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Specific detection of type II human chorionic gonadotropin beta subunit produced by trophoblastic and neoplastic cells

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ABSTRACT

Background: The sequence of the beta-subunit of human chorionic gonadotropin (hCGβ) varies depending on whether hCGβ is encoded by type I or type II genes. Type II genes are upregulated in trophoblast and cancer but hCGβ can be detected in the serum of nonpregnant women and healthy individuals. We aimed to determine whether monoclonal antibody (mAb) FBT11-II specifically detects hCGβ encoded by type II genes (type II hCGβ).
Methods: Competitive inhibition assays with synthetic peptides, immunocytochemical and immunohistochemical studies, type II hCGβ dosing immunoassays and sequencing of *CGB* genes were performed.
Results: Competitive inhibition assays determined that mAb FBT11-II recognizes the type II hCGβ derived peptide. CGB mRNA sequencing of JEG-3 (trophoblastic) and T24 (bladder) cell lines confirmed that JEG-3 expresses type II genes while T24 expresses exclusively type I. FBT11-II only recognizes JEG-expressed hCGβ. Placenta immunohistochemical studies confirmed that type II hCGβ expression is restricted to the syncytiotrophoblast. Immunoassays detected type II hCGβ in serum of patients with either nontrophoblastic cancers or fetal Down syndrome.
Conclusion: Type II gene expression can be detected using FBT11-II. This specific recognition could improve the clinical usefulness of assays aimed at either managing aggressive tumors or screening for Down syndrome.

1. Introduction

Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family, which also comprises LH, FSH, and TSH [1]. These hormones share a common α-subunit of 92 amino acids that is non-covalently associated with a hormone β-subunit. The β-subunit of hCG (hCGβ) contains 145 amino acids. In addition to its expression by trophoblastic cells during pregnancy, hCGβ is produced by normal tissues of different histological origins and is expressed by gonadal and nongonadal neoplasms [2,3]. While the hCG α-subunit (hCGα) is encoded by one gene on chromosome 12q21.1-23 [4], hCGβ is encoded by six non-allelic genes (*CGB* genes) clustered on chromosome 19q13.3 and named *CGB1* or β1, *CGB2* or β2, *CGB3* or β3, *CGB5* or β5, *CGB7* or β7 and *CGB8* or β8 [5–7]. Recent data show that the diversity of these genes is one of the highest reported for human genes and that high interindividual and intergenic differences in expression exist [7,8]. Genes β1 and β2 might encode a protein unrelated to hCG [9] while the remaining four genes encode the same protein, with the exception of the β7

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gene which encodes for a protein with an alanine at position 117 as opposed to an aspartic acid in the other three genes [6,10,11]. On the basis of the amino acid residues in position 117, genes encoding the hCGβ subunit were classified as type I genes if they encoded an alanine (of which only one exists: gene β7) or as type II genes if they encoded an aspartic acid (of which three exist: β3, β5, β8) [12]. Numerous normal nontrophoblastic tissues express preferentially type I gene, whereas, in addition to type I gene, normal pituitary, testis and trophoblast as well as malignant trophoblastic and malignant nontrophoblastic tissues of different histological types express type II genes [2]. These differences in expression offer the possibility of distinguishing between hCGβ expressed by most normal nontrophoblastic tissues (i.e. type I) from hCGβ produced only by normal trophoblast and by malignant cells (i.e. type II).

Studies to differentiate between type I and type II genes have concentrated on elegant techniques using molecular beacons or nested PCR and are able to detect a single nucleotide difference at position 117, i.e. GCC as opposed to GAC coding respectively for alanine or aspartic acid [2,13]. However, depending upon the techniques employed, different results were observed in tissues and in cell lines such as the T24 cell line [2,13]. On the other hand, a specific antibody able to distinguish between the free hCGβ subunits transcribed and translated from either type I or type II genes has never been described: fusion experiments using synthetic peptides analogous to the 114–122 region of hCGβ as immunogens and aimed at generating monoclonal antibodies capable of distinguishing an aspartic acid from an alanine at position 117 have been unsuccessful, at least in our laboratory.

In the present study, we show that mAb FBT11-II, which is specific to free hCGβ and recognizes the nicked form of this subunit [14,15] is capable of discriminating between hCGβ subunits encoded by type I and type II genes. Interestingly, the ELSA-FbHCG immunoradiometric assay is based on FBT11, the parental clone of FBT11-II, which suggests that this assay only measures type II hCGβ expression. This result has an impact on studies that use this assay since only hCG produced during either pregnancy and malignant processes will be preferentially measured. hCG produced by normal non-trophoblastic cells will be undetected.

2. Materials and methods

2.1. Subjects

Sera from pregnant women with fetal Down syndrome (n = 9) were obtained from the serum library of Hopital Cochin, Paris. These blood samples had been collected during the first trimester of pregnancy. Sera from patients with lung cancer (n = 30) or bladder cancer (n = 3) were obtained from the serum library of Institut Curie, Saint-Cloud. These blood samples had been collected according to protocols previously approved by the human studies committee of each institution. An informed consent had been obtained from each participant.

2.2. Cell lines

Human choriocarcinoma cell line JEG-3 was cultured in Eagle's Minimum Essential Medium and human bladder carcinoma cell line T24 was cultured in Dulbecco's MEM (4.5 g/l glucose). All media were supplemented with 10% fetal calf serum and 1× penicillin–streptomycin (Invitrogen, Cergy Pontoise, France). Cell culture supernatants from confluent JEG-3 or T24 cell lines were concentrated 10× using amicon ultra-15 centrifugal filter units (nunc, Thermo Fisher Scientific, Brebières, France).

2.3. Solid-phase peptide synthesis

Synthetic 7-mer peptides corresponding to residues 1 through 7 of the hCGβ subunit were synthesized as previously described [16] by the solid-phase method [17] in an Applied Biosystems Model 430 A peptide synthesizer. The sequences of the peptides were as follows: SKEPLRP (corresponding to residues 1 through 7 of hCGβ encoded by type II genes β3, β5 and β8); SREMLRP (corresponding to residues 1 through 7 of hCGβ encoded by type I gene β7); and SREPLRP (corresponding to residues 1 through 7 of the LHβ gene).

2.4. Antibodies

Monoclonal antibodies (mAbs) FB09, FB12 and FBT11-II were obtained as previously described [16–18]. MAbs FB09 and FB12, elicited against a synthetic peptide analogous to the COOH 109-145 terminal portion (CTP) of hCGβ, are directed against the 134–140 and 110–116 regions, respectively [18]. These mAbs are specific for either hCG or its hCGβ subunit and do not bind to LH or its LHβ subunit. MAb FBT11-II, elicited against purified hCGβ subunit (CR 129), is directed to a discontinuous epitope encompassing residues 1 through 7 and 82 through 92 of hCGβ [16]. FBT11-II is an IgG1 specific for the hCGβ subunit and does not bind to hCG nor to LH while its cross reactivity with the LHβ subunit is of 0.6% [14,16]. Mouse IgG1 isotype control MG100 (Invitrogen, Cergy Pontoise, France) was used as a control antibody. Polyclonal antibody A0231 from Dako (Trappes, France) was raised against the isolated beta-chain of hCG and reacts with free hCGβ and dimeric hCG.

2.5. Competitive inhibition assays with peptides

Competitive inhibition assays were performed as previously described [16]. Briefly, ^{125}I labeled hCGβ, containing both type I and type II hCGβ, (NIH-hCGβ (CR-125) labeled by the IODO-GEN method [19]) was employed as the tracer. All experiments were performed in 50 mM phosphate buffer, pH 7.5, containing 154 mM NaCl, 0.02% sodium azide, and 1% bovine serum albumin. First, we determined the dilution of FBT11-II which produced a 50% binding to ^{125}I-hCGβ (30,000 cpm) in the absence of peptide. Then, competitive inhibition assays were performed with the defined dilution of antibody. Briefly, displacement curves were generated in the presence of increasing concentrations of unlabeled peptides as follows: 100 μl of ^{125}I-hCGβ, 100 μl of monoclonal antibody, and 50 μl of the competitive inhibitor (at increasing concentrations) were incubated simultaneously at 4 °C for 18 h. The antigen–antibody complex was then precipitated by adding normal human serum diluted (1:3) in phosphate buffer (100 μl) and 1 ml of 20% polyethylene glycol. After centrifugation, radioactivity of the pellet was measured. Dose–response curves showed a half-maximal inhibitory dose for each molecule tested (ID_{50}).

2.6. Sequencing

RNA obtained from human bladder carcinoma cell line T24 was reverse-transcribed into cDNA with 400 units of SuperScript II RNase H-reverse transcriptase (Life Technologies, California, USA). Two microliters of this cDNA was used for 35 cycles of polymerase chain reaction (PCR) with 1.25 units of AmpliTaq Gold from Applied Biosystems (Courtaboeuf, France) with the CG Forward and CG Reverse primers [20] to obtain the CGB insert. The PCR products were purified by electrophoresis on 1% agarose gel using the S.N.A.P. gel purification kit from Invitrogen (Cergy Pontoise, France). Next, the inserts and pcDNA3 plasmid (Invitrogen) were digested with XbaI from New England Biolabs (Frankfurt, Germany) overnight at 37 °C and 19 pmol of plasmid was dephosphorylated using 0.4 units of calf intestinal alkaline phosphatase from Promega (Charbonnières-les-bains, France) following the manufacturer's instructions. After precipitation and ligation of the digested products, sequences were cloned using the TOP10F' chemically competent *E. coli* from Invitrogen following the manufacturer's instructions. Fragments were directly sequenced with sequencing primers T7 and Sp6 together with the hCG Forward and hCG Reverse primers using the ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (PE Biosystems, Courtaboeuf, France) on an

ABI PRISM 377 DNA sequencer according to the manufacturer's specifications. Primers were: CG Forward: 5′-TGTGCTCTAGATCATGACCAAG GATGGAGATGTTCCAG-3′; CG Reverse: 5′-GCACAGTCTAGATTATTGTG GGAGGATCGGG-3′; T7 (forward): 5′-TAATACGACTCACTATAGGG-3′; and Sp6 (reverse): 5′-GATTTAGGTGACACTATAG-3′.

2.7. Immunocytochemical studies

Indirect immunoperoxidase staining of fixed and permeabilized cells was performed using monoclonal antibodies FB12 and FBT11-II or MG100 IgG1 isotype control (Invitrogen). For immunocytochemical studies on cell lines, cells were grown in a permanox Lab-Tek chamber slide (nunc, Thermo Fisher Scientific, Brebières, France), fixed in 4% PFA in PBS for 20 min at room temperature (RT) and then permeabilized in methanol for 8 min at −20 °C. Slides were then either stained immediately or stored at −80 °C. Antibodies were diluted at 5 μg/ml in 1% BSA in PBS and staining was performed using the NovoLink detection system kit (A. Menarini diagnostics, Rungis, France) following the manufacturer's instructions. Between steps, slides were washed twice for 5 min in 50 mM TBS pH 7.6 and once in TBS-0.1% Tween 20. Cells were counterstained with Harris hematoxylin.

2.8. Immunohistochemical studies

For immunohistochemical studies on placentas, placental tissue of first trimester (8 to 12 WA; n = 3) or second trimester (16 to 19 WA; n = 4) pregnancy was obtained from legal abortion while placental samples from late pregnancy (39 WA; n = 1) were obtained at term from uncomplicated pregnancies. Use of tissues was approved by the local ethical committee. Tissues obtained were fixed in 4% buffered neutral formalin, dehydrated and embedded in paraffin. Sections, 5 to 6 μm in thickness, were deparaffinized, followed by standard histological techniques. Antibodies were diluted in 1% BSA in PBS and staining was performed using the NovoLink detection system kit (A. Menarini diagnostics, Rungis, France) following the manufacturer's instructions. Between steps, slides were washed twice in 50 mM TBS pH 7.6 for 5 min and once in TBS-0.1% Tween 20. Cells were counterstained with Harris hematoxylin.

2.9. Development of immunoassays

i) An enzyme linked immunosorbent assay (ELISA) was performed with Maxisorp nunc plates (Thermo Fisher Scientific, Brebières, France) coated with 0.75 μg of monoclonal antibody FB09 and/or 0.25 μg of monoclonal antibody FB12 in 0.1 M phosphate buffer pH 7.4, blocked with 1% bovine serum albumin in PBS and incubated with the hCGβ standards (CIS Bio International, France; 1 ng CIS = 1 mIU 1st IRP WHO 75/551) for 1 h at 37 °C. Bound hCGβ was detected with monoclonal antibody FBT11-II, coupled with biotin, for 1 h at 37 °C (Biotin Labeling Kit-NH_2 from Interchim, Montluçon, France). The plate was then incubated with Immunopure Streptavidin Horseradish Peroxidase conjugated (Pierce, Thermo Fisher Scientific, Brebières, France) for 15 min at RT. One-Step Ultra TMB-ELISA from Thermo Scientific was used as the substrate and the absorbance was read at 450 nm. Experiments were done in duplicate. The standard curve was constructed with the hCGβ standards used at increasing concentrations ranging from 0.1 to 47 ng/ml. Linearity was consistently shown in between run assays.

ii) A sandwich magnetic immunoassay (MIA) was developed following the guidelines previously described [21]. Seventy percent-porosity synthetic-polymer-fiber-based cylindrical wicks (Filtrona, Reinbek, Germany) were used as solid-phase supports. After washing with excess absolute ethanol and then 50% ethanol, each wick was coated with 2.5 μg of monoclonal antibody FB09 and 2.5 μg of monoclonal antibody FB12 in phosphate buffer pH 7.4. These solid phases were then inserted in MIAflo cartridges (Magnisense, Paris, France). In parallel, 200 nm-diameter carboxyl-modified superparamagnetic beads referred to as Estapor M1-020/50 (Merck Chimie, Fontenay sous bois, France) were coated with monoclonal antibody FBT11-II (50 mg/g) according to the manufacturer's instructions. They were then mixed with hCGβ standards to allow conjugation between hCGβ and the FBT11-II-covered magnetic beads. The mixture, containing 24 μg of magnetic beads per cartridge, was immediately passed back and forth through FB09 and FB12 coated-MIAflo cartridges a number of times for a total incubation time of 14 min. After four washings with a phosphate buffer containing 0.1% tween to remove unbound magnetic beads, MIAflo cartridges were measured by means of a MIAtek reader (Magnisense, Paris, France). A magnetic signal is recorded by the reader at a voltage frequency which is a linear combination of two excitation frequencies preset by the reader manufacturer. This signal is proportional to the amount of magnetic material present in the cartridges, thus to the number of sandwiched magnetic beads. Microplates (96-well) were used to hold reagents and washing solutions, to mix HGCβ standards and magnetic beads, and to collect waste. Experiments were performed in duplicate. The standard curve was constructed with 100 μl samples of hCGβ at concentrations ranging from 0.21 to 23.2 ng/ml.

3. Results

3.1. Competitive inhibition assays with synthetic peptides show specific recognition of type II hCGβ by FBT11-II

Type I and type II genes were described based on the residue difference in position 117 [6,10,11]. Interestingly, apart from the difference in amino acid 117, two other amino acids differ between type I and type II gene products: Arg2 and Met4 for type I as opposed to Lys2 and Pro4 for type II [7,10,22] (Fig. 1A). Since it was previously shown that FBT11-II recognizes residues 1 through 7 and residues 82 through 92 of the free hCGβ subunit [16] (Fig. 1B), we examined whether binding of FBT11-II was sensitive to the differences in the N-terminal sequence of hCGβ. To determine mAb FBT11-II's specificity, we performed inhibition assays with synthetic peptides spanning residues 1 through 7. We used three peptides analogous to sequences 1–7 of hCGβ encoded by type I gene (SREMLRP), 1–7 of hCGβ encoded by type II genes (SKEPLRP) and 1–7 of LHβ (SREPLRP). The results are shown in Fig. 2. First, we determined the dilution of FBT11-II which produced a 50% binding to ^{125}I-hCGβ in the absence of peptide. Then, competitive inhibition assays were performed with the defined dilution of antibody. Displacement curves were generated in the presence of increasing concentrations of unlabeled peptides.

Synthetic peptide SKEPLRP, corresponding to the N-terminal sequence encoded by type II genes, exhibited the highest potency in displacing bound ^{125}I-hCGβ from antibody FBT11-II. In striking contrast, peptide SREMLRP, differing only in two residues and corresponding to the N-terminal sequence encoded by the type I gene, was unable to inhibit the binding of the β-subunit to antibody FBT11-II. Peptide SREPLRP, corresponding to the 1–7 N-terminal sequence of LHβ and comprising only one residue change compared to the type II sequence, was able to inhibit binding of hCGβ to FBT11-II to a lesser degree than SKEPLRP. As FBT11-II displays a very low cross reactivity (0.6%) with LHβ [14], these observations, taken all together, demonstrate that FBT11-II binds with a high specificity to hCGβ encoded by type II genes.

3.2. Sequencing of hCGβ genes

In order to confirm that monoclonal antibody FBT11-II is specific for the hCGβ subunit encoded by type II genes (referred to as type II hCGβ), we selected cell lines that express either type I or type II genes as model systems. Human choriocarcinoma cell line JEG-3 expresses

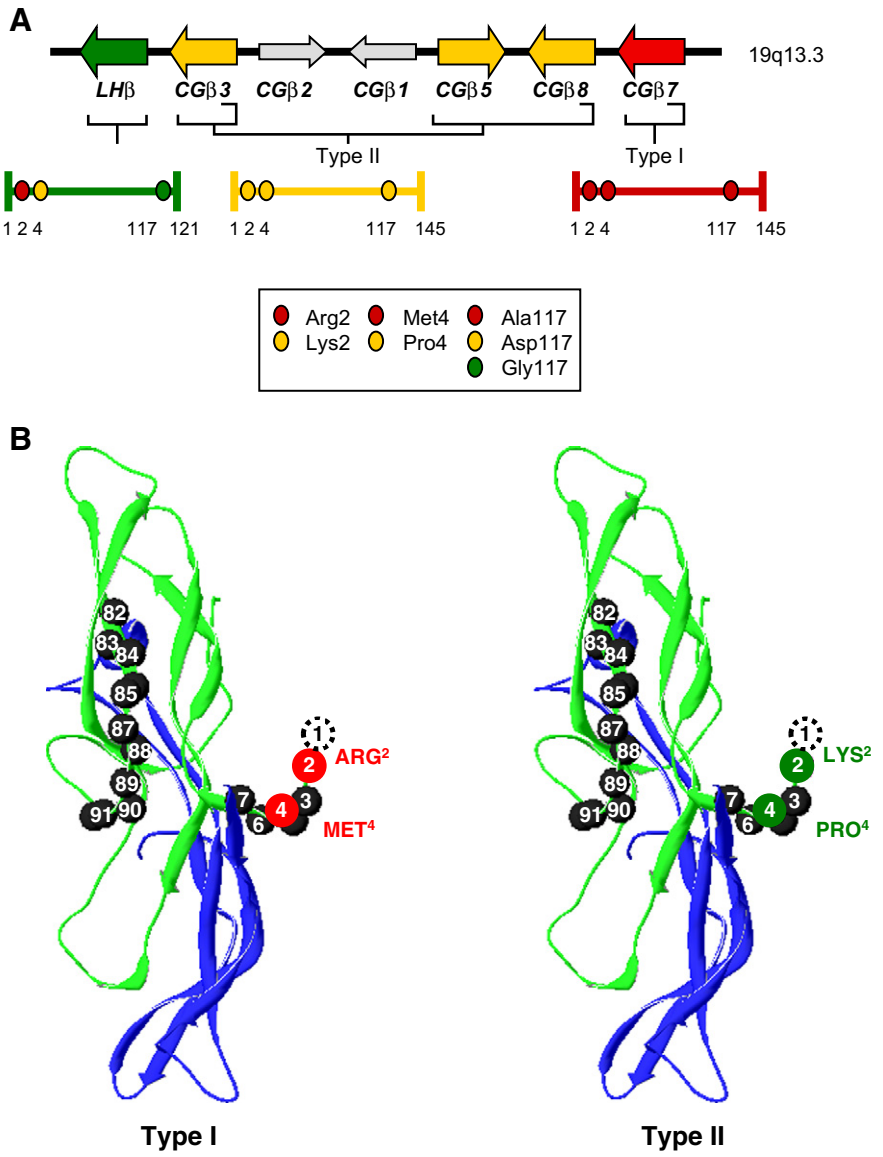

Fig. 1. The human chorionic gonadotropin beta subunit. A) Organization of the *CGβ/LHβ* gene cluster and amino acid sequences of expressed genes. Only genes *CGβ3*, *CGβ5*, *CGβ7* and *CGβ8* code for the hCGβ subunit. Type I gene codes for a mature protein with an arginine residue at amino acid 2, a methionine residue at amino acid 4 and an alanine residue at amino acid 117. Type II genes encode a mature protein with a lysine residue at amino acid 2, a proline residue at amino acid 4 and an aspartic acid at amino acid 117. Also shown is the *LHβ* gene, which encodes a mature LHβ protein with an arginine residue at amino acid 2, a proline residue at amino acid 4 and a glycine residue at amino acid 117. B) Localization of FBT11-II epitopes on the crystal structure of hCG. hCG is shown as a ribbon diagram (Protein Data Bank ID PDB: 1HRP) [44]. The alpha subunit is represented in blue and the beta subunit is represented in green. The discontinuous epitope recognized by mAb FBT11-II spans residues 1-7 and 82-92 and is indicated by black spheres, residues 2 and 4 are shown in green. As a comparison, type I hCGβ is shown with residues 2 and 4 indicated by red spheres, emphasizing the difference between type I and type II proteins concerning the FBT11-II epitope. Residue 1, while not appearing on the crystal structure, has been added for clarity. This image was obtained using the program Swiss-PdbViewer (http://www.expasy.org/spdbv/) [45].

preferentially β5 type II gene [23]. Therefore, this cell line was selected as prototypic of cell lines expressing type II hCGβ. It was initially described that the bladder cell line T24 expressed only the type I gene [2]. However, it was later argued that this cell line expressed only type II genes [13]. In order to clarify this issue, we sequenced the CG beta genes encoded by T24 cell line. The *CGB* mRNA of human bladder carcinoma cell line T24 was amplified by RT-PCR, cloned and sequenced. Our results show that T24 cell line transcribes only the β7 gene (type I *CGB* gene) (Fig. 3). We confirmed this result by quantitative real-time PCR as shown in [24].

3.3. FBT11-II specifically recognizes type II hCGβ in a cellular setting

In order to determine whether mAb FBT11-II specifically recognizes type II hCGβ in a cellular setting, detection of hCGβ on either human choriocarcinoma cell line JEG-3 or human bladder carcinoma cell line T24 was performed by immunocytochemistry using either mAb FB12, mAb FBT11-II or IgG1 isotype control at 5 μg/ml. Representative results are shown in Fig. 4. Monoclonal antibody FB12, directed against the 110–116 region, is specific for hCG and hCGβ, and its binding to hCGβ is unaffected by the presence of an alanine residue instead of an aspartic acid residue at position 117 [18]. Thus, FB12 recognizes both type I and type II hCGβ. Monoclonal antibody FBT11-II is specific for free hCGβ and had never before been tested for its specific recognition of either type I or type II hCGβ.

As expected, FB12 recognized type II hCGβ expressed by the JEG-3 cell line as well as type I hCGβ expressed by the T24 cell line (Fig. 4, left panels). In contrast, FBT11-II only reacted with hCGβ produced by the JEG-3 cell line indicating that this antibody specifically recognizes type II hCGβ (Fig. 4, center panels). Immunocytochemical analyses

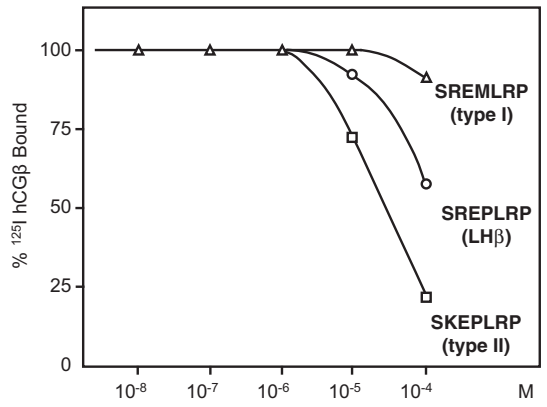

Fig. 2. Inhibition of ^{125}I-hCGβ binding to monoclonal antibody FBT-11-II by synthetic peptides corresponding to residues 1 through 7 of type I gene (triangles), type II genes (squares) or LHβ (circles).

using IgG1 isotype control antibody were uniformly negative (Fig. 4, right panels). Both FB12 and FBT11-II showed a heterogeneous staining of JEG-3 cells, which was also observed with polyclonal antibody A0231, directed to hCG, and on different batches of JEG-3 cell lines (data not shown).

3.4. FBT11-II recognizes type II hCGβ produced by trophoblasts during the course of gestation

In order to confirm that FBT11-II is able to recognize type II hCGβ produced by normal trophoblasts during the course of gestation, we performed immunohistochemical staining on different placenta samples (Fig. 5). In situ detection of either hCG or hCGβ was performed using mAb FB12, which recognizes both type I and type II hCGβ. In early placenta obtained at 9 weeks (Fig. 5, top row), immunostaining with FBT11-II was exclusively localized to syncytiotrophoblast (ST), whereas FB12 stained either both the cytotrophoblast (CT) and the ST or only the ST, depending on the individual villous structures. In the case of mid-term placenta (17 weeks) (Fig. 5, middle row), the cytological localization of type II hCGβ was similar to that in early placenta, but the staining intensity was less prominent. Interestingly, immunostaining with FB12 at this term was exclusively localized to the ST. In term placenta obtained at 39 weeks of gestation, both immunostaining with FBT11-II and FB12 were observed in the syncytiotrophoblast (Fig. 5, bottom row). Staining with a normal mouse IgG1 isotype control was consistently negative in all samples.

3.5. Development of immunoassays specific for type II hCGβ

FBT11 is already used in a commercial immunoradiometric assay (ELSA-FbHCG). In order to design alternative, radioactive free assays that specifically detect type II hCGβ, we developed an ELISA using FBT11-II as indicator antibody. In previous radiolabeled antibody binding experiments based on immunoradiometric assays (IRMAs), it was demonstrated that enhanced binding of indicator antibody to hCG was obtained using anti-C terminal peptide (CTP) antibodies FB09 and FB12 as capture antibodies [18]. In order to determine whether this synergistic effect for capturing hCG might also be observed for detecting type II hCGβ, an enzyme linked immunosorbent assay (ELISA) was designed. This assay uses mAbs FB09 and FB12, either alone or in combination, to capture hCGβ on a solid phase support, and biotinylated mAb FBT11-II as indicator antibody (Fig. 6A, right). Neither at a low dose (9.8 ng/ml) nor at a high dose of hCGβ (47 ng/ml), was any significant binding of hCGβ to FB09 detected, whereas hCGβ showed low level binding to FB12 at 47 ng/ml (Fig. 6A, left). By using both FB09 and FB12 as capture antibodies (50%, vol/vol), a dramatic synergistic effect was observed with a 20-fold increase of hCGβ-bound signal detected at 47 ng/ml.

Based on these results, this ELISA was further developed. An hCGβ standard curve is shown in Fig. 6B. The minimum detectable dose of hCGβ (analytical sensitivity) was determined to be 0.15 ng/ml. This assay was used to measure type II hCGβ in culture supernatants of JEG-3 and T24 cell lines. Using an assay based on mAb FBT10 both these cell lines were shown to secrete the hCGβ subunit [2]. However, since mAb FBT10 does not distinguish between type I and type II hCGβ [2], we were up to now unable to dose type II gene expression. Using the newly developed ELISA based on FBT11-II we were able to determine that concentrated supernatants from JEG-3 cells contained hCGβ type II (32 ng/ml ± 1.6, mean and SEM) while no type II hCGβ was detected in concentrated T24 supernatants. In order to improve the analytical sensitivity of immunoassays, we used a novel technique named magnetic immunoassay or MIA based on the highly sensitive detection of a magnetic signal [21]. This MIA showed an analytical sensitivity lower than 0.2 ng/ml (Fig. 6C). Serum levels of type II hCGβ were measured by ELISA in patients with nontrophoblastic neoplasms including bladder and lung cancers as representative examples. Low levels of type II hCGβ were detected as illustrated in Fig. 6D and E. In sera from healthy male and cycling, non-pregnant, female individuals hCGβ levels were consistently below 0.1 ng/ml (data not shown). In addition, levels of type II hCGβ were also measured during the first trimester of pregnancy in maternal serum from women with fetal Down syndrome and, as expected, high levels of type II hCGβ were observed (Fig. 6F).

4. Discussion

The main goal of this study was to determine whether an assay can specifically distinguish between hCGβ encoded by type I and type II *CGB* genes (type I and type II hCGβ). It is noteworthy that type I and type II hCGβ differ in their amino acid sequences at positions 2, 4 and 117 (Fig. 1) and that these differences might generate different epitopes. The hCGβ subunit contains at least 13 epitopes, named β1 to β13, of which β1 to β5 are exposed on the hCG heterodimer and β6 and β7

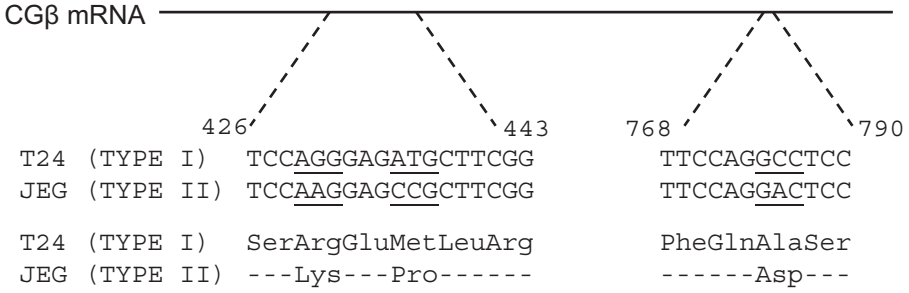

Fig. 3. Schematic representation of the oligonucleotide sequence corresponding to residues 2, 4 and 117 of the T24 cell line compared to the JEG-3 cell line (NCBI Reference Sequence: NM_033043.1). Codon differences are underlined and the corresponding amino acid residues are indicated below. For JEG-3, only the amino acids that are different to the T24 sequence are shown. Data were obtained from three independent experiments.

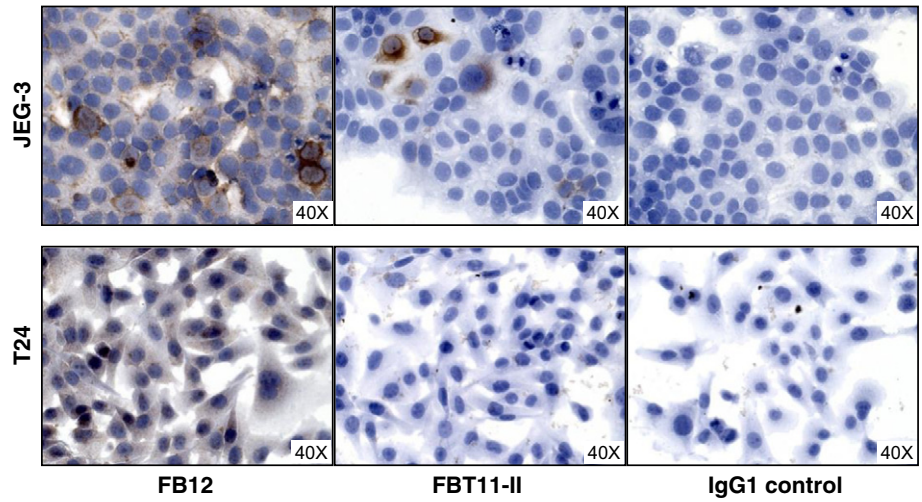

Fig. 4. Immunocytochemical staining of human choriocarcinoma cell line JEG-3 (top row) and human bladder carcinoma cell line T24 (bottom row) with hCGβ specific antibodies FB12 (left), FBT11-II (center) or normal mouse IgG1 control (right). Cells were counterstained with hematoxylin.

are specific for free hCGβ [25,26]. Linear epitopes β8 and β9 have been mapped to residues 109–145, which constitute the C-terminal peptide (CTP) of hCGβ and contain residue 117, but all other epitopes are discontinuous.

Since monoclonal antibody FBT11 is specific to free hCGβ and binds to a discontinuous epitope comprising residues 1 through 7 and 82 through 92 [14,15], we aimed to determine whether its derivative, FBT11-II, could differentiate between type I and type II hCGβ. Competitive inhibition assays with peptides show that FBT11-II recognizes the amino terminal sequence corresponding to type II hCGβ while it does not bind to the amino terminal sequence corresponding to type I hCGβ. FBT11-II is therefore directed against a highly specific and discontinuous epitope of hCGβ that does not belong to the β1 to β13 epitopes previously described and that we have called β14. This β14 epitope comprises two regions: region 1–7, with a lysine and a proline residue at positions 2 and 4 respectively, and region 82–92, and is only present in type II hCGβ. To confirm that FBT11-II specifically recognizes type II gene expression, T24 and JEG-3 cells were used as prototypic cells expressing hCGβ encoded by either type I or type II genes, respectively. Sequencing of *CGB* genes confirmed that the bladder cell line T24 expresses type I gene β7. Previous reports have shown that tumor progression in bladder tissues is characterized by different patterns of transcription of the

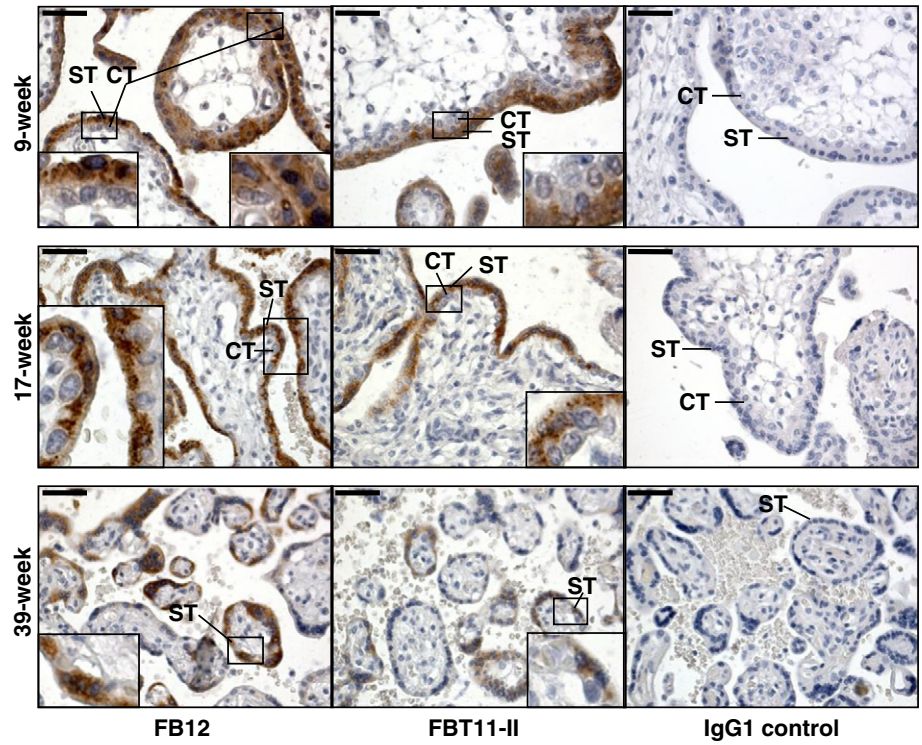

Fig. 5. Immunocytochemical localization of hCGβ in first, second and third trimester placentas. Formalin-fixed, paraffin-embedded sections of 9-week placenta (top), 17-week (middle) or 39-week (bottom) placenta were stained with hCGβ specific antibodies FB12 (left), FBT11-II (center) or normal mouse IgG1 control (right). Cells were counterstained with hematoxylin. FBT11-II showed staining for hCGβ in syncytiotrophoblast (ST), whereas cytotrophoblastic cells (CT) were negative. FB12 showed staining for hCGβ in ST and CT in early placenta, while mid and late placentas were CT negative. Representative results for first trimester (8 to 12 WA; n = 3), second trimester (16 to 19 WA; n = 4) or term pregnancy (39 WA; n = 1) are shown. Insets: higher magnification images of boxed regions. Scale bars represent 50 μm.

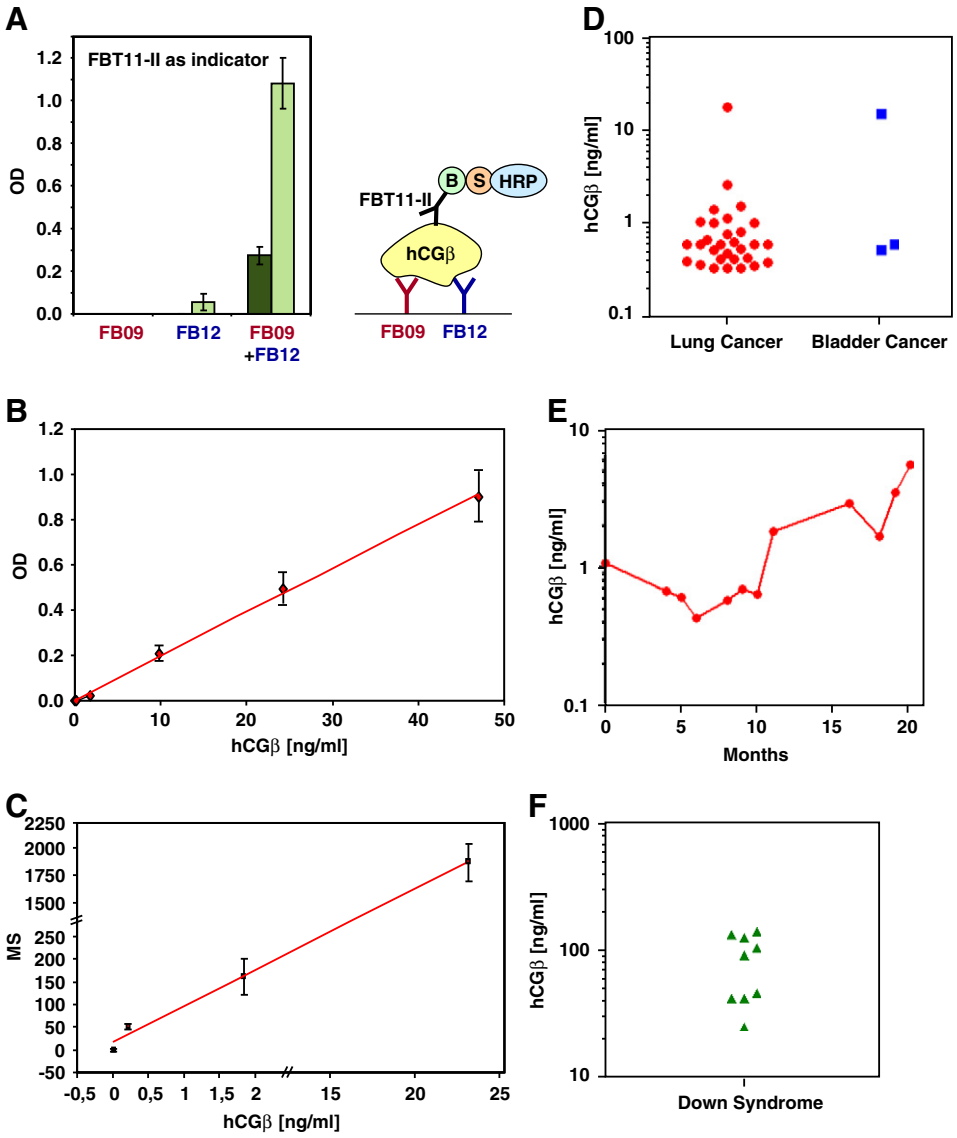

Fig. 6. Specific type II hCGβ immunoassays. A) Left: binding of biotinylated FBT11-II to hCGβ immobilized on an ELISA plate by anti-CTP antibodies FB09 and FB12 either alone or in combination. Data are mean results (±SEM) of three independent experiments and are expressed in optical density (OD). Two different concentrations of hCGβ were used: 9.8 ng/ml (indicated in dark green) and 47 ng/ml (indicated in light green). Right: schematic representation of the hCGβ capture method developed here. This assay uses two antibodies as capture antibodies, FB09 and FB12, while biotinylated antibody FBT11-II is used as indicator. B: biotin; S: streptavidin; HRP: horseradish peroxidase. B) Standard curve of the ELISA for mean values (±SEM) of hCGβ detected using mAbs FB09 and FB12 as capture antibodies and biotinylated FBT11-II as indicator antibody. Data are mean results of seven independent experiments. C) Standard curve of the sandwich magnetic immunoassay (MIA) for mean values of hCGβ detected using mAbs FB09 and FB12 as capture antibodies and FBT11-II conjugated to superparamagnetic beads as indicator antibody. Results are expressed in magnetic signal (MS). D) Representative examples of type II hCGβ serum levels in patients with either lung or bladder cancer as measured by ELISA. E) Type II hCGβ serum levels during the follow-up of a patient with non-small cell lung cancer. F) Representative examples of type II hCGβ serum levels in first trimester maternal serum from women with fetal Down syndrome.

CGB genes; type I gene β7 is the only gene transcribed in normal urothelia and early-stage non-invasive papillary carcinoma tumors (stage Ta) whereas, in addition to β7, type II genes were transcribed in more advanced tumors (stages T1 to T4) [27]. Thus, it was not unexpected that a bladder cell line preferentially expresses type I gene. Once type I gene expression was confirmed in T24 cells, immunocytochemistry assays were used to determine whether FBT11-II specifically detects type II hCGβ. We found that FBT11-II does not recognize type I hCGβ, expressed by T24 cells, and only recognizes type II hCGβ, expressed by JEG-3 cells. Lastly, immunohistochemical staining was carried out on placentas at different times of gestation, showing that FBT11-II consistently stains the syncytiotrophoblast as previously observed [28]. As normal trophoblastic cells express type II genes, these results are in line with previous observations.

The measurement of hCG protein or its variants is important for monitoring pregnancy, for prenatal screening for Down syndrome and for the diagnosis or follow-up of tumors [29,30]. Type I hCGβ is preferentially expressed by cells of nontrophoblastic origin, whereas trophoblastic and malignant nontrophoblastic tissues also express type II hCGβ. The expression of type I gene by numerous normal tissues might hinder the specific recognition of hCGβ expressed during pregnancy. As the measurement of hCGβ is useful for the screening for Down syndrome during the first trimester, it would be preferable to screen for this chromosomal abnormality by using an assay specific for type II hCGβ. As we show in this study, this is already the case for the immunoradiometric assay ELSA-FbHCG (by CIS, UK) used by Spencer et al., in their clinical trials [31]. This assay is based on FBT11, which is the parental clone from which FBT11-II is derived. Since FBT11-II

recognizes type II hCGβ, we can assume that FBT11 does too. Therefore, the assay used by Spencer and colleagues may owe its high detection rate of Down syndrome and low false positive cases to the specific detection of hCGβ of trophoblastic origin (type II) without cross-reaction with hCGβ of nontrophoblastic origin (type I).

Different variants of hCG have been described, including carbohydrate isoforms, nicked variants and truncated versions of the proteins and of individual subunits. Part of the hCG in urine, as well as its free β-subunit, has intrachain nicks at various positions between amino acids 44 and 48. These forms may also occur in the serum of cancer patients and in patients with trophoblastic disease [32–34]. Antibodies have been obtained against most of these variants and are used routinely in immunoassays. However, recent reports have demonstrated that because of hCG's heterogeneity, different immunoassays give differing results for the same specimens [35]. The data obtained from these tests can differ depending on the epitopes and isoforms recognized by the antibodies, for example depending on their sensitivity to the nicked form [15,25].

It is important to know the specificity of the method being used as pregnancy and cancer studies require different specifications. Indeed, during pregnancy, the predominant form of hCG in serum is intact hCG, whereas patients with gestational trophoblastic disease secrete intact hCG, free hCGβ, nicked hCG and nicked hCGβ [29]. Individuals harboring nongestational trophoblastic neoplasms, such as germ cell tumors of the testes and ovaries, frequently secrete hCGβ and lesser amounts of hCG, while patients with nontrophoblastic neoplasms secrete only hCGβ. Adding to this complexity, two different hCGβ subunits produced by cells of different origin have several amino acid changes, depending upon the genes expressed by these cells. The type I gene is preferentially expressed by cells of nontrophoblastic origin, whereas trophoblastic and malignant nontrophoblastic tissues also express type II genes. hCGβ expressed by type I or II genes differs in three residues located in positions 2, 4 and 117 (Fig. 1). Up to now, our team and others have tried to distinguish between type I and type II expression by focusing on residue 117. Indeed, these types of studies have concentrated on elegant techniques using molecular beacons or nested PCR and able to detect a single nucleotide difference at position 117, i.e. GCC as opposed to GAC coding respectively for alanine or aspartic acid [2,13]. However, depending upon the techniques, different results were observed in tissues and in cell lines as the T24 bladder cell line [2,13]. Moreover, these techniques did not exploit the N-terminal differences between type I and type II genes. In addition, a specific antibody able to distinguish between the free hCGβ subunits transcribed and translated from either type I or type II genes has never been described: fusion experiments using synthetic peptides analogous to the 114–122 region of hCGβ as immunogens and aimed at generating monoclonal antibodies capable of distinguishing an aspartic acid from an alanine at position 117 have been unsuccessful, at least in our laboratory. Thanks to FBT11-II, which binds to a discontinuous epitope comprising residues 1 through 7 and 82 through 92, we can now address the challenge of distinguishing type I from type II hCGβ using the differences located in the N-terminal end.

In order to specifically and easily detect type II hCGβ without using radioactive reagents, an ELISA and a magnetic immunoassay (MIA) were developed. These tests should allow us to discriminate between type I and type II expression. Both immunoassays are based on FB09 and FB12 mAbs directed to the carboxyl terminal portion of hCGβ as capture antibodies and on mAb FBT11-II as a tracer antibody binding only to type II hCGβ. The ELISA underlines the synergistic effects of FB09 and FB12 to capture hCGβ while both the ELISA and MIA display the required sensitivity to detect low amounts of type II hCGβ in clinical settings. To our knowledge, the current study is the first to describe an assay specific for type II beta subunit. This assay can be useful to determine the presence or absence of type II hCGβ in biological fluids. As a proof of concept, ELISA for type II hCGβ was performed on sera collected during the first trimester of pregnancy from women affected with fetal Down syndrome and on sera from patients with nontrophoblastic neoplasms. High levels of type II hCGβ were present in sera from Down syndrome pregnancies (Fig. 6F) while low levels were detected in sera from patients with lung and bladder cancers (Fig. 6D).

Recently, it was stated that even though most hCG assays are very reliable, there is still a need for better methods for diagnosis and monitoring of cancers [35]. Methods that detect type II hCGβ might respond to this need as it has been shown that type II genes are expressed in many nontrophoblastic malignancies including lung, thyroid, prostate, bladder and breast cancers [27,36–39]. In invasive bladder cancers, type II genes are predominantly expressed. In breast cancers, expression of type II genes has prognostic value for relapse-free survival. Moreover, it is well established that the more malignant forms of gestational trophoblastic diseases express excessive amounts of hCGβ [29] and that gonadal tumors might also express hCGβ. Thus, it would be useful to monitor patients with trophoblastic and nontrophoblastic tumors for the presence of type II hCGβ in biological fluids. As a representative example, Fig. 6E shows the monitoring of a patient with a lung cancer based on serum levels of type II hCGβ measured by ELISA.

Finally, it remained unclear why some men and women show positive in hCG screening tests when they are not pregnant, do not have cancer and are otherwise asymptomatic. In line with these observations, the group of Laurence Cole described a genetic defect designated "Familial hCG Syndrome" [40,41]. Members of families with inherited Familial hCG Syndrome produce only hCGβ, and hCG or hCGβ missing the β-subunit-C-terminal peptide. Of these molecules, 48 to 100% were described as missing the C-terminal peptide [42]. Taking together his observations and our findings, Laurence Cole elegantly suggests that men and women with unexpected positive hCG screening tests, including individuals with "Familial hCG syndrome", that produce what they had named "hCGβ missing the β-subunit-C-terminal peptide" may in fact produce hCGβ encoded by *CGB7* gene which would therefore not be detected by assays using FBT11 or FBT11-II. This seems to be supported by the fact that hCGβ missing the C-terminal peptide appears to have a normal β-subunit size by gel filtration [42]. Thus assays specific for both type II hCGβ and the C-terminal peptide, as are the ELISA and MIA assays based on FBT11-II, FB09 and FB12 antibodies, might help to measure hCGβ produced only by pregnant women and patients with cancer since these assays would not detect hCGβ produced by patients with Familial hCG Syndrome and encoded by the type I gene. This type of selectivity can prove useful in avoiding unnecessary chemotherapy of Familial hCG Syndrome patients before diagnosis, as what happened to two of the fifteen reported cases [40]. A different and altogether unexpected application of these assays could be found in the world of sports. Up to now, athletes with Familial hCG Syndrome were wrongly accused of doping with hCG (as was the case of five of the fifteen reported cases) since current tests do not differentiate between type I and type II hCGβ [42]. It is possible that using a type II specific assay would allow the doping agencies to avoid detecting cases of Familial hCG Syndrome.

Eighty-seven years after the first description of an hCG assay by Ascheim and Zondek [43], the specific recognition of type II hCGβ might continue to improve the clinical usefulness of such assays.

Declaration of authors' roles

L Aldaz-Carroll: acquisition of data; analysis and interpretation of data; drafting of the manuscript.

S Richon: acquisition of data; analysis and interpretation of data; technical and material support.

V Dangles-Marie: critical discussion.

M Cocquebert: technical and material support; critical discussion.

T Fournier: technical and material support; critical discussion; critical revision of the manuscript for important intellectual content.

F Troalen: acquisition of data; analysis and interpretation of data; technical and material support.

D Stevens: analysis and interpretation of clinical data.
B Guery: technical and material support.
A-M Hersant: technical and material support.
A Nordor: critical discussion; critical revision of the manuscript.
J Guibourdenche: analysis and interpretation of clinical data.
A Pecking: critical discussion; obtained funding.
D Bellet: study concept, design and supervision; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; obtained funding.

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Conflict of interest

Co-authors have no conflict of interest to declare.

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