Binding characteristics of pro-insulin-like growth factor-II from cancer patients: binary and ternary complex formation with IGF binding proteins-1 to -6

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Abstract

Many tumours secrete IGF-II in incompletely processed precursor forms. The ability of these pro-IGF-II forms to complex with the six IGF binding proteins (IGFBPs) is poorly understood. In this study, pro-IGF-II has been extracted from the serum and tumour tissue of two patients with non-islet cell tumour hypoglycaemia. These samples were used to study binary complex formation with IGFBPs-1 to -6 using competitive IGF-II binding assays and ternary complex formation with IGFBP-3 and IGFBP-5.

In each case, IGFBPs-1 to -6 showed little difference in their ability to form binary complexes with recombinant IGF-II or tumour-derived pro-IGF-II forms, when the preparations were standardised according to IGF-II immunoreactivity. As previously described, ternary complex formation by acid-labile subunit (ALS) with IGFBP-3 and pro-IGF-II was greatly decreased compared with complex formation with mature IGF-II. In contrast, ALS bound similarly to IGFBP-5 in the presence of pro-IGF-II and mature IGF-II.

These studies suggest that pro-IGF-II preferentially forms binary complexes with IGFBPs, and ternary complexes with IGFBP-5, rather than ternary complexes with IGFBP-3 as seen predominantly in normal serum. This may increase the tissue availability of serum pro-IGF-II, allowing its insulin-like potential to be realised.

Introduction

Mature insulin-like growth factor (IGF)-II is a 7·5 kDa peptide secreted by cells into the blood after enzymatic cleavage from a larger precursor pro-IGF-II. It is produced throughout the body tissues as shown by mRNA distribution and immunohistochemistry (Sussenbach et al. 1992). Pro-IGF-II consists of the mature growth factor of 67 residues with a carboxy-terminal extension of 88 amino acids known as the E-peptide. Various forms of this peptide have been reported as a result of incomplete cleavage of the carboxy-terminal end. Zumstein et al. (1985) found a 10 kDa molecule from human serum that contained a 21 residue E-domain extension. Gowan et al. (1987) purified a 15 kDa IGF-II molecule from human serum. An increased proportion of these higher molecular weight IGF-II forms has been reported in plasma and tissue extracts from patients with non-islet cell tumour hypoglycaemia (NICTH), a syndrome resulting from IGF-II overproduction by mesenchymal tumours of non-pancreatic origin (Daughaday et al. 1988, Daughaday & Kapadia 1989). More recent reports have shown that IGF-II overexpression and pro-IGF-II forms are more widespread than first thought in various cancers (Horiuchi et al. 1994, Singer et al. 1995, Hodzic et al. 1997, Van der Ven et al. 1997).

IGF-II circulates in the blood and competes with IGF-I for high affinity interactions with six IGF-binding proteins (IGFBPs-1 to -6). The IGFBPs have core molecular masses in the range of 23 000–31 000 daltons, although this may be increased by glycosylation, particularly in the case of IGFBP-3, with glycoforms of 40–45 kDa. In addition, the binary complexes which IGF-I and IGF-II form with IGFBP-3 and IGFBP-5, may also form a ternary complex with the glycoprotein called acid-labile subunit (ALS) (Baxter et al. 1989, Twigg & Baxter 1998). These complexes are involved in maintaining the homeostasis of glucose metabolism and availability of IGFs to perform other important cellular functions in the body (Baxter 1991, Jones & Clemmons 1995). Little is known about how the E-domain of pro-IGF-II affects its affinity for IGFBPs and the ability to form ternary complexes. IGFBPs that do not form ternary complexes with ALS have the potential to carry IGF-II from the circulation to
target tissues. Levels of IGFBP-2 and IGFBP-6 are markedly elevated in the serum of NICTH patients (Baxter 1996). In the serum of these patients, the occurrence of the 150 kDa ternary complex is reported to be reduced (Daughaday & Kapadia 1989) and 40–50 kDa IGF–IGFBP complexes are increased. It has been suggested that forms of pro-IGF-II disrupt the affinity of the ternary IGFBP-3 complexes, leading to hypoglycaemia (Baxter et al. 1995).

Knowledge of the ability of pro-IGF-II to form binary and ternary complexes in serum of these patients is important in understanding the significance of elevated secretion of pro-IGF-II by certain cancers. This report describes the characterisation of the forms of pro-IGF-II in NICTH tumour and serum, and the use of these preparations of pro-IGF-II to test their binding characteristics to the six IGFBPs and ALS.

Materials and Methods

Peptides

Recombinant human (rh) IGF-II was a gift from Kabi Peptide Hormones, Stockholm, Sweden. IGFBP-1 was purified from human amniotic fluid (Baxter et al. 1987); rhIGFBP-2 was a gift from Sandoz (Basel, Switzerland); IGFBP-3 was purified from human plasma (Martin & Baxter 1986); rhIGFBP-4 was purchased from Austral Biologicals, San Ramon, CA, USA. rhIGFBP-5 was expressed in our laboratory in 911 human embryonic retina cells by Dr S Firth, using an adenoviral vector, and purified essentially as described for IGFBP-3 (Firth et al. 1999). Its amino-terminus was confirmed by sequencing as that of authentic hIGFBP-5 and its concentration was determined by quantitative amino acid analysis (unpublished data). IGFBP-6 was purified from human fibroblast-conditioned medium (Martin et al. 1990). ALS was purified from human serum as previously described (Baxter et al. 1989).

IGF-II was iodinated with chloramine T and purified as previously reported (Baxter 1990). IGF-II tracer was cross-linked to IGFBP-5 and purified essentially as described for the cross-linking of IGF-I tracer to IGFBP-3 (Baxter & Martin 1986). In brief, the iodo-IGF-II in 50 mM Na phosphate, 0·05% bovine albumin, pH 7·0, was incubated with 5 µg rhIGFBP-5 for 16 h at 2 °C, then cross-linked for 30 min at 2 °C with 0·25 mM final concentration of disuccinimidyl suberate (Pierce, Rockford, IL, USA). The reaction was stopped with an excess of Tris–HCl, pH 7·8, and the cross-linked IGFBP-5–IGF-II was separated from unreacted IGF-II at 22 °C on a column of Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated and eluted in 0·5 M acetic acid, 0·1 M NaCl, 0·25% bovine albumin, pH 3·0.

Sources of pro-IGF-II

Serum from the same female patient with NICTH described by Baxter et al. (1995) was collected post-operatively. Pre-operative serum samples of an 87-year-old man with type II diabetes and a recurring NICTH tumour of the abdominal cavity were also collected. Subsequently, tumour samples from the male patient were collected in sterile Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St Louis, MO, USA) during surgery for the removal of the tumour.

Chromatographic fractionation of IGF-II

Tumour samples were solubilised in 6 M guanidine hydrochloric acid (Progen, Darra, Australia) and 1 tablet of complete enzyme inhibitor (Boehringer Mannheim, Mannheim, Germany) per 50 ml buffer for 16 h at 4 °C. The solubilised tissue was poured into 50 ml tubes (Falcon, Becton-Dickinson Labware, Lincoln Park, NJ, USA) and centrifuged at 4200 r.p.m. at 4 °C for 20 min. The supernatant was dialysed overnight in Spectra/Por 3 membrane (MWCO: 3500; Spectrum Medical Industries, Houston, TX, USA) against 5 changes of 0·1 M NaCl at room temperature. The diluent was then centrifuged as above and the supernatant fractionated on a 5 × 100 cm column of Sephadex G-100 fine (Pharmacia Biotech, Uppsala, Sweden) and eluted at 2 ml/min in 0·5 mol/l acetic acid and 75 mmol/l NaCl at room temperature. The diluent was then centrifuged as above and the supernatant fractionated on a 5 × 100 cm column of Sephadex G-100 fine (Pharmacia Biotech, Uppsala, Sweden) and eluted at 2 ml/min in 0·5 mol/l acetic acid and 75 mmol/l NaCl, collecting 10 ml fractions. IGF-II was measured in column fractions using a conventional IGF-II RIA (Baxter 1990). The peak fractions containing IGF-II reactive material were pooled and concentrated against phosphate-buffered saline pH 6·5 in an ultrafiltration chamber (Amicon 3000 molecular mass cut-off filter, Amicon, Beverly, MA, USA).

To remove further endogenous binding proteins and mature IGF-II, the G-100 column fractions and also the serum samples (1 ml) were fractionated on a 1·6 × 100 cm column of Bio-Gel P-60 fine (Bio-Rad, Richmond, CA, USA), 0·5 M acetic acid and 75 mM NaCl as described by Baxter et al. (1995). Concentrations were determined by IGF-II RIA, and pro-IGF-II forms from the peaks off the column were analysed by SDS-PAGE and Western blotting.

Western ligand blot

To detect the pro-IGF-II forms, tissue extracts, serum samples and column fractions were prepared for SDS-PAGE and detection on Western immunoblots. The acidified protein samples were freeze-dried in Corex tubes (Corning, NY, USA) and resuspended in 0·0625 M Tris–HCl buffer (pH 6·8) containing 4% sodium dodecysulphate (SDS) and 2·5% 2-mercaptoethanol. The samples were heated in a boiling water bath for 3–5 min, centrifuged, and the supernatant subjected to SDS-PAGE. The
proteins were separated on a 16% acrylamide separating gel and overlaid by a 4% stacking gel for SDS-PAGE of the proteins, using a PROTEAN II electrophoresis unit (Bio-Rad). Alternatively, column fractions were analysed on the Phastsystem (Pharmacia) by 20% homogeneous SDS-PAGE. Proteins were visualised by subsequent staining with silver stain.

For immunoblots, the proteins were transferred electrophoretically from SDS gels to polyvinylidene fluoride (PVDF) membrane (BioRad) and incubated with a rabbit antiserum raised to natural human IGF-II (Baxter 1990). Control incubations were carried out with preimmune serum. Antibody binding was visualised by an incubation with a secondary antibody, biotinylated donkey anti-rabbit IgG (Amersham, Amersham, Bucks, UK) followed by streptavidin–horseradish peroxidase conjugate (Boehringer Mannheim).

**Competitive binding curves for binary complexes**

Competitive binding assays were used to measure the formation of binary complexes between IGFBPs and IGF-II. Except for IGFBP-5, assays were performed in a final volume of 0.3 ml buffer containing 0.1 M sodium phosphate, 0.02% sodium azide and 0.25% bovine serum albumin pH 6.5. Incubations consisted of IGFBP (IGFBP-1, -2, -4, -6: 1 ng, approx. 35 fmol; IGFBP-3: 2-5 ng, approx. 60 fmol), increasing concentrations of recombinant IGF-II or extracted NICTH pro-IGF-II, and 125I-labelled IGF-II (10 000 c.p.m.). For assays involving IGFBP-3, -2 or -3 antiserum was added in 25 µl buffer to the appropriate binary complex. After 1 h at 22 °C, 2-5 µL goat anti-rabbit immunoglobulin in 25 µl buffer were added. After another hour at 22 °C, bound radioactivity was precipitated by adding 1 mL cold 60 g/l polyethylene glycol 6000 in 0.15 M NaCl, waiting 15 min, then centrifuging for 20 min at 4200 r.p.m. at 2 °C in a J6 centrifuge (Beckman, Palo Alto, CA, USA). Supernatants were decanted and radioactivity in the pellet was measured by gamma counting. It has previously been demonstrated for IGFBP-3 binding studies that the immunoprecipitation method of separating bound from free ligand gives identical results to the use of charcoal, or of concanavalin A (Martin & Baxter 1986). We have previously reported the use of immunoprecipitation for both IGFBP-1 (Baxter et al. 1987) and IGFBP-2 (Ho & Baxter 1997) binding assays, but have not compared it to alternative methods. For assays with IGFBP-4 and IGFBP-6, after 2 h incubation at room temperature, 1 mL 0.5% activated charcoal separation buffer was added to all incubations at 4 °C, which were then centrifuged for 10 min at 4000 r.p.m. at 2 °C. Half of the supernatant was then pipetted into a fresh tube and gamma counted for 2 min.

IGFBP-5 assays were performed in 44 mM sodium phosphate, 100 mM HEPES, 0.1% BSA and 0.1% Triton X-100 pH 6.0. Incubation for binary complex formation consisted of IGFBP-5 (5 ng/tube, approx. 175 pmol), increasing concentrations of recombinant IGF-II or pro-IGF-II sample, and 125I-labelled IGF-II (10 000 c.p.m.) in a total volume of 250 µL. After 2 h incubation at 22 °C, 250 µL 0.25% gamma globulin in assay buffer and 500 µL 25% polyethylene glycol (PEG) in ice-cold water were added for 15 min. The radioactive pellet was precipitated by centrifuging at 2200 r.p.m. for 15 min at 4 °C. Supernatants were decanted and the pellet was washed with 1 mL 6.25% polyethylene glycol in ice-cold water, and centrifuged in a J6 centrifuge for 10 min at 4200 r.p.m. at 4 °C. Supernatants were decanted and radioactivity in the pellet was measured by gamma counting.

**Ternary complex formation assays**

For IGFBP-3 ternary complex formation with IGF-II, 125I-labelled ALS tracer (10 000 c.p.m.) was incubated with 5 ng IGFBP-3 and increasing amounts of IGF-II or pro-IGF-II (0-10 ng/tube), in a total of 300 µL of the phosphate buffer as used in the binary complex formation assay. The conditions used for precipitation of the ternary complex were exactly the same as for the immunoprecipitation of binary IGFBP-3–IGF-II described in the section above.

Since a precipitating IGFBP-5 antiserum was not available, the ability of IGFBP-5 to form ternary complexes was analysed using a competition assay with a constant amount of ALS (25 ng) per tube, crosslinked 125I-labelled IGF-II–IGFBP-5 (10 000 c.p.m./tube), unlabelled IGFBP-5 (50 ng/tube) and an increasing amount of unlabelled IGF-II or pro-IGF-II (0.5–50 ng/tube). Controls without IGF-II were included in each assay. Incubations were in a total volume of 300 µL buffer containing 50 mM sodium phosphate, 0.25% bovine serum albumin, pH 6.5. A rabbit anti-human ALS antisemur, AL2/2, affinity purified on a column of Protein A–Sepharose (Pharmacia) was added at 25 µL of a 1:50 dilution to precipitate the complex. This antibody concentration had previously been shown to optimally bind the IGFBP-5 ternary complexes formed. After 1 h incubation at 22 °C, precipitating antibody (goat anti-rabbit immunoglobulin) was added as 25 µL of a 1:10 dilution. After 30 min, 1 mL cold polyethylene glycol 6000 in 0.15 M NaCl was added, and after 15 min, each tube was centrifuged at 4000 r.p.m. at 4 °C for 20 min, the supernatant decanted, and the pellet containing the precipitated complex counted in a gamma counter.

**Results**

**Characterisation of chromatographic fractionation of IGF-II by radioimmunoassay and Western blots using polyclonal IGF-II antibodies**

Serum from the female patient with NICTH was fractionated on a column of Bio-Gel P-60 to separate
pro-IGF-II from IGF-II. The resulting profile, with three major IGF-II immunoreactive peaks, is shown in Fig. 1a. The first peak corresponds to IGFBPs (>30 kDa) which bind the radiolabelled IGF-II in the RIA. Fractions in peaks 2 and 3 were pooled and subjected to SDS-PAGE, then immunoblotted using polyclonal antibodies to IGF-II to detect the various forms of IGF-II. Peak 2 contained two immunoreactive bands of ~10 kDa and ~20 kDa (Fig. 1b, lane 2) and peak 3, mature IGF-II (~7 kDa, Fig. 1b, lane 1). Recombinant IGF-II (200 ng) was used as a control and was detected at ~7 kDa (Fig. 1b, lane 3).

In contrast, using the polyclonal IGF-II antibody we detected several forms of pro-IGF-II from NICTH tumour tissue different from those in serum. A NICTH tissue extract prepared with SDS sample buffer contained a triplet of 13–15 kDa and a single band of ~11 kDa (Fig. 1b, lane 4). In addition, NICTH tissue extracts were fractionated first on Sephadex G-100 and then on Bio-Gel P-60, yielding two immunoreactive peaks (Fig. 2a). In peak 2, we detected a faint band at 14 kDa and two bands at 12 and 10·5 kDa (Fig. 2b) by IGF-II immunoblot. The amount of pro-IGF-II in these preparations was quantified by IGF-II RIA.

**Binary complex formation between IGF-II and IGFBPs-1 to -6**

Competitive binding assays were used to determine the ability of IGFBPs to form binary complexes with IGF-II. Figure 3 shows the binding curves of IGFBPs-1 to -6 of NICTH patient serum and tumour tissue extract of pro-IGF-II. Measured using an immunoprecipitation assay, radiolabelled IGF-II was displaced from IGFBP-1 slightly more efficiently by pro-IGF-II than by recombinant IGF-II in triplicate experiments (Fig. 3a), indicating a relative potency increase of up to twofold for pro-IGF-II compared with IGF-II. IGFBP-2 and IGFBP-3 interacted with recombinant IGF-II and pro-IGF-II with equal potency (Fig. 3b and 3c).

Measured using a PEG precipitation assay, the ability of IGFBP-5 to form a binary complex with pro-IGF-II was the same compared with recombinant IGF-II (Fig. 3d). A competitive charcoal binding assay was used to test the binding affinity of IGFBP-4 and IGFBP-6 with recombinant IGF-II and pro-IGF-II. No difference was observed between the binary complex formation of pro-IGF-II and IGFBP-4 and IGFBP-6 compared with that determined using recombinant IGF-II (Fig. 3d and 3f).
Ternary complex formation between ALS, IGFBP-3 and IGF-II

The ability of IGFBP-3 and pro-IGF-II in increasing concentrations to form ternary complexes was tested using radiolabelled ALS, precipitating complexes with IGFBP-3 antiserum. Figure 4a shows that, in triplicate experiments, IGFBP-3 (5 ng/tube) bound ALS tracer effectively in the presence of IGF-II, whereas pro-IGF-II from NICTH tumour extract was at least tenfold less effective than normal IGF-II in enabling ALS to bind to IGFBP-3. Since no precipitating IGFBP-5 antiserum was available, a different assay format was used to determine IGFBP-5 ternary complexes, involving their precipitation with ALS antiserum. Binding of covalent IGF-II–IGFBP-5 tracer to a fixed amount of ALS was competed by non-covalent binary complexes containing unlabelled IGFBP-5 and increasing concentrations of IGF-II or pro-IGF-II. Figure 4b shows that, in duplicate experiments, pro-IGF-II was, if anything, slightly less effective than recombinant IGF-II in displacing the crosslinked IGFBP-5–IGF-II tracer from ALS, demonstrating that, in contrast to IGFBP-3 complexes, ternary complexes with IGFBP-5 formed normally with pro-IGF-II.

Discussion

In this study, various forms of pro-IGF-II extracted from the serum and tumour of patients with NICTH have been described. We have also, for the first time, defined the binding characteristics of ALS and all six IGFBPs with pro-IGF-II as compared with IGF-II. Because the pro-IGF-II preparations used contain more than one species,
our results can only indicate the ‘average’ activity of the forms present. As described previously (Liu et al. 1993), the pro-IGF-II bands detected by immunoreactivity from a NICHT patient were larger than normal IGF-II. It is not known to what extent the difference in size of the pro-IGF-II forms is related to the sequence length of the E-peptide or the degree of glycosylation of the various forms. The E-domain is known to be subject to O-linked glycosylation (Hudgins et al. 1992, Daughaday et al. 1993, Duguay et al. 1998, Hizuka et al. 1998). In this study we found bands of 13–15 kDa and single bands of ~11 by SDS solubilisation of tumour tissue. In comparison, a faint band at 14 kDa and two bands at 10·5 and 12 kDa were detected in the same tumour tissue after size-fractionation on a P-60 gel permeation column. These forms of pro-IGF-II extracted from NICHT tumour tissue are similar to previous findings of IGF-II bands with molecular masses of 10 or 15 kDa in serum of NICHT patients (Gowan et al. 1987, Hudgins et al. 1992, Liu et al. 1993). Zumstein et al. (1985) showed a 10 kDa peptide from plasma protein fractions contained 21 amino acids of the E-domain of pro-IGF-II. The larger forms may be O-glycosylated at position 8 or 21 of the E peptide.

We used this partially purified mixture of pro-IGF-II forms from NICHT tumour tissue to define their binding characteristics with the six IGFBPs. The ability of IGFBPs to carry the excess IGF-II that occurs in the serum of NICHT patients is probably crucial in maintaining glucose homeostasis. IGFBPs that do not form ternary complexes with ALS have the potential to carry IGFs from the circulation to their target tissues.

As described by Baxter (1996), levels of IGFBP-2 and IGFBP-6 are markedly elevated in the serum of NICHT patients. Little is known of the levels of IGFBP-4 and IGFBP-5 in the serum of these patients due to the limited availability of specific immunoassays for these proteins. We have shown that pro-IGF-II binds to IGFBPs-1 to -6 with similar affinity to IGF-II. Presumably, these IGFBPs could act as carriers of excess IGF-II present in NICHT serum. In particular, those IGFBPs that are upregulated, such as IGFBP-2 and IGFBP-6 may have a significant role in IGF transport in NICHT.

It has previously been shown that ternary complex formation is greatly reduced in the presence of pro-IGF-II compared with normal IGF-II (Baxter et al. 1995). The present study confirms this result for IGFBP-3, and shows that the reduced ternary complex is not a result of disrupted binary complex formation, but appears to be due to reduced binding of IGFBP-3–pro-IGF-II to ALS. Recently, we reported that IGFBP-5 also forms complexes with ALS (Twigg & Baxter 1998). We have now shown that pro-IGF-II is similar to IGF-II in forming binary complexes with IGFBP-5 and, in contrast to IGFBP-3, binary complexes with IGFBP-5 interact with ALS at least as well in the presence of pro-IGF-II as with IGF-II. This implies that, in patients with high circulating pro-IGF-II levels, there may be a tendency for the ternary complexed pro-peptide to be bound to IGFBP-5 rather than to IGFBP-3. The actual distribution of IGF-II species between the two IGFBPs will, however, depend upon their relative concentrations. It has been postulated that the reduced formation of ternary complex is a contributing factor in the tumour-associated hypoglycaemia in NICHT patients. It has been shown previously that this is associated with reduced growth hormone (GH) secretion, leading to a decrease in serum ALS and IGFBP-3 (Daughaday et al. 1995). Our data support the evidence to date that the excessive hypoglycaemic activity of NICHT tumour IGF-II corresponds with the inability of pro-IGF-II to form predominantly the larger 150 kDa ternary complexes with IGFBP-3. Since GH secretion is suppressed, it is possible that levels of IGFBP-5 in the serum are also depressed, since serum levels of this protein are GH dependent (Ono et al. 1996). Therefore, the importance of our findings that IGFBP-5 can preferentially form ternary complexes in NICHT is difficult to quantitate. Since all six IGFBPs form binary complexes well with pro-IGF-II, and the concentrations of several of these proteins are elevated in NICHT, a shift to binary complexes is likely to be the major contributor to increased IGF bioavailability.

Similar basic carboxyl-terminal domains of IGFBP-3 (Firth et al. 1998) and IGFBP-5 (Twigg et al. 1998) are believed to be the preferential binding site for ALS. Pro-IGF-II does not prevent binary complex formation when compared with IGF-II, but the carboxy-terminal extension of the pro-IGF-II molecule is presumably responsible for the reduced ability to form ternary complex with IGFBP-3 and ALS. It is difficult to explain why such complexes still form normally with IGFBP-5. One possible explanation is that the heavy N-linked glycosylation of IGFBP-3, absent from IGFBP-5, may interact with O-linked carbohydrate on pro-IGF-II in a way which inhibits ALS binding. Consistent with this hypothesis, pro-IGF-II extracted from a NICHT patient serum, which was shown to lack the normal O-linked glycosylation, formed apparently normal ternary complex with IGFBP-3 (Daughaday et al. 1993).

High levels of IGF-II expression, seen in several human embryonal tumours such as Wilms’ tumour, rhabdomyosarcoma and hepatoblastoma (Reeve et al. 1985, Scott et al. 1985, Gray et al. 1987), are not always associated with hypoglycaemia. Gelato and Vassalotti (1990) showed that 60% of IGF-II from 7 phaeochromocytomas was in large molecular mass forms (8·7–10 kDa). However, serum IGF-II levels suggested very little tumour-derived IGF-II is released into the circulation. The precise combination of factors leading from IGF-II overproduction to hypoglycaemia is thus still incompletely understood. Our demonstration that tumour-derived pro-IGF-II binds all IGFBPs normally, and only inhibits ternary complex formation with IGFBP-3, will allow a better understanding of the
significance of altered IGFBP levels in patients with tumours overexpressing IGF-II.

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