

Dissertation

Molecular markers to characterise nephroblastoma and stage2 colorectal carcinoma

Submitted by

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I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all those individuals and affiliated organizations that have contributed to the presented work. Due acknowledgements have been made in the text in all the respective sections of the thesis.

Throughout this thesis and all related publications the guidelines of Good Scientific Practice were followed.

Please note that parts of this thesis have been already accepted for publication in the following journals:

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Summary

Nephroblastoma and colorectal carcinoma (CC) are two tumour entities that occur in children and adults, respectively. Nephroblastomas are embryonal neoplasms that histologically and ultrastructurally recapitulate developing kidney. CC is one of the leading causes of cancer deaths worldwide and treatment is determined according to clinic-pathological staging system. Herein, we have analysed different molecular markers by a variety of techniques to characterise nephroblastoma and stage2 CC. SIX2 is one of the characteristic genetic factors, which maintains renal progenitor cells in foetal kidney. Blastema showed a significantly higher level of mRNA expression of SIX2 compared to epithelial regions regardless of the subtype of nephroblastoma and a strong nuclear expression was detected in blastema. Upstream activators of SIX2 indicated that the signal transduction pathway was activated in most, but not all nephroblastomas. Increased percentage of cells in the S-phase and cellular migration was identified in cells overexpressing SIX2. Kidney enriched miRNAs; miR-192, miR-215 and miR-194 had a significantly lower expression in nephroblastomas regardless of the subtype compared to mature kidney. Kidney parenchyma had a significantly higher expression of miR-192, miR-194, miR-215 and miR-200c compared to nephrogenic rests (NRs). Activin receptor type 2B (ACVR2B), a member of the TGF- β pathway, was identified as single common target gene for miR-192, miR-215, miR-194, miR-141 and miR-200c *in-silico*. The interaction between miRNAs and ACVR2B was also verified by an *in-vitro* assay. A distinct mRNA and protein expression of ACVR2B was detected in nephroblastomas. Array CGH analysis revealed amplifications of HOXC6 in nephroblastomas and NRs. mRNA expression of HOXC6 V1 and V2 was concordant with HOXC6 amplifications. Overexpressing V1 and V2 in a nephroblastoma cell line identified distinct up or down regulation of target genes by V1 compared to V2.

Different molecular and immunohistochemical markers were analysed in a small cohort of stage2 CC patients. Mutations in PIK3CA exon 9 had a significant impact on disease free survival.

In conclusion, SIX2, based on a differential regulation of ACVR2B by miRNAs and HOXC6 V1, V2 appears to be an important molecular marker in the pathogenesis of nephroblastomas. Mutations in PIK3CA exon 9 are useful prognostic markers in stage2 CC.

Zusammenfassung

Nephroblastome und colorectale Karzinome (CC) sind Tumorentitäten, die überwiegend bei Kindern bzw. Erwachsenen auftreten. Nephroblastome sind typische embryonale Neoplasien, die histologisch und ultrastrukturell die Nierenentwicklung rekapitulieren. CC sind eine der häufigsten krebsbezogenen Todesursachen weltweit, ihre Behandlung richtet sich nach einem klinikopathologischen „staging“ System. Wir untersuchten unterschiedliche Marker, um Nephroblastome und CC im Stadium 2 mit verschiedenen molekularen Techniken zu charakterisieren. SIX2 ist ein charakteristischer Marker, der Vorstufenzellen der Nierenentwicklung im foetalen Zustand erhält. Das Nierenblastem zeigte eine signifikant höhere Konzentration von SIX2 mRNA im Vergleich zu epithelialen Regionen verschiedener Nephroblastom-Subtypen, im Zellkern zeigte sich eine starke Expression. Die Untersuchung von SIX2 Aktivatoren zeigte, dass dieser Signaltransduktionsweg in den meisten Nephroblastomen aktiviert ist. In Zellen, die SIX2 überexprimieren konnte ein erhöhter Prozentsatz von Zellen in S-Phase und eine verstärkte zelluläre Migration festgestellt werden. miR-192, miR-215 und miR-194 die in der Niere vermehrt nachgewiesen werden, waren in allen Nephroblastom-Subtypen vermindert. Normales Nierenparenchym zeigte eine signifikant stärkere Expression von miR-192, miR-194, miR-215 und miR-200c im Vergleich zu nephrogenen Resten. Der Activin Rezeptor Typ 2B (ACVR2B), ein Mitglied der TGF- β pathway Familie, konnte als gemeinsames Target von miR-192, miR-215, miR-194, miR-141 und miR-200c *in-silico* identifiziert werden. Die Interaktion zwischen den miRNAs und ACVR2B konnte auch in einem *in-vitro* Assay nachgewiesen werden. Ein spezifisches Muster der mRNA und Protein Expression von ACVR2B konnte in Nephroblastomen nachgewiesen werden. Eine Array CGH Analyse zeigte eine Amplifikation von HOXC6 in Nephroblastomen und nephrogenen Resten. Die mRNA Expression von HOXC6 V1 und V2 war konkordant mit der HOXC6 Amplifikation. Überexpression von V1 und V2 in einer Nephroblastom-Zelllinie zeigte eine deutlich unterschiedliche Regulation der Targetgene von V1 und V2. Unterschiedliche molekulare und immunhistochemische Marker wurden in einer kleinen Kohorte von Patienten mit Stage 2 CC analysiert. Mutationen im PIK3CA Exon 9 zeigten einen deutlichen Einfluss auf das krankheitsfreie Intervall. Zusammenfassend zeigte sich, dass SIX2, ein Regulator von ACVR2B in Abhängigkeit von miRNAs und HOXC6 V1 und V2, in Nephroblastomen ein wichtiger molekularer Marker in der Pathogenese zu sein

scheint. Mutationen im PIK3CA Exon 9 sind ein wichtiger prognostischer Marker in Stage 2 CC.

Chapter 1

General introduction

Kidney development

The kidney is one of the main organ systems that maintains a homeostatic environment in the human body. An interesting study by Grobstein (Grobstein, 1955) led to the use of the kidney as a model to study cellular mechanisms such as epithelial mesenchymal transition, epithelial cell differentiation and polarization.

The first step of kidney development occurs in the intermediate mesenchyme through the formation of a nephric cord. The nephric cord consists of a primary nephric duct and an epithelial tube. The epithelial tube is formed by the mesonephric mesenchyme and extends caudally along the anterior-posterior axis of the body till it reaches the metanephric mesenchymal cell population. The most anterior region of the nephric cord, termed the pronephric tubules, is completely degenerate in mammals. Mesonephros lies caudal to pronephros (Dressler, 2006) (Figure 1).

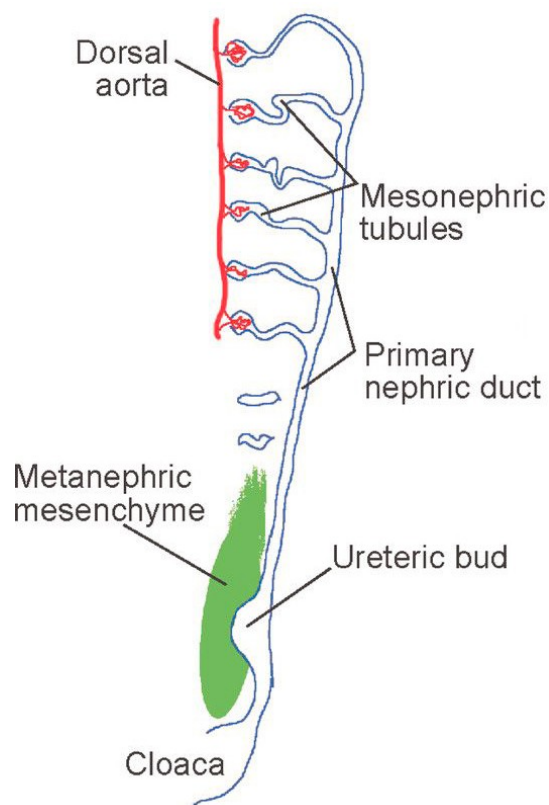


Figure 1: Kidney development in the mouse at E10.5 (Dressler, 2006)

The formation of the kidney can be divided into 5 development stages:

- The primary ureteric bud
- Cap mesenchyme
- Mesenchymal epithelial transition
- Glomerulogenesis and tubulogenesis
- Interstitial cells

At the 28th day of gestation within the human kidney, the ureteric bud of the primary mesonephric duct begins to develop (Yosypiv, 2008). Branching of this ureteric bud induces nephron formation at the tip of each branch, and these branches finally give rise to the collecting duct system, renal pelvis, ureter and bladder trigone (Reidy *et al.*, 2009a). Many genes play a role in origin, elongation and branching of the ureteric bud through their roles as transcription inducers [*GDNF* (glial cell derived neurotrophic factor), *Wnt11* (wingless-type MMTV integration site family, member 11), *PAX2* (paired box homeotic gene 2) or inhibitors (*BMP4* (bone morphogenetic protein 4), *Spry1* (protein sprouty homolog 1)] (Costantini, 2006; Yosypiv, 2008) (Figure 2).

Metanephric mesenchymal cells including progenitor cells (Boyle *et al.*, 2008) lead to the formation of a cap mesenchyme at the tip of the branches of the ureteric bud (Rosenblum, 2008). Cells in the cap mesenchyme undergo epithelial mesenchymal transition (Kispert *et al.*, 1998; Stark *et al.*, 1994), to form the epithelial structures of the nephron (Carroll *et al.*, 2005).

Many genes have been identified as involved in different stages of renal development such as *PAX2* (metanephric mesenchyme differentiation towards cap mesenchyme) (Batchelder *et al.*, 2009), *NCAM* (neural Cell Adhesion Molecule) (maintenance of progenitor cell population) (Metsuyanin *et al.*, 2009), *Lhx1* (LIM homeobox 1) (Dressler, 2009), *Eya1* (eyes absent homolog 1), *Six1* (SIX homeobox 1) (Li *et al.*, 2003), *Sall1* (sal-like1) (Nishinakamura, 2008), *Six2* (SIX homeobox 2) (Self *et al.*, 2006) and *WT1* (Wilms tumour 1) (mesenchymal epithelial transition) (Kreidberg *et al.*, 2008) have been identified (Figure 3).

Sema3a (Semaphorin-3A) gene is involved in glomerulogenesis (Reidy *et al.*, 2009b), *Notch1* (notch homolog 1, translocation-associated-Drosophila) in concert with *Notch 2* (notch homolog 2, translocation-associated-Drosophila) plays a role in the segmentation of comma shape bodies into “S” shape bodies (Surendran *et al.*, 2010).

WT1 and *PAX8* (paired box homeotic gene 8) genes influence differentiation and maturation of the renal tubules (Faa *et al.*, 2012).

In addition to the nephron-lineage cells, *Foxd1* (forkhead box D1) positive progenitor cells originate from metanephric mesenchyme. These cells can differentiate into medullary interstitium, renal capsule, mesangium and pericytes of the kidney (Faa *et al.*, 2012).

Recent studies have also shown that patterning, regulation and differentiation of medullary interstitium or stroma can be influenced by *HOX10* (homeobox 10) (Wellik, 2011).

The majority of nephrons are formed within the first 36 weeks of gestation in humans (Hoy *et al.*, 2008). The number of nephrons is between 300,000 to 1,800,000 (Georgas *et al.*, 2009).

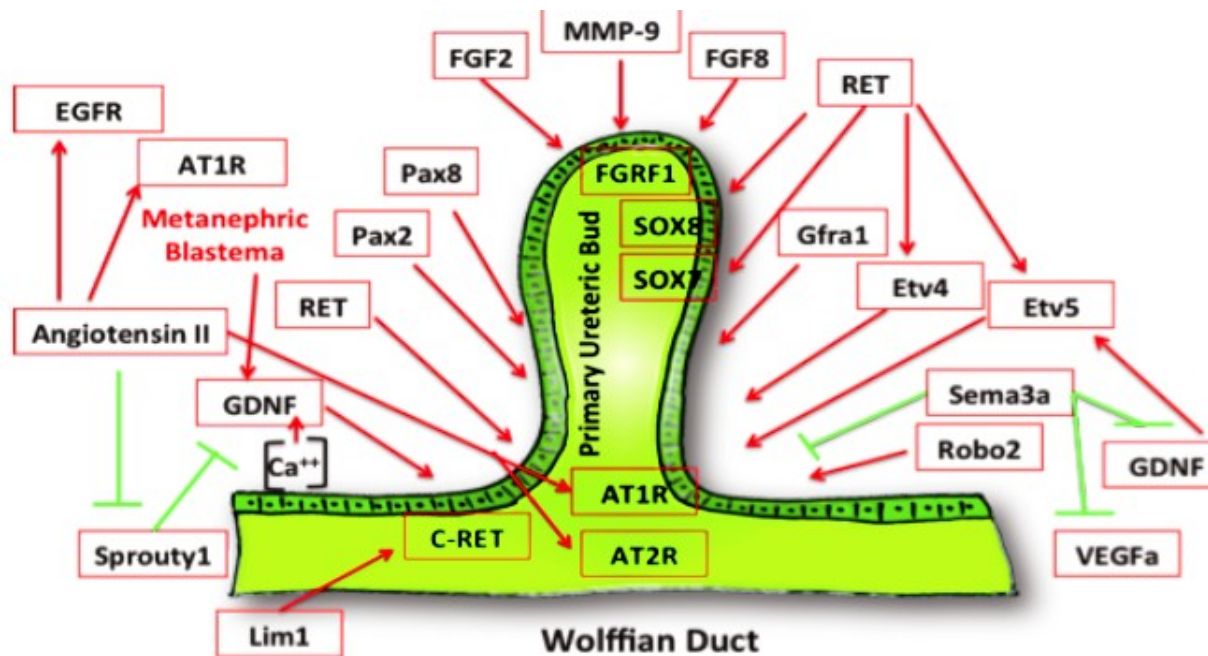


Figure 2: Molecular pathways involved in development and differentiation of the ureteric bud (Faa *et al.*, 2012)

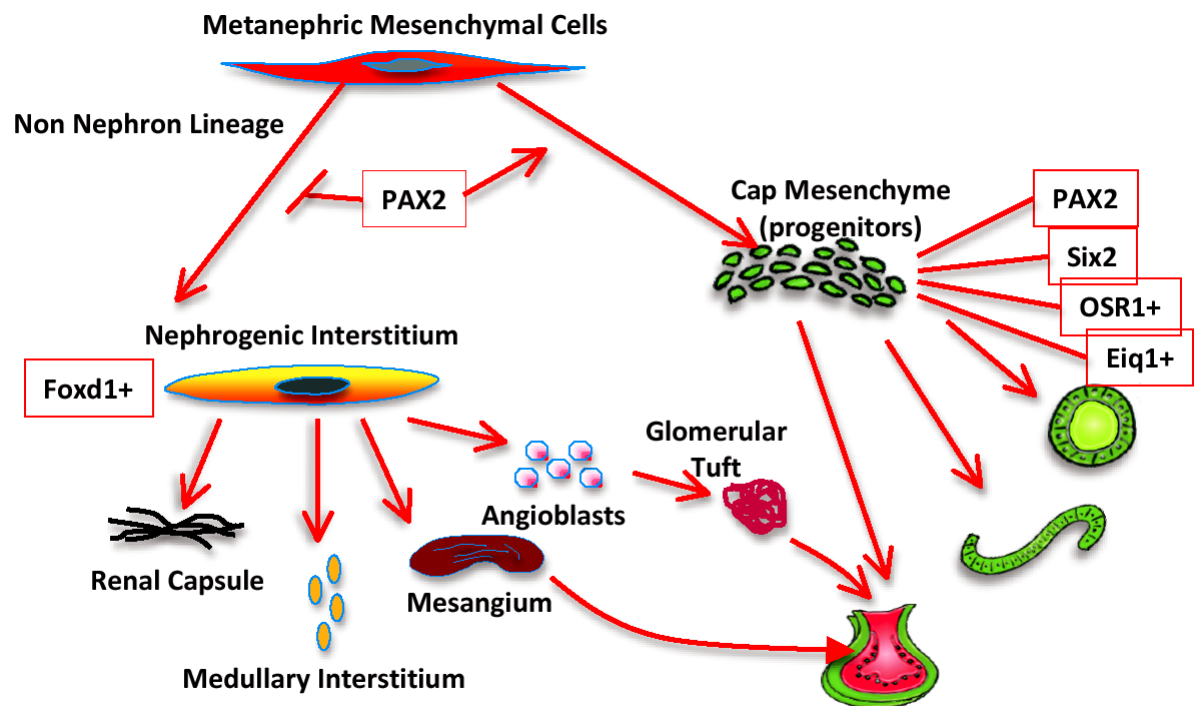


Figure 3: Differentiation of metanephric mesenchyme into nephrogenic and non-nephrogenic cell lineages (Faa *et al.*, 2012)

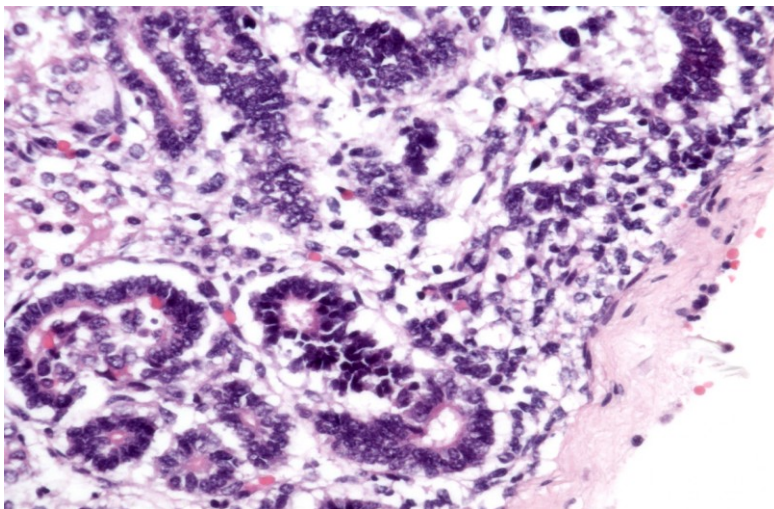


Figure 4: Histological section of foetal Kidney

Nephroblastoma

The most common abdominal tumour in children was described by Max Wilms over a century ago (Ellis, 1986) and it is currently referred to as nephroblastoma or Wilms tumour. Nephroblastomas are known to be the fourth most common paediatric malignancy (Miller *et al.*, 1995). These tumours have equal incidence in males and females and can be identified in the kidneys before the age of 5 (Tumour study committee Wilms tumour 1991). Combined treatments of surgery and chemotherapy is recommended in most cases; however, radiotherapy is considered for advanced stages of the disease (National Wilms' Tumor Study Committee) (1991).

Nephroblastomas are classified as a primitive, multiliniage malignancy of embryonal renal precursors (Bennington and Beckwith., 1975). These tumours consist of blastemal, epithelial and stromal regions (Birch *et al.*, 1987). Nephroblastomas can be classified as epithelial-type, blastema type, stroma type and mixed type, depending on the percentage of each of these different morphological regions (Vujanic *et al.*, 2002) (Figure 5).

Nephroblastomas can be sporadic or may develop in association with syndromes. The majority of nephroblastomas that develop in association with a syndrome have mutations in the *WT1* gene or genomic alteration related to the chromosome 11p15 locus. WAGR syndrome, Denys-Drash syndrome, Fraiser syndrome are few of the syndromes associated with *WT1* mutations (Breslow *et al.*, 2000). The genes at 11p15 play a role in Wilms tumourigenesis in Beckwith-Wiedemann syndrome (Koufos *et al.*, 1989; Ping *et al.*, 1989). Additionally, other disorders such as trisomy 18, Perlman syndrome and Li-Fraumeni syndrome are also associated with nephroblastomas (Domes and Huff., 2003).

Important prognostic factors for long term survival are anaplastic features of the tumours (Davidoff, 2009). European-wide studies on treatment and prognosis of nephroblastomas within the SIOP group have identified the blastemal part, especially if harbouring diffuse anaplasia, as the most malignant part of these embryonal tumours (Berrebi *et al.*, 2008). After preoperative chemotherapy, the residual blastema in nephroblastomas might be one of the most important prognostic parameters correlating to a worse prognosis if a large amount of blastema persists in post-chemotherapy specimens (Weirich *et al.*, 2001).

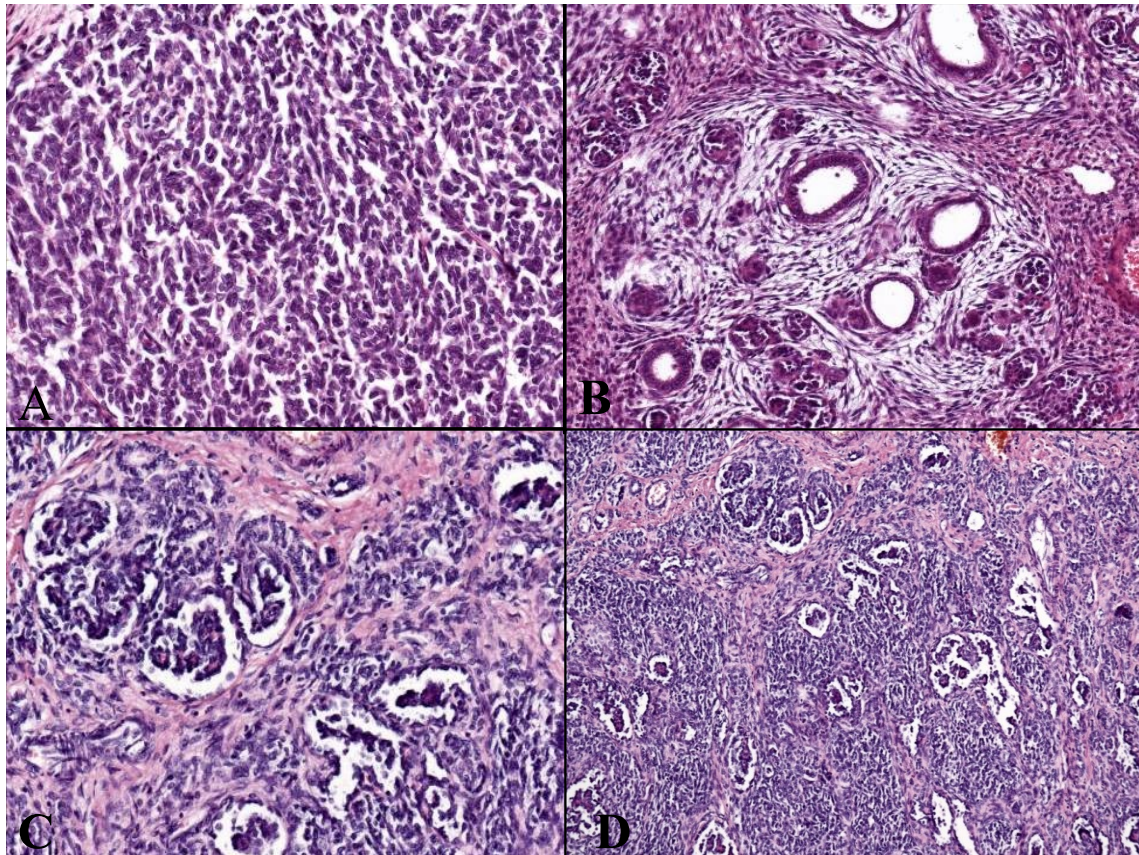


Figure 5: Different subtypes of nephroblastomas. Blastema-type (A), stroma-type (B), epithelial type (C), mixed type (D)

Nephrogenic rests (NRs)

Two distinct categories of NRs are currently recognized; perilobar NRs and intralobar NRs (Beckwith, 1993; Beckwith *et al.*, 1990). Perilobar NR (PLNR-Figure 6) are strictly confined to the periphery of the renal lobe, are sharply demarcated from kidney, and in their active phases are composed of blastemal or embryonal epithelial cell types. Intralobar NRs (ILNR-Figure 7) occur anywhere within the renal lobe, as well as within the renal sinus and the walls of the pelvicaliceal system. They usually contain multiple cell types including abundant immature or mature stroma that are interposed between normal nephrons.

PLNRs and ILNRs are distinct precursor lesions of nephroblastomas that show different epidemiological and clinical features. Their existence is thought to underscore the developmental heterogeneity of nephroblastoma, and helps to illustrate why it is unlikely that there is a single “Wilms tumour” gene.

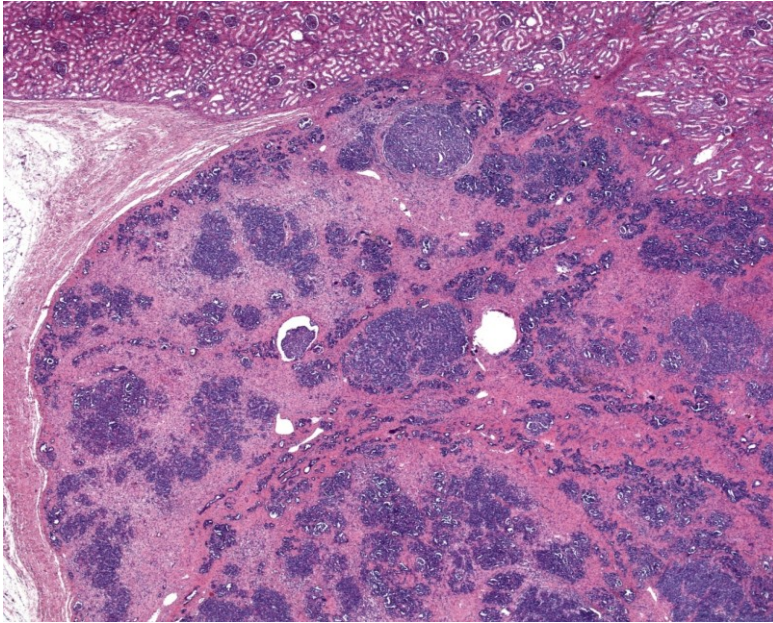


Figure 6: Histological section of perilobar NR

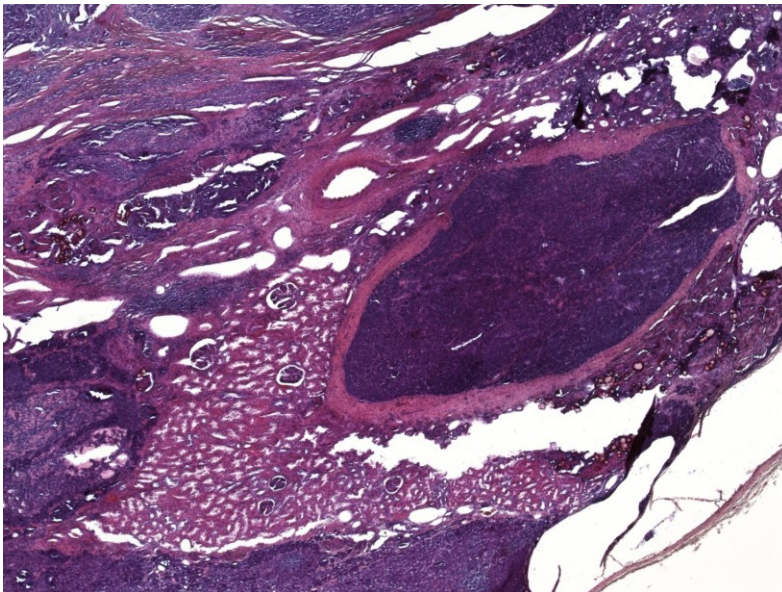


Figure 7: Histological section of an intralobar NR

Nephroblastomas recapitulate normal embryonal kidney development, when studied histologically and ultrastructurally (Mierau *et al.*, 1987). Not only the morphology of nephroblastomas mimics normal kidney development (Figure 4), but also the overall gene

expression of nephroblastomas is similar to an embryonal kidney at 8 weeks of gestation (Dekel *et al.*, 2002)

WT1 was the first gene identified to postulate a genetic link between tumorigenesis and embryogenesis of the kidney (Huff, 1998). Thereafter, more genes necessary for renal development such as *PAX2*, have been identified, and are shown to play a role in the development of nephroblastoma (Dressler *et al.*, 1992).

Chapter 1.1

The pluripotent stem cell regulator SIX2 is activated in renal neoplasms

Introduction

The SIX (Sine oculis homeobox) family

Drosophila sine oculis (so), is an essential gene in the development of the visual system in the fly and shows homology to the mammalian Six family of homeobox transcription factors (Cheyette *et al.*, 1994; Oliver *et al.*, 1995; Roederer *et al.*, 2005; Serikaku *et al.*, 1994; Wawersik *et al.*, 2000). The Six family includes 6 (Six1-Six6) members in mammals, all containing a homeodomain and a conserved amino-terminal 110-115 amino acids long six domain (Boucher *et al.*, 1996; Boucher *et al.*, 2000; Gallardo *et al.*, 1999; Granadino *et al.*, 1999; Kawakami *et al.*, 1996; Oliver *et al.*, 1995) (Figure 8).

Six domains are important for the protein-protein interactions and conserved regions are required for DNA binding activity (Kawakami *et al.*, 1996; Ohto *et al.*, 1999; Pignoni *et al.*, 1997).

The Six family is spread across different chromosomes. Human *SIX1*, *SIX4* and *SIX6* are located in chromosome 14. Though they are on the same chromosome, *SIX1* and *SIX4* lie on one strand and *SIX6* on the opposite strand. *SIX2* and *SIX3* are located on the 2nd chromosome opposite to each other, *SIX5* in chromosome 19 (Boucher *et al.*, 1996; Boucher *et al.*, 2000; Gallardo *et al.*, 1999; Granadino *et al.*, 1999; Kawakami *et al.*, 2000).

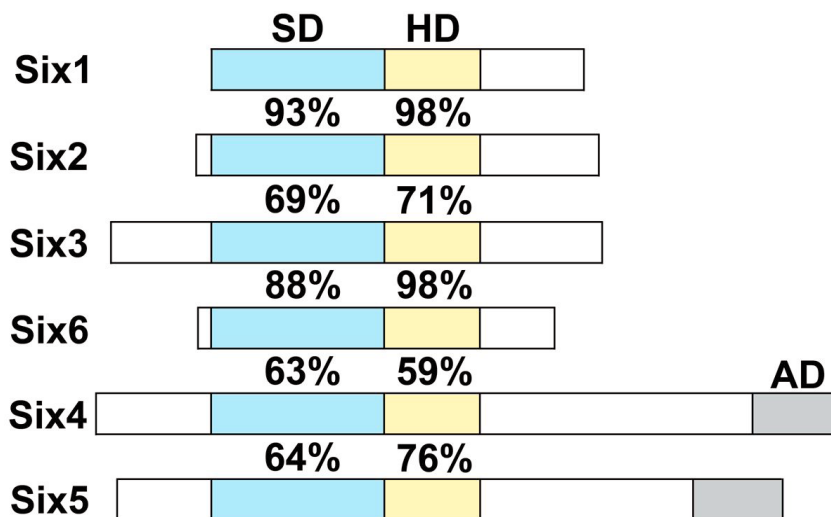


Figure 8: Structure and sequence homology of the Six family proteins. Blue colour indicates the amino acid homology of the Six domains (SD), homeodomains by yellow colour (HD) and gray colour indicates the activation domain (AD). Region homology is shown as percentages compared to Six1. (Kawakami *et al.*, 2000)

SIX2

Six2 is expressed in the nephric code, mesonephroic mesenchyme and neuroectoderm at stages E 10.5 and later at E 11.5 in metanephric mesenchyme, in limb buds of mice and mapped in chromosome 17 (Oliver *et al.*, 1995). Chromosome 2p15-p16 has been identified as the chromosomal location for *SIX2* in humans (Boucher *et al.*, 2000).

This gene plays a vital role during renal development for maintenance of pluripotent progenitor cells (Kobayashi *et al.*, 2008), which morphologically resemble blastema in nephroblastomas. *Six2* suppresses the signals for epithelial differentiation and maintains blastemal cells in an undifferentiated state (renal progenitor cells) (Self *et al.*, 2006). This feature is crucial for supporting the self-renewing potential of multipotent progenitor renal cells, as it disappears rapidly in *Six2* knockout mice (Kobayashi *et al.*, 2008) showing an increase in apoptosis (Self *et al.*, 2006). *Six2* deficiency leads to renal hypoplasia and dysplasia (Reidy *et al.*, 2009a) and reduced number of nephrons in animal models (Fogelgren *et al.*, 2009; Self *et al.*, 2006). An abnormal development of renal parenchyma in humans leads to multicystic renal dysplasia, which microscopically resembles some areas of nephroblastoma. Primitive ducts, that can also become cystic, are lined by undifferentiated epithelium and surrounded by embryonal mesenchyme (Bernstein, Risdon 1997). The role of *SIX2* in human disease has hardly been investigated. In a small pedigree of patients mutations of *SIX2* have been described as the cause of renal hypodysplasia (Weber *et al.*, 2008). Additionally, hypomethylation of the promotor region of *SIX2* was demonstrated in small number of primary nephroblastomas and foetal kidneys (Metsuyanin *et al.*, 2008).

Objectives

- To investigate a possible role for *SIX2* in the pathogenesis of a variety of renal neoplasms, ranging from nephroblastoma to clear cell and papillary carcinoma of the kidney and developmental abnormalities of the kidney.
- To ascertain the mode of activation of *SIX2* in nephroblastomas and consequences of *SIX2* activation in a cell culture model.

Materials and Methods

Materials

Formalin-fixed, paraffin-embedded (FFPE) tissue samples of nephroblastomas, renal clear cell carcinomas, renal papillary carcinomas, multicystic dysplastic kidneys and were included in these studies. Nephroblastomas were received from the Kiel Paediatric Tumour Registry, Department of Paediatric Pathology, University of Kiel, Germany and had been classified according to the current paediatric renal tumour classification system (Vujanic *et al.*, 2002). Cell culture experiments were carried out with WT-CLS1, a commercially available epithelial nephroblastoma cell line (Cell lines services, Heidelberg, Germany).

Quantitative Real-time polymerase chain reaction (qRT-PCR)

FFPE samples (3µm sections) were deparaffinised and stained with Mayer's hemalaun then manually dissected under a microscope (Guertl *et al.*, 2003). Total RNA was isolated using the RNAeasy FFPE kit (Qiagen, Hilden, Germany). Up to 500 ng of total RNA was reverse transcribed into cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). qRT-PCR was performed using primers specific for *SIX2*, *EYA1*, *HOXA11* (homeobox A11), *HOXC11* (homeobox C11) *HOXD11* (homeobox D11), *WNT9B* (wingless-type MMTV integration site family, member 9B), *18s* RNA, *E-Cadherin* (cadherin 1), *ZEB-2* (zinc finger E-box binding homeobox 2) and *SLUG* (snail homolog 2) (Table 1). QRT-PCR was performed on a 7900T fast-realtime-PCR-system (Applied Biosystems) using SYBR green as the detection fluorophore and reactions were carried out in triplicate. (PCR conditions Table 2). *18s* RNA was included as an internal control. Relative gene expression levels were calculated as delta delta CT.

Immunohistochemistry

Tissue sections (3-4µm) were deparaffinised. Antigens were unmasked by sodium citrate (pH6) treatment for 40 minutes at 20 Pascal in a pressure cooker (Dako, Glostrup, Denmark). Endogenous peroxidase was blocked with 3% H₂O₂ (Merck, Darmstadt, Germany) for 30 minutes at room temperature. *SIX2* antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were prepared in a 1:100 dilution in antibody diluent (Dako). Samples were incubated for 2 and half hours at room temperature followed by 15 minutes washing steps (3 times). After room temperature incubation with the

biotinylated secondary antibody (Dako) for 30 minutes, streptavidin-conjugated horseradish peroxidase (Dako) was added and incubated for a further 30 minutes at room temperature. DAB (3,3'-diaminobenzidine) chromogen substrate diluted 1:50 (Dako) was added (detection system) and incubated for a minimum of 5 minutes. Colour development was monitored under a microscope. Sections were counterstained with Mayer's hemalaun for 2 minutes, and finally mounted in Entellan (Merck).

Cell culture

Commercially available epithelial nephroblastomas cell line WT-CLS1 cells (Cell lines services) were cultured and maintained using Isocoves medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% penicillin and streptomycin (PAA, Pasching, Austria) and 2mM L-Glutamine (PAA) 15% foetal bovine serum (PAA); Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Transient transfections

Cells were grown to 70% confluence in 6 well or 24 well plates and covered with 1ml of fresh serum-free medium. Transfections were carried out using the plasmid pcMCV6-XL5, which contained the complete coding sequence of *SIX2* (Origene, Rockville, MD, USA). The plasmid pMAxFP-green-N containing a GFP (green-fluorescent-protein)-tag (Amara, Cologne, Germany) used as control. 140 ng/cm² of *SIX2* plasmid and 75ng/cm² of GFP plasmid were suspended in 0.6µl/cm² Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I reduced-serum medium (Invitrogen) to a final volume of 10µl/cm². Transfection solution was incubated at 37°C for 30 minutes and was added to the medium covering the cells. After 2 hours, 1 ml of medium containing serum was added. Cells were incubated for 24 hours and medium was changed.

Bromodeoxyuridine (BrdU) staining

1x10⁶ cells were harvested after 48 hours transfection and labelled with Bromodeoxyuridine (BrdU) for 1 hour. Subsequently, cells were stained with APC-conjugated anti-BrdU antibody and 7-amino-actinomycin D (7-AAD) following the manufacturer's instructions (APC BrdU Flow Kit, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The samples were analysed on a FACS LSR11 (Becton Dickinson) and FCS Express Software version 3 (DE Novo Software, Los Angeles, CA).

The analysis was performed by Ms. Heike Knausz and Dr. Strohmaier at the ZMF core facility, Medical University of Graz.

Invitro Scratch Assay

Scratches were made perpendicular to each other on a 100% confluent monolayer of cells in 24 well plates transfected with the vector containing the *SIX2* sequence or with the GFP control vector. Debris was removed with a washing step with PBS, and cells were covered with medium. Migration of cells was monitored using the live cell observer system (Carl Zeiss MicroImaging GmbH, Jena, Germany) every 2 hours for 24 hours. Distance differences between the initial and final gaps of the images were calculated with the AxioVision Rel. 4.5 software (Carl Zeiss MicroImaging GmbH).

Statistical analysis

All data were analysed using Graphpad Prism software V5 (Graphpad Inc., San Diego, CA, USA). Non-paired analysis of different mRNA expression of *SIX2* between epithelial structures and blastemal areas were analysed using the Mann-Whitney U test. Percentage of cells in the S phase and migration distance of cells overexpressing *SIX2* and the GFP control vector were compared with the continuous variables analysis method Student's t test. A two tailed P value of <0.05 was considered as significant (Balaguer *et al.*, 2010).

Results

SIX2 is expressed in blastemal regions of nephroblastomas

4 epithelial-type tumours, 4 blastema-type, 16 mixed nephroblastomas and 10 regressive nephroblastomas were included in the study (34 nephroblastomas).

Minimal viable tissue was observed in 2 regressive nephroblastomas. The remaining ones showed one or more different morphological areas. In summary, 7 out of 10 regressive nephroblastomas showed tubular structures. In addition, one was observed to have immature glomeruloid bodies, while another one had a blastemal area. 2 regressive tumours displayed viable structures of all 3 components. The 4 epithelial-type tumours showed tubular structures, 3 of them also displayed immature glomeruloid bodies. All of the mixed tumours contained tubular structures, and 7 of them also immature glomeruloid bodies in the epithelial regions.

Protein expression of SIX2 was evaluated in 3 epithelial-type nephroblastomas, 3 blastema-type tumours, 16 mixed and 10 regressive nephroblastomas by immunohistochemistry. Regardless of the subtype of the tumour, distinct nuclear positivity was detected in all blastemal regions. Epithelial, mesenchymal regions and more specialized structures, such as immature glomerular bodies, were completely negative throughout all the studied tumours (Figure 9).

mRNA (messenger RNA) expression of *SIX2* was studied in 10 nephroblastomas including 3 epithelial-type nephroblastomas and 3 blastema-type tumours and 4 mixed type tumours. Epithelial and blastemal areas were dissected resulting in blastemal and epithelial areas from 2 mixed nephroblastomas and 1 blastemal and 1 epithelial area from each of the remaining 2 tumours. *SIX2* had a significantly higher mRNA expression in the blastema of blastema-type and mixed nephroblastomas compared to that of the epithelial structures in epithelial-type and mixed tumors (Figure 10) ($P < 0.01$).

2 epithelial-type tumours, 2 blastema-type tumours and 4 mixed nephroblastomas were investigated for both RNA and protein expression of *SIX2* in parallel. In all cases, statistically significant increases in mRNA levels from the blastemal areas compared to epithelial and mesenchymal structures reflected the presence of nuclear protein regardless of the nephroblastoma subtype.

Clear cell carcinomas expressed SIX2 protein

Protein expression of 49 renal clear cell carcinomas and 19 papillary renal carcinomas were studied. 47 clear cell carcinomas showed distinct nuclear positivity (Figure 11A). 3 of these tumours contained areas with large tumour cells. Those cells were negative for SIX2.

2 clear cell carcinomas were completely negative. All papillary cell carcinomas were entirely negative (Figure 11B)

Multicystic dysplastic kidneys were completely negative for SIX2

11 samples of multicystic dysplastic kidneys were analysed. The dysplastic areas ranged from focal replacement of kidney parenchyma to entire multicystic dysplastic kidneys. All samples had the characteristic morphology of immature tubules and surrounding embryonal mesenchyme. All Multicystic kidneys were completely negative for *SIX2* (Figure 11C).

Expression of upstream genes of SIX2 in nephroblastomas

EYA1, *HOXA11*, *HOXC11*, *HOXD11*, *WNT9B* and *WNT11* genes have been described as important upstream activators of *SIX2* (Carroll *et al.*, 2005; Gong *et al.*, 2007; Karner *et al.*, 2011; Nishinakamura, 2008), we investigated their mRNA expression by qRT-PCR in the same nephroblastoma samples examined for *SIX2* mRNA expression.

In all three epithelial-type nephroblastomas the regulatory upstream genes of *SIX2*, including *EYA1*, *HOXA11*, *HOXC11*, *HOXD11*, *WNT9B* and *WNT11*, showed a moderately low expression compared to most of the blastemal areas. In agreement with these results all epithelial areas of mixed nephroblastomas had a lower expression compared to blastema of the same tumours of the genes listed above with one exception: *in one mixed tumour the expression of WNT11 in epithelial and blastemal areas did not differ distinctly.*

Two blastemal areas of mixed nephroblastomas showed a high expression of *EYA1*, *HOXC11* and *HOXD11*. One of them also had a high expression of *HOXA11*, *WNT9B* and *WNT11*, whereas the second showed comparatively low expression of these genes. In contrast, one blastemal area of a mixed nephroblastoma had a low expression of all upstream transcription factors in comparison to all the other tumours investigated.

Interestingly, high mRNA expression levels of *EYA1* and *HOXC11* were demonstrated in all 3 blastema-type nephroblastomas studied. Two of them also had a high expression of *HOXA11* and *WNT11*, whereas the third only showed a low level of expression of these

genes. One blastema-type tumour showed very high expression levels of *HOXD11* and *WNT9B*, in addition to the above mentioned genes, whereas the other two samples displayed only a low expression level of these transcription factors (Figure 12).

Overexpression of SIX2 increases cellular proliferation and migration.

The hypothesised main role of SIX2 in embryonal nephrogenesis is the maintenance of cells in an undifferentiated state. Therefore, we investigated whether a high level of SIX2 would affect the cell cycle or the characteristics of migration in a cell culture model.

Flow cytometry analysis was carried out to compare the percentage of cells in the S-phase of the cell cycle, when cells were transfected with *SIX2* (Figure 13B) versus control cells (Figure 13A) by staining with Brdu and 7-AAD. 15.39% of the control cells were identified in the S phase, in contrast to 18.66 % of cells expressing SIX2 (Figure 13C). The difference of 3.27% in the mean percentage of cells in S-phase was statistically significant ($P < 0.01$). However a phenotypic alteration of the cells was not identified.

The effects of SIX2 overexpression on cell migration was analysed by a scratch assay. Perpendicular scratches were made on a 100% confluent monolayer of cells transfected either with the vector containing *SIX2* or the GFP control vector. Migration of cells was observed with a cell observer system. The initial gap for the control was 522µm (Figure 13E) with a migrating speed of 34µm/h (Figure 13D), and was reduced to 260µm after 10 hours (Figure 13G). The scratch of the cells overexpressing SIX2 had an initial gap of 464µm (Figure 13F). In contrast to the control cells the gap was completely closed after 10 hours (Figure 13H) with a migrating speed of 39.4µm/h (figure 13D). The difference of the mean speed of migration was statistically significant ($P < 0.05$).

***SIX2* is affecting the mRNA expression of genes involve in epithelial mesenchymal transition**

mRNA expression of E-cadherin was down-regulated in cells expressing *SIX2* compared to control. However, *ZEB-2* and *SLUG* transcription factors show a comparatively high mRNA expression in *SIX2* overexpressing cells compared to control (Figure 14). Morphological changes were not identified.

Table 1: Primer sequences for QRT PCR

Gene	Forward primer	Reverse primer
<i>SIX2</i>	5'ACACAGGTCAGCAACTGGTTC3'	5'AGCTGCCTAACACCGACTTG3'
<i>EYA1</i>	5'CCCATCCAGGAGCAAACTA3'	5'TGCAACTTGAGGAAACAGCA3'
<i>HOXA11</i>	5'CGGCAGCAGAGGAGAAAG3'	5'GTATAGGGGCAGCGCTTTT3'
<i>HOXC11</i>	5'AACACAAATCCCAGCTCGTC3'	5'TGAAGAAAACTCTCGCTCCA3'
<i>HOXD11</i>	5'GGCCGAGAAGAGCAGCAG3'	5'AAAGAAAACTCGCGTTCCA3'
<i>WNT9B</i>	5'GCGAGGAGATGCTAGAGG3'	5'GGAAGGGCGTCAGGACTT3'
<i>WNT11</i>	5'AGACCGGCGTGTGCTATG3'	5'CACCTGTGCAGACACCAGAC3'
<i>18S</i>	5'GTAACCCGTTGAACCCCAT3'	5'CCATCCAATCGGTAGTAGCG3'
<i>E-Cadherin</i>	5'TGGACAGGGAGGATTTTGAG 3'	5' GTGTCCCTGTTCCAGTAGCAA 3'
<i>ZEB-2</i>	5'CCAATCCCAGGAGGAAAAAC3'	5'GTTGGCAATACCGTCATCCT3'
<i>SLUG</i>	5'TTCGGACCCACACATTACCT3'	5'GCAGTGAGGGCAAGAAAAAG3'

Table 2: PCR conditions

Stage 1	50 ⁰ C ; 2min
Stage 2	95 ⁰ C ; 10min
Stage 3	95 ⁰ C ; 15sec; 60 ⁰ C 1min 45 cycles
Dissociation stage	95 ⁰ C ; 15sec 60 ⁰ C; 15sec 95 ⁰ C ; 15sec

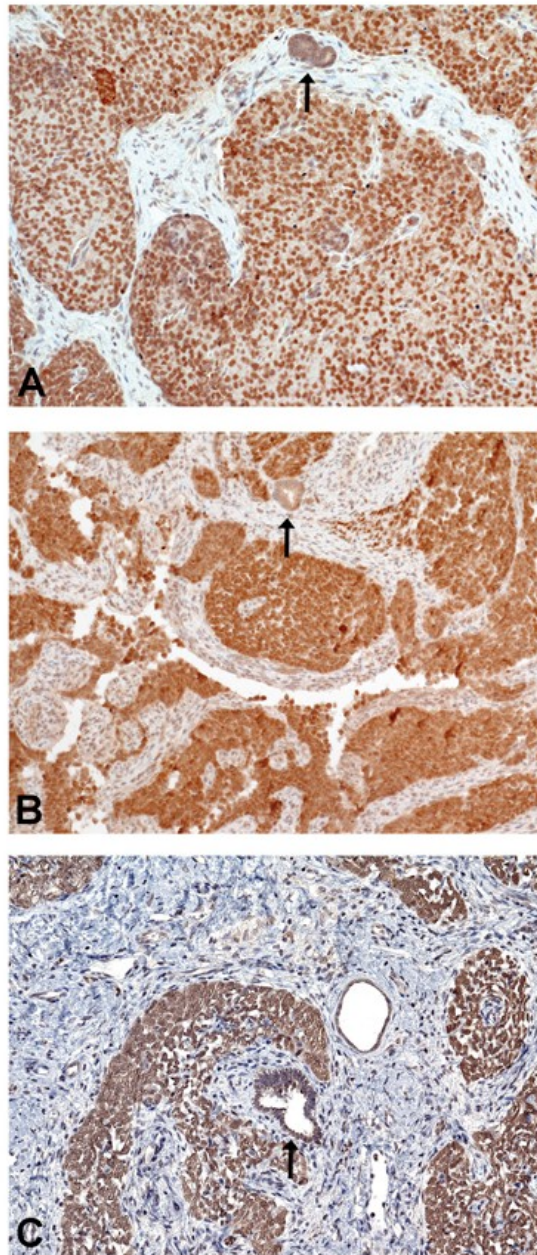


Figure 9:

SIX2 expression was assessed in 32 nephroblastomas by immunohistochemistry. All blastemal areas (A, B, C, mixed nephroblastomas 200x) had a distinct nuclear positivity for SIX2 whereas epithelial structures were completely negative (indicated by arrows).

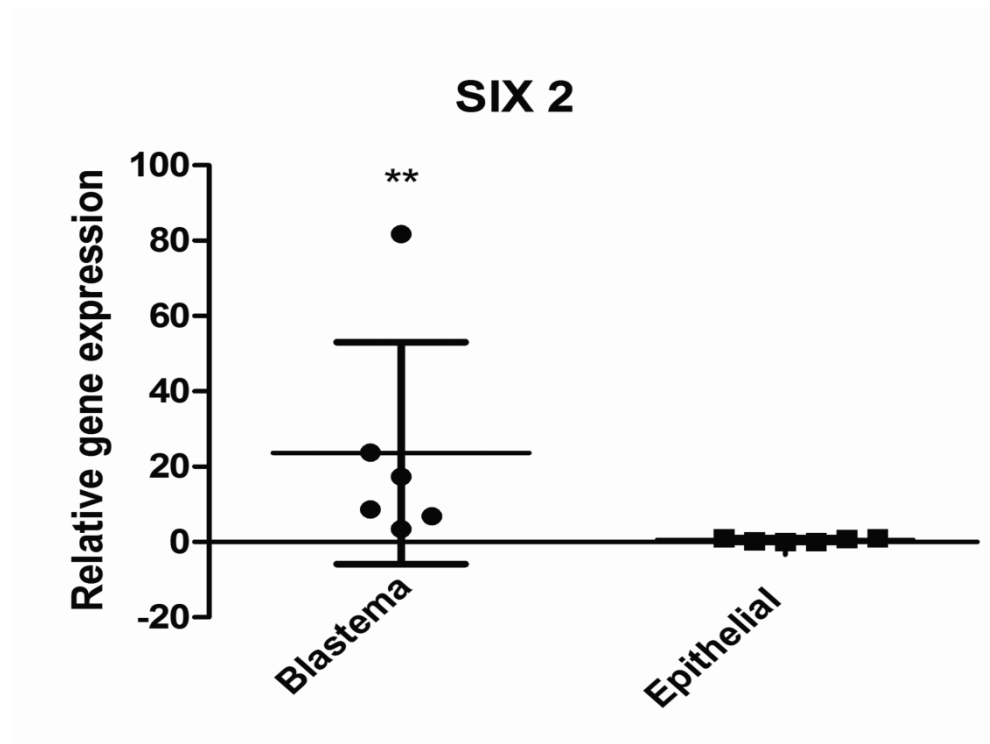


Figure 10:

Relative *SIX2* mRNA expression was measured in blastema (●) and epithelial structures (■) of nephroblastomas by qRT-PCR. Blastema showed statistically significantly higher expression of *SIX2* compared to epithelial structures regardless of the nephroblastoma subtype. Data compared with initial values using unpaired t-test followed by Mann Whitney test. Vertical bars represent +/- S.D. from the mean value (n=6, ** $p < 0.01$).

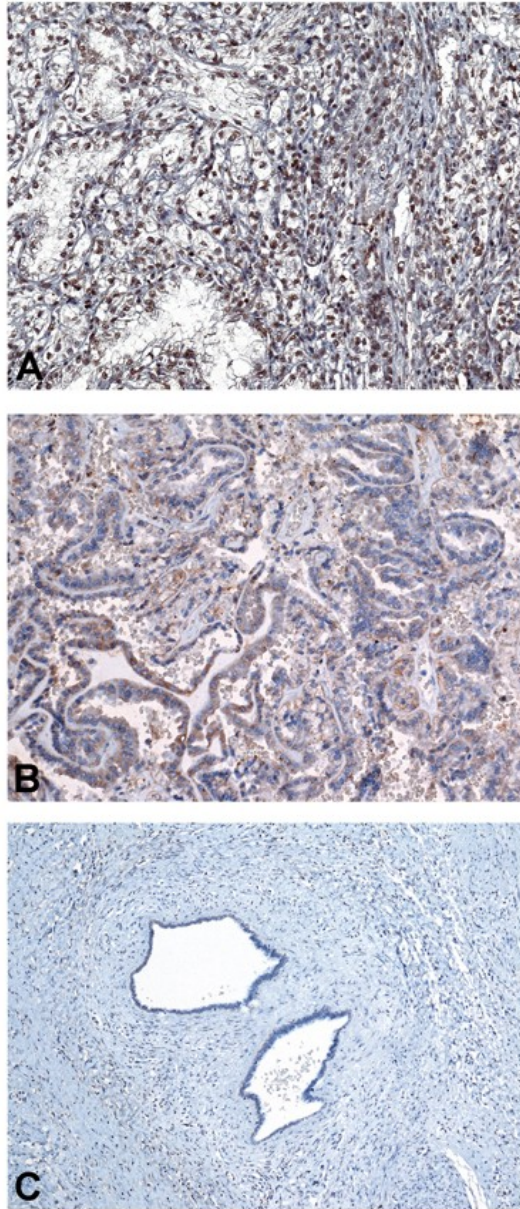
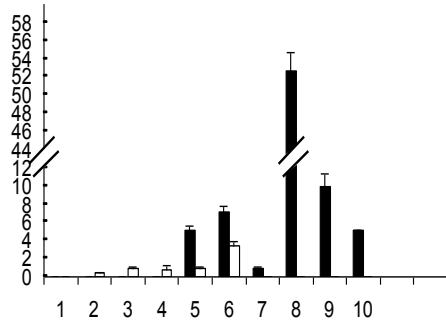


Figure 11:

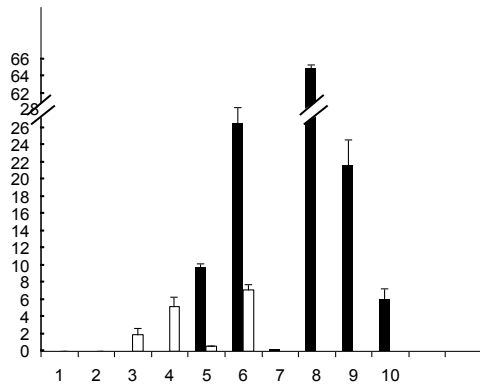
SIX2 protein expression was assessed in 49 clear cell carcinomas and 19 papillary cell carcinomas and 11 multicystic dysplastic kidneys, by immunohistochemistry. 47 clear cell carcinomas (A, 200x) showed distinct nuclear positivity for SIX2. Whereas papillary cell carcinomas (B, 200x) and multicystic dysplastic kidneys (C, 100x) were completely negative.

EYA 1



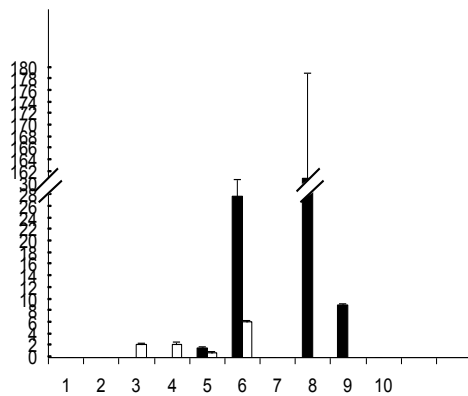
A

HOXC11



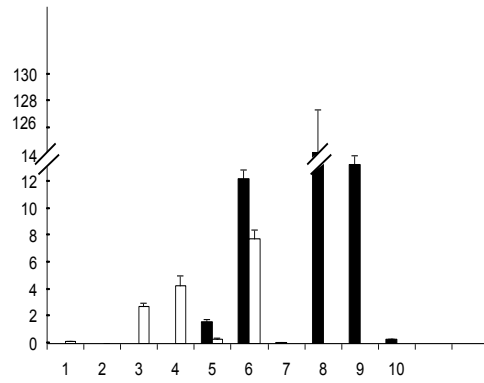
C

WNT9B



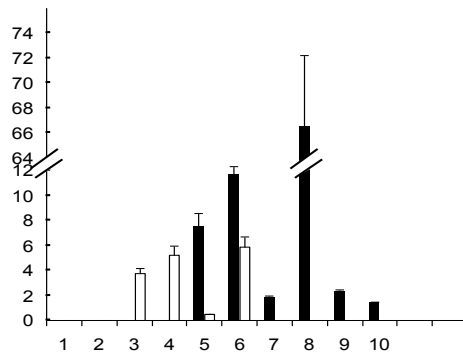
E

HOXA11



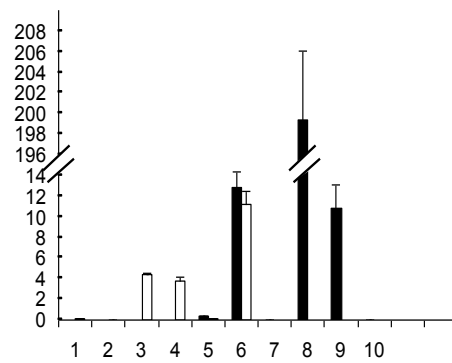
B

HOXD11



D

WNT11



F

Figure 12:

mRNA expression of *EYAI* (A), *HOXA11* (B), *HOXC11* (C), *HOXD11* (D), , *WNT9B* (E) and *WNT11* (F) genes was analysed in 3 epithelial-type tumours (1-3), one epithelial area of a mixed tumour (4), both epithelial and blastemal areas of 2 mixed tumours (5-6), 1 blastemal area of a mixed tumour (7) and 3 blastema-type tumours (8-10). The relative mRNA expression of blastemal areas was calculated in comparison to epithelial structures. The regulatory upstream genes showed comparatively low expression in all three epithelial-type tumours. Most of the upstream genes were upregulated in blastemal areas compared to epithelial structures of mixed nephroblastomas. However, one blastemal area of a mixed nephroblastoma also showed a very low expression of all the upstream genes. The highest mRNA expression values for all the upstream genes were observed in one blastema-type tumour. The X-axis indicates the case numbers and the Y-axis shows the relative gene expression. Vertical bars represent +S.D from the mean value of technical replicates (blastema ■, epithelial structures □, Y-axis broken by ).

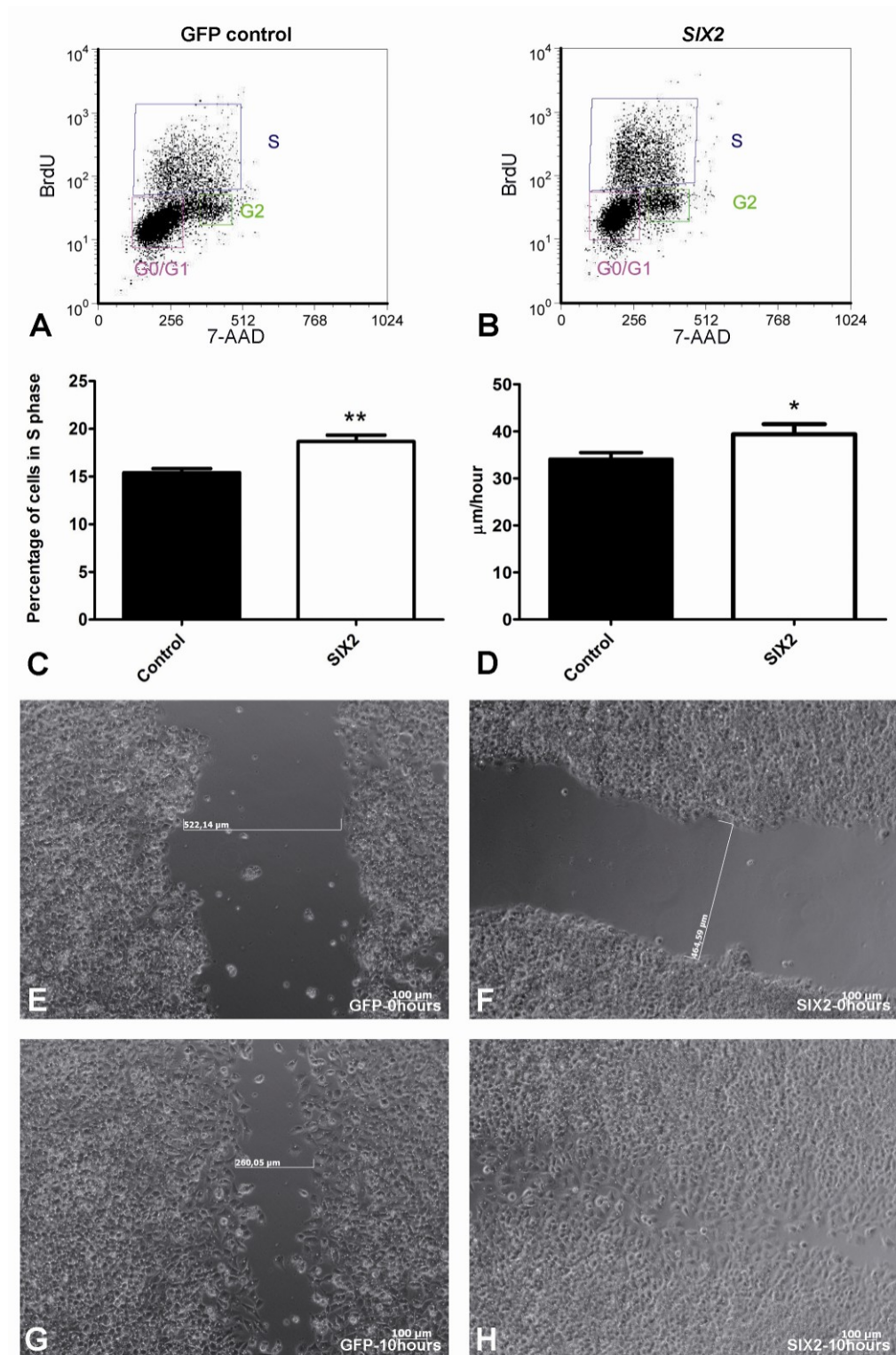


Figure 13:

The percentage of BrdU positive cells in *SIX2* (A) and GFP control (B) overexpressing cells compared by flow cytometry. Cells overexpressing *SIX2* (C) revealed a mean percentage (18.66%) of cells in the S-phase when compared with cells overexpressing a GFP control (15.39%), with a statistically significant degree of difference of 3.27% (n=6).

Control cells showed an initial gap of 522.14 μ m (E) and 260.05 μ m (G) after 10 hours. The scratch of the cells overexpressing SIX2 had an initial gap of 464.59 μ m (F) with a complete closure after 10 hours (H). The difference of the mean distance of migration is statistically significant (D) (n=16), (* p <0.05, ** p <0.01, error bars indicate + SEM).

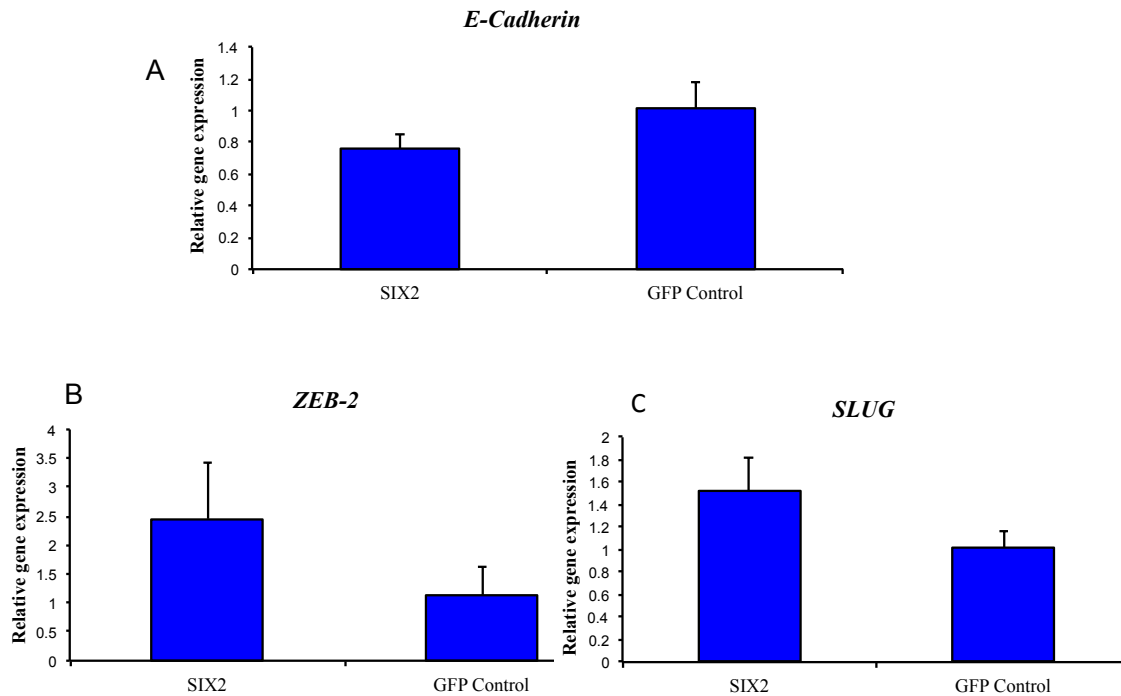


Figure 14:

Relative *E-Cadherin* (A), *ZEB-2* (B), *SLUG* (C) mRNA expression was measured in *SIX2* and GFP control overexpressing WT-CLS1 cells. *SIX2* expressing cells have low *E-Cadherin* expression and high *ZEB-2* and *SLUG* expression compared to control (n=3, error bars indicate + SD).

Discussion

Nephroblastomas are monoclonal regardless of the specific subtype (Guertl *et al.*, 2006). However, molecular background of these tumours is very heterogeneous. Literature provides multiple evidence for different genetic changes in subtypes of nephroblastomas. Stroma-type nephroblastomas with ectopic mesenchymal elements such as myogenic differentiation and cartilage show activation of the *WNT* pathway with nuclear translocation of *B-CATENIN* (Fukuzawa *et al.*, 2009). A very recent study showed that the epithelial-type nephroblastomas were most likely to be associated with mutations of *FBXW7* (F-box and WD repeat domain containing 7) (Williams *et al.*, 2010). Nephroblastomas consisting exclusively of differentiated epithelial structures lacked loss of heterozygosity on chromosomes 1p, 16q and 11p and demonstrated a gene expression pattern with arrest during the mesenchymal-to-epithelial transition in a microarray analysis (Sredni *et al.*, 2009).

Only a limited number of studies attempted to define genetic differences between morphologically different areas of nephroblastomas. Microarray analysis demonstrated an overrepresentation of genes involved in *WNT* signalling pathway in blastema (Maschietto *et al.*, 2008). In another study, immunohistochemistry of 2 nephroblastomas showed positivity for *SIX1* only in blastemal areas of the tumours (Li *et al.*, 2002). Recent study described *SIX1* as a marker for blastemal regions of nephroblastomas (Sehic *et al.*, 2012). These data indicate activation of different genetic pathways in morphologically different regions of nephroblastomas.

Our investigation focussed on *SIX2* expression in various subtypes of nephroblastomas and different regions within the same mixed nephroblastomas. In our series all samples regardless of the subtype of nephroblastomas showed a significant overexpression of mRNA and protein levels of *SIX2* in blastema. Our findings are in agreement with two published studies. Microarray analysis revealing a decreased level of *SIX2* in epithelial tubular structures in nephroblastomas compared to all subgroups (Sredni *et al.*, 2009) and strong signal for *SIX2* protein was identified in the blastemal compartments of tumour sections by immunofluorescence (Aiden *et al.*, 2010). Another study identified *SIX2* mRNA and protein expression remains active and quantitatively similar across all stages of nephroblastomas (Murphy *et al.*, 2012).

A cell culture model of triphasic nephroblastomas demonstrated different pattern of *SIX2* expression levels in adherent cells and sphere-like clusters (Pode-Shakked *et al.*, 2009).

These differences in expression levels might be caused by distinct morphological elements in triphasic nephroblastomas forming different types of clusters, as our results show distinct expression of *SIX2* in blastemal regions and complete negativity in epithelial elements.

The persistence of large amount of blastema after post chemotherapy in nephroblastomas might be one of the most important prognostic parameters indicating a worse prognosis (Weirich *et al.*, 2001). Therefore, identification of blastema in tumour specimens will become more important to predict the prognosis of nephroblastomas. Quantification and identification of blastema especially in mixed nephroblastomas might be possible in the future by assessing *SIX2* expression.

In vitro cell culture studies and transgenic mice demonstrated direct regulation of *Six2* by a protein complex containing *Hox11*, *Pax2* and *Eya1* during renal development (Carroll *et al.*, 2005; Gong *et al.*, 2007; Nishinakamura, 2008; Reidy *et al.*, 2009a). A recent study has shown a high expression of *EYA1* in nephroblastomas (Drake *et al.*, 2009; Li *et al.*, 2002). Wnt9b helps to maintain *Six2*-positive cells as renal progenitors whereas cells with reduced levels of *Six2* are induced to differentiate by Wnt9b (Karner *et al.*, 2011). Using these simple mechanisms, the kidney is able to balance progenitor cell expansion and differentiation insuring proper nephron endowment. We examined the expression of the upstream signals of *SIX2* in more detail to determine whether *SIX2* is upregulated alone or the whole signal transduction pathway is activated. Upstream signals of *SIX2* showed an increased expression in most blastemal areas of mixed and in most blastema-type tumours. All epithelial areas of mixed and all epithelial-type nephroblastomas had a low expression of transcription factors of upstream genes.

Our results demonstrate that *SIX2* is upregulated in blastema of large number of cases by activation of a signal transduction pathway although in some morphologically not discernable cases not all or none of the upstream genes are activated. These contradictory results are in accordance with the previous literature and heterogeneous nature of nephroblastomas.

Epigenetic regulatory mechanisms might play a role in regulation of *SIX2* in blastemal and epithelial regions of nephroblastomas. K4me3 (trimethylated lysine 4 of histone 3) is a marker of transcriptional initiation (Li *et al.*, 2007) and it is the largest domain that coincides with master regulators such as *OCT4* and *SOX2* in pluripotency (Mikkelsen *et al.*, 2007). One of the largest K4me3 domain overlaps with *SIX2* promoter in nephroblastomas, and additionally they have identified a strong *SIX2* protein expression in

blastemal regions (Aiden *et al.*, 2010). K27me3 (trimethylated lysine 27 of histone 3) is a repressive marker enriched in promoters of genes expressed in epithelial structures of mature kidney (Aiden *et al.*, 2010) and this modification is catalysed by polycomb repressor complex 1 (Sparmann *et al.*, 2006). Epigenetic role of polycomb repressor genes has been identified in primary nephroblastomas and nephroblastoma xenografts. Primary nephroblastomas and foetal kidneys demonstrated hypomethylation of *SIX2* promoter; progressive nephroblastoma xenografts showed hypermethylation of the promoter with an upregulation of polycomb repressor genes and downregulation of *SIX2* (Metsuyanin *et al.*, 2008). The Bivalent promoters are described as promoters simultaneously enriched for both K4me3 (active) and K27me3 (repressive), correlating to genes that are poised within a given cell population and these genes can be activated or silenced. Aforementioned histone modifications are markers of important developmental genes in embryonic stem cells (Azucara *et al.*, 2006; Bernstein *et al.*, 2006). Therefore, *SIX2* might have a bivalent promoter regulated by K4me3 and K27me3 histone modifications in blastemal and epithelial regions of nephroblastomas.

An upregulation of *PAX2* has been identified in blastema and some epithelial structures by in-situ hybridization (Eccles *et al.*, 1995) and in the epithelial component by Western blot and immunohistochemistry (Dressler *et al.*, 1992). It has been suggested that *PAX2* expression in nephroblastomas is rather a developmental phenomenon than an oncogenic activation (Dressler, 1996). Our results indicate that overexpression of *SIX2* seems to indicate reactivation of an embryonal signal transduction pathway leading to tumorigenesis in renal neoplasms.

We also investigated whether overexpression of *SIX2* in blastema indicates only a recapitulation of a differentiation process. Multicystic dysplasia of the kidney is benign malformation of kidney. Dysplastic renal tissue morphologically resembles some areas of nephroblastomas (Bernstein, Risdon 1997). *PAX2*, known to be an upstream activator of *SIX2* (Gong *et al.*, 2007) and is strongly expressed in dysplastic epithelia (Guertl *et al.*, 2011; Winyard *et al.*, 1996; Woolf *et al.*, 2000). All samples of multicystic dysplasia included in this study were completely negative for *SIX2* expression. A further study with a microarray analysis of renal dysplasia showed overexpression of multiple genes such as *WT1*, but not *SIX2* (Jain *et al.*, 2007). In contrast to neoplastic nephroblastomas, non-neoplastic multicystic dysplastic kidneys showed no expression of *SIX2*, but despite this *PAX2* is nevertheless expressed in the dysplastic epithelium (Guertl *et al.*, 2011; Winyard *et al.*, 1996; Woolf *et al.*, 2000).

This pattern of *SIX2* expression indicates that overexpression of *SIX2* might play a role in oncogenesis. Therefore, we investigated the expression of *SIX2* in adult renal neoplasms such as clear cell carcinomas and papillary renal cell carcinomas. These different histological types of carcinomas demonstrate different clinical behaviour that might be attributed to different molecular pathogenesis. In renal clear cell carcinomas mutations of *VHL* (von Hippel-Lindau) gene and deletions of chromosome 3p are frequent, while papillary carcinomas are characterized by aberrations of chromosomes 7, 17 and Y (Moch *et al.*, 2002). Expression of PAX2 has been identified in both entities (Guertl *et al.*, 2011; Memeo *et al.*, 2007; Ozcan *et al.*, 2010). *SIX2* is activated by PAX2 during renal development (Carroll *et al.*, 2005; Gong *et al.*, 2007; Karner *et al.*, 2011; Nishinakamura, 2008; Reidy *et al.*, 2009a); *SIX2* is only expressed in the majority of clear cell carcinomas and all papillary cell carcinomas showed negative in our study. Interestingly, large tumour cells were negative in 3 clear cell carcinomas, and 2 more clear cell carcinoma samples did not show any expression of *SIX2*.

We examined the mechanism of *SIX2* in an *invitro* model using an epithelial nephroblastomas cell line. Overexpression of *SIX2* in a cell culture model led to a statistically significant increase of the percentage of cells in the S-phase compared to the control. The main function of *SIX2* during renal development is maintaining cells in an undifferentiated state of pluripotent stem cells (Self *et al.*, 2006). This result reflects the role of *SIX2* in pluripotent progenitor cells, as some types of stem cells are considered to have an ability to increase proliferation (da Silva *et al.*, 2009; White *et al.*, 2005).

Another important fact of stem cells is their ability for regeneration (Morrison *et al.*, 1997). *SIX2* induced a statistically significant increase in cell migration in the scratch assay. Similar observations have been identified for a new oncogene, *LIMI*. Silencing of *LIMI* decreased the motility of cells of a clear cell carcinoma cell line (Dormoy *et al.*, 2011). These features might be important in regeneration induced by stem cells and in migration and invasion of tumour cells. Another recent study demonstrated that expression of markers of differentiation with *SIX2* after a period of time led to increased mitosis (Brunskill *et al.*, 2011).

SIX1 overexpression contributed to repression of *E- Cadherin* expression and promote epithelial mesenchymal transition by activating of *ZEB-1* (zinc finger E-box binding homeobox 2) in colorectal cancer (Ono *et al.*, 2012). Epithelial mesenchymal transition and stem cell like features were also identified in *SIX1* overexpressing transgenic mouse model bearing malignant neoplasms similar to breast carcinoma (McCoy *et al.*, 2009). In

our study, we identified upregulation of *ZEB-2*, *SLUG* and down regulation of *E-cadherin* mRNA expression in *SIX2* expressing cells compared to control. Therefore, in our view *SIX2* may also contribute to epithelial mesenchymal transition process in nephroblastomas.

Conclusions

In our study, we show that *SIX2* is overexpressed in different renal neoplasms, including blastemal structures in nephroblastomas and the majority of clear cell carcinomas. Expression of *SIX2* can therefore help to identify even minimal residual blastema in nephroblastomas after chemotherapy. Cell culture experiments demonstrated that overexpression of *SIX2* increased percentage of cells in S phase and cellular and migration.

Chapter 1.2

miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target gene ACVR2B is strongly expressed in nephroblastomas

Introduction

MicroRNA (miRNA)

miRNA genes are located within exons or introns of protein non-coding or coding genes. The first miRNA was discovered in the nematode, *Caenorhabditis elegans*, in 1993 (Lee *et al.*, 1993). The non-coding small RNA was 22 nucleotides in length. These small transcripts were considered as anomalies until the year 2000. After the identification of the second miR-let-7 in 2001, these RNAs were termed miRNAs (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee *et al.*, 2001; Ruvkun, 2001). The biogenesis of miRNA occurs in the nucleus and cytoplasm of a cell. miRNAs are transcribed as pri-miRNA in the nucleus, processed in to pre-miRNA, and transported into the cytoplasm. The terminal loop of pre-miRNA is then cleaved by RNase III Dicer, forming a 'mature' miRNA (Schaefer *et al.*, 2010).

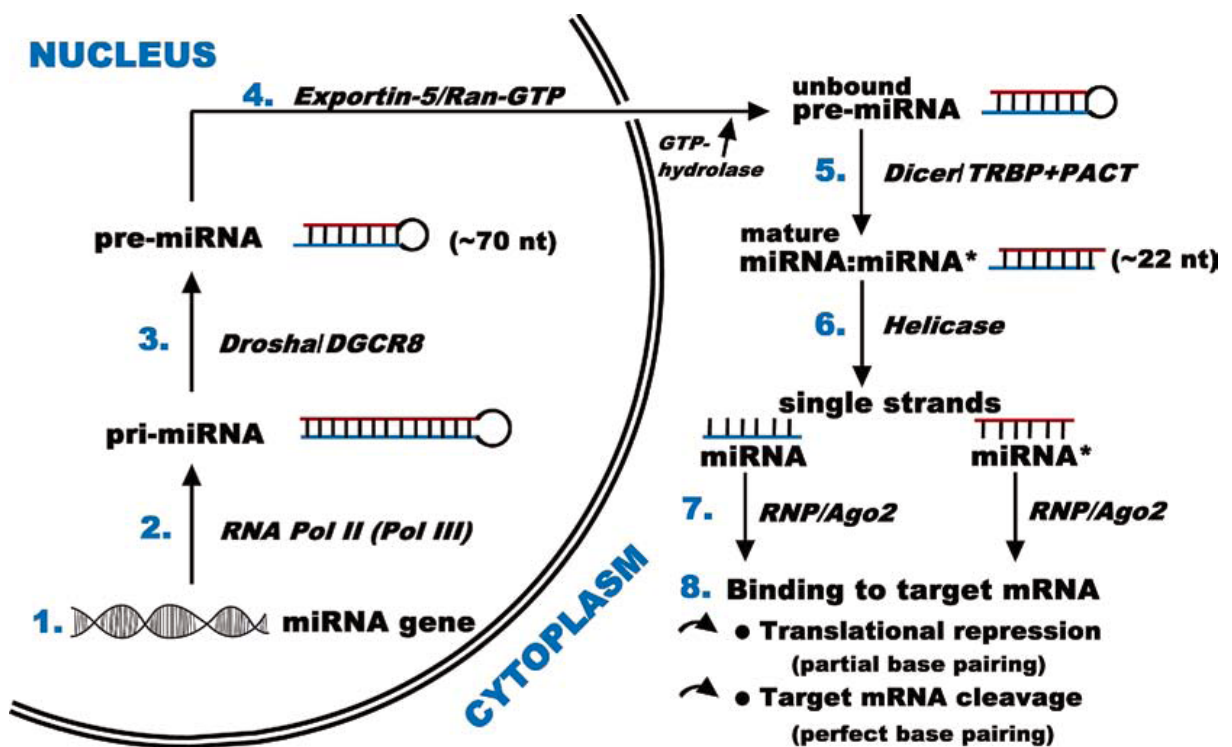


Figure 15: Biogenesis of miRNA (Schaefer *et al.*, 2010)

One strand of mature miRNA is incorporated in to ribonucleoprotein complex (miRNP), making the silencing complex miRISC. This miRISC complex then binds to the target site of mRNA, repressing mRNA translation and leading to mRNA decay and degradation.

Decapping can occur depending on the seed region of miRNA and 3' UTR of target region (Schaefer *et al.*, 2010) (Figure 15).

miRNA expression seems to play a role predominantly in later stages of renal development. In terminally differentiated epithelia multiple miRNAs can be detected. The miR-200 family has been implicated in the process of mesenchymal-epithelial transition during renal development (Wessely *et al.*, 2010). In an animal model, the enzyme responsible for maturation of miRNAs, Dicer, was specifically suppressed in podocytes. At three weeks the transgenic animals showed proteinuria and end-stage kidney disease (Harvey *et al.*, 2008). In an additional animal model with a Dicer deletion in podocytes, mmu-miR23b, mmu-miR24 and mmu-miR26b were identified as being responsible for glomerular filtration (Ho *et al.*, 2008).

Several miRNAs have been identified as being deregulated in nephroblastomas, many of them target genes that have known roles during renal development. In a small number of sporadic nephroblastomas chromosome 2q37.1 deletions associated with a decreased expression of miR-562 and its target gene EYA1 (Drake *et al.*, 2009). Overexpression of miR-483-3p was correlated with an increased level of IGF2 (Insulin growth factor 2) and this miRNA was shown to protect cells from apoptosis in *invitro* and *invivo* systems (Veronese *et al.*, 2010). MiR-185 identified as a tumour suppressor that repress Six1. Samples of ovarian cancers and nephroblastomas showed a significantly reduced expression of miR-185 (Imam *et al.*, 2010).

Analysis of miRNA expression patterns in various tissues demonstrated a distinct expression of miR-192, miR-194, miR-204, miR-215 and miR-216 in mature kidney (Sun *et al.*, 2004). A tissue profiling study identified an enrichment of a set of miRNAs in the kidney, including miR-10a/b, miR-200a, miR-196a/b and miR-335 (Saal *et al.*, 2009).

TGF beta pathway

The Transforming Growth Factor β (TGF- β) signalling pathway is important in several different cellular processes such as cell proliferation, recognition, differentiation, apoptosis and embryogenesis (Massague *et al.*, 2000; Ten Dijke *et al.*, 2002) (Figure 16).

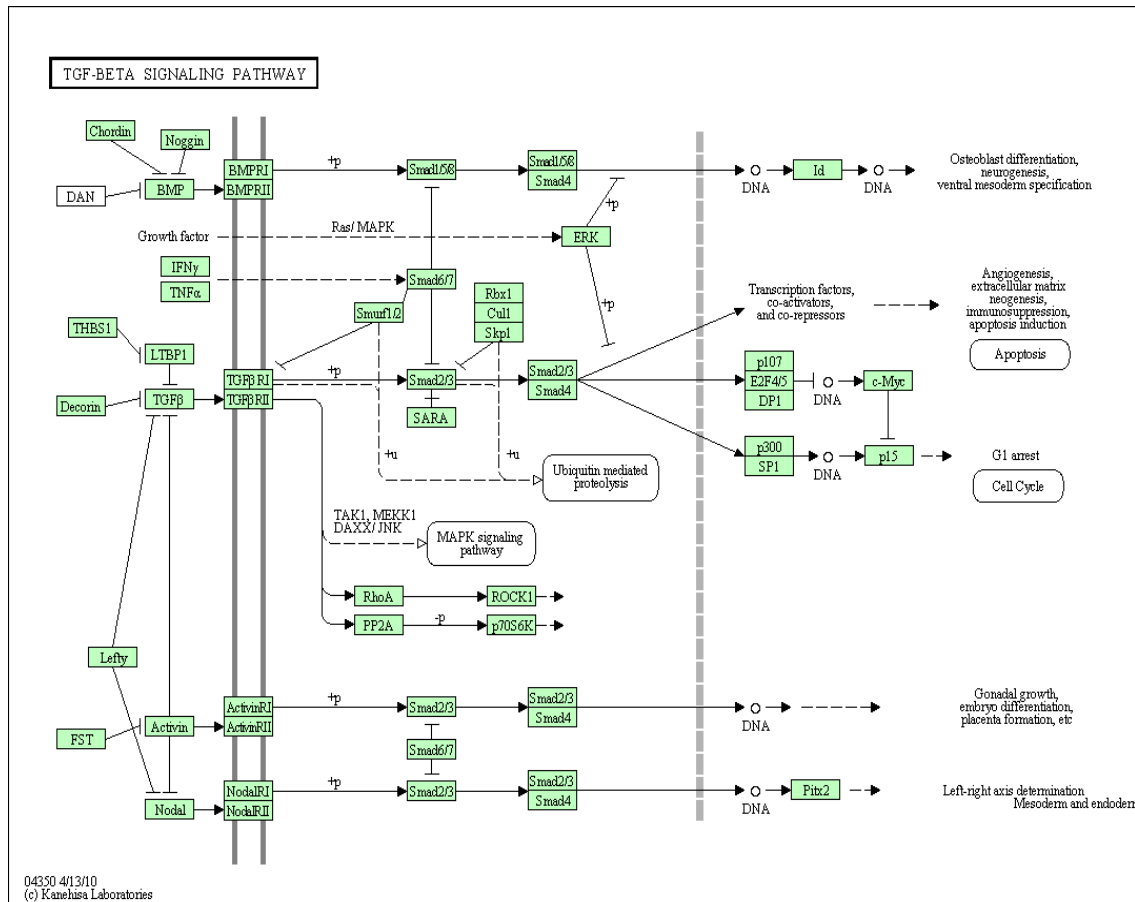


Figure 16: TGF beta pathway adopted from *KEGG*- Kyoto Encyclopedia of Genes and Genomes pathways programme (<http://www.genome.jp/kegg/pathway.html>).

The activin receptor type 2B (ACVR2B) receptor plays an important role in the activation of activin pathway, which is an important sub pathway of the (TGF)- β signaling pathway (Shi *et al.*, 2003). Members of the TGF- β superfamily are known to play a vital role during renal development. Various transcription factors regulate branching of the ureteric bud (Shah *et al.*, 2004), activin A is one of them. Dimerization of two different receptors, activin receptor type II and I can regulate the activity of activin and these two receptors are involve in TGF- β signalling pathway (Levy *et al.*, 2006; Ying *et al.*, 1997). The type II

receptor binds to activin and in turn phosphorylates and activates the type I receptor and this is followed by downstream activation of Smads (Levy *et al.*, 2006). mRNA expression of activin type II receptor was found in embryonal and adult kidneys of rats. ACVR2B protein expression was identified in the ureteric bud and metanephric mesenchyme at E13 and E16 (Maeshima *et al.*, 2003) and at 12, 13 and 18 weeks of gestation in human embryos. Human foetal kidney demonstrated predominant expression in the metanephric mesenchyme, S and comma shaped bodies, ureteric bud and parietal epithelium of foetal glomeruli but not in the stroma (Metsuyanin *et al.*, 2009). Activin type II receptors were identified in tubular cells of the proximal tubules, distal tubules and the collecting ducts in adult mature kidneys of rats (Maeshima *et al.*, 2001).

Hypothesis

Activation of the TGF- β pathway achieved by upregulation of the ACVR2B receptor via downregulation of miRNAs could play a role in the development of nephroblastomas. Therefore, we evaluated ACVR2B as a putative target gene of miR-192, miR-194, miR215, miR-200c and miR-141 and their expression in nephroblastoma.

Materials and Methods

Tissue samples

Formalin fixed paraffin embedded tissue samples were obtained from the Children's Tumour Registry at the Institute of Pathology, University of Kiel, Germany. 3µm sections were prepared for further analysis.

miRNA array

One microgram of pooled microdissected samples of blastemal, epithelial, NRs and mature renal tissues were used for the array. The array was performed by Exiqon (Exiqon, Vedbaek, Denmark). Samples were labeled using the miRCURY™ Hy3/Hy5 Power labeling kit (Exiqon) and hybridized on the miRCURY™ LNA Array (v.11.0 *hsa*, *mmu* and *rno*). Analysis of scanned slides showed that the labeling was successful; all capture probes for the control spike-in oligo nucleotides produced signals in the expected range. The quantified signals (background corrected) were normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

miRNA target prediction

Different DIANA lab web servers were used to predicted target genes and target regions. A single common target gene in the TGF β pathway for the analysed miRNAs was identified by the DIANA-mirPath programme. This programme is a tool to identify molecular pathways targeted by single or multiple miRNAs (Papadopoulos *et al.*, 2009). DIANA-mirPath programme based on DIANAmicro-T-4.0 (beta version) was used to recognise biological pathways targeted by miR-192, miR-194, miR-215, miR-141 and miR-200c (<http://diana.cslab.ece.ntua.gr/pathways/>). Computer mouse over 'option' was used to identify a common target gene for all 5 miRNAs in possible targeted biological pathways predicted by the programme (according to -ln p values).

The identification miR-192, miR-194, miR-215, miR-141 and miR-200c target sites in the 3'-UTR region of common target genes was performed by the DIANAmicro-T-3.0 programme (<http://Diana.cslab.ece.ntua.gr/gr/microT/>). miRNA target sites in the 3'UTR of the ACVR2B gene (Table 4) were identified by DIANA-microT 3.0 (Maragkakis *et al.*, 2009).

Cell lines

A commercially available, an epithelial nephroblastoma cell-line WT-CLS1 (Cell lines services, Heidelberg, Germany) were cultured in Isocoves medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 15% foetal bovine serum (PAA, Pasching, Austria), 1% penicillin, 1% streptomycine (PAA) and 2mM L-Glutamine (PAA). An embryonic kidney cell line, HEK293 cells (American type culture collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (PAA), 1% penicillin, 1% streptomycine (PAA) and 2mM L-Glutamine (PAA). Both cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

mRNA and miRNA isolation

Total RNA was isolated from formalin-fixed, paraffin-embedded (FFPE) samples using the RNAeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total cellular RNA from cultured cells was isolated using TRIZOL (Invitrogen) according to the manufacturer's instructions. Additionally, commercially available total RNA from foetal (pool of 18 and 22 weeks embryos) and mature kidney (70 years old) was purchased (Stratagene, La Jolla, CA, USA).

miRNA quantitative real time PCR (qRT-PCR)

Mature miR-192, miR-194, miR-215, miR-141, miR-200c expressions were analysed using the TaqMan miRNA Assay (Applied Biosystems, Carlsbad, CA, USA). RNU6B (Applied Biosystems) was used as endogenous control. All reactions were performed in triplicate. Relative miRNA expressions were calculated as delta delta CT.

mRNA qRT-PCR

A maximum of 500 ng of total RNA was reverse transcribed into cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturers' instructions. qRT-PCR was performed with primers specific to ACVR2B and 18s RNA (Table 3). All reactions were performed in triplicates. qRT-PCR was performed on a 7900T Fast Real Time PCR system (Applied Biosystems) using SYBR Green as the detection fluorophore. 18s RNA was used as the internal control. Relative gene expression levels were calculated as delta delta CT.

Immunohistochemistry

ACVR2B (Abnova, Taipei City, Taiwan) antibodies were diluted 1:25 in antibody diluent (Dako, Glostrup, Denmark). Immunohistochemical staining procedures were performed using the automated VENTANA staining system (Ventana, Tucson, AZ, USA). Experiment was performed by Mrs. Margit Gogg-Kamerer.

Transfections of miRNA precursor molecules and Luciferase reporter assays

WT-CLS1 cells were cultured in 6-well plates up to 50% confluence. Plasmid constructs for luciferase assays were created by ligating oligonucleotides containing the target sites of the ACVR2B 3'-untranslated region (UTR) downstream of the luciferase gene in the pMIR-REPORT™ Reporter Luciferase vector (Ambion, Carlsbad, CA, USA) (Table 3). Transfection was carried out using a solution containing 2µg firefly luciferase reporter vector containing the target oligonucleotide sequence, 500ng of pMIR-REPORT™ beta-gal control vector (Ambion) and pre-miRNA of miR-192, miR-194, miR-215, miR-141, miR-200c (Ambion) or pre-miR scrambled miRNA (Ambion). Pre-miRNA molecules were used at a final concentration of 100nM and mixed with 12µl lipofectamine 2000 (Invitrogen). A final volume of 100µl was obtained by adding Opti-MEM® I Reduced-Serum medium (Invitrogen) per well. The transfection solution was incubated at 37°C for 30 minutes and mixture was added to 900µl of serum free medium covering the cells. Another 1ml of medium with serum was added after 4 hours post-transfection. Medium was changed to standard growth media after washing the cells with PBS after 24 hours of transfection.

Luciferase activity was measured 50 hours post-transfection (Bright Glo Luciferase Assay System, Promega, Madison, WI, USA) using β-galactosidase for normalization (β-galactosidase Enzyme Assay System, Promega). Changes in luciferase activity were calculated by dividing the luciferase activity of the test miRNA by the luciferase activity of scrambled miRNA.

Western blot

HEK293 cells were cultured in 6 well plates up to 70% confluence and transfected with 100nM pre-miRNAs of miR-192, miR-194, miR-215, miR-141, miR-200c (Ambion) or pre-miR scrambled miRNA (Ambion). Transfections were carried out as described previously.

30ug of Total cellular proteins were isolated 60 hours after transfection. Cell pellets were resuspended in RIPA buffer (Thermo Scientific Inc, Rockford, IL, USA) containing 1x protease inhibitors (Halt protease and phosphatase inhibitor cocktail, Thermo Scientific Inc). Samples were kept on ice for 15 minutes and cells were sonicated. Protein extracts were cleared by centrifugation for 10 minutes at 13,000rpm. Supernatants were transferred into fresh tubes and protein concentrations were obtained by the Lowry protein assay.

Isolated protein extracts were loaded onto a pre-cast 4-12%, Nu Page Bis-Tris 1.0mm gel (Invitrogen). SDS-PAGE electrophoresis was performed using Nu-Page TM MOPS SDS (Invitrogen) running buffer. The proteins were transferred electrophoretically to a nitrocellulose membrane at 35V for 90 minutes using 1X NuPage transfer buffer (Invitrogen). The membrane was blocked with 5% (v/v) skimmed milk powder (Bio-Rad, Hercules, CA, USA) in TBST (0.2mM Tris, 15 mM NaCl, pH7.4, 0.2%(v/v) Tween 20 for one hour at room temperature. Next, the membrane was incubated with ACVR2B antibody diluted 1:100 (Abnova) for 1 hour at room temperature in 1% (v/v) skimmed milk powder with TBST for immunodetection, Subsequently the membrane was washed in TBST three times for 5 minutes each at room temperature and membrane was treated with HRP conjugated anti-rabbit secondary antibody (DAKO). The membrane was developed by exposing it to a photosensitive film using Pierce ECL western blotting substrate (Thermo scientific). Afterwards the membrane was stripped using a stripping buffer (Thermo scientific) and incubated with an antibody against GAPDH diluted 1:500 in 2% (v/v) skimmed milk powder with TBST and incubated overnight at 4°C for normalization of the protein content. The detection was carried out as described above. Densitometric analysis was performed to quantify protein expression (Balaguer 2010). Experiment was performed by Ms. Pooja Chowdhury and Ms. Upeka Senanayake.

Statistical analysis

Statistical analysis was performed using the Graphpad Prism softwareV5 (Graph pad Inc., San Diego, CA, USA). The comparison of miRNA and mRNA expression of nephroblastomas was calculated by the Mann-Whitney test (non-parametric analysis) and change in the luciferase activity by Student's t test (parametric analysis) $p < 0.05$ was considered as significant (Balaguer 2010).

Results

miRNA array

The miRNA profiling identified a subset (55) of the total number of miRNAs analysed by the miRCURY™ array that are differentially expressed in the different samples.

miR-192, miR-194, miR-215, miR-141 and miR-200c expression is downregulated in blastemal, epithelial and NR and upregulated in mature renal parenchyma (Figure 17).

miR-192, miR-194, miR-215, miR-141, miR-200c have a low expression in most nephroblastomas

The expression levels of miR-192, miR-194, miR-215, miR-141, and miR-200c in nephroblastomas in relation to normal kidney was analysed by qRT-PCR. Six blastema-type, 4 epithelial-type, 11 mixed type, 3 stroma-type, 4 corresponding samples of mature kidney and 3 additional samples of mature kidney were studied.

Regardless of the subtypes, miR-192, miR-194 and miR-215 had a significantly lower expression in all nephroblastomas compared to mature renal parenchyma. All mixed type and blastema-type nephroblastomas showed a significantly lower expression of miR-141 compared to mature renal parenchyma. The difference in expression of miR-141 in stroma-type nephroblastomas and mature renal tissue was not significant. Three epithelial-type tumours showed a similar level of expression as the other sub-types, however one epithelial-type nephroblastoma had a very high expression of miR-141. Therefore, the difference between epithelial-type tumours and mature renal parenchyma did not differ significantly. The same expression pattern was observed for miR-200c (Figure 18).

Nephrogenic rests have a lower expression of miR-192, miR-194, miR-215, miR-141 and miR-200c compared to most mature renal parenchyma, and a higher expression compared to most nephroblastomas

miR-192, miR-194, miR-215, miR-141, and miR-200c expressions were assessed in nine incidentally detected NRs. miRNA expression levels were compared across all subtypes of nephroblastomas and also with mature renal parenchyma. miR-192, miR-194 and miR-215 expression was significantly lower in all NRs compared to mature renal parenchyma; and significantly higher compared to blastema-type, mixed type and stroma-type nephroblastomas. However, there was no significant difference compared to epithelial-type nephroblastomas (Figure 18).

miR-141 expression was significantly higher in NRs compared to blastema-type nephroblastomas, but did not differ significantly compared to epithelial-type, mixed type, stroma-type nephroblastomas or mature renal parenchyma. One NR had a very high expression of miR-141 and miR-200c in comparison with all other NRs (Figure 18).

miR-200c had a significantly lower expression in NRs compared to mature renal parenchyma, but a significantly higher expression in relation to blastema-type tumours. Interestingly, the expression of miR-200c did not differ significantly between NRs and the other subtypes of nephroblastomas (Figure 18).

All five miRNAs are targeting a single common *ACVR2B* gene in the TGF beta pathway according to an *insilico* analysis

Diana web path software, which is a web-based computational tool, was used to predict the target genes for analysed miRNAs. (Maragkakis *et al.*, 2009; Papadopoulos *et al.*, 2009). Names of the miRNAs were included as input in the DIANA miRPath analysis based on DIANA-micro-T-4.0 (β -version). This web based computational programme performs enrichment analysis of data sets with all available pathways provided by the Kyoto Encyclopedia of Genes (Kanehisa *et al.*, 2000). Interactions of genes in different biological pathways are diagrammatically represented in the KEGG database. The enrichment analysis of the input miRNAs regarding pathways was carried out by a Pearson's chi-square test (Papadopoulos *et al.*, 2009). The combinatorial effect of the 5 miRNAs (Union data set) on the TGF- β pathway was 9.17 (-In p value). Computer mouse over 'option' displayed that miR-192, miR-194, miR-215, miR-141 and miR-200c are targeting a single common gene *ACVR2B*. The analysis of other pathways, which had - In p value of >9.17 , did not show a common target gene for analysed 5 miRNAs (Table 4).

Foetal kidney has a lower expression of miR-192, miR-194 and miR-215 compared to mature kidney and *ACVR2B* upregulated in foetal kidney

miRNA and mRNA expression was analysed using commercially available foetal and mature kidney RNA samples. Foetal kidney sample showed a higher expression of miR-192, miR-194 and miR-215 and a lower expression of miR-141, miR-200c compared to mature kidney (Figure 19). *ACVR2B* mRNA expression was higher in foetal kidney compared to mature kidney (Figure 20).

ACVR2B is upregulated in most nephroblastomas compared to mature renal parenchyma

24 nephroblastomas (4 blastema-type, 4 epithelial-type, 3 stroma-type and 13 mixed type one regressive nephroblastomas) as well as two corresponding mature kidney samples and four additional samples of mature kidney were analysed for *ACVR2B* mRNA expression. Nephroblastomas had a significantly higher ($p < 0.0013$) mRNA expression compared to mature renal parenchyma, (Figure 22).

Membrane and cytoplasmic protein expression for ACVR2B was assessed immunohistochemically in 55 tumours (20 mixed type, 4 epithelial-type, 4 blastema-type, 6 stroma-type and 21 regressive nephroblastomas). Additionally, ACVR2B protein expression of 6 incidentally identified NRs was analysed. Corresponding nephroblastomas of the NRs were two regressive, three mixed-type and one stroma-type nephroblastomas.

The expression pattern of ACVR2B was assessed in all the different morphological components of nephroblastomas. Importantly, ACVR2B protein expression was observed in the main morphological components of nephroblastomas, in all blastemal areas regardless of the nephroblastomas subtypes, and in all tubular structures with the exception of one mixed nephroblastoma, which showed negative tubular structures. Undifferentiated mesenchyme showed complete negativity in 19 and positivity in 9 tumours. A smaller number of nephroblastomas also contained more specialised structures such as immature glomeruloid bodies and skeletal muscle. ACVR2B expression was identified in immature glomerular bodies (3 out of 5 cases) and in skeletal muscle (4 out of 6 cases). No expression of ACVR2B was identified in fully differentiated structures such as hyaline cartilage and adipose tissue.

A direct comparison between staining intensity of the tumour and the adjacent renal parenchyma was possible in 25 cases. 21 tumours had a stronger ACVR2B protein expression compared to adjacent normal kidney parenchyma. Two cases showed a lower intensity of staining compared to adjacent non-tumoral kidney parenchyma. One regressive and one mixed nephroblastoma had the same staining intensity. Correlation of mRNA and protein expression levels showed that the tumours with high mRNA expression of ACVR2B also had a strong protein expression. In all other samples a direct comparison with non-tumoural renal parenchyma was not possible, as sections exclusively contained areas of tumour.

All NRs (6 samples) were positive for ACVR2B (Figure 21). A direct comparison of ACVR2B expression in the same section with corresponding nephroblastomas was

possible in 2 cases, both corresponding nephroblastoma and normal kidney parenchyma in 1 case and with normal parenchyma in 3 cases. In comparison, all NRs showed a higher expression than the corresponding normal parenchyma, and a lower expression than the corresponding nephroblastomas.

ACVR2B is a direct target of miR-215, miR-192, miR-194, miR-141 and miR-200c

Luciferase reporter assays were performed to conclude whether the 3'UTR regions of *ACVR2B* were definitely functional target regions of analysed 5 miRNAs. Luciferase reporter plasmids harbouring target regions for 5 different miRNAs were prepared and transient transfections were performed using a WT-CLS1 cell line. This nephroblastoma cell line has a low expression of *ACVR2B*.

The pre-miR-192 co-transfected with target regions 2582-2610, 8021-8049 and 9188-9216 respectively, led to a significant decrease in luciferase activity compared to the scrambled control (Figure 23). Also, the 2582-2610 and 9188-9216 target regions of miR-215 revealed a significant downregulation of luciferase activity following the transfection of the pre-miR-215 (Figure 23).

miR-194, 9328-9356 showed a downregulation of luciferase activity compared to scrambled control, but the luciferase activity of the 415-443 target region was not downregulated by miR-194 (Figure 23).

5632-5660 and 7567-7593 target regions of miR-200c showed significant downregulation following transient transfection with pre-miR-200c (Figure 23).

miR-141 had a single target region 1872-1900. Transfections with pre-miR-141 and its target region bearing reporter vector showed significant downregulation of luciferase activity compared to control (Figure 22). Additionally, the regulation of protein expression by all miRNAs was investigated in HEK293 cells, which has a high level of *ACVR2B* expression. Western blot analysis was performed after transfecting pre-miRNAs and pre-scrambled control miRNA. Experiment was performed once. Densitometric analysis showed downregulation of *ACVR2B* protein by miRNAs compared to scrambled control (Figure 24).

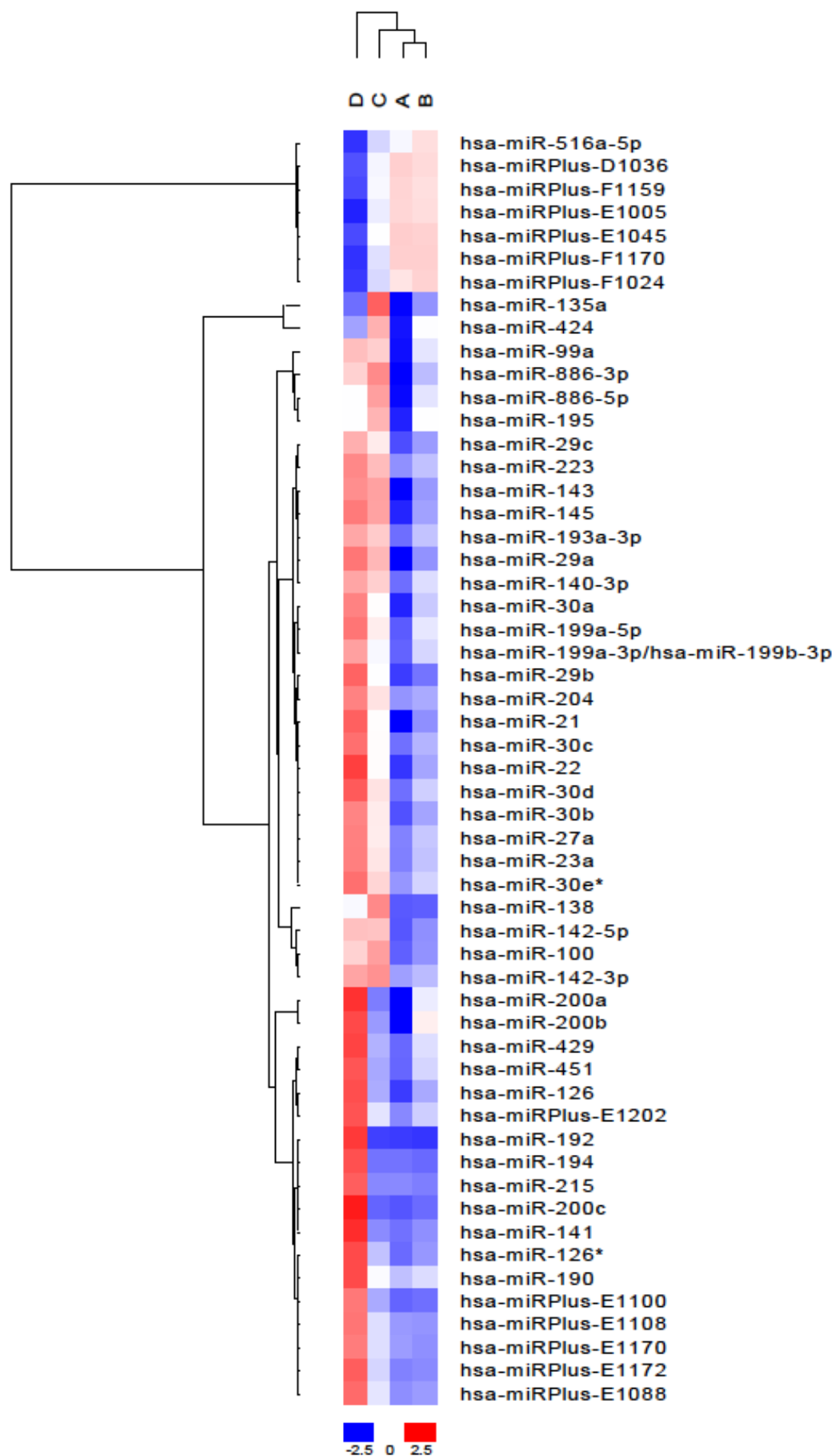


Figure 17:

miRNA expression profiles of blastemal (A), epithelial (B), NR (C) and mature renal parenchyma were analysed by a miRNA microarray. The heat map diagram shows the result of the two-way hierarchical clustering of miRNAs and samples. Each row represents a miRNA and each column represents a sample. The miRNA clustering tree is shown on the left. The colour scale shown at the bottom illustrates the relative expression level of a miRNA across all samples: red represents an expression level above the mean and blue represents expression lower than the mean.

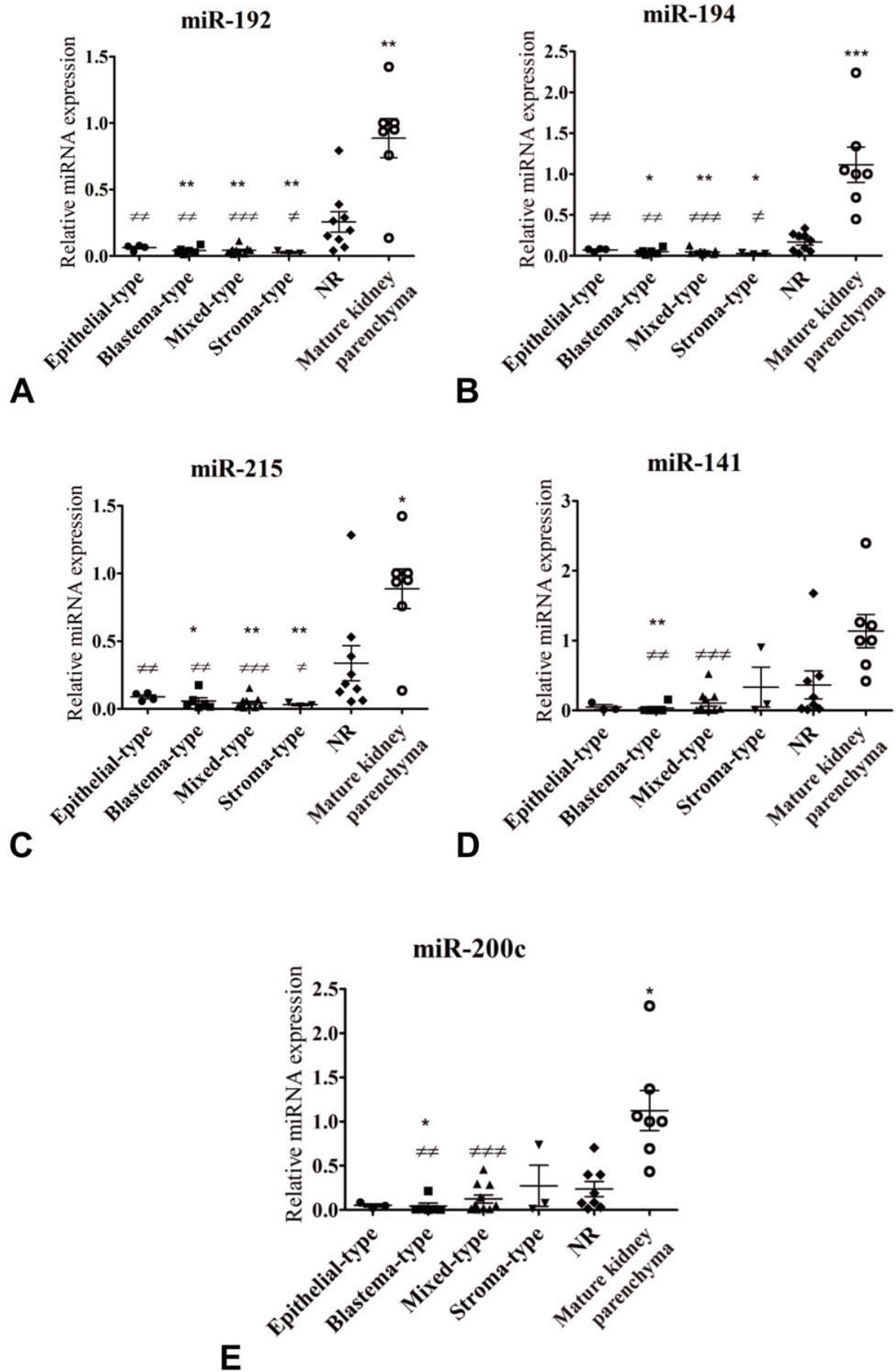


Figure 18:

Relative miRNA expression of miR-192, miR-194, miR-215, miR-141 and miR-200c was analysed by qRT-PCR in epithelial-type (n=4), blastema-type (n=6), mixed type (n=11), stroma- type (n=3) nephroblastomas, NRs (n=9) and mature kidney parenchyma (n=7). miR-192 has a significantly lower expression in all nephroblastoma subtypes compared to mature renal parenchyma. Similar type of results was obtained for miR-194 and miR-215. miR-141 and miR-200c have significantly lower expression in blastema- type and mixed-type tumours compared to mature renal parenchyma. There was no significant difference between the other tumour subtypes and the renal parenchyma ($\neq p < 0.05$, $\neq\neq p < 0.01$, $\neq\neq\neq p < 0.001$ vs mature renal parenchyma).

Blastema-type, mixed type and stroma-type nephroblastomas showed a significantly lower expression of miR-192, miR-194 and miR-215 compared to NRs. miR-141 and miR-200c expression was significantly lower in blastema-type tumours compared to NRs. Kidney parenchyma shows a significantly higher expression of miR-192, miR-194, miR-215 and miR-200c compared to NRs ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs NRs, vertical bars represent + SEM).

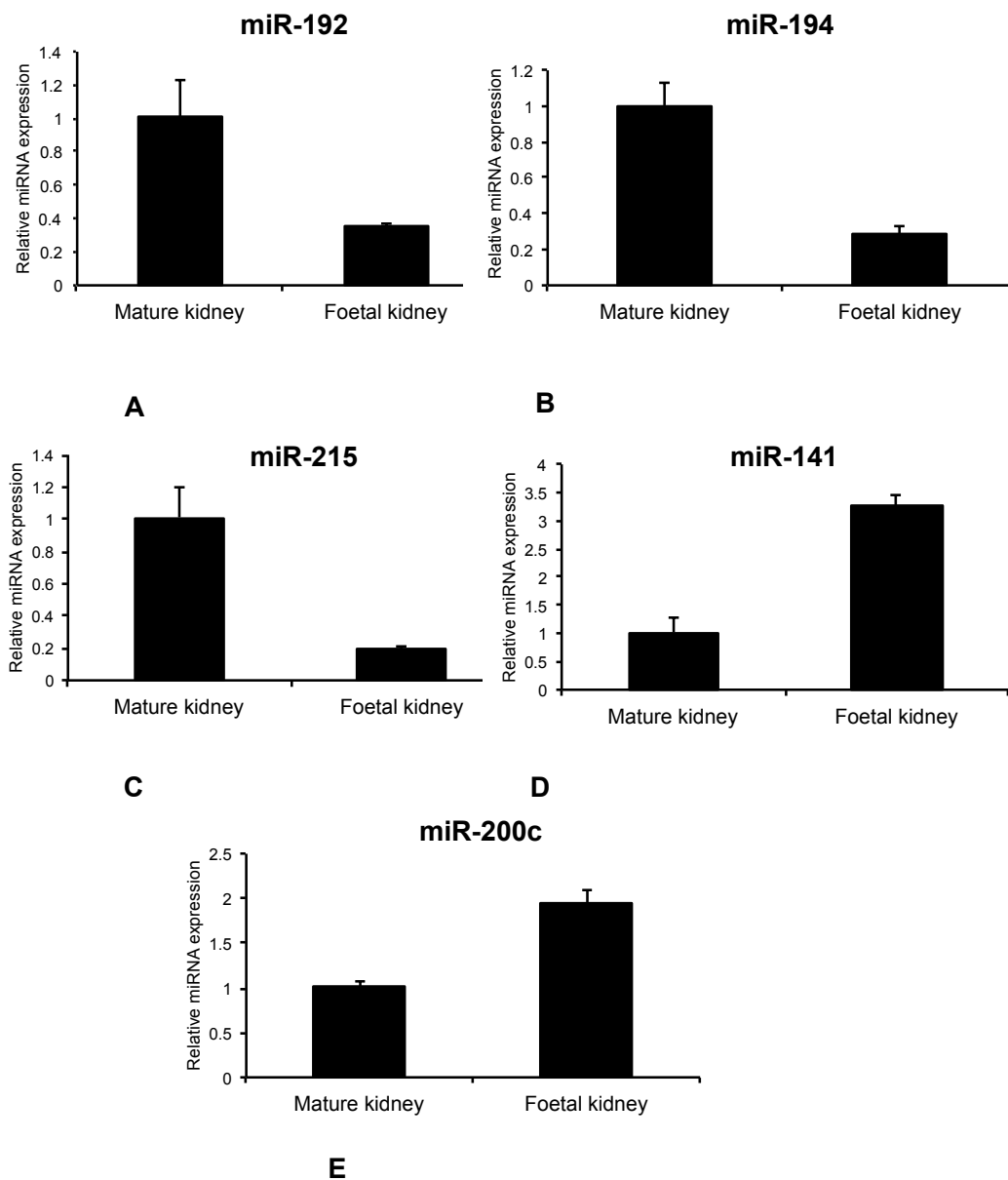


Figure 19:

Relative miRNA expression of miR-192 (A), miR-194 (B), miR-215 (C), miR-141 (D) and miR-200c (E) was analysed by qRT-PCR in commercially available foetal kidney and mature kidney RNA samples. Foetal kidney showed a higher expression of miR-192, miR-194 and miR-215 and a lower expression of miR-141, miR-200c compared to mature kidney (n=1, vertical bars indicate +SEM)

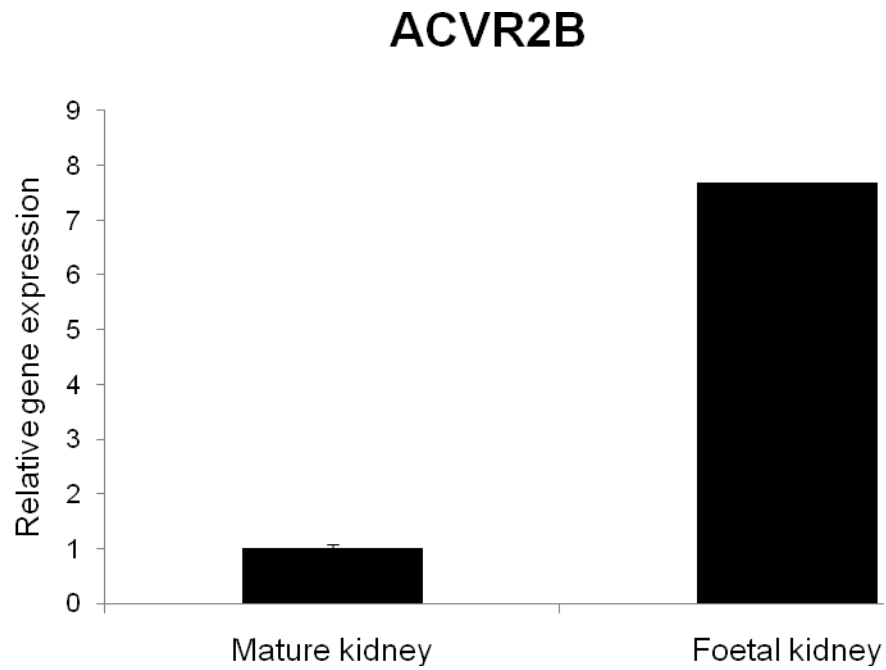


Figure 20:

Relative *ACVR2B* mRNA expression was measured in foetal kidney and mature kidney. *ACVR2B* showed high expression in foetal kidney compared to mature kidney. (n=1, error bars indicate + SEM).

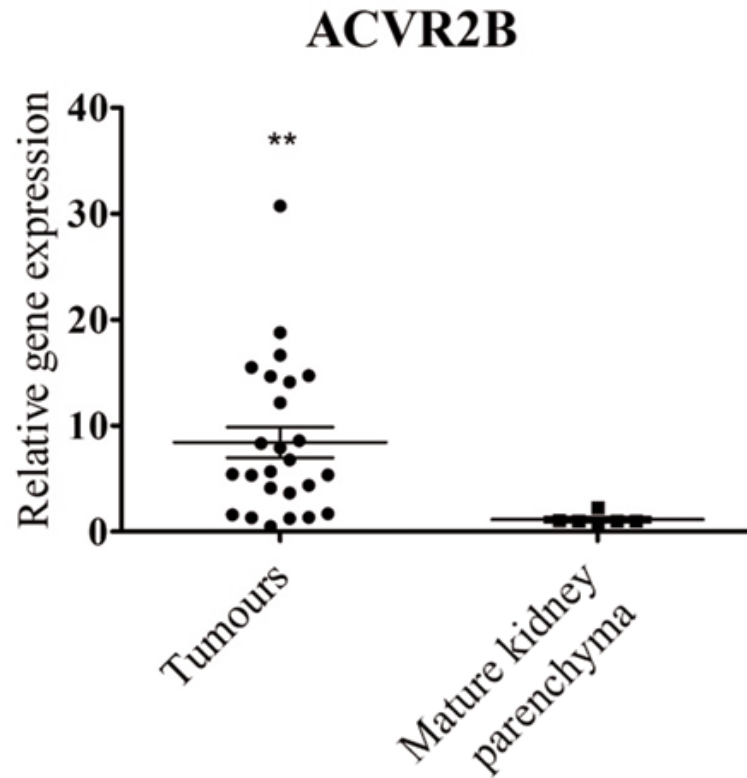


Figure 21:

Relative expression of *ACVR2B* was analysed in nephroblastomas (n=25) and mature renal parenchyma (n=6) by qRT-PCR. Tumours showed significantly higher *ACVR2B* expression compared to renal parenchyma (vertical bars represent + SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

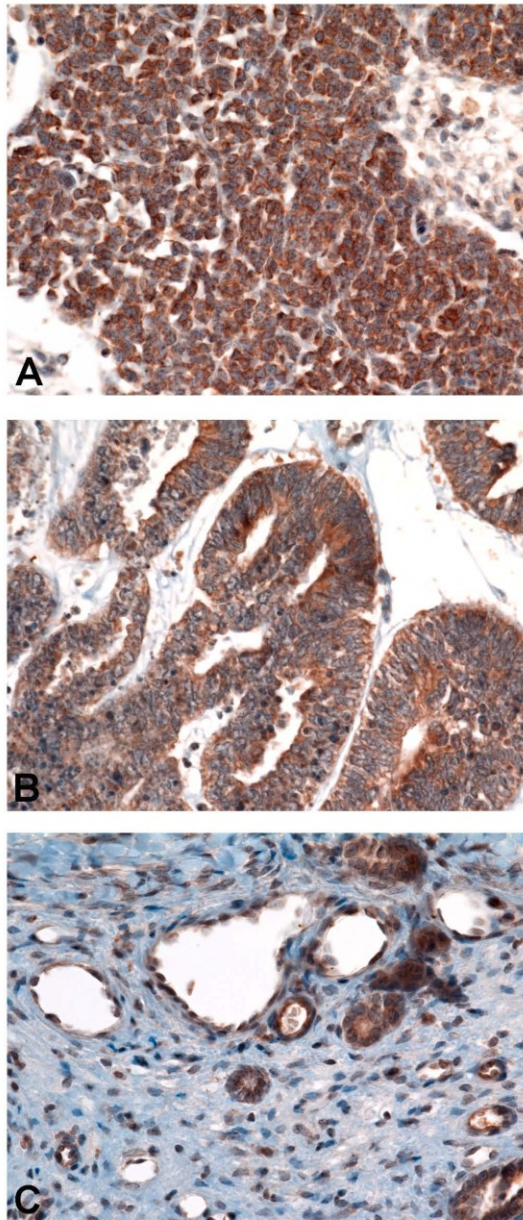


Figure 22:

ACVR2B protein expression was analysed by immunohistochemistry in different subtypes of nephroblastomas (n=55). Blastemal regions, epithelial regions, mesenchyme (mixed type tumour-A, mixed type tumour- B, 400x) and NRs (C, 400x) showed strong membrane and cytoplasmic staining for ACVR2B compared to corresponding mature renal parenchyma.

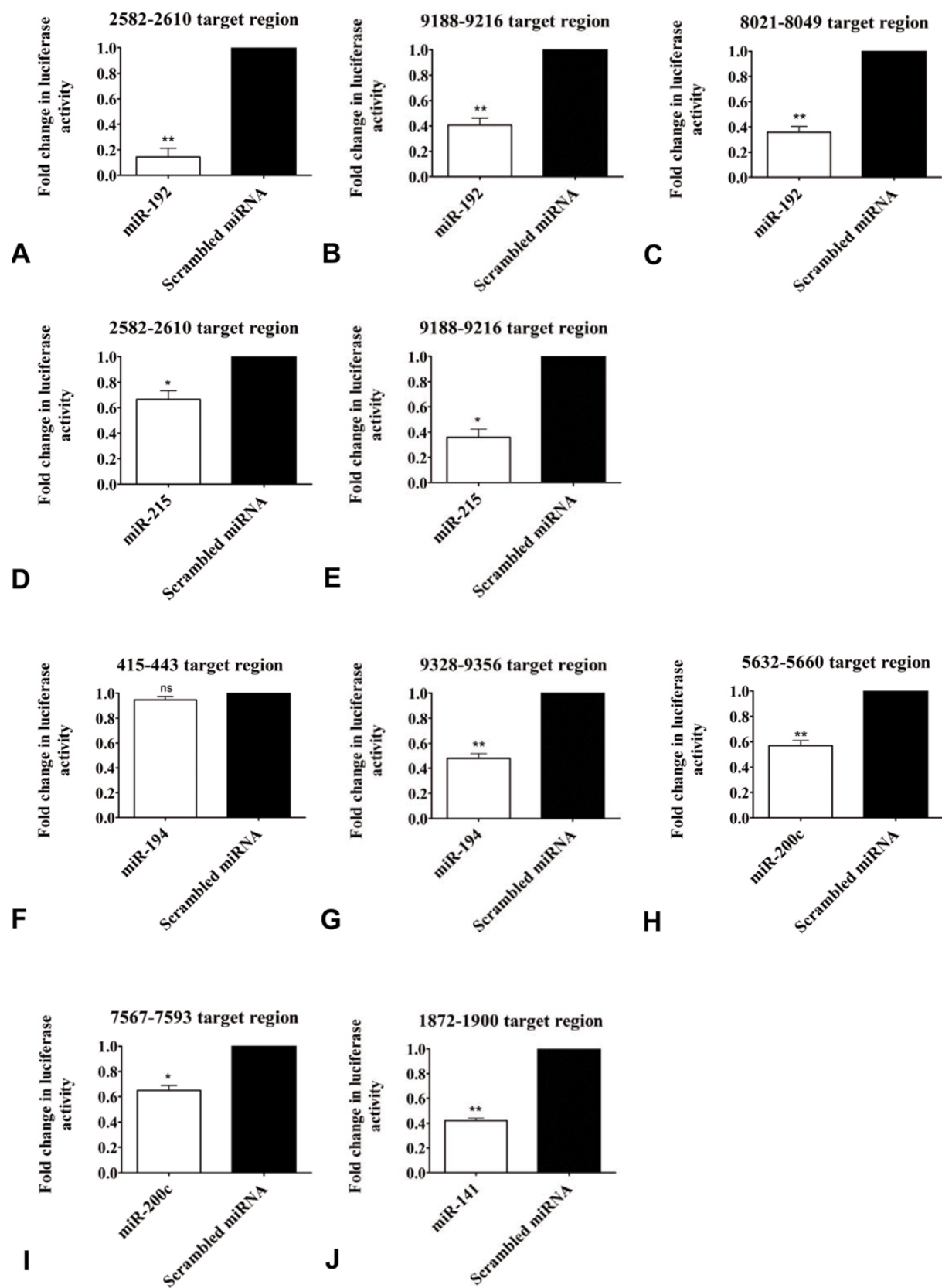


Figure 23:

Direct interaction with the 3' UTR of *ACVR2B* by miR-192, miR-215, miR-194, miR-141 and miR-200c was analysed by luciferase reporter assays. WT-CLS1 cells were transfected with firefly luciferase vector containing different target regions for miRNAs and pre-miRNAs for different target regions or scrambled miRNA. β -galactosidase activity was used to normalization. Results were depicted as fold-change in luciferase activity compared to scrambled control.

miR-192 (A, B, C), miR-215 (D, E), miR-200c (H, I) and miR-141 (J) significantly downregulated luciferase activity by binding to their target regions. miR-194 decreased the luciferase activity by binding with one target region out of the two predicted target regions (F,G).

Vertical bars represent + SEM, n=3, * p <0.05, ** p <0.01.

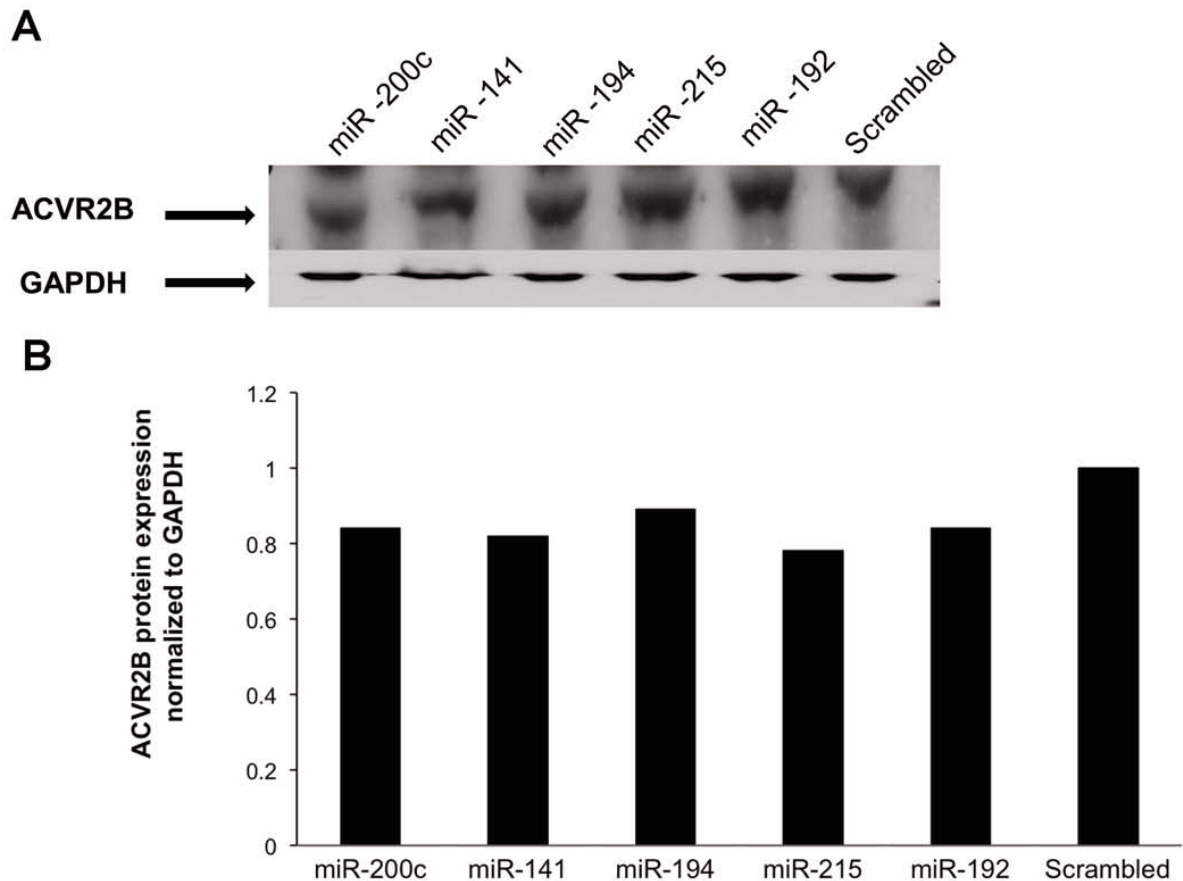


Figure 24:

Protein lysates were extracted following transfections of pre-miR-200c, pre-miR-141, pre-miR-194, pre-miR-215, pre-miR-192 and pre scrambled control miRNA, Proteins were separated using 4-12% SDS-PAGE, blotted and probed with anti ACVR2B antibody in 1:100 dilution (A-upper panel). The blot was stripped and reprobed using anti-GAPDH antibody in 1:500 dilution (A-lower panel). Densitometric analysis was performed to calculate the average protein expression of ACVR2B. All values were normalized to average protein expression of GAPDH. Normalized densitometric values are indicated in relation to scrambled control (B). Analysed miRNAs are downregulating protein expression of ACVR2B. Western blot and analysis was performed by Ms. Pooja Chowdhury and Ms. Upeka Senanayake.

Table 3: Oligonucleotide sequences for cloning and primers for QRT PCR

Target regions	Sequences	
2582-2610	5'-CTAGTACTGCAGGAATAGTTAGGTCATGCTCAGCA-3' 5'-AGCTTGCTGAGCATGACCTAACTATTCCTGCAGTA-3'	
2756-2784	5'- CTAGTGCGGTCAAGACACAGTACAGACAGGTCAGGCTCAGCA- 3' 5'- AGCTTGCTGAGCCTGACCTGTCTGTACTGTGTCTTGACCGCA-3'	
8021-8049	5'-CTAGTAAGGTCAGGCTCAGCA-3' 5'-AGCTTGCTGAGCCTGACCTTA-3'	
9188-9216	5'-CTAGTATGTAAATGTAGTAGGTCACGCTCAGCA-3' 5'-AGCTTGCTGAGCGTGACCTACTACATTACATA-3'	
415-443	5'- CTAGTACCTAGAGGAACCTTTGAAGACTGTTACAGCTCAGCA- 3' 5'-AGCTTGCTGAGCTGTAACAGTCTTCAAAGGTTCTCTAGGTA -3'	
9328-9356	5'-CTAGTATACAGAGTTAAGCTTGTTGCTGTTACCGCTCAGCA- 3' 5'-AGCTTGCTGAGCGGTAACAGCAACAAGCTTAACTCTGTATA- 3'	
7567-7595	5'-CTAGT CTGCCATTTTCAGTATTCGCTCAGCA-3' 5'-AGCTTGCTGAGCGAATACTGAAAATGGCAGA-3'	
5632-5660	5'-CTAGT ACATCTACAGAAGAGTATTAGCTCAGCA-3' 5'-AGCTTGCTGAGCTAATACTCTTCTGTAGATGTA-3'	
1872-1900	5'-CTAGTACTTGATCGGTCAGTGTTTCGCTCAGCA3' 5'-AGCTTGCTGAGCGAACACTGACCGATCAAGTA-3'	
Gene	Forward primer	Reverse primer
<i>ACVR2B</i>	5'-GGCTGCTGGCTAGATGACTT-3'	5'-AAGCGTTCGTTGCAGAAGTT-3'
<i>18S</i>	5'-GTAACCCGTTGAACCCCAT-3'	5'-CCATCCAATCGGTAGTAGCG-3'

Table 4: Predicted target regions

	Seed match	3'UTR position	Predicted consequential pairing of target region (top) and miRNA (bottom)			
hsa-miR-192	7mer (pos 2)	2582 - 2610	3'UTR: 5'	A	GAAUAGU	U 3'
				CUG CAG	UAGGUCA	
				.		
				GAC GUU	AUCCAGU	
			miRNA: 3'	CC A AAGU		C 5'
hsa-miR-192	7mer (pos 1)	8021 - 8049	3'UTR: 5'		A	3'
					AGGUCAG	
					UCCAGUC	
			miRNA: 3'	CCGACAGUUAAGUA		5'
hsa-miR-192	7mer (pos 2)	9188 - 9216	3'UTR: 5'	A A GUA		C 3'
				UGU AAU	GUAGGUCA	
					.	
				ACA UUA	UAUCCAGU	
			miRNA: 3'	CCG G AG		C 5'
hsa-miR-215	8mer (pos 1)	2582 - 2610	3'UTR: 5'	A	GAAUAGU	3'
				CUG CAG	UAGGUCAU	
				.		
				GAC GUU	AUCCAGUA	
			miRNA: 3'	CA A AAGU		5'
hsa-miR-215	7mer (pos 2)	9188 - 9216	3'UTR: 5'	A A GUA		C 3'
				UGU AAU	GUAGGUCA	
					.	
				ACA UUA	UAUCCAGU	
			miRNA: 3'	CAG G AG		A 5'
hsa-miR-194	8mer (pos 1)	415 - 443	3'UTR: 5'	A UAGA ACCU AAGA		3'
				CC GGA UUG	CUGUAA	
				GG CCU AAC	GACAAU	
			miRNA: 3'	A UGUA C		5'

hsa-miR-194	8mer (pos 2)	9328 - 9356	3'UTR: 5' A GAGUUAAGCUU C UACA GUUGCUGUUAC . GUGU CAACGACAAUG miRNA: 3' AG ACCU U 5'
hsa-miR-141	7mer (pos 2)	1872 - 1900	3'UTR: 5' A G U C CUU AUCGG CAGUGUU . . GAA UGGUC GUCACAA miRNA: 3' GGUA A U U 5'
hsa-miR-200c	7mer (pos 1)	5632 - 5660	3'UTR: 5' A AGAAG CAUC UAC AGUAUUA GUAG AUG UCAUAAU miRNA: 3' AG UA GGCCG 5'
hsa-miR-200c	7mer (pos 2)	7567 - 7595	3'UTR: 5' C AUUUU C UGCC CAGUAUU . AUGG GUCAUAA miRNA: 3' AGGUAGUA GCC U 5'

Discussion

miRNAs play a role in various biological cellular functions including differentiation, proliferation and apoptosis (Babashah *et al.*, 2011; Cheng *et al.*, 2005). In addition, abnormal expressions of miRNAs play a role in the pathogenesis, progression and metastasis of malignant tumours. miRNAs can have dual functions; they act as oncogenes or tumour suppressors and also signify targets for therapeutical approaches (Babashah *et al.*, 2011; Esquela-Kerscher *et al.*, 2006; Lu *et al.*, 2005; White *et al.*, 2010).

We have screened miRNA expression in blastemal and epithelial regions of nephroblastomas, NRs and mature renal parenchyma. Fifty-five miRNAs were differentially expressed in analysed samples. Our experiments were focused on a group of miRNAs upregulated in mature renal parenchyma and downregulated in nephroblastomas and NRs. A previous study has identified miR-192, miR-194 and miR-215 as enriched in mature kidneys (Sun *et al.*, 2004). They have performed a Clustal W analysis of miRNA-192 and miR-215 and identified that both have a high homology between their precursor sequence as well as 18bp common sequence (UGACCUAUGAAUUGACAG) in mature miRNA sequences (Sun *et al.*, 2004). miR-194 is transcribed by 2 loci (miR-194-1 and miR-194-2). miR-192 is located in chromosome 11 and 109 bp upstream of miR-194-2. miR-194-1 and miR-215 are located in chromosome 1 and the distance between them is only 195 bp. Therefore miR-192 and miR-194-2 as well as miR-215 and miR-194-1 are likely to regulate as common transcriptional units (Sun *et al.*, 2004). miR-141 and miR-200c belongs to miR 200 family and both miRNAs are located in chromosome 12 (Vrba *et al.*, 2010). Therefore, our subsequent experiments were focused on miR-192, miR-194, miR-215, miR-141 and miR-200c.

The analysis of miRNAs revealed a downregulation of miR-192, miR-194 and miR-215 in all nephroblastoma samples regardless of the subtype. The debatable role of these miRNAs has been discussed in other malignant tumours. In a recent study, a significantly reduced expression level of miR-192 in a rat model of adenocarcinomas has been demonstrated (Davidson *et al.*, 2009). miR-192, miR-194 and miR-215 were also shown to have a low expression in colon cancer samples in comparison to normal colon (Braun *et al.*, 2008). In contrast, a high level of miR-194 expression was identified in Barrett's epithelium and adenocarcinoma in comparison to normal gastric and oesophageal mucosa (Wijnhoven *et al.*, 2010). Our results also show that miR-200c and miR-141 are downregulated in the majority of nephroblastomas with the exception of stroma-type nephroblastomas and one

epithelial-type tumour. This epithelial type tumour consists of mature tubules and showed expression comparable to mature kidney parenchyma. Major published international studies have shown that stroma-type and highly differentiated epithelial-type nephroblastomas have a very good outcome (Verschuur *et al.*, 2010). This might also be reflected by a distinct gene expression patterns, since epithelial-type nephroblastomas with differentiated tubular growth pattern harboured a gene expression pattern corresponding to an arrest following mesenchymal to epithelial transition (Sredni *et al.*, 2009). It is therefore tempting to speculate about a role of miR-200c and miR-141 leading to a less aggressive biological behaviour of these entities. The role of miR-141 and miR-200c has also been investigated in several other tumour entities. Significantly reduced expression of miR-141 was identified in gastric cancer cell lines and gastric cancer samples, compared to adjacent non-neoplastic tissue (Du *et al.*, 2009). A comparison of clear cell renal carcinomas with corresponding non-malignant samples revealed a decreased expression of miR-200c and miR-141. A comparison of miRNA expression profiles also made a clear distinction between malignant and normal tissue (Jung *et al.*, 2009). Differences in expression levels of several miRNAs including miR-200c were also able to differentiate between oncocytoma and chromophobe carcinoma up to 93% (Fridman *et al.*, 2010). In contrast, high levels of miR-141 and miR-200c were expressed in renal clear cell carcinomas compared to normal renal tissue (Liu *et al.*, 2010). A comparison of ovarian cancer and normal ovarian tissue demonstrated an overexpression of numerous miRNAs including miR-200c and miR-141 (Iorio *et al.*, 2007).

miRNAs directly influence stem cell self-renewal and lineage specification during development. Dicer null embryonic stem cell showed abnormalities in differentiation (Kanellopoulou *et al.*, 2005). Our results showed a low expression of miR-192, miR-194 and miR-215 in foetal kidney (pooled 18 weeks and 20 weeks total RNA) compared to mature kidney. (Tang *et al.*, 2011). Results in a previous study revealed a completely contrasted result to our findings. They have identified a high expression of miR-194 and miR-192 in foetal kidney compared to mature kidney in their miRNA microarray, but the age of the analysed foetus was 30 weeks and adult kidney was 18 years (Tang *et al.*, 2011). The expression pattern of miR-141 and miR-200c showed an upregulation in foetal kidney compared to mature renal parenchyma. Similar pattern of miRNA expression profile was obtained for nephroblastomas in our study. These observations predict that miRNA expression pattern can be similar in both nephroblastoma and early stages of developing kidney.

The contrasting results of expression of these miRNAs in different studies and tumour entities might also be associated with the wide range of different functions and targets of miRNAs. Our *insilico* analysis showed that all five miRNAs with low expression in the large majority of the nephroblastomas and foetal kidney have a single common target, *ACVR2B*, which has not been identified as a target gene of these miRNAs before. *Invitro* Luciferase reporter assays and western blot analysis confirmed *ACVR2B* as a target gene of analysed five miRNAs.

Analysis of the expression of *ACVR2B* on mRNA level in foetal kidney, protein and mRNA level in nephroblastomas revealed a significant increased expression compared to mature kidney. Expression of activin type II receptor was already identified in embryonal kidneys of rats, humans (Maeshima *et al.*, 2003; Metsuyanin *et al.*, 2009) and in nephroblastomas. The two studies under reference which discuss the expression of *ACVR2B* in nephroblastomas were from the same study group. In their first study they have identified *ACVR2B* as a marker that overexpressed in stem like nephroblastoma xenografts (Li *et al.*, 2002). In the second study, 0.5-10% of *ACVR2B* expressing cells were sorted by fluorescent activated cell sorting in low passage primary nephroblastoma cell cultures and corresponding primary tumours, including one blastema-type tumour and 4 mixed type tumours. (Pode-Shakked *et al.*, 2009). Activins are part of the TGF- β pathway. They act on cell growth and differentiation in different biological functions. The activity of activin is regulated by dimerization of two different receptors; activin receptor type I and II (Ying *et al.*, 1997). Activation of the TGF- β pathway plays a vital role in human tumorigenesis by acting as tumour suppressor gene or oncogene (Derynck *et al.*, 2001; Grady *et al.*, 1999; Hahn *et al.*, 1996; Levy *et al.*, 2006). However, the role of *ACVR2B* in different malignant neoplasms is controversial. *ACVR2B* showed a reduced expression in colorectal carcinomas compared to normal epithelia. In addition, antibodies against *ACVR2B* were more abundant in normal serum compared to that in patients diagnosed with colorectal carcinoma (Babel *et al.*, 2009). *ACVR2B* had a higher expression in a well-differentiated endometrial adenocarcinoma cell line than in a cell line originated from a poorly differentiated endometrial adenocarcinoma (Di Simone *et al.*, 2002). In a breast carcinoma cell-line, oestradiol induced mitosis associated with a decreasing expression of *ACVR2B* (Takahashi *et al.*, 2008). In contrast, the ovarian cancer cell-line OVCAR-3 showed a higher expression of *ACVR2B* compared to normal ovarian surface epithelium and activin induced proliferation of cells (Choi *et al.*, 2001). A distinct expression of *ACVR2B* was also identified in four out of ten clear cell adenocarcinomas of

the ovary (Mabuchi *et al.*, 2006). On the basis of our results, nephroblastomas are by far the most homogenous group of neoplasms with regard to ACVR2B overexpression signifying an important pathogenic role in the process of tumorigenesis in nephroblastomas.

NRs are known to be precursor lesions of nephroblastomas (Beckwith, 1993). However, genetic evidence for this statement has so far been sparse (Vuononvirta *et al.*, 2008). Our results show that analysed miRNAs and their target gene ACVR2B exhibit different expression level in between nephroblastomas and mature renal parenchyma. Characteristically, precursor lesions of malignant tumours harbour some, but not all, genetic lesions identified in the tumours, for an example, atypical adenomatous hyperplasia of the prostate and prostate adenocarcinoma (Zhang *et al.*, 2011) or the adenoma to carcinoma sequence of the colon (Bodmer, 2006).

Conclusion

Our results demonstrated a significant role of ACVR2B expression and the TGF- β pathway in the tumorigenesis of nephroblastomas. Additionally, we have also demonstrated the regulation of ACVR2B by microRNAs.

Chapter 1.3

HOXC6 in the pathogenesis of nephroblastomas

Introduction

Nephroblastomas commonly occur as sporadic tumours, but they are also described as familial tumours (Breslow *et al.*, 1996) and tumours associated with congenital syndromes (Scott *et al.*, 2006). These tumours are genetically characterised by a non-random pattern of chromosomal aberrations in chromosomes 1, 2, 4, 7, 9, 11, 12, 14, 16, and 21 whose clinical significance is still not clearly defined (Austruy *et al.*, 1995; Hawthorn *et al.*, 2011; Mannens *et al.*, 1988; Schaub *et al.*, 2007; Wadey *et al.*, 1990).

NRs are predicted to be precursor lesions of nephroblastomas. Two distinct categories of NRs are currently recognised (Beckwith, 1993; Beckwith *et al.*, 1990). PLNRs are strictly confined to the periphery of the renal lobe and sharply demarcated by renal capsule. ILNRs occur anywhere within the renal lobe as well as within the renal sinus and the walls of the pelvicaliceal system (Beckwith, 1993; Beckwith *et al.*, 1990).

The involvement of homeobox transcription factors such as *PAX2* and *LIM1* regulating embryonal kidney development has been identified in recent years (Dressler *et al.*, 1992; Guertl *et al.*, 2011; Patterson *et al.*, 2004). The same activators play a role in the pathogenesis of nephroblastomas (Guertl *et al.*, 2011; Metsuyanin *et al.*, 2008).

The homeobox genes were first discovered in fruit fly *Drosophila melanogaster* and mutations of these genes led to homeotic transformations (Garcia-Bellido 1977, Bridges and Morgan 1923). Deregulation of homeobox genes can occur via several mechanisms. Translocations can result in fusion of DNA-binding regions of *PAX3* (paired box 3) and of *PAX7* (paired box 3) to the *FKHR* (forkhead in rhabdomyosarcoma) transcription factor as described in alveolar rhabdomyosarcoms (Davis *et al.*, 1994; Galili *et al.*, 1993). Several homeobox genes are localised in regions, which commonly undergo loss of heterozygosity in cancers, such as the *NKX3.1* (NK3 homeobox 1) gene located in the 8p21 region that is deleted in approximately 80% of prostate carcinomas (He *et al.*, 1997). Allelic losses in this region can also be seen in pre-cancerous lesions of the prostatic epithelium (Emmert-Buck *et al.*, 1995).

HOX genes are a subset of the family of homeobox genes associated with their spatio-temporal pattern of expression in developing vertebrates (Graham *et al.*, 1989). The 39 HOX genes are organized in 4 clusters located on chromosome 7, 17, 12 and 2 (Ruddle *et al.*, 1994). Abnormalities of HOX gene expression in leukemia (Celetti *et al.*, 1993) and their oncogenic potential have been identified in solid tumours (Calvo *et al.*, 2000; Chariot *et al.*, 1996a; De Vita *et al.*, 1993). The pattern of homeobox HOX gene family expression

has been reported in normal and neoplastic kidneys by Northern blot analysis. Several authors have identified an expression of HOXB5, HOXB9, and HOXC11 in nephroblastomas, which were not detectable in normal kidneys (Cillo *et al.*, 1992). Another group have identified an expression of HOXD10, HOXA9, and HOXC9 in both normal kidneys and nephroblastomas (Redline *et al.*, 1994).

Our aCGH results showed amplification of HOXC6 in tumours and NRs. Therefore, we hypothesised that HOXC6 might play a role in the pathogenesis of nephroblastomas.

Objectives

- To identify the expression pattern of HOXC6 V1 (Variant 1) and V2 (Variant 2) in nephroblastomas and NR
- To identify the downstream target genes which interact with HOXC6 variants

Materials and Methods

Samples

Samples of nephroblastomas were obtained from the children's tumour registry at the Institute of Pathology, University of Kiel, Germany. Tissue samples had been fixed in 10% buffered formaldehyde solution, and embedded in paraffin wax. Sections from paraffin blocks were obtained.

DNA Extraction.

Areas of NRs and nephroblastomas were selected for microdissection after deparaffinization and stained with Mayer's haemalaun. Areas of interest from tumours and NRs were dissected manually under microscopic control from 3-5 sections, pending on the size of the selected area, as described elsewhere (Guertl *et al.*, 2003). The microdissected tissue was directly transferred into Eppendorf tubes containing ATL buffer (QIAamp®DNA, QIAGEN, Hilden, Germany). Genomic DNA was extracted with the QIAamp®DNA mini kit (QIAGEN) according to the manufacturer's instructions.

Preparation of DNA for array CGH

Samples with less than 50ng/μl of DNA and hybridizing male reference DNA (Promega, Madison, WI, USA) were amplified with the Genomeplex single cell whole genome amplification kit (Sigma Aldrich, St Louis, MO, USA) and removal of excess nucleotides was performed with the GenElute PCR clean-up kit (Sigma Aldrich) according to the manufacturer's instructions. Quality of all samples was analysed by multiplex PCR, as described in the literature (van Beers *et al.*, 2006). Hybridizing reference DNA for non amplified samples was digested with restriction endonucleases Alu (NEB, Herts, UK) and RsaI (NEB) (Table 5). Completeness of DNA digestion was confirmed on an ethidium-bromide stained 1% agarose gel.

Array CGH (aCGH)

Samples were labeled using the Bioprime Array CGH Genomic Labeling Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, 250 ng of test DNA and reference DNA (Promega) was differentially labelled with dCTP-Cy5 and dCTP-Cy3, respectively (GE Healthcare, Piscataway, NJ, USA). Clean up of the labelled genomic DNA was performed according to the manufacturer's instructions (Agilent, Santa Clara, CA, USA) followed by measurement of the yield for each test and reference DNA with the nanodrop (Thermo scientific Inc, Rockford, IL, USA). Samples with equal yields

were chosen as the test and reference DNA for co-hybridization. The preparation of combined reference and test-labelled genomic DNA for hybridization was carried out according to the manufacturer's instructions (Agilent). Genome-wide analysis of DNA copy number changes was conducted using an oligonucleotide array containing 60,000 probes according to the manufacturer's protocol version 6 (Agilent). Microarray washing was carried out 24 hour after hybridization as described in the Agilent protocol. Slides were scanned using Agilent's microarray scanner G2505B and analysed using Agilent DNA Analytics software 4.0.76 (smoothing 5MB, threshold 8, aberration filters 50 0.3, Fuzzy zero off). Experiments were performed by Ms. Karin Flicker and Ms. Upeka Senanayake and the analysis performed by Ms. Karin Flicker.

Table 5: Conditions for Alu and RsaI restriction endonuclease digestion

37C	2-5 hours
65C	20 minutes
8C	Forever

mRNA qRT-PCR

500 ng of total RNA were reverse transcribed into cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed with primers specific for HOXC6 V1, V2 and 18s RNA (Table 6). All reactions were carried out in triplicates. qRT-PCR was carried out on a 7900T Fast real time PCR system (Applied Biosystems) using SYBR Green as the detection fluorophore. 18s RNA was used as the internal control. Relative gene expression levels were calculated as delta delta CT.

CNV assay

Copy number analysis of *HOXC4* 5, 6 was performed using the TaqMan® Copy Number Assay (Applied Biosystems). Genomic DNA was isolated using Tissue NucleoSpin Tissue XS kit (Macherey- Nagel, Düren, Germany) according to the manufacturer's instructions. DNA samples were diluted to a concentration of 5 ng/μl. Each PCR reaction mix contained 10 μl of 2× TaqMan® Genotyping Master Mix, 1 μl of the TaqMan Copy Number target assay (Assay ID- Hs02779403_cn), 1 μl of the TaqMan Copy Number reference assay

(RNaseP), which is known to exist only in two copies in a diploid Genome, 4 µl of Nuclease-free water, and 4 µl of DNA.

The reactions were processed in an ABI 7900 real time PCR System using a 96 well reaction plate, with each DNA sample analysed in four technical replicates on 95°C/10 min for 1 cycle followed by 92°C/ 15 s and 60°C/1 min for 40 cycles. Data were collected by the SDS software (version 2.3; ABI) using the standard absolute quantification method. After the reaction, raw data were analysed using a manual cycle threshold (CT) of 0.2 with the automatic baseline on, and then imported to the CopyCaller™ Software (version 1.0; ABI) for data analysis. The software estimates copy numbers compared to a calibrator sample with a known copy number of HOXC4, 5, 6. The analytical setting was identical for all experiments.

Affymetrix Genechip array

Transient transfections were performed using 2µg plasmid containing the open reading frame of HOXC6 V1 (Origene, Rockville, MD, USA), HOXC6 V2 (Origene) and pMAxFP-green-N containing a GFP (Amara, Cologne, Germany) plasmids as described in chapter 1.1. Total RNA was isolated 24 hours after transfections and DNA digestion was performed.

The quality checks for RNA and the Arrays were performed at the ZMF core facility, Medical University of Graz.

Data were scanned using the Affymetrix Scanner: GCS3000; AGCC (Command Console Software AGCC_3_1_1.), data processing was performed using the Gene expression Console v.1.1, Affymetrix (Expression Console EC_1_1_2) with Affymetrix default analysis settings for the generation of CEL-files.

Data pre-processing and filtering was performed by the partek Software, v.6.5: RMA (background correction, quantile normalization across all chips in the experiment, log2 transformation, median polish summarization); paired t-test was used to compare the gene expression differences in between groups. Arrays and analysis were performed by Ms. Karin Wagner at the medical University of Graz.

Results

HOXC6 is amplified in the majority of nephroblastomas and NRs.

Seven tumours including 4 mixed type tumours, 2 blastema type and 1 stroma type tumour were analysed by aCGH. In addition to the tumour samples, corresponding ILNR of 7 tumours, incidentally identified ILNR and an incidentally identified adenomatous PLNR were also included in the analysis.

Gains at chromosomes 1, 12, 16, 17, 19 were most often observed in tumour samples and NRs, whereas other changes only occurred in a low percentage of cases. Amplification of chromosome 22 was observed in NRs (Figure 25, 26, 27).

Amplified regions were further studied to identify genes of interest which might be involved in the pathogenesis of nephroblastomas. *HOXC6* was amplified in 4 tumours and 5 NRs (Figure 28). Confirmation of the amplified regions was performed by CNV assay using probes, which identify *HOXC4*, 5, 6 regions. The comparison was possible for 5 tumors (Figure 29A and 29C) and 8 NRs (Figure 29B and 29C) due to availability of DNA samples. Amplifications of *HOXC6* were identified with the CNV assay as observed with aCGH (Figure 29C.). One NR had 391 copies according to CNV assay (not included in the chart).

CNV assays were performed for 57 additional tumours including 17 mixed-type, 11 blastema-type, 7 epithelial-type, 9 stroma-type, 1 regressive, 7 with diffuse anaplasia, 3 with focal anaplasia and 2 tumour samples for which the morphological type was not available as well as 6 NRs (see figure 3C). Twenty out of 57 tumours and 3 out of 6 NRS showed an amplification of *HOXC6*. Seven tumours and one NR had deletions in this chromosomal region (Figure 29C).

HOXC6 V1 and HOXC6 V2 mRNA are differentially expressed in nephroblastomas

mRNA expression of *HOXC6* V1 and V2 in tumours was analysed by qRT-PCR in 26 tumours. Comparison of *HOXC6* aCGH results with the mRNA expression was possible in 5 tumours (3 mixed-type, 1 blastema-type and 1 stroma-type nephroblastomas). Results revealed that both variants were upregulated in all 3 cases with amplifications. One tumour with a balanced profile demonstrated a very low expression of both variants and the other tumour with a balanced profile showed overexpression of V2.

Further 21 tumours (3 blastema-type, 3 epithelial-type, 4 stroma-type, 9 mixed-type and 2 regressive-tumours) were analysed for *HOXC6*V1 and V2 mRNA expression and compared to 5 samples of renal parenchyma. The expression patterns of *HOXC6* variants differentiated in the tumours, 40% of tumours demonstrated high expression of both variants compared to mature kidney (Figure 30). One tumour had an extremely high expression of V2. Out of these 21 tumours, mRNA expression of 17 tumours was compared with the results of CNV assay. 75% of nephroblastomas showing amplification in the *HOXC6* region had an upregulation of one or both variants of *HOXC6* mRNA.

***HOXC6* V1 and V2 play a different role in regulating genes in a nephroblastoma cell line.**

HOXC6 V1, V2 and a control were transiently overexpressed in the epithelial nephroblastoma cell line WT-CLS1. Total RNA was isolated after 24 hours of transfections. Affymetrix array was performed to compare the common and different target genes of V1 and V2. Most of the genes were upregulated or downregulated by both variants (Table 7 and 8).

Table 6: Primer sequences

	Forward	Reverse
<i>HOXC6</i> V1	5' CCTTCCTTATCCTGCCACCT 3'	5'GCTGGAACCTGAACACGACAT 3'
<i>HOXC6</i> V2	5' GGGAGAGAGGGAGAGTGACA 3'	5' TCATAGGCGGTGGAATTGAG 3'

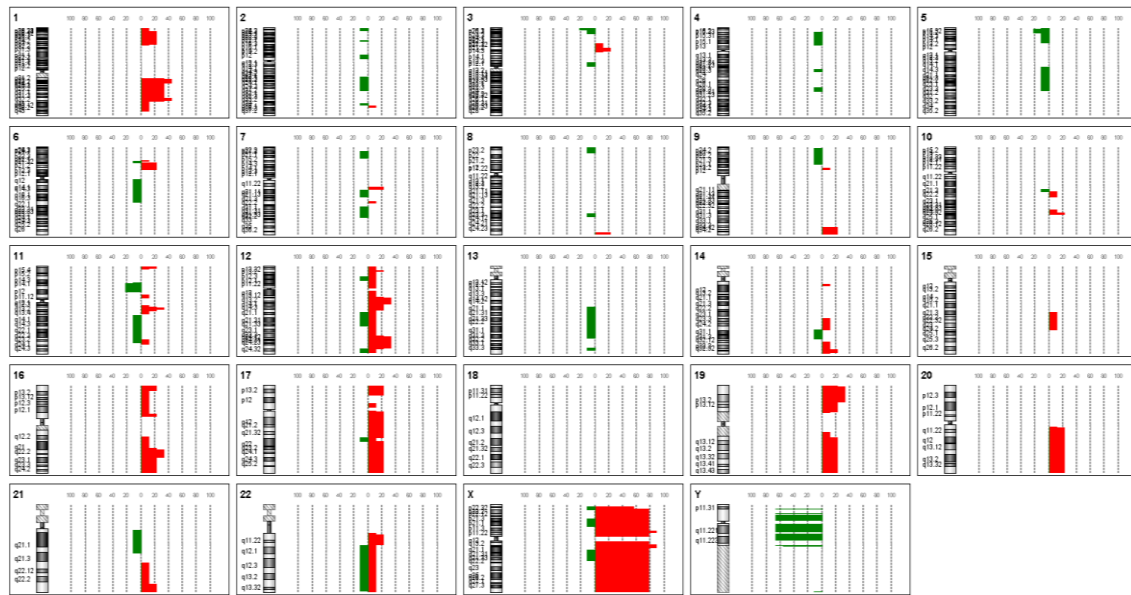


Figure 25:

Genomic aberrations in nephroblastomas detected by array CGH. The ideogram summarizes chromosomal gains and losses of 7 patients. The green and red bars on the either side of each chromosome indicate how often copy number changes were found. Losses are displayed in green in the left half of each figure while gains are shown in red in the right half. Gains at chromosomes 1, 12, 16, 17, 19 were most often observed, whereas other changes only occurred in a low percentage of cases. Experiments performed by Ms. Karin Flicker and Ms. Upeka Senanayake. Data was analysed by Ms. Karin Flicker.

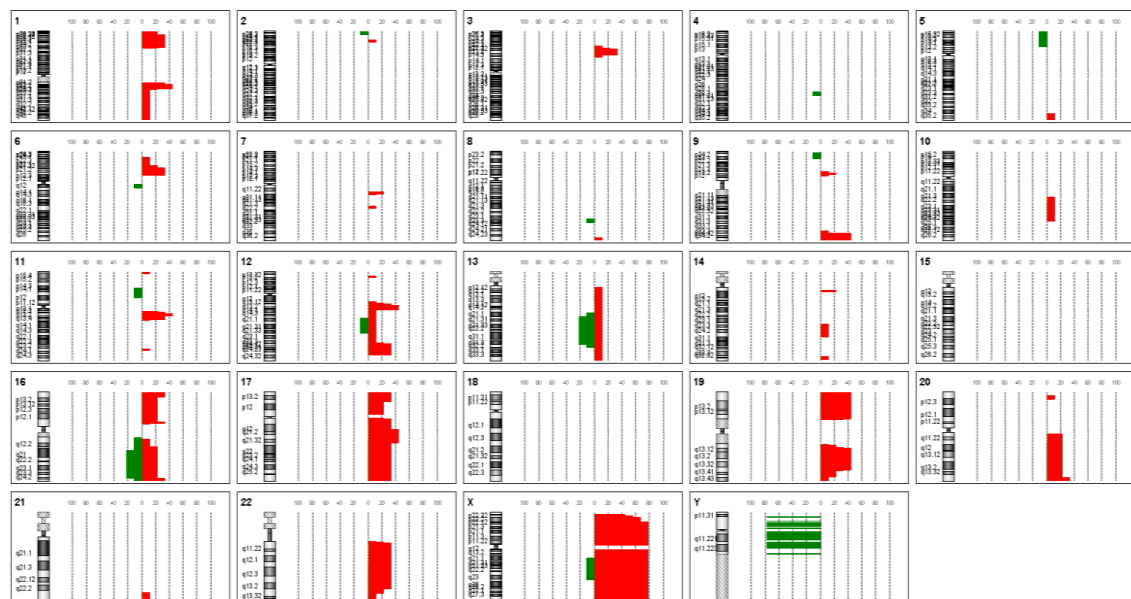


Figure 26:

Genomic aberrations in NR detected by array CGH. The ideogram summarizes chromosomal gains and losses of 9 patients. The green and red bars on the either side of each chromosome indicate how often copy number changes were found. Losses are displayed in green in the left half of each figure, and gains are shown in red in the right half. Gains at chromosomes 1, 12, 16, 17, 19, 22 were most often observed, whereas other changes only occurred in a low percentage of cases. Experiments performed by Ms. Karin Flicker and Ms. Upeka Senanayake. Data was analysed by Ms. Karin Flicker.

All sample overlay

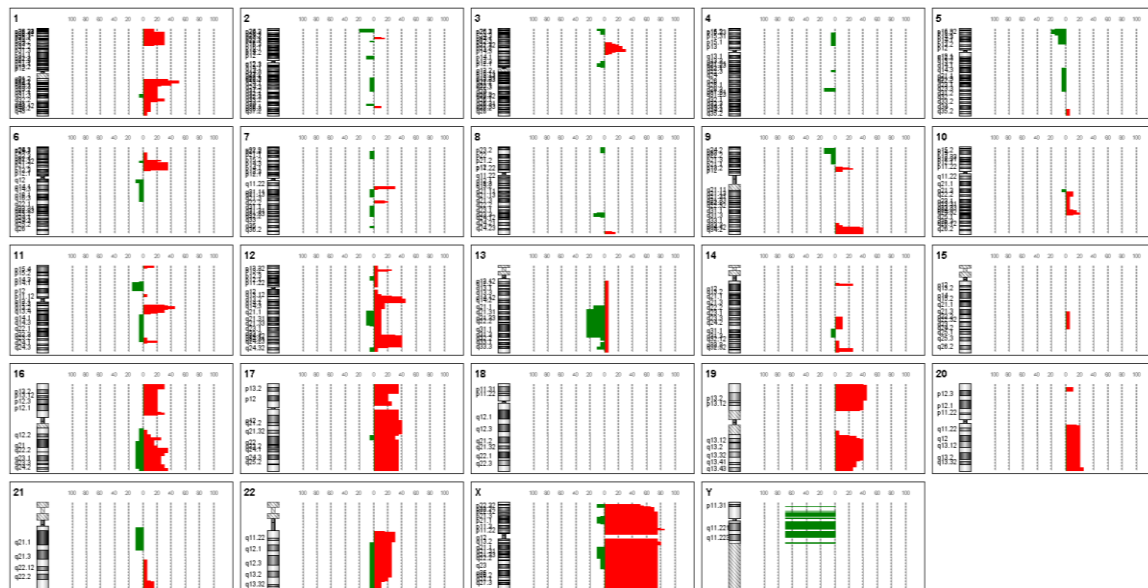


Figure 27: Overlay of all the samples (nephroblastomas and NR) indicates the areas, which may harbour an oncogene important for development of nephroblastomas. Experiments performed by Ms. Karin Flicker and Ms. Upeka Senanayake. Data was analysed by Ms. Karin Flicker.

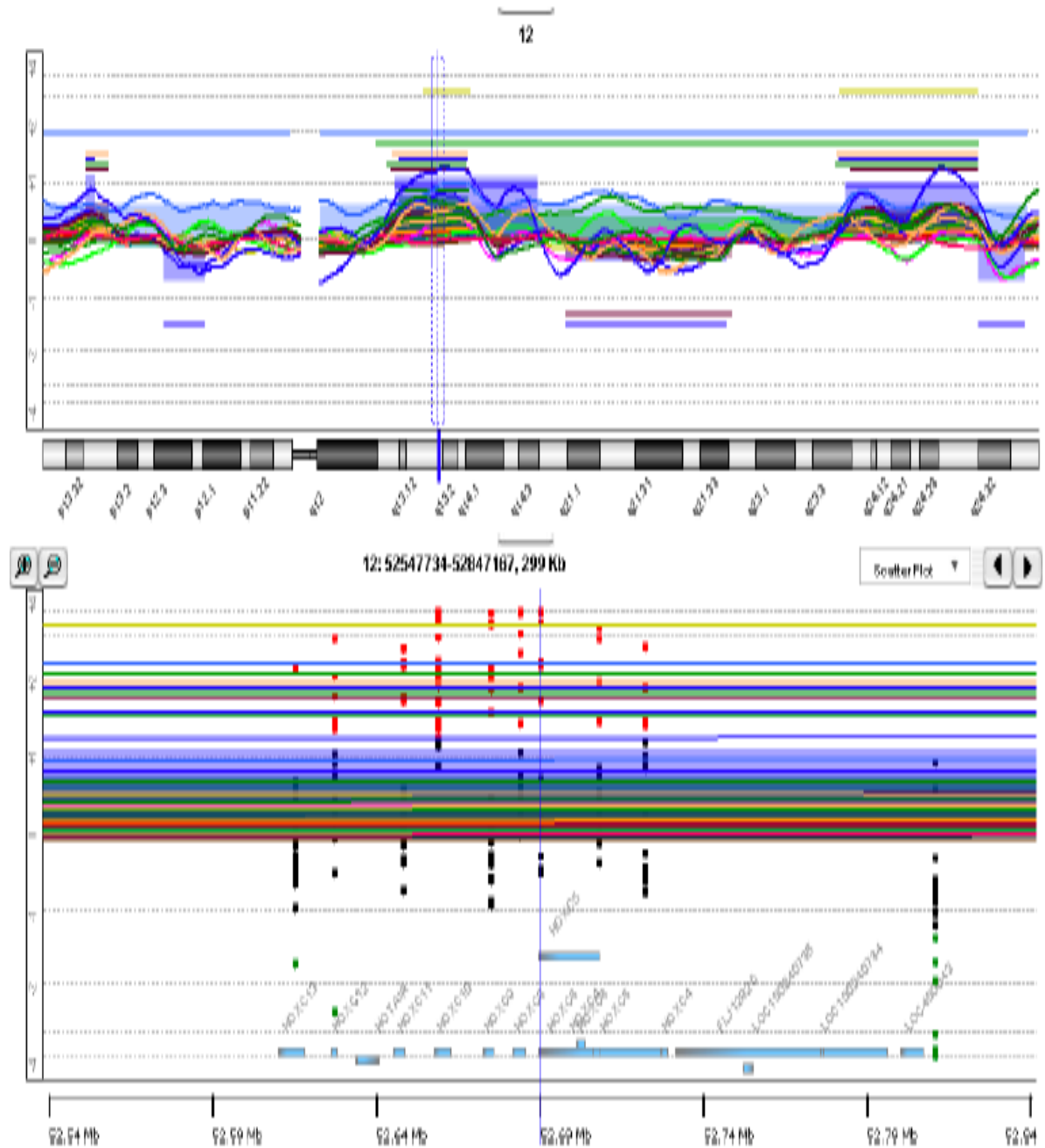


Figure 28: Overlay of amplified *HOXC6* regions (12q13.3) in tumours and NRs. Experiments performed by Ms. Karin Flicker and Ms. Upeka Senanayake. Data analysed by Ms. Karin Flicker.

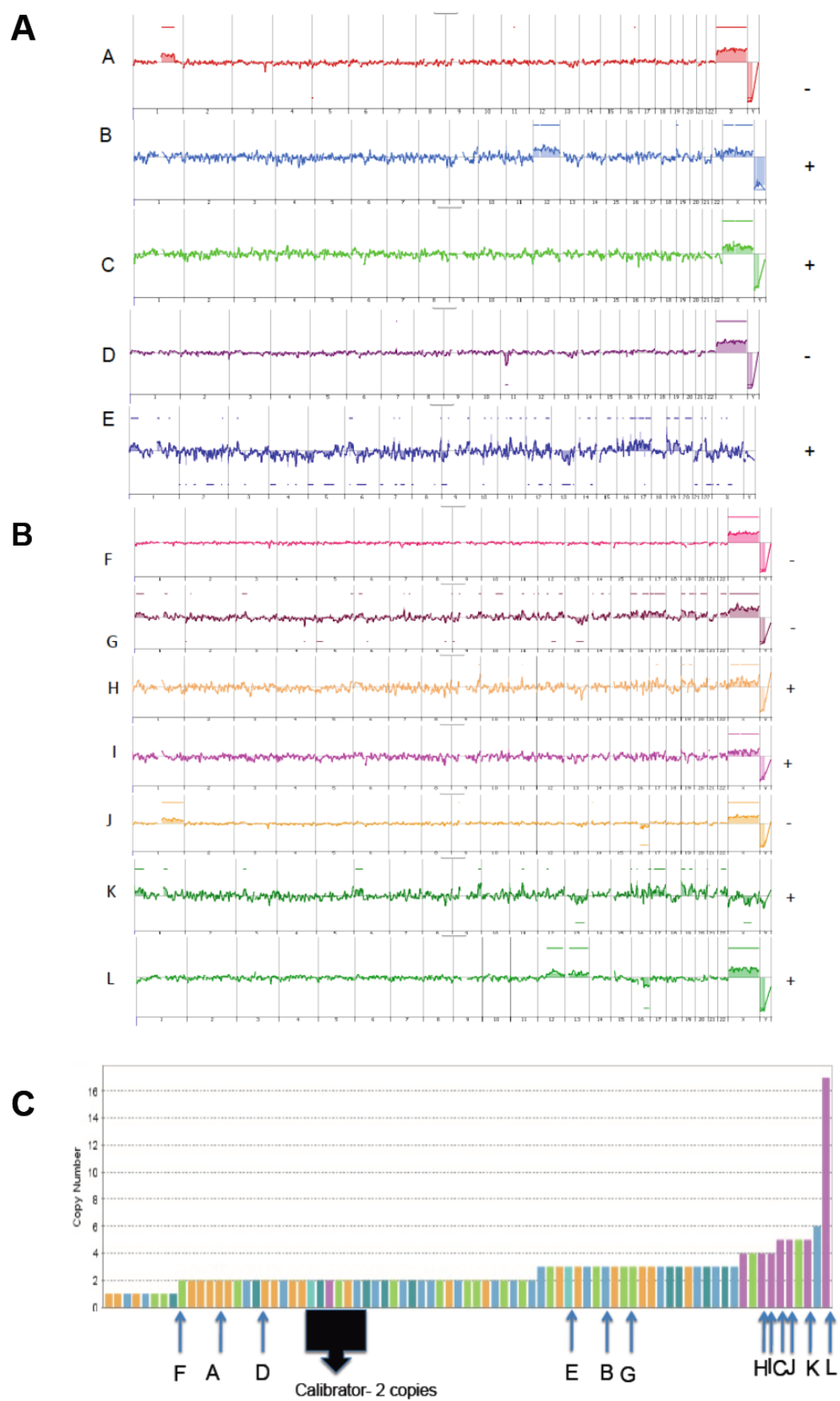


Figure 29: Amplification of chromosome 12q13.13 was identified in nephroblastomas (Figure A (A-E) and NRs (Figure B (F-L) + indicate gains, - indicate balanced profile). Experiments performed by Ms. Karin Flicker and Ms. Upeka Senanayake. Data was analysed by Ms. Karin Flicker. Nephroblastomas were also analysed by a CNV assay to identify the aberrations in *HOXC6* including the samples used for aCGH (Figure C). aCGH results were concordant with the CNV assay results except for 2 NRs.

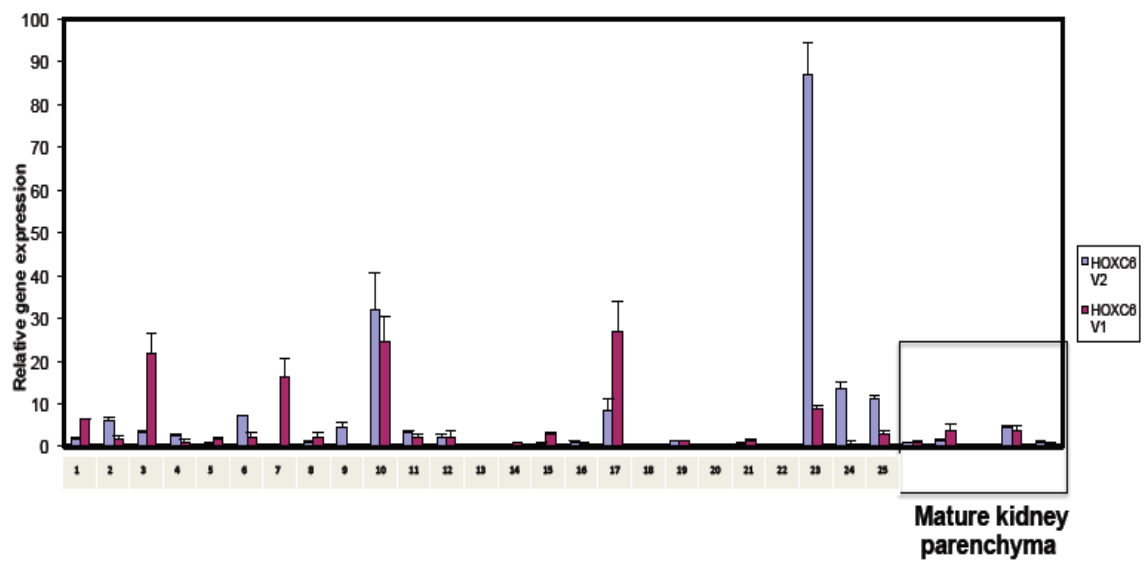


Figure 30: Relative *HOXC6* V1 and V2 mRNA expression was analysed in nephroblastomas and mature renal parenchyma by qRT-PCR. Differential expression of V1 and V2 was identified for each tumour (vertical bars represent + SEM)

gene_assignment	Gene Symbol	RefSeq	V2 and V1 vs. Ctrl			V2 vs. V1			V1 vs. Ctrl			V2 vs. Ctrl		
			p-value V2 vs V1 vs. Ctrl	Fold- Change V2 vs V1 vs. Ctrl	Fold-Change V2 and V1 vs. Ctrl (Description)	p-value V2 vs. V1	Fold- Change V2 vs. V1	Fold-Change V2 vs V1 (Description)	p-value V1 vs. Ctrl	Fold- Change V1 vs. Ctrl	Fold-Change V1 vs Ctrl (Description)	p-value V2 vs. Ctrl	Fold- Change V2 vs. Ctrl	Fold-Change V2 vs. Ctrl (Description)
NM_016932 // SIX2 // SIX homeobox 2 // 2p16-p15 // 10736 // ENTSIX2	NM_016932		1.46E-06	-13.44	V2 and V1 down vs Ctrl	0.842626	-1.03	V2 down vs V1	3.54E-06	-13.22	V1 down vs Ctrl	3.29E-06	-13.66	V2 down vs Ctrl
NM_003810 // TNFSF10 // tumor necrosis factor (ligand) superfamily, TNFSF10	NM_003810		8.52E-07	-8.45	V2 and V1 down vs Ctrl	2.44E-06	7.86	V2 up vs V1	1.93E-07	-23.68	V1 down vs Ctrl	9.18E-05	-3.01	V2 down vs Ctrl
NM_001773 // CD34 // CD34 molecule // 1q32 // 947 // NM_0010251CD34	NM_001773		2.81E-09	-7.99	V2 and V1 down vs Ctrl	2.18E-10	39.45	V2 up vs V1	1.49E-10	-50.15	V1 down vs Ctrl	0.001708	-1.27	V2 down vs Ctrl
NM_006424 // SLC34A2 // solute carrier family 34 (sodium phosphate)SLC34A2	NM_006424		1.02E-08	-7.20	V2 and V1 down vs Ctrl	1.19E-09	26.06	V2 up vs V1	6.53E-10	-36.73	V1 down vs Ctrl	0.000621	-1.41	V2 down vs Ctrl
NM_001142966 // GREB1L // growth regulation by estrogen in breast GREB1L	NM_001142966		2.39E-07	-5.91	V2 and V1 down vs Ctrl	3.7E-08	16.56	V2 up vs V1	1.75E-08	-24.07	V1 down vs Ctrl	0.003514	-1.45	V2 down vs Ctrl
NM_001128310 // SPARC1L // SPARC-like 1 (hevin) // 4q22.1 // 8404 SPARC1L	NM_001128310		2.78E-06	-5.85	V2 and V1 down vs Ctrl	5.16E-05	3.44	V2 up vs V1	1.11E-06	-10.84	V1 down vs Ctrl	7.74E-05	-3.16	V2 down vs Ctrl
NM_018672 // ABCA5 // ATP-binding cassette, sub-family A (ABCA1), iABCA5	NM_018672		3.01E-08	-5.40	V2 and V1 down vs Ctrl	4.7E-09	14.25	V2 up vs V1	2.2E-09	-20.40	V1 down vs Ctrl	0.00056	-1.43	V2 down vs Ctrl
NM_015345 // DAM2 // dishevelled associated activator of morphogDAM2	NM_015345		5.92E-07	-4.85	V2 and V1 down vs Ctrl	2.59E-07	8.13	V2 up vs V1	6.73E-06	-13.82	V1 down vs Ctrl	0.000701	-1.70	V2 down vs Ctrl
NM_001685 // CRYAB // crystallin, alpha B // 11q22.3-q23.1 // 1410 // CRYAB	NM_001685		3.99E-07	-4.58	V2 and V1 down vs Ctrl	4.21E-06	3.26	V2 up vs V1	1.34E-07	-8.27	V1 down vs Ctrl	1.67E-05	-2.54	V2 down vs Ctrl
---	---		0.000542	-3.96	V2 and V1 down vs Ctrl	0.030934	1.95	V2 up vs V1	0.000365	-5.53	V1 down vs Ctrl	0.004624	-2.84	V2 down vs Ctrl
NM_152390 // TMEM178 // transmembrane protein 178 // 2p22.1 // 11TMEM178	NM_152390		9.1E-06	-3.68	V2 and V1 down vs Ctrl	5.59E-06	5.13	V2 up vs V1	1.21E-06	-8.33	V1 down vs Ctrl	0.004353	-1.62	V2 down vs Ctrl
NM_001130064 // GAP43 // growth associated protein 43 // 3q13.1-q1GAP43	NM_001130064		0.000424	-3.24	V2 and V1 down vs Ctrl	0.021056	1.82	V2 up vs V1	0.000268	-4.38	V1 down vs Ctrl	0.004062	-2.40	V2 down vs Ctrl
NM_152666 // PLD5 // phospholipase D family, member 5 // 1q43 // 2PLD5	NM_152666		4E-05	-3.01	V2 and V1 down vs Ctrl	7.87E-05	3.10	V2 up vs V1	8.37E-06	-5.30	V1 down vs Ctrl	0.004107	-1.71	V2 down vs Ctrl
NM_000478 // ALPL // alkaline phosphatase, liver/bone/kidney // 1p36ALPL	NM_000478		3.14E-06	-2.90	V2 and V1 down vs Ctrl	2.65E-05	2.35	V2 up vs V1	1E-06	-4.45	V1 down vs Ctrl	0.000138	-1.89	V2 down vs Ctrl
NM_002038 // IFI6 // interferon, alpha-inducible protein 6 // 1p35 // 25IFI6	NM_002038		5.52E-06	-2.90	V2 and V1 down vs Ctrl	6.42E-06	3.32	V2 up vs V1	9.27E-07	-5.29	V1 down vs Ctrl	0.001281	-1.59	V2 down vs Ctrl
NM_198401 // ANKRD46 // ankyrin repeat domain 46 // 8q22.2 // 157ANKRD46	NM_198401		4.56E-07	-2.79	V2 and V1 down vs Ctrl	8.8E-06	4.76	V2 up vs V1	3.68E-06	-6.09	V1 down vs Ctrl	0.001192	-1.23	V2 down vs Ctrl
---	---		0.000454	-2.79	V2 and V1 down vs Ctrl	0.275577	1.23	V2 up vs V1	0.000601	-3.09	V1 down vs Ctrl	0.001728	-2.51	V2 down vs Ctrl
NM_080282 // ABCA10 // ATP-binding cassette, sub-family A (ABCA1),ABCA10	NM_080282		2.98E-05	-2.71	V2 and V1 down vs Ctrl	0.000111	2.33	V2 up vs V1	8.8E-06	-4.14	V1 down vs Ctrl	0.001384	-1.78	V2 down vs Ctrl
NM_015570 // AUTS2 // autism susceptibility candidate 2 // 7q11.22 iAUTS2	NM_015570		2.81E-10	-2.69	V2 and V1 down vs Ctrl	1.67E-11	6.24	V2 up vs V1	1.31E-11	-6.73	V1 down vs Ctrl	0.002084	-1.08	V2 down vs Ctrl
NM_000533 // PLP1 // proteolipid protein 1 // Xq22 // 5354 // NM_001PLP1	NM_000533		9.94E-05	-2.53	V2 and V1 down vs Ctrl	0.001719	1.88	V2 up vs V1	4.25E-05	-3.48	V1 down vs Ctrl	0.002032	-1.85	V2 down vs Ctrl
NM_080284 // ABCA6 // ATP-binding cassette, sub-family A (ABCA1), iABCA6	NM_080284		2.28E-05	-2.46	V2 and V1 down vs Ctrl	0.000201	2.03	V2 up vs V1	7.64E-06	-3.51	V1 down vs Ctrl	0.000827	-1.73	V2 down vs Ctrl
NM_001142343 // CMKLR1 // chemokine-like receptor 1 // 12q24.1 // CMKLR1	NM_001142343		0.000132	-2.39	V2 and V1 down vs Ctrl	0.001728	1.87	V2 up vs V1	5.29E-05	-3.27	V1 down vs Ctrl	0.002988	-1.75	V2 down vs Ctrl
NM_001135091 // MUC15 // mucin 15, cell surface associated // 1p1MUC15	NM_001135091		7.81E-05	-2.29	V2 and V1 down vs Ctrl	0.00116	1.79	V2 up vs V1	3.19E-05	-3.07	V1 down vs Ctrl	0.001778	-1.71	V2 down vs Ctrl
---	---		9.11E-05	-2.21	V2 and V1 down vs Ctrl	0.000431	2.00	V2 up vs V1	2.63E-05	-3.12	V1 down vs Ctrl	0.004073	-1.66	V2 down vs Ctrl
NM_001004019 // FBIN2 // fibulin 2 // 3p25.1 // 2199 // NM_001998 //FBIN2	NM_001004019		9.94E-06	-2.20	V2 and V1 down vs Ctrl	1.97E-05	2.24	V2 up vs V1	2.03E-06	-3.29	V1 down vs Ctrl	0.001238	-1.47	V2 down vs Ctrl
NM_172037 // RDH10 // retinol dehydrogenase 10 (all-trans) // 8q21.1RDH10	NM_172037		1.8E-06	-2.16	V2 and V1 down vs Ctrl	5.02E-07	3.01	V2 up vs V1	1.71E-07	-3.75	V1 down vs Ctrl	0.004302	-1.24	V2 down vs Ctrl
NM_145799 // SEPT6 // septin 6 // Xq24 // 23157 // NM_145800 // SE	NM_145799		4.29E-08	-2.14	V2 and V1 down vs Ctrl	1.34E-08	2.91	V2 up vs V1	4.22E-09	-3.65	V1 down vs Ctrl	0.000119	-1.26	V2 down vs Ctrl
NM_001111283 // IGF1 // insulin-like growth factor 1 (somatomedin C)IGF1	NM_001111283		0.000165	-2.08	V2 and V1 down vs Ctrl	0.001305	1.78	V2 up vs V1	5.71E-05	-2.77	V1 down vs Ctrl	0.004757	-1.56	V2 down vs Ctrl
NM_021800 // DNAJC12 // DnaJ (Hsp40) homolog, subfamily C, memberDNAJC12	NM_021800		1.87E-06	-2.06	V2 and V1 down vs Ctrl	4.89E-06	2.03	V2 up vs V1	4.11E-07	-2.94	V1 down vs Ctrl	0.000208	-1.45	V2 down vs Ctrl
NM_001014811 // ME3 // malic enzyme 3, NAD(P)+dependent, mitochondME3	NM_001014811		3.76E-07	-2.06	V2 and V1 down vs Ctrl	7.3E-08	3.00	V2 up vs V1	3.04E-08	-3.57	V1 down vs Ctrl	0.002676	-1.19	V2 down vs Ctrl

Table 7: *HOXC6* V1, V2 genes were transiently overexpressed in the cell line WT-CLS1. Total RNA was isolated 24 hours after transfections followed by cDNA microarray. Commonly downregulated genes by *HOXC6* V1 and V2 compared to control were identified. V1 has more impact on downregulating genes compared to V2.

gene_assignment	Gene Symbol	RefSeq	V2 and V1 vs. Ctrl			V2 vs. V1			V1 vs. Ctrl			V2 vs. Ctrl		
			p-value V2 vs. V1 vs. Ctrl	Fold- Change V2 vs. V1 vs. Ctrl	Fold-Change V2 and V1 vs. Ctrl (Description)	p-value V2 vs. V1 vs. Ctrl	Fold- Change V2 vs. V1 (Description)	Fold-Change V2 vs. V1 (Description)	p-value V1 vs. Ctrl vs. Ctrl	Fold- Change V1 vs. Ctrl (Description)	Fold-Change V1 vs. Ctrl (Description)	p-value V2 vs. Ctrl vs. Ctrl	Fold- Change V2 vs. Ctrl (Description)	Fold-Change V2 vs. Ctrl (Description)
NM_018099 // F1F1 // fatty acyl CoA reductase 2 // 12p11.22 // 55711 // FAR2	NM_018099		3.21E-05	10.00	V2 and V1 up vs Ctrl	0.000155	-7.50	V2 down vs V1	9.02E-06	27.39	V1 up vs Ctrl	0.001663	3.65	V2 up vs Ctrl
NM_006211 // PENK // proenkephalin // 8q23-q24 // 5179 // NM_00113FENK	NM_006211		6.95E-06	7.76	V2 and V1 up vs Ctrl	3.91E-05	-5.79	V2 down vs V1	1.99E-06	18.68	V1 up vs Ctrl	0.000381	3.22	V2 up vs Ctrl
NM_003866 // INP4B // inositol polyphosphate 4-phosphatase, type I // INP4B	NM_003866		3.0010E-05	7.62	V2 and V1 up vs Ctrl	0.000476	-5.96	V2 down vs V1	2.99E-05	11.89	V1 up vs Ctrl	0.004723	3.12	V2 up vs Ctrl
NM_005215 // DOC // deleted in colorectal carcinoma // 16p21.3 // 1630 // DOC	NM_005215		1.91E-07	6.62	V2 and V1 up vs Ctrl	4.21E-07	-6.78	V2 down vs V1	3.92E-06	17.20	V1 up vs Ctrl	2.81E-05	2.55	V2 up vs Ctrl
NM_001221 // CALM1 // calcium/calmodulin-dependent protein kinase C subunit 1	NM_001221		1.41E-07	5.94	V2 and V1 up vs Ctrl	2.08E-06	-3.70	V2 down vs V1	5.15E-06	18.51	V1 up vs Ctrl	4.95E-06	3.09	V2 up vs Ctrl
NM_00104587 // SLPN1 // schlafen family member 1 // 11p12.1 // 9165 // SLPN1	NM_00104587		1.45E-06	5.84	V2 and V1 up vs Ctrl	2.62E-06	-6.25	V2 down vs V1	2.75E-07	14.61	V1 up vs Ctrl	0.00022	2.34	V2 up vs Ctrl
NM_001058 // TACR1 // tachykinin receptor 1 // 2p12.1 // 6869 // NM_015TACR1	NM_001058		1.78E-05	5.70	V2 and V1 up vs Ctrl	0.000124	-4.19	V2 down vs V1	5.53E-06	11.67	V1 up vs Ctrl	0.000772	2.79	V2 up vs Ctrl
NM_016341 // PLEC1 // phospholipase C, epsilon // 1 // 10q23.1 // 51196 // PLEC1	NM_016341		1.06E-08	5.36	V2 and V1 up vs Ctrl	8.38E-09	-5.00	V2 down vs V1	1.56E-09	14.87	V1 up vs Ctrl	5.76E-06	1.96	V2 up vs Ctrl
NM_008229 // FBN3 // F-box protein 32 // 8q24.13 // 114907 // NM_1FBX032	NM_008229		7.42E-06	5.14	V2 and V1 up vs Ctrl	0.000157	-3.04	V2 down vs V1	3.11E-06	16.67	V1 up vs Ctrl	0.000182	2.95	V2 up vs Ctrl
NM_021072 // HON1 // hyperlocomotion activated cyclic nucleotide-gated ion channel 1	NM_021072		2.86E-08	5.10	V2 and V1 up vs Ctrl	1.31E-06	-2.69	V2 down vs V1	1.39E-08	8.38	V1 up vs Ctrl	5.86E-07	3.11	V2 up vs Ctrl
NM_003271 // AGPAT9 // 1-acylglycerol-3-phosphate O-acyltransferase AGPAT9	NM_003271		2.92E-05	4.88	V2 and V1 up vs Ctrl	4.01E-05	-6.66	V2 down vs V1	5.34E-06	11.62	V1 up vs Ctrl	0.004426	2.05	V2 up vs Ctrl
NM_001167674 // CADM2 // cell adhesion molecule 2 // 3p12.1 // 25355 // CADM2	NM_001167674		3.93E-06	4.88	V2 and V1 up vs Ctrl	3.63E-05	-3.49	V2 down vs V1	1.29E-06	9.11	V1 up vs Ctrl	0.000161	2.61	V2 up vs Ctrl
NM_001136529 // SERPINE2 // serpin peptidase inhibitor, clade E (nexin) SERPINE2	NM_001136529		4.08E-06	4.88	V2 and V1 up vs Ctrl	2.82E-05	-3.72	V2 down vs V1	1.23E-06	9.40	V1 up vs Ctrl	0.002022	2.53	V2 up vs Ctrl
NM_001013732 // C6orf138 // chromosome 6 open reading frame 138 // C6orf138	NM_001013732		1.82E-07	4.84	V2 and V1 up vs Ctrl	2.97E-06	-3.12	V2 down vs V1	6.84E-08	8.55	V1 up vs Ctrl	5.99E-06	2.74	V2 up vs Ctrl
NM_139072 // DNER // delta-homophilic-like EGF repeat containing // 2q36.3 // DNER	NM_139072		2.94E-06	4.79	V2 and V1 up vs Ctrl	9.41E-06	-4.41	V2 down vs V1	6.95E-07	10.07	V1 up vs Ctrl	0.00266	2.28	V2 up vs Ctrl
NM_004172 // SLC1A3 // solute carrier family 1 (glial high affinity glutamate) SLC1A3	NM_004172		2.06E-05	3.77	V2 and V1 up vs Ctrl	0.000917	2.77	V2 down vs V1	9.33E-07	7.95	V1 up vs Ctrl	0.00043	1.69	V2 up vs Ctrl
NM_178565 // RSPD2 // R-spondin 2 homolog (Xenopus laevis) // 8q23 // RSPD2	NM_178565		2.87E-06	4.69	V2 and V1 up vs Ctrl	1.68E-05	-4.12	V2 down vs V1	1.23E-06	9.53	V1 up vs Ctrl	0.000365	2.31	V2 up vs Ctrl
NM_001259 // CDK6 // cyclin-dependent kinase 6 // 12q12-q21 // 1021 // CDK6	NM_001259		4.82E-07	4.63	V2 and V1 up vs Ctrl	5.82E-07	-5.55	V2 down vs V1	8.05E-08	9.91	V1 up vs Ctrl	0.000131	1.97	V2 up vs Ctrl
NM_024081 // PRRG4 // proline rich Gln (G-carboxyglutamic acid) 4 (rat) PRRG4	NM_024081		8.40E-06	4.56	V2 and V1 up vs Ctrl	0.000354	-2.48	V2 down vs V1	4.23E-06	7.19	V1 up vs Ctrl	0.000149	2.90	V2 up vs Ctrl
NM_012454 // TIAM2 // T-cell lymphoma invasion and metastasis 2 // 6q1TAM2	NM_012454		1.07E-07	4.54	V2 and V1 up vs Ctrl	2.22E-07	-4.88	V2 down vs V1	2.19E-08	9.81	V1 up vs Ctrl	1.69E-05	2.10	V2 up vs Ctrl
NM_003474 // ADAMT2 // ADAM metalloproteinase domain 12 // 10q26.3 // ADAMT2	NM_003474		1.89E-06	4.53	V2 and V1 up vs Ctrl	1.88E-06	-5.73	V2 down vs V1	2.99E-07	10.85	V1 up vs Ctrl	0.000587	1.89	V2 up vs Ctrl
NM_015071 // ARHGAP28 // Rho GTPase activating protein 28 // 5p1 // ARHGAP28	NM_015071		1.55E-05	4.48	V2 and V1 up vs Ctrl	1.03E-06	-4.40	V2 down vs V1	2.08E-07	11.39	V1 up vs Ctrl	0.00065	1.77	V2 up vs Ctrl
NM_014782 // ARMC2 // armadillo repeat containing, X-linked 2 // Xq21 // ARMCX2	NM_014782		5.45E-06	4.37	V2 and V1 up vs Ctrl	9.76E-06	-4.67	V2 down vs V1	1.07E-06	9.45	V1 up vs Ctrl	0.0008	2.02	V2 up vs Ctrl
NM_012082 // ZFPM2 // zinc finger protein, multiple 2 // 8q23 // 23414 // ZFPM2	NM_012082		4.07E-05	4.33	V2 and V1 up vs Ctrl	0.000558	-2.88	V2 down vs V1	1.58E-05	7.35	V1 up vs Ctrl	0.001068	2.55	V2 up vs Ctrl
NM_005592 // MUSK // muscle, skeletal, receptor tyrosine kinase // 9q31MUSK	NM_005592		8.31E-07	4.28	V2 and V1 up vs Ctrl	2.83E-06	-3.92	V2 down vs V1	1.99E-07	6.48	V1 up vs Ctrl	7.72E-05	2.16	V2 up vs Ctrl
NM_0020310 // LIFR // leukemia inhibitory factor receptor alpha // 5p13p // LIFR	NM_0020310		4.74E-06	4.12	V2 and V1 up vs Ctrl	8.31E-05	-2.71	V2 down vs V1	1.88E-06	8.78	V1 up vs Ctrl	0.000132	2.50	V2 up vs Ctrl
NM_002223 // KRT12 // keratin 12 // 17p12 // 3859 // ENST0000025164KRT12	NM_002223		3.95E-08	4.07	V2 and V1 up vs Ctrl	5.84E-09	-3.36	V2 down vs V1	2.74E-08	12.46	V1 up vs Ctrl	0.000871	1.33	V2 up vs Ctrl
NM_005715 // UST // uronyl-2-sulfatase // 6q25.1 // 10090 // ENJUS1	NM_005715		5.72E-07	4.06	V2 and V1 up vs Ctrl	7.65E-07	-4.67	V2 down vs V1	9.95E-08	7.18	V1 up vs Ctrl	0.000136	1.88	V2 up vs Ctrl
NM_003999 // OSMR // oncostatin M receptor // 5p13 // 9180 // NM_0 // OSMR	NM_003999		1.91E-06	4.05	V2 and V1 up vs Ctrl	1.47E-05	-3.13	V2 down vs V1	5.92E-07	8.76	V1 up vs Ctrl	9.23E-05	2.29	V2 up vs Ctrl
NM_004840 // ARHGFE // Rac/Cdc42 guanine nucleotide exchange factor ARHGFE	NM_004840		3.41E-05	4.04	V2 and V1 up vs Ctrl	0.000121	-3.64	V2 down vs V1	8.66E-06	7.71	V1 up vs Ctrl	0.002022	2.12	V2 up vs Ctrl
NM_001338 // CXADR // cossack virus and adenovirus receptor // 21q // CXADR	NM_001338		2.97E-07	4.02	V2 and V1 up vs Ctrl	1.05E-06	-3.66	V2 down vs V1	7.14E-08	7.69	V1 up vs Ctrl	2.78E-05	2.10	V2 up vs Ctrl
NM_006206 // PDGFRA // platelet-derived growth factor receptor, family A PDGFRA	NM_006206		3.35E-07	3.97	V2 and V1 up vs Ctrl	6.24E-05	-3.69	V2 down vs V1	4.82E-06	7.84	V1 up vs Ctrl	0.001536	2.07	V2 up vs Ctrl
NM_012104 // C10orf1 // chromosome 10 open reading frame 41 // 10p11 // C10orf1	NM_012104		5.13E-07	3.91	V2 and V1 up vs Ctrl	2.86E-07	-5.73	V2 down vs V1	6.37E-08	9.38	V1 up vs Ctrl	0.0002	1.64	V2 up vs Ctrl
NM_001159702 // FHL1 // four and a half LIM domains 1 // Xq26 // 2273 // FHL1	NM_001159702		3.31E-07	3.86	V2 and V1 up vs Ctrl	1.33E-06	-3.43	V2 down vs V1	8.29E-05	7.15	V1 up vs Ctrl	2.78E-05	2.08	V2 up vs Ctrl
NM_199051 // FAM5C // family with sequence similarity 5, member C // FAM5C	NM_199051		1.13E-07	3.83	V2 and V1 up vs Ctrl	1.37E-06	-2.77	V2 down vs V1	3.91E-08	6.38	V1 up vs Ctrl	4.45E-06	2.30	V2 up vs Ctrl
NM_002068 // FAM38B // family with sequence similarity 38, member B // FAM38B	NM_002068		5.79E-07	3.80	V2 and V1 up vs Ctrl	3.4E-06	-3.14	V2 down vs V1	1.64E-07	6.74	V1 up vs Ctrl	3.55E-05	2.15	V2 up vs Ctrl
NM_004540 // NCAM2 // neural cell adhesion molecule 2 // 21q21.1 // 46NCAM2	NM_004540		1.10E-05	3.80	V2 and V1 up vs Ctrl	3.38E-05	-3.56	V2 down vs V1	2.62E-06	7.17	V1 up vs Ctrl	0.000923	2.01	V2 up vs Ctrl
NM_002068 // FAM38B // family with sequence similarity 38, member B // FAM38B	NM_002068		2.41E-06	3.77	V2 and V1 up vs Ctrl	2.05E-05	-2.89	V2 down vs V1	7.71E-07	6.41	V1 up vs Ctrl	0.000108	2.22	V2 up vs Ctrl
NM_001083112 // GPR12 // G-protein-coupled receptor, family C, group 5, GPR12	NM_001083112		1.24E-07	3.65	V2 and V1 up vs Ctrl	8.94E-07	-4.86	V2 down vs V1	1.51E-06	9.17	V1 up vs Ctrl	0.00362	1.69	V2 up vs Ctrl
NM_005221 // DLX5 // distal-less homeobox 5 // 7p22 // 1749 // ENST001DLX5	NM_005221		6.84E-08	3.68	V2 and V1 up vs Ctrl	9.13E-07	-2.65	V2 down vs V1	2.43E-08	5.99	V1 up vs Ctrl	2.58E-06	2.28	V2 up vs Ctrl
NM_016353 // ZDHHC2 // zinc finger, DHHC-type containing 2 // 8p21.3 // ZDHHC2	NM_016353		1.6E-06	3.66	V2 and V1 up vs Ctrl	9.24E-07	-6.18	V2 down vs V1	2.04E-07	8.34	V1 up vs Ctrl	0.001078	1.61	V2 up vs Ctrl
NM_002977 // SCN9A // sodium channel, voltage-gated, type IX, alpha // SCN9A	NM_002977		1.24E-05	3.63	V2 and V1 up vs Ctrl	4.17E-05	-3.41	V2 down vs V1	3.03E-06	6.63	V1 up vs Ctrl	0.000943	1.99	V2 up vs Ctrl
NM_002515 // TANC2 // tetrahydrocannabinol receptor, anykin repeat and coiled TANC2	NM_002515		7.04E-06	3.63	V2 and V1 up vs Ctrl	5.81E-05	-2.84	V2 down vs V1	2.28E-06	6.08	V1 up vs Ctrl	0.000297	2.16	V2 up vs Ctrl
NM_003403 // C6orf150 // chromosome 6 open reading frame 150 // 3p21 // C6orf150	NM_003403		6E-07	3.62	V2 and V1 up vs Ctrl	3.4E-06	-3.93	V2 down vs V1	4.78E-07	6.36	V1 up vs Ctrl	5.36E-06	2.06	V2 up vs Ctrl
NM_003979 // GPRC5A // G-protein-coupled receptor, family C, group 5, GPRC5A	NM_003979		6.37E-06	3.53	V2 and V1 up vs Ctrl	6.31E-06	-4.13	V2 down vs V1	8.05E-07	7.18	V1 up vs Ctrl	0.000125	1.74	V2 up vs Ctrl
NM_000201 // ICAM1 // intercellular adhesion molecule 1 // 19p13.3 // ICAM1	NM_000201		0.000113	3.53	V2 and V1 up vs Ctrl	0.00231	-2.29	V2 down vs V1	5.11E-05	5.34	V1 up vs Ctrl	0.00209	2.33	V2 up vs Ctrl
NM_198461 // LONRF2 // LON peptide-N-terminal domain and ring finger LONRF2	NM_198461		9.55E-07	3.50	V2 and V1 up vs Ctrl	5.33E-07	-4.93	V2 down vs V1	1.2E-07	7.76	V1 up vs Ctrl	0.000714	1.58	V2 up vs Ctrl
NM_001143676 // SGK1 // serum/glucocorticoid-induced kinase 1 // 6q // SGK1	NM_001143676		3.88E-05	3.49	V2 and V1 up vs Ctrl	0.000941	-2.26	V2 down vs V1	1.76E-05	5.24	V1 up vs Ctrl	0.000762	2.33	V2 up vs Ctrl
NM_002068 // FAM38B // family with sequence similarity 38, member B // FAM38B	NM_002068		8.01E-07	3.48	V2 and V1 up vs Ctrl	4.59E-06	-2.91	V2 down vs V1	2.23E-07	5.91	V1 up vs Ctrl	4.97E-05	2.03	V2 up vs Ctrl
NM_002268 // ALDH4 // aldehyde dehydrogenase 4 family, member A // ALDH4	NM_002268		3.75E-06	3.46	V2 and V1 up vs Ctrl	3.4E-06	-3.93	V2 down vs V1	4.78E-07	6.36	V1 up vs Ctrl	0.000902	1.74	V2 up vs Ctrl
NM_004263 // ATP8B4 // ATPase, class I, type 8B, member 4 // 15q21.2 // ATP8B4	NM_004263		3.57E-06	3.43	V2 and V1 up vs Ctrl	2.05E-06	-4.78	V2 down vs V1	4.57E-07	5.17	V1 up vs Ctrl	0.002173	1.57	V2 up vs Ctrl
NM_014689 // DOCK10 // dedicator of cytokinesis 10 // 2q36.2 // 55619 // DOCK10	NM_014689		1.61E-04	3.43	V2 and V1 up vs Ctrl	2.08E-07	-3.90	V2 down vs V1	2.74E-07	6.77	V1 up vs Ctrl	4.27E-05	1.73	V2 up vs Ctrl
NM_001013732 // C6orf138 // chromosome 6 open reading frame 138 // C6orf138	NM_001013732		5.93E-06	3.41	V2 and V1 up vs Ctrl	5.08E-06	-4.29	V2 down vs V1	8.88E-07	7.06	V1 up vs Ctrl	0.001974	1.65	V2 up vs Ctrl
NM_002317 // LOK // lysyl oxidase // 5q23.2 // 4015 // ENST000002310X LOK	NM_002317		2.77E-05	3.35	V2 and V1 up vs Ctrl	0.000888	-1.19	V2 down vs V1	1.29E-06	6.96	V1 up vs Ctrl	0.00091	2.26	V2 up vs Ctrl
NM_0057159 // LPAR1 // lysophosphatidic acid receptor 1 // 9q31.3 // 150PAR1	NM_0057159		2.28E-05	3.33	V2 and V1 up vs Ctrl	3.97E-05	-3.55	V2 down vs V1	4.44E-06	4.27	V1 up vs Ctrl	0.002912	1.77	V2 up vs Ctrl
NM_001130445 // SSTR2 // somatostatin receptor 2 // 10p11.2 // 130445	NM_001130445		1.59E-07	3.29	V2 and V1 up vs Ctrl	7.84E-07	-3.89	V2 down vs V1	7.84E-07	6.39	V1 up vs Ctrl	7.76E-06	1.75	V2 up vs Ctrl
NM_017933 // PDI // protein disulfide isomerase interaction domain containing 1 // PDI1	NM_017933		2.63E-06	3.22	V2 and V1 up vs Ctrl	2.88E-06	-3.80	V2 down vs V1	4.34E-07	6.29	V1 up vs Ctrl	0.000734	1.65	V2 up vs Ctrl
NM_182964 // NRG2 // neuron navigator 2 // 11p15.1 // 89797 // NM_14NANV2	NM_182964		3.7E-08	3.22	V2 and V1 up vs Ctrl	2.83E-08	-4.11	V2 down vs V1	5.19E-09	6.53	V1 up vs Ctrl	2.06E-05	1.59	V2 up vs Ctrl
NM_002736 // PKARZB // protein kinase, cAMP-dependent, regulatory PKARZB	NM_002736		3.64E-07	3.22	V2 and V1 up vs Ctrl	1.01E-07	-6.33	V2 down vs V1	3.43E-08	7.42	V1 up vs Ctrl	0.00109	1.39	V2 up vs Ctrl
NM_001814 // C11orf1 // cathenin C // 11q14.1-q14.3 // 1075 // NM_148CTSC	NM_001814		1.33E-06	3.15	V2 and V1 up vs Ctrl	5.77E-05	-2.27	V2 down vs V1	1.36E-06	4.74	V1 up vs Ctrl	0.000104	2.09	V2 up vs Ctrl
NM_001086213 // HRH1 // histamine receptor, human // 3p25 // 3269 // NM_HRH1	NM_001086213		1.23E-07	3.12	V2 and V1 up vs Ctrl	1.98E-07	-3.39	V2 down vs V1	2.23E-08	5.71	V1 up vs Ctrl	2.57E-05	1.70	V2 up vs Ctrl
NM_018010 // ITF1 // intracellular transport factor 1 // 10p11.2 // 18010	NM_018010		2.86E-07	3.09	V2 and V1 up vs Ctrl	2.36E-07	-2.72	V2 down vs V1	3.89E-07	6.01	V1 up vs Ctrl	0.000113	1.62	V2 up vs Ctrl
NM_001793 // CDH3 // cadherin 3, type 1, P-cadherin														

Table 8: *HOXC6* V1, V2 genes were overexpressed in the cell line WT-CLS1. Total RNA was isolated 24 hours after transfections followed by cDNA microarray analysis. Commonly upregulated genes by *HOXC6* V1 and V2 compared to control were identified. V1 has more impact on upregulating genes compared to V2.

Discussion

Various chromosomal aberrations have been identified in nephroblastomas (Hawthorn *et al.*, 2011; Mannens *et al.*, 1988; Schaub *et al.*, 2007; Wadey *et al.*, 1990), but the mechanisms involved in the pathogenesis of these tumours are not clearly defined. In our study we have identified gains in chromosome 1, 12, 16, 17, 19 in tumours and NRs by aCGH analysis. The majority of nephroblastoma samples contained a gain in 12q13.13 location harbouring a gene known as *HOXC6*. Previous evidences link this gene with the pathogenesis of carcinoid tumours (Fujiki *et al.*, 2008), prostate cancers (Miller *et al.*, 2003) and breast carcinomas (Chariot *et al.*, 1996b).

The expression and genomic organization of the *HOXC6* gene have been investigated in *Xenopus* (Cho *et al.*, 1988), mouse (Coletta *et al.*, 1991) and humans (Chariot *et al.*, 1996b; Simeone *et al.*, 1987). This gene has 2 promoters, PR1 and PR2. They are 9KB apart from each other and the differential use of PR1 and PR2 leads to the synthesis of a 2.2 kB long transcript translate in to short protein (HOXC6 V1) or a 1.8kB short transcript translate in to long protein (HOXC6 V2). Both variants use the same open reading frame but possess distinct N terminal regions due to use of different initial ATG. Both proteins have same DNA binding sequence (homeodomain) and pentapeptide regions that are important for binding to other proteins (Chariot *et al.*, 1998).

HOXC6 is important in spatio-temporal regulation during the anterior and posterior determination in *xenopus* and mouse embryos (Oliver *et al.*, 1988) while it is also important in cell differentiation from normal to malignant phenotype, such as breast carcinoma (Chariot *et al.*, 1996b).

Our aCGH analysis revealed that the corresponding NRs of tumours also harboured gains in the *HOXC6* regions. This suggests that this is an early genetic event in the pathogenesis of nephroblastomas.

HOXC6 amplification was concordant with mRNA expression in 75% of the tumour samples. However, *HOXC6* gains are not a prerequisite for upregulation of mRNA expression. For example *MYCN* (V-myc myelocytomatosis viral related oncogene, neuroblastoma derived) gains were identified in nephroblastomas and mRNA expressions were not upregulated in all samples (Schaub *et al.*, 2007). The expression pattern of *HOXC6V1* and *V2* varied in nephroblastomas with 40% of tumours showing overexpression of both variants compared to mature renal parenchyma. Similar findings

were observed in malignant gastrointestinal carcinoid tumours compared to normal pancreas and ileum (Fujiki *et al.*, 2008).

HOXC6 is expressed in metanephric mesenchyme during kidney development (Patterson *et al.*, 2004). The pattern of V1 and V2 expression is different in adult and embryonic tissues. *HOXC6V2* is expressed in 13 ½ days old mouse embryos (Sharpe *et al.*, 1988) whereas 2.2kB (*HOXC6V1*) is present in mature human kidney (Cillo *et al.*, 1992) and in mature mouse kidney (Sharpe *et al.*, 1988). Nephroblastomas consist of different morphological regions ranging from undifferentiated blastema to differentiated epithelial regions. Therefore, it is possible to obtain a different expression pattern of V1 and V2 as we have observed in our tumour samples.

Overexpression of *HOX* genes can induce upregulation of genes important in development such as fibroblast growth factor (Care *et al.*, 1996). In addition, these genes may also have non-transcriptional functions. For an example, HOX11 can control the G2 cycle checkpoint through its interaction with PP2 (Protein phosphatase 2) and PP4 (protein phosphatase 4) (Kawabe *et al.*, 1997). Therefore, it is important to identify the target genes or genes that can interact with V1 and V2. Moreover, the functions of *HOXC6* are tissue specific. It appears to regulate completely different genes in prostate carcinoma and carcinoid tumours (Fujiki *et al.*, 2008; McCabe *et al.*, 2008).

Therefore, we have carried out mRNA expression analysis using nephroblastoma cells overexpressing *HOXC6* V1 and V2. Both variants are involved in the up and downregulation of genes in different biological pathways. V1 may be the dominant isoform, which may up or downregulate the genes in nephroblastomas. Different abilities of these 2 variants were published in 1989 resulting in distinct phenotypes of *Xenopus* after microinjection experiments. Injecting antibodies against the HOXC6V1 protein into one-cell *Xenopus* embryos caused transformation of the anterior spinal cord into a hindbrain-like structure. Injections of V2 mRNA into one-cell embryos also showed spinal cord malformations similar to the one demonstrated by variant 1 (Wright *et al.*, 1989).

It is also possible to have an oncogenic and a tumour suppressing ability for a single gene product (Huff, 2011). WT1 gene acts as a tumour suppressor in nephroblastomas (Huff, 1998) and there are also reported nephroblastomas with WT1 overexpression (Huff, 2011). WT1 also act as a tumour suppressor gene and an oncogene in leukaemia (Miwa *et al.*, 1992; Pritchard-Jones *et al.*, 1994). This functioning as an oncogene or tumour suppressor gene depends apparently on the differentiation status of the cell (Huff V et al 2011). Our CNV assay results indicated deletions and amplifications in the chromosomal region of

HOXC6. Deletions are frequently observed in cancer genome and is associated with tumour suppressor inactivation (Xue *et al.*, 2012), whereas oncogenes are activated by gene amplifications (Cai *et al.*, 2012). Therefore, *HOXC6* might be a gene which act as a tumour suppressor and an oncogene in nephroblastomas.

The samples with amplifications and deletions in *HOXC6* may also indicate deletions and amplifications in *HOXC4* and *HOXC5*. The genomic location of *HOXC6* overlaps with *HOXC4* and *HOXC5* genes which share a 5' non-coding exon. Transcripts of these genes may include only the gene-specific exons or may include the shared exon spliced to the gene-specific exons (<http://www.ncbi.nlm.nih.gov/gene/3223>).

In conclusion, *HOXC6* is amplified in most nephroblastomas and NRs. Two different variants of *HOXC6* are differentially expressed in nephroblastomas and have distinctive capacity of regulating genes. Therefore, further investigation is required to identify the potential pathways regulated by these variants to draw the pathway of the progression from NRs to nephroblastomas.

Chapter 2

Molecular markers to characterise prognosis and therapy response in stage2 colorectal carcinoma

Introduction

Colorectal carcinoma (CC) is one of the most common solid malignancies in the western world. World health organization identified 1.23 million new CC cases worldwide in 2008 and this carcinoma ranks as the fourth most common cancer in men and third in women. The international Union against cancer (UICC) staging system Tumour Node Metastasis (TNM) classification is the most important factor in making therapy decisions in CC (Figure 31). The effects of adjuvant chemotherapy in tumours that penetrate through the bowel wall with regional lymph node metastasis and no distant metastasis (stage 3) were well studied and it is clearly recommended (Laurie *et al.*, 1989; Moertel *et al.*, 1990; Moertel *et al.*, 1995). Though adjuvant chemotherapy is clearly effective in stage 3 disease, the impact of overall survival in tumours confined to colon (stage2) disease is controversial. Early studies to analyse the effects of chemotherapeutic treatment in stage2 disease shows contradictory results for disease free survival (DFS) and overall survival (Andre *et al.*, 2004; Gray *et al.*, 2007).

In addition, the previous studies indicated that there might be a subset of stage2 patients at high risk of recurrences that might benefit from adjuvant chemotherapy. Identification of patients who can be treated by surgery alone is equally important in protecting them from significant side effects of chemotherapy. Considering all these facts, the necessity of identifying prognostic markers in stage2 CC is an emerging issue in the medical field.

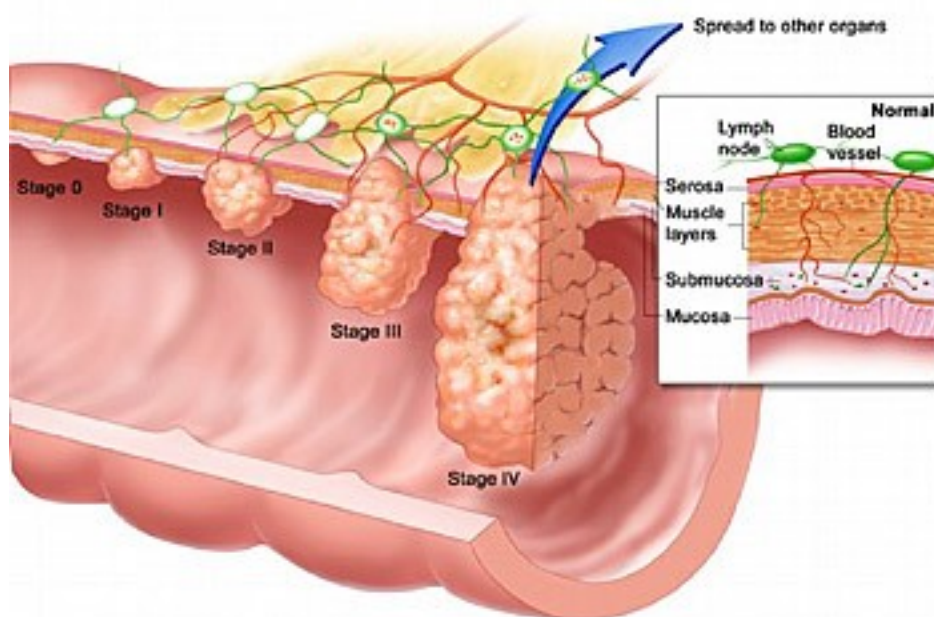


Figure 31: Tumour (T) Node (N) Metastasis (M) classification of colorectal carcinoma. Adopted from <http://fightcolorectalcancer.org>

A plethora of molecular markers to predict the prognosis and therapy response in metastatic CC has been studied. Epidermal growth factor receptor (EGFR) is a predictive marker, which can activate several pathways, involve in development of CC, and this receptor was also identified as an essential therapeutic target in CC (Cunningham *et al.*, 2004; Van Cutsem *et al.*, 2007). EGFR receptor activation is able to modulate cell proliferation, adhesion, angiogenesis, migration and cell survival via RAS/MAPK, STAT and PIK3/AKT signalling pathways (Hynes *et al.*, 2005; Mendelsohn *et al.*, 2006). Mutations in downstream genes of EGFR pathway can affect the prognosis and therapy response in CC. The *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) oncogene is a validated marker for lack of response to EGFR inhibitors (Benvenuti *et al.*, 2007; De Roock *et al.*, 2008; Di Fiore *et al.*, 2007; Lievre *et al.*, 2006), whereas prognostic power of *KRAS* in CC is reported conflictingly in different studies (Di Nicolantonio *et al.*, 2008; Laurent-Puig *et al.*, 2009).

Inflammatory immune cells were also identified as markers of prognostic relevance in CC (Chiba *et al.*, 2004; Funada *et al.*, 2003; Galon *et al.*, 2006; Guidoboni *et al.*, 2001; Menon *et al.*, 2004; Naito *et al.*, 1998; Petty *et al.*, 2002; Prall *et al.*, 2004). Increased cell density in CD3 (cluster of differentiation 3) cell population is associated with increased overall survival, DFS and reduced number of tumour recurrences (Galon *et al.*, 2006). Also, the percentage of patients with a high level of macrophage infiltration is reported to be significantly higher in vascular invasion negative cases and in lymph node metastasis negative cases (Funada *et al.*, 2003). Therefore, studies on CD68 (cluster of differentiation 68) and CD3 indicate the possibility of using immune cells as predictive marker to determine the prognosis of CC (Funada *et al.*, 2003; Galon *et al.*, 2006).

In addition to aforementioned reasons, genomic and epigenomic instability plays a role in prognosis of CC. Genomic instability occurs via 2 different mechanisms, Chromosomal instability (CIN) present in about 65-75% of CC patients (Miyazaki *et al.*, 1999) and microsatellite instability (MSI) in 15% of CC patients (Peltomaki, 2003). Interestingly, CIN+ cases have poorer prognosis than patients with CIN- (Walther *et al.*, 2008) whereas MSI+ have a better prognosis than MSI- CC (Popat *et al.*, 2005). MSI+ cases benefit from adjuvant chemotherapy (Ribic *et al.*, 2003). Although the studies related to CIN and MSI have analysed the prognostic effect separately, one study group stated that the prognostic

effect is not independent from each other (Sinicrope *et al.*, 2006).

Epigenomic instability can occur via the CpG island methylator phenotype (CIMP). CIMP defined as methylation at three or more specific marker loci (Weisenberger *et al.*, 2006). MSI+ patients who do not have a germline mutation in DNA repair genes are associated with CIMP (Weisenberger *et al.*, 2006). MSI+ cases also can be associated with *BRAF* V600E and in this case with CIMP-high. These patients have better prognosis (Ogino *et al.*, 2009a). CIMP phenotype is also associated with *KRAS* mutations and is termed as CIMP-low (Ogino *et al.*, 2006).

The molecular markers identified in advanced CC might be important in managing stage2 CC. Therefore, the objective of this study was to identify a marker or markers to predict the prognosis and treatment options for stage2 colorectal carcinoma patients.

Materials and Methods

Study Group

Patients. A total of 95 stage II colon cancer patients were included in this study. 65 patients had not been not treated with chemotherapy. These patients were part of a phase III multicenter study of the Austrian Breast and Colorectal Cancer Study Group (ABCSG) comparing adjuvant chemotherapy (5-FU/LV) with surveillance only in stage II colon cancer, which included a total of 535 patients. In brief, the phase III trial involved 31 participating hospitals and enrolled patients from 1993 to 2003. Patients were evaluated every 3 months during the first year post-randomization, every 6 months during years 2–5, and once yearly until year 10 after randomization. A physical examination with evaluation of body weight and performance status, determination of blood count, serum liver function parameters, carcinoembryonic antigen and tests for occult blood in stool, as well as chest radiography and ultrasonography of the liver were performed at each follow-up visit. Colonoscopy was required every 6 months during the first 6 years after randomization, then once a year. The present study was conducted at the Medical University of Graz (MUG) and approved by the Institutional Review Board of the MUG.

TMA (Tissue micro array)

TMA for samples outside the Graz were prepared by Oridis Biomed, Austria and the rest prepared at the Institute of Pathology, Medical University of Graz, Austria. Three samples of normal tissue and 3 samples of tumour core tissue biopsy sections were taken from specific areas from paraffin-embedded tissue blocks. These cylindrical cores of tissue were re-embedded into an arrayed blank recipient block. Tissue cores were arrayed in to a single slide (Figure 32).

Immunohistochemistry

Sections were deparaffinised. Antigens were unmasked by treating with PH6 Nacitrate buffer for 40 minutes at 270w in the microwave and cooling down for 20 minutes. Endogenous peroxidase was blocked with 1% H₂O₂ (Merck, Darmstadt Germany) for 10 minutes at room temperature for CD3, CD68, CD20 (B-lymphocyte antigen CD20) (Dako, Glostrup, Denmark), MLH1 (MutL homolog 1, colon cancer, nonpolyposis type 2) (Biocare Medical, Concord, CA, USA) and AVEN (apoptosis, caspase activation inhibitor) (Prosci incorporated, Poway, CA, USA) antibodies, an additional 0.1% protease treatment

for 10 minutes was performed for MIB1 antibody (Dako). CD3, CD68, CD20 and MIB1 antibodies were used in a 1:200, 1:3000, 1:200, 1:50, 1:1000 dilutions in antibody diluent (Dako) and incubated for 60 minutes at room temperature followed by washing steps. After incubation with the biotinylated secondary antibody (Dako) for 30 minutes at room temperature streptavidin-conjugated horse-reddish peroxidase (Dako) was added for another 30 minutes at room temperature. AEC (3,3'-diaminobenzidine) chromogen substrate diluted 1:50 or AEC substrate (Dako) was used as the detection system for a minimum of 5 minutes. Colour development was monitored under a microscope. Sections were counterstained with Mayer's hemalaun for 2 minutes. Finally sections were mounted in Aquatex (Merk, Darmstadt, Germany). Immunohistochemical analysis for MSH2 (mutS homolog 2, colon cancer, nonpolyposis type 1) (Ventana, Tucson, AZ, USA ready to use antibody) was performed by automated ventana system (Ventana). Immunohistochemistry for TMAs was performed by Mrs. Silvia Schauer. Intensity of staining analysed according to a scoring system (Table 9).

Table 9: scoring system for immunohistochemistry staining

	1	2	3
MIB1	<10% positive nuclei	10-50% positive cells	>50% positive cells
CD68	No/Few cells	Many cells	
CD3	No/Few cells	Many cells	
CD20	No/Few cells	Many cells	
MLH1	Positive	Negative	
MSH2	Positive	Negative	
AVEN	Weakly positive	Moderately positive	Strongly positive

DNA isolation

Tumour regions were microdissected manually under microscopic control. The microdissected tissue was directly transferred into Eppendorf tubes containing ATL buffer. Genomic DNA was extracted with the QIAamp®DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantity and quality of DNA was measured at OD260 and 280 using a photometer (BIO Rad, Hercules, CA, USA).

Mutation Analysis

DNA Mutation analysis was performed for the following target regions: *KRAS* (target region codon 12 and 13), *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) and *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide) Exon 9 and Exon 20 (target regions codons 542-546 and codons 1023, 1024 and 1047 respectively) by Dr. Christian Güllý at the ZMF core facility Medical University of Graz.

Amplification of target regions and high resolution melting analysis for *BRAF*

The samples were amplified in duplicate 20µl reactions using the Light Cycler 480 High Resolution Melting Master kit (Roche Diagnostics, Vienna, Austria) and analysed on a LC480 instrument I (Roche Diagnostics GmbH, Mannheim, Germany). The final reaction mix contained 1x Master Mix, 3mM MgCl₂, 4µM forward and reverse primer and 50ng of genomic DNA. For PCR the following cycling conditions were chosen: one cycle of 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 20 seconds. The amplicons were then denatured at 95°C for 1 min, cooled down to 40°C for 1 min and then melted from 65°C to 95°C with 25 signal acquisitions per degree.

Sequence variations for *BRAF* were detected using Gene Scanning Software v1.5 (Roche Diagnostics GmbH, Mannheim, Germany) samples were automatically grouped according to of their melting curves using the Auto Group mode.

The primers used to amplify the target regions are indicated in Table 10

Table 10: Primers used for *KRAS*, *BRAF*, *PIK3CA* mutation analysis

	Forward	Reverse
<i>KRAS</i>	ATAAGGCCTGCTGAAA ATGAC	GGTCCTGCACCAGT AATATGC
<i>BRAF</i>	GGTGATTTTGGTCTAG CTACA	AGTAACTCAGCAGC ATCTCAGG
<i>PIK3CA</i> exon 9	CTAGCTAGAGACAATG AATTAAGGGAAA	CATTTTAGCACTTAC CTGTGACTCCA
<i>PIK3CA</i> exon 20 (Codon 1047)	TGAGCAAGAGGCTTTG GAGT	GGTCTTTGCCTGCTG AGAGT
<i>PIK3CA</i> exon 20 (Codon 1023 and 1024)	CATTGCTCCAAACTG ACCA	TACTCCAAAGCCTCT TGCTC

DNA sequence analysis for *KRAS* and *PIK3CA*

DNA sequencing analysis was performed at the ZMF core facility.

PCR products were column purified using Sigma Spin Post-Reaction Purification columns (Sigma, Aldrich, St Louis, MO, USA) according to the manufacturer's instructions or microtiter plates (Marchery-Nagel, Düren, Germany), for amplicons with a length > 150bp. The purified PCR products were used as templates in DNA sequencing reactions using either BigDye Terminator v1.1 (*KRAS*, *PIK3CA* Exon 9) or v3.1 (*PIK3CA* Exon 20) chemistry (Applied Biosystems, Carlsbad, CA, USA). The 20µl reaction mix consisted of 2µl Big Dye terminator v1.1/3.1 premix, 4µl of 5x sequencing buffer, 10µM forward or reverse primer and 1µl purified PCR product (Sequencing PCR conditions: initial denaturation at 96°C for 1 min followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 30 seconds). After BigDye Terminator removal, using Sigma Spin Post Reaction Clean-up Plates, the samples were run on an ABI Prism AB3730 capillary sequencer with SeqScape v2.5 Software (Applied Biosystems) used for data analysis.

Pyrosequencing for *KRAS*

Pyrosequencing analysis was performed according to the manufacturer's instructions for PyroMark Q24 *KRAS* v2.0 assays. Briefly, 30ng of genomic DNA were used for analyses of *KRAS* codons 12/13.

DNA was amplified using the Pyromark (Qiagen) kit according to manufacturer's instructions. Cycling conditions were as follows: 95°C 5 minutes, 42 cycles (95°C 20 seconds, 53°C 30 seconds, 72°C 20 seconds), 72°C 5 minutes and 8°C forever. The quality of the amplified product was checked on a 2% agarose gel.

Depending on the size of the bands on PCR gel, 10µl or 17µl of biotinylated PCR product was immobilized on streptavidin-coated sepharose beads (GE Health care Bio-Sciences Corp., Piscataway, NJ, USA). Following washing step with the washing buffer (Qiagen), denaturation solution (Qiagen) and 70% EtOH (Qiagen), the purified biotinylated PCR product was released into the PyroMark Q24 plate with the sequencing primer. The cartridge was loaded with enzymes, substrate and nucleotides. PyroMark Q24 plate and cartridge was inserted in to the PyromarkQ24 machine and sequencing was performed. Analyses of the results were carried out by Pyromark Q24 software.

Ploidy analysis

From each of the paraffin blocks, 50µm thick two sections were cut and put in to glass tubes. These sections were deparaffinised two times 10 minutes in xylene and rehydrated in 100%, 90%, 70% and 50% ethanol. Then the samples were treated in pH6 Nacitrate buffer for 2 hours at 80C. After a cooling phase of 15 minutes at room temperature, samples were washed with PBS and digested with 1mg/ml pepsin (Sigma) at 37C for 15 minutes. Cells were filtered with a mesh nylon filter and pepsin was neutralized with cold PBS. Following another step of centrifugation, pellet was resuspended in 500µl of 1% bovine serum albumin (BSA) (Sigma) in PBS. Cells were stained by adding 500µl of DNA-prep stain (Beckman Coulter, Brea, CA) into the cells resuspended in BSA/PBS and left under dark for 20 minutes. The flow cytometric analysis was performed using Cytomics FC 500 (Beckman Coulter) and the calculation of DNA index and the percentage of cells in the S phase was done by Dr. Mannweiler.

DNA index 1 considered as diploid cycle, whereas >1 as diploid cycle with an aneuploid cycle.

CIMP analysis

CpG island methylator phenotype (CIMP) experiments were performed by Dr Martin Pichler at the department of Oncology, MUG.

Methylation status of 8 genes [*CACNA1G* (calcium channel, voltage-dependent, T type, alpha 1G subunit), *CDKN2A* (*p16*- cyclin-dependent kinase inhibitor 2A), *CRABP1* (cellular retinoic acid binding protein 1), *IGF2*, *MLH1*, *NEUROG1* (neurogenin 1), *RUNX3* (runt-related transcription factor 3), *SOCS1* (suppressor of cytokine signalling 1)] was analysed. Methylation status was categorized in to 3 phenotypes according to the number of methylated genes as CIMP-negative (none of the 8 genes methylated), CIMP-low (1-5 genes methylated), CIMP-high (6-8 genes methylated) ((Kawasaki *et al.*, 2008)).

Statistical Analysis

Statistical analysis was carried out by Prof. Andrea Berghold and her group at the Statistical Department of Medical University of Graz.

Results

Age of the patient has a significant effect on disease free survival

Thirty-six females and 29 males were included in the untreated group. Disease free survival analysis was carried out in the untreated 30 patients, which included 14 females and 16 females.

As a main clinical parameter, untreated patients were divided according to tumour grading and 26 cases out of 30 placed in G1 and G2.

Tumours were identified in caecum-3.3%, ascending colon-20%, right flexure-3.3%, transverse colon-6.7%, left flexure-16.7%, descending colon-10% and sigmoid-40% in untreated patients. Number of lymph nodes examined was also included as an additional clinical parameter.

Log rank test was performed to statistically compare the survival distribution for gender, grading, age, localisation of the tumour and number of lymph nodes in patients without chemotherapy.

Patients' aged from 65 years to 80 years had a significantly shorter disease free survival compared to patients less than 65 years of age ($P < 0.0495$). All other parameters did not show any effect on disease free survival (Figure 29)

Markers analysed immunohistochemically did not have any impact on disease free survival.

A large numbers of genes had been selected as potential prognostic markers at the initial stage of the project, from different resources. Twenty-three markers were identified with a published study (Wang *et al.*, 2004). Further to that, peroxisomal genes and their expression profiles in CC were identified by an *insilico* method. Genes responsible for adjuvant chemotherapy were identified in a comparative literature study. Experimental work was carried out in 10 markers.

Immunohistochemistry was performed for AVEA, MIB, CD68, CD3, CD20, MLH1 and MSH2 proteins. The expression of those proteins in tumour and normal tissue was analysed according to a scoring system. Additional immunohistochemical analysis was performed for MLH1 and MSH2 using the whole tissue samples to confirm the results obtained with the tissue microarray results.

Statistical analysis for disease free survival was possible for untreated 30 patients and findings revealed that there was no effect of AVEN, MIB, CD68, CD3 and CD20 in disease free survival (Figure 30).

MLH1 and MSH2 expression was assessed in both treated and untreated patients. MLH1 expression was negative in 14% of cases (80 patients analysed) and MSH2 negative in 8.8% cases (83 patients analysed).

Mutations in *PIK3CA* exon 9 decrease the disease free survival

KRAS codon 12 and 13 DNA sequencing was performed in 64 cases. Analysis revealed that 19.2% of treated patients and 11.8% of untreated patients had mutations.

DNA sequencing (95 patients) and high resolution melting analysis (66 patients) assessed the mutation in *BRAF*. Sequencing analysis showed that 3.2% of treated and 0% untreated had the *BRAF* V600E mutation whereas 10.4% treated cases and 16.7% untreated case was positive using the high resolution melting analysis.

PIK3CA codon 1023 (63 patients), 1047 (53 patients), 1043 (52 patients) and exon 9 mutations (66 patients) were analysed by DNA sequencing. None of the patients had *PIK3CA* 1023 mutation. *PIK3CA* 1047 and 1043 were positive in one treated patient. *PIK3CA* exon 9 mutations were positive in 3 untreated cases and one treated case.

Untreated group was statistically analysed for disease free survival associated with mutations in *KRAS*, *BRAF* and *PIK3CA*. *PIK3CA* exon 9 showed statistically significant association with disease free survival ($P < 0.0187$) (Figure 31).

BRAF and *PIK3CA* mutations were positive concomitantly in 2 patients, *BRAF* and *KRAS* in 1 patient, *KRAS* and *PIK3CA* in 1 patient and *BRAF*, *KRAS* and *PIK3CA* in 1 patient.

Ploidy and CIMP analysis

The cells isolated from 60 patient samples were stained with DNA prep staining and analysed by flow cytometry. Aneuploid cycles were identified in 33% cases. The percentage of cells in S-phase was also identified and 30 cases had >20% cells in S-phase.

CIMP analysis was performed in 67 cases. 20 cases were CIMP-negative, 41 cases CIMP-low and 6 cases CIMP-high.

Statistical analysis of these results in relation to survival analysis is under process.

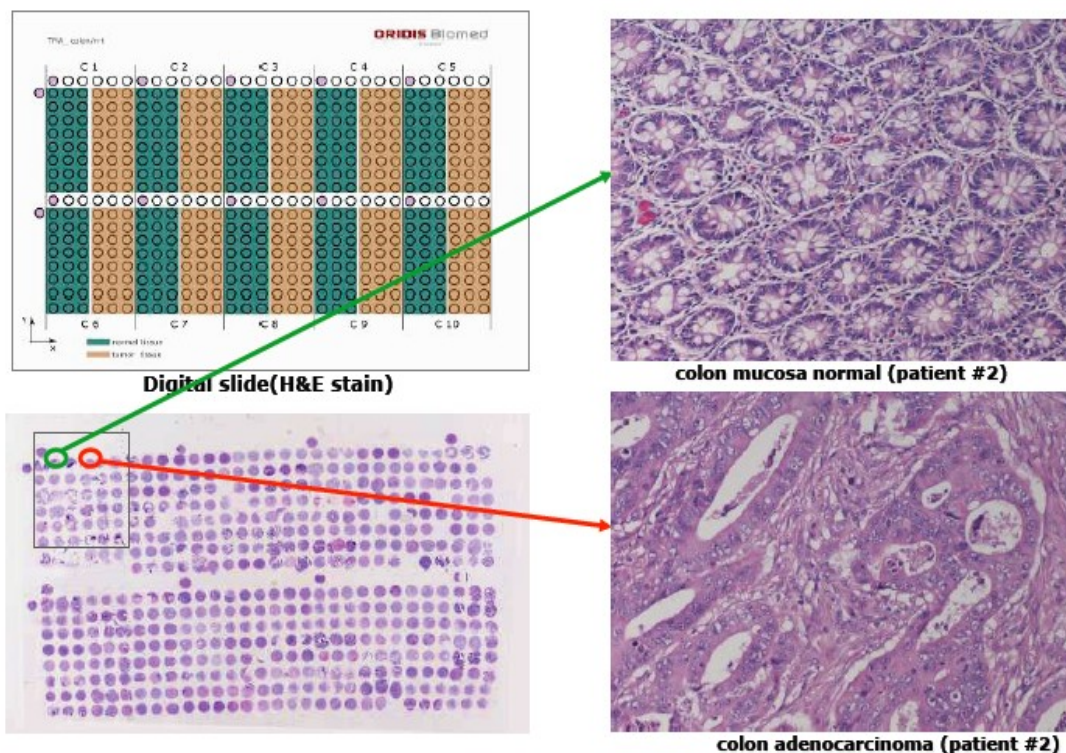


Figure 32:

Three samples of normal tissue and 3 samples of tumour core tissue biopsy sections were taken from specific areas from paraffin-embedded tissue blocks. These cylindrical cores of tissue were re-embedded into an arrayed blank recipient block. Tissue cores were arrayed in to a single slide. Tissue microarray with paired normal (green) and tumour tissue (red) shown.

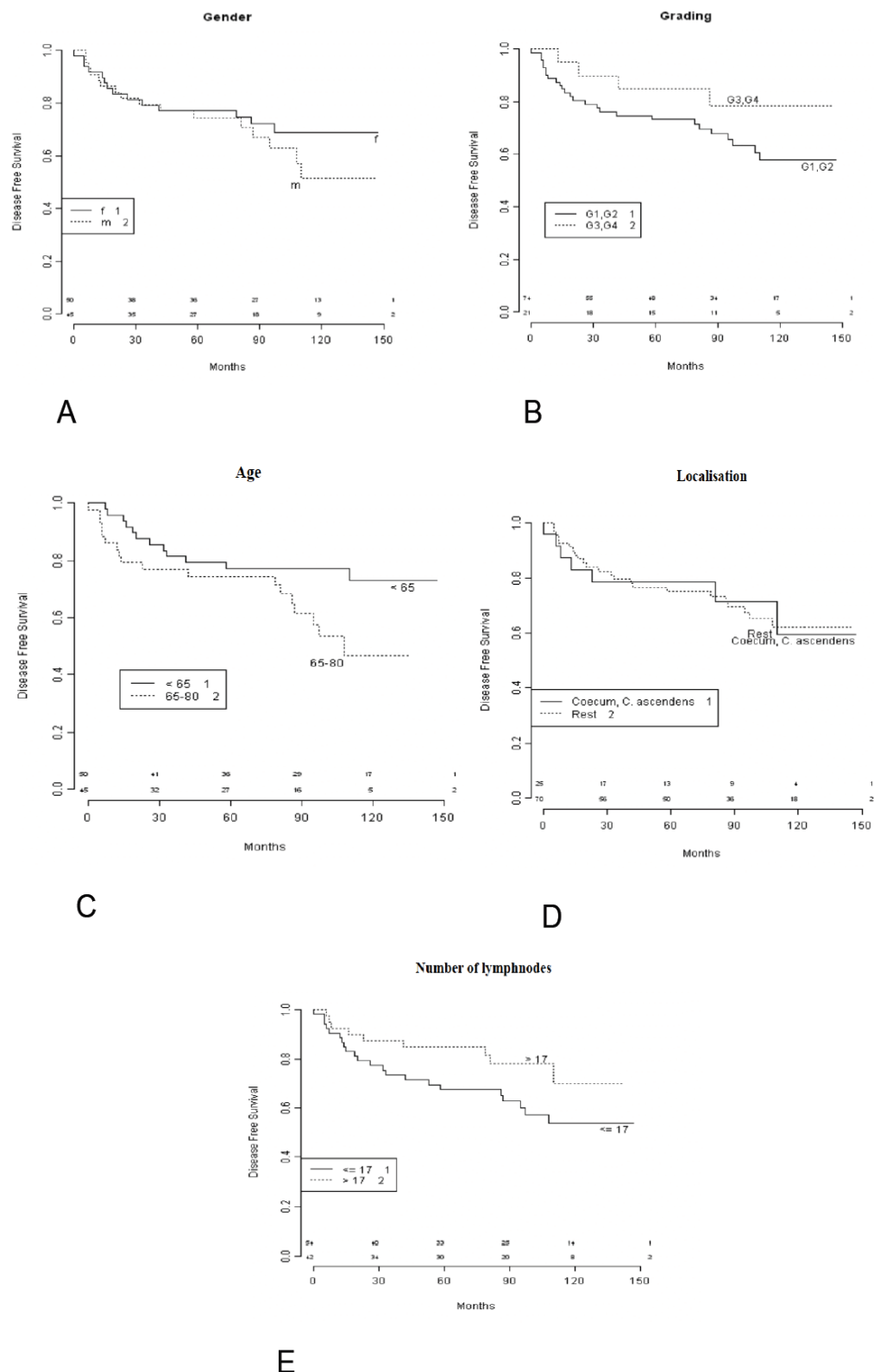


Figure 33: Kaplan-Meier survival curves for disease free survival associated with Gender (A), Grading (B), Age (C), Localization (D), Number of lymph nodes (E) was analysed. Age of the patient has a significant effect on disease free survival.

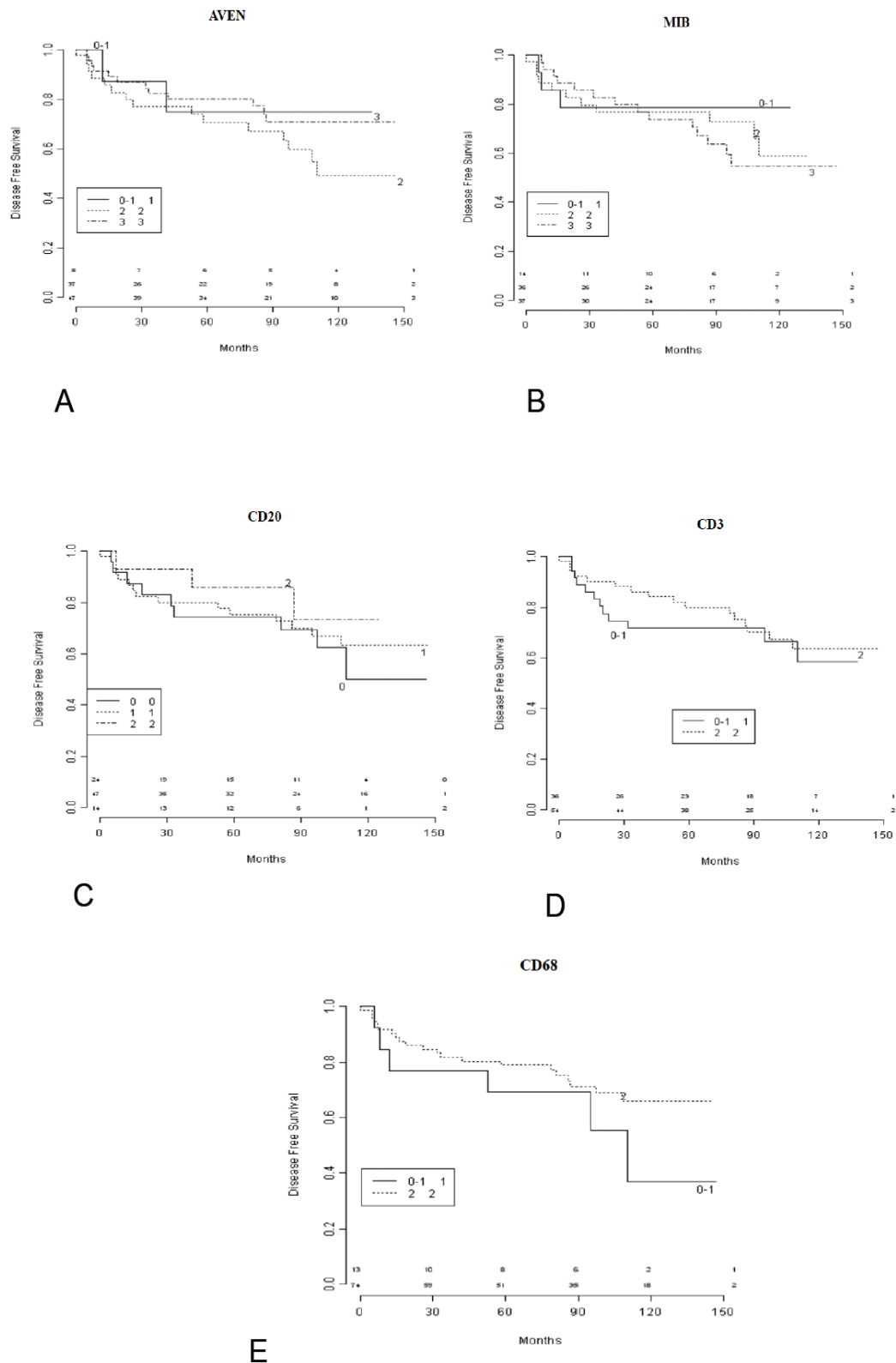


Figure 34: Kaplan-Meier survival curves for disease free survival associated with expression of AVEN (A), MIB (B), CD20 (C), CD3 (D), CD68 (E) was analysed. None of the markers had an impact on disease free survival.

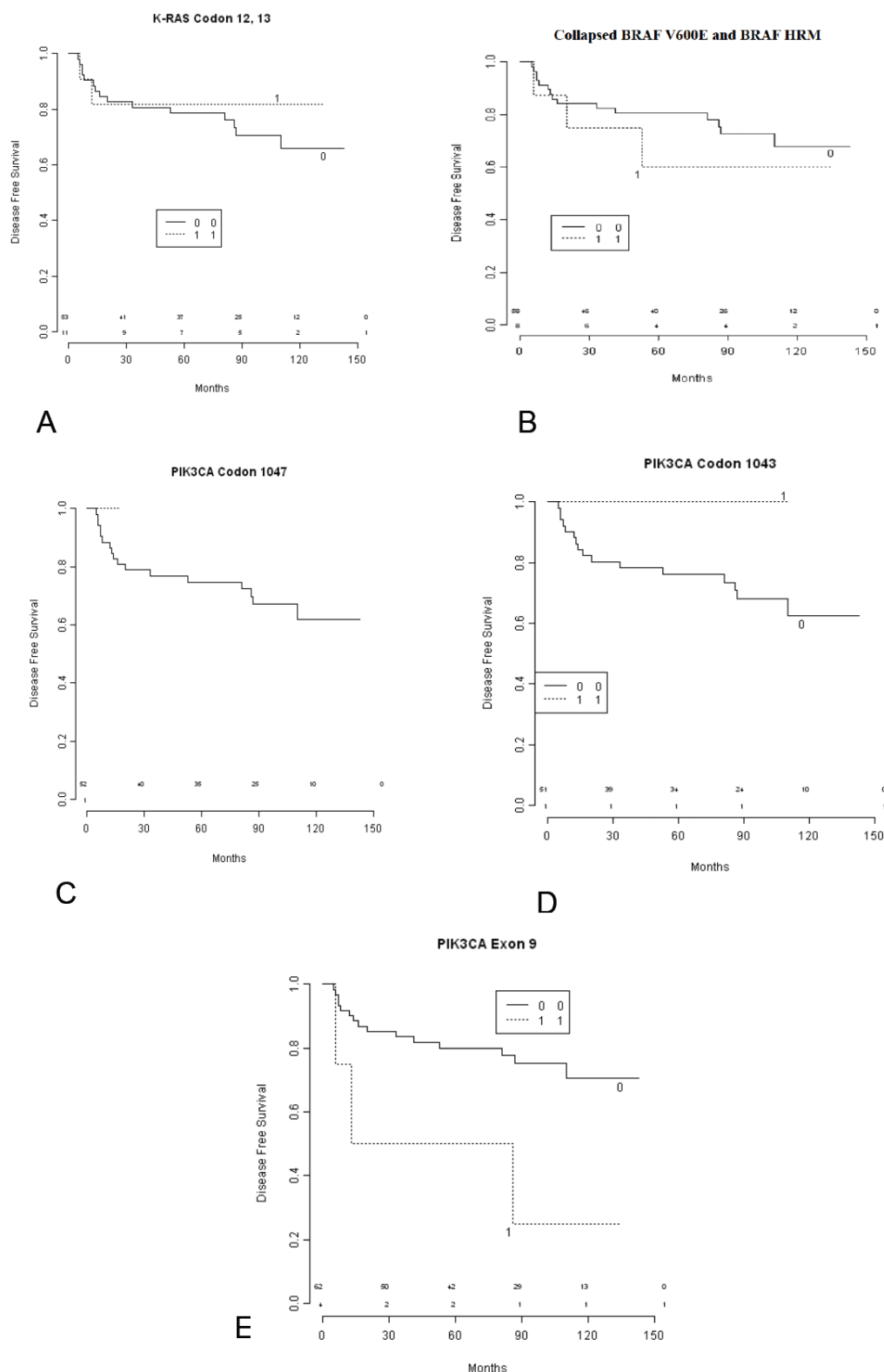


Figure 35: Kaplan-Meier survival curves for disease free survival associated with mutations in *KRAS* codon 12, 13 (A), collapsed *BRAF* V600E and *BRAF* HRM (B), *PIK3CA* codon 1047 (C), *PIK3CA* codon 1043 (D), *PIK3CA* exon 9 (E) was analysed. *PIK3CA* exon 9 mutations had a significant impact on disease free survival.

Discussion

The molecular profile of a tumour is likely to predict the clinical outcome (Rosenwald *et al.*, 2002). A large number of prognostic markers for CC are reported in literature. Most studies were focused on a single marker and few used several markers. Identification of prognostic markers would be particularly important in stage2 and stage3 CC, in which patients' choice of treatment will be surgery alone or surgery with adjuvant chemotherapy (Moertel *et al.*, 1990; Moertel *et al.*, 1995).

Our results indicated that in our cohort gender, grading, localization or the number of lymph nodes are not factors to predict the disease free survival. Patients over 65 years of age had a significantly shorter disease free survival according to Kaplan- Meier curves. This is in contrast to a study showing that young patients with stage 1 to 3 had a similar disease-free survival period as the older patients (Li *et al.*, 2011). However, this study has categorized "old" patients as over 40 years of age.

The analyses of immunohistochemical markers were performed according to a scoring system. This method is known to be a reliable system to establish the association with clinico-pathological findings (Zlobec *et al.*, 2007).

AVEN act as an antiapoptotic protein and is involved in cell cycle arrest following DNA damage (Gross, 2008). Disturbed apoptosis and genomic instability may induce oncogenic events by different mechanisms (Lowe *et al.*, 2004). AVEN has been identified as a prognostic marker in acute lymphoblastic leukaemia (Choi *et al.*, 2006), but its prognostic power in solid tumours is unknown. Our data suggest that the intensity of protein expression does not have an impact on disease free survival.

Cell proliferation is increased in premalignant and malignant colonic epithelium, indicating malignant progression of tumour (Moss *et al.*, 1996; Risio, 1992). MIB (Ki67- antigen identified by monoclonal antibody Ki67) is a nuclear protein that has been reported as a reliable marker of cell proliferation in gastrointestinal mucosa (Holt *et al.*, 1997; Lee, 1996). Ki67 has a prognostic value and predictive value in different tumour types such as breast carcinoma and lung carcinoma; however, in CC, the results in different studies seem to be conflicting (Brown *et al.*, 2002). The number of Ki67 positive cells in the tumour tissue was not associated with prognosis in our study.

The inflammatory markers did not also show any affect in disease free survival in our cohort. Nevertheless, previous studies indicated the importance of CD3 and CD68 as prognostic markers in colorectal cancer (Funada *et al.*, 2003; Galon *et al.*, 2006).

Distribution of inflammatory cells can be varied in a tumour sample. The combined analysis of inflammatory cells at the invasive margins and at the center of the tumour will increase the accuracy of the prediction of prognosis (Galon *et al.*, 2006). Expression of CD68 in the invasive margins can predict the risk of metachronous liver metastasis (Imano *et al.*, 2011). Several studies have shown associations between molecular features and clinico-pathological endpoints in TMAs using only one sample per tumour (Barlund *et al.*, 2000; Torhorst *et al.*, 2001). In our study we have analysed 3 samples from each patient sample, however, it is not known whether these core tissues do contain samples from invasive margins.

In accordance with some previous studies, *KRAS* mutations did not show have any effect on disease free survival in our study (Di Nicolantonio *et al.*, 2008; Laurent-Puig *et al.*, 2009). The number of *KRAS* mutated cases detected by DNA sequencing was less than the expected, which is 30% to 50% (Amado *et al.*, 2008; Di Nicolantonio *et al.*, 2008; Laurent-Puig *et al.*, 2009). Since the quality of DNA can affect DNA sequencing, pyrosequencing was performed to confirm the obtained results. Similarly *BRAF* mutations were less than anticipated according to HRM analysis. The expected percentage for *BRAF* is 10-15% (Di Nicolantonio *et al.*, 2008; Laurent-Puig *et al.*, 2009). Therefore, combinations of both results were included for disease free survival analysis.

KRAS wild type tumours with *PIK3CA* mutations are associated with poor prognosis, increased CC specific mortality (Baldus *et al.*, 2010; Ogino *et al.*, 2009b) and suggested to be a possible cause for lack of efficacy of EGFR therapy (Herrerros-Villanueva *et al.*, 2011).

In our study, we have identified mutations in *PIK3CA* exon 9 can significantly affect disease free survival. *PIK3CA* encodes for catalytic sub-unit p110 of phosphatidylinositol 3-kinase (PIK), which belongs to class 1A PI3K (Engelman *et al.*, 2006; Manning *et al.*, 2007). Majority of *PIK3CA* mutations occur in exon 9 and exon 20 in CC, but mutations in exon 1, 2, 4, 7, 12, 14 and 18 are reported in a few cases (Campbell *et al.*, 2004; Samuels *et al.*, 2004).

The percentage of *PIK3CA* occurrence in CC varies from 8% to 32% in the literature (Barault *et al.*, 2008; Benvenuti *et al.*, 2008; Board *et al.*, 2008; Herrerros-Villanueva *et al.*, 2011; Kato *et al.*, 2007; Nosho *et al.*, 2008; Ogino *et al.*, 2009b; Samuels *et al.*, 2004; Velho *et al.*, 2005).

The occurrence of a combination of *BRAF* and *KRAS* or *BRAF* and *PIK3CA* or *KRAS* and *PIK3CA* was reported in previously published material (Kato *et al.*, 2007; Velho *et al.*,

2005). None of the studies, however, revealed occurrence of all 3 mutations in one tumour. Therefore, our finding is likely to be a very rare incident in colorectal carcinoma study.

Immunohistochemical analysis of MLH1 and MSH2 is a rapid, cost effective and accurate method to assess the mismatch repair status of colorectal cancer (Cawkwell *et al.*, 1999; Dietmaier *et al.*, 1997; Ward *et al.*, 2001). The prognostic relevance of MLH1 and MSH2 has been studied in several research (Dietmaier *et al.*, 1997; Kruschewski *et al.*, 2002; Parc *et al.*, 2004; Perrin *et al.*, 2001). Patients with microsatellite instability have better prognosis for stage2 colorectal cancer, and chemotherapy should not be performed (Parc *et al.*, 2004). The percentage of tumours with a loss of MLH1 and MSH2 expression in our study is in agreement with the literature (Lanza *et al.*, 2006; Peltomaki, 2003).

By way of conclusion, our study indicates that whereas most of the commonly used immunohistochemical markers and molecular markers were not prognostic in our cohort of stage2 CC, markers related to genomic instability may still be useful as predictive indicators. Therefore, analysis of CIMP, ploidy and MSI data with the clinical parameters may lead us to identify novel prognostic markers for stage2 CC, requiring further studies.

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Curriculum Vitae

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EMPLOYMENT

Institute of Molecular Medicine (WIMM), University of Oxford,

Junior Postdoctoral Scientist; Nov 2011- Present

EDUCATION AND TRAINING

PhD in Molecular Medicine.

Medical University of Graz (MedUni), Graz, Austria.

Molecular diagnostics laboratory training, Institute of Pathology, Medical University of Graz, Austria

Research Projects:

Molecular Markers to predict Clinical Outcome and Therapy Response in Stage II Colon Cancer.

Advisors: Prof. Dr. Gerald Hoefler (MedUni-Graz); Prof. Dr. Michael Speicher (MedUni-Graz); Prof. Dr. Karoline Lackner (MedUni-Graz), Dr. Armin Gerger (MedUni-Graz)

Pathogenesis of Nephroblastoma- Reiteration of Kidney Development?

Advisors: Prof. Dr. Gerald Hoefler (MedUni-Graz); Prof. Dr. Michael Speicher (MedUni-Graz); Dr. Barbara Gurtel (MedUni-Graz).

Core Techniques:

RNA and DNA isolations, mRNA/ miRNA Quantitative real time PCR, Gel electrophoresis, Cell culture, Transient transfections, Immunohistochemistry, Pyrosequencing, Array CGH, Ploidy analysis by Flow cytometry, Cloning, Luciferase reporter assays, Microdissections, knowledge in handling laboratory animals and xenograft experience

Awards:

Poster prize at the Pannonia Congress of Pathology, 2010.

MSc. in Parasitology and Vector Biology. September 2008.

Universities of Manchester, Salford & Keele, Manchester, U.K.

Thesis: ***Regulation of helminth-driven Intestinal Inflammation.***

Supervisors: Prof. Kathryn Else (Univ. of Manchester); Prof. Geoff Hide (Univ. of Salford)

Grade: Distinction. Overall Average 74.4%, Dissertation 76%.

Awards: South Asian Postgraduate Scholarship, University of Salford. U.K. 2007-08

MBBS - Bachelor of Medicine; Bachelor of Surgery. September 2006.

University of Peradeniya, Peradeniya, Sri Lanka

Core Modules: Anatomy, Physiology, Biochemistry, Parasitology, Microbiology, Pharmacology, Pathology, Forensic Medicine, Community medicine, Medicine, Surgery, Obstetrics & Gynecology, Pediatrics.

Grade: Second Class Honours in 3rd MBBS Parts I and II. Distinction in Parasitology.

Awards: 'Prize for 'Best Physiology Project' at Faculty of Medicine. 2002-03.

CLINICAL APPOINTMENTS

University Teaching Hospital, Peradeniya, Sri Lanka. (2005-06)

Professorial Appointments in Medicine, Surgery, Obstetrics & Gynecology, Pediatrics.

NHS Teaching Hospitals, Kandy, Sri Lanka. (2003-05)

Clinical Rotations in Medicine, Surgery, Dermatology, Chest medicine, Neurology, Rheumatology, Clinical Pathology, Radiology, Oncology, Ophthalmology, Otolaryngology and Orthopaedic Surgery.

ACADEMIC PRESENTATIONS

Senanayake.U, Leuschner.I, Hoefler.G, Guertl.B

SIX2 in renal neoplasms

Congress of Pathology, Leipzig, Germany. Jun. 2011. (Oral)

Senanayake, U; Das, S; Leuscher, I; Schauer, S; Vesely, P; Hoefler, G; Guertl, B.

Six2 stem cell regulator and oncogene.

Doctoral and ZMF Day, Medical University of Graz, Graz, Austria. Nov. 2010. (Oral)

Senanayake, U; Das, S; Leuscher, I; Schauer, S; Vesely, P; Hoefler, G; Guertl, B.

Six2 stem cell regulator and oncogene.

Intercongress Meeting of the European Society of Pathology, Krakow, Poland. Sep. 2010. (Oral)

Nusshold, E; Senanayake, U; Guelly, C; Ebner, B; Leuschner, I; Hoefler, G; Guertl, B.

The role of Lim1 in renal development and renal neoplasms.

Pannonia Congress of Pathology, Graz, Austria. Mar. 2010. (Poster)

Senanayake, U; Das, S; Schauer, S; Vesely, P; Hoefler, G; Guertl, B

Six2 embryonically active transcription factor with oncogenic properties.

Pannonia Congress of Pathology, Graz, Austria. Mar. 2010. (Poster)

Senanayake, U; Flicker, K; Obenauf, A; Speicher, M; Hoefler, G; Guertl, B.

Nephrogenic rests - genetic precursor lesions of nephroblastomas.

Pannonia Congress of Pathology, Graz, Graz, Austria. Mar. 2010. (Poster)

Senanayake, U; Schauer, S; Lackner, K; Gerger, A; Staber, P; Guelly, C; Hoefler, G.
Molecular markers to predict clinical outcome and therapy response in stage 2 colorectal cancer. Doctoral and ZMF Day, Medical University of Graz, Graz, Austria. Nov. 2009.
(Poster)

PUBLICATIONS

The pluripotent stem cell regulator SIX2 is activated in renal neoplasms and influences cellular proliferation and migration.

Upeka Senanayake, Karin Koller, Martin Pichler, Ivo Leuschner, Heimo Strohmaier, Ulreike Hadler, Suman Das, Gerald Hoefler, Barbara Guertl. Human Pathology accepted 2012

miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms.

Senanayake, U; Das, S; Vesely, P; Alzoughbi, W; Fröhlich, LF; Chowdhury, P; Leuschner, I; Hoefler, G; Guertl, B. Carcinogenesis. 2012; 33(5):1014-1021

Lim1, an embryonal transcription factor, is absent in multicystic renal dysplasia, but reactivated in nephroblastomas.

Barbara Guertl, Upeka Senanayake, Elisa Nussold, Ivo Leuschner, Sebastian Mannweiler, Birgit Ebner, Gerald Hoefler. Pathobiology 2011;78(4):210-9

MANUSCRIPTS PENDING SUBMISSION

First authored

Prognostic relevance of PIK3CA exon 9 and intra-tumoral density of immune cells in stage 2 colorectal cancer

TECHNICAL AND THEORETICAL SKILLS

Windows/Mac word, excel, power point, Bioinformatics, Image J, GraphPad Prism and Copycaller software. Integrated approach to biomedical research using clinical, *invivo* and *invitro* models.

LANGUAGES

Fluency in English.