New insights in tailored treatment of gastrointestinal stromal tumours

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Stellingen behorende bij het proefschrift

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1. Men dient zich ervan bewust te zijn dat bij patiënten met een GIST, met name in het geval van progressieve ziekte, paraneoplastische hypoglykemieën kunnen optreden (dit proefschrift).

2. 'Big'-IGF-II en de insuline receptor spelen een rol bij de overleving van GIST cellen (dit proefschrift).

3. De waargenomen overexpressie van IGF-1R in GISTen zonder *KIT* en *PDGFRA* mutaties zou kunnen betekenen dat deze subklasse van tumoren geschikt is voor behandeling met monoklonale antilichamen tegen IGF-1R, hetgeen nader onderzocht dient te worden (dit proefschrift; Tarn *et al*, Proc Natl Acad Sci U S A 2008; Janeway *et al*, Int J Cancer 2010).

4. De death receptor Fas is een potentiële target voor therapie bij GIST (dit proefschrift).

5. Er zijn geen overtuigende aanwijzingen dat imatinib gepaard gaat met cardiotoxiciteit (dit proefschrift).

6. Het *KIT* en *PDGFRA* genotype van een GIST zal naar verwachting in de toekomst een belangrijke rol spelen bij de keuze voor de behandeling met een specifieke tyrosinekinaseremmer.

7. Ondanks het frequente gebruik van FDG-PET ter evaluatie van tumorrespons na het starten van systemische behandeling dient de exacte waarde hiervan bij GIST nog prospectief te worden vastgesteld.

8. Imatinib zorgt voor sterke botten (Vandyke *et al*, Blood 2010).

9. Hoewel GIST als kanker en gist als micro-organisme niks met elkaar van doen hebben, moet men rekening houden met een zeer sterke aantrekkingskracht tussen beide in een kweekflaconnetje.

10. Het voornemen van de minister van volksgezondheid om arts-assistenten zelf bij te laten dragen aan de kosten van hun opleiding tot medisch specialist doet afbreuk aan hun productiviteit en gaat voorbij aan de ondoorzichtige besteding van het geld uit de 'rugzakjes'.

11. Lachen is lief! (Loek Rikhof)

Bart Rikhof
Groningen, 19 mei 2010

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Chapter 1

General introduction

Introduction

Gastrointestinal stromal tumours (GISTs) are mesenchymal tumours with a spindle cell or epithelioid morphology. They arise primarily in the muscle wall of the gastrointestinal tract, most commonly the stomach or small bowel, but they also occur in the colon, rectum, mesentery and omentum. GISTs tend to metastasize to the liver, very rarely to the lungs, and intra-abdominal by spreading over serosal surfaces [1]. Although they are the most common malignant mesenchymal tumours of the gastrointestinal tract, their incidence is very low. The incidence in the Netherlands is estimated at 13 per million people annually, which is in agreement with the reported incidence in other countries [1,2]. GISTs are typically detected in adults over the age of 40 with a peak age around 60 years but they may also occur in children and adolescents.

GIST has only recently been identified as a separate tumour entity. Until the late 1990s, these tumours were often classified as smooth muscle tumours. The recognition that a subset of mesenchymal tumours originating from the stomach or bowel showed marked morphological similarities, including over-expression of the receptor tyrosine kinase KIT, with a population of cells located in the gut wall called interstitial cells of Cajal, defined GIST as an unique tumour group [3,4]. KIT appeared to be mutated in the far majority of GISTs and, a few years later, also mutations in the closely related platelet-derived growth factor receptor α (*PDGFRA*) was reported to occur in a small proportion (~5 percent) of GISTs [5,6]. These mutations are considered as initiating steps in the tumorigenesis of GIST and successive genetic changes are needed for a stromal tumour to develop towards malignancy [7]. In about 10 to 15 percent of GISTs no mutations of *KIT* or *PDGFRA* are found. In these so called 'wild-type' tumours other, yet unidentified oncogenic events may occur that probably activate similar intracellular signal transduction pathways as mutated KIT or PDGFRA [1].

Surgical resection is the only curative treatment option for GIST so far. Until recently, no further treatment options were available for patients with non-resectable and/or metastastic disease as GISTs are highly resistant to conventional single-agent or combination chemotherapy and radiotherapy. Since imatinib, a drug originally developed for treatment of chronic myelogenous leukaemia, was introduced as a therapy for GIST in 2001, the perspectives for patients with GIST have improved dramatically.

Imatinib is a small-molecule tyrosine kinase inhibitor that is active against a number of kinases including KIT, PDGFRA and Bcr-Abl. Clinical trials showed that imatinib is highly effective in patients with advanced GIST achieving disease control in 70 to 80

percent of the patients with a median progression-free survival up to 20-24 months [8,9]. Nowadays, imatinib is the standard therapy for advanced GIST. Despite these successes, complete responses are almost never seen in patients with imatinib. Furthermore, some GISTs are intrinsic resistant to this compound, while almost all patients finally develop disease progression after an initial tumour response. Therefore, alternative or additional treatment strategies are needed.

In this thesis, studies exploring new molecular targets for the treatment of GIST are described. Furthermore, studies evaluating potential side-effects of imatinib and markers for response to imatinib are presented.

Outline of this thesis

The identification of critical cellular signalling pathways, in addition to those activated by mutated KIT or PDGFRA is an important issue for the development of new targeted therapies in GIST. The insulin-like growth factor (IGF) signalling system could play a role in the tumorigenesis of GIST. **Chapter 2** describes the current knowledge about the IGF system and highlights the experimental and clinical evidence supporting the role of IGF signalling in the cellular transformation and progression of several types of malignant mesenchymal tumours arising from bone and soft tissues, including GIST.

Patients with sarcomas occasionally suffer from a paraneoplastic syndrome called non-islet cell tumour-induced hypoglycaemia (NICTH). NICTH is characterized by recurrent fasting hypoglycaemia and is associated with the secretion of incompletely processed precursors of insulin-like growth factor-II ('big'-IGF-II) by the tumour. **Chapter 3a** focuses on the tumour types and symptoms associated with NICTH as well as the pathogenesis, diagnosis and treatment of this rare paraneoplastic phenomenon. Two illustrative cases of patients suffering from NICTH caused by a solitary fibrous tumour and a haemangiopericytoma, respectively, are reported in this chapter. In **chapter 3b**, a case of a GIST patient suffering from NICTH is described.

As patients with a GIST tend to be at risk for the development of NICTH, we studied the clinical relevance of determining the plasma levels of 'big'-IGF-II and other IGF-related proteins in GIST patients in **chapter 4**. In this study, we determined the plasma levels of IGF-I, total IGF-II, pro-IGF-IIE [68–88] ('big'-IGF-II), IGF binding protein (IGFBP)-2, IGFBP-3 and IGFBP-6 for a cohort of 24 patients with a GIST, before and during treatment with imatinib. The predictive values of these parameters with respect

to the occurrence of hypoglycaemia, disease progression and response on treatment were subsequently evaluated. In addition, we assessed whether the observed changes in plasma levels of pro-IGF-IIE[68-88] and IGFBP-2 were associated with expression of these proteins in the corresponding tumour samples from these patients.

Following the observations in the previous chapter, the biological role of 'big'-IGF-II secretion in GIST was investigated in **chapter 5**. IGF-II (both in its mature and its partially processed form) has been shown to act as an autocrine growth and survival factor in several tumour types by binding to the IGF receptor type 1 (IGF-1R) and/or the insulin receptor isoform A. We hypothesized that 'big'-IGF-II secretion by GIST could provide a pro-survival signal in addition to constitutively activated KIT or PDGFRA. Therefore, the expression of 'big'-IGF-II and its cognate receptors was evaluated in a comprehensive cohort of GIST tissues. The effects of down-regulation of autocrine produced 'big'-IGF-II and silencing of the insulin receptor with short interference RNA on cellular survival and apoptosis was subsequently investigated in GIST cell lines.

Apoptosis can be induced through several mechanisms. One of these mechanisms involves the inhibition of receptor tyrosine kinases such as constitutively activated KIT or 'big'-IGF-II-activated insulin receptor isoform A in GIST. Another option is the activation of the cellular extrinsic apoptosis pathway through activation of death receptors. In **chapter 6**, we studied whether the death receptor Fas could be used as therapeutic target in GIST. We therefore evaluated the effectiveness of MegaFasL, a recently developed hexameric form of soluble Fas ligand (FasL), as an apoptosis-inducing agent in a panel of imatinib-sensitive and imatinib-resistant cell lines, alone and in combination with imatinib. In addition, the expression of Fas and FasL was evaluated in 45 primary GISTs with immunohistochemical stainings.

Although imatinib inhibits KIT and PDGFRA in GIST, it can also inhibit these or other targets in normal tissues which may result in side-effects. Case reports of patients treated with imatinib and data from animal studies suggested that this tyrosine kinase inhibitor may induce cardiomyopathy. Consequently, careful cardiac monitoring was advocated for clinical studies. The purpose of the study described in **chapter 7** was to evaluate whether imatinib induces early, subclinical, cardiac toxicity. In 55 patients with locally advanced and/or metastatic GIST, serial plasma N-terminal pro B-type natriuretic peptide and serum cardiac troponin T measurement was performed before, and one and three months after the start of imatinib treatment. These measurements were related to patient medical history and physical examinations.

Recently, several case reports and cross-sectional studies suggested that imatinib might influence normal tissue homeostasis inducing alterations in bone and mineral metabolism and causing hypogonadism in male patients. In the study described in C**hapter 8,** hormonal and electrolyte changes during the first 6 months of treatment with imatinib were analysed. In addition, the observed changes were related to tumour response to imatinib as evaluated with CT scanning in an attempt to explore additional early predictive biomarkers.

Findings from this thesis are summarized and discussed in **chapter 9.**

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Chapter 2

The insulin-like growth factor system and sarcomas

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Abstract

Sarcomas are a diverse group of malignant mesenchymal tumours arising from bone and soft tissues. The identification of critical cellular signalling pathways in sarcomas is an important issue for the development of new targeted therapies. This review highlights the experimental and clinical evidence supporting the role of the insulin-like growth factor (IGF) signalling system in the cellular transformation and progression of several types of sarcoma, including rhabdomyosarcoma, synovial sarcoma, leiomyosarcoma, Ewing's sarcoma and osteosarcoma. Preclinical data suggest that the IGF system could be a promising target for therapy in these sarcomas. Currently, therapies interrupting IGF signalling have been or are being developed. In recent phase 1 clinical studies with humanized monoclonal antibodies directed against IGF receptor type 1 (IGF-1R), objective tumour responses were observed in several patients with Ewing's sarcoma encouraging further clinical testing in Ewing's sarcoma and other sarcoma (sub)types. Moreover, the occasional occurrence of paraneoplastic hypoglycaemia as a result of the secretion of incompletely processed forms of pro-IGF-II by sarcomas is discussed.

Introduction

Sarcomas are rare tumours of mesenchymal origin comprising about 1% of all adult malignancies and 12% of paediatric cancers [1,2]. They form a heterogeneous group of solid tumours arising from soft tissue or bone. There are more than 50 histological (sub)types of soft tissue and bone sarcomas. These subtypes display different biological characteristics, clinical behaviour and therapeutic options [1]. On the basis of cytogenetics, sarcomas have been divided into two major classes. The first class consists of sarcomas with simple karyotypes and specific genetic alterations, including chromosomal translocations or specific gene mutations. The second category is characterized by a complex unbalanced karyotype with non-specific genetic alterations [3]. Overall, there is a poor response of sarcomas from both categories to chemotherapy. Recent advances in the treatment of gastrointestinal stromal tumours (GIST) with the tyrosine kinase inhibitor imatinib [4] and Ewing's sarcoma with the insulin-like growth factor receptor type 1 (IGF-1R) antagonist R1507 [5] have shown that understanding of the biology of sarcomas and identification of critical signalling pathways involved in the aetiology and progression of these tumours, can lead to the recognition of molecular targets for therapy. Research performed in the last two decades has demonstrated that the insulin-like growth factor (IGF) system plays an important role in tumorigenesis of several sarcoma (sub)types. Therefore, this system could be a target for antisarcoma therapy. Furthermore, mesenchymal neoplasms are occasionally associated with paraneoplastic hypoglycaemias as a result of pro-IGF-II secretion by the tumour. The purpose of this review is to highlight the biological, therapeutical and clinical role of the IGF system in sarcoma.

Components of the IGF signalling system

The IGF system consists of three ligands (IGF-I, IGF-II and insulin), four cell-membrane receptors (IGF-1R, insulin receptor isoform A (IR-A), hybrid receptors and IGF receptor type 2 (IGF-2R)), and six IGF binding proteins (IGFBP-1 through -6; figure 1).

IGFs

The majority of circulating IGF-I is produced by the liver and its expression is stimulated by growth hormone (GH). Other organs can also produce IGF-I. In this way, IGF-I

functions as an endocrine growth factor but also as an autocrine or paracrine factor [6]. Mature IGF-I consists of 70 amino acid residues (7.65 kDa) and shares 62% homology in amino acid sequence with IGF-II [7].

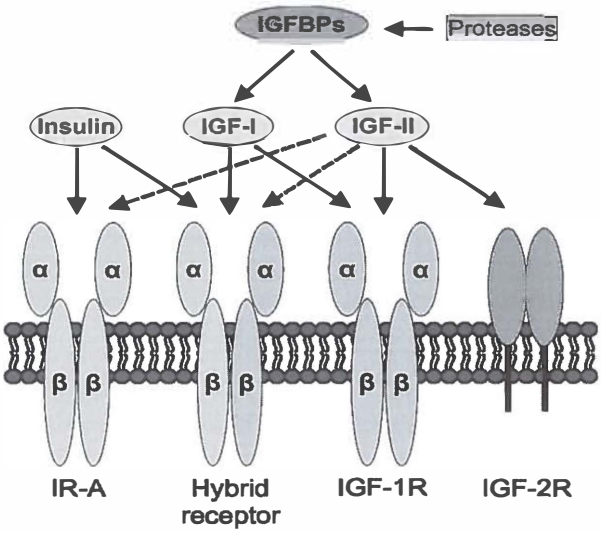

Figure 1. The components of the IGF system. IGF-I, IGF-II and insulin interact with their cognate receptors as indicated. IGF-II has a high affinity for the insulin receptor isoform A (IR-A) but not for IR-B. IGF-1R and insulin receptor isoforms can form heterodimers, called hybrid receptors. The function of IGF-I and IGF-II is modulated by IGF binding proteins (IGFBP-1 to -6) which in their turn are subjected to cleavage by proteases.

Similar to IGF-I, IGF-II is expressed in the liver and extrahepatic sites but its expression, in contrast, is not regulated by GH. In humans, IGF-II is the predominant circulating IGF with plasma levels 3- to 7-fold higher than IGF-I [8]. Mature IGF-II is a single-chain polypeptide consisting of 67 amino acids (7.47 kDa). It is synthesised as a 180 amino acid preprohormone with a 24 amino acid signal peptide at the N-terminal end and a C-terminal extension of 89 amino acids called the E-domain. During intracellular processing the signal peptide and the E-domain are cleaved in several steps from the precursor protein resulting in mature IGF-II [9]. Due to incomplete processing of pro-IGF-II, larger forms of IGF-II can be formed that still contain 21 amino acids of the E-domain. These forms of incompletely processed IGF-II are called 'big'-IGF-II and

constitute less than 10% of total human serum IGF-II. However, some malignancies, especially sarcomas, secrete large amounts of 'big'-IGF-II resulting in high circulating levels of this protein with associated hypoglycaemia (see below) [10].

The human *IGF2* gene is located at the short arm of chromosome 11 (locus 11p15.5). The transcription of *IGF2* is regulated by four separated promoters (P1-P4). These four promoters are active in a developmental and tissue specific manner [11]. In most non-malignant tissue, *IGF2* is transcribed from the paternal allele with the maternal allele being imprinted and thus silent. The imprinting of the *IGF2* gene is influenced by the neighbouring downstream *H19* gene, which encodes a non-translated RNA. *IGF2* and *H19* are oppositely imprinted. On the paternal chromosome, the promoter region of *H19* is imprinted by methylation causing silencing of the gene and providing access of several enhancers, located 3' of *H19*, to the *IGF2* promoters. The opposite occurs on the maternal gene [12]. Loss of imprinting (LOI) is one of several mechanisms that cause enhanced expression of IGF-II in human malignancies including some types of sarcomas. In this way, transcription occurs from two copies (both paternal and maternal) of the *IGF2* gene, leading to elevated mRNA levels. Some sarcoma subtypes (e.g. embryonal rhabdomyosarcoma) show loss of the maternal 11p15.5 locus causing loss of heterozygosity (LOH). LOH is associated with duplication of the paternal allele of *IGF2* (paternal isodisomy), resulting in the expression of two paternal genes. Furthermore, enhanced expression of transcription initiators, for example AP-2, or loss of inhibitors of IGF-II expression, such as p53, could play a role in increased expression observed in several sarcoma subtypes [13].

IGF receptors

IGF-1R is a transmembrane receptor formed by two extracellular ligand binding α subunits and two β subunits comprising the transmembrane and tyrosine kinase catalytic domains. IGF-1R is highly expressed in many human cancers including sarcomas. The expression of IGF-1R is predominantly determined at the transcriptional level and increased expression in cancers results from loss of transcriptional repressors, such as p53, or increased expression of transcriptional activators such as chimeric transcription factors found in, for example, alveolar rhabdomyosarcoma [14]. Amplification of the *IGF1R* locus is an uncommon event, although very recently amplification of *IGF1R* was found in a subset of GISTs [14,15]. Both IGF-I and IGF-II can bind and activate IGF-1R. Binding of these ligands causes a conformational change followed by autophosphorylation of the receptor. Subsequently, several adaptor proteins, such as the insulin receptor substrates

(IRS-1 to -4) and Src homology 2 domain-containing protein (Shc) are recruited to the phosphorylation sites in the cytoplasmic domain that activate at least two major signal transduction pathways. One of these routes is the phosphatidylinositol-3-kinase (PI3K) pathway, the other involves the mitogen-activated protein kinase (MAPK) pathway (figure 2) [16]. Depending on the cellular context, activation of these pathways results in cell proliferation and inhibition of apoptosis. Furthermore, IGF-1R signalling has been implicated in the induction of malignant transformation, the regulation of cell adhesion and motility, and stimulation of differentiation [17].

There are two isoforms of the insulin receptor (A and B) that arise due to alternative splicing of exon 11. IR-A, lacking the 12 amino acids encoded by exon 11, can bind to IGF-II with almost the same affinity as to insulin [18]. Under normal physiological conditions, IR-A is predominantly expressed in fetal cells, while IR-B is primarily expressed in adult tissue to mediate the metabolic effects of insulin [19]. Increased expression of IR-A has been reported in carcinomas of breast, colon, lung, thyroid and ovary [18]. As for IGF-1R, the expression of the insulin receptor is principally regulated at the transcriptional level. The factors contributing to the switch from isoform B into isoform A in cancer are poorly understood [18,20]. By using similar signalling pathways as IGF-1R, IR-A induces cell proliferation, survival and migration upon IGF-II and insulin binding [21,22].

As IGF-1R and insulin receptor are closely homologous, they are able to interact with each other to form heterodimers. Both insulin receptor isoforms are able to form these hybrid receptors by binding to IGF-1R. Hybrid receptors containing IR-B bind IGF-I with high affinity. In contrast, hybrid receptors with IR-A not only bind IGF-I but also IGF-II and insulin with a high affinity resulting in receptor autophosphorylation and activation of downstream signalling pathways [20]. However, the exact role of hybrid receptors in tumour formation and progression needs further investigation.

IGF-2R is a multifunctional receptor that lacks an intracellular signalling domain. It is also known as the cation-independent mannose 6-phosphate receptor which binds a diverse group of mannose 6-phosphate-tagged proteins for endosomal trafficking and degradation by the lysosome [23]. IGF-2R binds to extracellular IGF-II and causes internalization and subsequent clearance by the lysosome. In this way IGF-2R is involved in the regulation of the extracellular concentration of IGF-II [23]. Many tumours have been found with loss of IGF-2R expression or function by either LOH or inactivating mutations [24,25]. In addition, over-expression of IGF-2R leads to reduced tumour growth and delayed tumorigenesis in *in vivo* models suggesting that IGF-2R functions as a tumour suppressor [26,27].

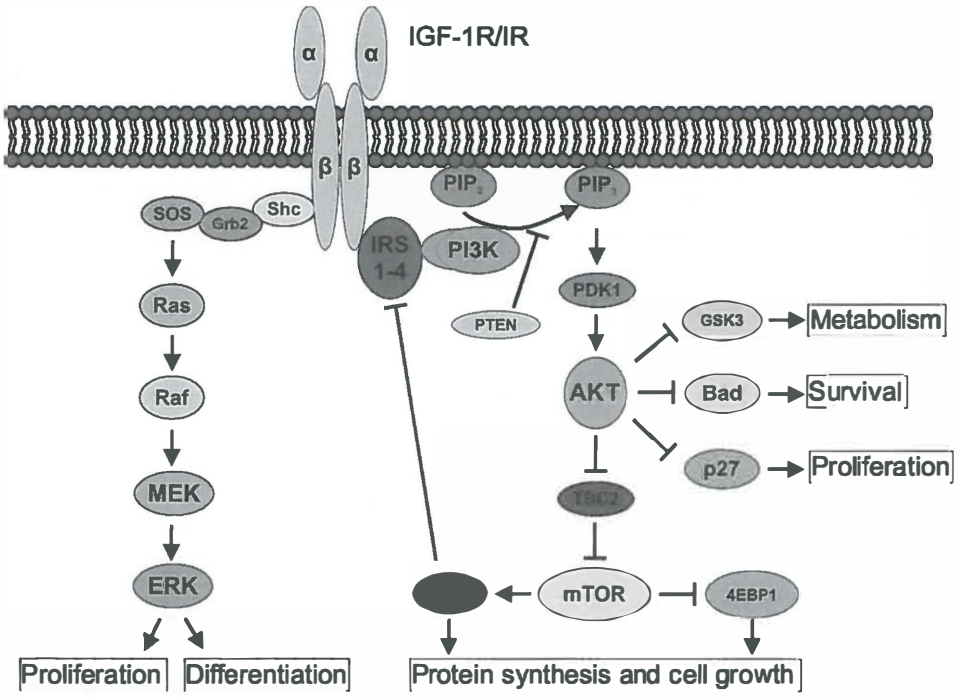

Figure 2. Schematic overview of the major IGF-1R and insulin receptor signalling pathways. Following ligand binding, IGF-1R and insulin receptor become activated resulting in receptor autophosphorylation and recruitment of several adaptor proteins such as the insulin receptor substrates (IRS-1 to -4) and the Src homology collagen-like (Shc) adaptor protein that bind to specific phosphorylated residues on the cytoplasmic domains of the receptors. IRS couples the receptors to the phosphatidylinositol-3-kinase (PI3K) pathway, while Shc couples the receptors to the mitogen-activated protein kinase (MAPK) pathway. In the PI3K pathway, binding of the p85 and p110 subunits of PI3K to IRS induces the conversion of phosphatidylinositol-4,5-biphoshate (PIP_2) to phosphatidylinositol-3,4,5-biphoshate (PIP_3) that subsequently activates phosphoinositide-dependent kinase-1 (PDK1). PDK1 phosphorylates the serine/threonine kinase AKT resulting in its activation. AKT has many substrates that are inhibited or activated by its phosphorylation such as glycogen synthase kinase-3 (GSK3), the Bcl-2 family member Bad, and $p27^{Kip1}$. These substrates are involved in the diverse cellular functions of AKT which include regulation of cell survival, proliferation, and metabolism. In addition, AKT activates the mammalian target of rapamycin (mTOR) by releasing the inhibitory activity of tuberous sclerosis complex-2 (TSC2) through phosphorylation. Activated mTOR stimulates protein synthesis and cell growth via at least two downstream targets, 4E-binding protein-1 (4EBP1) and S6 kinase (S6K). Several mechanisms exist that inhibit PI3K signalling. The phosphatase

and tensin homologue (PTEN) negatively regulates the formation of PIP_3. Another mechanism includes the phosphorylation of serine residues on IRS-1 by S6K which targets IRS-1 for degradation. In the MAPK pathway, Shc, growth factor receptor-bound protein-2 (Grb2) and son of sevenless (Sos) cooperate to activate Ras by catalysing the replacement of GDP with GTP. Activated Ras subsequently activates the kinase activity of Raf. This protein phosphorylates the MAPK/ERK kinases (MEK1 and MEK2), which phosphorylates and activates extracellular signal-regulated kinase-1 and -2 (ERK1 and ERK2). ERK has numerous target proteins in both the cytosol and the nucleus. These target proteins regulate processes such as proliferation, survival, differentiation and migration.

IGFBPs

The effects of IGF-I and -II are modulated by IGFBPs, a family of six homologous multifunctional proteins. In the circulation, IGF-I and IGF-II are mainly bound to IGFBP-3 which is the most abundant IGFBP in serum. About 75-80 percent of the IGFs circulate as 150 kDa ternary complexes that also consist of IGFBP-3, or to a much lesser extent IGFBP-5, and a third protein called acid-labile subunit (ALS). The remainder of IGFs exists as 40-50 kDa binary complexes bound solely to one of the six IGFBPs. Ternary complexes are not able to cross the capillary endothelial barrier due to the high molecular mass of these complexes. In this way, these large complexes are restricted to the vascular compartment prolonging the half-life of IGF-I and -II in the circulation (15-20 h). In contrast, binary complexes (and unbound IGFs) can easily leave the circulation (estimated half life ~30 min) facilitating the biological effects of IGFs to the tissue compartments [28,29].

On the cellular level, the interaction of IGFs with their receptors is influenced by the presence of IGFBPs. Depending on the cellular context and the IGFBP present, the actions of IGFs can be either enhanced or inhibited. In addition, IGF-binding capacity of IGFBPs is regulated by the presence of a wide range of proteolytic enzymes. Thus, there is a complex situation of local regulation of IGF activity. Interestingly, more and more evidence suggests that binding proteins also have IGF-independent functions (for review see Firth *et al.* [30]). For example, it has been shown that IGFBP-3 itself has a proapoptotic function although the exact signalling mechanism still needs to be elucidated.

The role of the IGF system in sarcoma

The current literature about the involvement of IGF signalling in the development and progression of various sarcomas will be discussed in the following sections and is summarised in table 1.

Rhabdomyosarcoma

Rhabdomyosarcoma is a malignant soft tissue tumour showing varying degrees of skeletal muscle differentiation. It is the most common soft tissue sarcoma of childhood. Two major histologic subtypes exist, alveolar and embryonal rhabdomyosarcoma. Embryonal rhabdomyosarcoma mainly affects children less than 10 years of age. Alveolar rhabdomyosarcoma is predominantly found in adolescents and young adults and is associated with a poorer prognosis than embryonal rhabdomyosarcoma [31]. Both rhabdomyosarcoma subtypes have distinct genetic alterations that have been shown to play a role in the pathogenesis of these tumours. Alveolar rhabdomyosarcomas carry a characteristic non-random translocation involving the DNA binding domain of *PAX3* on the long arm of chromosome 2 or *PAX7* on the short arm of chromosome 1, and the transactivation domain of *FKHR* on the long arm of chromosome 13 (encoding for the PAX3-FKHR and PAX7-FKHR fusion proteins) [32]. No consistent chromosomal rearrangements have been identified in embryonal rhabdomyosarcoma. However, embryonal rhabdomyosarcoma is characterized by the frequent LOH at the 11p15.5 locus [33,34].

Consistent over-expression of IGF-II has been identified in both embryonal and alveolar rhabdomyosarcoma tissue samples, and in cell lines derived from these tumours [35-38]. Both tumours and cell lines expressed IGF-1R [39]. Increased expression of this receptor was recently found to be associated with a poor survival of patients [40]. Rhabdomyosarcoma cell lines secreting IGF-II were able to grow in serum free media. Treatment of these cells with an antagonistic antibody against IGF-1R inhibited cell growth under serum-free conditions suggesting that IGF-II functions as an autocrine/ paracrine growth factor in rhabdomyosarcoma [39]. Furthermore, IGF-II stimulated the motility of rhabdomyosarcoma cells [36,39]. In vivo, tumour formation and growth of rhabdomyosarcoma cells were inhibited by treating the mice with an antagonistic antibody against IGF-1R [41]. Also other approaches targeting IGF-1R by antisense RNA or expression of a kinase deficient form of this receptor have resulted in the suppression of tumour formation [42,43].

Table 1. Involvement of IGF ligands and receptors in sarcomas

Tumour type	Cytogenetic characteristics	Fusion gene	Ligand expression	Receptor expression	Relevance
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) or t(1;13)(p36;q14)	*PAX3-FKHR* *PAX7-FKHR*	IGF-II	IGF-1R	IGF-II functions as an autocrine growth factor IGF-II/IGF-1R signalling provides a second hit after PAX-FKHR-induced transformation
Embryonal rhabdomyosarcoma	Complex karyotype, often LOH of 11p15.5	–	IGF-II	IGF-1R	IGF-II functions as an autocrine growth factor IGF-II/IGF-1R signalling is involved in the pathogenesis amongst other oncogenic events
Leiomyosarcoma	Complex karyotype	–	IGF-II	IGF-1R	The PI3K pathway is highly activated possibly through IGF-1R signalling
Synovial sarcoma	t(X;18)(p11;q11)	*SS18-SSX1* *SS18-SSX2*	IGF-II	IGF-1R	IGF-II/IGF-1R is involved in SS18-SSX-induced transformation and tumour progression
Kaposi's sarcoma	Complex karyotype	–	?	IGF-1R, IR-A	IR-A is involved in KSHV-induced transformation
Gastrointestinal stromal tumour	*KIT* or *PDGFRA* mutations	–	IGF-I, IGF-II	IGF-1R	GIST lacking *KIT*/*PDGFRA* mutations express IGF-1R IGF-I and IGF-II expression is associated with a poor prognosis
Solitary fibrous tumour	simple to complex karyotype	–	IGF-II	IR-A	IR-A is constitutively activated possibly by IGF-II autocrine signalling
Desmoplastic small round cell tumour	t(11;22)(p13;q12)	*WT1-EWS*	?	IGF-1R	IGF-1R is transcriptionally activated by EWS-WT1
Congenital fibrosarcoma	t(12;15)(p13;q25), trisomy of chromosome 11	*ETV6-NTRK3*	IGF-II	IGF-1R	IGF-1R is required for ETV6-NTRK3-induced transformation
Ewing's sarcoma	t(11;22)(q24;q12)[1]	*EWS-FLI1*	IGF-I	IGF-1R	IGF-1R is required for EWS-FLI1-induced transformation Ewings sarcoma cells are highly dependent on IGF-1R signalling both *in vitro* and *in vivo*
Osteosarcoma	Complex karyotype	–	IGF-I, IGF-II	IGF-1R	Activation of IGF-1R by IGF-I stimulates osteosarcoma cell growth *in vitro* and *in vivo*

[1] Also other, less common, translocations have been described

Activation of the PI3K pathway downstream of IGF-1R and insulin receptor is subjected to negative feedback regulation by mammalian target of rapamycin (mTOR) and S6 kinase (S6K) through inhibition of IRS function (figure 2) [44]. In accordance with these observations, an IGF-1R activation dependent increase in AKT activation was found in rhabdomyosarcoma cells upon inhibition of mTOR by rapamycin. Therefore, an even more efficient inhibition of rhabdomyosarcoma growth both *in vitro* and *in vivo* was observed when an IGF-1R antagonistic antibody was combined with rapamycin [45,46]. Interestingly, a recent study found that patients with a poor survival may have tumours with a disruption in the feedback mechanisms between mTOR and IRS resulting in an increased phosphorylation of AKT in contrast to a group of patients with a favourable outcome [40].

Rhabdomyosarcoma probably arises as a consequence of regulatory disruption of the growth and differentiation of myogenic precursor cells. PAX3 and PAX7 are transcription factors that are both required for normal muscle development [47]. Also IGF-II is implicated in normal skeletal muscle growth and differentiation [48]. The PAX3-FKHR fusion protein is a much stronger transactivator than PAX3 and is able to transform chicken fibroblast and inhibits myogenic differentiation of C2C12 mouse myoblasts [49,50]. Mixed results were reported about the forced over-expression of IGF-II in mouse myoblast cells. One study found that IGF-II induced enhanced differentiation of these cells [51]. However, another study showed that mouse myoblasts transfected with IGF-II alone or cotransfected with PAX3 or PAX3-FKHR, transform *in vitro* and can form tumours *in vivo*. Benign tumours were formed in all groups except for those cells transfected with both IGF-II and PAX3-FKHR. These cells developed invasive, poorly differentiated tumours with higher microvessel density and lower apoptotic rate [52]. These results suggest that IGF-II and PAX3-FKHR can cooperate in the formation of alveolar rhabdomyosarcoma. Of interest, PAX3-FKHR fusion protein can induce both IGF-II and IGF-1R expression resulting in enhanced IGF signalling [53,54].

Mouse models have been established in which spontaneous embryonal rhabdomyosarcomas develop. A mouse model for nevoid basal cell carcinoma syndrome, an overgrowth syndrome associated with tumour development including rhabdomyosarcoma, was created by introduction of a heterozygous deletion of the gene Patched (*Ptch*), a tumour suppressor gene involved in sonic hedgehog signalling. Nine percent of these mice developed embryonal rhabdomyosarcoma and the tumours expressed elevated levels of IGF-II [55]. Interestingly, when female heterozygous *Ptch*-knockout mice were crossed with male heterozygous *IGF2*-knockout mice, those mice with an *IGF2*-deficient

background did not develop rhabdomyosarcoma [56]. These data suggest that IGF-II is required for rhabdomyosarcoma formation in mice with loss of *Ptch* function.

More evidence about the role of IGF-II in the development of embryonal rhabdomyosarcomas comes from the observation that patients with Beckwith-Wiedemann syndrome, a rare disease characterized by fetal overgrowth, have an increased risk of developing embryonal tumours such as rhabdomyosarcoma. Inherited alterations of the 11p15.5 locus, including LOI and LOH of *IGF2*, are found in this syndrome leading to an increased expression of IGF-II [57,58]. Also in many sporadic rhabdomyosarcomas, of both embryonal and alveolar histology, transcription occurs from two copies of the *IGF2* gene by both LOH and LOI [59]. As stated above, most of the embryonal rhabdomyosarcomas show loss of the maternal 11p15.5 locus with duplication of the paternal allele of *IGF2* [60]. LOH has also been reported in alveolar rhabdomyosarcoma but LOI of *IGF2* is more often found [34,61,62].

Also other mechanisms are involved in the deregulation of IGF-II expression. P53 mutations and loss of p53 function as a result of MDM2 over-expression have frequently been found in rhabdomyosarcoma [63]. As p53 inhibits the expression of IGF-II, loss of p53 could increase the expression of IGF-II. Restoration of wild-type p53 in an alveolar rhabdomyosarcoma cell line expressing mutant p53 resulted in a significant decrease in IGF-II expression [64]. Furthermore, the levels of the transcription factor AP-2 are increased in rhabdomyosarcoma causing elevated IGF-II expression by binding to the P3 promoter [65].

In summary, IGF-II appears to function as an autocrine/paracrine growth factor in rhabdomyosarcoma by signalling through IGF-1R. In both embryonal and alveolar rhabdomyosarcoma, up-regulation of IGF-II appears to provide a secondary signal in the malignant transformation of myogenic precursor cells. As the IGF-II/IGF-1R signalling pathway plays an important role in the initiation and progression of rhabdomyosarcoma, disruption of this pathway could potentially be used as a therapeutic target.

Leiomyosarcoma

Leiomyosarcoma is a malignant tumour composed of spindle cells displaying features of smooth muscle differentiation. They account for 5 to 10% of all soft tissue sarcomas [66]. Leiomyosarcomas develop principally in adults and are most common in women. According to their localization, they can be divided into different subgroups: deep soft tissue tumours (retroperitoneum, abdominal cavity, and intramuscular), uterine tumours, (sub)cutaneous tumours, and vascular leiomyosarcomas. These subgroups

also differ in their clinical characteristics, with cutaneous tumours showing a better prognosis [67]. It has been reported that about half of the leiomyosarcomas studied express IGF-II at a high level compared with normal myometrium and leiomyomas. In some uterine leiomyosarcomas, LOI has been implicated in increased IGF-II expression [68,69]. In addition, equal to lower levels of IGF-1R were expressed in leiomysarcomas compared with non-malignant smooth muscle tissues and IGFBP-3 levels were decreased [68,70,71]. The latter could result in facilitation of IGF signalling. Indeed, constitutive activation of IRS-1 has been reported [72]. Furthermore, the PI3K pathway was highly activated in leiomyosarcomas, which appeared to be a critical event in the formation of leiomyosarcoma in mice [73]. Recently, Siacca *et al*. reported the production of IGF-II by several leiomyosarcoma cell lines. These cell lines expressed the insulin receptor, predominantly as IR-A, and variably expressed IGF-1R. It was shown that IR-A was able to mediate IGF-II autocrine signalling [22]. Still, more studies are needed to investigate the contribution of the IGF signalling system to the development and progression of leiomyosarcoma.

Synovial sarcoma

Synovial sarcoma is a mesenchymal spindle cell tumour which displays variable epithelial glandular differentiation. It accounts for 5 to 10% of all soft tissue sarcomas and can occur at every age but is most common in young adults. Synovial sarcomas are characterized by a specific reciprocal translocation between chromosome 18 and the X-chromosome resulting in the fusion of the genes *SS18* and *SSX1*, *SSX2* or *SSX4*. These SS18-SSX fusion proteins act as aberrant transcriptional regulators [74]. High expression levels of IGF-II were found in synovial sarcoma samples by microarray analysis [75,76]. Two studies investigating the effect of the *SS18-SSX1* or *SS18-SSX2* fusion gene in immortalised fibroblasts or kidney cells on gene expression found that *IGF2* was the most up-regulated gene [77,78]. This up-regulation was associated with changes in histone acetylation en methylation within the *IGF2* promoter and increased methylation of the *IGF2* imprinting control region [78]. LOI was found in a limited number of primary synovial sarcomas indicating that the SS18-SSX protein induces IGF-II expression in an epigenetic fashion [77]. Increased IGF-II production in *SS18-SSX* transfected cells protected the cells against apoptosis while growing under anchorage-independent conditions. Furthermore, it was shown that these transfected cells form synovial sarcoma-like tumours *in vivo*, in contrast to transfected cells in which IGF-II was down-regulated by siRNA [77]. IGF-1R seems to be involved in IGF-II signalling as down-regulation or inhibition of this receptor

leads to increased numbers of apoptotic cells in *SS18-SSX* transformed cells or synovial sarcoma cell lines [77,79]. In addition, a study investigating the IGF-1R expression in 35 synovial sarcomas found that there was an association between IGF-1R expression and an increased incidence in lung metastasis [80]. These data suggest that the IGF-II/IGF-1R pathway is involved in the development and aggressiveness of synovial sarcomas.

Kaposi's sarcoma

Kaposi's sarcoma is a locally aggressive endothelial tumour that typically presents with cutaneous lesions but may also involve mucosal sites, lymph nodes and visceral organs. Kaposi's sarcoma is the most common neoplasm that occurs in patients with AIDS and is uniformly associated with Kaposi's sarcoma-associated herpes virus (KSHV, also known as human herpes virus 8) infection [1,81]. IGF-1R and insulin receptor expression has been detected in Kaposi's sarcoma tissue samples [82,83]. IR-A, strongly up-regulated in dermal microvascular endothelial cells after infection with KSHV, was necessary for KSHV-induced transformation in contrast to IGF-1R [84]. Interestingly, IGF-2R was also up-regulated after infection of endothelial cells with KSHV and was required for transformation by retaining cathepsin B intracellular [85]. The role of IGF-1R in Kaposi's sarcoma is rather unclear. One study found that IGF-II stimulated proliferation and inhibit apoptosis via IGF-1R in an autocrine manner in a Kaposi's sarcoma cell line [82]. Furthermore, it has been suggested that activation of IGF-1R in Kaposi's sarcoma cells could contribute to the vascular phenotype of this tumour [86].

GIST

GISTs are the most common sarcomas of the gastrointestinal tract. These tumours are characterized by the over-expression of the receptor tyrosine kinase KIT. Activating mutations in this receptor or, less frequently, in the platelet-derived growth factor receptor α (PDGFRA) play a fundamental role in the pathogenesis of GIST. A subset of tumours lacks these mutations and in these, so called, wild-type GIST, other oncogenic events could play an important role in the development of these tumours [87]. Recently, it was demonstrated that wild-type GISTs express much higher levels of IGF-1R than mutant GISTs, which was associated with *IGF1R* gene amplification [15]. Also in paediatric GIST, a very rare disease with typically wild-type tumours, markedly increased expression levels of IGF-1R mRNA were observed that even exceeded the expression in wild-type adult tumours [88,89]. Immunohistochemical analysis revealed that IGF-I and/or IGF-II were expressed in the majority of (mutant and wild-type) GISTs and the expression of

each ligand was associated with a more aggressive phenotype and decreased disease-free survival [90]. To date, there are numerous reports about the occurrence of paraneoplastic hypoglycaemia with associated high plasma levels of 'big'-IGF-II in patients with a GIST [10,91-95]. This implies that large amounts of ('big'-)IGF-II are secreted by at least some tumours. Together, these data suggest that the IGF system could be involved in the pathogenesis of GIST, at least in wild-type tumours.

Ewing's sarcoma

Ewing's sarcomas may arise in either bone or soft tissues. It is the second most common cancer of bone in children and adolescents with a peak incidence during the second decade of life. Ewing's sarcomas are characterized by a recurrent chromosomal translocation resulting in the fusion of the *EWS* gene on chromosome 22 with one of several genes of the ETS family of transcription factors. The most common fusion, detected in approximately 85% of the cases, occurs with the *FLI1* gene resulting from the translocation t(11;22)(q24;q12) [1]. EWS-ETS fusion proteins promote oncogenesis in a dominant fashion in model systems and are necessary for continued growth of Ewing's sarcoma cell lines by acting as aberrant transcription factors [96]. Recent studies suggest that primary bone marrow-derived mesenchymal progenitor cells may be involved in Ewing's sarcoma development as expression of EWS-FLI-1 in these cells leads to Ewing's sarcoma-like transformation [97-99]. In association with this transformation, it was found that IGF-I was up-regulated and that transformed cells were dependent on IGF-1R signalling [99]. In addition, it has been shown that IGF-1R expression is required for EWS-FLI-1 mediated transformation of mouse fibroblasts [100]. In agreement with these findings suggesting an important role of IGF signalling in these Ewing's sarcoma models, consistent expression of IGF-I and IGF-1R was demonstrated in Ewing's sarcoma cell lines and tumour tissues [101,102]. Cases of Ewing's sarcomas that express type 1 EWS-FLI-1 are associated with a favourable prognosis and with lower IGF-1R expression compared to cases with non-type 1 EWS-FLI-1 translocations. In addition, the rate of IGF-1R expression in Ewing's sarcoma samples was shown to correlate with the proliferative index [103]. Many studies have investigated the effect of blocking the function of IGF-1R in preclinical models. Targeting the IGF-1R pathway in Ewing's sarcoma cell lines *in vitro* and/or *in vivo* by using dominant negative receptors [104], antisense strategies [105], antagonistic antibodies [102,106,107], small-molecule receptor tyrosine kinase inhibitors [108-110], or inhibitors of downstream signalling molecules (PI3K or MEK) [111,112] all appeared to be effective. Recently, it was shown that EWS-FLI-1 can bind to the *IGFBP-3*

promoter *in vitro* and *in vivo* and can repress its activity. Up-regulation of IGFBP-3 caused by EWS-FLI-1 inactivation induced apoptosis in Ewing's sarcoma cells in both an IGF-I-dependent and independent manner suggesting that repression of IGFBP-3 rescues Ewing's sarcoma cells from apoptosis [113].

These data imply that IGF-1R signalling contributes to Ewing's sarcoma development and progression with IGF-I as a possible autocrine factor with increased activity due to loss of IGFBP-3 expression. However, it is noteworthy that the age associated with the peak incidence of Ewing's sarcoma coincides with augmented IGF-I secretion in bone as a result of a burst in GH production. IGF-I induction could therefore provide a survival signal that is essential during early cell transformation by EWS-FLI-1 [114].

Osteosarcoma

Osteosarcoma is the most common primary malignant tumour of the bone. It most frequently occurs in the second decade of life, although one third of the cases are observed in patients over 40 years of age [1]. Osteosarcomas are characterized by a complex unbalanced karyotype. Although no characteristic reciprocal translocations are found in these sarcomas, non-random chromosomal abnormalities are observed, affecting for example the p53 and retinoblastoma tumour suppressor pathways [115]. The IGF system plays an important role in the formation and homeostasis of bone [116,117]. Differential expression of IGF-I and IGF-II and a more consistent expression of IGF-1R in osteosarcoma tissue samples and cell lines has been observed [118,119]. LOI of *IGF2* has also been reported [120]. However, there is no clear evidence that IGF-I or IGF-II function as a dominant autocrine growth or motility factor in osteosarcoma [119]. As for Ewing's sarcoma, the peak incidence of osteosarcoma coincides with the burst of GH release during puberty suggesting a causal relationship [115]. Indeed, osteosarcoma cells appeared to be IGF-I dependent for growth *in vitro* [121,122]. Furthermore, delayed tumour growth and inhibition of metastatic behaviour of murine osteosarcoma was observed in hypophysectomized mice [121]. In addition, inhibition of GH release by somatostatin analogs or antagonists of GH-releasing hormone in mice decreased serum IGF-I levels and inhibited tumour growth probably as a result of a combination of reduced IGF-I stimulation and direct effects on cellular growth by these compounds [123,124]. However, a phase 1 clinical trial with a long-acting somatostatin analog showed that serum IGF-I levels could be lowered but no clinical responses were observed [125]. An alternative therapeutic strategy would be targeting of IGF-1R. Both

antagonistic antibodies and tyrosine kinase inhibitors appeared to be effective in several preclinical models [107,108].

Recently, a study investigated whether single nucleotide polymorphisms in genes of several components of the IGF system could influence the risk of osteosarcoma development. A single nucleotide polymorphism in *IGF2R* was identified that was associated with an increased risk of osteosarcoma. This might suggest that a variant of IGF-2R is involved in the pathogenesis of osteosarcoma [126].

Other sarcomas

Solitary fibrous tumours are fibroblastic tumours that are anatomically ubiquitous. Although most tumours are benign, about 10 to 15% of these tumours behaves in a locally aggressive or malignant, sarcoma-like, manner [1]. Solitary fibrous tumours are frequently associated with hypoglycaemia due the expression and secretion of 'big'-IGF-II [127,128]. It has been reported that solitary fibrous tumours express the insulin receptor, predominantly as isoform A, in contrast to IGF-1R that was not detected. Furthermore, the insulin receptor and its downstream signalling pathways were constitutively active, suggesting that IR-A is the receptor involved in potential IGF-II autocrine signalling [129].

Desmoplastic small round cell tumours (DSRCT) are characterized by the recurrent chromosomal translocation t(11;22)(p13;q12) that results in the fusion of the *EWS* gene to the *WT1* gene. The WT1 protein plays a role in transcriptional regulation. EWS-WT1 chimeras are heterogeneous because of fusions of different regions of the *EWS* gene to the *WT1* gene. The various isoforms of the EWS-WT1 fusion protein are able to transactivate the *IGF-1R* promoter and to stimulate its expression, although to a various extent [130-132]. However, the exact role of EWS-WT1 induced up-regulation of IGF-1R in the aetiology of DSRCT needs to be determined.

Congenital fibrosarcoma and cellular mesoblastic nephroma are closely related malignancies that are characterized by the t(12;15)(p13;q25) reciprocal translocation resulting in the *ETV6-NTRK3* fusion gene. The chimeric ETV6-NTRK3 protein functions as a constitutively active tyrosine kinase. IGF-1R and IRS-1 are required for ETV6-NTRK3 induced transformation of murine fibroblasts *in vitro* [133,134]. In addition, these tumours showed increased expression of IGF-II, probably as a result of trisomy of chromosome 11 that is another characteristic cytogenetic abnormality of congenital fibrosarcoma and cellular mesoblastic nephroma [133]. Taken together these results imply that intact IGF signalling plays an important role in ETV6-NTRK3 induced transformation.

The IGF system as a therapeutic target in sarcomas

Current systemic therapies with chemotherapy for sarcomas of bone and soft tissues result in limited response rates. Childhood sarcomas, mostly embryonal rhabdomyosarcomas, form an exception as chemotherapy greatly improved overall survival of these patients. However, also in this group of patients, metastatic disease reflects a poor prognosis [135]. Furthermore, chemotherapy is characterized by a narrow therapeutic index showing toxicity on the short but also on the long term, which is of importance for the often young sarcoma survivors. New systemic therapies are therefore warranted to increase the survival of patients with sarcomas together with a decrease in the overall toxicity. As the IGF system plays an important role in the biology of several sarcomas, as discussed in this review, targeting IGFs or its receptors could be a promising therapeutic option.

Several (preclinical) strategies have been developed to decrease the bioavailability of IGF-I or -II to tumours. These include approaches to reduce ligand availability by somatostatin analogues or antagonists of GH-releasing hormone, neutralising antibodies and recombinant human IGFBPs [136]. Somatostatin analogues or antagonists of GH-releasing hormone could be safe for clinical use [137]. As stated above, these compounds were effective in preclinical osteosarcoma models, but no tumour responses were observed in a phase 1 clinical trial [123-125].

Another approach is to target IGF-1R. The expression of this receptor can be reduced by anti-sense and RNA interference strategies, or inhibited by antagonistic antibodies and small-molecule receptor tyrosine kinase inhibitors. At this moment, antibodies and tyrosine kinase inhibitors appear to be the most promising strategies to block IGF-1R function in patients. Recently, a phase 1 clinical trial was conducted with the single-agent R1507, a human anti-IGF-1R antibody. It was reported that four of the eight heavily pretreated patients with Ewing's sarcoma included in this study demonstrated clinical benefit and two patients achieved a durable partial response [5]. Another phase 1 study with an antibody against IGF-1R, AMG479, reported a complete response in a patient with Ewing's sarcoma [138]. A partial response in a patient with this type of sarcoma was also observed in a phase 1 study with the human monoclonal antibody CP-751,871 [139]. The drug was well tolerated in these studies and the maximum tolerated dose was not achieved. One of the most frequently reported adverse events was hyperglycaemia that could be managed with standard oral diabetic therapy [140]. Currently, several phase 2 studies with IGF-1R directed antibodies are running globally. Phase 2 studies with R1507, AMG479 and CP-751,871 have been initiated studying the effects of these agents

specifically in patients with Ewing's sarcoma but also in patients with other types of sarcoma (ClinicalTrials.gov identifiers NCT00642941, NCT00563680, and NCT00560235). All these recently developed antibodies against IGF-1R have similar mechanisms of action. They block the interaction of IGF-1R with IGF-I and IGF-II and, most importantly, they induce down-regulation of the receptor by internalization and degradation via the endosome [141]. It has also been reported that most of these antibodies can bind to and induce internalization of hybrid receptors [142]. However, the insulin receptor is unaffected providing a theoretical escape mechanism for mitogenic IGF-II and insulin signalling.

In addition to antibodies, a number of low molecular weight compounds have been developed that inhibit the tyrosine kinase activity of IGF-1R by targeting its intracellular kinase domain. Because the kinase domains of IGF-1R and insulin receptor share a very high protein sequence identity, the development of specific kinase inhibitors of IGF-1R appeared to be a major challenge [136]. Specificity of these inhibitors is an important issue, because the potential co-inhibition of the insulin receptor (isoform B) is expected to result in hyperglycaemia. Several compounds have been claimed to be selective towards IGF-1R, such as NVP-AEW541 and NVP-ADW742 [143,144]. These inhibitors were active in preclinical models of musculoskeletal sarcomas [108-110]. Small-molecule inhibitors of IGF-1R have not yet entered phase 1 clinical testing.

As IGF-1R signalling is involved in the protection of tumour cells from many types of insults, targeting IGF-1R could result in the sensitization of cells to chemotherapy or other anticancer drugs [145]. Many preclinical studies using different cancer types, including Ewing's sarcoma, showed an increased sensitivity toward chemotherapy using inhibitors of IGF-1R [109,146,147]. Also combinations with other targeted therapies, such as mTOR inhibitors, could be of interest in sarcomas [145]. Further clinical testing will have to reveal the optimal combination schemes for each sarcoma type.

Non-islet cell tumour-induced hypoglycaemia

As already stated above, several types of (soft tissue) sarcoma are relatively often associated with hypoglycaemias due to the massive secretion of incompletely processed forms of pro-IGF-II, called 'big'-IGF-II. This form of paraneoplastic hypoglycaemia is called non-islet cell tumour-induced hypoglycaemia (NICTH). In general, NICTH is an uncommon phenomenon and the incidence is unknown. However, about half of the

reported cases occurred in patients with a mesenchymal malignancy [128]. Many different sarcomas have been associated with NICTH. Solitary fibrous tumours are one of the most common mesenchymal tumours associated with hypoglycaemia. In a case series of 223 patients with a solitary fibrous tumour of the pleura, 3% percent with benign and 11% with malignant tumours developed hypoglycaemia [127]. Other sarcomas frequently associated with NICTH are mesotheliomas, leiomyosarcomas, and fibrosarcomas [128]. Tumours causing hypoglycaemia are usually large (i.e. a diameter more than 10 cm) [148]. It is unknown whether the existence of NICTH is of prognostic value with respect to predicting the degree of malignancy of a tumour and the (progression-free) survival of the patient [128].

With regard to the pathogenesis of NICTH, 'big'-IGF-II appears to have properties that do not allow the proper formation of the ternary 150 kDa complex with IGFBP-3 and ALS in the circulation, and competes with mature IGF-II and IGF-I for binding to IGFBP-3 and other IGFBPs. As a consequence, 'big'-IGF-II, mature IGF-II and IGF-I mainly circulate either as smaller binary complexes with IGFBPs or in the unbound, free form. In contrast to the ternary complexes, the binary complexes and free IGFs can easily cross the capillary membrane, hence increasing the bioavailability of IGFs to the tissue compartments. Finally, this may lead to hypoglycaemia as a result of excessive stimulation of the insulin receptor. In addition, IGFBP-3 and ALS levels are lowered since their GH-dependent production by the liver is suppressed, presumably due to an increased feedback inhibition by IGFs [149].

Increased 'big'-IGF-II production by tumour cells could simply be the result of an inadequate capacity of intracellular enzymes to process the relatively high amounts of pro-IGF-II [150]. However, increased production of 'big'-IGF-II (and resulting hypoglycaemia) might reflect the existence of an autocrine loop, because, similar to mature IGF-II, it is able to activate IGF-1R and insulin receptor. Furthermore, 'big'-IGF-II could have properties that are of more benefit for tumour cells than mature IGF-II. Sparse data suggests that 'big'-IGF-II is at least as mitogenic as mature IGF-II [151-154] which possibly depends on the amount of glycosylation of 'big'-IGF-II [154]. However, the exact role of 'big'-IGF-II production by sarcomas needs further delineation.

Conclusion

The IGF-1R/IR-A pathway is a commonly activated pathway in a variety of sarcomas. This pathway is either activated by endocrine, paracrine or autocrine produced IGF-I or ('big'-) IGF-II. Targeting this pathway or its ligands could therefore be an attractive therapeutical option. Recent successes reported in patients with Ewing's sarcoma treated wit an anti-IGF-1R antibody highlights the importance of the IGF system in this type of sarcoma and encourages further (pre)clinical studies investigating the benefit of targeting IGF signalling in other bone and soft tissue sarcomas.

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Chapter 3a

Non-islet cell tumour-induced hypoglycaemia: a review of the literature including two new cases

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Abstract

This review focuses on the tumour types and symptoms associated with non-islet cell tumour-induced hypoglycaemia (NICTH) as well as the pathogenesis, diagnosis and treatment of this rare paraneoplastic phenomenon. In addition, we report two illustrative cases of patients suffering from NICTH caused by a solitary fibrous tumour and a haemangiopericytoma, respectively. In the first case NICTH resolved following complete resection of the tumour, but in the second case the patient needed long term treatment aimed at controlling hypoglycaemia because of non-resectable metastases. Many tumour types have been associated with NICTH. The initiating event in the development of NICTH seems to be over-expression of the *IGF-II* gene by the tumour. NICTH is characterized by recurrent fasting hypoglycaemia and is associated with the secretion of incompletely processed precursors of insulin-like growth factor-II ('big'-IGF-II) by the tumour. This induces dramatic secondary changes in the circulating levels of insulin, growth hormone (GH), IGF-I and the insulin-like growth factor binding proteins (IGFBPs), resulting in an insulin-like hypoglycaemic activity of 'big'-IGF-II.

Introduction

Hypoglycaemia is a common medical emergency, mostly as a result of a complication of therapy with insulin and/or oral hypoglycaemic agents in diabetes mellitus. In rare cases hypoglycaemia can be a manifestation of neoplastic disease. Tumours related to the occurrence of hypoglycaemia can, as a general rule, be divided into three groups. First, tumours can produce excess insulin such as pancreatic insulinomas or ectopic insulin producing tumours. Second, hypoglycaemia can be caused by tumour-related factors such as destruction of the liver and adrenal glands by massive tumour infiltration. Finally, hypoglycaemia rarely can be induced by the production of substances interfering with glucose metabolism including insulin-receptor antibodies (in Hodgkin's disease and other haematological malignancies) [1], various cytokines including tumour necrosis factor-alpha and interleukin-1 and -6 [1,2], catecholamines (in phaeochromocytomas), secretion of insulin-like growth factor (IGF)-I [3], and tumours that secrete partially processed precursors of IGF-II ('big'-IGF-II) [1,4]. The latter condition is also known as non-islet cell tumour-induced hypoglycaemia (NICTH).

This review will focus on NICTH. So far, the literature on this rare and complex biochemical syndrome involving many types of tumours is mostly limited to case-reports. For this reason, besides a brief description of two new cases of NICTH, this paper reviews the available literature on this paraneoplastic phenomenon with respect to its pathogenesis, diagnosis and treatment.

The IGF system

IGF physiology

The IGF system is composed of two IGF ligands (IGF-I and IGF-II) and two IGF receptors (the IGF-I receptor (IGF-1R) and the IGF-II/mannose-6-phosphate-receptor (IGF-2R)) [5]. The majority of circulating IGF-I and IGF-II are produced by the liver, although various tissues and cell types are also capable to synthesize these peptides locally. The synthesis of IGF-I by the liver and various other organs largely depends on its stimulation by growth hormone (GH) through the GH-receptor, whereas the synthesis of IGF-II is relatively independent of GH action. The GH/IGF-I axis is the primary regulator of postnatal growth while IGF-II appears to have an important role during foetal development, cell proliferation and apoptosis [6-8].

Both IGF-I and IGF-II are structurally and functionally related to insulin. Most of the biological actions of IGF-I and IGF-II are thought to be mediated via IGF-1R as reviewed extensively by others [8-13]. However, IGFs may also interact with the insulin receptor which contributes to the pleiotrophic nature of IGF activity in the body.

The IGF-1R is a tyrosine kinase receptor consisting of two extracellular ligand binding α-subunits and two β-subunits comprising the transmembrane and tyrosine kinase domains [14]. IGF-1R signalling can induce differentiation, malignant transformation and regulate cell-cell adhesion [8-13].

Due to alternative splicing of exon 11 of the insulin receptor gene, there are two isoforms of the insulin receptor (isoform A and B). The insulin receptor-A isoform, is preferentially expressed in foetal tissues and in certain human malignancies, whereas the insulin receptor-B isoform is mainly expressed in important target tissues for the metabolic effects of insulin including liver, muscle and fat. IGF-I has low affinity for these receptors, but IGF-II binds with high affinity (comparable to its affinity for IGF-1R) and activates isoform A [15]. This interaction leads predominantly to mitogenic effects [15-17]. In contrast, the low-affinity binding of IGF-II to the B-isoform of the insulin receptor results in insulin-like, metabolic effects.

Insulin and IGF-I receptors are structurally homologous - both exhibit intrinsic tyrosine kinase activity and in part interact with various similar intracellular signal transduction mediators [8,18,19]. The structural homogeneity allows formation of hybrid receptors of IGF-1R and insulin receptor-A or -B. When IGF-1R and insulin receptor are co-expressed on the same cell, receptor hybrids form by random assembling and the least abundant receptor is drawn predominantly into hybrid receptors [20]. IGF-II can bind these hybrid receptors with high affinity but the biological role of these hybrid receptors remains largely unknown.

In addition, IGF-II also binds to the IGF-2R. Besides its role in the transport of lysosomal enzymes from the Golgi-apparatus to the lysosomes, this receptor is thought to function primarily as a scavenger receptor, promoting the endocytosis and degradation of extracellular IGF-II, thus regulating local IGF-II levels [6,8].

The glucose-lowering effect of IGFs is approximately 10 times lower than that of insulin, but in healthy subjects the serum concentration of IGFs is about 1000 times higher than insulin [21,22]. However, in contrast to insulin and proinsulin, in the circulation most (>90%) of the IGFs are tightly bound to IGF-binding proteins (IGFBPs). A total of six different high-affinity binding proteins have been identified (IGFBP-1-6). Although the majority of the circulating IGFBPs are derived from the liver, many other organs also

produce one or more IGFBPs [5,9,23]. Under normal circumstances, IGFBP-3 is the most abundant IGFBP in serum and binds more than 95% of the IGFs [8,23]. In normal human serum, approximately 70-80% of the IGFs forms a 150 kDa ternary complex with either IGFBP-3 or (to a much lesser extent) IGFBP-5, and an acid-labile subunit (ALS), a leucine-rich glycoprotein of approximately 85 kDa. Most of the residual IGFs are associated with IGFBP-1 to -6 (predominantly IGFBP-2 and -3) as smaller ~40-50 kDa binary complexes [23-25]. Only less than 1% of the IGFs circulates in the free form [23,26,27]. Due to its large molecular mass, the ternary complex is not able to pass the capillary membrane. Hence, the IGFs captured within this type of complex have a rather extended half life in the circulation ($T_{1/2}$ ~15 h) compared to the various binary complexes ($T_{1/2}$ ~25 min) or the free unbound IGFs ($T_{1/2}$ ~10 min). Thus, the unbound IGFs and the pool of IGFs associated with binary complexes in the circulation are considered to exchange relatively rapidly with the tissue compartments [28] and are more readily available for binding to IGF receptors and insulin receptors [29,30].

The IGF-II gene and protein

The *IGF-II* gene is one of the few genes known to have parental allele-specific expression. As such, it is referred to as an imprinted gene. The gene for IGF-II, together with two putative tumour suppressor genes, *H19* and $p57^{KIP2}$, is located on chromosome 11p15. In normal cells, the *IGF-II* gene is maternally imprinted in that it is expressed only from the paternal copy of the gene while *H19* and $p57^{KIP2}$ are expressed from the maternal allele. *H19* and $p57^{KIP2}$ are implicated in conserving imprinting of *IGF-II* [10,31,32]. The *IGF-II* gene consists of nine exons, including six non-coding ones, with four promoters (Figure 1). Promotor usage seems to be tissue specific and developmentally regulated which leads to multiple transcripts that all encode the same monomeric primary IGF-II translation product, pre-pro-IGF-II [33]. Pre-pro-IGF-II consists of 180 amino-acids including a N-terminal signal peptide of 24 amino acid residues, the 67 amino acids long mature IGF-II (7.5 kDa), and an 89 residue extension at the C-terminus. The latter has been designated the E-domain. Post-translational processing of pre-pro-IGF-II involves removal of the N-terminal signal sequence, addition of sialic acid containing oligosaccharides through O-linkage to one or more threonine residues of the E-domain, followed by sequential proteolysis of the latter extension into the mature protein. During this process a relatively stable intermediate is formed, pro-IGF-IIE[68-88], that may be secreted by the cell [34,35].

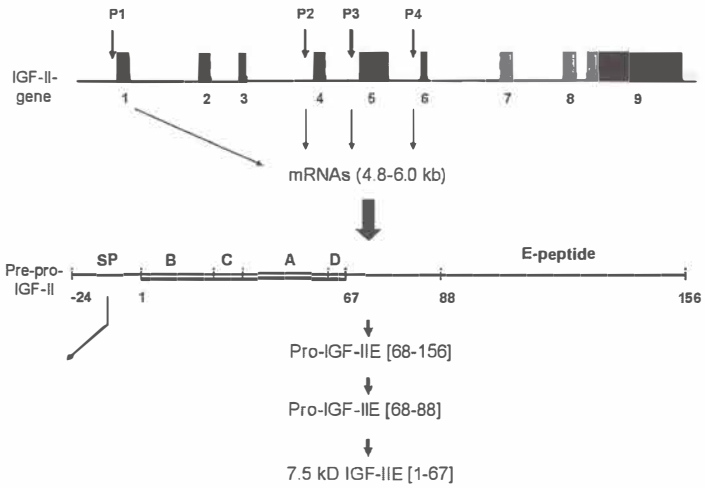

Figure 1. Structure of the insulin-like growth factor (IGF)-II gene and the precursor-IGF-II protein. The IGF-II gene has four promoters (P1-P4). Only exons 7, 8 and a part of 9 (depicted in gray) encode for the pre-pro-IGF-II protein. The N-terminal signalling peptide (SP) is enzymatically cleaved leading to the formation of pro-IGF-II. The E-domain is degraded through several steps into the mature 7.5 kDa IGF-II. Pro-IGF-IIE[68-88] is a relatively stable intermediary in this process.

Clinical features of non-islet cell tumour-induced hypoglycaemia

NICTH is a rare paraneoplastic phenomenon. It was first described in 1929 in a patient with a hepatocellular carcinoma [36]. Since then, many tumour types have been associated with hypoglycaemia. In 1988, Daughaday *et al* showed for the first time that tumour-induced hypoglycaemia was associated with the aberrant production of pro-IGF-II ('big'-IGF-II) resulting in a persistent insulin-like activity [37]. The two new cases we describe next, illustrate the clinical course and therapeutic problems that can be encountered in patients presenting with NICTH.

Case reports

Case 1

A 83-year-old man was admitted with confusion and lethargy without loss of consciousness. Over the last months he had lost 8 kg in weight. His medical history revealed atrial fibrillation and epilepsy for which he was treated with acenocoumarol

and carbamazepine. On admission, serum glucose was 1.1 mmol/L (normal fasting glucose: 4.0-5.4 mmol/L). Serum levels of insulin and C-peptide were suppressed. Sulfonylurea derivatives or insulin antibodies were not detected and the presence of a phaeochromocytoma was excluded. The concentration of serum IGF-I was reduced considerably whereas that of total IGF-II was within the normal range (molar ratio between total IGF-II and IGF-I: 17.7; reference value: <10). The serum levels of pro-IGF-IIE[68-88] ('big'-IGF-II) were markedly elevated (Table 1). Computed Tomography demonstrated a large tumour mass in the right kidney which histologically proved to be a solitary fibrous tumour. No metastases were found. Serum levels of glucose, IGF-I and pro-IGF-IIE[68-88] levels normalised after nefrectomy. Histochemical analysis of tumour tissue by in situ hybridization, using digoxigenin-labelled IGF-II cRNA probes [38,39] revealed an abundant and high expression level of IGF-II mRNA (Figure 2).

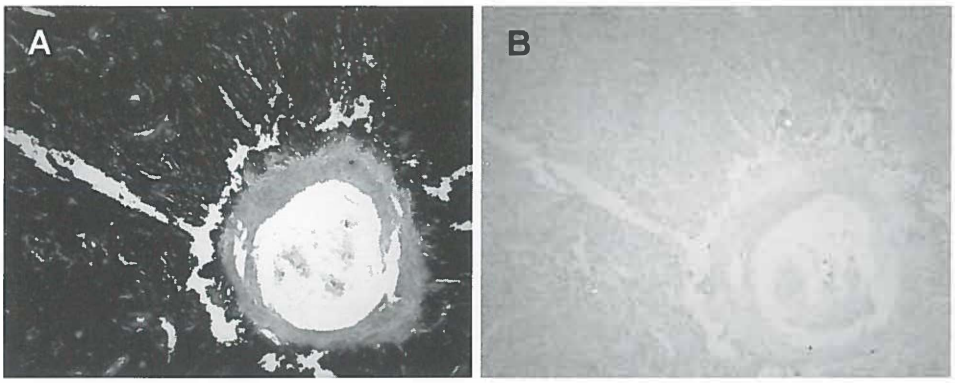

Figure 2. In situ hybridization of insulin-like growth factor (IGF)-II mRNA in a solitary fibrous tumour of the right kidney from patient 1, demonstrating high expression of the IGF-II gene (A). IGF-I is not expressed (B).

Case 2

A 48-year-old woman was admitted in coma. She was diagnosed with haemangiopericytoma of the meninges at the age of 35 for which she underwent surgery. She had two recurrences that were treated with radiosurgery. Five months prior to presentation, a large liver metastasis was histologically confirmed. Further medical history was uneventful. On admission serum glucose was 0.8 mmol/L and serum insulin was suppressed with normal levels of C-peptide. IGF-I, IGFBP-3 and ALS levels were below normal (Table 1). The amount of total IGF-II was within normal range. However, levels

of 'big'-IGF-II were markedly elevated. She recovered quickly after administration of intravenous glucose. A carbohydrate rich diet could hardly prevent more hypoglycaemic events and prednisolone 100 mg per day was started. Eventually, 40 mg daily was needed as maintenance therapy to prevent recurrent hypoglycaemias. Furthermore, she started with dacarbazine 800 mg/m^2 once every three weeks. After six courses she developed lung metastases and she switched to doxorubicin 50 mg/m^2 every three weeks which provided stable disease after six courses and, combined with steroids, kept her free of hypoglycaemic episodes for almost a year.

Table 1. Biochemical features of both patients with non-islet cell tumour-induced hypoglycaemia

	Patient 1	Patient 2
IGF-I (nmol/L)	3.4 (-5.5)	<1.6 (<-7.4)
Total IGF-II (nmol/L)	60.1 (0.9)	44.4 (-1.4)
Pro-IGF-IIE[68-88] (nmol/L)	37.7 (9.3)	22.9 (5.4)
IGFBP2 (nmol/L)	n.d.	21.2 (2.6)
IGFBP3 (nmol/L)	n.d.	22.3 (-3.9)
Insulin (mE/L)	1.6	18
C-peptide (nmol/L)	0.03	0.31
ALS (nmol/L)	n.d.	44.2 (-5.4)

Serum levels of IGF-I, total IGF-II, pro-IGF-IIE[68-88], and IGFBP-3 were determined as described previously [38,57]. Data are also expressed as SD scores for age and gender (in parenthesis). Reference ranges for insulin and C-peptide are <20 mE/L and 0.18-0.63 nmol/L, respectively.

Incidence and tumour types

Data on the exact incidence and prevalence of NICTH are not available. It has been estimated that NICTH is four times less common than insulinoma, but the true incidence is probably higher since many cases go unrecognised, especially concerning patients with disseminated disease [1]. NICTH can arise in virtually every benign and malignant tumour. However, it mainly occurs in patients with solid tumours of mesenchymal and epithelial origin, but rarely also in patients with tumours of haematopoietic and neuroendocrine origin (Table 2) [1,30,40-42]. In general, the mesenchymal tumours have in common that they are well differentiated and slowly growing, although many usually weigh between 2 and 4 kg at diagnosis.

Table 2. Non-islet cell tumours associated with hypoglycaemia

Tumour	% of total
Tumours of mesenchymal origin	*41*
Mesothelioma	8
Haemangiopericytoma	7
Solitary fibrous tumour	7
Leiomyosarcoma/Gastrointestinal stromal tumour	6
Fibrosarcoma	5
Others	8
Tumours of epithelial origin	*43*
Hepatocellular	16
Stomach	8
Lung	4
Colon	4
Pancreas (non-islet cell)	3
Prostate	2
Adrenal	2
Undifferentiated	2
Kidney	1
Others	1
Tumours of neuroendocrine origin	*1*
Tumours of haematopoietic origin	*1*
Tumours of unknown origin	*14*

Data extracted from Marks and Teale [1], Fukuda *et al* [41], Tsuro *et al* [42], Frystyk *et al* [30] and Zapf [40].

Symptoms

Hypoglycaemia

In unconscious cancer patients without signs of vascular events or brain metastases, NICTH should be considered. Subtle symptoms of hypoglycaemia, especially when they occur between meals and in the morning, can point towards the diagnosis. NICTH is thought to be a fasting hypoglycaemia characterized by: 1) diminished hepatic glucose production due to inhibition of glycogenolysis and gluconeogenesis [40,43,44]; 2) diminished lipolysis in adipose tissue resulting in low serum free fatty acids levels [40] and 3) increased peripheral glucose consumption [40,43-45]. These phenomena point to an enhanced insulin like activity in the body. Furthermore, glucose consumption by the tumour itself might contribute to hypoglycaemia [40]. As we also encountered in the present two cases, insulin levels are generally low or immeasurable in NICTH [1,37,40,46]. In NICTH, the onset of symptoms is frequently gradual with lethargy,

sweating, diminished motor activity and somnolence before a progressive drift into a coma. Recovery may occur spontaneously but is accelerated by the intake of carbohydrates or the administration of parenteral glucose or glucagon [1,46].

Several studies on NICTH indicate that a major part of the glucose intake is rapidly disposed into peripheral tissues, especially skeletal muscle, rather than consumed by the tumour. Suppression of hepatic glucose production or fat oxidation does occur but seems to play a minor role in the development of hypoglycaemia [44,45,47]. Indeed, in contrast to the liver, skeletal muscle contains large numbers of both the IGF-1R and insulin receptors [48] and the effect of 'big'-IGF-II on peripheral tissue was greater than its effect on the liver [47].

Other symptoms

In addition to hypoglycaemic symptoms, acromegaloid skin changes such as skin tags, excessive oiliness of the skin and rhinophyma have been described in patients with NICTH [49,50]. Elevated serum levels of total IGF-II are frequently found in acromegalic patients and it is known that prolonged activation of the IGF-1R by IGF-II may contribute to the development of acromegaloid features [51,52]. Therefore it is conceivable that the secretion of high molecular weight forms of IGF-II by tumour tissue into the circulation might play a role in the development of external signs of acromegaly in some NICTH patients.

Clinical course

NICTH can be either the presenting symptom of a tumour or present in patients with a history of a neoplasm. In a large study from Japan, describing 78 patients, the clinical course of patients with NICTH was analysed [41]. In 48% of these cases, a hypoglycaemic episode was the initial sign that led to the diagnosis and the discovery of a tumour. In the remainder of the patients, hypoglycaemia was detected during the period of observation and treatment of the underlying malignancy. Furthermore, 13% of the NICTH patients investigated had a histologically identical tumour resected in the past without any evidence of hypoglycaemia at that time. *Vice versa*, and quite surprisingly, hypoglycaemia does not always reappear when a previously NICTH-causing tumour recurs and grows back to its former size.

(Pro-) IGF-II is expressed in a broad spectrum of malignant (and benign) tumours and IGF-II may act as an autocrine growth factor through binding to the IGF-1R or the isoform A of the insulin receptor which conduct a strong mitogenic and antiapoptotic

activity [53,54]. Although both IGF-II and 'big'-IGF-II are capable of inducing the phosphorylation of partially purified preparations of both the insulin receptor and IGF-1R [55,56], it has not been unambiguously demonstrated that 'big'-IGF-II indeed stimulates tumour progression [35]. Furthermore, it is not known whether the existence of NICTH is of prognostic value with respect to predicting the degree of malignancy of a tumour or the (disease-free) survival of the patient.

Pathogenesis of non-islet cell tumour-induced hypoglycaemia

Involvement of IGFs

The incongruity between the clear insulin deficiency in patients with NICTH on the one hand and metabolic features pointing to enhanced insulin action on the other hand, suggests that IGFs play a major role in NICTH. In contrast to insulin and IGF-I which are under endocrine control, IGF-II production is predominantly autocrine and paracrine. The level of IGF-I in serum of NICTH patients is usually decreased. On the other hand, circulating levels of total IGF-II, as determined by conventional immunometric or receptor assays, may be either increased, decreased or within the normal range [38,55,57].

These puzzling observations were clarified by Daughaday *et al* in 1988. The concentration of total IGF-II levels in serum from a NICTH patient with a leiomyosarcoma investigated by them was within the normative range as measured by both radioimmunoassay and radioreceptorassay. However when the patient's serum was subjected to Biogel P-60 column chromatography at acidic pH, about 70% of the total IGF-II appeared to be recovered in a higher molecular weight (10-17 kDa) fraction. The remainder of the IGF-II was in the mature 7.5 kDa form. In contrast, in normal serum, high molecular forms of IGF-II ('big IGF-II') contributed only in about 10-20% to the total IGF-II pool. Furthermore, the tumour contained high concentrations of IGF-II mRNA. The authors also demonstrated that after removal of the tumour, 'big'-IGF-II levels in the patient's serum normalised [37]. These findings suggested that the IGF system indeed was involved in pathogenesis of NICTH.

Aberrant IGF expression in NICTH

As illustrated in the case of patient 1, NICTH-causing tumours abundantly express IGF-II mRNA. *IGF-II* gene over-expression is more widespread than first thought and appears to occur in various malignancies, albeit to a variable extent [58,59]. Nowadays it

is generally accepted that IGF-II is involved in oncogene-induced tumorigenesis [32,60]. Over-expression of *IGF-II* in tumours has been mainly attributed to either a loss of imprinting or mutations in tumour suppressor genes [32,61,62]. The mechanisms leading to IGF-II mRNA over-expression in NICTH have rarely been studied [50,58]. Hodzic *et al* reported loss of imprinting of the *IGF-II* gene in a mesothelioma causing hypoglycaemia [58]. In addition, Bertherat and colleagues studied allele specific expression of the *IGF-II* gene in a pleural fibrosarcoma causing NICTH. They observed a loss of imprinting of both parental alleles causing increased expression of the *IGF-II* gene and decreased expression of the genes encoding the tumour suppressors *H19* and $p57^{KIP2}$ [50]. Besides elevated levels of mRNA for IGF-II, in some cases tumours causing NICTH occasionally also express mRNAs for either IGFBP-4, -5 or -6 [57,63-65]. Patients with NICTH usually exhibit elevated levels of IGFBP-2 in their circulation but the source (with exception of one reported case with a IGFBP-2 expressing tumour) [65] and pathophysiologic role of this IGFBP are not established [57,63-65].

'Big'-IGF-II and NICTH

Not all tumours that overexpress the *IGF-II* gene cause NICTH and many NICTH cases involve pre-existing tumours. It is not clear whether serum pro-IGF-II levels in patients with these tumours are already elevated prior to the first signs of hypoglycaemia. In non-islet cell tumours causing hypoglycaemia, post-translational processing of pro-IGF-II is abnormal [66-69]. As depicted in Figure 1, over-expression of the *IGF-II* gene causes overproduction of pro-IGF-II [66]. Incompletely processed pro-IGF-II accounts for 10–20% of the total IGF-II in the normal human serum [34] and is O-glycosylated [70,71]. In serum of patients with NICTH, a much higher proportion (usually >60%) of IGF-II is in a higher molecular weight form that seems to be mainly non-glycosylated and consists primarily of IGF-II with a 21 amino acid extension of the E-domain (pro-IGF-IIE[68-88]) [37,68,70]. Glycosylation may therefore be a targeting signal for cleavage of the E-domain peptide and contribute to the size heterogeneity observed in 'big'-IGF-II. It seems likely that in many neoplastic cells the levels of the various enzymes involved in post-translational processing are not sufficient to handle the relatively high amounts of pro-IGF-II produced adequately [1,37,38,40,67,72-78].

As emphasized previously, 'big'-IGF-II is biologically active and is present in relatively high amounts in the serum of NICTH patients. In most cases the serum level of total IGF-II is not elevated. Therefore, it seems that 'big'-IGF-II must have specific biochemical properties, being different from those of mature IGF-II, that lead to an

enhanced bioavailability and, consequently, increased insulin-like activity in the body [68].

'Big'-IGF-II has equal affinity for the IGFBPs compared to fully processed IGF-II and can therefore form the binary complex with all IGFBPs [27,68]. However, although the exact mechanism is still unknown, 'big'-IGF-II seems to possess properties, which do not allow the proper formation of a 150 kDa complex together with IGFBP-3 and ALS (Figure 3). It seems that in NICTH the binary complex of 'big'-IGF-II and IGFBP-3 has a strongly reduced affinity for ALS since a deficiency in or dysfunction of ALS do not occur [79-81]. Possibly, the heavy N-linked carbohydrate moiety of IGFBP-3, which is absent from IGFBP-5, may interact with the E-domain of 'big'-IGF-II leading to steric interference and consequently reduction of the affinity for ALS [27,81]. Indeed, IGFBP-5 is still capable of forming ternary complexes with IGF-I and ALS [27]. As a consequence of impaired formation of the 150 kDa complex, tumour-derived 'big'-IGF-II primarily forms smaller binary complexes with IGFBPs and a greater fraction may stay in the free unbound form [25,68,82]. These smaller complexes have a greater capillary permeability and thus are thought to increase IGF bioavailability to the tissues, resulting in hypoglycaemia through action on the insulin receptors and IGF-1R. In light of this, it is fascinating that tumours causing NICTH display elevated serum levels of particular IGFBPs [57,63-65]. Apparently, some tumours can be more or less self sufficient in delivering IGF-II from the circulation to its target tissues causing hypoglycaemia.

Serum of patients with NICTH has been shown to contain 4 and 20 times the concentration of free IGF-I and free total IGF-II (these measurements do not discriminate between free mature and free 'big'-IGF-II), respectively, as normally present in serum, although serum levels of total IGF-I and IGF-II were lower [30]. A possible explanation (besides the impaired formation of 150 kDa complexes) may be that increased production of 'big'-IGF-II by the tumour displaces free IGFs from the IGFBPs leading to increased serum concentrations of free, unbound IGFs. The significant positive correlation between 'big'-IGF-II and free total IGF-II observed in the serum of NICTH patients supports such hypothesis [30]. Highly elevated levels of free IGF-I and free IGF-II most likely imply an enhanced hypoglycaemic insulin-like activity, and may, through negative feedback, contribute to the marked suppression of GH secretion by the anterior pituitary gland as observed in NICTH. As a consequence of reduced GH release, the concentrations of the GH-dependent proteins IGF-I, IGFBP-3 and ALS, decrease.

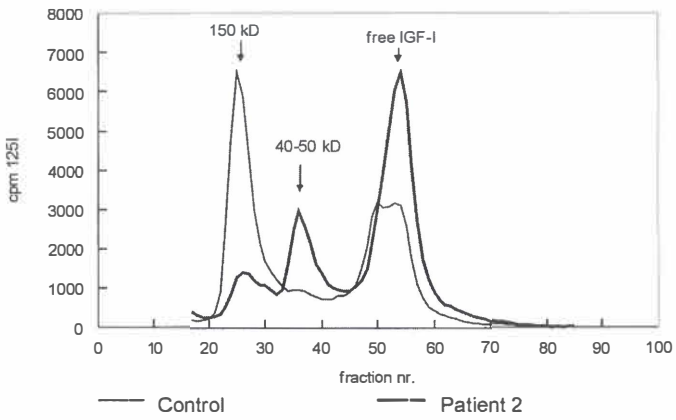

Figure 3. Analysis of complex formation in patient 2 using S200 column chromatography. An aliquot of patient's serum (250 µl) or normal control serum was incubated with 50 µl 125I-labelled purified human insulin-like growth factor (IGF)-I (100 000 c.p.m.) and the different complexes were subsequently separated. In patient's serum, 150 kDa complex formation is reduced and, instead, relatively higher proportions of [125I]-IGF-I are associated with the smaller 40–50 kDa binary complexes and remain unbound.

Thus, as summarised in figure 4, it can be hypothesised that excessive production of 'big'-IGF-II by a tumour leads in fact to a vicious circle whereby the impaired formation of ternary complexes is gradually worsened by an increasing feedback inhibition of GH production that reduces the amounts of IGFBP-3 and ALS available for complex formation further. Quite probably, hypoglycaemia occurs when the various counter-regulatory processes cannot compensate anymore for the increasing insulin-like activity.

Other causes of hypoglycaemia

Of course, other combinations of factors may occur as well. Decreased hepatic glucose output associated with destruction of the liver by tumour infiltration may also be a critical factor in the onset of hypoglycaemia. In addition, sometimes patients with NICTH do not have elevated 'big'-IGF-II levels and hypoglycaemia may be caused by the increased secretion of mature instead of 'big'-IGF-II [40], IGF-I [3], insulin or other peptides with insulin-like activity [1,83] or a combination of cachexia, renal and hepatic dysfunction, and glucose consumption by the tumour [84].

'Big'-IGF-II and other disease entities

There are other disease entities in which abnormal processing of pro-IGF-II plays a role in the aetiology and/or pathophysiology. For example, Daughaday *et al* reported that individuals who have immunologic markers of hepatitis B virus infection may exhibit an increased proportion of partially processed pro-IGF-II in their circulation. However, these patients had no evidence of hypoglycaemia [85].

Patients with hepatitis C-associated osteosclerosis (HCAO) have a specific increase in circulating 'big'-IGF-II and IGFBP-2 levels. However, HCAO patients do not exhibit hypoglycaemia, nor have NICTH patients been reported to have osteosclerosis. The predominant circulating forms of 'big'-IGF-II in HCAO and NICTH are clearly different, including pro-IGF-IIE[1–104] and pro-IGF-IIE[1–88], respectively, perhaps accounting for the development of osteosclerosis in one syndrome and hypoglycaemia in the other [86]. Furthermore, in HCAO, the 150 kDa ternary complexes in serum are formed normally and there is no increase in serum free IGFs [87].

Diagnosis

NICTH suppresses insulin secretion by beta-cells, lipolysis, and ketogenesis [40,43,44], leading to low C-peptide, and inappropriately low GH and beta-hydroxybutyrate (β-OHB) concentrations in the circulation [1,46]. In case of hypoinsulinaemic hypoglycaemia the assessment of elevated serum levels of 'big'-IGF-II or E[68-88]-peptide, in combination with increased levels of IGFBP-2 by specific immunometric assays is of high diagnostic value [38]. Size-exclusion acid chromatography, a very time-consuming procedure, has been considered the gold standard method for detection of 'big'-IGF-II in NICTH. However, measurement of the serum concentration of 'big'-IGF-II determined by immunoblot analysis of 'big'-IGF-II and mature IGF-II after 16.5% tricine-sodium dodecyl sulphate-polyacrylamide gels has proven to be a more rapid, reproducible and equally sensitive method and a useful laboratory evaluation of patients with a clinical diagnosis of NICTH [88].

Since GH secretion is restrained with subsequent lowering of GH dependent IGF-I and IGFBP-3 production by the liver (figure 4), reduced levels of the latter proteins in serum represent useful additional markers, as well as an increased ratio between total IGF-II and IGF-I. Despite hypoglycaemia, levels of glucagon are often within the normal range suggesting a suppressive effect of 'big'-IGF-II on glucagon secretion [45,89].

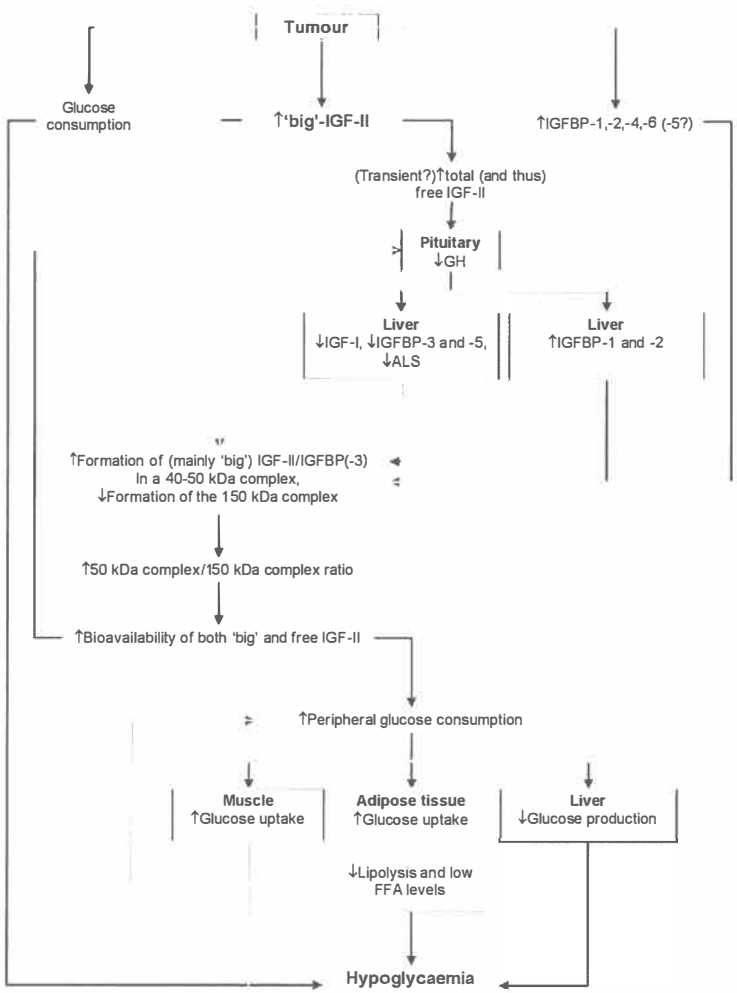

Figure 4. Proposed mechanism of the development of non-islet cell tumour-induced hypoglycaemia (NICTH). It is likely that tumour cells cannot process the augmented amounts of pro-insulin-like growth factor-II (pro-IGF-II) synthesized, resulting in a substantial release of 'big'-IGF-II into the circulation. 'Big'-IGF-II competes with the mature IGF-II and IGF-I for binding to IGF-binding proteins (IGFBPs). However, the formation of the ternary complex between 'big'-IGF-II, IGFBP-3 and the acid-labile subunit (ALS) in the circulation is hampered. As a consequence, primarily 40-50 kDa binary complexes are formed. In addition, both free fractions of IGF-I and total IGF-II are increased. Since the binary complexes and free IGFs can pass the capillary membrane relatively easily when compared to the 150 kDa ternary complex, the concentrations of IGFs, presumably especially 'big'-IGF-II, at the tissue level will

rise inducing a strong insulin-like effect via the insulin receptors, causing hypoglycaemia. Moreover, because of an increasing negative feedback on growth hormone (GH) production by the anterior pituitary gland, the synthesis of GH-dependent peptides such as IGF-I, IGFBP-3, IGFBP-5 and ALS is decreased. This leads to even further declined formation of ternary complexes. Abbreviations: FFA, free fatty acids.

However, in the absence of diffuse liver metastases, tumour-induced hypoglycaemia often is associated with increased, rather than depleted, hepatic glycogen stores [18,45]. Furthermore, hypokalaemia is often associated with hypoglycaemia, presumably due to the insulin-like activity of ('big'-) IGF-II [41].

Since tumours causing NICTH are usually very large, they can be readily detected by conventional radiological imaging using computed tomography or magnetic resonance imaging. However, one has to bear in mind that functional tumour imaging techniques such as fluorodeoxyglucose-positron emission tomography may lead to false-negative results [90]. Presumably this is due to an accelerated uptake of fluorodeoxyglucose by especially the heart and skeletal muscle that competes with the slower rate of uptake of tracer by tumour tissue. Other nuclear tracers such as radiolabelled tyrosine can provide an alternative [91].

Treatment

Curative and palliative measures

The long-term therapeutic strategies in NICTH involve complete removal of the tumour or reduction of the tumour mass. The metabolic alterations caused by NICTH are fully reversible after successful surgical removal of the 'big'-IGF-II producing tumour [37,40,68], as also demonstrated in case 1.

In many cases, including patient 2, the malignancy causing NICTH is a large mass infiltrating into surrounding tissue and often accompanied by disseminated disease. Alleviating hypoglycaemia is subsequently a therapeutic challenge. When curative resection is no longer possible, various approaches to the treatment of NICTH have been tried with the initial aim of relieving the hypoglycaemic symptoms. Chemotherapy directed against the tumour or selective embolisation of tumour mass can also reduce the occurrence of hypoglycaemic events. However, when the tumour has only partially disappeared, hypoglycaemia is likely to reoccur when the tumour grows again [92].

Increasing serum glucose

In order to treat hypoglycaemia, a short-term beneficial effect is best achieved with (continuous) parenteral administration of glucose and dietary guidelines. However, especially when taken into account the condition of the patient, these measures are sometimes difficult to realise. As in insulinomas, diazoxide-chlorothiazide treatment may improve NICTH-symptoms [1]. Correction of hypoglycaemia also has been attempted successfully by the administration of glucagon. It does so primarily by increasing hepatic glucose output [18]. Interestingly, Hoff and Vassilopoulou-Sellin reported that a glycaemic response to a glucagon stimulation test predicted good response to long term treatment with glucagon (0.06-0.3 mg/hour), via continuous intravenous infusion [93].

Somatostatin analogues

The presence of somatostatin receptors has been demonstrated previously in a pleural fibroma causing NICTH [94] and in approximately 40-55% of hepatocellular carcinomas [95,96]. However, in NICTH the administration of somatostatin analogues such as octreotide, generally does not restore glucose levels, probably because somatostatin receptors, if present at all in the hypoglycaemia-causing tumour, are non-functional [94,97,98]. Nonetheless, in a case of an intra-abdominal haemangiopericytoma, the prolonged infusion of somatostatin appeared to reduce the secretion of 'big'-IGF-II by the tumour [45].

Glucocorticosteroids

Glucocorticosteroid treatment seems to be the most effective one in terms of long-term relief from hypoglycaemia by stimulating glyconeogenesis and suppressing, although not in all cases, the production of 'big'-IGF-II and correcting the attendant biochemical abnormalities involving the GH–IGF axis [63,94,99,100]. Moderate to high doses of glucocorticosteroids may cause shrinkage of the tumour. A recent study extended these findings by demonstrating that the beneficial effects of glucocorticosteroids are dose-dependent and reversible when treatment is withdrawn or when the dose falls below a critical level [101].

Growth hormone

In a recent review [102], the stimulation of IGFBP-3 and ALS production by the liver after the administration of rhGH was considered to be beneficial in the treatment of NICTH. However, in two cases of NICTH associated with pleural solitary fibrous tumours,

successful GH treatment lead to only moderate increments of circulating IGF-I and IGFBP-3 levels [103]. This would suggest that alternative, possible direct, mechanisms of action of GH in alleviating hypoglycaemia most likely play a role. Although increases in serum concentrations of IGFBP-3 and ALS indisputably occur, it seems that either the production rise is insufficient and/or the amount of tumour-derived 'big'-IGF-II is still sufficient to inhibit formation of ternary complexes [65,100]. Analogous to the induction of glucose intolerance in acromegaly, stimulation of hepatic gluconeogenesis and glycogenolysis may be an important aspect of the effect of (recombinant) GH. As demonstrated previously [99,103], GH can alleviate hypoglycaemia. Aside from their influence on serum insulin and glucose the diverse metabolic effects of GH (in protein sparing) and glucocorticosteroids (in tumour suppression) suggest that their combined use may be feasible in the treatment of NICTH [63,100,101,104].

Conclusion

In patients with a mesenchymal or malignant epithelial tumour suffering from hypoglycaemic episodes or unconsciousness, NICTH should be considered. NICTH follows the production of partially processed forms of pro-IGF-II, called 'big'-IGF-II, which, apart from direct insulin-like biological activity, fails to form inactive ternary complexes with IGFBPs and ALS and would increase bioavailability of IGFs. At the target tissues, ('big'-)IGF-II interacts with IGF-1R and insulin receptors resulting in hypoglycaemia. Low serum insulin in combination with elevated levels of 'big'-IGF-II and an increased IGF-II:IGF-I ratio would confirm the diagnosis. Removal of the tumour can cure NICTH but when that is no longer possible, treatment with glucocorticosteroids, GH or combinations thereof can suppress NICTH and alleviate symptoms.

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Chapter 3b

Non-islet cell tumour hypoglycaemia in a patient with a gastrointestinal stromal tumour

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Tumour-induced hypoglycaemia is a rare paraneoplastic phenomenon. Insulinomas are the most common tumours associated with hypoglycaemia. The incidence of these insulin secreting tumours is estimated at 0.5 to 4 per million people per year [1,2]. However, hypoglycaemia may also occur in solid tumours of epithelial or mesenchymal origin. Hypoglycaemia caused by these types of tumours is referred to as non-islet cell tumour hypoglycaemia (NICTH). NICTH is generally attributable to the secretion of large amounts of incompletely processed insulin-like growth factor II (IGF-II), also called 'big'-IGF-II [3].

In this report, we describe the case of a patient with metastatic gastrointestinal stromal tumour (GIST) who presented with a loss of consciousness due to hypoglycaemia caused by increased concentrations of 'big'-IGF-II.

Case report

A 50-year-old man was admitted to the hospital because of loss of consciousness. He was known for a large retroperitoneal GIST diagnosed three years earlier as a recurrence of a previously resected sarcoma, at that time interpreted as a leiomyosarcoma. In the summer of 2001, the patient was enrolled in a clinical trial with the tyrosine kinase inhibitor imatinib mesylate (gleevec®). From that moment, stable disease was established during a year followed by local tumour progression. At that time, debulking of the tumour was performed. Three months later, a CT-scan revealed multiple liver metastases. Subsequently, the patient was included in a phase I study. However, during the following months he showed further disease progression.

On admission, physical examination revealed a comatose patient with a Glascow Coma Score of 1-4-1. A CT-scan was performed because a neurological cause was considered. However, his serum glucose level turned out to be 1.0 mmol/L (normal fasting glucose 4.0-5.4 mmol/L). Further laboratory investigation showed normal kidney function, slightly abnormal liver function tests and low insulin (<2 mU/L) levels. He recovered quickly after glucose infusion. A carbohydrate rich diet could hardly prevent more hypoglycaemic events and prednisolone 30 mg per day was started. The patient died a month later.

Because NICTH was suspected, serum samples obtained during the phase of stable disease, disease progression and a month prior to admission were analysed. IGF-I and IGF-binding protein 3 (IGFBP-3) levels were reduced and the concentration of IGFBP-2

was elevated a month prior to admission (table 1). In contrast, the levels of these proteins at the other time points fell within the normal range. The amount of total IGF-II was not deviated from normal control at all the time points. However, levels of pro-IGF-IIE[68-88] ('big'-IGF-II) were markedly elevated a month before admission.

Table 1. IGF and IGFBP levels at various time points[1]

	Stable disease	Disease progression	Prior to admission
IGF-I (nmol/L)	10.6 (-1.19)[2]	19.5 (1.10)	3.9 (-4.56)
IGF-II (nmol/L)	49.4 (-0.83)	71.2 (1.16)	48.5 (-0.92)
Pro-IGF-IIE[68-88] (nmol/L)	6.24 (0.16)	7.13 (0.35)	21.9 (5.23)
IGFBP-2 (nmol/L)	12.8 (1.89)	11.6 (1.65)	23.6 (3.27)
IGFBP-3 (nmol/L)	44.7 (-1.49)	55.0 (-0.64)	22.3 (-4.44)

[1] Serum concentrations were determined by Jaap van Doorn, Laboratory of Endocrinology, Wilhelmina Children's Hospital, Utrecht, The Netherlands

[2] Standard deviation scores (Z-scores) are given between hedges.

Discussion

There are several mechanisms by which solid tumours can cause hypoglycaemia: (1) insulin secreting insulinomas, (2) non-islet cell tumours secreting aberrant IGF-II and (3) liver and adrenal failure due to tumour invasion [2]. Our patient had elevated levels of 'big'-IGF-II that is associated with NICTH and had no signs of liver failure.

Both IGF-II and IGF-I are structurally and functionally related to insulin [4,5]. They can exert an insulin-like activity by binding to the insulin receptor and IGF receptor type 1 [6]. This insulin-like activity is only 5% of insulin but the total serum concentration of IGFs is about 1000-fold higher than the mean insulin concentration. Hypoglycaemia does not occur, however, because of binding to IGFBPs. Under physiological conditions, 75-80 percent of the IGFs are bound to IGFBPs, mostly IGFBP-3, and an acid-labile subunit (ALS). The remainder is bound to IGFBP-3 alone as a binary complex or, less than 1 percent, circulates in a free form. The ternary complex has a molecular mass of about 150 kDa and is not able to pass the capillary membrane. In this way the availability of IGFs to their target tissues is limited [7]. IGF-I, IGFBP-3 and ALS are produced by the liver and their production is stimulated by growth hormone (GH) secretion. GH, in turn,

is negatively regulated by IGF-I and IGF-II. In contrast, IGF-II is produced by various tissues which is GH-independent [6].

Non-islet cell tumours causing hypoglycaemia contain elevated levels of mRNA for IGF-II [8,9]. IGF-II is thought to act as an autocrine growth factor in various tumours [8,10]. It is produced as a pre-prohormone. During intracellular processing, tumour cells fail to cleave the large E-domain from pro-IGF-II, which leads to the secretion of an extraordinary large amount of uncleaved peptide, the so called 'big'-IGF-II [11]. In NICTH, high serum concentrations of 'big'-IGF-II are associated with a shift in the distribution of IGFs from high-molecular-weight ternary complexes to binary complexes and to the unbound form [9,12]. Therefore, it seems likely that abundant 'big'-IGF-II leads to an impaired formation of the ternary complex. This could be due to several factors. In the first place, It has been reported that the aberrant form of IGF-II and IGFBP-3 have the inability to form complexes with ALS [13]. Furthermore, an increase in circulating 'big'-IGF-II levels could suppress GH secretion. As a consequence, IGFBP-3 and ALS production by the liver is decreased, leading to a further decrease in ternary complexes [9]. Binary complexes and unbound IGF can easily cross the capillary membrane. 'Big'-IGF-II has the same affinity to the insulin and IGF receptor type 1 as normal IGF-II [9]. Excessive receptor stimulation causes increased peripheral glucose uptake and suppressed hepatic glucose output, resulting in hypoglycaemia [14].

Laboratory investigation can confirm the diagnosis NICTH. Low insulin and C-peptide levels are detected, related to a low glucose concentration. GH, IGF-I and IGFBP-3 levels are typically decreased. An elevated level of IGFBP-2 is also a characteristic finding in patients with NICTH, although the mechanisms by which it is increased are unclear [15]. The concentration of E-domain containing forms of IGF-II is increased [11]. However, total IGF-II levels are usually within the normal range, which is probably due to increased turnover rates. Furthermore, an IGF-II:IGF-I molar ratio >10 is also considered pathognomic of NICTH [2]. All these characteristic laboratory findings were retrospectively found in our patient, including markedly elevated big-IGF-II levels (standard deviation score 5.23) and a IGF-II:IGF-I molar ratio of 12.4 (table 1).

Data on the exact incidence of NICTH are not available. It has been estimated that NICTH is four times less common than insulinoma, but the true incidence is probably higher [2]. In most of the cases, NICTH is caused by a tumour of mesenchymal origin. These tumours are usually large and can be both benign and malignant. The most common histological types causing hypoglycaemia are fibrosarcomas, mesotheliomas, leiomyosarcomas, and haemangiopericytomas [3].

Our patient suffered from a large GIST with multiple liver metastases. GISTs are a group of mesenchymal neoplasms, showing differentiation towards the interstitial cells of Cajal, also known as the pacemaker cells of the gastrointestinal tract. Virtually all GISTs over-express the receptor tyrosine kinase KIT that, in most cases, contains mutations leading to ligand-independent activation of the receptor. Until recently, stromal tumours of the gastrointestinal tract were regarded as leiomyomas, leiomyosarcomas or leiomyoblastomas. However, these tumours do not express KIT and nowadays GISTs are considered as a distinct clinicopathological entity [16]. The introduction of imatinib mesylate, an inhibitor of KIT, has dramatically improved the life expectancy of patients with metastatic GIST [17]. Recently, Beckers *et al* have described a patient with NICTH due to a GIST [18]. This report is the second one about this topic. There are several reported cases about hypoglycaemia caused by a leiomyosarcoma of the stomach or bowel [19,20]. Advances in KIT immunostaining has revealed that leiomyosarcomas of stomach and bowel are extremely rare. Therefore, it is very suggestive that these tumours should have been GIST and probably more cases associated with hypoglycaemia will be reported in the near future.

In order to treat the hypoglycaemia, a short-term effect is best achieved with infusion of glucose and dietary guidelines. The best long-term treatment is to reduce the tumour by surgery, irradiation or chemotherapy [6]. In our patient, frequent carbohydrate rich meals could not prevent the recurrence of new hypoglycaemic events. When tumour reduction can not be established and dietary measurements and infusion of glucose is not sufficient, administration of glucocorticoids, glucagon and high dose GH can be tried [21-23].

In conclusion, in unconscious cancer patients – especially those diagnosed with mesenchymal tumours – apart from vascular events and brain metastases, NICTH should be considered.

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Chapter 4

Insulin-like growth factors and insulin-like growth factor-binding proteins in relation to disease status and incidence of hypoglycaemia in patients with a gastrointestinal stromal tumour

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Abstract

Objective: Patients with a gastrointestinal stomal tumour (GIST) suffering from non-islet cell tumour-induced hypoglycaemia (NICTH), being associated with increased plasma levels of pro-insulin-like growth factor (IGF)-IIE[68-88], have been reported occasionally. We studied the clinical relevance of pro-IGF-IIE[68-88] and other IGF-related proteins in GIST patients.

Patients and methods: Twenty-four patients were included. Plasma samples were collected before, 1 week, and median 5 months after start of treatment with imatinib, and levels of IGF-I, total IGF-II, pro-IGF-IIE[68-88], IGFBP-2, -3 and -6 were determined. GIST specimens from 17 patients and tumour cyst fluid from two patients were analysed for IGF-II and IGFBP-2.

Results: Before treatment and/or during follow-up, 3/24 (13%) patients showed increased plasma levels of pro-IGF-IIE[68-88]. All three developed NICTH. Overall, patients with either metastatic disease, elevated serum LDH activity, or total tumour size >12 cm had the highest pro-IGF-IIE[68-88] levels. Most patients had increased plasma IGFBP-2 levels and these levels were significantly higher in patients with progressive disease. (pro-)IGF-II was expressed in 82% of GISTs, and IGFBP-2 only in one case.

Conclusions: We identified pro-IGF-IIE[68-88] as a marker that may be used in the surveillance of GIST.

Introduction

Gastrointestinal stomal tumours (GISTs) are the most common mesenchymal malignant tumours of the gastrointestinal tract. GISTs are characterised by the over-expression of the receptor tyrosine kinase KIT. In about 80 percent of the GISTs, *KIT* gain-of-function mutations are found, while 5-7 percent of the tumours harbour platelet-derived growth factor receptor α (*PDGFRA*) activating mutations [1]. Patients with metastatic or unresectable GIST are successfully treated with imatinib, an inhibitor of KIT and PDGFRA [2].

Non-islet cell tumour-induced hypoglycaemia (NICTH) is a paraneoplastic syndrome characterised by recurrent fasting hypoglycaemia. It is associated with the secretion of incompletely processed precursors of insulin-like growth factor-II (IGF-II) by the tumour into the circulation. These precursors consist mainly of mature IGF-II with a 21-residue carboxyl extension of the E-domain of pro-IGF-II, called pro-IGF-IIE[68-88] or 'big'-IGF-II (for review, see [3]). Patients with NICTH usually exhibit low circulating levels of IGF-binding protein-3 (IGFBP-3) and total IGF-I, whereas those of IGFBP-2, (occasionally) IGFBP-6, and free unbound IGFs are increased [3,4].

Recently, we and others have reported on the occurrence of NICTH in patients with a GIST [5-10]. Based on these relatively high number of cases reported so far, it seems that patients with a GIST are at risk for developing NICTH. In the present study, we determined the plasma levels of IGF-I, total IGF-II, pro-IGF-IIE[68-88], IGFBP-2, -3, and -6 for a cohort of patients with a GIST, before and during treatment with imatinib. The predictive values of these parameters with respect to the occurrence of hypoglycaemia, disease progression and response on treatment were evaluated.

Patients and methods

Patients

Twenty-four consecutive newly treated patients at the department of Medical Oncology of the University Medical Center Groningen were included in this study. Patients were eligible in case of advanced unresectable or metastatic histologically proven GIST, as characterised by CD117 positivity, for which treatment with imatinib was indicated. Every patient underwent a routine computed tomography (CT) scan prior to the start of therapy, 8 weeks after start of therapy, and every 3 months thereafter until progression

of disease. In addition, routine 2-[fluorine-18]fluoro-2-deoxy-D-glucose (FDG)–positron emission tomography (PET) scanning was performed a few days before and one week after the start of imatinib. EDTA-plasma samples were collected prior to the start of treatment, 1 week after start of therapy, and during the first year (median 5 months, range 2-12 months) of treatment. For those patients who developed hypoglycaemia during clinical follow-up, routine, non-fasting, EDTA-plasma samples were taken to diagnose NICTH.

Overall tumour size was determined by the sum of the longest diameter for all target lesions as defined by RECIST [11]. Initial overall response to therapy was defined when at least two of the following events were noted in an individual patient: (1) improvement of clinical symptoms, (2) CT scan response (partial response according to RECIST, *i.e.* ≥30% decrease in the sum of the longest diameter of target lesions based on CT before and 8 weeks after start of treatment [11], or (3) >25% decrease in FDG uptake on PET scanning on day 8 compared with the pretreatment scan [12,13]. The examination of the PET images was performed as described previously by using standardized uptake value (SUV) analysis [14]. Disease progression after initial response was defined as (1) the occurrence of a new lesion, (2) increasing size of the pre-existing lesions (as defined by RECIST), or (3) development of an intratumoral nodule and/or an increase in 'solid' tissue, in the background of a hypodense lesion [13].

The study protocol was approved by the Medical Ethics Committee of the University Medical Center Groningen and all patients gave their informed consent.

Measurement of plasma levels of IGFs and IGFBPs

Concentrations of IGFBP-2, IGFBP-3, IGFBP-6, IGF-I, pro-IGF-IIE[68-88], and total IGF-II (i.e. both mature 7.4 kDa IGF-II and pro-IGF-IIE[68-88] are detected in this radioimunnoassay with nearly equal potency) in plasma and cyst fluid were determined as described previously [15-17].

Cyst fluid, GIST specimens and histological examinations

For two patients cyst fluid was aspirated from metastatic lesions using a routine procedure to relieve abdominal tension.

From 17 of 24 patients paraffin-embedded tumour samples were available. From each tumour sample three (distinct) representative 0.6 mm cores were taken and transferred to a standard-size recipient paraffin block and immunohistochemistry was performed as described previously [18]. Antigen retrieval was performed by boiling slides in 0.01 M

citrate buffer (pH 6.0). Primary antibodies were rabbit anti-IIE[68-88] antiserum (1:200; WKZ6279) [16] and polyclonal goat anti-IGFBP-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, Ca, USA). As a negative control, a serial section was processed by replacement of primary antibody with preimmune primary antisera or normal goat IgG. The methods used for in situ hybridisation with digoxigenin-labeled hIGF-II complementary RNA probes have been described previously [19]. Negative controls for in situ hybridisation were prepared using sense probes. For both immunohistochemistry and in situ hybridisation, normal tissue samples derived from first trimester placenta served as positive controls [20]. The intensity of histochemical staining was evaluated by two independent observers (B.R. and A.J.H.S). Tissue cores from a particular tumour were judged to be positive when there was a clear cytoplasmic staining in at least two of the three cores.

Tumours from patients with NICTH were analysed for *KIT* and *PDGFRA* mutations as described in the supplementary materials and methods section and the supplementary table.

Statistics

As the plasma levels of most of the IGFs and IGFBPs are age- and sex-dependent, the concentrations were compared with age- and sex-dependent references and expressed as standard deviation scores (SDS) [15-17]. The Wilcoxon signed ranks test was used to compare plasma levels with the reference population or to analyse paired data. Differences between two groups were studied with the Mann-Whitney U test. Correlations were described by the Spearman's rank correlation coefficient (r). The log rank test was applied to analyse the impact of variables on survival. A double-sided P-value <0.05 was considered statistically significant using SPSS for windows version 12.0.

Results

General clinical characteristics of the GIST patients

Patient characteristics are summarised in table 1. Two third of the patients had metastatic disease and the median total tumour size was 14 cm. The initial overall response to imatinib was 75% with a median progression-free survival of 25 months which is in agreement with previous reports [2]. Three patients encountered episodes of symptomatic hypoglycaemia, with fasting plasma glucose levels lower than 3.0 mmol/L, either during

treatment with a tyrosine kinase inhibitor or in the course of follow-up after treatment. These patients will be presented in more detail in the last paragraph.

Table 1. Patient characteristics (n=24)

	n (%)	Median (range)
Sex		
Male	16 (67)	
Female	8 (33)	
Age (years)		62 (24 – 82)
Primary tumour localisation		
Stomach	8 (33)	
Small bowel	10 (42)	
Colon	3 (13)	
Other site	3 (13)	
Disease stage		
Primary disease only	8 (33)	
Metastatic disease	16 (67)	
Total tumour size (cm)		14 (4 – 47)
Serum LDH (U/L)		263 (117 – 902)
Serum albumin (g/L)		37 (28 – 45)
Initial overall response		
Response	18 (75)	
No response	6 (25)	
PET-response		
Response	19 (79)	
No response	5 (21)	
CT-response		
Partial response	7 (29)	
Stable disease	16 (67)	
Progressive disease	1 (4)	
Clinical symptoms		
Improvement	16 (67)	
No improvement	3 (13)	
No symptoms	5 (21)	
Progression-free survival (months)		25
Follow-up (months)		37 (4 – 85)

LDH, lactate dehydrogenase; PET, positron emission tomography; CT, computed tomography.

Pretreatment plasma levels of IGFs and IGFBPs

Pretreatment plasma samples were available from 22 of 24 patients included in this study. The median SDS values of the IGFs and IGFBPs, as determined prior to imatinib treatment, are shown in table 2. Eight patients (36%) showed plasma pro-IGF-IIE[68-88] levels higher than 1.0 SDS, including one patient with a plasma level higher than 2.0 SDS. This patient developed NICTH. The median concentration of IGFBP-2 in the plasma of GIST patients was markedly higher than that observed in healthy individuals. For 14 patients (64%), the plasma IGFBP-2 levels exceeded 2.0 SDS. In contrast, the median IGFBP-3 SDS for GIST patients was slightly but significantly reduced compared to the reference population.

Table 2. Plasma levels of IGFs and IGFBPs, expressed as standard deviation scores, for patients (n=22) before the start of treatment with imatinib

	Median (range)	P-value (vs. normal)[1]
IGF-I	0.07 (-2.26 – 2.66)	0.95
Total IGF-II	-0.07 (-2.99 – 2.05)	0.63
Pro-IGF-IIE[68-88]	0.47 (-2.17 – 7.08)	0.077
IGFBP-2	2.20 (-0.62 – 3.65)	<0.001
IGFBP-3	-0.58 (-1.84 – 2.38)	0.009
IGFBP-6	0.08 (-1.47 – 2.11)	0.88

[1]Wilcoxon signed ranks test, the P-value indicates whether a median SDS is different from normal (i.e. 0 SDS).

IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; SDS, standard deviation scores.

Patients with a relatively high tumour load (i.e. total diameter >12 cm [21]) exhibited significantly higher pretreatment plasma levels of pro-IGF-IIE[68-88] than subjects with a lower tumour load. Furthermore, plasma levels of pro-IGF-IIE[68-88] were higher in patients with metastatic disease than those with single primary (unresectable) tumours. In addition, we encountered that increased serum LDH (i.e. >235 U/L) predicted a relatively high plasma pro-IGF-IIE[68-88] level (table 3). Serum LDH correlated well with total tumour size ($r=0.848$, $p<0.001$).

We found a significant inverse correlation between serum albumin levels and plasma IGFBP-2 SDS values ($r=-0.559$, $p=0.007$). No associations were found between IGF-I, total IGF-II, IGFBP-3 and IGFBP-6 plasma levels and any of the clinical parameters studied. Elevated plasma levels (i.e. either SDS >1.0 or >2.0) of any component of the IGF system investigated did not predict progression-free survival. In addition, we analysed the

Table 3. Association between patient and tumour characteristics and baseline plasma pro-IGF-IIE[68-88] levels, expressed as standard deviation scores

	Pro-IGF-IIE[68-88], median (range)	P-value[1]
Sex		
Male (n=15)	-0.08 (-2.81 – 1.83)	0.056
Female (n=7)	1.29 (-0.39 – 7.08)	
Age		
≤62 years (n=11)	0.22 (-0.43 – 1.28)	0.65
>62 years (n=11)	0.72 (-2.17 – 7.08)	
Primary localisation		
Small bowel (n=9)	0.92 (-0.75 – 1.64)	0.24
Other sites (n=13)	0.22 (-2.17 – 7.08)	
Disease stage		
Primary disease only (n=7)	-0.32 (-2.17 – 1.25)	0.017
Metastatic disease (n=15)	1.02 (-0.75 – 7.08)	
Tumour size		
≤12 cm (n=8)	-0.09 (-2.17 – 1.25)	0.029
>12 cm (n=14)	1.02 (-0.75 – 7.08)	
Serum LDH		
≤235 (U/L) (n=9)	-0.25 (-2.17 – 1.25)	0.009
>235 (U/L) (n=13)	1.12 (-0.75 – 7.08)	
Serum albumin		
<34 (g/L) (n=6)	0.37 (-2.17 – 1.64)	0.54
≥34 (g/L) (n=16)	0.67 (-0.39 – 7.08)	
Initial overall response		
Response (n=16)	0.23 (-2.17 – 1.64)	0.12
No response (n=6)	0.90 (0.21 –7.08)	
PET-response		
Response (n=17)	0.42 (-2.17 – 1.64)	0.32
No response (n=5)	0.52 (0.21 – 7.08)	
CT-response		
Partial response (n=6)	0.47 (-0.75 – 1.25)	0.91
Stable/progressive disease (n=16)	0.47 (-2.17 – 7.08)	
Clinical symptoms		
Improvement (n=14)	0.01 (-2.17 – 1.64)	0.20
No improvement (n=3)	0.72 (0.21 – 7.08)	

[1]Mann-Whitney U test.

IGF, insulin-like growth factor; LDH, lactate dehydrogenase; PET, positron emission tomography; CT, computed tomography.

mutual relationships among the pretreatment levels of IGFs and IGFBPs. IGF-I SDS correlated with total IGF-II SDS and IGFBP-3 SDS (r=0.521, p=0.013 and r=0.475, p=0.025, respectively). Total IGF-II SDS correlated negatively with IGFBP-2 SDS (r=-0.562, p=0.007).

Plasma levels of IGFs and IGFBPs in patients during treatment with imatinib

In the total group of patients, the plasma SDS values of the various IGFs and IGFBPs did not change significantly after one week of imatinib treatment. After median 5 months, only IGFBP-3 showed a significant decline compared to pretreatment scores (median pretreatment SDS: -0.64 (range: -1.84 – 2.38) *vs.* median follow-up SDS: -1.18 (range -3.27 – 0.78); p=0.005; n=20). Cross-sectional analysis revealed that, during the first year of treatment, the plasma IGFBP-2 levels in patients with progressive disease were significantly higher compared to those in patients without progressive disease (table 4). Within the first treatment year, two additional patients (besides the one who already showed a high pretreatment value) had plasma pro-IGF-IIE[68-88] levels that exceeded 2.0 SDS, and subsequently also developed NICTH (table 5).

Table 4. Plasma levels of IGFs and IGFBPs, expressed as standard deviation scores, during treatment with imatinib for patients with either progressive (n=4) or non-progressive disease (n=18)

	Progressive disease	Non-progressive disease	P-value[1]
Interval since start of imatinib, median months (range)	5.7 (1.9 – 9.5)	4.6 (1.6 – 12.0)	0.77
IGF-I, median (range)	-0.27 (-1.9 – 0.4)	-0.46 (-4.8 – 2.11)	0.59
Total IGF-II, median (range)	-0.60 (-1.33 – 1.68)	-0.76 (-2.21 – 1.42)	0.40
Pro-IGF-IIE[68-88], median (range)	1.13 (-1.62 – 8.64)	-0.29 (-2.68 – 2.49)	0.11
IGFBP-2, median (range)	3.50 (2.47 – 4.46)	1.38 (-1.02 – 3.97)	0.002
IGFBP-3, median (range)	-1.68 (-3.27 – -1.22)	-0.98 (-2.13 – 0.78)	0.40
IGFBP-6, median (range)	-0.79 (-1.77 – 1.37)	0.01 (-1.41 – 3.31)	0.49

[1]Mann-Whitney U test.

IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein.

IGF-II and IGFBP-2 in GIST specimens and tumour cyst fluid

Sixteen of the 17 (94%) tumours studied showed significant IGF-II mRNA expression. Fourteen of the mRNA positive samples stained also positive for IIE[68-88] containing peptides. The GIST specimens of the patients with hypoglycaemia, who exhibited elevated pro-IGF-IIE[68-88] plasma levels, showed a pronounced expression of both IGF-

II mRNA and protein (figure 1). In contrast, positive staining for IGFBP-2 protein was observed for only one GIST. Curiously, the patient from whom this tumour specimen was obtained showed only moderately elevated plasma IGFBP-2 levels (2.09 SDS).

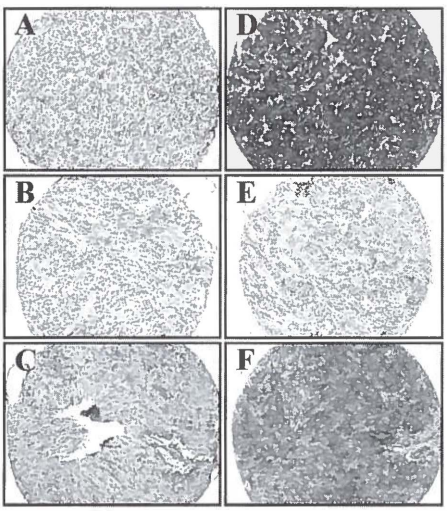

Figure 1. Expression of the IIE[68-88] containing pro-IGF-II protein and IGF-II mRNA in GISTs causing hypoglycaemia. (A-C) Immunohistochemistry for the IIE[68-88] protein in paraffin-embedded GIST sections obtained from patients (numbers 1, 2 and 3, respectively) who appeared to develop NICTH. All samples showed diffuse cytoplasmic staining. (D-F) In situ hybridisation for hIGF-II mRNA expression in the same tissue sections. (Dark)blue staining indicates the presence of hIGF-II mRNA (magnification, x200). *See page 190 for colour figure.*

Analysis of cyst fluid revealed that the concentrations of pro-IGF-IIE[68-88] in cyst fluid and the corresponding plasma sample were nearly similar, i.e. 6.9 nmol/L and 7.3 nmol/L (= 0.3 SDS), respectively, in one case. For the second patient, who exhibited an elevated level of pro-IGF-IIE[68-88] in plasma (14.5 nmol/L, corresponding to 3.5 SDS), the molar ratio of pro-IGF-IIE[68-88] between cyst fluid and plasma was markedly higher, i.e. 1.6. For the two patients investigated, molar ratios of IGFBP-2 between cyst fluid and plasma were 1.1 and 0.8.

Patients with NICTH

The first patient was a 50-year old man and has been described previously [6]. In short, this patient had a large recurrent retroperitoneal GIST with a *KIT* exon 9 mutation. After

an initial response to imatinib, he suffered from progressive disease and developed a hypoglycaemic coma. The second patient was an 83-year old woman with type 2 diabetes mellitus treated with insulin and a recurrent unresectable GIST with a *KIT* exon 9 mutation. During imatinib treatment, her daily insulin doses had to be reduced continuously to maintain acceptable glucose levels. After 3 years of imatinib treatment, she had disease progression, switched to sunitinib and developed nocturnal episodes of hypoglycaemia, without insulin treatment. She died comatose at home while having progressive disease. The third patient was an 82-year old woman with liver metastases from a gastric tumour with a *PDGFRA* exon 18 (Asp842Val) mutation. She did not respond to imatinib and developed fasting hypoglycaemias 8 weeks after the start of the treatment. At the time hypoglycaemia became manifest, markedly elevated plasma levels of both pro-IGF-IIE[68-88] and IGFBP-2 and low concentrations of IGFBP-3 and IGF-I were observed in all patients (table 5). Patient 2 showed a plasma IGFBP-6 level that exceeded 2.0 SDS, a phenomenon that has been encountered previously for some patients suffering from NICTH [22]. Notably, patient 3 showed a strikingly elevated concentration of pro-IGF-IIE[68-88] already before and 1 week after the start of imatinib (table 5). Furthermore, patient 1 and 2 had slightly increased pro-IGF-IIE[68-88] within the first year of treatment while they still responded clinically to imatinib therapy. None of the patients had signs of liver- or (ad)renal failure or cachexia at the time of NICTH. All three patients died of progressive disease within 2 months after the first documented episode of hypoglycaemia.

Table 5. Plasma levels of IGFs and IGFBPs, expressed as standard deviation scores, for patients with NICTH at baseline, during imatinib treatment and when suffering from hypoglycaemia

	Patient								
	1			2			3		
Time point	0[1]	2 mo[2]	hypo[3]	0	8 mo	hypo	0	1 wk	hypo
IGF-I	0.34	-0.46	-4.56	-2.26	-4.80	-5.54	-0.41	-0.52	0.40
Total IGF-II	1.08	-0.22	-0.92	-1.40	-2.21	-6.15	1.39	-0.20	1.68
Pro-IGF-IIE[68-88]	1.17	2.49	5.23	1.40	2.21	4.55	7.08	6.53	8.64
IGFBP-2	1.43	0.66	3.27	2.85	3.97	4.32	2.09	1.83	2.47
IGFBP-3	-0.32	0.15	-4.44	-0.68	-2.07	-4.49	-1.69	-2.80	-3.27
IGFBP-6	0.50	-0.53	–	0.19	1.07	2.42	0.09	-0.53	1.37

[1]Baseline values; [2]Plasma levels after 2 months of imatinib treatment for patient 1, after 8 months for patient 2, and after 1 week for patient 3; [3]Plasma levels when suffering from hypoglycaemia.

IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; Hypo, hypoglycaemia; NICTH, non-islet cell tumour-induced hypoglycaemia.

Discussion

This study was performed to investigate the relationship between plasma levels of IGF-I, -II, pro-IGF-IIE[68-88], IGFBP-2, -3, and -6 and disease status of patients with a GIST before and during treatment with imatinib. In our cohort of patients we noticed that plasma levels of pro-IGF-IIE[68-88], IGFBP-2 and -3 tend to differ considerably from normative range values.

It appeared that at any moment before or during treatment, 11 of the 24 (46%) patients studied exhibited pro-IGF-IIE[68-88] levels that were higher than 1.0 SDS. Three of these patients showed values of pro-IGF-IIE[68-88] that even exceeded 2.0 SDS. Later on, they developed hypoglycaemia. These findings and the various previous case reports on this subject [5,7-10], indicate that elevated plasma concentrations of pro-IGF-IIE[68-88] are not uncommon in patients with a GIST and would predict a high risk for the development of hypoglycaemia. Histological examination and analysis of cyst fluid of the various GISTs revealed that it is highly likely that most of the pro-IGF-IIE[68-88] detected in plasma is secreted by tumour tissue. Indeed, a relatively high tumour mass and/or increased serum LDH appeared to be associated with significantly higher plasma levels of pro-IGF-IIE[68-88]. In addition, our data suggest that metastatic GISTs tend to produce more pro-IGF-IIE[68-88]. Presumably, when a certain threshold level of pro-IGF-IIE[68-88] in the circulation has been exceeded, GH secretion by the anterior pituitary gland will become reduced due to an increased feedback inhibition [3]. Moreover, the formation of 150-kDa IGF-IGFBP-3-acid-labile subunit complexes in the circulation is increasingly hampered. This cascade of events results in an increased insulin-like activity in the body that may ultimately overrule the various counter regulatory mechanisms for hypoglycaemia.

The three patients with hypoglycaemia reported in this study did not suffer from conditions such as (ad)renal failure, hepatic dysfunction or cachexia that could alternatively explain the occurrence of hypoglycaemia [23]. However, there are some reports that suggest that imatinib, and also the second-line tyrosine kinase inhibitor sunitinib, could influence glucose metabolism in patients with type 2 diabetes mellitus and possibly also in non-diabetic patients with a disturbed glucose homeostasis, like NICTH [8,24,25]. We also observed an improvement of fasting blood glucose levels in a patient with type 2 diabetes mellitus during treatment with imatinib, and later on with sunitinib, which allowed a consequent reduction of her insulin dosage before she finally suffered from NICTH. The latter could have been facilitated by the use of a KIT kinase inhibitor as suggested by Hamberg *et al* [8].

Within our total patient population, we noticed that plasma IGFBP-3 levels, being already slightly reduced before treatment, declined further during prolonged imatinib therapy. Theoretically, this could increase the tissue availability of IGFs, including pro-IGF-IIE[68-88], facilitating the occurrence of hypoglycaemia and reducing insulin resistance in type 2 diabetes mellitus [26]. Recently, it was reported that IGFBP-3 mRNA was induced in GISTs that responded to imatinib according to FDG-PET several days after initiation of treatment. In contrast, IGFBP-3 mRNA was suppressed in non-responding GISTs [27]. In our evaluation, however, we noticed no significant changes in IGFBP-3 plasma levels after one week of imatinib treatment, irrespective of the PET-response (data not shown) suggesting that an eventual induction of IGFBP-3 mRNA and protein in responsive GISTs does not influence the plasma levels of this IGFBP.

Mutation analysis carried out on tumour tissues obtained from the three patients with NICTH revealed that two patients had a tumour with a *KIT* exon 9 mutation and one had a *PDGFRA* exon 18 mutation. These mutations have a much lower incidence (*i.e.* approximately 10 and 6 percent, respectively) than those that affect *KIT* exon 11, being by far the most frequent mutation found in GISTs (~60%) [1]. So far, only one NICTH GIST case with a *KIT* exon 9 mutation has been reported [9]. Interestingly, Braconi *et al* recently reported that a decreased disease-free survival in patients with a GIST was independently associated with IGF-II expression by the tumour. In addition, they found that IGF-II was more frequently expressed in tumours with *KIT* exon 9 mutations than in those with other mutations suggesting that mutational status could influence IGF-II expression and possibly the occurrence of NICTH [28].

In the majority of the patients, plasma IGFBP-2 levels were markedly elevated. Several studies have shown that circulating IGFBP-2 levels may be elevated in various malignancies [29-33] and may be associated with increasing tumour size, disease stage and adverse prognosis [29,30,32]. There were no such associations in our study, although patients with progressive diseases had significantly higher plasma levels of IGFBP-2 than patients who responded to imatinib. It has been suggested that elevated circulating IGFBP-2 levels found in patients with aforementioned malignancies are caused by the secretion of this protein by the tumour. However, our analysis of tissue and cyst fluid suggests that the elevated plasma levels of IGFBP-2, as found for most of the GIST patients, are due to other, systemic, as yet unknown processes. Increased levels of IGFBP-2 have also been observed in other disease conditions such as AIDS, diabetes mellitus and malnourishment [30,34]. As we did not evaluate specifically the nutritional status of our patients, we were not able to establish whether malnutrition

could have contributed to the increased IGFBP-2 levels in most of our patients. In spite of this, we did find a significant negative correlation between plasma IGFBP-2 SDS and serum albumin levels which is a rather non-specific marker for nutritional status [35]. On the other hand, IGF-I levels are also influenced by nutritional status [26]. In our patients, however, IGF-I levels generally did not differ from normative range values. All together, an eventual role for IGFBP-2 as a laboratory marker for disease progression in GIST patients remains to be established.

In conclusion, we identified pro-IGF-IIE[68-88] as a marker that can potentially be useful in the surveillance of patients with a GIST. Symptoms of hypoglycaemia may be non-specific. Hence, we expect that in daily clinical practice cases of NICTH are being missed. Two of our patients even became comatose, indicating the serious implications of NICTH. We would therefore suggest that patients with a GIST, especially those with progressive disease, are monitored for plasma levels of pro-IGF-IIE[68-88] to identify patients who are at high risk for the development of hypoglycaemia.

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Supplementary Material

Material and methods

KIT and PDGFRA mutation analysis. DNA was extracted from formalin-fixed and paraffin-embedded (FFPE) tumour samples. DNA extraction from FFPE tissues was performed by affinity-purification of the DNA (QIAamp DNA micro handbook, QIAGEN GmbH, Germany) after treatment with proteinase K. DNA sample concentration and quality were assessed spectrophotometrically (260/280 nm using the NanoDrop) and by PCR-amplification using the BIOMED-2 Control gene primer set [1]. All extracted DNAs allowed amplification of at least the 200 bp amplicon of the Control gene PCR. *KIT* exon 9, 11, 13 and 17 and *PDGFRA* exon 12, 14 and 18 were amplified by PCR with the primers indicated in the supplementary table. All primers contain an M13 forward or reverse consensus sequence. The PCRs were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems), PCR buffer II and 2.5 mM $MgCl_2$. The cycling conditions were standardised: after denaturation at 94°C for 5 min, DNA was amplified for a total of 40 cycles (annealing temperature at 60°C). After the last cycle, a final extension step of 5 min at 72°C was performed. Sequencing reactions were performed from two independent PCR-reactions, using the forward and the reverse primers, and assessed on an ABI 3730 platform (Applied Biosystems). Sequencing results from the cases were compared to sequences from normal individuals (*KIT*: NM_000222.2 and *PDGFRA:* NM_006206.3).

Supplementary table. Primers used for *KIT* and *PDGFRA* mutation analysis

	Primer sequence 5'-3'
KIT exon 9-1	
Forward	TGT AAA ACG ACG GCC AGT GTA TGC CAC ATC CCA AGT G
Reverse	CAG GAA ACA GCT ATG ACC TGC CCA CAT CGT TGT AAG C
KIT exon 9-2	
Forward	TGT AAA ACG ACG GCC AGT GCT TCT GTA CTG CCA GTG G
Reverse	CAG GAA ACA GCT ATG ACC GTA GAC AGA GCC TAA ACA TC
KIT exon 11	
Forward	TGT AAA ACG ACG GCC AGT GTG CTC TAA TGA CTG AGA C
Reverse	CAG GAA ACA GCT ATG ACC GTG TAC CCA AAA AGG TGA C
KIT exon 13	
Forward	TGT AAA ACG ACG GCC AGT CAT CAG TTT GCC AGT TGT GC
Reverse	CAG GAA ACA GCT ATG ACC CCT GAC AGA CAA TAA AAG G
KIT exon 17	
Forward	TGT AAA ACG ACG GCC AGT GGT TTT CTT TTC TCC TCC
Reverse	CAG GAA ACA GCT ATG ACC CCT TTG CAG GAC TGT CAA GC
PDGFRA exon 12	
Forward	TGT AAA ACG ACG GCC AGT GAC TTT GGT AAT TCA CCA G
Reverse	CAG GAA ACA GCT ATG ACC CAA GGG AAA AGG GAG TCT TG
PDGFRA exon 14	
Forward	TGT AAA ACG ACG GCC AGT GGT AGC TCA GCT GGA CTG
Reverse	CAG GAA ACA GCT ATG ACC CAC AAC CAC ATG TGT CCA G
PDGFRA exon 18	
Forward	TGT AAA ACG ACG GCC AGT GCT ACA GAT GGC TTG ATC C
Reverse	CAG GAA ACA GCT ATG ACC AAG TGA AGG AGG ATG AGC C

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Chapter 5

'Big'-insulin-like growth factor-II signaling is an autocrine survival pathway in gastrointestinal stromal tumors

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Submitted

Abstract

Background & Aims: Insulin-like growth factor (IGF)-II acts as an autocrine factor in several tumor types by binding to IGF receptor type 1 (IGF-1R) and/or the insulin receptor (IR) isoform A. Previously, we reported that unprocessed pro-IGF-II, called 'big'-IGF-II, is expressed in gastrointestinal stromal tumors (GISTs). The aim of the present study was to investigate the putative role of 'big'-IGF-II in GIST.

Methods: Secretion of ('big'-)IGF-II by GIST cell lines, GIST882 and GIST48, was analyzed by ELISA and western blotting. Expression of IGF-1R and IR isoforms in cell lines and frozen tumor samples were assessed with RT-PCR and western blotting. Effects of down-regulation of IGF-II or IR with short interference RNA were evaluated with cytotoxicity assays and western blotting. IGF-II expression was analyzed in 60 primary GISTs by RNA in situ hybridization and immunohistochemistry.

Results: Both cell lines secreted high levels of IGF-II, predominantly as 'big'-IGF-II. Expression of IR isoform A but not of IGF-1R was demonstrated in the *KIT* mutant cell lines and in the *KIT* and *platelet-derived growth factor receptor* α mutant GIST specimens. Down-regulation of either 'big'-IGF-II or IR affected AKT and MAP kinase signaling and reduced survival. Disruption of 'big'-IGF-II signaling in combination with imatinib had an additive cytotoxic effect. IGF-II mRNA was present in 91% of primary GISTs. ('big'-) IGF-II protein expression was observed in the majority of GISTs, especially in high-risk tumors.

Conclusions: Our data put forward the 'big'-IGF-II-IR isoform A axis as an autocrine survival pathway and potential therapeutic target in GIST.

Introduction

Gastrointestinal stromal tumors (GISTs) are the most common soft tissue sarcomas of the gastrointestinal tract and are characterized by the over-expression of the receptor tyrosine kinase KIT. Various *KIT* genomic mutations occur in at least 80% of GISTs. In addition, about 5% of GISTs have mutations in the platelet-derived growth factor receptor α (*PDGFRA*) gene. These mutations lead to ligand-independent activation of either KIT or PDGFRA, which is considered as an initiating step in the development of GIST [1]. Although most patients with advanced GIST are successfully treated with imatinib, a small-molecule tyrosine kinase inhibitor active against KIT and PDGFRA, some patients are initially resistant to this compound and almost all patients finally show disease progression after an initial response [2]. Therefore, new treatment targets need to be identified.

IGF-II is a regulatory polypeptide, which is critical for normal growth and differentiation. In many types of cancer, IGF-II is over-expressed and involved in tumor progression and metastasis [3]. It is synthesized as a 180 amino acid preprohormone with a 24 amino acid signal peptide at the N-terminal end and a C-terminal extension of 89 amino acids called the E-domain. During intracellular processing the signal peptide and the E-domain are cleaved in several steps from the precursor protein resulting in mature IGF-II consisting of 67 amino acids (Mw ~7.5 kDa). However, many tumors have been found to secrete larger forms of IGF-II (Mw 10-18 kDa) that still contain at least the first 21 amino acids of the E-domain, which may be differentially glycosylated [4]. These larger forms of IGF-II are called pro-IGF-IIE[68-88] or 'big'-IGF-II. Mature IGF-II and 'big'-IGF-II have been shown to act as autocrine growth factors in several tumor types. It can exert its mitogenic and anti-apoptotic effects by binding to the IGF receptor type 1 (IGF-1R) and the insulin receptor (IR) isoform A [5].

Recently, we have reported on the occurrence of hypoglycemia in patients with a GIST being associated with increased circulating levels of tumor-derived 'big'-IGF-II [6]. This observation prompted us to study the role of 'big'-IGF-II as an autocrine factor in *in vitro* GIST model systems. We also evaluated the incidence of IGF-II expression in GIST specimens. Our findings indicate that 'big'-IGF-II secretion by GIST provides a pro-survival signal that is relevant for the maintenance of this malignancy.

Materials and Methods

Cell Culture

The GIST cell lines GIST882 en GIST48 were kindly provided by Jonathan Fletcher (Brigham and Women's Hospital, Boston, MA). GIST882 was developed from a primary, untreated, GIST with a *KIT* exon 13 mutation and was maintained in RPMI 1640 medium (Invitrogen, Praisley, UK) supplemented with 15% heat inactivated fetal calf serum (Bodinco, Alkmaar, The Netherlands) and 1 mmol/L L-glutamine (Invitrogen) [7]. GIST48 was established from a progressive tumor during imatinib therapy and harbors a primary *KIT* exon 11 mutation and a secondary exon 17 mutation. The GIST48 cells were maintained in F-10 medium (Invitrogen) supplemented with 10% fetal calf serum and 0.5% mito+ serum extender (VWR International, Roden, The Netherlands) and 1% bovine pituitary extract (VWR International) [8]. The rhabdomyosarcoma cell line RD was cultured in Dulbecco's modified eagle medium high glucose (Invitrogen) supplemented with 10% fetal calf serum.

Table 1. Primers used for RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Number of cycles RT-PCR	Product size (bp)
IGF-II	CCT CCT GGA GAC GTA CTG TGC TA	TCA TAT TGG AAG AAC TTG CCC A	60	30	117
IGF-I	AGC AGT CTT CCA ACC CAA TTA TTT A	AGA TGC GAG GAG GAC ATG GT	55	36	83
Insulin	GCA GCC TTT GTG AAC CAA CAC	CGT TCC CCG CAC ACT AGG TA	60	36	71
IGF-1R	GCC CGA AGG TCT GTG AGG AAG AA	GGT ACC GGT GCC AGG TTA TGA	55	30	555
Insulin receptor[1]	CTG AAG GAG CTG GAG GAG TC	CGC TGG TCG AGG AAG TGT TG	55	30	205/169
GAPDH	CAC CCA CTC CTC CAC CTT TG	CCA CCA CCC TGT TGC TGT AG	60	30	110

[1]This primer pair detects both insulin receptor isoforms resulting in PCR products of 169 bp representing isoform A and of 205 bp representing isoform B.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Cell lines and frozen tumor samples were resuspended in 1 ml of Trizol (Invitrogen) and RNA was isolated and converted to complementary DNA (cDNA) [9]. For PCR, 1 μL cDNA was amplified using primers (see table 1 for primer sequences) in a total volume of 25 μL using 1.25 U Taq polymerase (Invitrogen). Universal human reference RNA (Stratagene, La Jolla, CA, USA) was used as a positive control for IGF-I and insulin expression.

Determination of IGF-II secretion

GIST882, GIST48 and RD cells were cultured in 6-well plates until a 75% confluence was reached. After washing the cells twice, they were cultured in 1 ml of cell line specific maintenance medium for an additional 48 h. The final numbers of cells were counted. The conditioned cell culture medium was collected, centrifuged for 10 min at 110 g, and the supernatant stored at -20°C until further analysis. IGF-II concentrations in the supernatant were determined by a non-extraction IGF-II enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic Systems Laboratories, Sinsheim, Germany) according to the manufactures instructions and related to the number of cells in the culture. This ELISA does not cross-react with bovine IGF-II (according to the manufacturer and our own testing) and measures both human mature and 'big'-IGF-II.

To determine the forms of IGF-II secreted by GIST882 and GIST48 by western blotting, cells were cultured under serum-free conditions in RPMI 1640 or F10 with bovine pituitary extract, respectively, supplemented with 10 μg/mL transferrin (Invitrogen), 5 ng/mL sodium selenite (Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 mmol/L L-glutamine (Invitrogen), 0.1% bovine serum albumin (Invitrogen). Conditioned media was lyophilized and reconstituted in demi-water. The total IGF-II concentration was determined by ELISA. For western blotting, an equal volume of 4% (w/v) sodium dodecyl sulfate (SDS) sample buffer (0.5 mol/L Tris-HCl pH 6. 8, containing 20% glycerol, 0.002% bromophenol blue, and 10% 2-β-mercaptoethanol) was added and the samples were boiled for 10 min. Unconditioned serum-free media and rhIGF-II (R&D systems, Abingdon, UK) were processed in parallel as a negative and positive control, respectively.

Western blot analysis

Frozen primary GIST samples were pulverized and dissolved in ice cold PBS. After centrifugation at 18,000 g for 1 min to remove debris, the supernatant was collected. The protein concentration was determined according to the Bradford method and 4% SDS

sample buffer was added. Cell lines cultured in 6-well plates were serum starved for 4 h and then incubated for 5 min with rhIGF-II (20 nmol/L) or insulin (20 nmol/L; Novo Nordisk, Alphen aan den Rijn, The Netherlands). Alternatively, cells were treated with siRNA as described below. After treatments, cells were lysed in ice cold 2% (w/v) sample buffer and boiled immediately for 10 min. The protein concentration was determined with a detergent-compatible protein assay (Bio-Rad). Western blotting was performed as described previously [10]. Goat anti-IGF-II (polyclonal, 1:500; R&D Systems), rabbit anti-IIE[68-88] antiserum (1:1000; WKZ6279) [11], rabbit anti-phospho-IGF-1R(Tyr1135/1136)/IR(Tyr1150/1151), rabbit anti-IGF-1R, rabbit anti-phospho-AKT(Ser473), rabbit anti-AKT and rabbit anti-extracellular signal-regulated kinase-1 and -2 (ERK1/2; 1:1000; Cell Signaling Technology, Bioké, Leiden, The Netherlands), mouse anti-phospho-ERK1/2(Thr202/Tyr204) (clone E10, 1:2000; Cell Signaling), rabbit anti-IR (polyclonal, 1:200; Santa Cruz, Heerhugowaard, The Netherlands), and mouse anti-actin (clone C4, 1:20,000; ICN Biomedicals, Zoetermeer, The Netherlands) served as primary antibodies.

Flow cytometry

IGF-1R and IR membrane expression was determined in GIST cells by flow cytometry [10], using phycoerythrin (PE)-conjugated mouse anti-IGF-1R monoclonal antibody (clone 1H7, 1:10; BD Pharmingen, Alphen aan de Rijn, The Netherlands) and PE-conjugated mouse anti-IR monoclonal antibody (clone 3B6, 1:10; BD Pharmingen). PE-conjugated mouse $IgG_{1\kappa}$ (BD Pharmingen) served as an isotype control.

Short interference RNA (siRNA) transfections

Synthetic siRNA duplexes were purchased from Eurogentec (Maastricht, The Netherlands). The sense sequence for IGF-II siRNA was 5′-GCA UCU UCA AAC AUG UAC AAA dTdT-3′and the antisense was 5′-UUU GUA CAU GUU UGAAGA UGC dTdT -3′. The sense sequence for the IR was 5′-GGC UCC UUC GGC AUG GUG UAU dTdT -3′ and the antisense was 5′-AUA CAC CAU GCC GAA GGA GCC dTdT-3′. Plated cells were transfected using siRNAs (final concentration 50 nM) with oligofectamine (Invitrogen) and OptiMEM (Invitrogen) according to the manufacturer's instructions. After 4 h incubation, the medium was changed to complete culture medium. As a negative control, scrambled, non-silencing siRNA (Eurogentec) was used.

Cell viability and apoptosis assays

Cells were plated at $3x10^4$ cells per well in 96-well plates and cultured in normal media for 2 days before siRNA transfection or treatment with the IR tyrosine kinase inhibitor HNMPA-$(AM)_3$ (Biomol Research Laboratories, Plymouth Meeting, PA, USA) [12,13]. Seventy-two h after transfection or treatment, cell viability was assessed with thiazolyl blue tetrazolium bromide (MTT) [10]. Alternatively, 24 h after siRNA transfection, GIST882 cells were washed with and incubated in RPMI 1640 supplemented with transferrin, sodium selenite, L-glutamine and bovine serum albumin, as described above. After 48 h incubation under serum-free conditions, cell viability was evaluated with MTT. To evaluate the combined effects of KIT inhibition and either IGF-II or IR silencing, cells were incubated with 0.05 or 0.1 μmol/L imatinib for 48 h, starting 24 h after siRNA transfections. All MTT assays were performed three times in quadruplicate. To evaluate apoptosis, $1.5x10^4$ cells per well in 96-well plates were treated with siRNA. Apoptosis was determined 48 and 72 h after siRNA transfection with the acridine orange assay [14]. All apoptosis assays were performed in duplicate and repeated three times.

Tissue collection and tissue microarray construction

Sixty primary paraffin-embedded tumor tissues of GISTs and seven frozen GIST samples were retrieved from the files of the Department of Pathology of the University Medical Center Groningen. All tumors were surgically resected between 1985 and 2006 and diagnosed as described previously [14]. Tumors were categorized into risk groups, based on tumor size, mitotic index and primary localization of the tumor, according to the recently modified consensus classification for prediction of metastatic potential [15]. Mutation analysis of *KIT* exons 9, 11, 13 and 17, and *PDGFRA* exons 12, 14 and 18 was performed as described previously [6]. Patient and tumor characteristics are summarized in table 2. Based on the corresponding H&E-stained slides, several representative regions were selected of each individual paraffin-embedded tumor specimen. Subsequently, from each of these regions three distinct 0.6 mm tissue cores were taken and transferred to a standard-size recipient paraffin block to create a tissue microarray [14]. All tumor samples used in this study were handled according to the guidelines of the Dutch Federation of Biomedical Scientific Societies (FMWV) as described in "Code Proper Secondary Use of Human Tissue".

Table 2. Patient (n=59) and tumor (n=60) characteristics[1]

	n (%)	
Sex		
Male	28 (47)	
Female	31 (53)	
Age (years), median (range)		66 (24–87)
Risk classification		
Very low risk	4 (8)	
Low risk	15 (25)	
Intermediate risk	10 (17)	
High risk	31 (52)	
Primary site		
Stomach	32 (53)	
Small intestine	23 (38)	
Colon	4 (7)	
Unknown	1 (2)	
Histology		
Spindle cell	48 (80)	
Epithelioid	7 (12)	
Mixed	5 (8)	
Tumor size		
≤10 cm	41 (68)	
>10 cm	19 (32)	
Mitotic figures		
≤5 per 50 HPF	45 (75)	
>5 per 50 HPF	15 (25)	
Mutations		
KIT exon 9	6 (10)	
KIT exon 11	31 (52)	
PDGFRA exon 12	2 (3)	
PDGFRA exon 18	7 (12)	
No mutations found	8 (13)	
Not evaluable	6 (10)	

[1]One patient had two primary GISTs, which were a high-risk epithelioid gastric tumor with a *PDGFRA* exon 18 mutation and a low-risk spindle-cell small intestine tumor with a *KIT* exon 11 mutation, respectively.
Abbreviations: HPF, high power fields; PDGFRA, platelet-derived growth factor receptor α.

RNA in situ hybridization and immunohistochemistry

The methods used for in situ hybridization with digoxigenin-labeled hIGF-II complementary RNA probes have been described previously [16]. β-actin digoxigenin-labeled complementary RNA probes were used to evaluate the preservation of RNA in the tissue specimens studied. Negative controls for in situ hybridization were prepared using sense probes. Immunohistochemistry was performed as described previously [14]. Antigen retrieval was performed either by boiling slides in a microwave in 0.01 mol/L citrate buffer (pH 6.0) for 15 min or by autoclaving in blocking solution (2% blocking reagent (Roche Diagnostics, Mannheim, Germany) and 0.2% SDS in maleic acid buffer (pH 6.0)) at 115°C for three times 5 min for the detection of IIE[68-88] or total IGF-II, respectively. Primary antibodies were rabbit anti-IIE[68-88] antiserum (1:200; WKZ6279) [11] and goat anti-IGF-II (1:50; R&D Systems). As a negative control, a serial section was processed by replacement of primary antibody with either preimmune rabbit antiserum or normal goat IgG. For both immunohistochemistry and in situ hybridization, normal tissue samples derived from first trimester human placenta served as positive controls [17]. Results for immunohistochemistry and in situ hybridization were scored independently in a semi-quantitative fashion for intensity of staining by two observers (B.R. and A.J.H.S). Tissue cores from one tumor were judged to be positive when there was a clear cellular cytoplamic staining (*i.e.* comparable or more intense staining than placenta) in at least two of the three cores. Tumors with less than two assessable cores were excluded from the analysis.

Statistical analysis

The student t-test was used to analyze the differences between groups. Associations between IGF-II expression and tumor characteristics were evaluated by the Chi-square or Fisher's exact test, where appropriate. A two-tailed P-value of <0.05 was considered to be significant.

Results

'Big'-IGF-II secretion by GIST cell lines

IGF-II expression was evaluated in imatinib-sensitive GIST882 cells and in imatinib-resistant GIST48 cells. As shown in figure 1A, both GIST cell lines expressed IGF-II mRNA equally to the RD rhabdomyosarcoma cell line that served as a positive control

[18]. Furthermore, the expression of IGF-II mRNA in the GIST cell lines was comparable to those found in the various GIST samples and also to the expression in a primary GIST cell culture. No IGF-I or insulin mRNA expression could be detected in GIST882 and GIST48 indicating that IGF-II is the only IGF ligand that is significantly expressed by these cell lines (figure 1B). After culturing the cells for 48 h, both GIST cell lines secreted significant amounts of IGF-II into the culture medium, which largely exceeded the IGF-II production by the RD control cell line (figure 1C). IGF-II was predominantly secreted as high molecular weight forms containing the IIE[68-88] domain peptide of pro-IGF-II, which can be differentially glycosylated [19]. GIST882 secreted mainly ~11 kDa 'big'-IGF-II and two other less abundant high molecular weight forms of ~12 and ~18 kDa. In addition, a faint ~8 kDa band was observed, corresponding to fully processed IGF-II. GIST48 only produced 'big'-IGF-II and at least three different forms between ~12-18 kDa were detected (figure 1D).

IGF-1R and IR expression in GIST specimen and cell lines

The expression of the cognate receptors of 'big'-IGF-II was evaluated in frozen tumor samples and GIST cell cultures (figure 2A and B). In six of the seven GIST tumor samples, all harboring mutated *KIT* or *PDGFRA*, neither IGF-1R mRNA nor protein could be detected. Only the tumor with wild-type *KIT* and *PDGFRA* showed significant expression of IGF-1R mRNA and protein. Among the three GIST cell cultures, only GIST48 expressed IGF-1R mRNA, albeit at a relatively low level compared to the positive tumor. No IGF-1R protein could be detected in GIST48 or the other GIST cell cultures. In contrast, all GIST tissue samples and cell cultures expressed mRNA of both isoforms A and B of the IR (figure 2A), and expressed IR protein (the antibody used did not discriminate between both isoforms; figure 2B). IR was expressed at the cell surface of the GIST882 and GIST48 cell lines. No IGF-1R membrane expression could be detected in these GIST cell lines in contrast to the highly positive control MCF-7 (figure 2C). Stimulation with IGF-II or insulin induced phosphorylation of IR in GIST cells (figure 2D).

Effects of 'big'-IGF-II signaling inhibition

The role of 'big'-IGF-II secretion by GIST cells was further investigated using siRNA strategies to evaluate the effects of IGF-II and IR silencing. The expression of both IGF-II and IR could be effectively down-regulated with siRNA in GIST882 and GIST48 (figure 3A). Furthermore, a marked reduction in the secretion of total IGF-II could be observed for both cell lines after IGF-II siRNA transfection (figure 3B). In both cell lines, down-

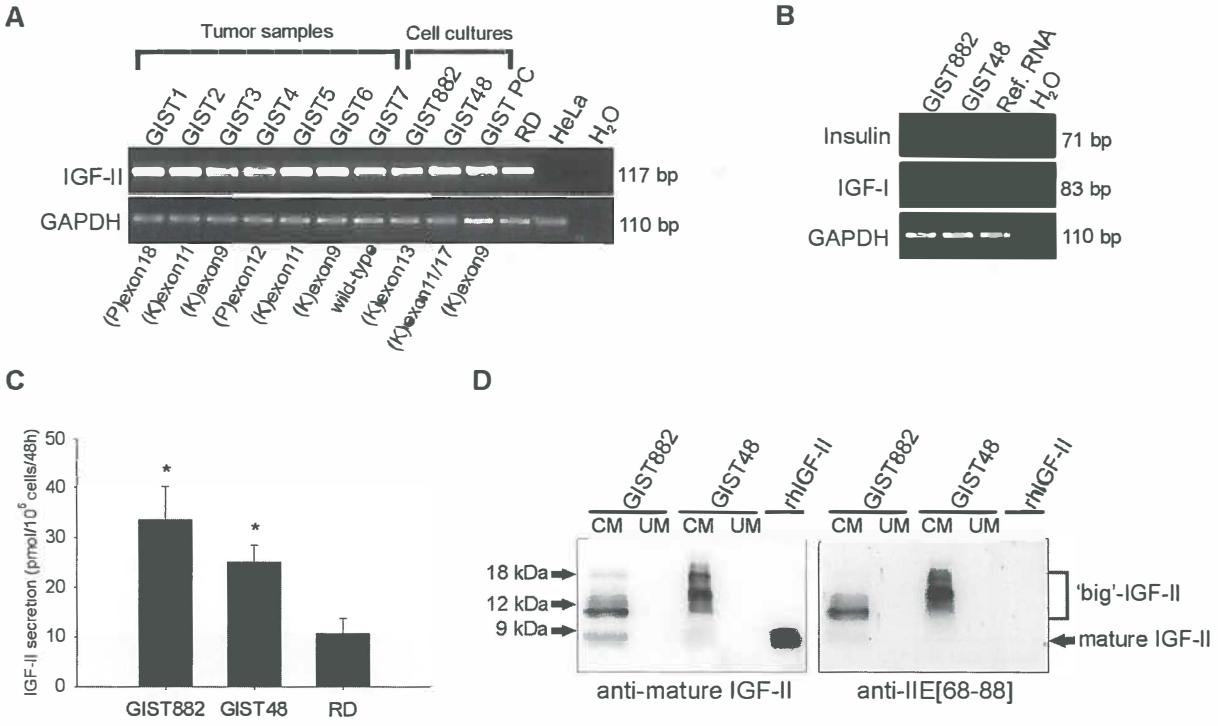

Figure 1. IGF-II expression and secretion by GIST cell lines. (A) IGF-II mRNA expression in GIST samples and cell cultures with RT-PCR. RD was used as a positive control and HeLa was used as a negative control [40]. GIST PC is a primary cell culture (passage 3) with a *KIT* exon 9 mutation established from a tumor that was progressive during imatinib therapy. *KIT* (K) and *PDGFRA* (P) mutations are indicated for each GIST. GAPDH was used as a loading control. (B) Insulin and IGF-I mRNA expression in GIST cell lines. Human reference RNA served as a positive control. (C) GIST882, GIST48 and RD cells were cultured for 48 h and total IGF-II secretion (both mature and 'big'-IGF-II) was measured by ELISA. RD served as a positive control. *$p<0.01$ compared with RD. (D) Lyophilized serum-free conditioned media (CM) and unconditioned media (UM) obtained from GIST882 and GIST48 cells were subjected to western blotting. Membranes were probed with an antibody against mature IGF-II and with an antibody against IIE[68-88]. Recombinant human (rh) IGF-II served as a positive control for mature IGF-II immunoblotting.

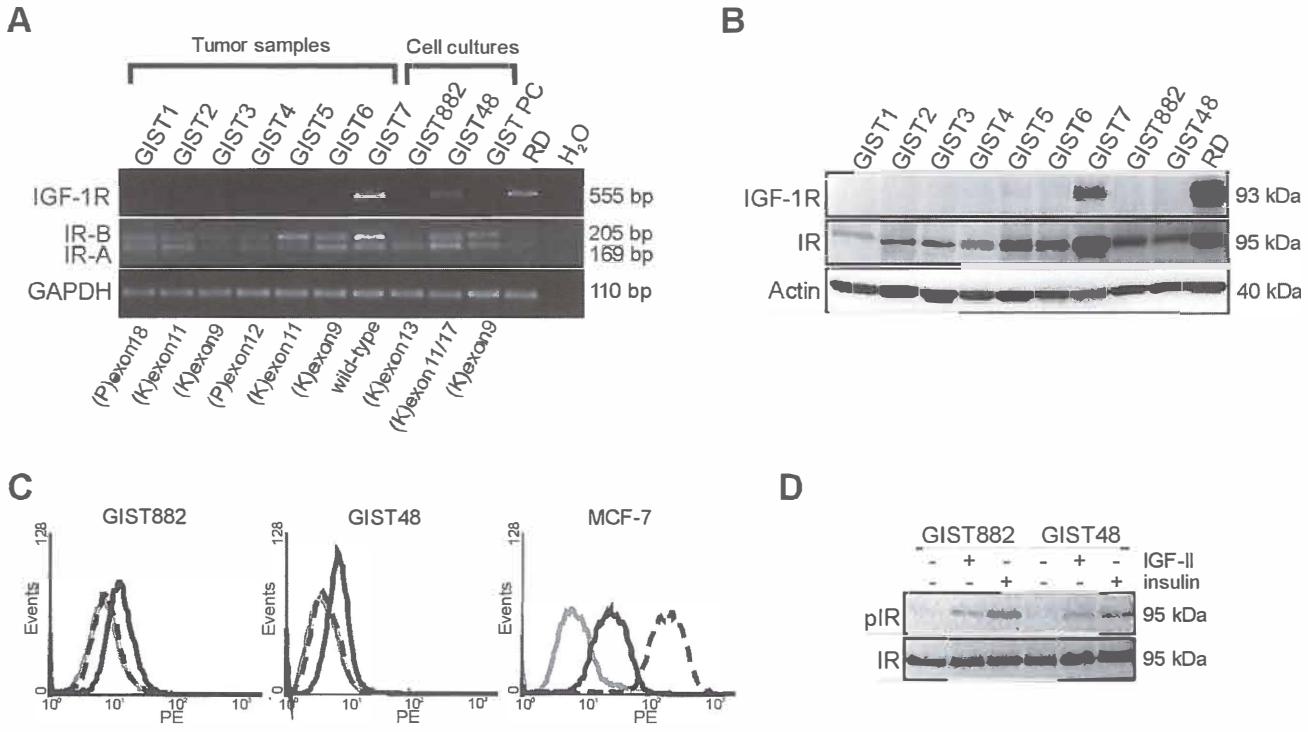

Figure 2. IGF-1R and IR expression in GIST. (A) IGF-IR and IR mRNA expression in GIST samples and cell lines with RT-PCR. The primer pair for the IR detects both isoforms reflecting isoform A (IR-A) as a 169 bp band and isoform B (IR-B) as a 205 bp band. RD was used as a positive control. *KIT* (K) and *PDGFRA* (P) mutations are indicated for each GIST. GAPDH was used as a loading control. (B) Western blot analysis of IGF-1R and IR expression in GIST samples and cell lines, and RD cells (positive control). Actin served as a loading control. (C) Analysis of IGF-1R and IR membrane expression by flow cytometry. The gray line represents the IgG control, the black line the anti-IR antibody and the black dotted line the anti-IGF-1R antibody. MCF-7 breast carcinoma cells were used as a positive control. (D) Serum starved cells were stimulated with 20 nmol/L recombinant human IGF-II or insulin for 5 min. IR phosphorylation was evaluated with western blotting.

regulation of either IGF-II or IR caused a marked reduction in ERK1/2 phosphorylation. Transfection of GIST882 and GIST48 cells with IR siRNA in both cases reduced AKT phosphorylation. Only in GIST882 cells, the same phenomenon was seen after transfection with IGF-II siRNA (figure 3A). IR or IGF-II siRNA transfection did not influence KIT expression nor KIT phosphorylation in both cell lines (data not shown).

Treatment with IGF-II siRNA caused a reduction in cell viability with ~30-35 percent that was nearly similar for GIST882 and GIST48. For both cell lines, down-regulation of IR expression also decreased the viability of the cells but the effect was most pronounced in GIST48 (figure 4A). The GIST882 cell line has the capacity to survive and even grow in serum-free media (data not shown). This may be due to an autocrine action of 'big'-IGF-II on IR. In order to investigate this possibility, the same experiments with siRNA were performed with GIST882 but now under serum-free conditions. A slightly larger effect of IGF-II siRNA on the cell viability of GIST882 cells was observed under serum-free conditions compared to the viability of these cells when cultured in serum containing media (figure 4A and B). For both cell lines, transfection with IGF-II siRNA resulted in a significant proportion of apoptotic cells after 48 h, which had further increased after 72 h. Silencing of the expression of IR for 48 h resulted in a marked induction of apoptosis in GIST48 cells only (figure 4C). The simultaneous treatment with the KIT tyrosine kinase inhibitor imatinib and transfection of the cells with either IGF-II or IR siRNA had an additive effect on cell viability of the imatinib-sensitive GIST882 cells (figure 4D).

In addition to the siRNA approach, the effects of HNMPA-$(AM)_3$, a well-known IR tyrosine kinase inhibitor, was evaluated. HNMPA-$(AM)_3$ decreased cell viability in GIST882 and GIST48, the latter being more sensitive to this compound, which is in line with the IR silencing experiments (figure 5).

('Big'-)IGF-II expression in GIST specimen

For a large cohort of paraffin-embedded GIST specimens the expression of IGF-II in relation to tumor characteristics was investigated. Fifty-two of 57 evaluable primary tumors (91%) were positive for IGF-II mRNA as revealed by in situ hybridization (figure 6). All four very-low-risk tumors were negative for IGF-II mRNA, whereas the tumors from the other risk groups were positive for IGF-II mRNA, except for one high-risk tumor. To investigate ('big'-)IGF-II expression at the protein level, immunohistochemistry with two different antibodies, an antibody raised against the IIE[68-88] domain peptide of pro-IGF-II and an antibody against fully processed IGF-II (i.e. detecting both mature and high molecular weight forms of IGF-II), was performed (figure 6). Thirty-six of 56

evaluable primary tumors (64%) stained positive for IIE[68-88] and 17 of 53 evaluable primary tumors (32%) showed positive staining for total IGF-II (table 3). All tumors positive for total IGF-II were also positive for IIE[68-88], except for one. With both antibodies, a more frequent expression of ('big'-)IGF-II was observed in high-risk tumors (i.e. tumors with the highest malignant potential) in contrast to tumors with a lower risk classification (table 3).

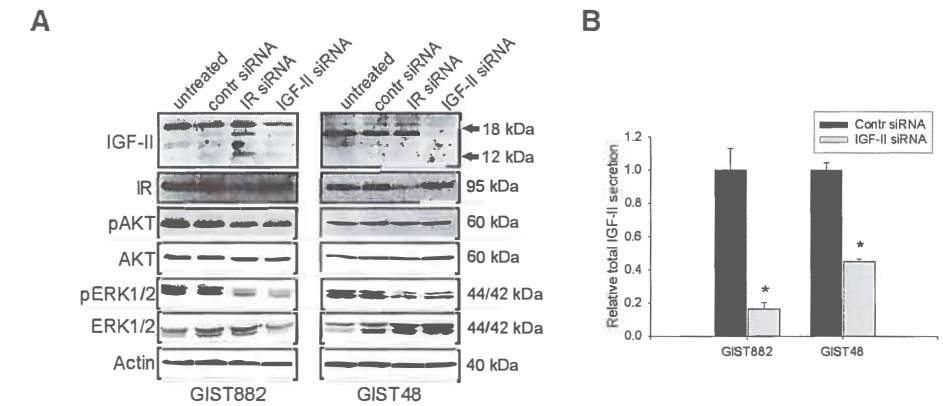

Figure 3. Effects of down-regulation of IGF-II or IR on downstream signaling. (A) GIST cells were either treated with scrambled control siRNA or siRNA directed against IGF-II or IR. IR and IGF-II down-regulation and changes in phosphorylation of AKT and ERK1/2 were investigated with immoblotting 48 h after siRNA treatment. Actin served as a loading control. (B) Twenty-four h after siRNA transfection, cells were washed and incubated for 48 h in maintenance culture media. Total IGF-II concentrations were determined with ELISA and adjusted to the number of cells. The relative (i.e. normalized to transfections with scrambled siRNA) IGF-II secretion after siRNA transfection, was calculated for each cell line. Data represent the mean values (± SD) of 3 independent experiments. *p <0.01 compared with scrambled control siRNA.

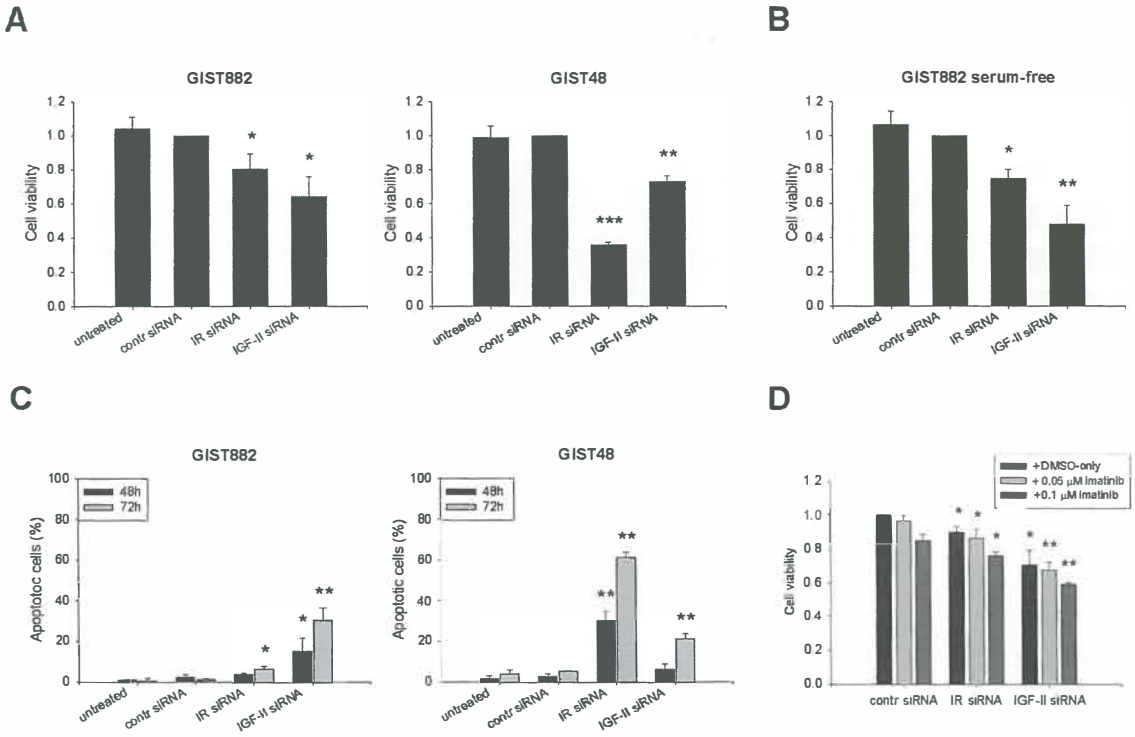

Figure 4. Effects of IGF-II and IR down-regulation on cellular survival. (A) The impact of IR and IGF-II down-regulation on cell viability was determined in GIST cells 72 h after siRNA transfection with MTT. The data were normalized to scrambled siRNA treated control cells. (B) GIST882 cells were switched to serum-free media 24 h after siRNA transfection and cell viability was determined with MTT after an additional 48 h. (C) The apoptotic response after siRNA treatment was determined in GIST cells with acridine orange apoptosis assays 48 and 72 h after transfection. (D) IR or IGF-II siRNA transfection of GIST882 was combined with imatinib treatment. The effects on cell viability were determined with MTT. The data were normalized to scrambled siRNA/DMSO-treated control cells. All data represent the mean values (± SD) of 3 independent experiments. *p <0.05, **p <0.01 and ***p <0.001 compared with scrambled control siRNA.

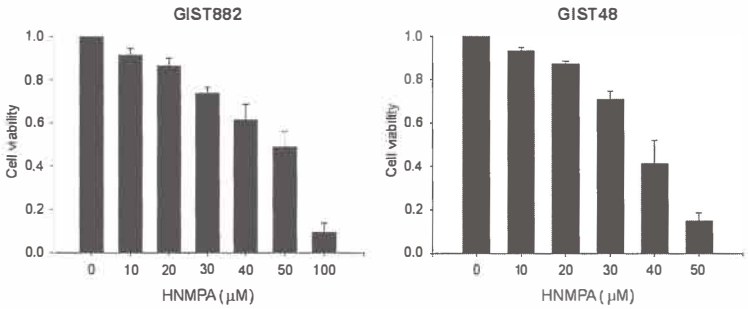

Figure 5. Effects of insulin receptor inhibition by the tyrosine kinase inhibitor HNMPA-$(AM)_3$ on GIST cell viability. Viability was determined 72 h after treatment with HNMPA-$(AM)_3$ with MTT. The data were normalized to untreated (DMSO-only treated) controls. Data are shown as mean values (± SD) of 3 independent experiments.

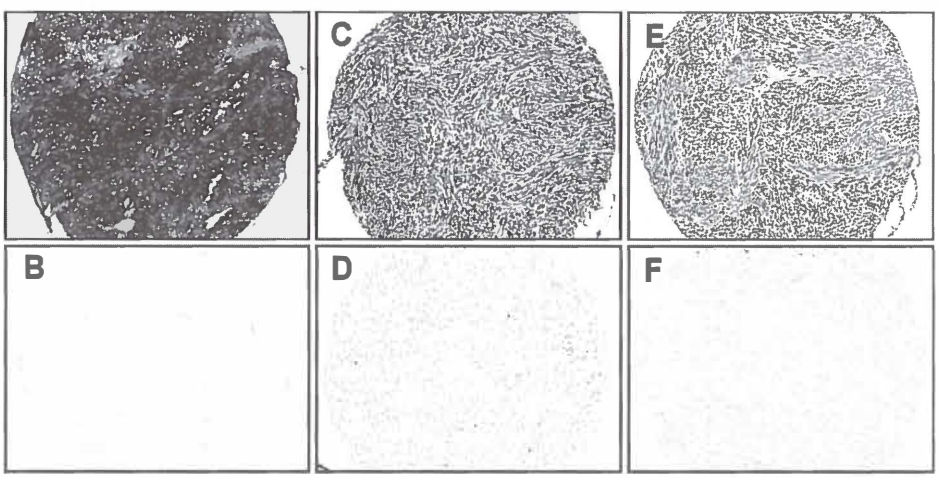

Figure 6. Representative examples of RNA in situ hybridization and immunohistochemical stainings. (A) In situ hybridization for IGF-II mRNA expression in a GIST tissue microarray core. (Dark)blue staining indicates the presence of IGF-II mRNA. (B) Sense probes were used as a negative control for in situ hybridization showing no staining in first trimester human placenta. (C) An example of GIST that showed immunostaining of IIE[68-88] being part of the E-domain of IGF-II. (D) No staining was observed using preimmune serum as a negative control for IIE[68-88] immunohistochemistry. (E) An example of a GIST showing diffuse cytoplasmic staining of total IGF-II, with no staining when normal goat IgG was applied as a negative control (F); (A-F, magnification x200). *See page 190 for colour figure.*

Table 3. IGF-II expression and tumor characteristics

	Pro-IGF-IIE[68-88] positive		Total IGF-II positive	
	n (%)	p[1]	n (%)	p
All primary tumors	36/56[2] (64)		17/53 (32)	
Risk category[3]				
(very) low/intermediate risk	12/26 (46)	0.012	3/23 (13)	0.016
High risk	24/30 (80)		14/30 (47)	
Primary site				
Stomach	16/31 (52)	0.022	2/28 (7)	<0.001
Small intestine/colon	20/24 (83)		15/24 (63)	
Histology				
Spindle cell	26/44 (59)	0.18	11/41 (27)	0.17
Epithelioid/mixed	10/12 (83)		6/12 (50)	
Tumor size				
≤10 cm	22/38 (58)	0.23	9/34 (26)	0.36
>10 cm	14/18 (78)		8/19 (42)	
Mitotic figures				
≤5/50 HPF	25/42 (60)	0.33	9/39 (23)	0.042
>5/50 HPF	11/14 (79)		8/14 (57)	
Mutation				
KIT Exon 11	16/29[4] (55)	0.14	5/26 (19)	0.066
No *KIT* exon 11	17/22 (77)		10/22 (45)	

[1]P-values are derived from the Chi-square or Fisher's exact test, where appropriate.
[2]Number of evaluable cases (tumors with less than 2 evaluable cores were excluded from the analysis).
[3]The risk group classification is based on size, mitotic index and primary localization of the tumor [15].
[4]Tumors that were not evaluable for mutation analysis were not included in this analysis.
Abbreviation: HPF, high power fields

Discussion

KIT and *PDGFRA* gain-of-function mutations are found in the far majority of GISTs. These mutations are considered as initiating steps in the development of a GIST. Additional oncogenic events could then contribute to the malignant outgrow of GIST [1]. The identification of critical pathways that cooperate with oncogenic KIT and PDGFRA signaling in the development of a malignant GIST could be useful for the development of new therapeutics for the treatment of this cancer. The existence of additional KIT signaling-dependent and KIT signaling-independent pathways has been implied in GIST tumors as well as in GIST cell lines [20,21]. We have previously reported that 'big'-IGF-II

is expressed in GISTs [6]. Since the IGF signaling system is involved in the pathogenesis of several sarcoma (sub)types [5], the purpose of this study was to investigate the putative biological role of 'big'-IGF-II in GIST.

We demonstrated that IGF-II is actively secreted by GIST cells *in vitro*, predominantly as 'big'-IGF-II. These results would supplement our recent findings that increased plasma levels of 'big'-IGF-II frequently occur in GIST patients. This is probably due to excessive production of 'big'-IGF-II by tumor tissue which may finally result in the induction of fasting hypoglycemia [6]. Many tumors, including sarcoma cells of different origin, have been shown to secrete increased amounts of incompletely processed forms of pro-IGF-II that still contain the first 21 amino acids of the E-domain. The latter peptide may be differentially *O*-glycosylated leading to the size heterogeneity of 'big'-IGF-II as we also observed for the cultured GIST cells in the present study [4,22]. The change in post-translational processing of pro-IGF-II in tumor cells could simply be the result of an inadequate capacity of intracellular enzymes to process the relatively high amounts of pro-IGF-II produced. Similar to mature IGF-II, 'big'-IGF-II is able to activate IGF-1R and IR isoform A. Sparse data suggest that 'big'-IGF-II is at least as mitogenic as mature IGF-II, which possibly depends on the amount of glycosylation of 'big'-IGF-II [5]. Nonetheless, in GIST, 'big'-IGF-II appeared a potent protein capable of providing an autocrine signal.

In several sarcoma types such as rhabdomyosarcoma, synovial sarcoma and osteosarcoma, IGF-II acts as an autocrine growth and survival factor [18,23,24]. IGF-II is involved in the pathogenesis of these tumors, amongst other oncogenic events, by signaling through IGF-1R. Recently, it has been demonstrated that a subset of GISTs lacking *KIT* and *PDGFRA* mutations, so called wild-type GISTs, express much higher levels of IGF-1R than the mutated GISTs, which was associated with *IGF1R* gene amplification [25]. Also in rarely occurring pediatric GIST, typically associated with wild-type tumors, markedly increased IGF-1R mRNA levels have been observed that even exceeded those in wild-type adult tumours [26]. IGF-1R expression has been detected in GISTs with *KIT* and *PDGFRA* mutations although to a lower level than in wild-type tumors, an observation that was primarily based on immunohistochemical stainings [25,27]. Indeed, in the wild-type GIST specimen investigated by us, we also encountered significant IGF-1R expression both at the mRNA and protein level. However, in our limited cohort of *KIT* or *PDGFRA* mutated GISTs, IGF-IR expression appeared to be undetectably low. Our data are nevertheless consistent with those very recently reported by Pantaleo *et al* [28]. At present, this apparent discrepancy with data from two previous studies on this subject remains unclear. Recently, it has been recognized that the IR isoform A also serves

as a high affinity receptor for IGF-II which mediates IGF-II-dependent signaling [29]. The IR (isoform A) actually was detectable in tumor specimens of our cohort of mutated GISTs suggesting that this receptor is the most likely candidate for autocrine 'big'-IGF-II signaling.

In order to inhibit autocrine 'big'-IGF-II signaling in GIST cell lines, we used siRNA against either IGF-II or IR. For both cell lines, the reduction in cell viability following down-regulation of IGF-II or IR expression with siRNA was more or less in the same order of magnitude as the extent of induction of apoptosis, suggesting that autocrine 'big'-IGF-II is mainly involved in eliciting an anti-apoptotic survival signal rather than a proliferation stimulus. Notably, GIST882 expressed the IR predominantly as isoform A, whereas in GIST48 both isoforms were expressed. Interestingly, GIST48 cells were highly sensitive towards IR silencing. This phenomenon could be due to differences in the cellular context of insulin receptor signaling between GIST48 and GIST882 cells or (partial) dependence on an exogenous IGF ligand, for example insulin, that is present in the enriched media these cells are cultured in. In both cell lines, down-regulation of IGF-II expression resulted in a markedly decreased phosphorylation of ERK, whereas phosphorylation of AKT was affected to a variable and much lesser extent. Recent studies have shown that the phosphatidylinositol-3-kinase (PI3K)/AKT pathway is an important survival pathway in several GIST cell lines, including GIST882 and GIST48. Specific inhibition of the mitogen-activated protein (MAP) kinase/ERK pathway, however, also reduced survival, albeit to a lesser extent, suggesting that both the PI3K/AKT and the MAPK/ERK pathway are involved in the survival of GIST cells [30]. Several studies showed that the MAPK/ERK pathway in GIST cells is only partially or not at all depending on activated KIT in contrast to the PI3K/AKT pathway [31,32]. Presumably, stimulation of other tyrosine kinase receptors contribute to the activation of intracellular signaling pathways in GIST as well, including the 'big'-IGF-II/IR autocrine loop. Nonetheless, despite the fact that AKT phosphorylation was only marginally inhibited by IR siRNA treatment in GIST48 cells, apoptosis was readily induced in these cells, when compared to the more sensitive GIST882 cells. This is in agreement with a previous study showing that specific inhibition of PI3K only in GIST48 induced high levels of apoptosis, even though AKT phosphorylation was not completely inhibited [30].

Our results imply an association between IGF-II protein expression and the potential aggressive behavior of GISTs. Similar observations have been reported by Braconi *et al* and Steigen *et al* who found that IGF-II expression was associated with decreased disease-free survival and decreased overall survival, respectively [27,33]. Furthermore,

microarray analysis performed in 181 different sarcomas, revealed that *IGF2* was one of the top discriminating genes associated with GIST [34]. Recent studies showed that minute (1 to 10 mm) GIST-like lesions are a common finding in the stomach of the adult population. In the majority of these lesions, *KIT* or *PDGFRA* mutations were found [35-37]. These findings suggest that GISTs could arise from premalignant lesions initially caused by *KIT* or *PDGFRA* mutations but which require additional oncogenic events. In our study, only a very small number of GISTs were classified as very-low-risk with an extremely low malignant capacity [15]. Three of these tumors were even smaller than 10 mm in size and in each of these tumors, *KIT* or *PDGFRA* mutation have been detected (data not shown). We noticed that all four very-low-risk tumors did not show any detectable IGF-II mRNA expression in contrast to tumors of the other risk groups except for one high risk tumor. This suggests, together with our *in vitro* findings, that gain of IGF-II (over-)expression could be an additional oncogenic event contributing to the malignant potential of GIST.

From a therapeutic point of view for those (wild-type) GISTs expressing IGF-1R, inhibition of this receptor would be an attractive option as several humanized monoclonal antibodies are currently (successfully) being tested in clinical studies [38]. Blocking of IR would be an interesting option in mutant *KIT* or *PDGFRA* GISTs, although the induction of hyperglycemia in patients with this strategy could be of concern. Alternatively, 'big'-IGF-II could be a direct target for therapy in both imatinib-sensitive and imatinib-resistant wild-type and mutant GISTs. IGF-II neutralizing antibodies have been developed but are not used in the clinics yet [39], but based on our results 'big'-IGF-II neutralizing antibodies may even have more tumor selective properties.

In conclusion, we showed that IGF-II is commonly expressed in GIST. Our data suggest that secreted 'big'-IGF-II is involved in the pathogenesis of GIST by providing a pro-survival signal in an autocrine manner. Targeting its receptors or directly neutralizing the action of 'big'-IGF-II might be attractive new therapeutic options in the fascinating area of GIST treatment, still being the role model for targeted therapy in the 21st century.

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Chapter 6

Abundant Fas expression by gastrointestinal stromal tumours may serve as a therapeutic target for MegaFasL

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Abstract

Although the tyrosine kinase inhibitor imatinib has been shown to be an active agent in patients with gastrointestinal stromal tumours (GIST), complete remissions are almost never seen and most patients finally experience disease progression during their course of treatment. An alternative therapeutic option is to target death receptors such as Fas. We showed that a panel of imatinib-sensitive (GIST882) and imatinib-resistant (GIST48, GIST430 and GIST430K-) cell lines expressed Fas. MegaFasL, a recently developed hexameric form of soluble Fas ligand (FasL), appeared to be an active apoptosis-inducing agent in these cell lines. Moreover, MegaFasL potentiated the apoptotic effects of imatinib. Immunohistochemical evaluations, in 45 primary GISTs, underscored the relevance of the Fas pathway: Fas was expressed in all GISTs and was expressed strongly in 93%, whereas FasL was expressed at moderate and strong levels in 35% and 53% of GISTs, respectively. Fas and FasL expression were positively correlated in these primary GISTs, but there was no association between Fas or FasL expression and primary site, histological subtype, tumour size, mitotic index, risk classification, and *KIT* mutation status. The abundant immunohistochemical Fas and FasL expression were corroborated by western blot analysis. In conclusion, our data implicate Fas as a potential therapeutic target in GIST.

Introduction

The observation that nearly all spindle cell and epithelial tumours of the stomach and bowel highly express the receptor tyrosine kinase KIT has led to the characterisation of gastrointestinal stromal tumours (GIST) as a distinct clinicopathological entity different from other gastrointestinal mesenchymal tumours. Various *KIT* genomic mutations occur in about 80% of GISTs. In addition, about 5% of GISTs have mutations in the platelet-derived growth factor receptor α (*PDGFRA*) [1]. These mutations lead to ligand-independent activation of KIT or PDGFRA, which plays an essential role in the development and progression of GIST [2,3]. Since GISTs are insensitive to conventional chemotherapy, the introduction of imatinib, a small-molecule receptor tyrosine kinase inhibitor active against KIT and PDGFRA, has been a major therapeutic breakthrough. Imatinib therapy has dramatically improved the survival of patients with unresectable or metastatic GIST [4]. Despite these successes, about 10% of the patients show initial resistance to imatinib. Moreover, complete remissions are almost never seen and most patients experience disease progression after a median period of approximately 2-3 years [4]. To date, sunitinib, an inhibitor of multiple receptor tyrosine kinases including KIT, PDGFRA, vascular endothelial growth factor receptor (VEGFR) and fms-related tyrosine kinase 3 (FLT3), is used as a second line treatment providing clinical benefit in patients with imatinib-resistant GIST for a limited time period [5]. However, there is urgent need for development of new therapeutics acting via pathways complementary to those targeted by KIT kinase inhibitors such as imatinib and sunitinib.

Fas (CD95) and Fas ligand (FasL; CD95L) belong to the TNF family of death receptors and ligands [6,7]. At the molecular level, binding of FasL to Fas induces receptor trimerisation, followed by the binding of Fas-associated death domain (FADD) with caspase 8 and/or 10 to the intracellular death domain of Fas. Caspase activation within this complex initiates cleavage and activation of an intracellular cascade of effector caspases (*e.g.* caspase 3, 6 and 7), eventuating in cleavage of specific death substrates and apoptosis [8]. Thus, in tumours expressing Fas, targeting of Fas-mediated apoptosis could be a promising therapy. Although many *in vitro* and *in vivo* cancer models have shown sensitivity towards Fas agonistic antibodies, clinical application of these antibodies is hampered because of severe liver toxicity [9]. Furthermore, resistance to Fas-mediated apoptosis because of inhibitory mechanisms along the apoptotic signalling pathway has been observed in other cancer models [10]. Soluble forms of FasL (sFasL) are potentially less toxic [11], and a hexameric form of sFasL, produced by fusing the

dimer-forming serum protein stalk of human ACRP30 to the trimeric portion of human FasL, has recently been developed. This compound, called MegaFasL, is more cytotoxic to tumour cells compared to trimeric sFasL [12-14]. MegaFasL is currently tested in a phase-I clinical trial (ClinicalTrials.gov identifier: NCT00437736).

This study was initiated given the lack of data on expression of Fas and FasL in GIST. Therefore, the sensitivity of GIST cell lines towards Fas activation and the potentiating effect of imatinib on this activation were investigated. In addition, the expression of Fas and FasL in primary GIST samples was studied. Together, these data implicate Fas as a potential therapeutic target in GIST.

Materials and Methods

Cell Culture

The GIST cell line GIST882 was developed from a primary, untreated, GIST, with a homozygous *KIT* exon 13 mutation [15]. Cells were maintained in RPMI 1640 (Invitrogen, Praisley, UK) supplemented with 15% heat inactivated fetal calf serum (FCS; Bodinco, Alkmaar, The Netherlands) and 1 mM L-glutamine (Invitrogen). The GIST cell lines GIST48 and GIST430 were established from tumours that were progressive during imatinib therapy after an initial clinical response [16]. GIST48 harbours a homozygous primary *KIT* exon 11 mutation and a heterozygous secondary exon 17 mutation. GIST430 has a heterozygous primary *KIT* exon 11 and a secondary heterozygous exon 13 mutation. The KIT-negative GIST430K- cell line was derived from GIST430 cells. The GIST48 and GIST430 cells were maintained in F-10 (Invitrogen) supplemented with 10% and 15% FCS, respectively, and 0.5% mito+ serum extender (VWR International, Roden, The Netherlands) and 1% bovine pituitary extract (VWR International). The cervical carcinoma cell line HeLa was maintained in 1:1 DMEM/HAM supplemented with 10% FCS.

Flow cytometry

Fas membrane expression was determined in GIST cells by flow cytometry as described previously [17]. Phycoerythrin (PE)-conjugated mouse anti-Fas monoclonal antibody (clone DX-2, 1:10; BD Pharmingen, Alphen aan de Rijn, The Netherlands) was used. PE-conjugated mouse IgG_1 (BD Pharmingen) served as an isotype control.

Acridine orange apoptosis assay

Cells were treated with MegaFasL (kindly provided by Apoxis, Lausanne, Switzerland) for 6 hr. To investigate the effect of MegaFasL on imatinib-induced apoptosis, cells were pretreated with imatinib (kindly provided by Novartis, Basel, Switzerland) for 24 hr followed by 24 hr incubation with MegaFasL without removing imatinib. Alternatively, cells were first treated with MegaFasL for 24 hr with subsequent treatment with imatinib for 24 hr. Control cells were incubated with DMSO, which was used as a solvent for imatinib, where appropriate, or a single-drug. After these treatments, acridine orange (10 mg/ml) was added to each well to distinguish apoptotic cells from vital cells. Apoptosis was defined as the appearance of apoptotic bodies and/or chromatin condensation, using a fluorescence microscope. Results were expressed as the percentage of apoptotic cells in a culture by counting at least 300 cells per well. All apoptosis assays were performed three times in duplicate.

Western blot analysis

After treatment with MegaFasL, as described above, GIST882 and GIST48 cells were lysed and the protein concentration was determined according to the Bradford method. Western blotting was performed as described previously [17]. Primary antibodies were goat anti-Fas (polyclonal, 1:400; Santa Cruz, Heerhugowaard, The Netherlands), mouse anti-FasL and mouse anti-caspase 3 (clone 33 and clone 19 respectively, 1:1000; BD Transduction Laboratories, Alphen aan den Rijn, The Netherlands), rabbit anti-cleaved caspase 3 and rabbit anti-caspase 6 (1:1000; Cell Signalling Technology, Bioké, Leiden, The Netherlands), mouse anti-caspase 8 (clone 1C12, 1:500; Cell Signalling) and mouse anti-actin (clone C4, 1:20,000; ICN Biomedicals, Zoetermeer, The Netherlands).

Tissue collection

The study group consisted of 45 primary GISTs derived from 44 patients. Paraffin-embedded tumour tissues of primary GISTs were retrieved from the files of the Department of Pathology of the University Medical Center Groningen. Tumours were surgically resected between 1985 and 2005. All tumours were reviewed by a pathologist with special expertise in soft tissue tumours (A.J.H.S.). The diagnosis of GIST was based on the typical histological features of a cellular spindle cell or epithelioid mesenchymal tumour of the gastrointestinal tract. In addition, all tumours were positive for CD117 (KIT) by immunohistochemistry. Tumours were categorised into risk groups, based on tumour size and mitotic index, according to the consensus classification for prediction of

metastatic potential [18]. Tumour size was obtained from the pathology reports. Mitotic index was counted in 50 consecutive high power fields (x 40 objective; field diameter 0.55 mm). Clinicopathological data collected included sex and age of the patient at diagnosis, primary tumour site and histological subtype. For the detection of *KIT* mutations, genomic DNA was extracted from paraffin-embedded tumour samples and *KIT* exon 9 or 11 were amplified by PCR. Both forward and reverse PCR products were sequenced and results were compared with normal sequences. One patient had two primary GISTs, which were a high risk epithelioid gastric tumour lacking a *KIT* exon 9 and 11 mutation and a low risk spindle-cell small intestine tumour with a *KIT* exon 11 mutation, respectively. Patient and tumour characteristics are summarised in table 1.

For the detection of Fas and FasL by western blotting, six frozen primary GIST samples were pulverized and dissolved in ice cold PBS. After centrifugation at 18,000 g for 1 min to remove debris, the supernatant was collected. Western blot analysis was performed as described above.

All tumour samples used in this study were handled according to the guidelines of the Dutch Federation of Biomedical Scientific Societies (FMWV) as described in "Code Proper Secondary Use of Human Tissue"

Tissue micro-array construction

Representative regions of the paraffin-embedded primary tumours were selected using H&E-stained slides and arrayed into a tissue micro-array (TMA), as described previously [19]. Briefly, three 0.6 mm tissue cores were taken from (distinct) representative areas of each tumour specimen using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) and then transferred to a standard-size recipient paraffin block. The array contained 161 tissue cores including 45 tumour samples in triplicate and 13 normal tissues in duplicate, the latter serving as an internal control for immunohistochemistry.

Immunohistochemistry

Sections of 4 μm were taken from each array block and deparaffinised in xylene. Immunohistochemistry was performed as previously described [20]. Antigen retrieval was carried out by autoclaving sections in blocking solution (2% blocking reagent (Roche Diagnostics, Mannheim, Germany) and 0.2% SDS in maleic acid buffer (pH 6.0)) at 115°C for three times 5 min. Primary antibodies were mouse anti-Fas (clone CH-11, 1:100; Upstate Biotechnology, Lake Placid, NY) and mouse anti-FasL (clone 33, 1:160; BD Transduction Laboratories). As a negative control, a serial section was processed without

the addition of primary antibody. Normal tissue samples within the TMA block derived from liver and kidney served as positive controls for Fas and FasL staining [21,22].

Table 1. Patient (n=44) and tumour (n=45) characteristics

	n (%)	
Sex		
Male	19 (43)	
Female	25 (57)	
Age (years), median (range)		65.5 (36-87)
Risk classification		
Very low risk	4 (9)	
Low risk	13 (29)	
Intermediate risk	13 (29)	
High risk	15 (33)	
Primary site		
Stomach	29 (64)	
Small intestine	12 (27)	
Colon	3 (7)	
Unknown	1 (2)	
Histology		
Spindle cell	37 (82)	
Epithelioid	7 (16)	
Mixed	1 (2)	
Tumour size		
≤5 cm	19 (42)	
5-10 cm	17 (38)	
>10 cm	9 (20)	
Mitotic figures		
≤5 per 50 HPF	38 (84)	
>5 per 50 HPF	7 (16)	
KIT mutation		
Exon 11 mutation	28 (62)	
Exon 9 mutation	1 (2)	
No exon 11 or 9 mutation	15 (33)	
Unknown	1 (2)	

Abbreviation: HPF, high power fields

Staining analysis

Immunohistochemistry results were scored independently by two observers (B.R. and A.J.H.S.) without knowledge of the clinicopathological data. As in each individual core all tumour cells showed the same staining, cores were scored in a semi-quantitative fashion for staining intensity: no staining (0), weakly positive staining (1), positive staining (2), strong positive staining (3). Discrepant scoring results were discussed under a multi-headed microscope to achieve consensus. If cores from one tumour differed in staining intensity, the median score of the three related cores determined the score of this tumour. Furthermore, the staining pattern was determined from each core.

Statistical analysis

Data analysis was performed using SPSS 12.0 software package for windows (SPSS Inc., Chicago, IL). Differences in distributions of Fas or FasL staining intensity and the different tumour characteristics were evaluated by the Chi-square test. To determine the correlation between Fas and FasL staining, the spearman's rank test was used. A two-tailed P-value <0.05 was considered to be significant.

Results

MegaFasL-induced apoptosis in GIST cells

To evaluate whether Fas could be used as a target in GIST, we first investigated Fas membrane expression in a panel of imatinib-sensitive (GIST882) and imatinib-resistant (GIST48, GIST430 and GIST430K-) cell lines. The cervical carcinoma cell line HeLa is responsive to MegaFasL and was used as positive control [12]. Flow cytometry analysis revealed that all the GIST cell lines had a high level of Fas membrane expression (figure 1A). We therefore tested the effectiveness of MegaFasL, a hexameric form of sFasL. After as little as 6 hr of MegaFasL treatment, dose dependent apoptosis was observed in all the GIST cell lines. MegaFasL induced substantially higher levels of apoptosis in GIST882, GIST430 and GIST430K- than in HeLa, while nearly equal amounts were observed in GIST48 compared to HeLa (figure 1B and C). Treatment of GIST882 and GIST48 with MegaFasL resulted in caspase 8 activation and the disappearance of the inactive proform of caspase 3 and 6. The active p19/p17 fragments of caspase 3 were detected in both cell lines although to a lesser extend in GIST48 which is in agreement with the observed difference in apoptosis levels between these two cell lines when treated with 50 ng/ml

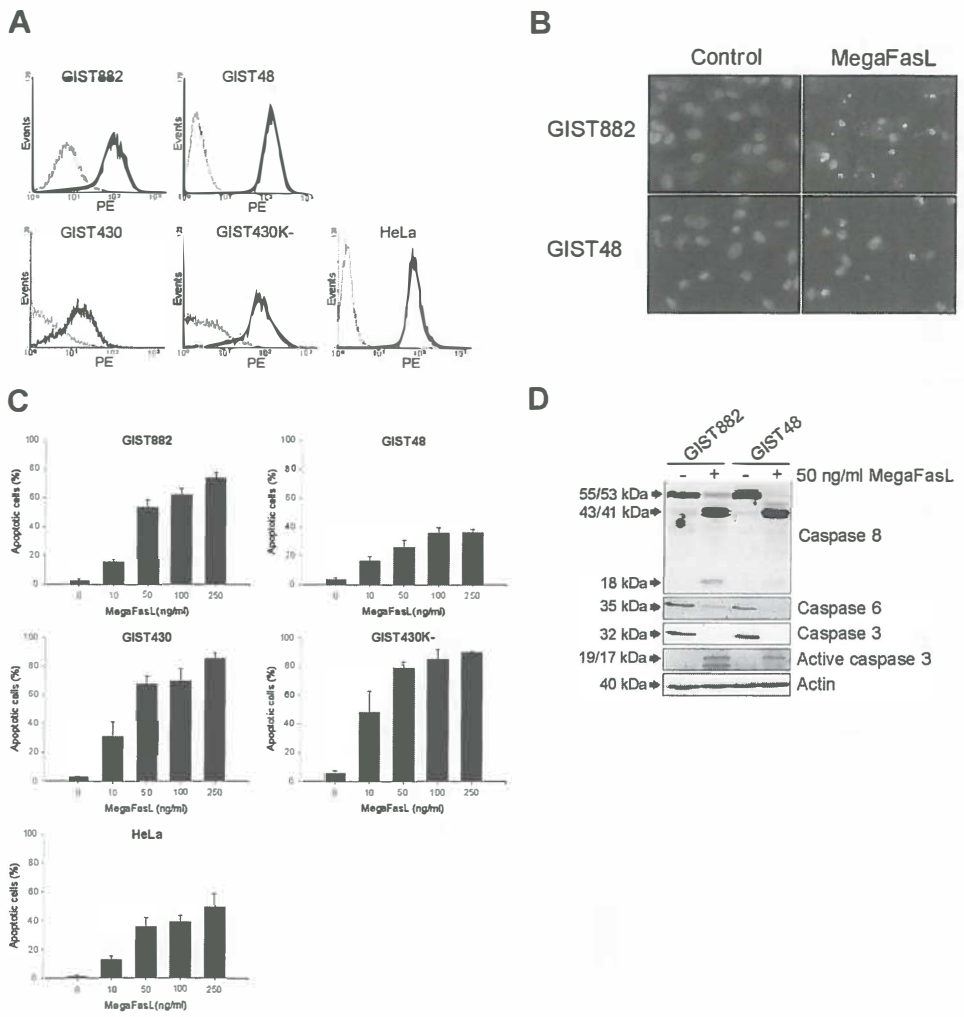

Figure 1. Apoptosis induction by MegaFasL in GIST cell lines. (A) Analysis of Fas membrane expression by flow cytometry. The thin grey line represents the IgG control and thick black line reflects the anti-Fas antibody. HeLa was used as a positive control. (B) Representative images of acridine orange staining of GIST882 and GIST48 with or without treatment with 50 ng/ml MegaFasL for 6 hr. Nuclear fragmentation is seen in cells treated with MegaFasL. (C) GIST cell lines, and HeLa, were incubated with different concentrations of MegaFasL for 6 hr. Apoptosis was quantified with acridine orange staining. Data represent the mean ± SD of 3 independent experiments. (D) Expression of caspase 8, caspase 6, caspase 3, and cleaved caspase 3 after treatment of GIST882 and GIST48 cells with 50 ng/ml MegaFasL for 6 hr, analysed by western blot. Immunoblotting of actin was used as a loading control. *See page 191 for colour figure.*

MegaFasL (53% apoptosis in GIST882 and 25% in GIST48; figure 1C and D). The effect of MegaFasL was dependent on caspase activation as zVAD-fmk, a pan-caspase inhibitor, completely blocked apoptosis induction by this compound (data not shown).

The effect of MegaFasL on imatinib-induced apoptosis

Following the identification of MegaFasL as a potent apoptosis-inducing agent in GIST cells, we investigated its effect in combination with imatinib. GIST882 pretreatment with MegaFasL followed by the addition of imatinib, appeared to be the most effective schedule. In this way, low concentrations of MegaFasL for 24 hr followed by the addition of imatinib for another 24 hr substantially increased the amount of apoptosis compared to levels seen with either MegaFasL or imatinib treatments alone (figure 2A). When imatinib was administered before MegaFasL, no synergistic but rather an additive effect was observed (data not shown).

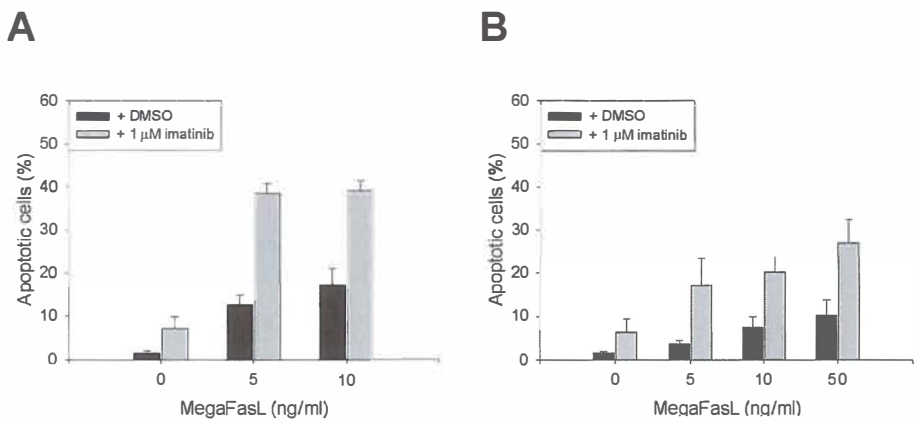

Figure 2. The effect of MegaFasL on imatinib-induced apoptosis. (A) GIST882 cells were pretreated with MegaFasL, as indicated, for 24 hr followed by incubation with either DMSO-only or 1 μM imatinib for an additional 24 hr. Apoptosis was determined by acridine orange staining. (B) GIST48 cells were treated with MegaFasL and/or imatinib, as described in (A). Data represent the mean ± SD of 3 independent experiments.

Recently, Bauer *et al* showed that GIST48 cells are relatively resistant towards imatinib [16]. We therefore also evaluated the combination of MegaFasL and imatinib in GIST48 by using the same treatment schedule as GIST882. As in GIST882, synergistic apoptosis induction was seen for the combination of MegaFasL and imatinib, although higher

concentrations of MegaFasL were necessary to induce a substantial amount of apoptosis (figure 2B).

Fas and FasL expression in GIST by immunohistochemistry

As MegaFasL appeared to be an active agent in GIST cells, we studied the expression of Fas and FasL in 45 GIST samples by immunohistochemistry using a TMA. Table 2 shows the overall staining characteristics of Fas and FasL in the 45 GISTs tested. Fas was detectable in all the tumour samples studied and was strongly expressed in 62%. FasL expression was discerned in 89% of the tumours with 27% staining strongly positive. A significant correlation between Fas and FasL expression was found (spearman's correlation coefficient = 0.4, p=0.006). Further statistical analysis revealed that Fas and FasL staining intensity was not associated with (1) risk classification, (2) primary site, (3) histological subtype, (4) tumour size, (5) mitotic index, and (6) *KIT* mutation status.

Both Fas and FasL immunohistochemical staining was predominantly cytoplasmic. The staining pattern for Fas was diffuse, in contrast to FasL which was mainly granular (figure 3).

Table 2. Expression of Fas and FasL in GIST

Staining intensity	Fas, n (%)	FasL, n (%)
0	0 (0)	5 (11)
1	3 (7)	16 (36)
2	14 (31)	12 (27)
3	28 (62)	12 (27)
Total	45 (100)	45 (100)

Fas and FasL expression in GIST by western blot analysis

In addition to immunohistochemistry, Fas and FasL protein expression in six GIST samples and the GIST882 cell line was evaluated by western blot analysis. HeLa was used as a positive control [23]. As shown in figure 4, protein products corresponding to the predicted molecular mass of ~36 kDa for FasL were detected in HeLa, GIST882, and in all six GISTs. The ~48 kDa Fas protein product was also detectable in GIST882 and in all GIST samples. Furthermore, most GIST samples – as well as the HeLa cell line – showed a lower band of ~43 kDa. In addition, most of the GISTs featured an additional band that migrated more slowly than the two bands shown in HeLa (Figure 4). This additional ~52 kDa band could represent a highly sialylated but functional form of Fas [24,25].

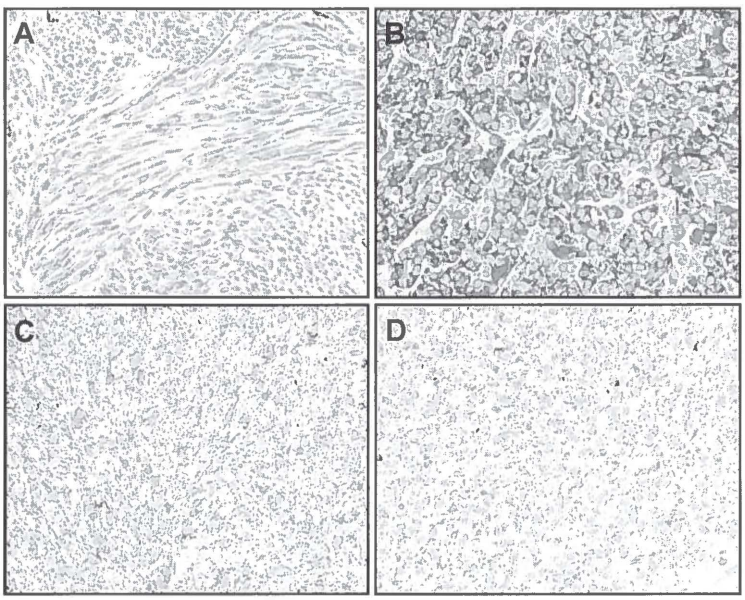

Figure 3. Immunohistochemical staining for Fas and FasL in paraffin-embedded GIST samples. Representative examples of immunostaining for Fas (A and B) showing predominantly diffuse cytoplasmic staining and FasL (C and D) showing granular cytoplasmic staining (magnification, 400x). *See page 191 for colour figure.*

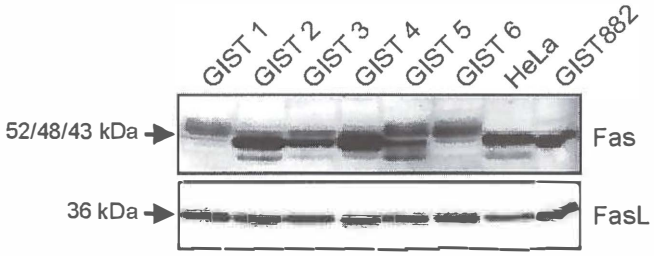

Figure 4. Expression of Fas and FasL in six resected GISTs and the GIST882 cell line as determined by western blot analysis. HeLa served as a positive control. GIST 2, 3 and 4 harbour a *KIT* exon 11 mutation, GIST 1, 5 and 6 have no *KIT* exon 9 or 11 mutations.

Discussion

The results of this study indicate that Fas may be a promising therapeutic target in GIST. The GIST cell lines in our panel were found to be highly sensitive to MegaFasL, which proved to be an active apoptosis-inducing agent. Moreover, MegaFasL sensitised GIST cells to imatinib-induced apoptosis. In addition to these *in vitro* data, we found that each of 45 primary GISTs expressed Fas, and most expressed FasL.

This is the first study investigating the role of death receptors in the treatment of GIST. Both imatinib-sensitive (GIST882) and imatinib-resistant GIST cell lines (GIST48, GIST430 and GIST430K-) were responsive to MegaFasL. Furthermore, MegaFasL sensitivity appeared to be independent of KIT expression because the GIST430 cell line that expresses KIT was as sensitive to MegaFasL as its KIT-negative daughter cell line. We found that low concentrations of MegaFasL sensitised GIST cells to imatinib-induced apoptosis. Notably, it appeared that the sequence of drug delivery was important. Pretreatment of cells with imatinib followed by incubation with MegaFasL had no more than an additive effect. Reversing this schedule, however, resulted in synergistic apoptosis induction. The underlying mechanism for this observation remains unclear. Nimmanapalli *et al* showed that the sensitising effect of imatinib to TRAIL in Bcr-Abl-positive leukaemia cells was reduced when TRAIL was administered after exposure to imatinib [26]. Although imatinib causes cell cycle arrest, it is unlikely that this mechanism is involved in the reduced potentiation of MegaFasL-induced apoptosis by imatinib pretreatment as Fas-mediated apoptosis is not cell cycle dependent [27,28]. Possibly, KIT inhibition by imatinib alters the membrane distribution of Fas, making it less accessible for MegaFasL. Alternatively, the activated caspase cascade by MegaFasL may induce cleavage of proteins involved in cellular resistance to imatinib.

In our patient cohort, which was representative for an unselected GIST population with regard to sex, age, tumour localisation, histology, risk classification, and *KIT* exon 11 mutations [1,29], Fas and FasL were abundantly expressed. Western blot analysis confirmed these findings. Acquisition of FasL expression by tumour cells is one of the mechanisms involved in tumour immune escape. Various tumour types have been reported to express FasL, which can be negatively correlated with prognosis as described for colon and breast carcinomas [30,31]. In our study, 89% of the GISTs expressed FasL, but no correlation was found with tumour size and mitotic index or risk groups based on these prognostic factors which predict metastatic behaviour. The FasL staining pattern in the cytoplasm was granular, an expression pattern that has also been observed in ovarian

carcinoma and melanoma, where FasL is stored in cytoplasmic microvesicles and, upon release, induces apoptosis in Fas-bearing immune cells [20,32,33]. FasL could therefore be involved in the protection of GIST cells against the immune system. In addition, we found a significant correlation between Fas and FasL expression in GIST, which has been observed in many other tumour types as well [20]. This co-expression has been implicated in tumour progression, since FasL can act in an autocrine or juxtacrine Fas-dependent manner to promote tumour growth [10,34]. Further studies are needed to determine whether such mechanisms also apply in GIST.

GIST patients generally respond to therapy with imatinib, which inhibits the KIT and PDGFRA oncoproteins that appear to be initiating oncogenic events in most GISTs. However, in the long run, most patients develop resistance to imatinib therapy, as manifested by tumour progression. It appears that, although imatinib treatment induces apoptosis and causes cell cycle arrest of GIST cells, a fraction of the cells usually survive, and these surviving cells may subsequently form the nidus of an imatinib-resistant GIST, often containing secondary *KIT* mutations [35,36]. Therefore, novel systemic therapeutic approaches are needed to maximise GIST cell death. Targeting death receptors, such as Fas, is a promising anticancer strategy by which apoptotic cell death can be induced. Unfortunately, the introduction of therapies targeting Fas with agonistic antibodies has been hampered by liver toxicity [9]. However, the recently developed MegaFasL is potentially less toxic and has been shown to be active in several *in vitro* and *in vivo* models [12-14]. MegaFasL is formed by cross-linking two sFasL trimers resulting in a hexameric protein that much more efficiently induces clustering of Fas on the cell surface leading to a higher degree of multimerisation of activated receptors as compared with sFasL. As a consequence, a high local concentration of the intracellular death domains might be formed, leading to more efficient activation of caspase 8 after binding to the adaptor molecule FADD [12].

We found that very low doses of MegaFasL potentiate the effect of imatinib. Therefore, even when the therapeutic window is low, systemic use of MegaFasL could still have a beneficial effect in combination with imatinib. As many GISTs tend to metastasise in the abdominal cavity, potential liver toxicity by MegaFasL might be circumvented by intraperitoneal applications to prevent MegaFasL from reaching high levels in the circulation [14,37]. In this way, MegaFasL might be applied in combination with systemic imatinib. The abundant Fas expression in GISTs suggests that also MegaFasL alone might be an option for patients with primary or acquired resistance to imatinib. However, besides Fas membrane expression, one should take in account the expression

of intracellular components affecting sensitivity to Fas-mediated apoptosis in these GISTs.

In conclusion, we have identified Fas as a uniformly expressed receptor in GIST, which may be used as a therapeutic target. This is supported by our *in vitro* studies, in which MegaFasL is a potent apoptosis-inducing agent that is even more efficient in combination with imatinib.

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Chapter 7

Results of plasma N-terminal pro B-type natriuretic peptide and cardiac troponin monitoring in GIST patients do not support the existence of imatinib-induced cardiotoxicity

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Abstract

Background: Recently, case reports of patients treated with imatinib (imatinib mesylate; Gleevec®; Glivec®) indicated that this tyrosine kinase inhibitor may induce cardiomyopathy. Consequently, careful cardiac monitoring was advocated for clinical studies. The purpose of this study was to prospectively evaluate whether imatinib (Gleevec®) induces early, subclinical, cardiac toxicity.

Patients and methods: History and physical examination were carried out with special attention for symptoms of heart failure. Additionally, assessments of serial plasma N-terminal pro B-type natriuretic peptide (NT-proBNP) and serum cardiac troponin T (cTnT) measurement before and one and three months after the start of imatinib treatment (400-800 mg daily) were done in patients with advanced and/or metastatic gastrointestinal stromal tumours (GIST).

Results: A total of 55 GIST patients were enrolled. Only one patient, with a normal pretreatment NT-proBNP, showed an increase in NT-proBNP to above age-specific normal values during imatinib treatment and developed symptomatic heart failure due to pre-existent cardiac valvular disease. cTnT levels remained stable.

Conclusions: In our study population, imatinib treatment for GIST was not associated with an increase in plasma NT-proBNP levels, indicating that the risk of subclinical cardiac toxicity is limited with the use of this agent. These results do not support the current strategy to standard cardiac monitoring in all patients. This may be restricted to GIST patients with a history of cardiac disease.

Introduction

Imatinib (imatinib mesylate; Gleevec®; Glivec®, Novartis, Basel, Switzerland), a small molecule tyrosine kinase inhibitor, is applied successfully as standard therapy for the treatment of chronic myelogenous leukaemia (CML) and gastrointestinal stromal tumours (GIST) [1]. A recent publication of Kerkela *et al* [2], however, without giving a patient denominator, suggested a risk of cardiotoxicity induced by imatinib, which was unexpected, given the previous toxicity data of large patient studies. Subsequent specific reports indicated a low incidence (if any) of New York Heart Association (NYHA) class III-VI heart failure due to imatinib, varying from 0.2% to 1.8% [3-5]. Although the clinical problem of imatinib-related cardiotoxicity may be limited, lower NYHA class, as well as subclinical cardiotoxicity, may be more frequently observed in patients treated with imatinib if paid attention to [6].

Natriuretic peptides are neurohormones which are increased in plasma in case of cardiac dysfunction. N-terminal pro B-type natriuretic peptide (NT-proBNP) is the prohormone of BNP and is secreted by cardiomyocytes in response to ventricular dilation or local wall stress, and can be used as a sensitive biochemical marker to detect left ventricular systolic dysfunction [7].

To evaluate whether imatinib induces (subclinical) cardiac toxicity, we prospectively evaluated cardiac function, including history, a physical examination, and determination of plasma NT-proBNP and serum cardiac troponin T (cTnT) concentrations in GIST patients treated with this agent.

Patients and methods

Patients and treatments

As part of surveillance programmes, plasma and serum from patients treated with imatinib for locally advanced and/or metastatic GIST were collected after informed consent before treatment and at 1 and 3 months thereafter. Imatinib (Novartis) was administered orally at 400-800 mg daily, continuously. Treatment was continued until disease progression or unacceptable toxicity.

Cardiac evaluation

A cardiac evaluation, including a history and physical examination with special attention to signs and symptoms related to heart failure, was carried out before the start of imatinib treatment and 1 and 3 months after the start of treatment. If present, heart failure severity was classified according to the NYHA scale [8].

Natriuretic peptides and cTnT concentrations

For the determination of plasma NT-proBNP and serum cTnT levels, peripheral blood samples were collected as serum or lithium-heparin plasma specimens using standard sampling tubes. Serum or plasma was separated and stored at -80°C until determination. NT-proBNP and cTnT levels were measured with an electrochemiluminescence immunoassay (Elecsys proBNP assay and Elecsys Troponin T assay, respectively; Roche Diagnostics, Mannheim, Germany). NT-proBNP has an upper limit of normal of 125 ng/L [9]. Age-dependent cut-off values are as follows: ≤50 years: 450 ng/L; 50-75 years: 900 ng/L, >75 years: 1800 ng/L [10]. cTnT values of >50 ng/L indicate myocardial injury.

Statistics

Values are given in mean (±standard deviation) for normally distributed variables and median (range) for variables with a skewed distribution. Paired analysis was carried out with a Wilcoxon paired samples test. All *P*-values are two-sided and *P*-values <0.05 indicate statistical significance.

Results

Patients

Serum en plasma were obtained from 55 patients (34 male) with a mean age of 62 (±12) years. The mean follow-up duration was 9 (±3.7) months. Two patients had received prior chemotherapy; one had received 4 cycles of bleomycin, etoposide and cisplatin for a testicular germ-cell tumour, 18 years before imatinib, and the other 6 months of adjuvant capecitabine for colon carcinoma, 6 months before imatinib.

Cardiac evaluation and cardiac marker measurements

Figure 1 represents individual plasma NT-proBNP values during imatinib treatment. Before the start of imatinib, two patients had chronic heart failure. One patient had

NYHA class III symptoms on the basis of atrial fibrillation and mitral regurgitation, which remained stable during treatment. She had an elevated pretreatment NT-proBNP level, which increased from 2384 to 4014 ng/L after 3 months. The second patient had NYHA class IV symptoms at the start of imatinib therapy due to idiopathic dilated cardiomyopathy. The use of diuretics markedly improved his symptoms and lead to a decrease in plasma NT-proBNP from 19229 before treatment to 294 ng/L after 3 months.

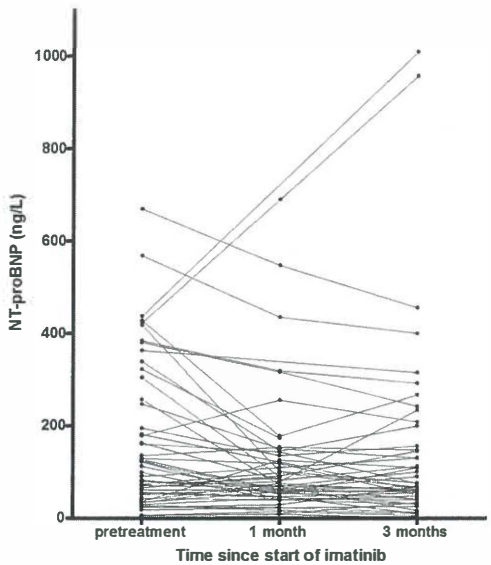

Figure 1. Individual N-terminal pro B-type natriuretic peptide (NT-proBNP) values before, 1 month after the start and 3 months after the start of imatinib treatment. Two patients with a plasma NT-proBNP level above the upper limit of normal are not shown due to the scale. One patient had a pre-treatment value of 19229 ng/L, that decreased to 2358 ng/L at 1 month after the start of imatinib and to 294 ng/L after three months. In the other patient, NT-proBNP before imatinib was 2384 ng/L, 2303 ng/L after one month imatinib, and 4014 ng/L after 3 months of treatment (see explanation in the text). Two patients showed an increase in NT-proBNP during treatment; however, in only one of these two, NT-proBNP increased to above the age-adjusted cut-off value.

Only one of the patients with normal pretreatment NT-proBNP levels developed a plasma NT-proBNP level above the age-specific cut-off level (from pretreatment 437 to 1009 ng/L). This 58-year-old woman, with pre-existing asymptomatic mitral valve regurgitation, developed NYHA class II heart failure after 5 months. As she had had

heart failure in the past, the relapse of symptoms was considered to be due to the mitral valve regurgitation. No other patient presented symptoms of cardiac failure during the study period.

Serum cTnT levels remained below the lower limit of normal for all patients during the follow-up period.

Discussion

Imatinib is currently widely and successfully applied in the treatment of patients with CML or GIST. The recent report by Kerkela *et al* [2], indicating that the use of imatinib could induce cardiac dysfunction, alarmed many oncologists treating these patients. In the current study, we found that plasma NT-proBNP levels remained stable during the first three months of treatment. Only 1 of 55 patients developed symptomatic, drug-unrelated heart failure during imatinib treatment, coinciding with a marked increase in plasma NT-proBNP value. An increase in serum cTnT was not observed at the various time points, as compared to pretreatment values.

The alarming data of Kerkela *et al* [2], who did not mention the total number of patients at risk, have not been confirmed in subsequent reports with large patient numbers. For example, in the EORTC-ISG-AGITG phase III trial, the frequency of cardiomyopathy in 942 GIST patients treated imatinib at a dose of either 400 or 800 mg daily was 0.2% [5], indicating that treatment with imatinib is not related to an increased risk of heart failure. Our data are in line with those, and may indicate that short-term imatinib treatment is not related to an increased rate of (subclinical) cardiotoxicity. In combination with the other studies reported that indicated that the few imatinib-related cardiac events can also occur after only a few days of treatment, indicating that if any, the drug rather than the cumulative dose may be responsible, our data confirm that standard cardiac monitoring does not appear required.

During the last years, the value of NT-proBNP has been studied in the oncological setting. For instance, plasma NT-proBNP measurement in anthracycline-treated breast cancer patients, proved to be a useful early marker for a decrease in left ventricular ejection fraction [11]. Interestingly, measurement of plasma BNP has recently been shown to be a practical and useable marker for the detection of imatinib-related cardiotoxicity [12].

Next to NT-proBNP, we also measured serum cTnT. In oncology, serum cTnT may be a useful marker for detecting subclinical cardiotoxicity induced by doxorubicin in adults [13,14], although most data are currently derived from studies carried out in children. In our study, we measured for the first time cTnT prospectively in patients treated with imatinib and could not demonstrate any change in time.

A limitation of our study may be the fact that no objective measures of left ventricular function were included in the cardiac assessment. Another limitation of the current study may be our relatively small study population, considering the low reported incidence of cardiomyopathy during treatment of GIST patients with imatinib. Additionally, the follow-up duration was short.

In conclusion, plasma NT-proBNP and serum cTnT levels did not change during imatinib treatment, further indicating that treatment with imatinib is not related to an increased incidence of subclinical cardiotoxicity. Standard cardiac monitoring does not appear required with this agent.

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Chapter 8

Hormonal and electrolyte changes in patients with a gastrointestinal stromal tumour during imatinib treatment

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In progress

Abstract

Background: The tyrosine kinase inhibitor imatinib is the first-line treatment for patients with an advanced gastrointestinal stromal tumour (GIST) or chronic myeloid leukaemia (CML). Several studies in small series of patients with CML treated with imatinib reported changes in bone and mineral metabolism and occurrence of hypogonadism in male patients. The objective was to assess hormonal and electrolyte changes in patients with GIST during treatment with imatinib and to analyse their relation with clinical outcome.

Patients and methods: Patients with GIST treated with imatinib at the University Medical Center Groningen between February 2001 and February 2009 were eligible for this study. Levels of calcium, phosphate, magnesium, PTH, TSH, FT4, FT3, LH, FSH, testosterone, estradiol and prolactin were determined in serum samples obtained before start of imatinib and up to 6 months after start of imatinib. Progression-free survival was determined for all patients.

Results: 36 patients were eligible. Compared to baseline, permanent significant decreases in serum calcium and phosphate were observed within one week and one month of treatment, respectively. A long-term increase in PTH was seen after one week of imatinib. No consistent changes in thyroid hormones were detected. The sex hormones did not change over time, except a small but significant increase of FSH in male patients. Patients, who developed hypophosphataemia after 3 months of treatment, had a decreased progression-free survival.

Conclusions: Imatinib causes hyperparathyroidism in patients with advanced GIST but does not affect thyroid and gonadal function. The development of hypophosphataemia is associated with a worse clinical outcome.

Introduction

The tyrosine kinase inhibitor imatinib is an inhibitor of KIT, platelet derived growth factor receptor (PDGFR), c-Abl and Bcr-Abl. Nowadays, imatinib is the first-line treatment for patients with Bcr-Abl positive chronic myeloid leukaemia (CML) and advanced gastrointestinal stromal tumours (GISTs) [1,2]. GISTs are neoplasms of mesenchymal origin arising from the gastrointestinal tract. The majority of GISTs (~80%) are characterized by an activating mutation in the *KIT* proto-oncogene. In a small proportion of GISTs, an activating mutation in *PDGFR* α (PDGFRA) gene is present. These mutations play a central role in the development and progression of GIST [3].

Several case reports and studies with small series of patients described hormonal changes during imatinib treatment. Secondary hyperparathyroidism was found in patients with GISTs as well as in patients with CML [4-6]. Studies have addressed imatinib-associated alterations in bone metabolism as an important factor that influenced serum calcium, phosphate and parathyroid hormone (PTH) levels [4,6-8]. In patients with CML treated with imatinib, development of hypophosphataemia was associated with response to treatment suggesting that "off-target" effects of imatinib could be predictive for tumour response [5].

Other effects of imatinib have been reported to be likely attributable to inhibition of its molecular targets in normal tissues including hypogonadism in male patients [9] and hypothyroidism in patients on thyroid hormone suppletion after thyroidectomy [10].

Given the limited data regarding the occurrence of the above mentioned changes in patients with GIST, we studied hormonal and electrolyte alterations in a single centre cohort of GIST patients during the first 6 months of treatment with imatinib. In addition, we analysed whether these changes were associated with clinical outcome.

Patients and methods

Patients

Newly diagnosed patients with advanced unresectable or metastatic GIST, histologically proven by CD117 positivity, who started systemic treatment with imatinib at the Department of Medical Oncology of the University Medical Center Groningen between February 2001 and February 2009, were eligible for this study. Imatinib was administered orally at 400-800 mg daily, continuously. The study protocol was approved by the Medical

Ethics Committee of the University Medical Center Groningen and all patients gave their informed consent.

Tumour response evaluation

Every patient underwent a routine contrast enhanced CT scan prior to the start of therapy, 8 weeks after start of therapy, and every 3 months thereafter until progression of disease. Disease progression was defined as (1) the occurrence of a new lesion, (2) increasing size of the pre-existing lesions (as defined by RECIST version 1.0 [11]), or (3) development of an intratumoral nodule and/or an increase in 'solid' tissue, in the background of a hypodense lesion [12]. Progression-free survival was defined as the time from imatinib initiation until disease progression.

Biochemical analyses

Blood samples were collected prior to the start of imatinib therapy, and 1 week and 1, 3 and 6 months after initiation of treatment. After the samples were drawn, tubes were placed on ice immediately and serum was separated within 30 min of collection by centrifugation at 4°C. All serum samples were stored at -20°C until analysis. Serum calcium, phosphate, magnesium, and albumin were measured on a Roche Modular automated analyser. Albumin-adjusted serum calcium was calculated using the formula Ca = serumCa [mmol/L] + 0.02 (43 – serumAlb [g/L]). Serum concentrations of prolactin were determined using an electrochemiluminescence immunoassay (Cobas, Roche diagnostics, Mannheim, Germany). Serum thyroid stimulating hormone (TSH), free thyroxine (FT4), free triiodothyronine (FT3), follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol were measured using a fluoroimmunoassay (AutoDELFIA, PerkinElmer, Zaventem, Belgium). Sex-hormone binding globulin (SHBG) was measured using an in-house binding assay and total testosterone was determined with a radioimmunoassay [13]. Serum intact PTH levels were analysed with a two-site chemiluminescent enzyme-labeled immunometric assay (Immulite 2500, Siemens, The Hague, The Netherlands).

KIT and *PDGFRA* mutation analysis

Mutation analysis of *KIT* exons 9, 11, 13 and 17 and *PDGFRA* exons 12, 14 and 18 was performed as described previously [14].

Statistics

Changes in serum levels were evaluated with the paired samples t-test or the Wilcoxon signed ranks test, as appropriate. The log rank test was applied to analyse the impact of changes of biochemical markers during imatinib treatment on progression-free survival. For this, patients were divided into groups according to quartile changes from baseline for each marker at each time point, or, alternatively, into groups based on the normal reference values. Correlations were described by the Pearson's correlation coefficient (r). A double-sided P-value <0.05 was considered statistically significant using SPSS for windows version 16.0.

Results

Thirty-six patients treated with imatinib entered this study. Patient characteristics are summarized in table 1. All patients were evaluable for tumour response assessment. The median follow-up was 32 (range 4 – 87) months and the median progression-free survival was 23 months.

Calcium, phosphate, magnesium and PTH

One patient was on calcium and vitamin D supplements at start of treatment and was excluded from analysis. Before treatment, the serum concentrations of calcium, phosphate, magnesium and PTH were within the normal reference range. During treatment with imatinib, significant sustainable changes in serum levels of calcium, phosphate and PTH were observed (figure 1 and table 2). A decrease in albumin-adjusted serum calcium levels was observed already 1 week after start of imatinib with a further decline after 1 month, which remained stable thereafter. The mean (±SD) calcium reduction at one month was 0.14 (±0.11) mmol/L with 27 percent of the patients developing a mild hypocalcaemia (i.e. a calcium level below normal). A drop in serum phosphate levels was seen from 1 month after start of treatment, which remained reduced at 3 and 6 months. The mean (±SD) decrease in serum phosphate at 1 month was 0.20 (±0.15) mmol/L while one patient developed hypophosphataemia after 1 month. In 25 of 32 patients (78%), who had both calcium and phosphate levels analysed at 3 months, both electrolytes were decreased. A transient small increase in serum magnesium was observed after one week of treatment. A rise in serum PTH levels was observed after one week of treatment inclining after 1 month and remaining stable at this increased level at the later time points. Up to 50

percent of the patients developed hyperparathyroidism (i.e. serum PTH values above the normal upper limit of 8.7 pmol/L; table 2).

Table 1. Patient characteristics (n=36)

	n (%)	Median (range)
Sex		
Male	23 (64)	
Female	13 (36)	
Age (years)		62 (24 – 81)
Imatinib dose		
400 mg	34 (94)	
800 mg	2 (6)	
Primary tumour localization		
Stomach	13 (36)	
Small bowel	14 (39)	
Colon	3 (8)	
Other site	6 (17)	
Disease stage		
Primary disease only	10 (28)	
Metastatic disease	26 (72)	
Mutation		
KIT exon 9	5 (14)	
KIT exon 11	16 (44)	
PDGFRA exon 18	3 (8)	
Wild-type	2 (6)	
Not evaluable	10 (28)	
Progression-free survival (months)		23
Follow-up (months)		32 (4 – 87)

When analysing the mutual relationships between the changes in calcium, phosphate and PTH levels, there was no significant correlation between the changes of any of these factors at the different time points (r between -0.2 and 0.2, and $p > 0.10$ for all correlations).

There were no associations between changes in serum levels of calcium or PTH and progression-free survival. Patients who developed hypophosphataemia after 3 months of treatment had a worse progression-free survival than those with a phosphate level within normal range ($p=0.013$; figure 2). In contrast, no associations were found between the degree of decrease in phosphate levels and progression-free survival.

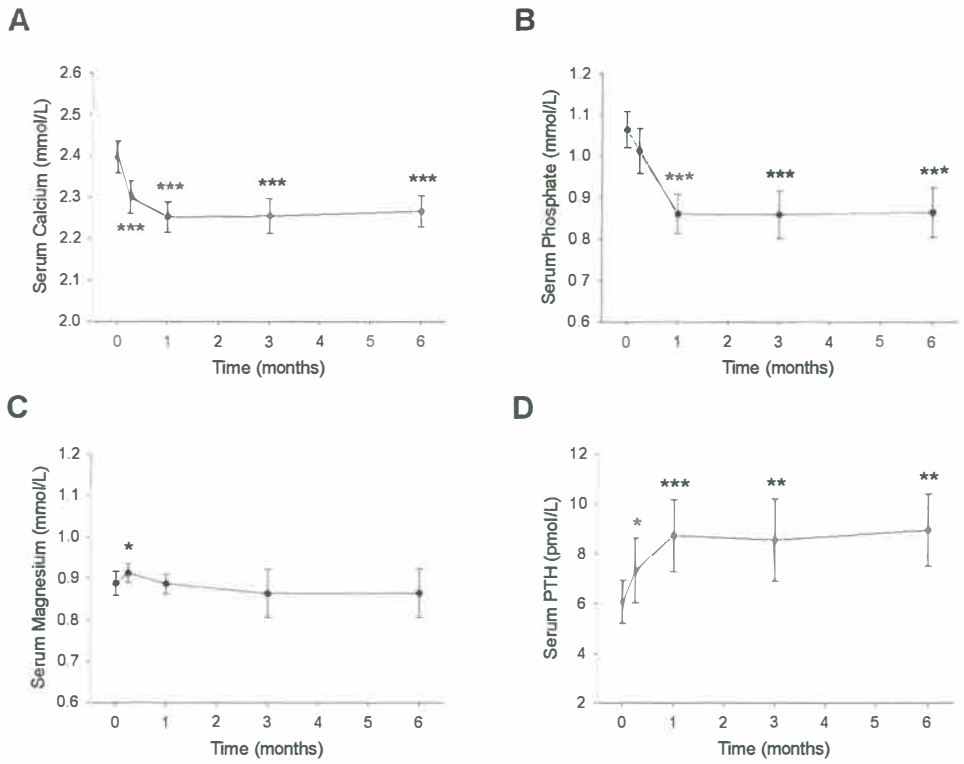

Figure 1. Electrolyte and PTH serum levels during imatinib treatment. (A) Albumin-adjusted serum calcium, (B) serum phospate, (C) serum magnesium, (D) serum PTH. Data are mean (95% confidence interval). *, p<0.05; **, p<0.01, ***, p<0.001 vs baseline.

Thyroid hormones

Three patients with known thyroid disorders (one with hypothyroidism and two with hyperthyroidism) were excluded from this analysis. The values of TSH, FT4 and FT3 at baseline and at one month are shown in table 3. TSH and FT3 did not change during treatment. An increase in FT4 with median 0.90 (range -7.40 – 1.90; p=0.014) pmol/L and 1.00 (range -7.80 – 2.00; p=0.012) pmol/L was found after one week and one month of treatment, respectively. At 3 and 6 months, the levels of FT4 did not differ from baseline. There were no associations found between changes in the levels of FT4 and progression-free survival.

Table 2A. Levels of albumin-adjusted serum calcium (mmol/L)

Time point (months)	Cases	Mean (SD)	Median (range)	Cases below lower reference value (%)	Cases above upper reference value[1] (%)
0	35	2.40 (0.11)	2.40 (2.15–2.68)	1 (3)	1 (3)
0.25	34	2.30 (0.11)	2.30 (2.10–2.58)	6 (18)	0 (0)
1	34	2.25 (0.11)	2.26 (2.03–2.46)	9 (27)	0 (0)
3	34	2.25 (0.12)	2.26 (2.04–2.56)	12 (35)	0 (0)
6	32	2.27 (0.10)	2.25 (2.11–2.53)	9 (28)	0 (0)

[1] Reference values: 2.20-2.60 (mmol/L)

Table 2B. Levels of serum phosphate (mmol/L)

Time point (months)	Cases	Mean (SD)	Median (range)	Cases below lower reference value[1] (%)
0	35	1.06 (0.13)	1.06 (0.77–1.24)	0 (0)
0.25	30	1.01 (0.15)	1.00 (0.79–1.33)	0 (0)
1	30	0.86 (0.13)	0.86 (0.56–1.29)	1 (3)
3	33	0.86 (0.16)	0.86 (0.50–1.20)	5 (15)
6	30	0.87 (0.16)	0.86 (0.55–1.29)	3 (10)

[1] Reference values: 0.70-1.50 (mmol/L)

Table 2C. Levels of serum PTH (pmol/L)

Time point (months)	Cases	Mean (SD)	Median (range)	Cases above upper reference value[1] (%)
0	35	6.1 (2.6)	5.8 (1.5–12.0)	6 (17)
0.25	30	7.3 (3.5)	6.0 (3.2–17.0)	8 (27)
1	30	8.7 (3.9)	7.4 (4.0–22.0)	12 (40)
3	33	8.6 (4.7)	7.7 (3.5–26.0)	10 (30)
6	30	9.0 (3.9)	8.8 (3.5–18.0)	15 (50)

[1] Reference values: <8.7 (pmol/L)

Abbreviation: PTH, parathyroid hormone

Sex hormones

One male patient was excluded from this analysis because he suffered from a prolactinoma. The values of the various sex hormones at baseline and at three months are shown in table 4. Six male patients (27%) showed testosterone levels below the normal reference value at baseline, which was accompanied with an increased LH in two of these patients. No changes in testosterone, estradiol, SHBG and prolactin were observed in the total group of patients or in the male or female subgroups. In the group of 22 male

patients, an increase in FSH but not of LH was detected. Median FSH levels at baseline were 7.33 U/L (range 2.84-23.90) and increased after 1 week and after 1, 3 and 6 months with median 0.83 (range -1.25 – 10.01), 0.88 (range -2.00 – 8.30), 1.02 (range -5.30 – 8.60) and 1.18 (-1.90 – 9.60) U/L, respectively ($P < 0.05$ for all time points vs. baseline). Notably, 50 percent of the male patients had FSH levels above the upper reference limit of 7.2 U/L at baseline already and this percentage did not change during treatment. There was no association between alterations in the levels of FSH and progression-free survival.

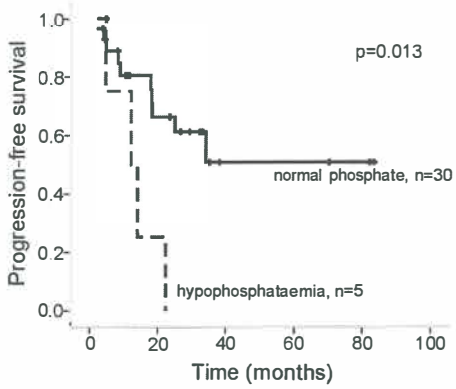

Figure 2. Progression-free survival for patients with GIST developing hypophosphataemia versus patients remaining serum phosphate levels within normal range after 3 months of treatment with imatinib.

Table 3. Thyroid hormones in GIST patients (n=33)[1] at baseline and after one month of imatinib treatment

	Median value (range) at baseline	Median value (range) at 1 month	P-value[2]	Reference values
TSH (mU/L)	1.30 (0.34 – 19.00)	1.30 (0.56 – 14.00)	0.85	0.5 – 4.0
FT4 (pmol/L)	14.2 (8.3 – 25.0)	14.8 (10.8 – 23.1)	0.012	11.0 – 19.5
FT3 (pmol/L)	5.7 (3.3 – 7.6)	5.6 (3.3 – 7.7)	0.27	4.4 – 6.7

[1]Three patients with thyroid disorders were excluded from analysis.

[2]Paired samples test

Abbreviations: TSH, thyroid stimulating hormone; FT4, free thyroxine; FT3, free triiodothyronine.

Table 4. Sex hormones in male (n=22)[1] and female (n=13) patients at baseline and after three months of imatinib treatment

	Median value (range) at baseline	Median value (range) at 3 months	P-value[2]	Reference values
Testosterone (nmol/L)				
Male	14 (3 – 26)	16 (1 - 23)	0.87	12 – 40
Female	1.7 (0.1 – 4.5)	1.6 (0.0 – 3.3)	0.07	0 – 3.5
Estradiol (nmol/L)				
Male	0.10 (0.03 – 0.13)	0.08 (0.05 – 0.13)	0.66	0.06 – 0.13
Female	0.05 (0.02 – 0.25)	0.05 (0.02 – 0.34)	0.34	0.03 – 0.09
SHBG (nmol/L)				
Male	36 (13 – 61)	35 (17 – 89)	0.75	12 – 30
Female	39 (27 – 99)	49 (27 – 95)	0.93	10 – 100
Prolactin (mU/L)				
Male	165.3 (92.6 – 371.8)	189.8 (100.5 – 429.4)	0.13	<300
Female	201.0 (63.7 – 729.1)	205.8 (77.0 – 518.5)	0.75	<500
FSH (U/L)				
Male	7.33 (2.84 – 23.9)	8.12 (3.53 – 32.20)	0.036	1.8 – 7.2
LH (U/L)				
Male	5.61 (2.72 – 27.0)	5.31 (2.57 – 23.50)	0.17	2.1 – 11.2

[1]One male patient with prolactinoma was excluded from analysis.

[2]Paired samples test

Abbreviations: SHBG, sex-hormone binding globulin; FSH, follicle stimulating hormone; LH, luteinizing hormone.

Discussion

Most GIST patients are effectively treated with imatinib. Many patients achieve long-lived responses resulting in a significant number of patients (~30 percent) using imatinib for even more than 5 year [2,15]. In general, imatinib is well tolerated and dose limiting toxicity is seen in a limited number of patients using the standard daily dose of 400 mg [16]. As imatinib is used as a long-term, chronic therapy, the recognition of side-effects is of importance for preventing discontinuation of therapy and maintaining quality of life. Most of the oncogenes that are targeted by imatinib, such as KIT, PDGFR and c-ABL, are not only expressed in malignancies but also in normal tissues, *e.g.* in the testis [17,18]. As a result, imatinib could influence normal tissue homeostasis as a reflection of its pharmacodynamic activity. Recent reports, predominantly studying patients with CML,

suggested that imatinib can affect bone metabolism and male gonadal function [4-9]. In the present study, we investigated the biochemical effects of imatinib in GIST patients in relation to bone metabolism and gonadal and thyroid function. In addition, we evaluated whether these effects could predict clinical outcome.

Changes in bone and mineral metabolism in patients with CML during treatment with imatinib have been reported. These changes were associated with a decrease in serum phosphate and calcium and the development of hyperparathyroidism, which coincide with alterations in markers of bone turnover [4-6,8,19]. It appears that imatinib stimulates bone formation causing an increase in trabecular bone volume and bone mineral density [6,8,19]. It has been suggested that imatinib stimulates osteoblast differentiation and function via inhibition of PDGFR and, on the other hand, suppression of osteoclast function as a consequence of inhibition of c-Fms signal transduction [7,8,20]. This increase in bone formation results in the sequestration of calcium and phosphate to bone. As a consequence, decreased serum calcium stimulates PTH secretion which decreases renal phosphate resorption, leading to hypophosphataemia [4,8]. The results of our study in a cohort of GIST patients are consistent with those found in CML patients. For so far, only a limited number of patients with GIST, while on imatinib therapy, have been studied regarding changes in calcium phosphate metabolism [4,21]. Our study is by far the largest study performed on this topic in GIST. We noticed in our GIST population that changes in serum calcium and PTH were already detectable after one week of imatinib treatment suggesting an immediate action on bone metabolism. Although serum phosphate and calcium decreased in the fast majority of patients, the levels of most patients remained within the normal range. Furthermore, most patients developed a mild hyperparathyroidism, which is in agreement with previous reports [5,6]. Apparently, the magnitude of these biochemical effects are rather small and critical electrolyte deficiencies, which are clinical significant are not to be expected in GIST patients treated with imatinib.

Although we observed a transient increase in FT4 shortly after start of therapy, there were no persistent changes in thyroid hormones during imatinib treatment. This is consistent with the findings reported by Dora *et al* [22]. There were also no changes observed in the sex hormones except for a mild increase in FSH levels. This is in contrast with a study in CML patients suggesting that imatinib influences serum testosterone and free testosterone, at least in a subset of male patients developing gynaecomastia [9]. These differences might be attributable to the fact that almost all our male patients were treated with 400 mg imatinib daily in contrast to the 600-800 mg daily dose that

was associated with the development of hypogonadism and gynaecomastia described by Gambacorti-Passerini *et al*. Furthermore, these patients with CML were pre-treated with interferon alpha, a drug that has been reported to have testosterone lowering effects [23,24].

Given the lack of predictive biomarkers for the response of patients with GIST to imatinib, we evaluated whether changes in hormones and electrolytes could predict clinical outcome. Predictive biomarkers can provide information on different aspects of the efficacy of a therapeutic intervention. Upfront predictive markers can be used for patient selection, while early predictive markers give information shortly after start of treatment [25]. Mutations in *KIT* and *PDGFRA* in GIST are upfront predictive markers for the response to imatinib and for progression-free survival [26,27]. Mutational status will probably guide the choice and the dose of tyrosine kinase inhibitor treatment in patients with GIST in the near future. Early predictive markers for efficacy of imatinib treatment in patients with GIST are not available yet, although monitoring changes in glucose uptake in GIST lesions with 2-[fluorine-18]fluoro-2-deoxy-D-glucose-positron emission tomography scanning is an interesting approach [28,29]. Hormonal and electrolyte changes during imatinib treatment may potentially be novel biomarkers. We noticed that patients developing hypophosphataemia after 3 months of treatment had a decreased progression-free survival. This is in contrast with the results published by Osorio *et al* who found the largest reduction in phosphate in CML patients exhibiting a major genetic response to imatinib [5]. Theoretically, imatinib might influence the microenvironment of CML stem cells within the bone marrow by altering bone metabolism. In this way, changes in bone metabolism might contribute to the treatment effect of imatinib in CML as reflected by serum phosphate levels [30]. Nonetheless, changes in serum phosphate levels shortly after initiation of imatinib treatment as an early predictive marker for imatinib treatment need to be studied further in patients with GIST and CML.

In conclusion, imatinib affects calcium and phosphate homeostasis and causes mild hyperparathyroidism in patients with GIST. Furthermore, our data suggest that imatinib does not influence thyroid or male gonadal function. The development of hypophosphataemia appears to predict a worse clinical outcome.

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Chapter 9

Summary and general discussion

Summary

Gastrointestinal stromal tumours (GISTs) are mesenchymal neoplasms that arise in the wall of the gastrointestinal tract. GISTs represent a continuum from incidentally discovered small nodules with a benign or extremely low malignant potential to large highly malignant sarcomas. Characteristically, virtually all GISTs (>95 percent) over-express the receptor tyrosine kinase and proto-oncogene KIT, which is used as an immunophenotypical marker for the diagnosis of GIST. Moreover, oncogenic mutations that affect *KIT* or, much less often, the closely related platelet-derived growth factor receptor α (*PDGFRA*), have been detected in 85-90 percent of GISTs. These mutations result in ligand-independent, constitutive activation of these receptors leading to activation of cellular signal transduction cascades involved in cellular proliferation and survival. *KIT* and *PDGFRA* mutations are mutually exclusive and play a fundamental role in the development of GIST. The tyrosine kinase inhibitor imatinib blocks the activity of KIT and PDGFRA. It has become the standard first-line treatment of patients with unresectable or metastasized GIST. Imatinib therapy results in high objective response and disease stabilisation rates but none of the patients are cured with this compound. The same holds true for second-line treatment with sunitinib, a tyrosine kinase inhibitor of KIT, PDGFR but also vascular endothelial growth factor (VEGFR) and FMS-like tyrosine kinase 3 (FLT3), of which clinical benefit, i.e. response and disease stabilisation, has been described but which is effective just as imatinib for a limited period of time. Therefore, new therapeutic strategies need to be developed.

The first aim of this thesis was to explore new molecular targets for the treatment of GIST. Secondly, potential side-effects of imatinib and markers for response to this drug were evaluated.

The first part of this thesis focuses on the role of the insulin-like growth factor (IGF) signalling system in GIST. **Chapter 2** reviews the current knowledge about the IGF system. Special attention was drawn to the function of IGF signalling in the pathogenesis of several types of sarcomas. In GIST, it has been demonstrated that tumours without *KIT* or *PDGFRA* mutations, so called wild-type GIST, express high levels of IGF receptor type 1 (IGF-1R), suggesting that this IGF receptor could be involved in the pathogenesis of this subgroup of GISTs. Currently, therapies inhibiting IGF signalling are being developed and tested. Humanized monoclonal antibodies directed against IGF-1R have entered phase 2 and 3 clinical testing and objective tumour responses were reported in several

patients with Ewing's sarcoma encouraging further clinical testing in Ewing's sarcoma and other sarcoma entities, including possibly wild-type GISTs.

Non-islet cell tumour-induced hypoglycaemia (NICTH) is a paraneoplastic syndrome occasionally observed in patients with sarcomas. NICTH is characterized by the occurrence of recurrent fasting hypoglycaemia. Tumours involved in NICTH secrete high amounts of incompletely processed precursors of IGF-II, called 'big'-IGF-II, leading to markedly increased levels of 'big'-IGF-II in the circulation. Subsequently, this induces alterations in the circulating levels of insulin, IGF-I and the insulin-like growth factor binding proteins (IGFBPs), causing an insulin-like hypoglycaemic activity of 'big'-IGF-II. **Chapter 3a** discusses the tumour types and symptoms associated with NICTH as well as the pathogenesis, diagnosis and treatment of this rare paraneoplastic phenomenon. In addition, two illustrative cases of patients suffering from NICTH caused by a solitary fibrous tumour and a haemangiopericytoma, respectively, are reported. In **chapter 3b**, an illustrative case of a patient with a GIST suffering from NICTH is described. The conclusion of this case-report is that NICTH should be considered in patients with GIST, especially in those with loss of consciousness.

In **chapter 4**, the prognostic relevance of plasma levels of 'big'-IGF-II and other IGF-related proteins in patients with a GIST before and during imatinib treatment was investigated. It appeared that before or during treatment, 3 of 24 included patients (13%) showed increased plasma levels of 'big'-IGF-II. Later on, all three patients developed hypoglycaemia. In the total group of patients, the highest 'big'-IGF-II levels were found in patients with either metastatic disease, elevated serum lactate dehydrogenase activity, or a relatively high tumour mass. Most patients had increased plasma IGFBP-2 levels and these levels were significantly higher in patients with progressive disease during treatment. Analysis of tumour tissues and cyst fluid for 'big'-IGF-II and IGFBP-2 revealed that it is likely that most of the 'big'-IGF-II detected in plasma is secreted by tumour tissue in contrast to IGFBP-2. All together, these results indicate that elevated plasma concentrations of 'big'-IGF-II are not uncommon in patients with a GIST and are prognostic for the development of hypoglycaemia. 'Big'-IGF-II could therefore be useful as marker in the surveillance of GIST. An eventual role for IGFBP-2 as a biomarker in GIST remains to be established.

The observations in the previous chapter regarding the production of 'big'-IGF-II by GIST prompted us to investigate the biological role of this secretion in *in vitro* GIST models described in **chapter 5**. IGF-II (both in its mature and immature forms) acts as an autocrine factor in several tumour types by binding to IGF-1R and/or insulin receptor

isoform A. Analysis of IGF-II secretion by two GIST cell lines revealed that both cell lines secreted high amounts of IGF-II, predominantly as 'big'-IGF-II. At the mRNA and protein level, expression of the insulin receptor isoform A but not of IGF-1R was demonstrated in the *KIT* mutant GIST cell lines and in *KIT* and *PDGFRA* mutant GIST specimen. Down-regulation of 'big'-IGF-II production or insulin receptor expression with short interference RNA reduced the survival of both cell lines by affecting AKT and mitogen-activated protein kinase signalling. Disruption of 'big'-IGF-II signalling in combination with imatinib had an additive cytotoxic effect in GIST cells. Finally, IGF-II expression was investigated in an extended cohort of 60 paraffin-embedded tumours of various malignant potential most having activating mutations in *KIT* or *PDGFRA*. IGF-II mRNA was present in 52 of 57 (91%) primary GISTs and absent in all four very-low-risk tumours. IGF-II protein was expressed in the majority of these GISTs, far most in high-risk tumours, and its expression was irrespective of *KIT* or *PDGFRA* mutation status. These findings indicate that 'big'-IGF-II secretion by GIST provides an autocrine pro-survival signal by interacting with insulin receptor isoform A that is relevant for the pathogenesis of GIST.

In **chapter 6**, the possibility of targeting the death receptor Fas with MegaFasL, a recently developed hexameric form of soluble Fas ligand (FasL), was explored. MegaFasL appeared to be an active apoptosis-inducing agent in a panel of imatinib-sensitive and imatinib-resistant cell lines which all expressed Fas. Moreover, MegaFasL potentiated the apoptotic effects of imatinib. Histological evaluations, in 45 primary GISTs, underscored the relevance of the Fas pathway: Fas was expressed in virtually all GISTs, independent from the presence of certain *KIT* mutations, whereas FasL was expressed in the majority. These results show that Fas is a potential therapeutic target in GIST.

The last part of this thesis addresses potential side-effects of imatinib. In **chapter 7**, the suggested risk of imatinib-induced cardiotoxicity was evaluated. Therefore, plasma N-terminal pro B-type natriuretic peptide (NT-proBNP) and serum cardiac troponin T (cTnT) levels were used as markers for cardiotoxicity. Both markers were determined before treatment with standard dose of imatinib and up to three months after start of treatment in 55 patients with locally advanced and/or metastatic GIST. NT-proBNP and cTnT levels remained stable in all patients except one. This patient developed symptomatic heart failure during the study period that was likely caused by pre-existing cardiac valvular disease. We concluded that, in our study population, the risk on early subclinical cardiac toxicity with the use of imatinib was not present.

Several studies in small series of patients with chronic myeloid leukaemia reported imatinib-associated changes in bone and mineral metabolism, and occurrence of hypogonadism in male patients. Hence, the influence of imatinib on various serum electrolytes and hormones was studied in GIST patients in **chapter 8**. In 36 patients with advanced GIST, serial analysis from baseline up to 6 months after start of therapy revealed that serum calcium and phosphate significantly decreased within one week and one month of treatment, respectively. Imatinib caused hyperparathyroidism and an increase in serum parathyroid hormone was detected already after one week of treatment. On the other hand, imatinib did not affect thyroid and gonadal function. Patients, who developed hypophosphataemia after 3 months of treatment, had a decreased progression-free survival. Whether changes in phosphate levels might be used as a predictive marker for response should be explored prospectively in larger patient groups.

General discussion

GIST is considered as the role model for successful application of targeted therapy in solid tumours. Apparently, most GISTs are tumours driven by oncogenic *KIT* and *PDGFRA* gain-of-function mutations, which are clearly targets for therapy. The KIT and PDGFRA tyrosine kinase inhibitor imatinib has revolutionized the treatment of GIST making this drug one of the most successful new agents introduced for the treatment of a solid tumour in the last decade. However, clinical resistance to this drug either initially or during a prolonged period of treatment has been proven to be a significant problem.

Therapeutic targets in GIST

In current clinical practice, optimal inhibition of constitutively activated KIT and PDGFRA signalling is the mainstay of systemic treatment of GIST. This is also the main focus of most of the present (pre)clinical research performed in GIST. The recognition that the type of *KIT* or *PDGFRA* mutation has an important predictive value for the response of GIST to a particular kinase inhibitor has tailored the way for further individualization of treatment [1]. For example, tumours with *KIT* exon 11 mutations respond much better to imatinib than tumours with *KIT* exon 9 mutations (or wild-type tumours) [2,3]. Sunitinib was approved for the treatment of imatinib-resistant GIST. Interestingly, it was shown that this drug is much more effective than imatinib in GISTs with *KIT* exon 9 mutations [4]. Currently, other KIT and PDGFRA targeting compounds, such as sorafenib and

nilotinib, with an inhibitory preference for certain *KIT* or *PDGFRA* mutations that are partly different from those targeted by imatinib, are also clinically tested [5,6]. It might, therefore, be possible that future therapies for GIST patients will largely be directed by the GIST genotype using tyrosine kinase inhibitors that have the most potent inhibitory activity towards a particular *KIT* or *PDGFRA* mutation. This is also of importance when resistance to imatinib or sunitinib has developed, which most often occurs through the development of secondary mutations in *KIT* or *PDGFRA* [7]. Several of the new tyrosine kinase inhibitors are able to inhibit these secondary receptor alterations, which makes these kinase inhibitors potentially suitable to target progressive imatinib and sunitinib-resistant GISTs that have acquired particular secondary mutations [8]. Of major concern with respect to resistance in GIST, however, is the occurrence of several different secondary mutations in distinct tumour lesions of individual patients whose tumours progressed during imatinib treatment [9,10]. This heterogeneity of secondary receptor mutations could hamper the application of successful universal targeted therapy with tyrosine kinase inhibitors when resistance against standard treatment has been developed. Alternative strategies could then be the use of surgery of focal progressive lesions, which policy should be further explored, or agents that influence the expression of KIT and PDGFRA such as heat shock protein 90 inhibitors or inhibitors of downstream signalling cascades such as phosphatidylinositol-3-kinase or mammalian target of rapamycin inhibitors [11-15].

The main focus of this thesis was to identify therapeutic targets other then those involved in activated KIT and PDGFRA. Although these two receptors and their cellular signal cascades are highly important in the pathogenesis of GIST, also other oncogenic signalling cascades are likely to be of importance. For the development of GIST, genetic aberrations and activation of oncogenic pathways are a requisite for further progression of prematurely transformed GIST-progenitor cells towards a malignant tumour [16]. To date, little is known about these secondary events in the development of malignant GIST. Several successive chromosomal alteration have been described such as the loss of 14q and 22q [17]. However, knowledge about the involvement of alternative oncogenes and tumour suppressors in GIST oncogenesis is limited. Identification of such secondary hits could lead to the identification of drugable targets.

The studies presented in this thesis put forward that gain of IGF-II expression is an oncogenic event involved in the pathogenesis of GIST. Therefore, targeting 'big'-IGF signalling could be useful as a new treatment option. The recognition that the IGF signalling system is involved in many cancer types has encouraged the development of

new therapeutics that can block the different members of this system [18]. One strategy interesting for GIST would be 'big'-IGF-II neutralizing antibodies. Such antibodies have been developed and show preclinical potential [19]. For those wild-type GISTs expressing IGF-1R, receptor-specific blocking antibodies or small-molecule tyrosine kinase inhibitors, which are currently in early clinical trials, could be a potential therapy [20]. However, as shown in this thesis, most GISTs, carrying *KIT* and *PDGFRA* mutations, do not express IGF-1R but show the expression of an alternative 'big'-IGF-II receptor, that is the insulin receptor isoform A. In line with other malignant tissues, GISTs have a preference for the expression of the isoform A splice variant of the insulin receptor rather than the isoform B, which is much less responsive to IGF-II than isoform A. Isoform B is commonly expressed by normal insulin-sensitive tissues in adults and has dominant effects on cell metabolism. In contrast, the isoform A has important effects on cellular survival and proliferation [21]. Knowledge about the mechanisms involved in the preferred expression of isoform A in cancer cells is limited. This is also the case for the spatial effects of the 12 amino acid difference between both isoforms of the insulin receptor that leads to differences in ligand affinities and intracellular signalling [21]. Targeting both isoforms of the insulin receptor as an anticancer strategy could result in serious metabolic toxicity. It would therefore be a challenge to develop compounds that selectively target isoform A. Until now, only small molecule tyrosine kinase inhibitors have been developed that inhibit both isoforms of the insulin receptor together with IGF-1R [22]. In preclinical xenograft models, such agents caused (transient) hyperglycaemia. Further (pre)clinical testing will reveal whether these compounds will be beneficial for the treatment of GIST.

Activation of the extrinsic apoptosis pathway through activation of members of the tumour-necrosis factor receptor family of death receptors has been shown to induce apoptosis in a wide variety of cancer cells [23]. Strategies activating the death receptor Fas, as described in this thesis, were an alternative therapeutic approach we explored in GIST cells. Inhibition of KIT only results in modest levels of apoptosis and causes predominantly cell cycle arrest in the majority of cells in a GIST [24]. In this way, a significant number of cells are able to survive and give rise to the development of resistance towards imatinib or other tyrosine kinase inhibitors. Systemic therapeutic approaches that maximize GIST cell death, such as MegaFasL, could therefore be a promising treatment. Although agonistic antibodies against Fas appeared to be highly toxic in mice, hexameric Fas ligand (MegaFasL; also called APO010) showed only modest transient toxicity in animal studies [25,26]. Currently, MegaFasL has entered phase 1

clinical testing. The results from these clinical studies will have to reveal whether this compound comes further into sight for studying its efficacy in GIST patients.

Aspects involving personalised treatment

Besides the development of new treatment strategies, also other aspects are involved in optimizing the treatment of the individual patient. These include the identification of prognostic and predictive factors, as well as the elucidation of markers of response to a given treatment, or toxicity-related issues.

The type and dosing of systemic treatment in GIST is ideally guided by predictive factors. The presence of molecular targets including KIT and PDGFRA will likely guide future therapies including those targeting alternative signalling pathways. Till now, the *KIT* and *PDGFRA* genotype of a GIST is the most important factor predicting the response to imatinib and sunitinib [8]. Identification of other biomarkers could result in further fine-tuning of KIT/PDGFRA targeting agents. In daily practice, CT scanning is used to evaluate tumour response to imatinib or other tyrosine kinases and has been shown to be of value to discriminate between progressive and nonprogressive disease [27]. 2-[fluorine-18]fluoro-2-deoxy-D-glucose-positron emission tomography scanning has been shown to be an additional valuable tool for response evaluation in GIST, but well powered prospective studies regarding its exact place in daily clinical practice of GIST patients are lacking [28]. The identification of tumour-derived proteins, measured in serum or plasma of patients and serving as a reliable tumour marker, could simplify and be cost effective for the follow-up of GIST patients. No such markers are nowadays available. Candidates for further research on this topic could be the soluble KIT [29,30] and DOG-1 (discovered on GIST-1) [31] proteins that are extensively expressed in GIST, but also 'big'-IGF-II.

The occurrence of toxicity by tyrosine kinase inhibitors is also important for the tailoring of treatment of GIST. Although imatinib, and similarly sunitinib, as targeted cancer therapy is aimed at inhibition of constitutively activated KIT and PDGFRA in GIST, these receptor tyrosine kinases are also expressed in normal tissues playing a role in organ physiology. Therefore, these therapies could influence normal tissue homeostasis resulting in toxicity. In the last few years, several unexpected toxicities have been reported that were associated with imatinib, including heart failure, testicular insufficiency and hypophosphataemia [32-34]. Although the studies presented in this thesis showed that these toxicities are of limited significance during imatinib treatment, the recognition of side effects and the identification of those patients at risk of toxic

effects have important implications for the prolonged application of this drug. The development of a risk calculator for predicting imatinib-induced toxicity is an example of a useful initiative to guide the dosage of this compound [35]. The same principles hold for sunitinib that, in general, has a toxicity profile associated with more adverse events than imatinib, including hypertension, hypothyroidism and cardiotoxicity. This is likely the result of the broad range of tyrosine kinases, such as VEGFR and FLT3 in addition to KIT and PDGFR, that are inhibited by this compound [36]. Close monitoring of side effects of imatinib, sunitinib and new agents in the treatment of GIST will optimize the treatment success of these agents.

Conclusions

In conclusion, this thesis provides new insights in the pathogenesis of GIST. As for many other sarcomas, the IGF signalling system is involved in the development of GIST. We identified 'big'-IGF-II as an autocrine factor that is relevant for the maintenance of this malignancy by signalling through the insulin receptor isoform A. Furthermore, 'big'-IGF-II could be useful for the surveillance of GIST, at least for those patients at risk for developing NICTH. In addition, we implied the death receptor Fas as a promising new treatment target. Finally, issues regarding imatinib-associated toxicity and their value as predictive markers were addressed. Hopefully, these studies form the basis for further research involving improvement and tailoring of GIST treatment.

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Chapter 10

Nederlandse samenvatting

Samenvatting

Gastro-intestinale stromaceltumoren (GIST) vormen een zeldzame groep van mesenchymale tumoren uitgaande van het maag-darmkanaal. GISTen kunnen voorkomen als kleine, incidenteel ontdekte tumortjes met een nagenoeg benigne karakter tot grote, sterk maligne tumoren. Ze worden gekenmerkt door de expressie van de receptortyrosinekinase KIT, die als belangrijke pathologische marker fungeert bij de diagnostiek naar GIST. In circa 85% van de gevallen worden oncogene mutaties aangetroffen in het *KIT* gen. Daarnaast wordt in een klein deel van de tumoren mutaties in het platelet-derived growth factor receptor α (*PDGFRA*) gen gevonden. Deze mutaties zorgen ervoor dat het KIT of PDGFRA eiwit zichzelf activeert onafhankelijk van ligandbinding met als gevolg dat er continu intracellulaire signaalcascades worden geactiveerd die betrokken zijn bij de proliferatie en overleving van tumorcellen. *KIT* en *PDGFRA* mutaties spelen een fundamentele rol bij de ontwikkeling van GIST. De tyrosinekinaseremmer imatinib remt de activiteit van beide receptoren en wordt gebruikt als standaard eerstelijnsbehandeling bij patiënten met irresectabele of gemetastaseerde GIST. Bij het merendeel van de patiënten die met imatinib behandeld worden, wordt objectieve tumorrespons dan wel ziektestabilisatie waargenomen. Deze behandeling leidt echter niet tot genezing van GIST. Hetzelfde geldt voor sunitinib, een tyrosinekinaseremmer die eveneens de activatie van KIT en PDGFRA blokkeert, maar tevens andere receptoren zoals vascular endothelial growth factor receptor (VEGFR) en FMS-like tyrosinekinase 3 (FLT3). Ook van dit middel zijn klinische effecten in de vorm van tumorrespons en ziektestabilisatie beschreven, maar net als bij imatinib is deze werking niet blijvend. Om deze reden bestaat er de noodzaak tot de ontwikkeling van nieuwe therapeutische strategieën.

Het eerste doel van het onderzoek beschreven in dit proefschrift was om nieuwe moleculaire aangrijpingspunten voor de behandeling van GIST te identificeren. In de tweede plaats werden mogelijke bijwerkingen van imatinib en markers die de tumorrespons op dit middel zouden kunnen voorspellen nader onderzocht.

Het eerste deel van dit proefschrift richt zich op de rol van het insulineachtige groeifactor (IGF) systeem bij GIST. **Hoofdstuk 2** zet de huidige kennis over het IGF systeem uiteen. Hierbij wordt de nadruk gelegd op de rol van IGF signalering in de pathogenese van diverse typen sarcomen. Bij GIST is aangetoond dat tumoren zonder *KIT* of *PDGFRA* mutaties, de zogenaamde 'wild-type' GISTen, de IGF receptor type 1 (IGF-1R) in hoge mate tot expressie brengen. Dit suggereert dat deze IGF receptor betrokken is bij de pathogenese van deze subgroep van tumoren. Recent zijn verscheidene middelen

onderzocht en getest, die gericht zijn op de remming van IGF signalering. Humane monoclonale antilichamen gericht tegen IGF-1R bevinden zich momenteel voor diverse tumortypen in klinisch fase 2 en 3 onderzoek. Bij verscheidene patiënten met een Ewing sarcoom zijn in deze studies objectieve tumorresponsen gezien, hetgeen een belangrijke stimulus vormt voor verder klinisch onderzoek naar het effect van deze antilichamen bij de behandeling van het Ewing sarcoom en andere typen sarcomen, zoals bijvoorbeeld 'wild-type' GISTen.

Non-islet cell tumour-induced hypoglycaemia (NICTH) is een paraneoplastisch syndroom dat incidenteel wordt gediagnosticeerd bij patiënten met een sarcoom. NICTH wordt gekenmerkt door het bij herhaling optreden van hypoglykemieën bij vasten. Tumoren waarbij NICTH optreedt, scheiden grote hoeveelheden onrijp IGF-II uit, genaamd 'big'-IGF-II. Dit leidt tot een aanzienlijk verhoogde spiegel 'big'-IGF-II in de circulatie. Hierdoor veranderen de concentraties van circulerend insuline, IGF-I en IGF-bindende proteïnen (IGFBPs), waardoor een hypoglykemische werking van 'big'-IGF-II kan optreden, vergelijkbaar met het effect van insuline. In **hoofdstuk 3a** worden de verschillende tumortypen en symptomen die geassocieerd zijn met NICTH, beschreven, alsmede de pathogenese, diagnose en behandeling van dit zeldzame paraneoplastische fenomeen. Tevens worden twee illustratieve casus gepresenteerd van patiënten met NICTH, veroorzaakt door respectievelijk een solitaire fibreuze tumor en een hemangiopericytoom. In **hoofdstuk 3b** wordt de casus van een patiënt met een GIST en NICTH beschreven. De conclusie van deze casuïstiek is dat NICTH overwogen dient te worden bij patiënten met GIST, zeker bij het optreden van bewustzijnsdalingen.

In **hoofdstuk 4** wordt de studie naar de prognostische relevantie van de bepaling van 'big'-IGF-II en andere IGF-gerelateerde eiwitten in het plasma van patiënten met een GIST voor en tijdens behandeling met imatinib weergegeven. Bij drie van de 24 geïncludeerde patiënten (13%) bleek de plasmaspiegel van 'big'-IGF-II vóór of tijdens behandeling verhoogd. Later ontwikkelden deze drie patiënten hypoglykemieën. In de totale groep patiënten werden de hoogste 'big'-IGF-II spiegels gemeten bij patiënten met gemetastaseerde ziekte, een verhoogd LDH of een relatief grote tumormassa. Verder hadden de meeste patiënten verhoogde IGFBP-2 plasmaspiegels en waren die significant hoger bij patiënten met progressieve ziekte tijdens behandeling met imatinib. Analyse van 'big'-IGF-II en IGFBP-2 in GIST tumorweefsel en cystevloeistof liet zien dat het merendeel van de in plasma gemeten 'big'-IGF-II waarschijnlijk wordt uitgescheiden door de tumor, in tegenstelling tot IGFBP-2. Deze resultaten geven aan dat verhoogde plasmaspiegels van 'big'-IGF-II niet zelden voorkomen bij patiënten met een GIST en

een prognostische factor zijn voor het ontwikkelen van hypoglykemieën. De rol voor IGFBP-2 als eventuele biomarker in GIST moet nog nader onderzocht worden.

Na de observatie dat GIST 'big'-IGF-II kan produceren, hebben we verder onderzocht wat de biologische rol is van 'big'-IGF-II bij *in vitro* GIST modellen. Dit onderzoek wordt in **hoofdstuk 5** beschreven. Bij meerdere tumortypen is aangetoond dat IGF-II (zowel in rijpe als onrijpe vorm) als autocriene factor fungeert door te binden aan IGF-1R en/of insuline receptor isovorm A. De door ons uitgevoerde bepaling van IGF-II secretie door twee GIST cellijnen met *KIT* mutaties liet zien dat beide cellijnen grote hoeveelheden IGF-II uitscheidden, voornamelijk in de vorm van 'big'-IGF-II. Bij deze GIST cellijnen en bij een cohort tumoren met eveneens *KIT* of *PDGFRA* mutaties werd zowel op mRNA als op eiwitniveau expressie van de insuline receptor isovorm A aangetoond, in tegenstelling tot IGF-1R. Het uitschakelen van de expressie van 'big'-IGF-II of de insuline receptor met korte interferentie RNA (siRNA) verkorte de overleving van beide cellijnen middels beïnvloeding van AKT en MAP kinase signalering. Het onderbreken van 'big'-IGF-II signalering in combinatie met imatinib had een additief cytotoxisch effect op GIST cellen. Tot slot werd de ('big'-)IGF-II expressie onderzocht in een uitgebreid cohort van 60 primaire tumoren, ingebed in paraffine, waarvan de maligniteitsgraad varieerde en waarvan het overgrote merendeel *KIT* of *PDGFRA* mutaties bevatte. IGF-II mRNA was bij 52 van de 57 (91%) evalueerbare GISTen aanwezig en afwezig bij alle vier zeer-laag-risico tumoren. IGF-II eiwit werd door de meerderheid van deze GISTen tot expressie gebracht, met name bij hoog-risico tumoren, en deze expressie was onafhankelijk van de aanwezigheid van *KIT* of *PDGFRA* mutaties. Onze bevindingen in cellijnen en tumoren wijzen erop dat de secretie van 'big'-IGF-II een autocrien signaal kan geven via interactie met de insuline receptor isovorm A dat de overleving van GIST cellen stimuleert. 'Big'-IGF-II secretie lijkt daarom relevant te zijn in de pathogenese van GIST.

In **hoofdstuk 6** wordt onderzocht of de death receptor Fas een mogelijk therapeutisch aangrijpingspunt is bij GIST. Hierbij werd de effectiviteit van MegaFasL onderzocht. Dit is een hexamere vorm van Fas ligand (FasL), dat de eigenschap heeft de "death" receptor Fas te activeren. MegaFasL bleek zowel in imatinib gevoelige als in imatinib resistente GIST cellijnen in hoge mate geprogrammeerde celdood (apoptose) te induceren. Daarnaast versterkte MegaFasL het apoptotische effect van imatinib in GIST cellen. Histologisch onderzoek van preparaten afkomstig van 45 primaire GISTen bevestigde de relevantie van de Fas route zoals die was gevonden in de GIST cellijnen, namelijk dat in nagenoeg alle tumoren Fas expressie werd gevonden en in het merendeel FasL. De expressie van Fas en FasL in deze GISTen was onafhankelijk van de aanwezigheid van bepaalde *KIT*

mutaties. Deze resultaten suggereren dat Fas een potentieel aangrijpingspunt is voor therapie bij GIST.

Het laatste deel van dit proefschrift richt zich op mogelijke bijwerkingen van imatinib. In **hoofdstuk 7** wordt de gesuggereerde toxiciteit van imatinib op de hartspier nader bestudeerd. Om dit verder te onderzoeken zijn de plasma spiegels van N-terminal pro B-type natriuretic peptide (NT-proBNP) en serum cardiaal troponine T (cTnT) gebruikt als markers voor cardiotoxiciteit. Beide markers werden bepaald in 55 patiënten met een lokaal uitgebreide dan wel gemetastaseerde GIST voorafgaand aan de behandeling met imatinib en vervolgens gedurende de eerste drie maanden van behandeling met dit middel, dat gegeven werd in een standaard dosering. Afgezien van één patiënt, bleven de NT-proBNP en cTnT concentraties stabiel in de patiënten tijdens de studieperiode. De patiënt waarbij een stijging in de markers werd waargenomen, ontwikkelde symptomatisch hartfalen tijdens de studieperiode, dat meest waarschijnlijk veroorzaakt werd door pre-existent hartkleplijden. Kortom, in deze studiepopulatie werd geen vroegtijdige cardiale toxiciteit door imatinib waargenomen.

Verschillende studies die betrekking hadden op met name kleine groepen patiënten met chronische myeloïde leukemie, waarbij imatinib ook de eerstekeusbehandeling is, hebben gesuggereerd dat dit middel veranderingen veroorzaakt in bot- en mineraal-metabolisme, en tevens hypogonadisme teweegbrengt in mannelijke patiënten. Om deze reden werd in **hoofdstuk 8** de invloed van imatinib op verschillende serum elektrolyten en hormonen onderzocht in 36 patiënten met GIST, die in aanmerking kwamen voor behandeling met dit medicament. Seriële serumbepalingen bij deze patiëntengroep gedurende 6 maanden behandelen lieten zien dat de serum calcium en fosfaat spiegels respectievelijk na één week en na één maand significant daalden. Daarnaast veroorzaakte imatinib (milde) hyperparathyreoïdie, wat na reeds één week behandelen kon worden waargenomen. Er werden geen veranderingen in schildklier- en gonadale functies gevonden. Patiënten die na 3 maanden behandeling met imatinib een hypofosfatemie ontwikkelden, hadden een slechtere progressievrije overleving dan zij die dat niet ontwikkelden. Nader (prospectief) onderzoek zal moeten uitwijzen of een verlaging in serum fosfaat concentraties kan worden gebruikt als voorspellende marker voor verminderde overleving.

Algemene discussie

GIST wordt beschouwd als hét rolmodel voor de succesvolle applicatie van doelgerichte therapie (targeted therapy) bij solide tumoren. De meeste GISTen worden gedreven door oncogene mutaties in de *KIT* en *PDGFRA* genen. De geactiveerde eiwitten die hieruit voortkomen, vormen duidelijke aangrijpingspunten voor therapie. De tyrosinekinaseremmer imatinib heeft een revolutie teweeg gebracht bij de behandeling van GIST en is daarmee een van de meest succesvolle nieuwe medicijnen die in het afgelopen decennium zijn geïntroduceerd voor de behandeling van een solide tumor. Helaas blijkt er bij een (beperkte) groep patiënten initiële resistentie tegen dit middel te bestaan, terwijl het overgrote deel van de GIST patiënten vroeg of laat geconfronteerd wordt met ongevoeligheid voor imatinib.

Therapeutische aangrijpingspunten in GIST

Het optimaal remmen van continu geactiveerde KIT en PDGFRA signaleringsroutes is in de huidige klinische praktijk het belangrijkste doel bij de systemische behandeling van GIST. Dit is ook het voornaamste doel van het meeste (pre)klinische onderzoek dat momenteel verricht wordt bij GIST. De recente waarneming dat het type *KIT* of *PDGFRA* genmutatie een belangrijke voorspellende waarde heeft voor de respons van een GIST op een bepaalde tyrosinekinaseremmer, maakt het mogelijk om de behandeling verder te individualiseren [1]. Zo laten tumoren met *KIT* exon 11 mutaties een veel betere respons op imatinib zien dan tumoren met *KIT* exon 9 mutaties of tumoren met 'wild-type' *KIT* [2,3]. Sunitinib is geregistreerd voor de behandeling van imatinib resistente GIST. Dit middel is bij GIST met *KIT* exon 9 mutaties effectiever gebleken dan imatinib [4]. Momenteel worden er verschillende klinische studies verricht met andere KIT en PDGFRA remmende middelen, zoals sorafenib en nilotinib. Deze medicamenten hebben een voorkeur voor het inhiberen van bepaalde KIT of PDGFRA mutanten die deels verschillen van de door imatinib geremde mutanten [5,6]. Het zou daarom goed mogelijk kunnen zijn dat toekomstige therapieën bij GIST patiënten in belangrijke mate gestuurd gaan worden door het genotype van de tumor, waarbij er gekozen wordt voor de tyrosinekinaseremmer met de meest potente activiteit richting een bepaalde mutatie. Dit lijkt ook van belang te zijn bij het ontstaan van resistentie tegen imatinib of sunitinib, wat meestal het gevolg is van het ontwikkelen van secundaire mutaties in *KIT* of *PDGFRA* [7]. Enkele van de nieuwe tyrosinekinaseremmers zijn, ondanks de aanwezigheid van bepaalde secundaire mutaties, in staat om de functionaliteit van KIT en PDGFRA

te remmen. Hierdoor zijn ze mogelijk geschikt voor het behandelen van tumoren die door secundaire mutaties resistent zijn geworden voor imatinib en sunitinib [8]. Bron van zorg is echter de mogelijkheid dat gedurende langdurige behandeling met imatinib verschillende secundaire mutaties in diverse tumorlesies in een individuele patiënt kunnen optreden [9,10]. Deze heterogeniteit wat betreft secundaire mutaties kunnen het succes van doelgerichte therapie belemmeren zodra er resistentie is ontwikkeld tegen de standaard tyrosinekinaseremmers. Alternatieve behandelingsstrategieën kunnen dan zijn het toepassen van chirurgie op focaal progressieve lesies, of het gebruik van middelen die de expressie van KIT en PDGFRA eiwit op de celmembraan beïnvloeden, zoals heat shock protein 90 remmers, of remmers van intracellulaire eiwitten in de signaaltransductiecascade van KIT en PDGFRA, zoals phosphatidylinositol-3-kinase of mammalian target of rapamycin remmers [11-15].

De kern van het onderzoek dat in dit proefschrift is beschreven, is de zoektocht naar nieuwe aangrijpingspunten voor therapie, anders dan geactiveerd KIT en PDGFRA. Want hoewel deze twee receptoren en hun signaleringseiwitten zeer belangrijk zijn voor de pathogenese van GIST, is het zeer aannemelijk dat ook andere oncogene signaalroutes van belang zijn. Het ontstaan van multipele genetische afwijkingen en activatie van oncogene signaalroutes ligt waarschijnlijk aan de basis van de ontwikkeling van prematuur getransformeerde GIST voorlopercellen tot een maligne tumor [16]. Tot op heden is er echter weinig bekend over deze secundaire gebeurtenissen bij de ontwikkeling van een GIST. Zo zijn er verscheidene opeenvolgende chromosomale veranderingen beschreven zoals het verlies van 14q en 22q [17]. Over de betrokkenheid van alternatieve oncogenen en tumorsuppressors bij de oncogenese van GIST is echter weinig bekend. De identificatie van dergelijke eiwitten zou kunnen leiden tot de ontwikkeling van nieuwe medicijnen gericht tegen deze eiwitten of tegen de route waar die eiwitten onderdeel van zijn.

Verschillende studies in dit proefschrift laten zien dat het verkrijgen van IGF-II expressie een rol speelt bij de ontwikkeling van GIST. Het aangrijpen op 'big'-IGF-II signalering zou daarom gebruikt kunnen worden als nieuwe behandelingsmogelijkheid. Het feit dat het IGF systeem is betrokken bij vele kankersoorten, heeft geleid tot de ontwikkeling van nieuwe middelen die de verschillende leden van dit systeem kunnen blokkeren [18]. Van deze middelen zijn de recent ontwikkelde neutraliserende antilichamen tegen 'big'-IGF-II erg interessant als potentiële behandeling van GIST [19]. Voor 'wild-type' GISTen die IGF-1R tot expressie brengen, zouden receptorspecifieke antagonistische (blokkerende) antilichamen of klein-moleculaire tyrosinekinaseremmers in aanmerking

komen [20]. Zoals echter naar voren komt in dit proefschrift, brengen de meeste GISTen, namelijk de tumoren met *KIT* of *PDGFRA* mutaties, geen IGF-1R tot expressie maar een alternatieve receptor, de insuline receptor isovorm A. In overeenstemming met andere maligniteiten hebben GISTen de voorkeur voor de expressie van de insuline receptor isovorm A variant boven de isovorm B variant. De insuline receptor isovorm B heeft veel minder affiniteit voor IGF-II dan isovorm A. Daarnaast wordt de isovorm B normaalgesproken tot expressie gebracht door normale, niet-foetale weefsels waar het een dominante rol heeft op gebied van het stimuleren van het celmetabolisme. De isovorm A daarentegen speelt een belangrijke rol bij het stimuleren van de proliferatie en overleving van cellen [21]. De kennis over de mechanismen, die betrokken zijn bij de geprefereerde expressie van isovorm A in kankercellen, is beperkt. Dit geldt ook voor het ruimtelijke effect veroorzaakt door een verschil van 12 aminozuren tussen beide isovormen, dat klaarblijkelijk het verschil in affiniteit voor bepaalde liganden en zo de activering van intracellulaire signaaltransductieroutes beïnvloedt [21]. Het remmen van beide isovormen van de insuline receptor als antikankerbehandeling zou kunnen leiden tot ernstige metabole toxiciteit. Om deze reden zou het een uitdaging zijn om middelen te ontwikkelen die selectief isovorm A inhiberen. Tot op heden zijn er alleen klein-moleculaire tyrosinekinaseremmers ontwikkeld die zowel beide isovormen van de insuline receptor als IGF-1R remmen [22]. In preklinische diermodellen veroorzaakten dergelijke middelen (transiënte) hyperglykemie. Verder (pre)klinisch onderzoek zal moeten uitwijzen of deze middelen geschikt zijn voor de behandeling van GIST.

Door het activeren van de extrinsieke apoptoseroute via activatie van 'death' receptoren, die behoren tot de tumornecrosefactorreceptorfamilie, kan apoptose worden geïnduceerd in een grote verscheidenheid aan kankercellen [23]. In dit proefschrift is beschreven hoe activatie van de death receptor Fas *in vitro* een beloftevolle benadering is. Het remmen van KIT door imatinib induceert slechts in beperkte mate apoptose en veroorzaakt vooral celcyclusarrest in de meerderheid van de GIST cellen [24]. Hierdoor is een belangrijk deel van de cellen in staat om te overleven en daarmee bij te dragen aan de ontwikkeling van resistentie tegen imatinib en andere tyrosinekinaseremmers. Therapeutische benaderingen die celdood veroorzaken, zoals het gebruik van MegaFasL, zouden daarom veelbelovend kunnen zijn bij de behandeling van GIST. Ondanks het feit dat agonistische antilichamen tegen Fas uiterst toxisch zijn voor muizen, blijkt de toxiciteit van de hexamere vorm van Fas ligand (MegaFasL, ook wel APO010 genoemd) in muizen erg mee te vallen [25,26]. Momenteel wordt MegaFasL onderzocht in een

klinische fase 1 studie. De resultaten van dit onderzoek zullen bepalend zijn voor het eventueel verder bestuderen van de effecten van dit middel bij patiënten met GIST.

Aspecten met betrekking tot gepersonaliseerde behandeling

Naast de ontwikkeling van nieuwe medicamenteuze behandelingen zijn er ook andere aspecten die relevant zijn voor het optimaliseren van de behandeling van de individuele patiënt. Het gaat hierbij om het identificeren van predictieve en prognostische factoren, het vaststellen van responsmarkers, of toxiciteit gerelateerde kwesties.

De keuze voor en de dosering van een bepaald type medicijn bij GIST wordt idealiter gestuurd door predictieve factoren. De aanwezigheid van bepaalde moleculaire aangrijpingspunten kan bijvoorbeeld bepalend zijn voor het starten van toekomstige therapieën. Tot op heden is het *KIT* en *PDGFRA* genotype de meest belangrijke voorspeller wat betreft de (progressievrije en totale) overleving en tumorrespons bij het gebruik van imatinib en sunitinib [8]. De identificatie van andere biomarkers zou voor een verdere verfijning van de behandeling met KIT/PDGFRA remmers kunnen zorgen. In de hedendaagse klinische praktijk worden CT-scans gebruikt voor de evaluatie van de respons van een GIST op imatinib of een andere tyrosinekinaseremmer [27]. Met behulp van 2-[fluorine-18]fluoro-2-deoxy-D-glucose–positron emission tomography (FDG-PET) zou additionele informatie verkregen kunnen worden wat betreft tumorrespons. Het ontbreekt momenteel echter aan goede prospectieve studies die kunnen aangeven wat exact de plaats is van FDG-PET bij de behandeling van GIST [28]. Onderzoek naar eiwitten afkomstig van de tumor, die gemeten kunnen worden in serum of plasma en die zouden kunnen dienen als betrouwbare tumormarkers, zou een bijdrage kunnen leveren aan het vergemakkelijken van de follow-up van patiënten met GIST. Momenteel zijn dergelijke biomarkers niet beschikbaar. Kandidaat markers voor verder onderzoek op dit gebied zijn oplosbare KIT [29,30] en DOG-1 (discovered on GIST-1) [31], maar ook 'big'-IGF-II.

Bij de individualisering van de behandeling van GIST speelt het optreden van toxiciteit als gevolg van de behandeling met een tyrosinekinaseremmer ook een belangrijke rol. Ondanks het feit dat imatinib, net als sunitinib, zogenaamde doelgerichte antikankertherapie is, zijn deze middelen wel gericht tegen receptoren die ook in normale weefsels voorkomen. Daardoor kunnen deze middelen tevens de homeostase van normale weefsels beïnvloeden, met als gevolg het optreden van toxiciteit. In de afgelopen jaren zijn er meerdere vaak onverwachte bijwerkingen van imatinib gemeld, waaronder hartfalen, testiculaire insufficiëntie en hypofosfatemie [32-34]. Hoewel onderzoek in dit

proefschrift laat zien dat deze bijwerkingen slechts van beperkte betekenis lijken te zijn, is de herkenning van bijwerkingen en het identificeren van patiënten met een verhoogd risico hierop van belang bij langdurige behandeling met imatinib. De ontwikkeling van een risicocalculator die het optreden van imatinib geïnduceerde toxiciteit voorspelt, is hierbij een waardevol initiatief die richting kan geven bij de keuze voor een bepaalde dosering van dit middel [35]. Dezelfde principes gelden voor sunitinib, een middel dat over het algemeen meer toxiciteit laat zien dan imatinib, zoals hypertensie, hypothyreoïdie en cardiotoxiciteit. Dit is meest waarschijnlijk het gevolg van het feit dat sunitinib naast KIT en PDGFR multipele andere tyrosinekinases remt, waaronder VEGFR en FLT3 [36]. Kennis van en vroegtijdig inspelen op de toxiciteit van imatinib, sunitinib en andere nieuwe middelen bij de behandeling van GIST zal het therapeutische succes van deze middelen ten goede komen.

Conclusie

In dit proefschrift worden nieuwe inzichten in de pathogenese van GIST gepresenteerd. Gelijk als bij veel andere typen sarcomen is het IGF systeem ook betrokken bij de ontwikkeling van GIST. We hebben hierbij laten zien dat 'big'-IGF-II fungeert als een autocriene factor die via een interactie met de insuline receptor isovorm A de overleving van GIST cellen kan bevorderen. Daarnaast zou 'big'-IGF-II gebruikt kunnen worden als marker voor het vervolgen van met name die patiënten die het risico lopen op het ontwikkelen van NICTH. Verder blijkt uit dit proefschrift dat de death receptor Fas bruikbaar zou kunnen zijn als nieuw aangrijpingspunt voor therapie bij GIST. Tenslotte is ingegaan op factoren die samenhangen met imatinib gerelateerde toxiciteit en de predictieve waarde van veranderingen in elektrolyten en hormonen. Deze studies dienen als uitgangspunt voor verdere verbetering en individualisering van de behandeling van GIST.

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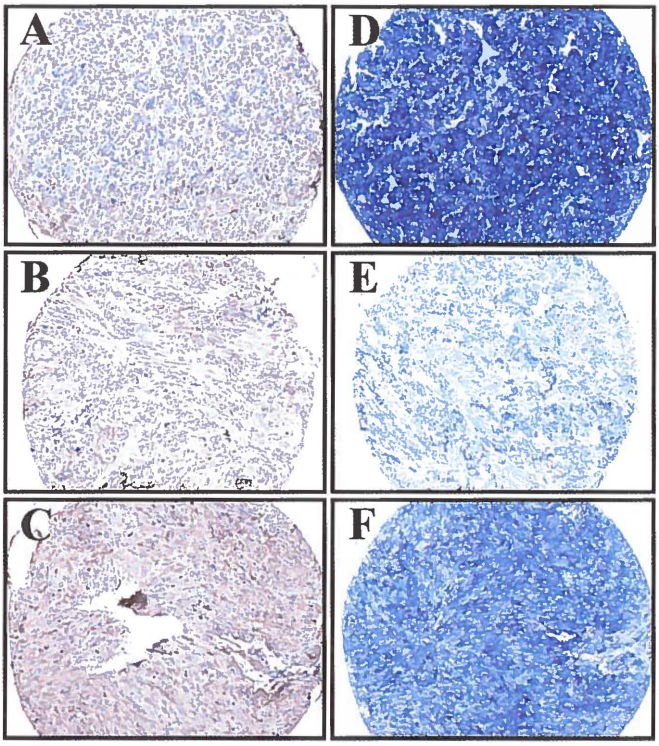
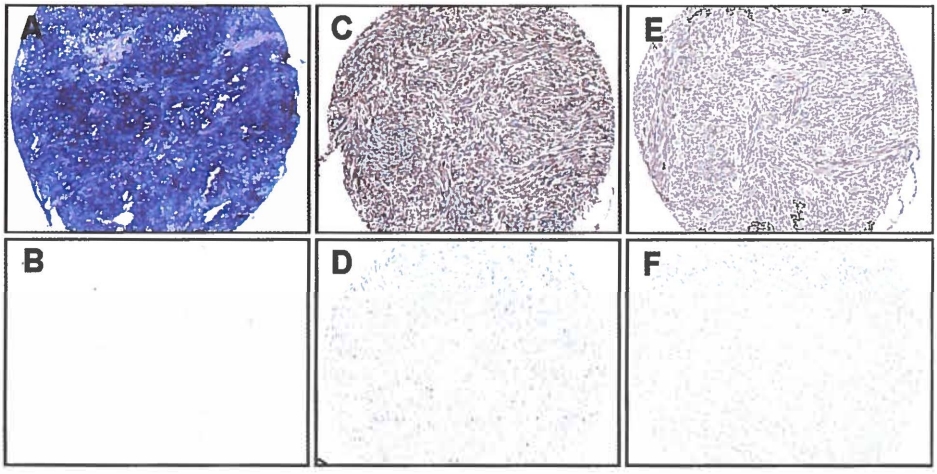
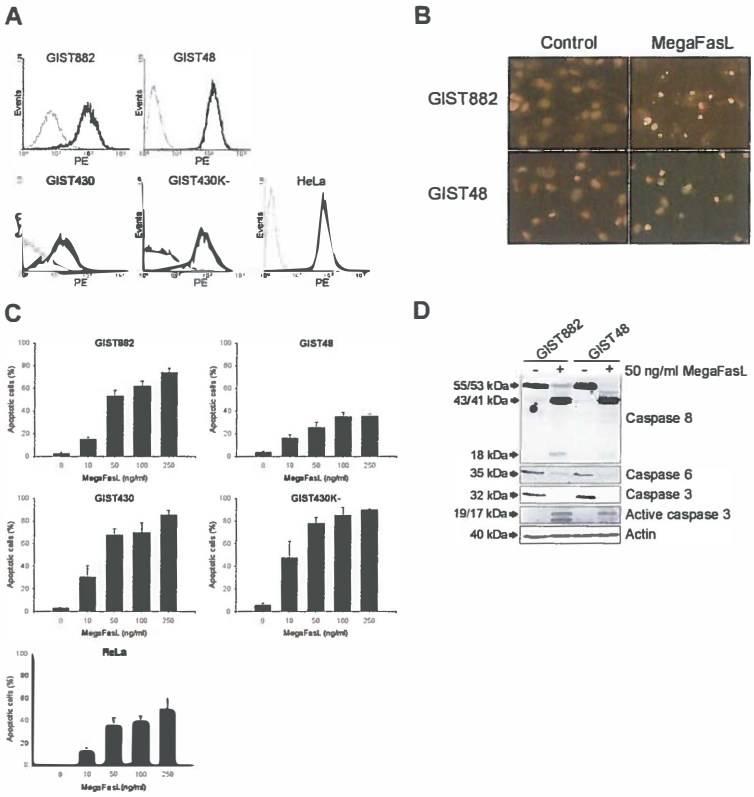
Colour figures



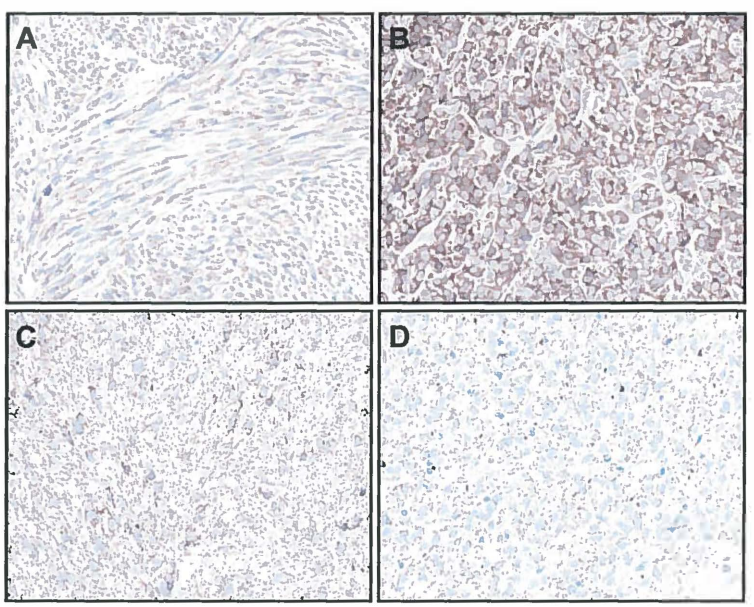
Chapter 4 (figure 1) see page 88.



Chapter 5 (figure 6) see page 112.



Chapter 6 (figure 1) see page 129.



Chapter 6 (figure 3) see page 132.