amplification and structural copy number alterations often undergo clonal sweeps during neuroblastoma evolution, indicative of a strong survival benefit from these alterations [103].

One of the most important genes affected by mutations in neuroblastoma is the *Anaplastic Lymphoma Kinase (ALK)*. Germline *ALK* mutation can be a trigger of hereditary neuroblastoma [104] and it has been reported that *ALK* promotes cell proliferation [105, 106]. Therefore, if the presence of *ALK* mutations is confirmed they could be a treatment target. In fact, there is a phase I clinical trial for childhood neoplasms including neuroblastoma for the usage of crizotinib (an ALK inhibitor), and 11 neuroblastoma patients with the presence of *ALK* mutations were evaluated (totally 34 patients were treated, but 23 patients had unknown *ALK* status). It should be noted that only one patient showed complete response and three patients obtained stable disease among the 11 *ALK* mutated patients [107]. Since 4/11 patients with the presence of *ALK* mutation responded to crizotinib, tyrosine kinase inhibitors might be a treatment option for *ALK* amplified or mutated neuroblastoma. However, intra-tumour heterogeneity with respect to *ALK* status and other targetable mutations remains a potential threat to effective targeted therapy because clones lacking mutations will not be sensitive to the treatment. How the genetic landscape changes over treatment of neuroblastoma as a function of intra-tumour heterogeneity is the final topic addressed in this thesis.

The present study

Aims

The overall aim of the present thesis was to investigate illustrative examples of how tumours emerge as complex ecosystems from a combination of factors that include the tumour microenvironment (Article I), tumour cell differentiation states (Article II), and evolving clonal landscapes (Articles III and IV).

The specific aims covered by each article were as follows:

Article I - To investigate cell to cell communication between epithelial ovarian cancer cells and cancer-associated mesothelial cells via chemokines
Article II - To explore the roles of IRX3 and IRX5 in determining the maturation of normal kidney cells and how these proteins are dysregulated in the differentiation block resulting in Wilms tumour
Article III - To assess tumour evolution under progression of malignant rhabdoid tumour under treatment, with a particular focus on management by immune checkpoint blockade
Article IV - To investigate the clonal evolution of neuroblastoma by tracing clonal ancestries under chemotherapy treatment

Materials and methods

Briefly, we have analysed clinical samples for cancer phenotype in Article I and Article II. For genomic analyses in Article III and Article IV, DNA was extracted from clinical samples. Cancer model systems using primary cells (Article I), conventional cell lines (Article I, Article II and Article IV) and patient-derived tumour xenograft (PDX) cells (Article IV) were applied for further experiments.

Handling of clinical samples (Article I, Article II, Article III and Article IV)
Ascites from ovarian cancer and benign ovarian tumours were obtained at surgery (Article I). Immunohistochemistry was conducted using formalin-fixed paraffin-embedded tumour blocks from clinical samples (Article I, Article II and Article III). The sections were cut from tumour blocks and were deparaffinized. After blocking endogenous peroxidase, each section was incubated with a primary antibody and staining was optimised by a secondary antibody. For assessment, staining intensity and the percentage of positively stained cells were measured to result in a final score in Article I. For Article II, tissue micro arrays from primary Wilms tumours were used and the presence or absence of IRX3 and IRX5 were assessed. To approximate the situation of diagnostics by core needle biopsy in Article III, each tissue section was subdivided into 20 mm^2 squares in a grid system to simulate core needle biopsy equivalents. Tumour infiltrating cytotoxic lymphocyte expressing CD8/PD-1 and tumour cells expressing programmed death-ligand (PD-L) 1 were evaluated. For DNA extraction, fresh frozen tumour samples or formalin-fixed paraffin blocks were used (Article III and Article IV). DNA was extracted by standard methods and was subjected to single nucleotide polymorphism (SNP) array and sequencing (see below). These samples were obtained from the Lund University Hospital pathology archives. For the malignant rhabdoid tumour project (Article III), DNA from

all tumours were obtained from fresh frozen samples. For the neuroblastoma project (Article IV), both fresh frozen and formalin-fixed paraffin blocks were used. Due to the presence of genetic inter- and intra-tumour heterogeneity, we conducted multiple samplings of the tumours. All studies were approved by the Ethics committee of each university/regional authority.

Cancer model systems

Human primary cells (Article I)

Primary mesothelial cells for ovarian cancer research were taken from the omentum obtained by omentectomy performed as a standard surgical procedure. Collected cells were cultured until they became confluent in each cultured plate in order to replicate the peritoneal surface covered by mesothelial cells. As a model for advanced ovarian cancer, we transformed normal mesothelial cells to cancer-associated mesothelial cells by the use of TGF-β [71].

Established cell lines (Article I, Article II and Article IV) and Patient-derived Tumour Xenograft (PDX) models (Article IV)

We employed the ovarian cancer cell lines SKOV-3, A-2780, OVCAR-3 and ES-2 for Article I, and the Wilms tumour cell line WiT49 for Article II for phenotypic analyses. Although cell lines are used frequently as cancer models, there are several limitations using conventional cell lines such as contaminations of other cancer cell lines [108]. Further it has been reported that cultured cell lines have changed their genomic profile compared to the primary tumour [109]. Thus, we have used patient-derived tumour xenograft (PDX) cells for Article IV in order to mimic conditions more relevant to clinical situations [110]. Because PDX cells from neuroblastoma can still be limited in their clonal diversity, we complemented these studies with the high risk neuroblastoma cell line IMR-32, which contains complex copy number aberrations grouped into multiple subclones for Article IV [111]. We used PDX cells for the neuroblastoma study to reproduce genomic landscapes that we have assessed in neuroblastoma patients. Further, it has been reported that PDX models respond in a relevant way to drugs [112]. We employed a previously established PDX cell line from a high risk neuroblastoma patient [113, 114] to assess how the genome of cancer cells evolve under cisplatin exposure.

Analysis of cancer phenotype (Article I and Article II)

Briefly we have analysed cancer phenotype, i.e. invasion for Article I and proliferation for Article II and examined signalling pathways by Western-blot analysis for Article I and ribonucleic acid (RNA) sequencing for Article II.

Chemokine array

We used cancer-associated mesothelial cells as a model of advanced ovarian cancer. We aimed to compare the protein levels of different chemokines in the conditioned medium secreted from these cells. Since the type of chemokines present in the conditioned medium was unknown, we conducted an array based experiment to detect them [115]. The intensity of each chemokine was measured using the ImageJ software [116] by which the expression landscape of chemokines was assessed.

Expression analysis of protein

The amount of CCL2 protein in the medium and in ascites in Article I was analysed using enzyme-linked immunosorbent assay (ELISA) and Western blotting analysis for other proteins which were not present in medium or ascites. For ELISA, we employed the sandwich method because of its high sensitivity [117], which enabled us to validate the low levels of protein in the clinical samples, i.e. ascites. For Western blot analysis, collected proteins were separated by SDS-PAGE, transferred to membrane and incubated with antibodies towards the protein of interest. Since the same membrane was also used for assessing the phosphorylation status (Article I), the membrane was washed by stripping buffer before the phosphorylation analysis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and RNA sequencing

RT-PCR is a method used to detect targeted gene expression and RNA sequencing has the benefit of examining the transcriptional landscape. After extraction of RNA from mesothelial cells by standard methods, expression of CCL2 was examined by RT-PCR in Article I. The cDNA was generated by reverse transcription and amplified using specific primers [118]. The whole transcriptome was assessed by RNA sequencing in Article II. Gene expression data was analysed by gene set enrichment analysis, and comparisons between *IRX3* deleted cells and wildtype or *IRX5* deleted cells and wildtype were performed. Since we were interested in the signal pathways in *IRX3* knockout cells and *IRX5* knockout cells, especially regarding genes related to nephrogenesis, the WNT and Hippo signalling pathways were assessed in targeted pathway analyses, and genes were subjected to hierarchical clustering [119].

Live cell analyses

We conducted a scratched wound-healing migration/invasion assay (Article I) and a proliferation assay (Article II) using a live cell analysing machine, i.e. IncuCyte Zoom [120]. A microscope inside the incubator captured and measured the movements or territories of cells to assess migration/invasion and proliferation. Since the investigator does not decide the location to assess, one strength of this analysis is that we can remove observation biases. Another advantage is that live cell analyses can be used to observe the entire experiment from the beginning to the end, while ordinary experiments only observe a certain time point. On the other hand, the weakness of live cell analysis is the high cost compared to ordinary analysis due to the equipment. In addition, we can only analyse scratching migration/invasion assays and are not able to assess chemotactic affects between an upper chamber and a lower chamber as when using a conventional trans-well chamber [121].

Analysis of cancer genotype (Article III and Article IV)

Briefly, genomic analyses were performed using SNP array for analysing copy number alterations and whole-exome sequencing for the detection of single nucleotide variants and short insertions and deletions (indels). Clonal deconvolution was performed for the detection of subclones, when possible followed by clone size estimations. These data were transformed into matrices containing the presence or absence of copy number aberrations or mutations. These matrices were systematically organised into phylogenies using the maximum-likelihood approach to examine the evolutionary history of each tumour.

Whole-genome copy number genotyping

SNP array is a highly robust method to assess copy number aberrations. Digested genomic DNA is subjected to ligation with adapters and fragments were amplified by PCR followed by labelling. These labelled DNA fragments bind to allele-specific probes on the surface of the SNP array's chip. The hybridized array is scanned and fluorescent signals are measured, giving information of whole-genome copy number genotyping such as gain, amplification, loss, and CNNI [122]. Intensity of gain and loss was obtained as logarithm values ($\log_2$ ratio), and the frequency of B-alleles compared to all alleles (B-allele frequency) was also available from SNP array analysis.

Whole-exome sequencing

This sequencing technology is a commonly used type of massive parallel sequencing or next-generation sequencing. Massive parallel sequencing using short reads assembly is capable of analysing small variants such as single nucleotide variations and short indels. Sample DNA is subjected to ligation and sequencing libraries containing DNA fragments with adapters were prepared [123]. There are multiple methods for sequencing. For instance, by synthesis method, each nucleotide is given a corresponding unique fluorescently tag, depending on the type of nucleotide. This nucleotide binds to a template strand through synthesis of DNA and the fluorescence is detached after synthesis. Fluorescent signals are captured and are accumulated until the end of sequencing. Because each fluorescent signal corresponds to a unique nucleotide, we can identify the DNA sequence by analysis of accumulated fluorescent signals. For sequencing in our projects, paired-end sequencing was performed using Illumina sequence technology. The human reference genome was used for mapping of the paired end reads. Our aim was to analyse single nucleotide variants and short indels in exome regions and evaluate the evolutionary history of cancer cells by mutational profiles after subclonal validation. We analysed clinical tumour samples (Article III) and PDX tumours (Article IV) by whole-exome sequencing. Somatic variants were called by filtering against normal samples from each patient and by using variant calling algorithms [124].

Clonal deconvolution and phylogenetic analysis

The "TAPS" (Tumor Aberration Prediction Suite), an R package designed for copy number data [125], was used to validate whether events were clonal or subclonal. The clone size for specific copy number alterations was estimated using the logarithm value for DNA content and the allelic imbalance computed from the B-allele frequency [103]. The tumour cell fraction that harboured single nucleotide variants and indels detected by whole-exome sequencing were obtained by combining the allelic information from SNP array and variant allele frequencies of sequencing data. The fraction of mutations, defined as tumour cell fraction (Article III) or mutated clone fraction (Article IV) was calculated using mathematical formulas described in each paper. Since we have analysed multiple samples, a mathematical method is necessary to integrate all data to reveal each tumour's evolutionary history. We decided to employ phylogenetic analysis and there are lots of phylogenetic methods [126]. It should be noted that there is no perfect method. Here, we employed probabilistic estimation in the form of the maximum-likelihood method implemented by the widely used R package "phangorn" [127].

Results

Article I

We first demonstrated the characteristic morphological change from epithelioid to fibroblastic appearance in cancer-associated mesothelial cells. Loss of tight junctions between these cells suggested that cancer-associated mesothelial cells could be a suitable target location for peritoneal metastasis. We employed SKOV-3 cells for these experiments because SKOV-3 is regarded as an ovarian serous carcinoma. [128, 129]. We examined the ability of cancer cells to adhere and to do trans-mesothelial migration [130], and the cancer-associated mesothelial cells promoted adhesion and migration of cancer cells through a significant induction of lost junctions between the mesothelial cells. We next explored the expression of chemokines in conditioned medium of cancer-associated mesothelial cells and found that the highest expressed chemokine was CCL2. CCL2 concentration in malignant ascites was also higher compared to benign tumours. These results suggest that CCL2 plays an important role in the tumour microenvironment. CCR2 is regarded as the most important receptor for CCL2 [131]. We used SKOV-3 for further phenotypic analyses because of its high expression level of CCR2. Conditioned medium of cancer-associated mesothelial cells promoted invasiveness of cancer cells compared to that of normal mesothelial cells, and the addition of an antibody towards CCL2 inhibited invasiveness of tumour cells. Thus, CCL2 in conditioned medium from cancer-associated mesothelial cells accelerated the invasiveness of cancer cells. We finally explored whether there is a correlation between clinical outcome and expression of CCR2 in tumour regions using clinical samples. We found that a high CCR2 expression in the tumour cells was associated to poor clinical outcome parameters, including overall survival and progression-free survival. Our results revealed the clinical importance of CCL2 and CCR2 in the ovarian tumour microenvironment.

Article II

We found that IRX3 was expressed mainly in the epithelial region of Wilms tumours, whereas IRX5 was expressed in the blastemal, epithelial and stromal parts. The number of maturing epithelial structures, including comma-shaped bodies and s-shaped bodies decreased in heterozygous *IRX3/IRX5* deleted mice and they were also reduced in homozygous *IRX3/IRX5* deleted mice compared to those in wild type mice. This inferred that IRX3 and IRX5 are involved in nephron formation. The different locations of IRX3 and IRX5 expression in Wilms tumour samples implied that IRX3 and IRX5 had distinct roles in tumorigenesis. It has been reported that WiT49 is the best suited cell line to mimic tumour morphology when used as an orthotopic Wilms tumour xenografts [132]. Thus, we employed the WiT49 cell line for further explorations and we established *IRX3* or *IRX5* knockout WiT49 cells. In vivo, WiT49 xenograft tumours confirmed that *IRX3* knock out tumours contained less tumour tubules, implying a function related to maturation of tumour cells. On the one hand, *IRX5* knock out tumours showed significantly less proliferation, while *IRX3* knockout tumours did not. Moreover, the global gene expression pattern of *IRX3* knockout cells and *IRX5* knockout cells were distinct. Loss of *IRX3* or *IRX5* showed an opposite pattern of WNT and Hippo signalling. Interestingly, WNT5A, a significant molecule in mice nephrogenesis [133], was expressed differentially in the respective knock-out systems. *IRX3* knockout cells showed lower expression of *WNT5A*, whereas *IRX5* knockout cells expressed high levels. This

supported that IRX3 promotes tumour maturation and IRX5 promoted oncogenic features such as tumour proliferation and immaturity. Our findings clarified that IRX3 and IRX5 play key roles for Wilms tumour differentiation.

Article III

We aimed to assess the evolutionary history of progressive malignant rhabdoid tumours under treatment by analyses of multiple tumour regions from two patients using SNP array and whole-exome sequencing. We found that the evolutionary pattern analysed by both SNP array and whole-exome sequencing showed early branching followed by linear progressive evolution at each metastatic site, since the clones of the offspring inherited genomic abnormalities from a common ancestral clone. Clonal mapping, which denotes the spatial and phylogenetic relationship between subclones in the tumour regions, revealed that three subclones coexisted in the primary tumour and also in the metastasis in patient 1, suggesting polyclonal seeding of the metastasis. In patient 2, the mapping inferred that metastases were each formed by a single cell ancestor with different mutational status, but we could not compare this with primary tumour because of a necrotic primary sample. Also, an interesting finding was that the tumour mutation burden, which was defined as the sum of mutations, increased in the metastasis along with the number of predicted neoantigens. We also found from selection analysis that pulmonary metastases in patient 2 showed ongoing positive selection of subclones. Since a high mutation burden and a high number of predicted neoantigens suggest potential effectiveness of immune checkpoint blockade according to several studies of adult cancers [134, 135], we explored the status of immune checkpoint activation by immunohistochemistry. There was a positive correlation between the proportion of PD-L1 positive tumour cells and the number of CD8 positive T cells/PD-1 positive T cells. However, the checkpoint status was regionally heterogeneous, warning against using small single tumour samples, such as core needle biopsies, to predict response to immunotherapy in this group of patients.

Article IV

We here analysed the neuroblastoma tumour genome from pre-treatment, post-treatment, and metastatic relapse samples using SNP array for copy number aberrations, followed by clonal deconvolution and phylogenetic estimations. We found two distinct evolutionary patterns, i.e. linear evolution and collateral evolution. In progressive disease, metastatic clones could be traced back to a common ancestral clone in the untreated primary tumour. In the treatment responsive group, a similar common ancestor in the untreated tumour could not be found for islands of tumour cells surviving treatment in the primary or for later relapses – a pattern we term collateral clonal replacement. This result suggested that a common ancestor to clones detected before and after therapy emerged early, from which metastatic relapses evolved with accumulation of genomic abnormalities, which the dominant clones of the primary tumour did not contain neither before, nor after therapy.

We defined an index of genomic diversity and assessed the degree of inter- and intra-tumour heterogeneity over time. Post-treated tumours showed a higher diversity than pre-treatment tumours and metastatic relapses, suggesting that tumour cells surviving treatment evolved in divergent ways under selective pressure by chemotherapy-induced

changes in the microenvironment. It is previously known that the copy number status of the *MYCN*-amplification can sometimes be heterogeneous [136-138], and we further showed that also the segments from chromosome band 2p24 including the *MYCN* amplicon is heterogeneous when it comes to break points. Another finding in the analysis of the clinical cohort was an amplification of *ALK* identified in two cases. One case harboured *ALK* amplification at the post-treated, primary sample which was not found in the pre-treatment primary sample. Post-treated, primary tumour and pre-treated, metastatic tumour did not contain *ALK* abnormalities in another case. However, a metastatic relapse encompassed *ALK* amplification.

We then assessed whether it was possible to reproduce the patterns of linear evolution and collateral evolution found in clinical analysis in standardized model systems. Since copy number aberrations are relevant to clinical outcome and PDX tumours follow clinical genetic evolution better than conventional cell lines, we first analysed a PDX model of progression with or without chemotherapy treatment (cisplatin only) using SNP array. We could reproduce linear evolution under progression in which PDX tumours treated by insufficient treatment evolved the most, compared to tumours that grew without treatment or were stationary under treatment. In the progression model, we also analysed single nucleotide variants and short indels using whole-exome sequencing for the purpose of subclonal analysis by a different method. Whole-exome sequencing enabled us to identify the presence of subclones in mother cultured cells that SNP array could not validate. Similar to the SNP array, we found that tumours that progressed under treatment accumulated more mutations than tumours growing untreated or that were stationary.

We further evaluated whether a PDX system of effective multimodal chemotherapy treatment (rapid COJEC) could reproduce some of the features of treatment responsive tumours in the clinical cohort. We indeed found that subclonal diversity increased in a cohort of tumours after treatment compared to a parallel cohort of PDXs left to grow freely without treatment. Because repeated sampling over time was difficult to perform in the PDX system for both practical and ethical reasons, we used the conventional cell line, IMR-32 for the purpose of replication in vitro of collateral evolution under effective therapy. We found the collateral evolutionary pattern in the treatment responsive groups exposed by single high-dose cisplatin and multiple high-dose cisplatin treatments. Our results from these experiments confirmed both the evolutionary patterns observed in clinical analyses, indicating that clinical phylogeny could be summarized into two distinct appearances of evolutionary history associated to treatment response.

Discussion

Here I would like to focus on five general topics based on our results. A deeper discussion of our findings can be found in each paper.

Are our results derived from phenotypic analyses relevant to treatment?

We explored how mesothelial cells affected cancer cells in the ovarian tumour microenvironment in Article I. Although the role of cancer-associated fibroblasts has widely been studied in ovarian cancer [139, 140] and various other cancer types [141-143], investigation of the relationship between cancer cells and cancer-associated peritoneal mesothelial cells has been little explored in ovarian carcinoma or other tumour types [144]. High grade serous carcinoma is considered to emerge from precursors in the fallopian tubes, so-called serous tubal intra-epithelial carcinomas, harbouring alterations of *TP53*, *BRCA1* and *BRCA2* [145]. Therefore, the vast surface of the peritoneal cavity covered by mesothelial cells could be a location easy to reach for metastasis. The investigation of crosstalk between cancer cells and mesothelial cells became the aim for our study. We used cancer-associated mesothelial cells exposed to TGF-β as our cancer model.

The weakest point of this modelling is that the presence of cancer-associated mesothelial cells has not directly been shown in clinical samples. Thus, verification of the role of cancer-associated mesothelial cells in the clinical context is urgent. Otherwise, our finding becomes just a hypothesis based on the inferred cancer-associated mesothelial cells, and we cannot conclude that our model reflects the clinical reality.

The second weakness was the low number of clinical ascites samples analysed. Our results suggested that ascites of advanced stage contains a higher amount of CCL2 than those of early stage. However, we could not investigate the comparison of CCL2 concentration in ascites between early and advanced stage of ovarian cancer.

Finally, we could not evaluate the role of the CCL2/CCR2 axis by analysing clinical samples due to the lack of paired samples from ascites and formalin-fixed paraffin blocks from the same patients. We only showed that CCL2 promoted invasiveness of high-grade serous carcinoma cell lines, and higher expression of CCR2 in the tumour cells correlated to poor clinical outcome. A recent report has shown that expression of chemokine receptors actually works without the presence of a coupled major chemokine [146]. Another report suggested that only overexpression of chemokine receptor promoted cell proliferation. Thus, chemokine receptors could have a function to alter the cancer phenotype and the presence of coupled chemokine is not necessarily required for phenotypic change [147]. Further, it has been shown that CCL2 has an anti-fibrotic effect on human fibroblasts independent of CCR2 [148]. Thus, we do not know if CCL2 affects cancer cells independently or dependently of CCR2, and if CCR2 works as a prognostic factor with independence or dependence on CCL2.

As an important point, chemokines and their receptors do not have a one-to-one relationship. It is well known that chemokines generally bind to multiple chemokine receptors. While the main receptor of CCL2 is regarded to be CCR2 and many articles

investigated the role of the CCL2/CCR2 axis [149, 150], CCL2 also binds to CCR4 and CCR11 [151, 152]. In fact, one paper mentioned that blocking of the CCL2/CCR4 axis in T cells led to inhibition of tumour growth in mice [153]. Since we did not examine the expression of CCR4 and CCR11 in the tumour cells, further exploration between CCL2 and CCR4/CCR11 expression in tumour cells and its correlation to patient outcomes using clinical materials might be a next step.

These validations of chemokines and receptors to determine which molecules contributed the most to tumour progression is significant for discussions of novel treatment strategies. While there are plenty of antibodies to target specific molecules which have been accepted by the National Institute of Health Sciences in Japan, only an antagonist of CCR4, i.e. mogamulizumab [154] has been accepted among the CCL2 receptor blockers. As a current established medication, mogamulizumab has been approved for adult T cell leukaemia and lymphoma, and human T-lymphotropic virus type 1 (HTLV1) associated myelopathy [155, 156]. Our results suggest that this already established therapy targeting CCR4 could be introduced to ovarian cancer treatment if CCR4 is an essential part of CCL2 signalling in the ovarian tumour microenvironment.

For Article II, while loss of the IRX cluster in 16q is associated to inferior rates of relapse-free survival in Wilms tumour, this association is not observed in other high risk childhood renal neoplasms such as clear-cell sarcoma of the kidney and malignant rhabdoid tumour of the kidney [157]. Therefore, aberrations of *IRX3* and *IRX5* might be unique to Wilms tumour. In our previous study, we showed that IRX3 was expressed in epithelial elements of Wilms tumour, suggesting that IRX3 has a significant role in tubular differentiation [81]. However, little was known about IRX5 in nephrogenesis before our Article II was published.

Wilms tumour is considered to emerge from a disruption of the mesenchymal-epithelial transition that is critical for normal nephrogenesis [158]. Thus, investigation of how the proteins IRX3 and IRX5 are involved in kidney cell differentiation could be important to understand Wilms tumour pathogenesis and also point to possibilities for novel treatment strategies. An example of how factors guiding differentiation can be used for therapeutic purposes is 13-cis-retinoic acid in neuroblastoma [96], a molecule that has so far been limited in use in Wilms tumour to rare cases with multifocal lesions [159].

Thus, we aimed to explore the roles of IRX3 and IRX5 in this study. We found that the number of maturing epithelial structures decreased in heterozygous or homozygous *IRX3/IRX5* deleted mice, possibly mimicking the effects of 16q deletion in Wilms tumour patients. These results inferred that both deletions of *IRX3* and *IRX5* correlate negatively to tumour differentiation. However, from cell line experiments in the presented study, we could only create *IRX3* or *IRX5* knock out cells, and our results suggested that loss of *IRX3* and retained *IRX5* would in theory be most efficient for tumorigenesis, leading to a contradiction between our model systems and clinical data. More specifically, loss of *IRX5* seems to suppress tumour development due to less tumour proliferation.

According to our previous study, chromosomal regions harbouring both *IRX3* and *IRX5* were identified to be deleted in anaplastic tumour elements which are regarded as a high

risk element in Wilms tumour [81]. Thus, the role of *IRX5* was contradictory between the results from previous analyses based on clinical data and the experiments of the current study's cell line experiments, while for *IRX3* there was no contradiction. Notably, the deletion that we found in the previous study was not a homozygous deletion. Therefore, deletion in one allele might not correspond to *IRX5* gene knockout, i.e. homozygous deletion completely. One possibility to resolve this contradiction would be to create human cells with both genes knocked out, in order to explore the interaction between the expression of the two proteins. However, we could not succeed in deleting both *IRX3* and *IRX5* from our Wilms tumour cell line.

A better way to examine differentiation might be to treat cancer cells using 3D culturing methods in order to avoid differentiation during culturing. It was shown that 3D culturing in stem-cell promoting medium inhibited tumour differentiation that was induced in serum containing medium [113]. Another paper suggested that 2D culturing promoted differentiation of mouse embryonic stem cells more than 3D culturing [160]. Since 3D culturing is regarded better then 2D cell culturing for retaining clinical features [161] and the cancer cell line for Wilms tumour was studied only in 2D culturing system, the former might induce different results of tumorigenesis of *IRX5* than those presented in Article II.

In summary, our results suggested that overexpressing *IRX3* signalling or inhibiting *IRX5* signalling might promote differentiation of Wilms tumour cells, and treatments leading to differentiation might be alternative therapy that can reduce adverse events compared to current intensive chemotherapy in Wilms tumour.

Do our cancer models reflect clinical features?

There are several limitations of cancer phenotypic and genetic analyses using conventional cell lines and PDX models. Established cell lines are widely used as cancer model systems. However, many reports supported by genomic analyses have suggested that some cancer cell lines poorly reflect clinical tumour samples. Regarding driver mutations, they are often retained in cell lines. Thus, it is considered that they do not easily lose the most important genetic abnormalities, such as *MYCN* amplification or *ALK* amplification for instance in neuroblastoma cell lines. However, the generation time for cancer cell lines is shorter than that of clinical tumours. Thus, genetic events related to genomic instability, such as copy number aberrations are easily induced by prolonged cell culturing [108, 162]. Further, cells cultured for long time periods change their phenotype from the original tumours and may also undergo substantial alterations in genotype through genetic bottlenecks and accumulate more mutations [163].

We used mainly the SKOV-3 ovarian cancer cell line for Article I and WiT49 cells as a mimic of Wilms tumour for Article II. I will discuss whether these cell lines have clinical relevance, here.

SKOV-3 has been regarded as a model of high-grade serous carcinoma. However, recent genetic analyses have revealed that SKOV-3 has a mutation of *ARIDIA* which is more typical of high-risk clear cell carcinoma. Further, SKOV-3 does not contain *TP53*

mutation which is very frequent in high grade serous carcinoma [164]. The overall genomic profile of SKOV-3 does not resemble high grade serous carcinoma [165] and is closer to clear cell carcinoma. Thus, one paper defined SKOV-3 as an unclassified ovarian carcinoma cell line [166]. High grade serous carcinoma is the main important subtype of ovarian cancer as it causes 70% of ovarian cancer deaths [167]. Thus, for mimicking serous carcinoma it might be better to use some of the other cell lines that were used in Article I, for instance, OVCAL-3 with the presence of *TP53* mutations.

Regarding WiT49 cells, it has been reported that 29/30 Wilms tumours with diffuse anaplasia and 7/17 blastemal tumours harboured *TP53* mutations according to genetic analyses using Wilms tumour patients treated by the SIOP 2001 protocol [168]. Therefore, *TP53* mutation is regarded as one of the most significant genetic alterations of high-risk Wilms tumour. WiT49 harbours a *TP53* mutation and the highly complex genomic profile typical for diffuse anaplasia [169]. Thus, we can say that genetically WiT49 cells reflect high-risk Wilms tumours. However, WiT49 cells were generated not from a primary Wilms tumour but from a pulmonary metastatic tumour [169]. We should take this into account when interpreting tumour differentiation because WiT49 cells might not function under the same biological limitations as primary cells from Wilms tumours would.

One way to overcome the issues from conventional cell lines might be to use PDX cells. Clinical tumours are heterogeneous which is also true for paediatric tumours whose mutations are fewer compared to adult cancers [170]. PDX tumours likely maintain more of the tumour heterogeneity than established cell lines [171]. Thus, it may be better to use PDX cells to model clinical tumours [172]. However, a recent report analysed copy number alterations in numerous PDXs and revealed that even PDXs often evolved mouse specific genetic alterations [173].

Then the question is what is the best cancer model? The conflicting situation between conventional cell lines and PDX cells is actually a difficult issue that is not solved easily from our current knowledge and experiences. In fact, a recent paper suggested that cancer cell lines are more heterogeneous than what could be expected. Therefore, conventional cell lines could be regarded as an useful experimental tool to study intra-tumour heterogeneity in a systematic fashion [174].

In Article IV, we used both PDX cells and IMR-32 cell line for reproducing our phylogenies of neuroblastoma. PDX cells could well mimic linear evolution under progression, while they poorly reflected collateral clonal replacement because of a limited repertoire of subclones from the beginning of the experiment. Therefore, we shifted to IMR-32 cells, which were more heterogeneous from the start and thus similar to the clinical situation of neuroblastoma primary tumours [103]. IMR-32 did indeed very well mimic collateral evolution under therapy, but of course does still not capture the great heterogeneity that can exist between different neuroblastomas in different patients.

In summary, there is still no perfect cancer model and choice of the best cell lines would depend on our scientific questions.

Do our genetic analyses contribute to the understanding of clinical scenarios?

Tumours evolve during their lifespan. Intra-tumour heterogeneity in the form of multiple subclones has been associated to inferior prognosis in multiple cancer types [170, 175-177]. To remove all clones by surgical resection as much as we can and to treat remaining clones by chemotherapy is the simplest strategy to cure cancer under the current paradigm. In fact there is evidence that show improvement of prognosis by performing radical surgery even in the most advanced ovarian cancer, which is a highly genetically heterogeneous disease [178]. However, if we cannot succeed to treat all clones and some clone survives, this clone could be an ancestral clone for future recurrence.

Eliminating all clones with long-term replicative potential is probably achieved already today for tumours that show high overall survival such as low- and intermediate-risk Wilms tumour [76]. However, there are tumours which have shown low survival rate even when diagnosed early, such as ovarian high grade serous carcinoma that has an 84% five-year overall survival even at stage I [179], or malignant rhabdoid tumour where the four-year overall survival of even localized tumour was reported to be only 40.1% [83]. In these tumour types, cancer cells are likely to spread and hide in the body, but we could not detect them at diagnosis. These hiding cells might be origins for future metastatic relapses.

To provide useful data to the clinic, we have to correctly assess tumour heterogeneity and tumour evolution. The presence of subclones makes tumour bulk sequencing data complex. Thus, delineating subclonal landscapes is an important step for deeper understanding of tumour evolution, preferably by clonal deconvolution by which exact clone sizes are calculated. For this, we used multiple mathematical formulas (see each article). But we were also forced to sometimes use less precise assessments, based on operational rules that were just the best of available options based on probabilistic reasoning. For example, we employed a rule by which small subclones was always placed in the population with largest size although they could in fact be nested in several populations in the clonal landscape. This rule was based on the assumption that it was the most likely outcome under a constant mutation rate across clones. But this assumption might be wrong and a small number of copy number events or variants might in reality be subdivided differently across multiple clones than what we presented in Articles III and IV. Interestingly, SNP array and whole-exome sequencing showed slightly different subclonal landscapes in Articles III and IV. Whole-exome sequencing could identify some subclones not detected by SNP array in the clinical material in Article III and in cultured PDX cells in Article IV. Complete integration of clonal deconvolution by both SNP array and whole-exome sequencing might enable us to interpret more accurately tumour heterogeneity and tumour evolution.

All methods of clonal deconvolution from sequencing data is based on frequencies of variants [180]. We have analysed variant data from whole-exome sequencing in this thesis. However whole-exome sequencing only obtains sequencing data of exonic regions. We could get more variant data from more comprehensive methods such as whole-genome sequencing. Therefore, whole-genome sequencing might lead us to conduct more accurate clonal deconvolution based on variant frequencies, while also allowing a fairly

confident copy number analysis. In fact, subclonal selection analysis is now routinely applied using variants data obtained from whole-genome sequencing [181]. However, the financial costs of employing these analyses to multiple samples from multiple tumours still is a limiting factor.

Further, we could only validate the single nucleotide variants and short indels from short read DNA sequencing in our projects. Recently, third-generation, long read sequencing technology has been developed. When third-generation sequencing enables us to detect large structural variants, clonal landscapes and clonal deconvolution might lead different conclusions regarding subclonal landscapes [182].

We aimed to explore tumour evolution by the phylogenetic analysis model called maximum-likelihood estimation in Articles III and IV. Tumour phylogeny models including maximum-likelihood estimation and the others have now been applied for evolutionary models in various cancer types [61, 183-186] and might sometimes even suggest treatments based on tumour evolutionary tracks [32]. There are multiple methods for the creation of tumour cell phylogenies. Method based on parsimony that make phylogenies by selecting the minimum number of total evolutionary events, i.e. maximum-parsimony method [187] has also been widely used for phylogenetic analysis in various tumour types. However, this is based on the hypothesis that genetic events are rare for cancer cells, which is a contradiction against the large number of mutations found in many cancers and genomic variations that lead human genetic diversity [188-190].

A further limitation of the maximum-parsimony method is that multiple results can be obtained from the same complex dataset, because it creates phylogenetic trees whose total number of events are at a certain minimum and this method is not an estimation of the most probable tree. I would like to illustrate this by a simple case. A subclone should inherit mutations from a main clone and is detected in different proportions of tumour cells across two samples – in one sample as a population of the same size as the main clone (100% for both), in the other as a smaller population (say 100% for main clone, 50% for subclone). Then two types of maximum parsimony trees can be created in, one where ***subclonal events are added to the major clone making the subclone the descendants of the main clone*** or another tree can be created with ***loss of subclonal events by which the major clone ends up as the descendant of the subclone***. In this case, the subclone should be derived from the major clone. Therefore, the former version corresponds to the clinical situation and it is not difficult to choose the first tree in this case. But the question is, if there are numerous phylogenetic trees generated by the computer and the situation is more complex - how can we choose the fittest tree? There, a bias can enter if we choose a model most consistent with our hypothesis and it is not honest to science.

Thus, we decided to employ a method based on probability, i.e. maximum-likelihood estimation [127]. The strength of the maximum-likelihood method is that we could obtain a single phylogenetic tree because there is generally only one tree representing the highest likelihood, i.e. a parametric approach. However, the weak point of maximum likelihood phylogenetic trees is that we do not know the true number of events on branches when we analyse complex data [191]. Because we analysed childhood tumours whose genomic

events are quite few compared to adult tumours [192], our data-set did not contain so much copy number alterations and mutations, and we could assign all events to their corresponding branches in our phylogenetic trees. In contrast, adult cancers might be difficult to adapt to maximum-likelihood estimation in all cases because of the difficulty to validate all events causing the different branches.

Importantly, whatever phylogenetic trees are used, the method is just a model for interpretation of cancer evolution and there is no perfect phylogeny. Thus, we cannot guarantee that phylogenetic tree follows the real truth of cancer evolution. But the final question is what is the truth of tumour evolution? We have not learnt the real truth of cancer evolution yet. When we succeed to cure patients whom we cannot treat now by our phylogenetic analysis, we might say that we could grasp the truth.

Are our findings from phylogenetic analysis relevant to the present treatment strategy?

Immune therapy has dramatically improved the outcome for several refractory adult tumours in the last decade. This includes in particular melanoma and non-small lung tumours by usage of immune checkpoint blockade [193-195]. Overall, tumour mutation burden is higher in melanoma and lung cancers, including squamous cell cancer and adenocarcinoma than in most other adult tumours [196, 197]. When we see the evolutionary trees of melanoma or lung carcinoma, they contain numerous mutations in their stem followed by shorter branching events, suggesting accumulation of a large number of mutations before branching evolution [198, 199]. The high tumour mutation burden in the stem are considered to be important for the effect of immune checkpoint blockade, as they correspond to a large number of clonal neoantigens present in all tumour cells [200].

In this thesis, we aimed to investigate how the tumour mutation burdens and predicted neoantigens vary over time in a highly refractory childhood tumour, i.e. malignant rhabdoid tumour. Our results indicated that tumour mutation burden increased in metastases. Further, recent bioinformatic methods enabled us to predict neoantigens from sequencing data [201], and these predicted neoantigens also increased over time in malignant rhabdoid tumour along with mutation burden. However, their specific nature (i.e. which specific neoantigens were present) varied locally.

The weakest point in this Article III was the number of patients. The total number of patients we analysed was only two, since malignant rhabdoid tumour is uncommon and there were no more available patients that we could analyse by multiregional sampling. Further, the primary tumour of one patient could not be analysed because the sample was too necrotic. Thus, our conclusion was based on an inferred assumption from our results. These facts are actual huge limitations.

However, because of increasing tumour mutation burden and predicted neoantigens, we then assessed immune checkpoint activation. Our result showed that PD-1 expressed T cell and PD-L1 expressed tumour cells have a positive correlation, suggesting that some tumour cells were going to escape from cytotoxic T cells by expressing PD-L1. The Food

and Drug Administration in the United States of America suggested usage of immune checkpoint blockade for first line therapy when more than 50% of tumour cells express PD-L1 and for second line therapy if over 1% show expression [202]. Since there were multiple regions in each metastasis above the 1% baseline, immune checkpoint inhibitors could be a second line treatment option for at least progressive malignant rhabdoid tumours that progressed under first line therapy. However, there were also regions in the same patients with very low expression of PD-L1.

Since efficacy of immune checkpoint blockade has in some studies been reported to be independent of the degree of PD-L1 expression if the expression was at least greater than or equal to 1% [195], our results of heterogeneous immune checkpoint activation might not cause a problem for the efficacy in malignant rhabdoid tumour. However, our phylogenetic trees showed early branching evolution, and not the later branching evolution seen for melanoma and lung adenocarcinoma. Further, the tumour mutation burden of malignant rhabdoid tumour is much lower than for those tumours and it might be difficult to expect dramatic improvement of patient prognosis by immune checkpoint inhibitors. Furthermore, the shifting neoantigen profiles of different tumour regions, as well as the regional variation on PD1/PD-L1 and CD8+ T cells argues that the effects of checkpoint blockade should be localised rather than effective on all tumour cells in patients with similar patterns to the ones we studied.

However, I personally believe that immune checkpoint blockades should be considered for progressive malignant rhabdoid tumour patients if the PD-L1 expression is assessed and shows greater than 1%, because there is no available established treatment option for them. Our results indicating the heterogeneity of immune checkpoint activation and showing neoantigens confined in phylogenetic branches should be taken into account at the clinical evaluation of immune checkpoint activation in malignant rhabdoid tumour. A paper to assess PD-L1 expression in non-small cell lung cancer has shown that at least four biopsies are necessary in order to perform accurate evaluation of PD-L1 expression [202]. Therefore, multiple samplings over several distinct regions should be performed in cases where immune checkpoint blockade is considered.

There is a clinical study to assess immune checkpoint inhibitors for various paediatric tumours, including malignant rhabdoid tumour. One patient of the two malignant rhabdoid tumour cases enrolled in this study showed a partial response to pembrolizumab which is one of the immune checkpoint blockades between PD-1 and PD-L1/PD-L2 [203]. This result inferred a positive efficacy by immune checkpoint blockade for some malignant rhabdoid tumours. Since it might be difficult to perform a prospective randomized study to assess the efficacy of immune checkpoint blockade for malignant rhabdoid tumours because of the rarity of this disease, accumulation of clinical cases that are treated by immune checkpoint blockade in the future will prove whether this medication also works in the tumour types which encompass low rates of mutations and follow early branching evolution, as long as PD-L1 expressed tumour cells are over at least 1%.

In our phylogenetic analysis in Article IV, we found two distinct evolutionary patterns, i.e. linear evolution and evolution by collateral clonal replacement. Unexpectedly, all

progressive disease patients in Article III and Article IV showed linear evolution as the disease progressed. In linear progression, there is an identified ancestral clone and later emerging clones inherit all genomic alterations which the ancestral clone harbours. This inferred that the ancestral clone might survive under treatment and the offspring evolved to develop further genomic alterations. The phylogenetic tree obtained from progressive disease samples in Article IV, does not denote later branching as seen for melanoma or lung cancer, but instead we see early branching.

PD-LI expression is often confirmed in as less than 1% of the cells in neuroblastoma according to a clinical trial, while malignant rhabdoid tumour contained some tumour regions whose PD-L1 expression was over 1% according to our results. Only one of 15 tested neuroblastoma patients showed over 1% expression of PD-L1, suggesting no treatment benefit by immune checkpoint inhibitor, unfortunately. In fact, nivolumab, one of the immune checkpoint inhibitors, did not lead to an objective result in this study [204]. This poor effect could be because copy number aberrations are more important than mutations for the tumorigenesis of neuroblastoma [100], while copy number alterations in malignant rhabdoid tumours is rare. Therefore, we need to explore different treatment strategies than checkpoint blockade for most treatment refractory neuroblastomas whose phylogenetic trees follow a linear progressive pattern. These neuroblastomas might still have a small number of targetable neoantigens present in the phylogenetic stem, i.e. in all cancer cells. Such patients might be candidates for chimeric antigen receptor-redirected T cell therapy instead of targeting immune checkpoint activation, something we have not investigated in our study.

In fact, because disialoganglioside (GD2) is expressed in neuroblastoma and the expression is restricted in normal tissue, a clinical study has been performed where they have targeted GD2 in neuroblastoma with the presence or absence of pembrolizumab, the other PD-1 inhibitor and 5/11 patients showed stable disease over short-term follow-up and 2/11 patients reached complete response [205]. We found that the PDX model in Article IV was a reproductive model for linear clonal evolution under progression. PDX cells are also known to better reflect drug sensitivity than conventional cell lines [112] and to mimic clinical features in neuroblastoma [110]. The copy number status of our PDX cells applied for Article IV were shown to be relatively stable [113]. It might be worth to assess how immunotherapy treatment will work on PDX treated mice. However, a major drawback here is that PDXs have so far been tested for growth only in immunodeficient animals.

Another evolutionary pattern termed collateral clonal replacement was found in the first line treatment responsive group in Article IV. This pattern arises from the disappearance of some clones while novel clones emerge to be detected after treatment. This suggested that the treatment was effective for the major clones, but that new clones with different genetic profiles arose over time. Some of the novel branches contained important clonal alterations, such as *ALK* amplification. To target novel clonal events over time, in this case *ALK* amplicon, might be a future treatment option. A phase I clinical trial to assess efficacy of the first-generation tyrosine kinase inhibitor, crizotinib in neuroblastoma has already been performed. Further, it should be noted that the second-generation tyrosine kinase inhibitor of *ALK*, alectinib overcomes resistance to crizotinib and a treatment

benefit of alectinib over crizotinib has been found in a previous study [206]. Alectinib or other later generation inhibitors might improve prognosis of neuroblastomas harbouring *ALK* mutations or *ALK* amplicons more than crizotinib.

In one case in our Article IV, this *ALK* abnormality was only confirmed in post-treated primary tumour and was not identified in the pre-treatment primary tumour. A previous report has shown that subclonal *ALK* mutations expanded at relapse and there were also presumably novel *ALK* mutations in relapsed tumours [207]. According to the current INRG criteria, clinicians only determine treatment protocol by pre-treatment tumour sampling, and we do not necessarily examine histologically or genetically post-treated tumour or metastatic relapses. Analysing genomic alterations in the treated, primary tumour could provide a novel target that genomic analysis of the diagnostic pre-treatment tumour sampling could never point out. Further, phylogenetic trees constructed from samples obtained both before and after therapy inferred a more likely common ancestor for all clones than just samples from the primary tumours. This result suggests that the common neoantigens of this inferred common ancestor might be therapeutic targets by chimeric antigen receptor-redirected T cells therapy for cases that do not harbour treatment targets such as mutated or amplified *ALK*.

In conclusions, the phylogenetic tree of progressive disease showed linear evolution and that of treatment responsive disease followed a pattern of collateral clonal replacement. Thus, these phylogenetic trees closely reflected clinical scenarios and phylogenetic analysis has the possibility to advocate new treatment options.

Is it possible to track evolutionary history by integration of phenotypic and genetic analysis?

The appearance of a phylogenetic tree can be highly dependent on the methodology. As we have seen above, phylogenetic trees provide understanding of tumour evolution and could preferably advise new treatment suggestions. We have only reconstructed tumour phylogeny based on genetic analyses in this thesis. However, phenotypic results are also significant to prognosis. Phenotype, including protein expression, for instance intensity of CCR2 expression in ovarian carcinoma, is associated to prognosis as shown in Article I. Therefore, applying phenotypic results to phylogenetic reconstruction along with genetic results might be considered. Immunohistochemical assessment could be integrated with DNA profiles from the same paraffin block. If the genomic alterations points to a targetable candidate gene, for example in the stem, adding the immunohistochemical staining related to that gene's expression could be interesting to assess the potential of treatment success.

Conclusions

The following statements are conclusions from the studies presented in this thesis:

-Phenotypic conclusions

- CCL2 was inferred as an important chemokine which promoted ovarian cancer invasiveness in the ovarian tumour microenvironment
- Expression of IRX3 was confirmed in the epithelial region of Wilms tumours, and IRX5 expression was confirmed in blastemal, epithelial and stromal regions
- *IRX3* knockout cells correlated to tumour immaturity while *IRX5* knockout cells showed less tumour proliferation, and increased maturity via distinct signalling pathways in our Wilms tumour model

-Genetic conclusions

- The tumour mutation burden and predicted neoantigens of malignant rhabdoid tumour increased in the metastasis
- Immune checkpoint activation was regionally heterogeneous in malignant rhabdoid tumours
- Progressed neuroblastoma and progressive malignant rhabdoid tumour followed linear evolution
- Treatment responsive neuroblastoma showed clonal replacement based on collateral evolution from an inferred early ancestral clone
- The genomic diversity in neuroblastoma increased under the pressure of chemotherapy
- Multiregional analysis of neuroblastoma uncovered a previously unreported heterogeneity of *MYCN* amplicon structures within patients
- *ALK* amplification in primary tumour that was not confirmed at diagnosis was identified in post-treated tumour removed by surgery
- We could reproduce evolutionary trajectories identified in clinical neuroblastoma samples using PDX cells and an established cell line
- Whole-exome sequencing identified more subclones than SNP array in the PDX model

Popular science summaries in Japanese, in Swedish and in English

私の学位論文を、より多くの方へ

がん患者さんを治療し治したい、医師になって以降の私の強い思いです。しかし、臨床医を続けていると、どうしても治癒しない患者さんを診療いたします。効果を認めた抗がん剤治療がだんだん効かなくなり、最終的には治療抵抗性に至ります。がん細胞が進化することが原因です。4 年前、なぜ、またどのようにがん細胞が時空を超えて進化するのか、臨床経験しかない私にはわかりませんでした。

この疑問に答えるために、博士課程に進学し、長年の疑問に一歩でも近づくためにこの学位論文は書かれています。より多くの方に私の学位論文を楽しんでいただくため、出来るだけ専門用語は使わないよう努めました。このような記事のことを popular science summary と呼びます。日本語で読まれる方はこの要約を、スウェーデン語で読まれる方は次の要約を、英語で読まれる方はどうぞ最後の要約をご覧ください。

日本でもおなじみのチャールズ・ロバート・ダーウィンは安政 6 年、著書「種の起源」において生物は選択を受けることによって進化し、結果として周囲の環境へ適応していくという学説を打ち立てました。当時は遺伝子という概念はありませんでしたが、その概念が確立した現在、実はがん細胞にも同じことが言えます。腫瘍を取り巻く環境は非常に微細で微小環境と呼ばれています。この微小環境はがん細胞の遺伝的変異をもたらし、もっとも環境に適したがん細胞が選択され、結果として進化をもたらします。私の研究における目的は、どのように腫瘍微小環境ががん細胞の行動に変化を与え、そしてどのように遺伝学的変化を引き起こすのか、を探求することでした。

この学位論文は四つの論文より構成されています。一つ目の論文では、卵巣癌に焦点を当てました。卵巣がんでは正常組織、特に腹腔内へのがん細胞の播種が癌死に関わっています。私は微小環境が、どのように卵巣がん細胞の腹腔内播種に関わっているのかどうかを模索しました。中でも腹腔内を構成する細胞が分泌する CCL2 という小さな分子に着目しました。がん細胞がこの CCL2 の刺激を受けた際、腹腔内の壁により浸潤しようと反応することを突き止めました。私は、この発見が将来における CCL2 とその関連分子を目標とする卵巣がんの新規治療へと発展することを心より願っています。

次に、通常の細胞はより成熟した細胞へと成長する分化という過程が存在します。分化すると細胞増殖が止まるため、悪いがん細胞は周囲からこの分化を促進するように求められることを嫌います。より悪性度が高いがん細胞は、人間で言えば大人へと成長するのではなく、自由で利己的なまるで幼い子供のままでいようとするのです。二つ目の論文では私たちは二つの遺伝子がもっとも子供の腎臓のがんで多いウィルムス腫瘍の分化に影響を与えるかどうかを模索しました。我々はそのうち一つの遺伝子である IRX3 が腫瘍の分化を引き起こし、もう一つの遺伝子である IRX5 が腫瘍の成長を促進することを突き止めました。今日におけるウィルムス腫瘍の化学療法は非常に強力であり、子供達の体に大きな負担をかけます。我々の発見が腫瘍細胞の分化を促進する治療へと発

展し、将来の子供達が現在のような強力な化学療法を受けなくて済むような治療法が開発されることを強く望んでいます。

三つ目および四つ目の論文では、腫瘍が異なる時相と異なる部位で、腫瘍細胞にとっては化学療法という厳しい環境に置かれた場合、生き残ったがん細胞がどのような進化を辿るのかを研究しました。もっとも私が臨床医時代に抱いていた疑問です。この研究のため、同じ小児の腫瘍でも二つの相異なるがんを用いて研究しました。一つ目は悪性ラブドイド腫瘍という極めて悪性度が高く、多くは赤ちゃんにできる腫瘍。もう一つは、血液にできるがんである白血病を除いた固形がんで頭蓋骨の外に発生するがんのうちもっとも頻度が高いとされる、神経芽腫と言われる腫瘍です。腫瘍の進化を辿るため、我々は同じ患者さんの体内に時と空間を超えて発生した腫瘍のゲノムを網羅解析しそれぞれのゲノム情報を統合するという手法にこだわり、遺伝学的進化を追い求めました。

まず、悪性ラブドイド腫瘍の研究では、化学療法下に成長したがん細胞は新たな遺伝学的変化を蓄積しながら進化していくことを突き止めました。腫瘍内のある部分では、この進化したがん細胞を攻撃するため免疫細胞が反応し、さらにそこからがん細胞が逃避を図ろうとしていることがわかりました。この反応は腫瘍内で認められる箇所もあれば、認められない箇所もあり、場所によって偏在しておりました。したがって近年成人腫瘍で用いられている免疫チェックポイント阻害剤を使用した場合、効果が認められる部位と、他の部分はもしかしたら効果がないかもしれない、という基礎的な見地を得ました。

最後の四つ目の論文では、神経芽腫のがん細胞では化学療法に反応したのか、もしくは反応しなかったのか、治療への反応の仕方に沿う形で二つの異なる遺伝学的進化を辿ることを明らかにしました。もし治療に抵抗してがんが進行していく場合、がん細胞のゲノムは元からの遺伝学的異常に加えて、新たな遺伝学的変化を蓄積して進化していく。反面、一旦治療が功を奏し腫瘍細胞をある程度倒すことに成功した場合、再発するがんでは初めに認められたがん細胞より遠い遺伝学的異常を持つ細胞が化学療法に耐え抜き、全く新たな遺伝学的異常を蓄積し進化をするということを発見しました。我々の発見は異なる時相のがん細胞のゲノムを解析することによって得られた遺伝学的進化図を念頭に置きながら、新たな治療の標的となり得る対象を柔軟に追求していく必要性を示唆しました。

楽しんでいただけたでしょうか。これが私の学位論文の要約になります。より学術的探求は学位論文の各部署、およびそれぞれの原著論文に盛り込まれています。研究者、臨床に関わる医療従事者、患者さん、ご家族、そして研究に少しでも興味を持っていただける方々が、私のした仕事を理解していただけることを心からお祈りしています。ここまでお読みいただきありがとうございました。

POPULÄRVETENSKAPLIG SAMMANFATTNING

Denna avhandling utforskar interaktionen mellan miljö och arvsmassa under utvecklingen av cancerceller. Charles Robert Darwin framkastade 1859 i sin bok "Om arternas uppkomst" hypotesen att levande varelser utvecklas genom naturligt urval och därigenom anpassar sig till den omgivande miljön. För cancerceller sker på samma sätt ett urval från mikromiljön av de starkaste varianterna av tumörceller, vilket resulterar i evolution. Målet med mina studier var att undersöka hur tumörmikromiljön påverkar cancercellers beteende och hur tumörgenomet förändras till följd av urvalet.

Avhandlingen baseras på fyra vetenskapliga artiklar. I Artikel I undersökte jag hur den lokala miljö som våra normala celler skapar påverkar beteendet hos äggstockscancerceller som har spridit sig till insidan av buken. Jag fokuserade på en liten molekyl som heter CCL2, utsöndrad av celler i magen. När cancerceller stimuleras av CCL2, reagerar de genom att migrera och invadera bukväggen. Detta är ett sätt att skapa metastaser, vilket är en vanlig dödsorsak hos cancerpatienter. Jag hoppas att våra resultat kan leda till behandling av äggstockscancer inriktad på CCL2 och relaterade molekyler i framtiden.

Förutom förmågan att migrera till mer gynnsamma förhållanden för att överleva måste tumörceller också undvika signaler från miljön som driver dem att mogna och sluta dela sig. Man kan säga att cancerceller på ett sätt strävar mot att förbli som små barn, fria och självviska, snarare än att utvecklas till ansvarsfulla vuxna. I Artikel II undersökte vi hur två gener påverkar mognad av cancerceller i Wilms tumör, den vanligaste formen av njurcancer hos barn. Vi fann att uttrycket av en av dessa gener (IRX3) driver tumörmognad, medan uttrycket av en annan gen (IRX5) främjar tumörtillväxt. Dagens behandling för Wilms tumör består av starka cellgifter och vi hoppas att våra resultat kan leda till metoder för att få cancerceller att mogna, så att framtida barn kan få mindre cellgifter och ändå överleva sin cancer.

I Artiklarna III och IV undersökte jag hur tumörer utvecklas över tid och rum under exponering för cellgifter. Jag studerade två cancerformer, malign rhabdoid tumör, en sällsynt men extremt aggressiv cancer som vanligtvis drabbar spädbarn, och neuroblastom, som är den vanligaste maligna tumörformen utanför hjärnan hos barn. För att utforska tumörcellernas evolution undersökte vi från samma tumör flera prover utsprida i tiden och över olika anatomiska områden och jämförde deras arvsmassa.

Vid malign rhabdoid tumör fann vi att när tumören fortsätter att växa och sprida sig under behandling, utvecklas den genom att ansamla nya genomförändringar, som varierar mellan metastaslokaler. På vissa platser utlöser dessa nya mutationer ett immunsvar som tumörceller i sin tur blockerar genom en specifik mekanism. Vår studie förutspår att läkemedel som aktiverar patientens immunceller genom att hämma denna mekanism, kan fungera mot vissa men vanligtvis inte alla metastaslokaler hos barn med spridd malign rhabdoid tumör.

I artikel IV visade vi att utvecklingen av neuroblastomceller under cellgiftsbehandling kan röra sig i två olika riktningar, beroende på svaret på behandling. Om tumören växer

vidare under behandling utvecklas det klonala landskapet genom att helt enkelt ansamla fler mutationer. Om behandlingen är effektiv och dödar de flesta tumörceller, kan andra tumörceller, avlägset besläktade med de första cellerna, överleva och ibland återväxa till en ny tumör. Vår upptäckt av en mycket föränderlig arvsmassa i neuroblastom över tid förespråkar att behandlingsstrategier som riktar sig mot tumörcellernas mutationer måste vara tillräckligt flexibla för att följa tumörens evolution.

Detta var en kort sammanfattning av min avhandling. Jag hoppas att forskare, kliniker, patienter, anhöriga och andra personer med ett visst intresse för forskning förstår vad jag gjort.

POPULAR SCIENCE SUMMARY

The present thesis explores the environment and evolution of cancer cells. Charles Robert Darwin in 1859 hypothesized in his "On the Origin of Species" that living creatures evolve by natural selection, thereby adapting to the surrounding environment. For cancer cells, the tumour microenvironment similarly selects the fittest cancer cells from a substrate of genetic variation, resulting in tumour evolution. The aim of my studies was to explore how the tumour microenvironment affects cancer cell behaviour and how the tumour genome changes as a consequence of selection.

The thesis is based on four scientific papers. In Article I, I explored how the local environment that our normal cells create, affects the behaviour of ovarian cancer cells that have spread to the inside of the abdomen. I focused on a small molecule called CCL2, secreted by cells lining the abdomen. When cancer cells are stimulated by CCL2, they react by moving and invading the abdominal wall. This is one way to create metastasis, which is a common cause of death from cancer. I hope that our findings could lead to ovarian cancer treatments targeting CCL2 and related molecules in the future.

Besides the capacity to move into more favourable conditions, tumour cells also need to avoid signals from the environment that push them to mature and stop dividing. Cancer cells in essence strive to stay as young children, free and selfish, rather than growing into responsible adults. In Article II, we investigated how two genes affect cancer cell maturation in Wilms tumour, the most common childhood kidney cancer. We found that the expression of one of these genes (IRX3) drives tumour maturation, while the expression of another gene (IRX5) promotes tumour growth. Today's chemotherapy treatment for Wilms tumour is intensive and we hope our findings can lead to methods to drive cancer cells to mature, which could reduce the strong chemotherapy for future children.

In Articles III and IV, I explored how tumours evolve over time and space under exposure to chemotherapy. I studied two cancers, malignant rhabdoid tumour, which is a rare but extremely aggressive cancer usually affecting infants, and neuroblastoma, which is the most common solid cancer outside the brain in children. In order to explore tumour evolution, we examined multiple sampled specimens over space or over time from each tumour and compared their genomes.

Our finding in malignant rhabdoid tumour is that when this tumour keeps growing under therapy, it evolves by accumulating new genomic alterations, which vary across metastatic locations. In some sites, these new mutations trigger an immune response, that tumour cells in turn escape from. Our study predicts that medications activating the patient's immune cells to target the cancer cells by inhibiting this evasion, may work on some but usually not all metastatic sites in children with malignant rhabdoid tumour.

In Article IV, we demonstrated that the evolution of neuroblastoma cells under treatment can move in two different directions, depending on the response to treatment. If the tumour progresses under treatment, the clonal landscape evolves by simply accumulating more mutations. If the treatment is effective and kills most tumour cells, other tumour

cells, distantly related to the first cells, can survive and sometimes regrow into a new tumour. Our finding of a highly plastic genome in neuroblastoma over time advocates that treatment strategies that target specific mutations must be flexible enough to follow tumour evolution.

This was a short summary of my thesis. I hope that researchers, clinicians, patients, patient families and other people with some interest in research will understand my work.

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