Mechanisms and Consequences of Chromosomal Instability in Malignant tumours

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MECHANISMS AND CONSEQUENCES

OF CHROMOSOMAL INSTABILITY IN

MALIGNANT TUMOURS

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ORIGINAL ARTICLES

This thesis is based on the following original articles, referred to in the text by their Roman numerals (Articles I-IV):

I. <u>Ylva Stewénius,</u> Ludmila Gorunova, Tord Jonson, Nina Larsson, Mattias Höglund, Nils Mandahl, Fredrik Mertens, Felix Mitelman, and David Gisselsson. Structural and numerical chromosome changes in colon cancer develop through telomere mediated anaphase bridges, not through mitotic multipolarity. Proceedings of the National Academy of Sciences of the United States of America. 2005. 102:5541-6.

II. <u>Ylva Stewénius,</u> Hans J. Tanke, Joop Wiegant, and David Gisselsson. Cryptic terminal chromosome rearrangements in colorectal carcinoma cell lines detected by subtelomeric FISH analysis. Cytogenetic and Genome Research. 2006. 114:257-62.

III. <u>Ylva Stewénius,</u> Yuesheng Jin, Ingrid Øra, Jan de Kraker, Johannes Bras, Attila Frigyesi, Jan Alumets, Bengt Sandstedt, Alan K. Meeker, and David Gisselsson. Defective chromosome segregation and telomere dysfunction in aggressive Wilms tumours. Clinical Cancer Research. 2007. 13:6593-602.

IV. <u>Ylva Stewénius,</u> Yuesheng Jin, Ingrid Øra, Ioannis Panagopoulos, Emely Möller, Fredrik Mertens, Bengt Sandstedt, Jan Alumets, Måns Åkerman, Johannes H.M. Merks, Jan de Kraker, and David Gisselsson. High resolution molecular cytogenetic analysis of Wilms tumours highlights diagnostic difficulties among small round cell kidney tumours. (Submitted).

TABLE OF CONTENTS

ABBREVIATIONS

BAC	Bacterial artificial chromosome
CD99	Cluster of Differentiation 99
CGH	Comparative genomic hybridisation
CIMP	CpG island hypermethylation phenotype
COBRA	Combined binary and ratio labelling
DNA	Deoxyribonucleic acid
FISH	Fluorescence *in situ* hybridisation
Mb	Mega base
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
RT-PCR	reverse transcriptase polymerase chain reaction
SIOP	International Society of Paediatric Oncology
TUNEL-assay	Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling assay

PREFACE

Chromosomal aberrations are found in a majority of human cancers and can be divided into primary and secondary aberrations. Primary aberrations are most often balanced rearrangements; they are found in all cells of a neoplasm and can be used for clinical purposes. However, it may be difficult to detect them, not only because many methods cannot detect balanced chromosomal aberrations but they may be hidden among the many secondary chromosomal aberrations especially found in carcinomas. The secondary chromosomal changes may vary in nature within the neoplasm and may, when clonal, reflect tumour progression. One potential cause of chromosomal aberrations and intra-tumour heterogeneity is chromosomal instability, defined as an increased mutation rate affecting whole chromosomes or substantial parts of the chromosomes. This enhanced mutation rate also may increase the risk of therapy resistance.

The aim of this thesis is to better understand chromosomal instability, both the mechanisms behind it and its consequences for the clinical features of the tumour. Within the aims is also to evaluate the potential of a high resolution combination of cytogenetic techniques for detecting breakpoints in simple and complex cancer karyotypes.

The thesis is divided into four sections. The first is an introduction to the field of chromosomal aberrations and chromosomal instability, a brief overview of tumour cytogenetic techniques and a general description of the tumour types that have been studied. The second is a summary of the specific materials and methods employed in the studies and a summary of the aims of the thesis and the results achieved in articles I-IV. The third comprises a discussion of the results and the fourth includes the original articles on which this thesis is based.

Lund, January 2008

INTRODUCTION

A neoplasm has been defined by the pathologist Rupert Willis as "… an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change" (Willis 1952). *Tumor* in Greek means swelling and solid tumours comprise many different entities. Benign tumours can grow large but do not possess the ability to metastasise. Most benign tumours thus do not kill their hosts but if they do, it is usually because of an unfortunate location. Conversely, malignant tumours have the ability to infiltrate the surrounding tissues and spread to other locations in the body. By disrupting the normal functions of the body, or by emitting toxic substances, malignant tumours eventually kill their hosts. While most epithelial tumours typically display a progression from benign to malignant, this is more uncommon among other types of solid tumours.

Second to cardiovascular disease, cancer is the most common cause of death in Sweden and every third Swede will develop a malignant tumour. Solid tumours are most often treated with combinations of chemotherapy, radiotherapy and surgery. Other therapies such as hormone inhibitors, immunotherapy, bone marrow and stem cell transplantation, and tyrosine kinase receptor inhibitors may also be effective (Holland and Frei 2003). The relative ten-year survival of patients diagnosed with cancer is approximately 50% in Sweden which means that many cancer patients are in fact cured of their disease (Talbäck, et al. 2003). The majority of cancers affect individuals older than 65 years and cancer in individuals younger than 15 years of age is rare and only account for 0.6% of all cancer cases in Sweden (Dreifaldt, et al. 2004; The National Board of Health 2007). Haematological malignancies, tumours of the central nervous system, neuroblastoma, Wilms

tumour, osteosarcoma and Ewing family tumours account for most cases of paediatric malignancies. Generally, paediatric malignancies are treated in the same way as adult neoplasms but as children are still growing and their organs are under development, they are generally very sensitive to therapies targeting dividing cells (Holland and Frei 2003). Children are also more at risk of developing secondary malignancies. The survival of children affected by neoplastic disease has improved in the past decades and the relative ten-year survival is now 75% in Sweden (Talbäck, et al. 2003).

Chromosomal aberrations in cancer

Most neoplasms have acquired somatic chromosomal aberrations (Heim and Mitelman 1995) which are generally believed to be important for tumour development. Tumours with normal karyotypes may have microsatellite instability, with point mutations not detectable in the karyotype (Thibodeau, et al. 1998), or have epigenetic aberrations. Some types of cancer, commonly haematological malignancies and bone and soft tissue tumours, often have only one or few chromosomal aberrations. Epithelial tumours on the other hand may have extremely complex karyotypes (Mitelman, et al. 2004). Chromosomal aberrations can be divided into two categories. Primary chromosomal aberrations are observed in all clones of a malignancy (Heim and Mitelman 1995), are often tumour-specific and are observed alone or together with only few other chromosomal aberrations. Secondary chromosomal aberrations may or may not be clonal and, although non-random, are not tumour-specific and are often not observed as sole anomalies. Primary chromosomal aberrations are typically balanced structural rearrangements and give rise to fusion genes. Such gene fusions have been found in 20% of all malignant tumours (Mitelman, et al. 2007) and exert their tumourigenic effect either by overexpressing one of the genes involved in the fusion or by generating a chimaeric protein with parts from both the original proteins (Rowley 2001).

Although primary chromosomal aberrations are fundamental for the development of cancer, they do not alone seem to be able to cause a malignancy (Hahn, et al. 1999). Other mutations or epigenetic events are also necessary. In both haematological and epithelial malignancies a multistep mutagenesis has been observed. It is exemplified by the accumulation of chromosomal aberrations over time, accompanied by a progressively more malignant phenotype (Ried, et al. 1999). Based on the cells' base-line mutation rate and the time it usually takes to develop a malignancy, it was hypothesised that 4-6 mutations are required for the development of a malignant tumour (Armitage and Doll 1954). A cell must achieve a number of properties in order to become cancerous, *i.e.* to be able to invade and to metastasise, as well as to continuously adapt to the changing tumour environment (Hanahan and Weinberg 2000). Based on a screening of sequences from approximately 13,000 genes in breast and colon cancer it has recently been estimated that these tumours harbour a much larger number of mutations (Sjöblom, et al. 2006). On average, breast and colon carcinomas displayed approximately 90 mutations in coding genes of which 14-20 were mutations in genes important for tumour development. Furthermore, primary genetic aberrations may be observed in the cells of healthy individuals; *e.g.* the *BCR-ABL1* fusion gene is present in bone marrow cells from a high percentage of individuals (Janz, et al. 2003), only a small minority of whom will develop chronic myeloid leukaemia. Adenomatous polyps, often showing trisomies (Heim and Mitelman 1995), are present in a substantial percentage of patients who undergo endoscopic screening but only few of them, in their life-time, will develop colorectal carcinoma (Imperiale, et al. 2000; Lieberman, et al. 2000). The genome has a low base-line mutation rate due to the high accuracy of the replication processes and the efficiency of the DNA repair systems (Kunkel 2004).

Although the normal mutation rate together with clonal expansion could account for several genomic changes seen in tumour cells (Simpson 1997), it is probably not high enough to explain the many mutations seen in most malignancies (Loeb 1991). However, if the function of any of the genes that monitor the genome is lost, the mutation rate could increase and chromosomal aberrations accumulate. Such protective genes can be divided into "gate keepers" that regulate cell growth and make sure that cells with damaged DNA do not continue through the cell cycle, and "caretakers" that repair the genome (Kinzler and Vogelstein 1997). When the "caretaker" function is lost, mutations are allowed to accumulate, and if the "gatekeeper" function is lost as well, these cells will proliferate and generate a malignant clone.

Genetic instability

A genome with a mutation rate higher than the base-line mutation rate is considered unstable and genomic instability together with clonal expansion could account for the accumulation of mutations that are necessary for cancer to develop (Loeb 1991). There are two main types of genomic instability described in cancer: microsatellite instability (MSI) and chromosomal instability (Lengauer, et al. 1998). MSI is caused by mutations and epigenetic changes in mismatch-repair genes and it in turn causes a dramatic increase of point mutations (Thibodeau, et al. 1993; Kane, et al. 1997). Chromosomal instability is seen both in inherited and sporadic disorders and is defined as an increased rate of losses and/or gains of whole chromosomes or parts of chromosomes (Lengauer, et al. 1997). Chromosomal instability in all cells of the body can be caused by inherited syndromes leading to congenital abnormalities, immunodeficiency and increased cancer susceptibility (Eyfjord and Bodvarsdottir 2005). Individuals affected by the ataxia teleangiectasia and Nijmegen breakage syndromes have chromosomal instability due to the failure

of cell cycle checkpoints, which should be activated in response to double stranded DNA breaks. Individuals with Bloom syndrome and Fanconi anemia instead have an increased formation of DNA breaks, which, in the first case, leads to an increased rate of sister chromatid exchanges and, in the second, to DNA cross linking. In sporadic malignancies, chromosomal instability has been found to have many different causes (Gollin 2005). Several single gene mutations have been associated with chromosomal segregation defects, cell cycle disturbances and defects in the DNA repair systems. One of the causes of chromosomal instability is telomere dysfunction (Artandi, et al. 2000; Gisselsson, et al. 2001). Telomeres are composed of tandem TTAGGG repeat sequences at the ends of eukaryote chromosomes that, when intact, protect the integrity of the chromosomes (Müller 1938; Blasco 2005). The telomere is 10-15 kilo bases long in humans and its protective ability is proposed to be generated by a single stranded loop formation supported by telomere specific protein complexes. Abnormally short or otherwise dysfunctional telomeres may lose their protective function and fuse to other chromosome ends, creating dicentric chromosomes, *i.e.* chromosomes with two functional centromeres. At mitosis, each of the two centromeres of a dicentric chromatid may attach to different centrosomes and at subsequent anaphase, the chromosome material may be stretched between the spindle poles of the mitotic cell as an anaphase bridge. The chromosome may then detach, break or inhibit the mitotic process (McClintock 1941). If it breaks, chromosome rearrangements may give rise to novel dicentric chromosomes, continuing the process in a breakage-fusion-bridge cycle (Sabatier, et al. 2005). Breakage-fusion-bridge cycles may also be caused by exogenous DNA damage such as irradiation (McClintock 1941). The breakage-fusion-bridge cycle and other types of chromosomal instability may cause many different chromosomal rearrangements such as numerical aberrations, translocations, amplifications, deletions and inversions (Murnane 2006).
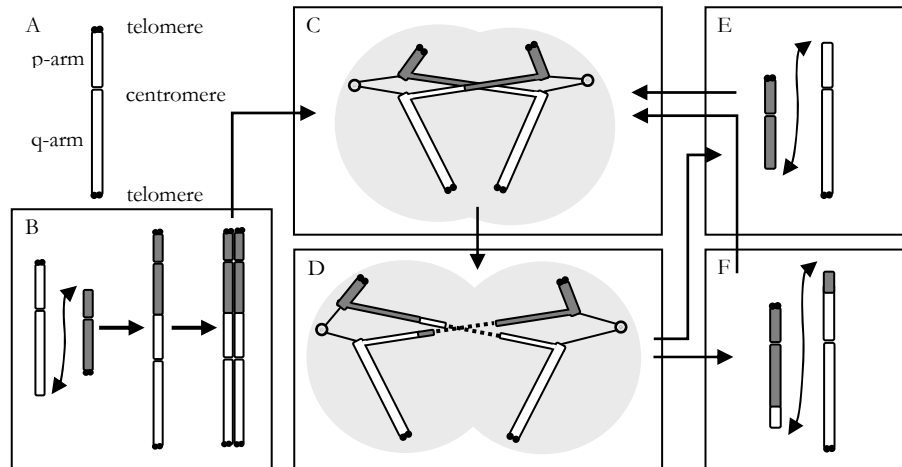
The breakage-fusion-bridge cycle
(A) Basic description of a chromosome. (B-F) The breakage-fusion-bridge cycle. (B) Chromosome arms without functional telomeres act like double stranded DNA-breaks and may thus fuse to other unprotected chromosome arms forming dicentric chromosomes. (C) If the two centromeres of the dicentric chromatids are both functional and each attaches to a different centrosome, the middle parts of the chromosomes between the centromeres become stretched out between the spindle poles in the anaphase and form an anaphase bridge. (D) As the anaphase bridge becomes more stretched out it eventually breaks. (E and F) The broken chromosomes ends are adhesive and may attach to other chromosome ends without protective telomeric sequences. These dicentric chromosomes may form new anaphase bridges as the daughter cells divide, continuing the breakage-fusion-bridge cycle.

A tumour exhibiting this type of chromosomal instability often displays a complex karyotype (Gisselsson, et al. 2000). While some of the chromosomal aberrations in such complex karyotypes undoubtedly are important for tumourigenesis others could represent background noise (Heim and Mitelman 1995).

Mitotic multipolarity is often observed together with the breakage-fusion-bridge type of chromosomal instability (Gisselsson 2005). Instead of dividing in a bipolar fashion, cells undergoing multipolar mitosis divide into three or more daughter cells. Supernumerary centrosomes have been shown to be a cause of multipolar mitoses (Lingle and Salisbury 1999; Saunders, et al. 2000). The multipolar mitotic figures can potentially give rise to daughter cells with an unequal chromosome copy-number (Jin, et al. 2007). Gains and losses of whole chromosomes are frequently seen in malignant tumours and mitotic multipolarity is one possible explanation of these phenomena.

Paediatric tumours

Whereas most adult malignancies are epithelial in origin (Jemal, et al. 2006), paediatric malignancies most often originate from haematopoietic cells, bone, soft tissues, or neural tissues (Gatta, et al. 2005). Many paediatric tumours, in fact, resemble embryonic cell populations which could possibly be explained by an inhibited maturation of foetal cells (Scotting, et al. 2005). Due to their similar histologic appearance at eosin-hematoxylin staining, some embryonic paediatric malignancies are sometimes referred to as small round cell tumours or small blue cell tumours (Variend 1985; Holland and Frei 2003). These tumours include, among other tumours, neuroblastoma, Ewing family tumours, rhabdomyosarcoma and lymphoma (McManus, et al. 1996; Cohn 1999; Parham 2001). Also blastemal type Wilms tumour may resemble this group of tumours (Jimenez, et al. 2002). Often these tumours are only distinguishable by genetic analysis and/or immunohistochemistry. Not even results from such studies are, however, always conclusive and a final diagnosis may be difficult to establish.

Wilms tumour

Wilms tumour affects approximately 1/100 000 children under the age of 15 per year (Pastore, et al. 2006), which makes it the most common paediatric kidney tumour. It constitutes 6% of all paediatric cancers and most frequently affects children under the age of five. Because the tumour grows from the kidney, situated behind the peritoneum, it can grow very large before causing symptoms (Cotran, et al. 1989). Wilms tumour is made up of immature epithelial and mesenchymal cells, of blastemal cells that resemble immature kidney cells and occasionally of anaplastic cells (Cotran, et al. 1989). The different cell types represented in Wilms tumours indicate that the tumours develop from the pluripotent cells of early nephrogenesis (Rivera and Haber 2005). The kidney arises from the middle germ layer, the mesoderm, and evolves though rudimentary epithelial and mesenchymal structures (blastema) to the functional kidney (Dressler 2006). Nephrogenic rests are foci with embryonic kidney cells that remain after the 37th week of gestation (Beckwith, et al. 1990). These can be found by routine histologic examinations in 1% of diseased infants. In 40% of unilateral Wilms tumours and 100% of bilateral Wilms tumours, the tumours are either found to have emanated from nephrogenic rests or nephrogenic rests are found in the same kidney. This combined with the fact that the nephrogenic rest and the adjacent Wilms tumour often exhibit the same pattern of mutations (Charles, et al. 1998), have lead to the conclusion that nephrogenic rests may be tumour precursor lesions. Approximately 1% of all nephrogenic precursor lesions are believed to develop into tumours (Beckwith, et al. 1990).

Genes that are of importance for the embryonic development of the kidney are also of importance for the development of Wilms tumour (Rivera and Haber 2005). By mRNA expression analysis it has been shown that the tumours have a profile similar to the early embryonic kidney (Li, et al.

2002; Li, et al. 2005). About 90% of Wilms tumours occur sporadically while 10% are either familial or associated with syndromes (Breslow, et al. 1993). The Wilms tumour 1 gene (*WT1*) is important for kidney development and the maturation of kidney cells (Kreidberg, et al. 1993). Syndromes associated with a hemizygous loss of *WT1* function typically include severe genitourinary malformations. Loss of *WT1* function could lead to an inhibition of the maturation of embryonic kidney cells, leaving a pool of rapidly dividing embryonic cells that accumulate additional mutations (Davies, et al. 2004). *WT1* mutations are frequently associated with *CTNNB1* mutations (Maiti, et al. 2000). *CTNNB1* is a component of the Wnt pathway which is important for embryogenesis and cancer development (Clevers 2006). Mutations in *CTNNB1* render the corresponding protein resistant to degradation. This causes the protein to accumulate in the nucleus and triggers an inappropriate inactivation of target genes that may be of importance to Wilms tumour development (Li, et al. 2004; Zirn, et al. 2006b). The "Wilms tumour gene on the X chromosome" (*WTX)* tumour suppressor gene has recently been shown to be mutated to a high percentage (30%) of Wilms tumours (Rivera, et al. 2007). Normally it is expressed at the same time as *WT1* in kidney development, and loss of *WTX* function could trigger tumour development in the same way as the loss of the *WT1* function.

Many overgrowth syndromes such as the Beckwith-Wiedemann syndrome also increase the risk of Wilms tumour (Koufos, et al. 1989; Ping, et al. 1989; Scott, et al. 2006). This syndrome is caused by either genetic or epigenetic aberrations at the *WT2* locus leading either to the overexpression of the paternally inherited *IGF2* and/or an underexpression of the maternally inherited *H19* (Steenman, et al. 2000). Tumour predisposition syndromes such as Li-Fraumeni syndrome and Fanconi anemia, and the presence of constitutional chromosomal abnormalities such as trisomies 13 and 18, and

2q37 deletions, also increase the risk of Wilms tumour (Scott, et al. 2006). Only 1-2% of Wilms tumours are familial and their occurrence has been linked to loci at chromosome 17 and 19 (Rahman, et al. 1996; McDonald, et al. 1998). The syndromes associated with Wilms tumour are thus in most cases due to *de novo* mutations. The low incidence of familial tumours is probably in part due to the low survival rates of the patients before modern therapy and in part because the syndromes associated with Wilms tumour often include severe, genitourinary malformations (Rivera and Haber 2005).

Wilms tumours are made up of immature kidney cells at various stages of differentiation. The blastemal cell components resemble embryonic cells by being small, round, and blue by hematoxylin-eosin staining (Rivera and Haber 2005). The epithelial component consists of more or less intact tubuli. The mesenchymal cells are commonly stromal cells but may also display other types of mesenchymal differentiation. One, two or three of these components exist concurrently in the tumours. It is the proportion of these cell components that determine the histopathological tumour subtype. In agreement with the European International Society of Paediatric Oncology (SIOP) protocol, tumour subtype together with the stage of the tumour form the basis of therapy (Vujanic, et al. 2002). According to the SIOP protocol the tumours are pre-treated with chemotherapy to shrink the tumour and the histological diagnosis is then made after excision of the pre-treated tumour. The tumours are divided into low-risk, intermediate-risk and high-risk tumours. Low-risk tumours are primarily completely necrotic tumours, high-risk tumours are blastemal type tumours and tumours with diffuse anaplasia. The other tumours are of intermediate risk. The stage represents the tumour spread and stages I, II and III represent tumours that are confined to the pelvis and abdomen, stage IV includes distant metastases and stage V includes bilateral tumours. Within the SIOP protocol only histology and stage are used

for prognostication, though quantification of telomerase expression (Dome, et al. 2005), global gene expression analysis (Zirn, et al. 2006a), and cytogenetics have been used in attempts to stratify prognostic subgroups further. Gain of 1q and loss of 16q and chromosome 22 have been associated with poor outcome (Hing, et al. 2001; Bown, et al. 2002; Kullendorff, et al. 2003). Loss of heterozygosity for 1q and 16q have also been suggested as adverse prognostic factors for non-anaplastic tumours (Grundy, et al. 2005).

In many other paediatric solid tumours, recurrent balanced translocations have proved tumour-specific and are useful diagnostic tools (Burchill 2003). Among the 408 Wilms tumour cases reported so far, no such recurrent balanced translocation has been identified (Mitelman 2007). Instead gains and losses of whole chromosomes are the most common karyotypic features, together with structural aberrations of chromosomes 1, 7 and 16 (Höglund, et al. 2004). No correlations between histology and karyotypic features have been established, except perhaps in some cases where complex karyotypes have been associated with anaplastic tumours (Gow and Murphy 2002). Inherent in the definition of anaplasia in Wilms tumour are multipolar mitoses and large, hyperchromatic nuclei (Vujanic, et al. 2002), features associated with an increased nuclear DNA content (Cotran, et al. 1989; Saunders, et al. 2000) which could be caused by chromosomal instability (Lengauer, et al. 1997). Telomeric fusions and amplifications have been reported in Wilms tumour (Fett-Conte, et al. 1993; Goldstein, et al. 2003; Tretiakova, et al. 2006), and there are cases with very complex karyotypes which could be caused by telomere dependent chromosomal instability. However, before the studies included in this thesis, the presence of chromosomal instability in Wilms tumour had not been explored.

Methods in tumour cytogenetics

In the studies included in this thesis, chromosomal instability was explored in colorectal cancer model systems and in Wilms tumour. It was also investigated whether the level of chromosomal instability, *i.e.* the rate at which chromosomal aberrations are formed, is associated with the histopathological and clinical features of Wilms tumour. A detailed characterization of chromosomal aberrations in tumour cells with chromosomal instability was also made. The current chapter is an overview of the techniques employed in these studies. This part of the thesis will also focus on the methodological and conceptual problems that may arise due to intratumour heterogeneity.

Methods for detecting chromosomal aberrations

Chromosome banding techniques, *e.g.* G-banding by Wright's stain which gives each chromosome a specific pattern of light and dark bands (Rooney 2000), are commonly used to screen for chromosomal aberrations. These techniques are economical and can detect balanced chromosomal aberrations in contrast to comparative genomic hybridisation (CGH) techniques which can detect only genomic imbalances. By fluorescence *in situ* hybridisation (FISH), fragments of DNA are labelled with fluorescent molecules (Trask 1991) and used to detect their complementary DNA sequences in the genome of metaphase or interphase cells. The regions detected can vary in size from a few thousand bases to entire chromosomes. The telomeres can be targeted by probes for TTAGGG repeats, making it possible to visualise all chromosome termini simultaneously (Meyne and Moyzis 1994; Lansdorp, et al. 1996). By multicolour FISH, probes for each chromosome are labelled by a specific colour and the entire chromosome complement can thus be analysed (Schröck, et al. 1996; Speicher, et al. 1996; Eils, et al. 1998; Tanke, et al. 1999). G-banding and multicolour FISH are reliable genomic screening tools; however, small chromosomal aberrations can

remain undetected, especially if the chromosome quality is low or the aberrations involve the distal parts of the chromosomes (Lee, et al. 2001). G-banding has a resolution of five Mb (Knight and Flint 2000) while multicolour FISH has a maximal resolution of 1-2.8 Mb depending on method (Schröck, et al. 1996; Tosi, et al. 1999). Translocations by definition involve chromosome ends and adding subtelomeric FISH, a method targeting the chromosome ends, enhances the resolution when screening for translocations (Tosi, et al. 1999). This method has been most commonly used in screening for constitutional subtelomeric rearrangements in patients with mental retardation and dysmorphology (Lamb, et al. 1989).

To pin-point breakpoints in balanced translocations, DNA sequences incorporated in bacterial artificial chromosomes (BACs) can be utilized as FISH probes. The cloned sequences are amplified by bacterial growth. The extracted DNA is then labelled by fluorochromes and hybridized to metaphase spreads revealing the locations of the probes' complementary DNA sequences. These sequence positions can be found in databases based on information from the Human Genome Project. To investigate further if the balanced translocations give rise to a fusion transcript, reverse transcriptase polymerase chain reaction (RT-PCR) can be performed with opposing primers and the amplified product is observed as a band in an agarose gel. To analyse the fusion product on base pair level, sequence analysis can then be performed.

Tumour material

From a cytogenetic or a histopathologic perspective it is the tumour cells, either resected or aspired, that is of most interest for diagnostic, prognostic and scientific purposes. The studies included in this thesis were performed on primary tumours, established tumour cell lines and tumour tissue sections. Solid tumours have, in most cases, a monoclonal origin (Cotran, et al. 1999).

However, during the existence of the tumour, cells acquire new mutations which, if the mutations give the cells proliferative advantages, will make the cells expand clonally. This multistep process of carcinogenesis creates tumours with genetic variation, *i.e.* intratumour heterogeneity. Histopathologically, tumour heterogeneity may be reflected by cellular pleomorphism (Bignold 2002) and regional variations in differentiation within the tumour that may complicate the diagnostic process. To reduce the risk of missing important information at histopathologic analysis, the analysis can be made on multiple tumour sections from different parts of the tumour. Nevertheless, for practical and economical reasons, frequently only a small fraction of a large tumour is analysed.

By cytogenetic analyses of multiple samples of a tumour, cytogenetically different clones are often found (Örndal, et al. 1994). However, normally only one sample of the tumour is used for analysis. The selection of cells for cytogenetic studies is also affected by the cell culturing which is necessary to obtain metaphase chromosome preparations. Tumour cells may be subject to other proliferative signals *in vitro* than *in vivo,* and consequently genetic aberrations that could be important for tumour development may go undiscovered (Jin, et al. 1993). Established cell lines have, by definition, grown for an extended period of time *in vitro* and can over time, due to genetic instability and the selection due to specific proliferative advantages *in vitro*, acquire features that make them different from the original tumour. Established cell lines have acquired immortality, either *in vivo* or *in vitro*, spontaneously by the activation of telomerase to elongate the telomeres, or by tumour viruses that inactivate check-point genes (Toouli, et al. 2002; Strachan and Read 2004). Furthermore it has been reported that one sixth of established cell lines have been cross-contaminated by other cell lines (MacLeod, et al. 1999; Lacroix 2008). Primary tumour material is therefore often preferred for cytogenetic analysis though it has qualities that sometimes make it less suitable.

Because of telomere attrition and a culture environment that may activate check-point genes, primary cells can usually grow for only a limited time *in vitro* (Hayflick and Moorhead 1961; Ben-Porath and Weinberg 2004). This makes primary tumours less suitable when a large quantity of cellular material is needed or when the cells must to be grown for an extended period of time. For cytogenetic analysis, cells should be dividing when harvested. Different subclones present in the culture may enter mitosis at different times and clones may thus be erroneously excluded from analysis. Although the genetic changes that are most important for tumourigenesis ought to be present in all neoplastic cells, other genetic changes, still significant for tumour development, may not be. Because of this, the validity of cytogenetic findings, even from short-term cultures, can to some extent be questioned.

Colon cancer as a model system for genomic instability

Colorectal cancer arises within the lumen of the bowel and is ideal as a model for studying cancer. It arises from the mucous membrane of the colorectum, progressing through a benign adenoma, and becomes increasingly more malignant until the invasive carcinoma is a fact (Cotran, et al. 1999). The precursor lesions of colorectal carcinoma are easily diagnosed compared with precursor lesions from other malignant tumours as they can be detected and resected by endoscopy. Tumours of different stages can thus be studied. From this adenoma-carcinoma sequence considerable knowledge of the sequential gains of oncogenes and losses of tumour suppressor genes has been obtained (Fearon and Vogelstein 1990). There are also several hereditary germline mutations associated with an increased risk of colorectal cancer and with the help of knowledge about these syndromes, conclusions have been made about sporadic cancers (Rustgi 2007). Hereditary non-polyposis colon cancer is caused by the loss of function of the mismatch repair system and causes MSI

which has been observed also in sporadic colorectal cancer. This is also the case with mutations of the *APC* gene, which cause familial adenomatous polyposis.

Established colorectal cancer cell lines have frequently been used to study genomic instability. Some of these cell lines were established in the 1970s and 1980s and have been thoroughly studied regarding, *e.g.* gene expression, telomerase expression, karyotype, gene sequences and loss of heterozygosity. The relationship between the two best known types of genomic instability, MSI and chromosomal instability, were first characterized in established colorectal cancer cell lines (Lengauer, et al. 1997) and to explore this relationship further, five of the same cell lines were used as model systems in this thesis.

Studies of chromosomal instability

As previously mentioned, chromosomal instability is defined as an enhanced mutation rate on the chromosome level (Lengauer, et al. 1997) and can be approximated by studying intercellular differences in chromosomal aberrations within a tumour. It is not sufficient to study the chromosomal aberrations of the tumour stemline, because this reveals little about the rate at which new chromosomal aberrations are accumulated. The ideal situation would be to follow an individual cell over time and study its accumulation of chromosomal aberrations but this is not yet technically possible. With respect to telomere-dependent chromosomal instability, the presence of excessive telomere shortening and anaphase bridging can be used to determine whether chromosomal instability is present in a sample and also to make an approximation of its level. As it is the shortest telomere that drives the development of chromosomal instability (Sabatier, et al. 2005), it is important to measure the telomeres of each chromosome individually. Although it is the shortened telomeres that initiate the breakage-fusion-bridge cycle, instability

cannot be studied only by quantifying telomere length. Telomeres become prone to attachment to other chromosomes when shorter than 12.8 TTAGGG repeats (Capper, et al. 2007), but it is not certain that all telomeres shortened to this degree cause anaphase bridges. The link between telomere length and chromosomal aberrations is the anaphase bridges. If these are present, so is chromosomal instability (Gisselsson, et al. 2001; Montgomery, et al. 2003) but what causes each individual mitosis with a bridge and what effect this mitosis has on the chromosomes is still not known and therefore several cells must be analysed. It is also possible that cells affected by telomere instability do not survive and no novel mutations are formed. Based on these considerations, the frequency of anaphase bridges together with telomere status and the pattern of cytogenetic changes has become an established strategy to estimate the level of telomere dependent chromosomal instability in a cell population (Gisselsson and Höglund 2005). It is a necessary simplification because of the current technical limitations, and it makes the analysis as straightforward as would be preferred in a clinical setting.

THE PRESENT STUDY

This section includes a summary of the materials and methods, the specific aims, and the results of the thesis. A general discussion with emphasis on the methods and results is given at the end of this section.

MATERIALS AND METHODS

The established colorectal cancer cell lines LoVo, DLD1, HCT116, HT29, and SW480, and the established embryonic kidney tumour cell line SK-NEP-1 (HTB-48) were obtained from the American Type Culture Collection. The established Wilms tumour cell line WiT 49 was donated by Dr. Yeger at the Laboratory of Medicine and Pathobiology, University of Toronto, Canada. Fresh Wilms tumour samples were received from patients treated at the University Hospital in Lund, Sweden, and from The Academic Medical Center, Amsterdam, the Netherlands. Wilms tumour tissue sections were from tumour material received for histopathologic analysis from patients treated at the University Hospital in Lund, Sweden between 1992 and 2005. Cell culture was performed according to standard procedures. To screen for chromosomal aberrations, G-banding, the multicolour FISH technique combined binary and ratio labelling (COBRA; Tanke, et al. 1999) and subtelomeric FISH (Lamb, et al. 1989; Knight, et al. 2000) were performed. Chromosomal instability was evaluated by FISH for TTAGGG repeat sequences, light microscopy studies of mitotic configurations and cytogenetic analysis. Anaphase bridges were defined as strings of chromatin between chromosomes at opposite poles of the anaphase, either connecting them or spanning at least two-thirds of the distance. Metaphase or anaphase cells with chromosome configurations indicating three or more spindle poles were classified as multipolar cell divisions. TUNEL-assay was used to detect double stranded DNA breaks. Break point mapping was done by fluorescent labelling of DNA extracted from BACs and gene fusion transcripts were analysed by RT-PCR and sequencing.

AIMS AND RESULTS

The aims of this thesis were to explore further the relationship between different kinds of genomic instabilities in a well known tumour model system from colorectal cancer, and to evaluate whether these instabilities were correlated with clinicopathological features in a well standardised set of patients with Wilms tumour. Within the aims was also to evaluate the efficacy of a combined molecular cytogenetic screening platform for detecting recurrent balanced chromosomal aberrations and the more complex aberrations caused by chromosomal instability. In addition, the focus of this thesis includes the diagnostic difficulties encountered in paediatric kidney tumours.

Before the initiation of this thesis project, chromosomal instability and MSI were considered mutually exclusive with few exceptions in the model system most frequently used to study genome instability: established colorectal cancer cell lines (Lengauer, et al. 1998). Although the cause of MSI was known, the cause of chromosomal instability in colorectal cancer had been little studied. In Article I, we analysed the correlation of telomere length to anaphase bridging and mitotic multipolarity in five established colorectal cancer cell lines, three with MSI and two without MSI. It was observed that not only was telomere length associated with and a possible cause of anaphase bridges and multipolar mitoses in these cell lines; chromosomal instability was present in all tumour cell lines, meaning that MSI and chromosomal instability are not mutually exclusive.

In Article I telomere shortening and anaphase bridges were proposed to cause both structural and numerical chromosomal aberrations. By the detection of double stranded DNA breaks by TUNEL-assay, extensive DNA fragmentation was observed in 0.1-3% of interphase cells. This fragmentation was typically found in protrusions of the nuclear membrane or

in chromatid strings between cells. These are nuclear structures which have been found to contain remains of broken anaphase bridges (Gisselsson, et al. 2001a). In >90% of the strings and protrusions, the fragmentation began just distal to the centromeric region. This was reflected in the cell line karyotypes as 20 of 54 breakpoints were pericentromeric. Anaphase bridges were observed to detach unilaterally from the mitotic spindle, potentially leading to non-disjunction and loss of chromosomes in one or both daughter cells. Bilateral detachment of chromosomes (anaphase lagging) was present in a third of the anaphase cells with bridging but in less than one cell of 100 normal anaphase cells. In one of the MSI negative cell lines, there was a high rate of intercellular copy-number variation of chromosome 7. One of its arms was observed to have an extremely short telomeric sequence and chromosome 7 was more frequently observed in anaphase bridges and involved in structural chromosomal aberrations than chromosomes with less intercellular copy-number variations chosen for comparison. The critically short telomere in one of the chromosome arms could thus be the underlying cause of the numerical instability of this particular chromosome and could also be an explanatory mechanism for the chromosomal instability seen in the other cell lines.

Multipolar cell divisions have been hypothesised to cause numerical chromosomal aberrations in cancer (Saunders, et al. 2000; Hardy and Zacharias 2005). In the colorectal cancer cell lines examined in Article I, multipolar mitoses were invariably present in the cell lines where anaphase bridges were found. When the rate of anaphase bridges was increased by irradiation of the cell lines and by telomerase inhibition, the rate of multipolar mitoses was also elevated. Mitotic multipolarity, however, could be evoked by polyploidisation without an increase in the rate of anaphase bridging. This indicated that anaphase bridging could be one cause of multipolar mitoses. To evaluate the outcome of multipolar cell divisions in colorectal cancer cell lines,

the chromosome segregation was evaluated by FISH with centromeric probes for chromosome X and 18 in multipolar anaphase and telophase cells in the cell line SW480. Nullisomies for the two studied chromosomes were observed in 18% and 9% respectively of the daughter cells indicating that nullisomy of any given chromosome is a frequent phenomenon. Except for the Y chromosome, nullisomies are thought to be lethal to the cells. By microdissection of 29 live multipolar mitoses and the culturing of their subsequent daughter cells from one of the MSI negative cell lines, it was observed that these cells did not form a viable daughter cell population, in contrast to daughter cells from anaphase figures with bridges, which had a colony forming capacity similar to that of normal anaphase cells. In this study it was thus observed that microsatellite and chromosomal instability are not mutually exclusive in colorectal cancer and that telomere dependent chromosomal instability is one cause of the complex chromosomal rearrangements often seen in colorectal cancer cell lines, including both numerical and structural aberrations. It was also observed that daughter cells from multipolar mitotic figures did not form clones in culture, possibly because of the severe aneuploidy which is the result of multipolar mitosis.

Telomere deficiency commonly gives rise to structural rearrangements with terminal breakpoints (Gisselsson, et al. 2001b; Scheel, et al. 2001) that are difficult to detect by standard cytogenetic techniques, and to a genetic heterogeneity that is difficult to assess by array-CGH techniques. In Article II, the efficiency of subtelomeric FISH, combined with G-banding and multicolour FISH, to detect chromosomal aberrations both in complex and simple karyotypes was evaluated in the same five established colorectal cancer cell lines that were studied in Article I. Forty-one of the totally 86 detected breakpoints in the cell line karyotypes were not detected with G-banding and multicolour FISH only but required subtelomeric FISH. Regarding the 20

terminal breakpoints, 14 were not detected without subtelomeric FISH. High resolution cytogenetic karyotyping was proposed as an effective tool to detect chromosomal breakpoints not observed by other standard cytogenetic screening or array techniques. Chromosomal instability has been little explored in paediatric tumours. Wilms tumour karyotypes typically exhibit trisomies and a relatively small number of structural rearrangements (Mitelman 2007). In Article III, cells from eleven primary Wilms tumours and one established Wilms tumour cell line were studied by FISH for telomeric sequences and for rates of anaphase bridges and multipolar mitoses. In two of the primary tumours and in the cell line, chromosome ends lacking telomeric FISH signals were observed, indicating that the telomeres on these chromosomes were abnormally shortened. In the same tumours the rate of anaphase bridging and mitotic multipolarity was increased. These three tumours were all classified as high-risk tumours indicating that chromosomal instability might be associated with a poor outcome. To explore this further, the presence of chromosomal instability, approximated from the rate of anaphase bridges and multipolar mitoses present in tumour tissue sections, was studied in 43 Wilms tumours of high risk and intermediate risk and correlated with clinicopathological features. Two tumours were excluded because too few mitotic figures were found. Chromosomal instability was found in eight of the tumours. Of these patients, six died of disease, one of complications to therapy and one wase alive at the time of the latest follow-up. In the group of 33 tumours without chromosomal instability, one patient died and 32 were alive at the time of the latest follow-up. Of the tumours with chromosomal instability, six were of high-risk histology while only one of the tumours without chromosomal instability was of high-risk histology. The presence of chromosomal instability was thus found to be an indicator of poor survival and correlated well with high-risk histology whereas the absence of chromosomal instability was an indicator of a

favourable prognosis and correlated with intermediate-risk histology. In the Wilms tumour tissue sections, abnormal mitotic figures were only found in the blastemal and anaplastic components of the tumours. To evaluate whether this could be caused by variations of telomere length in the different tumour components, telomere length was measured in tissue sections from five Wilms tumours by quantitative fluorescence *in situ* hybridisation. Telomeres were found to be generally shorter in the anaplastic and blastemal components of the high-risk tumours than in the stromal and epithelial components, potentially explaining the presence of the abnormal mitotic figures. Telomere dependent chromosomal instability thus seemed to be a predictor of poor outcome in Wilms tumours.

Within the aims of this thesis was also to use the combined cytogenetic techniques previously applied to colorectal cancer cell lines to screen for balanced recurrent chromosomal aberrations in Wilms tumour. Fifteen primary kidney tumours histopathologically diagnosed as Wilms tumours and two established cell lines from tumours reported to be Wilms tumours were screened. Fifty-six of the 117 detected breakpoints and one reciprocal translocation could not be detected or fully described with G-banding and multicolour FISH only, but required subtelomeric FISH analysis. Reciprocal translocations with deletions in the breakpoints were found in two primary tumours, of which one led to a deletion of the putative tumour suppressor gene *HACE1*. The other tumour with a reciprocal translocation, and one of the cell lines, proved to have structural rearrangements involving chromosome 22, leading to the fusion genes *EWSR1/ERG* and *EWSR1/FLI1*, respectively. Chimaeric genes involving *EWSR1,* are found in the majority of Ewing family tumours but have not been reported in Wilms tumour. Histopathologically, the primary tumour consisted of small, blue, round cells. Complementary immunohistochemical analysis performed after

the genetic analysis, showed that the tumour cells were negative for WT1 and positive for CD99. This together with the *EWSR1* rearrangement and the clinical features of the patient, she was 15 at the time of diagnosis, led to the conclusion that this tumour was most probably a Ewing family tumour. The established cell line with the *EWSR1* rearrangement was reported to be derived from a Wilms tumour of anaplastic histology (Fogh and Trempe 1975; Smith, et al. 2006). By gene expression analysis performed by another group it was found to have an expression pattern similar to Ewing family tumours and an *EWSR1-FLI1* was found (Smith, et al. 2006). Our finding of a der(11)t(11;22) at chromosome analysis of the cell line supports these result. Our genomic screening approach thus proved efficient in finding structural chromosomal aberrations but even though two reciprocal translocations were found, no fusion genes were detected in the Wilms tumours of this study.

DISCUSSION

Summary

In the studies included in this thesis, chromosomal instability has been investigated in established colorectal cancer cell lines and in Wilms tumour. Telomere deficiency was found to be a mechanism of chromosomal instability in both these tumour types. In colorectal cancer it was observed that the two previously described genomic instabilities, MSI and chromosomal instability, were not, as previously thought, mutually exclusive, and that chromosomal instability was present also in MSI positive cell lines. In contrast to telomere-dependent anaphase bridging, it was observed that multipolar cell divisions might not be a common mechanism of acquiring chromosomal aberrations, because they caused frequent nullisomies and the viability of daughter cells was low. In Wilms tumour it was found that telomere dependent chromosomal instability was present in a subset of aggressive tumours and that the presence of chromosomal instability was associated with a poor outcome. In the studied colorectal cancer cell lines, chromosomal instability was found to be a potential cause of extensive karyotypic complexity and heterogeneity. The cell line karyotypes were analysed with a combination of G-banding, COBRA and subtelomeric FISH. The combination proved very efficient in finding breakpoints, especially breakpoints involving terminal parts of the chromosomes. The method was then used on a set of Wilms tumours in the pursuit of recurrent balanced translocations creating fusion genes. Here also, breakpoints not detected by G-banding were found; however, no recurrent balanced translocations were seen in the Wilms tumours. Instead fusion genes typical of Ewing family tumours were found in two tumours that had been misclassified as Wilms tumours initially.

Can the results be trusted?

Because of the stepwise accumulation of mutations and the selection of clones with the highest growth advantage, many tumours are heterogeneous (Cotran, et al. 1999). Tumour heterogeneity can be observed both on the histopathologic and the genetic levels and may bias results by the selection of cells for analysis. In articles I-IV established cell lines were used and in articles III and IV, both established cell lines and primary tumour material were used. The established cell lines were used as model systems when a large volume of tumour material was required and no direct conclusion about the individual tumour of origin was made. Primary tumours on the other hand were used to study chromosomal instability and fusion genes in the individual tumours and to make correlations with the clinicopathological phenotype. It was not known for how many cell doublings or passages the established cell lines had undergone since their establishment, but the primary tumours were only cultured for between two and five population doublings. The level of chromosomal instability may have been affected by the *in vitro* environment but the fusion gene analysis on the other hand ought not to have been affected because these chromosomal changes are early changes and should thus be present in all tumour cells (Heim and Mitelman 1995). In Article III, the estimations of chromosomal instability, defined as the ratio of mitoses with bridges or multipolarity to normal mitotic figures, were made in the tissue sections originally used for histopathological diagnosis and prognostication. For each tumour all available tissue sections were used in an attempt to reduce the impact of tumour heterogeneity on the result. However, there may still be a bias in the results, because of the initial selection made by the pathologist. This may have led to an under- or overestimation of the rate of chromosomal instability. Most of the results have been obtained by microscopic analysis. In an attempt to avoid inter-individual differences in the interpretation of the results,

especially regarding the mitotic figures, strict definitions were used. However, when tissue sections were analysed, the analysis was more problematic because the quality of the material was poorer than in material obtained from cultured cells, and oblique sectioning of tissue blocks could generate confusing morphology.

The diagnostic process in Wilms tumour is based on histopathology and in some cases immunohistochemistry. In Article III, one tumour included in the study was later, in Article IV, observed to be biologically more similar to Ewing family tumours. This tumour was originally classified as a high-risk Wilms tumour of blastemal type and was included in the *in vitro* (case 2) and the *in vivo* (P5) study showing chromosomal instability. The patient subsequently died of the disease. Although it was, neither in the *in vitro* nor in the *in vivo* study, the only tumour with chromosomal instability and a poor outcome it was used to support the conclusion that chromosomal instability was associated with a poor outcome. It had, as all tumours included in our two studies of Wilms tumours, been reviewed by a local pathologist and a SIOP reference pathologist. In cancer research, as in the clinical setting, the diagnostic process is fundamental, and in this case we have trusted the established protocols for clinical diagnosis in which immunohistochemistry is not required.

Are different genomic instabilities mutually exclusive?

It has previously been suggested that in colorectal cancers, MSI and chromosomal instability are rarely present simultaneously. By observing telomere shortening, mitotic abnormalities and intercellular karyotypic variability in colorectal cancer cell lines both with and without MSI, the results in Article I showed that chromosomal instability was present also in MSI-positive colorectal cancer cell lines.

It has been observed that besides colorectal tumours with MSI and tumours with chromosomal instability, there is a group of cancers that do

not display either type of genetic instability (Matsuzaki, et al. 2005). These tumours have been found to exhibit regional CpG island hypermethylation. This has been defined as the CpG island hypermethylation phenotype (CIMP; Toyota, et al. 1999) and it is also found in all MSI-positive tumours (Weisenberger, et al. 2006) and in some tumours with chromosomal instability. The genetically stable CIMP tumours are located in the proximal colon, have a poor prognosis, and are thought to arise from serrated adenomas (Issa 2004). The observation of CIMP in colorectal cancers shows that the subdivision of cancer-promoting mechanisms in these tumours seems to be more complex than previously thought.

The investigations in Article I were performed on established cell lines which make it difficult to state with certainty that the observed telomere-dependent chromosomal instability was not an *in vitro* artefact. However, our results are supported by several studies that have shown that chromosomal aberrations are frequently present in MSI positive colorectal cancers *in vivo* (Li, et al. 2003; Jones, et al. 2005; Gaasenbeek, et al. 2006; Trautmann, et al. 2006). As has been pointed out in the introduction, chromosomal instability is not equivalent to the presence of chromosomal aberrations but rather reflects the rate at which these are formed. By this definition, there are so far no other studies on chromosomal instability in MSI positive tumours to our knowledge. The fact that the two types of genomic instability are not mutually exclusive does not necessarily exclude the possibility that they can both independently give rise to cancer. Because telomere shortening can be a result of *e.g.* cellular growth (Harley, et al. 1990) or of telomere damage caused by oxidative stress (von Zglinicki 2002) chromosomal instability may only be a symptom of the tumour process. On the other hand, an enhanced mutation rate also on the chromosomal level may have an effect additive to the MSI and further increase the acquisition of tumourigenic genomic aberrations.

Do anaphase bridges and multipolar mitoses generate chromosomal changes?

In Article I, it was observed that anaphase bridges gave rise not only to structural but also to numerical aberrations. This has been confirmed in other colorectal cancer cell lines where anaphase bridges have been observed to detach from the mitotic spindle (Shimizu, et al. 2005) and in urothelial cancer, anaphase bridges have also been associated with whole-chromosome loss (Jin, et al. 2007). In the latter study it was also found that non-disjunction, without telomere dependant chromosomal instability, could give rise to numerical chromosomal aberrations. Gains and losses of whole chromosomes have also been associated with multipolar mitoses (Saunders, et al. 2000). These abnormal cell divisions are typically caused by supernumerary (three or more) centrosomes (Lingle and Salisbury 1999; Saunders, et al. 2000) and have the potential of generating daughter cells with a high variability in chromosome number. However, in Article I, daughter cells from multipolar cell divisions were followed for 30 days in parallel with daughter cells from mitoses with anaphase bridges and normal mitoses. While most daughter cells from bipolar mitoses, both with and without anaphase bridges, gave rise to colonies, daughter cells from multipolar mitoses did not. To our knowledge, the fate of daughter cells of multipolar mitoses has not been studied by other groups. The daughter cells of multipolar mitoses would theoretically display a high variability of chromosome copy-number. If multipolar mitoses frequently gave rise to viable daughter cells this would be reflected also in the tumour karyotypes which would display cells with highly different copy-numbers than that of the stem-line karyotype (Jin, et al. 2007). No such clones were observed in the established cell lines or primary tumours included in these studies, although the established cell lines displayed high rates of mitotic multipolarity.

The most common chromosomal aberrations in Wilms tumour are trisomies and a few structural aberrations involving *e.g.* chromosomes 1, 7 and 16 (Höglund, et al. 2004). In article III, the level of chromosomal instability was assessed in Wilms tumours and only found in a subset of tumours. Anaphase bridging thus does not provide an explanation for all chromosomal changes in these cases indicating that there are other mechanisms for acquiring chromosomal aberrations in Wilms tumours. Based on studies of uniparental disomies in hyperdiploid acute lymphoblastic leukaemia (Paulsson, et al. 2005), it was hypothesised that the most likely mechanism of the generation of the trisomies observed in these malignancies, was a single abnormal mitotic event occurring early in the neoplastic process. Perhaps a similar mechanism is also present in Wilms tumour.

In Articles I and III, the frequency of multipolar mitoses was inversely correlated with telomere length and cells with anaphase bridges and multipolar mitoses were always present in the same cell samples. When the rate of anaphase bridges was enhanced by radiation and telomerase inhibition, the rate of multipolar mitoses was also increased. However, when the rate of multipolar mitoses was increased by inducing polyploidy, this did not have an effect on the frequency of anaphase bridges. We hypothesised that an anaphase bridge could mechanically inhibit a cell from undergoing cytokinesis and that this would lead to a duplication of the cellular genome as well as the centrosomes. The cell would then divide in a multipolar fashion in the next cell division (Meraldi and Nigg 2002). In colorectal cancer cell lines, an inhibitory effect of anaphase bridging on cytokinesis has indeed been observed (Shimizu, et al. 2005). Instead of dividing into two daughter cells, two nuclei develop within the same cytoplasmic membrane. This lends some support to the theory that telomere deficiency could cause both anaphase bridges and multipolar mitoses.

Does chromosomal instability correlate with tumour phenotype?

Several features associated with chromosomal instability such as reduced or altered telomere content (Bisoffi, et al. 2006), complex karyotypes (Ninomiya, et al. 2006), expression of genes associated with complex karyotypes (Carter, et al. 2006), losses of heterozygosity (Watanabe, et al. 2001; Ninomiya, et al. 2006), and aneuploidy (Risques, et al. 2003) have been associated with a more malignant tumour phenotype. In urothelial cancer, the level of chromosomal instability has been measured by inter-cellular chromosomal variability (Yamamoto, et al. 2006) and by abnormal mitotic figures in tissue sections (Jin, et al. 2007), and a high rate of chromosomal instability has been found in more aggressive tumours. After observing that frequencies of anaphase bridges in tissue sections were associated with karyotypic complexity, it was suggested that such measurements could be used clinically to distinguish different prognostic sub-groups of cancers (Montgomery, et al. 2003).

In article III, chromosomal instability in Wilms tumour was studied. Chromosomal instability was present in a subset of aggressive tumours *in vitro*, and in tissue sections anaphase bridges and multipolar mitoses were only observed in the blastemal and anaplastic tumour components. In five tumours, telomere length was measured and was found to be significantly shorter in the blastemal and anaplastic components of the tumours than in the other tumour components. This could explain the abnormal mitotic figures in these elements. Furthermore, in this study, scoring anaphase bridges or multipolar mitoses in tissue sections was as accurate in predicting clinical outcome as histopathological risk stratification.

The results in Article III led us to perform a follow-up study of anaphase bridges and multipolar mitoses in Wilms tumour tissue sections. The study is still on-going and the results have not been fully analysed although

some preliminary data have been obtained. To evaluate inter-individual differences in the interpretation of the mitotic figures, two persons analysed slides from 20 randomly selected cases of the 110 Wilms tumours from patients treated at The Academic Medical Center in Amsterdam, the Netherlands between 1992 and 2006. Only in one of the cases the observers reached different conclusions regarding the presence of chromosomal instability. Six of the 110 tumours displayed chromosomal instability by the criteria used in Article III and seven patients died of the disease. Only two of the patients who died of the disease had tumours with chromosomal instability. Hence, the quantification of anaphase bridges and multipolar mitoses may not be a universal predictor of outcome. However, in this second set of 110 tumours, there was also a poor correlation between histopathology and outcome. Three of the patients who died had high-risk tumours while four had intermediate-risk tumours. If the use of chromosomal instability as a prognostic factor in Wilms tumour is to be further explored, methods more objective and less time consuming than scoring the rates of anaphase bridges and multipolar mitoses in tissue sections need to be developed. Based on our observation that chromosomal instability in Wilms tumour is caused by telomere deficiency, measuring telomere lengths could provide a more objective measurement. However, this has to be done in individual cells from different histopathological tumour components and it is important to ensure that non-neoplastic cells are not included in the analysis.

Chemotherapeutical pre-treatment affects the histopathological spectrum of the tumour components in Wilms tumour (Kalapurakal, et al. 2004). Among tumours that have been pre-treated, the proportion of tumours of blastemal predominance is lower than in the tumours that have not been pre-treated. Chemotherapeutically pre-treated tumours of the blastemal type are associated with a poorer prognosis than tumours of the same histological

subtype in untreated tumours. Consequently blastemal predominance is considered being of high-risk histology in pre-treated tumours and of favorable histology in non pre-treated tumours. Thus, chemotherapeutically untreated blastemal type tumours can be divided into two kinds: tumours that will respond well to chemotherapy and therefore have a good prognosis and tumours that will not and therefore have a poor prognosis (Beckwith, et al. 1996). Because all tumours included in this study were chemotherapeutically pre-treated, a comparative study of the levels of chromosomal instability between blastemal tumours that respond well to chemotherapy and those that do not, was not possible to carry out. Chromosomal instability could be associated with therapy resistance because of the inferred high mutation rate. It is thus possible that chromosomal instability is present in the blastemal type tumours that respond poorly to therapy while it is not present in those that respond well. The epithelial and mesenchymal tumour components have a moderate telomere shortening according to data in Article III but no mitotic aberrations were seen in these components. Is it possible that the chemotherapy sensitive blastema is a developmental defect similar to nephroblastomatosis that gives rise to immature epithelial components and stromal components of the tumours while the other type of blastema has acquired more mutations and has become more malignant? Hypothetically, it could be from this chromosomally unstable blastema that anaplastic cells develop as the telomeres become progressively shorter, because these two tumour components share the feature of therapy resistance and telomere-dependent chromosomal instability.

Are chromosomal instability and fusion genes both important for tumorigenesis?

In Article II, five colorectal cancer cell lines were analysed by a combination of G-banding, multicolour FISH and subtelomeric FISH. In article IV the same approach was used to study the chromosomes in the pursuit of reciprocal translocations in 15 primary tumours and two established cell lines, all initially classified as Wilms tumours. In both studies, it proved to be an effective method to detect chromosomal breakpoints, especially breakpoints involving the distal parts of the chromosomes. In Article IV, however, the only fusion genes found were *EWSR1* rearrangements, indicating that two tumours were Ewing family tumours that had been misclassified. In the remaining Wilms tumours, one reciprocal translocation, involving a deletion of the putative tumour suppressor gene *HACE1,* was found. Because no recurrent reciprocal translocations were found in our set of tumours, it seems unlikely that a high proportion of Wilms tumours have a common pathognomonic reciprocal translocation. However, it does not exclude other arrangements by which fusion genes can be formed, *i.e.* inversions, deletions or duplications.

Array-CGH methods can detect smaller imbalances than our combined cytogenetic approach but still cannot detect completely balanced chromosomal aberrations nor describe clonal heterogeneity because array methods study the average gene copy-number or gene expression level of all the cells in a sample. However, as was observed in article IV where two detected reciprocal translocations were accompanied by deletions, and as has been reported by other groups, reciprocal translocations are often not balanced on the DNA level and may be associated with deletions and duplications (Moon, et al. 2007). These minute imbalances could potentially be detected by CGH array-techniques. Fusion genes have also been detected by observing abnormalities in gene expression and then confirming the suspected gene

fusions with FISH analysis in prostate and non-small cell lung cancer (Tomlins, et al. 2005; Soda, et al. 2007). Our combined approach detected twice the number of breakpoints as G-banding alone did and was especially efficient at detecting terminal chromosomal aberrations. However, the combined technique was time consuming and requires metaphase preparations of high quality. Considering the rapid development of array based and genomic screening techniques, it can be questioned whether high resolution cytogenetics will be of importance in the future search for new fusion genes.

Malignancies with fusion genes often display relatively few karyotypic changes (Heim and Mitelman 1995). However, fusion genes have recently been found also in 70% of prostate cancer cases although these tumours have been shown to have complex karyotypes (Mitelman 2007), intercellular chromosomal variability (Beheshti, et al. 2001), telomere shortening (Meeker, et al. 2002) and breakage-fusion-bridge cycles (Vukovic, et al. 2007). It has been hypothesised that paediatric tumours need fewer mutations for their development (Knudson 2001) than adult tumours. Embryonic cells have a high capacity to grow and invade and their failure to mature might alone increase their malignant potential. Genes or loci often shown to be mutated or epigenetically altered in Wilms tumours, *i.e. WT1*, *WT2* and *WTX*, are important for normal kidney development. Losses of function of these genes may inhibit the maturation of immature kidney cells and provide a pool of embryonic cells that may act as tumour precursors. Alterations in kidney differentiation genes could act in a similar way to gene fusions by being early changes that lead to a growth advantage. However, for a minority of Wilms tumours chromosomal instability seems to follow the initial event causing the inhibition of the maturation of kidney cells. It is thus possible that at least some Wilms tumours have a primary aberration, such as a fusion gene or a single gene change, and then develop chromosomal instability

which increases the likelihood of acquiring additional mutations. By increasing the tumour's possibilities of becoming resistant to therapy, chromosomal instability leads to clinical problems. Anaplastic Wilms tumours have at the time of diagnosis not spread further than intermediate-risk tumours; however, they respond poorly to therapy and are in almost half the cases associated with a deadly outcome (Beckwith, et al. 1996; Weirich, et al. 2004). Perhaps by targeting genetic instability, *e.g.* by telomerase inhibition leading to excessive telomere shortening, cancer therapy can become more efficient, both by enhancing the efficiency of therapy and by increasing the genetic instability to a level where it is lethal to the tumour.

CONCLUSIONS

In this thesis, telomere deficiency with subsequent anaphase bridging was found to be associated with chromosomal instability in established colorectal cancer cell lines and in Wilms tumour. In colorectal cancer cell lines, anaphase bridging was observed to generate both numerical and structural chromosomal aberrations and was also associated with the presence of multipolar mitoses. In contrast to cells having undergone anaphase bridging, daughter cells from these multipolar mitotic figures were observed not to form clones in culture, possibly because of the severe aneuploidy which is the result of multipolar mitosis. Chromosomal instability was observed also in colorectal cancer cell lines with mutations in the mismatch repair genes. In Wilms tumour, chromosomal instability was found to be associated with an aggressive tumour phenotype and poor survival. Telomere shortening was more pronounced in the immature tumour components, which could explain the fact that anaphase bridges and multipolar mitoses were only observed in these tumour elements. Because of breakage-fusion-bridge cycles, chromosomal instability is associated with karyotypes with extensive structural chromosomal rearrangements. By applying a combination of subtelomeric FISH, G-banding and multicolour FISH, a high resolution for cytogenetic analysis of tumours with chromosomal instability could be obtained. This combined approached was used also to search for reciprocal translocations leading to fusion genes in Wilms tumour. Although the technique proved efficient, no recurrent reciprocal translocation was found in Wilms tumour. Our results indicate that telomere dependent chromosomal instability is present in both colorectal cancer and Wilms tumour and could be an important prognostic factor in Wilms tumours.

SUMMARY IN SWEDISH

Cancer är en sjukdom som uppkommer när kroppens egna celler börjar tillväxa ohämmat och inte längre svarar på kroppens egna tillväxtkontrollerande signaler. Cancercellerna invaderar intilliggande vävnad och skickar ut metastaser till andra delar av kroppen antingen genom blod- eller lymfbanan.

I varje cell i kroppen (utom röda blodkroppar) finns kromosomer som består av arvsmassan, dvs. de gener som kontrollerar vilka ämnen som ska tillverkas i cellerna. Cellerna förökar sig genom att kromosomerna kopieras och delas upp i två identiska dotterceller. Mutationer är förändringar i arvsmassan och kan innebära att cellens funktion ändras. Om en cell får en mutation som gör att den tillväxer snabbare än de omkringliggande kommer det att finnas fler kopior av denna muterade cell än de övriga cellerna. Detta kan i förlängningen orsaka cancer. Man har kunnat analysera kromosom-uppsättningarna i många olika cancertyper och sett att kromosomerna i tumörer ofta skiljer sig från normala kromosomer både med avseende på antal och på utseende. Kromosomförändringar kan uppkomma tidigt i cancer-utvecklingen och ge upphov till fusionsgener som bildas av att två olika gener sätts samman och genen uttrycks på ett nytt sätt. Dessa förändringar finns i en tumörs alla celler och används ibland kliniskt för diagnostisering och behandling. Senare kromosomförändringar kan variera i olika delar av tumören men de tillför ytterligare förändringar som krävs för utvecklingen av en cancercell. För att en cell ska kunna ansamla dessa mutationer kan i många fall krävas att arvsmassan är instabil, dvs. att mutationsfrekvensen är förhöjd. Kromosomal instabilitet är en förhöjning av mutationsfrekvensen på kromosomnivå, dvs. en ökad förlust och/eller tillkomst av delar av och/eller hela kromosomer. Kromosomal instabilitet kan orsakas av att det speciella området på kromosomernas ändar som ser till att kromosomerna är intakta, telomererna, skadas och inte kan skydda kromosomerna. Istället fastnar kromosomerna i

varandra vilket leder till att de i celldelningen skapar anafasbryggor som sedan går sönder och fastnar i nya kromosomer (se bild på s. 11). På detta sätt ökar tillkomsten av kromosomförändringar i cellerna. Genom att titta på celldelningar i mikroskop kan anafasbryggor observeras och nivån av kromosomal instabilitet kan uppskattas. När en tumör är kromosomalt instabil bildas kontinuerligt nya förändrade kromosomer och det kan vara svårt att göra en analys av en cells kromosomer och att hitta de kromosomförändringar som kan användas kliniskt.

I avhandlingen har studier utförts på tjocktarmscancer och på barncancerformen Wilms tumör. Målsättningen har varit att studera uppkomsten av kromosomförändringar och om hastigheten med vilken de bildas är av betydelse för utgången av sjukdomen. Dessutom har en kombination av metoder för att studera kromosomförändringar utvärderats på tjocktarmstumörer för att sedan användas för att hitta tidiga kromosomförändringar som skulle kunna vara av klinisk nytta i Wilms tumör.

Eftersom man kan hitta godartade och elakartade tumörer av olika stadier i tjocktarmen har denna typ av cancer ofta använts för att studera utvecklingen av mutationer som leder till cancer. Begreppet kromosomal instabilitet är myntat efter studier av tjocktarmscancer. Därför studerade även vi denna cancerform. I Artikel I såg vi att telomerer även i tjocktarmscancer kan ge upphov till kromosomal instabilitet och att anafasbryggor kan leda till olika typer av kromosomförändringar. Dessutom förekom anafasbryggor samtidigt och i samma tumörer som andra avvikande celldelningar, t.ex. multipolära celldelningar, dvs. när cellen delar sig åt mer en ett håll. I Artikel III studerades effekten av kromosomal instabilitet på patientens överlevnad i Wilms tumör. Genom att analysera färgade snitt från tumörer kunde vi studera celldelningar och såg att anafasbryggor och multipolära delningar fanns i ett fåtal av dessa tumörer. Patienterna med dessa tumörer hade sämre överlevnad än patienterna med tumörer utan kromosomal instabilitet. Från studier av

telomerer både i odlade tumörceller och i tumörsnitt såg vi att dessa var förkortade i de tumörer som hade kromosomal instabilitet vilket pekar på att det skulle kunna vara orsaken till den kromosomala instabiliteten även i Wilms tumör.

I Artikel II studerades tjocktarmscancer för att utvärdera en kombination av metoder för att upptäcka kromosomförändringar. Metoden visade dig vara mycket effektiv, speciellt för att upptäcka förändringar som involverar kromosomernas ändar. Denna typ av förändringar är vanliga men ofta svåra att upptäcka med konventionella metoder. Metoden tillämpades sedan i Artikel IV på Wilms tumörer. Vi letade efter kromosomförändringar som, i andra cancertyper, har visats ge upphov till de första mutationerna och som används diagnostiskt och för behandling i vissa fall. Kombinationsmetoden visade sig vara effektiv även här men trots att många avvikande kromosomer upptäcktes, hittades inga sådana specifika kromosomförändringar i Wilms tumör.

Sammanfattningsvis har anafasbryggor visats kunna ge många olika typer av kromosomförändringar och även vara en möjlig orsak till multipolära celldelningar. I Wilms tumör har förekomsten av anafasbryggor visats bero på telomerförkortning samt vara associerad med en sämre prognos. En ny kombination av metoder för att studera kromosomförändringar utvärderades och befanns vara mycket effektiv. Trots det kunde inga tumörspecifika, kliniskt användbara kromosomförändringar hittas i Wilms tumör.

Tumörer av många olika typer har visats ha kromosomal instabilitet och den kromosomal instabiliteten skapar kliniska problem vid behandlingen eftersom den ökade mutationsfrekvensen förmedlar läkemedelsresistens. Genom att inrikta sig på kromosomal instabilitet, t.ex. genom att förhöja den till en nivå där den är dödlig för cancercellerna, kan man både behandla cancern som sådan och minska läkemedelsresistensen. Mätning av den kromosomala instabiliteten kan potentiellt komma till nytta, både som ett sätt att dela upp tumörer i prognostiska subgrupper men också för att hitta tumörer med kromosomal instabilitet som kan ha nytta av läkemedel som specifikt riktar sig mot kromosomal instabilitet.

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