Novel Insights into the Expression of *CGB1* & *2* Genes by Epithelial Cancer Cell Lines Secreting Ectopic Free hCGβ

BEATA B. BURCZYNSKA[1,2*], LATIFA KOBROULY[1*], STEPHEN A. BUTLER[1*], MAHMOUD NAASE[1,3] and RAY K. ILES[1,4,5]

[1]*Centre for Investigative and Diagnostic Oncology, Middlesex University, London, U.K.;*
[2]*Department of Cell Biology, Poznan University of Medical Sciences, Poznan, Poland;*
[3]*Department of Health Sciences, College of Arts and Sciences, Qatar University Doha, Doha, Qatar;*
[4]*ELK Foundation for Health Research, AnScoil Monzaird, Crieff, Scotland, U.K.;*
[5]*Onconexus Corp., Houston, TX, U.S.A.*

Abstract. *Background: Ectopic secretion of human chorionic gonadotrophin free beta (hCGβ) by epithelial cancer is associated with aggressive tumors which more readily metastasize, possibly by acting as an autocrine anti-apoptotic agent. hCGβ is encoded by six homologous CGB genes, with poorly-understood variable transcriptionally active expression profiles; CGB1 and CGB2 have always been considered pseudogenes. However, transcripts from CGB1 and -2 can be detected in placental, testicular and pituitary tissues. The expression and function of these genes in cancer is less well-known. Materials and Methods: Expression profiles of CGB genes in epithelial cancer cells by quantitative polymerase chain reaction (qPCR) were explored, along with the consequence of specific siRNA silencing of CGB1 and 2. Immunohistochemical and immunoassay techniques were used to detect the translation and secretion of hCGβ in these cells. Results: CGB1 and -2 gene transcripts were only detected in cells which secreted hCGβ. siRNA-mediated silencing of CGB1 and -2 transcripts significantly reduced secreted protein in concordance with a reduction in cell survival to a greater degree than that of other CGB genes. Conclusion: CGB genes 1 and 2, previously considered as pseudogenes, are notably expressed by epithelial cancer cell lines. The transcription of these genes, but not other CGB genes, correlates with a functionally expressed protein and propensity for cancer growth.*

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*These Authors contributed equally to this work.

Correspondence to: Dr. Ray K. Iles, 4550 Post Oak Place, Suite 224, Houston, TX, U.S.A. Tel: +1 7136228400, e-mail: ray@iles.net

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Ectopic expression of human chorionic gonadotropin free beta-subunit (hCGβ) by many types of epithelial cancer is now a recognised phenomenon, overall, in ~32% of carcinomas (9-55% in different cancer subtypes) (1, 2). Such expression is associated with poor prognosis in 87% of cases where prognosis was investigated (1). However, the underlying molecular mechanisms of expression and subsequent influence on oncogenesis are poorly-understood. We reported that hCGβ acts as an autocrine growth factor (3) by inhibiting apoptosis (4). Homodimers of hCGβ have been identified (5) but are not necessarily required for autocrine activity or receptor binding (6). The target receptor for free beta subunit of hCG has not yet been established (7). However, it has been shown that the hormonal luteinizing hormone (LH)/hCG receptor is not implicated (8). It has been inferred that hCGβ-positive cells have a selective survival advantage and potentially undergo further replication and mutation as a result of *CGB* gene expression.

The beta subunit of hCG is encoded by six *CGB* genes: *CGB* (originally *CGB3*), *CGB5*, *CGB7* and *CGB8* (classical hCGβ genes), located on chromosome 19q13.3 in close proximity to *CGB1* and *CGB2* and the gene for LHβ (*CGB4*) (9) (Figure 1). While classical *CGB* genes have been shown to be functional, genes *CGB1* and *CGB2* were always assumed to be pseudogenes (10).

Unlike the classical CG genes, which each produce a single mRNA transcript, *CGB1* and *CGB2* potentially have four splice variants (11) (Figure 1). The four variants include a predominant transcript coding a theoretical 132-amino-acid protein (which has never been isolated or characterised to date), and three other splice variants: +47, +166, +176 bp that contain additional DNA sequences (47 bp, 166 bp and 176 bp respectively) taken from within intron 1. An alternative theoretical form of +47 bp variant would result from the re-shift of the open reading frame (ORF) to that of the classical

hCGβ-coding transcripts, and would therefore produce an additional short transcript (essentially another exon, which has been termed exon 1b) from the middle of intron 1 (12, 13). Alternative mRNAs +166 bp and +176 bp may hypothetically code for a shorter polypeptide of about 60 amino acids in length (12, 14). Additionally, in testes, a novel rare splice variant form was detected which contains an extra 87-bp exonic sequence (13).

The incorporation of *CGB1* and *CGB2* within the human genome is believed to have evolved in the cluster as a result of a DNA fragment insertion (736 bp for *CGB1* and 724 bp for *CGB2*) that replaced the 52-bp sequence at the proximal end of the promoter, and also the entire 5'-UTR of the ancestral hCGβ-subunit coding gene fragment, which is still present in classical CGB genes (11, 15). Replacement of the *CGB5*'-UTR provided a novel putative promoter segment, 5-UTR and exon 1 (which codes for a lead sequence cleaved during synthesis). A single bp frameshift in the ORF for exons 2 and 3 in genes *CGB1* and *-2* has been proposed to result in an optional earlier stop codon and shorter exon 3 in the transcripts. Thus, expression of *CGB1* and *-2* genes may serve as a template for a hypothetical protein that is entirely different from the hCG beta subunit of pregnancy, one that lacks similarity to any known hCG protein (11). Previously *CGB1* and *-2* transcript mixtures have been detected in the placenta (11, 16), testes (17), pituitary gland (18) and other non-cancerous, non-trophoblastic tissues (12). The mRNAs of *CGB1* and *-2* were observed in brain tissue at a level equal to that found in placenta (19). However, all were detected at much lower levels than that of classical *CGB* genes expressed by placenta.

In cancer, overexpression of *CGB* genes does not necessarily correlate with hCGβ protein levels (1). However when *CGB* genes are silenced, both the secretion and effect of hCGβ are negatively impacted (20, 21). More specifically, the genes *CGB*, *CGB5* and *CGB8* are found to be variably expressed in cancer tissue such as of the breast, thyroid, and prostate, but not in non-cancerous tissues of corresponding organs (22), and the genes *CGB8*, *CGB5*, *CGB* and *CGB7* (in order of decreasing expression) were found unregulated in invasive breast tumors. *CGB7* was also detected in normal breast tissue (23). Additionally, overexpression of *CGB*, *CGB5* and *CGB8* in bladder cancer cell lines but lower expression of *CGB7* was shown by others (24, 25). In all these studies, neither *CGB1* nor *CGB2* were specifically addressed within the experimental design, in some cases, perhaps, because they have always been considered pseudogenes and therefore of no significance (10).

In the present study, we re-examined *CGB* gene expression in non-trophoblastic cancer cells, with a particular focus on the often disregarded genes *CGB1* and *-2*, to explore their potential function in cellular oncogenesis.

Materials and Methods

Cell culture and controls. The human bladder cancer cell lines RT112, SCaBER and T24, breast cancer cell lines C2235 and C2238, and prostate cancer LN-CAP and PC-3 were obtained from the American Type Culture Collection (Manassas, VA, USA) and from the European Collection of Animal Cell Cultures (Porton Down, Dorset, UK). Cells were cultured in RPMI-1640 medium (Invitrogen, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) with 5% antibiotics at 37˚C in 5% CO_2. Positive and negative controls were normal-term placental tissue (placenta obtained after vaginal delivery in the 39th week of pregnancy) and fresh water shrimp, which has no *CG*-like genomic sequence, respectively.

Immunohistochemical staining. Cancer cells were grown, as above, on cover slips until they reached 80% confluence, and then fixed in 4% paraformaldehyde followed by incubation in 3% H_2O_2 and washing in phosphate buffered saline (PBS). For permabilization, 0.1% Triton in PBS was added for 8 min, then cells were washed three times in PBS, and then blocked in 50% horse serum for 15 min. The cells were incubated for one hour with 1:100 (diluted in PBS) primary mouse monoclonal anti-hCG beta epitope 7 antibody (INN-hCG-68; Abcam, Cambridge, Cambridgeshire, UK) followed by washing in PBS. This method has been described previously (26). This antibody does not cross-react with intact hCG or hLH/LH free beta-subunit (17, 27). In the control samples, primary antibodies were excluded and substituted with PBS. For detection, we applied the ABC technique and used Vectastain Universal Elite ABC kit (Vector Laboratories, Peterborough, Cambridgeshire, UK) according to the protocol described by the manufacturer. Peroxide/3,3'-Diaminobenzidine (DAB) solution was applied until required stain intensity develops. The cell nuclei were counterstained with haematoxylin for 2 minutes.

Positive control paraffin-embedded sections of term placenta were de-paraffinised and rehydrated. Antigens were retrieved by heating in a microwave in citrate buffer (10 mM, pH=6.0) followed by blocking in horse serum and sequence of incubations as described above for cancer cells.

The immunoreactivity score for hCGβ immunohistochemistry was graded by two researchers independently and then agreed as negative (–ve), uncertain (+/–); weakly positive (+), strongly positive (++) and intensely positive (+++), as previously described (26).

RNA extraction and cDNA synthesis. Total RNA was isolated from all cells using SV Total RNA Isolation System (Promega, Southampton, Hampshire UK). One microgramme of total mRNA was used for cDNA first-strand synthesis by Verso cDNA Kit using oligo-dT primer and random hexamers in a 1:3 ratio (Thermo Scientific, Loughborough, Leicestershire, UK).

Quantitative real-time PCR. Relative quantification of *CGB* gene expression for the cells was carried out in Quantica thermocycler (Techne, Stone, Staffordshire, UK) using ABsolute™ Blue QPCR SYBR® Green Mix kit (Thermo Scientific) with ROX passive reference dye as recommended by the manufacturer using 2 μl of cDNA sample. The amplification program consisted of 1 cycle of 95˚C 10 min, followed by 40 cycles of 95˚C for 10 sec, annealing 60˚C for 5 sec, and 72˚C for 9 sec. After termination of 45 cycles, a dissociation curve analysis was performed.

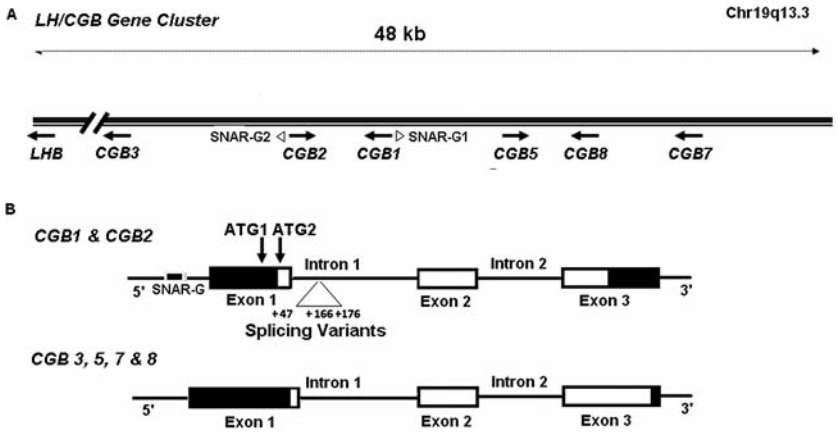

Figure 1. *Diagrammatic representation of the hCGβ gene cluster and comparison of different hCGβ genes. A. hCGβ-LHβ gene cluster; diagrammatic representation showing relative positions of the CGB genes, LHB and SNARs. Straight arrows show direction of transcription, and open arrowheads represent SNAR-G genes. Start codons – ATG_1 and ATG_2 correspond to alternative open reading frame. B: Schematic of CGB1 and -2 genes compared to CGB, CGB5, CGB7, CGB8 gene structures, illustrating exon and the intron regions of the CGB genes and indicating the potential three main splice variants reading through into intron 1 (+47 bp, +166 bp, +167 bp). Translated regions are depicted as black boxes; open reading frames are shown as open boxes. SNAR-Gs are located in the upstream region of exon 1 of genes CGB1 and -2 and are shown as a smaller black box along the gene line.*

All primers were designed in NCBI/Primer3 – BLAST tool and purchased from Sigma (Sigma Life Science, Gillingham, Dorset, UK). One set of primers were specific for *CGB1* and *-2*: forward primer (5'-CGTCCAACACCCCTCACTCC-3') designed to anneal to nucleotides 118-137 in mRNAs of human *CGB1* and mRNA of human *CGB2* (GenBank accession no. NM_033377 and NM_033378, respectively), and reverse-primer (5'-GGCAGCCCTCCTTCTCCAC-3') to nucleotides 329-347 of the mRNAs. This set of primers span intron 1 and consequently all major known spliced variants (Figure 1) would be detected. The second set of primers (F 5'-CAGCA CCTTTCTCGGGTCAC-3' and R 5'- CAGGGAGTAGGGTGTAGG AAGG-3') were specific for classical *CGB* gene mRNAs and designed to anneal to nucleotides 10-30 and nucleotides 73-95 (GenBank accession no. NM_033183). In order to check the integrity of the reverse transcriptase reaction, the expression of the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) amplified using a specific primer set (F 5'-CATGGGTGTGAACC ATGAGAAG- 3' and R 5'-GTGCTAAGCA GTTGGTGGTGC-3') was used as an endogenous control (GenBank accession no. NM_002046) in all reactions. Non-emplate control (NTC) contained complete qPCR master mix, but no cDNA.

Separation and sequencing of PCR products. Real-time PCR products were detected by Microchip Electrophoresis System MCE-202 MultiNA (Shimadzu, Milton Keynes, Buckinghamshire, UK) according to manufacturer instructions for DNA-500 kit (Shimadzu). The precise size estimation length of DNA products was based on separation of 25-bp DNA ladder (Invitrogen) on MultiNA electrochip system. As confirmation, qPCR products were also separated on 1% agarose gel, excised and purified with PureLink™ Quick Gel Extraction Kit (Invitrogen), and sequenced by GATC Biotech (London, UK).

Relative quantification of the level of CGB mRNA transcripts. For the calculation of relative quantity of *CGB* genes, we applied the $\Delta\Delta C_p$ method described by Livak and Schmittgen (28). The level of transcription of *CGB1* and *-2* and classical *CGB* genes was normalised against housekeeping gene *GAPDH* levels and calculated to relative gene expression in placental tissue (calibrator). Mean crossing point values (from triplicate reactions) were used in relative quantification. Final results are expressed as *N*-fold differences in *CGB* expression relative to calibrator gene expression level equal to 1.0.

siRNA silencing of CGB gene expression. The siRNA duplexes (Sigma-Aldrich, Gillingham, Dorset, UK) were designed primarily to target mRNA arising from the published sequences for *CGB2* (GenBank accession no. NM_033378) and *CGB8* (GenBank accession no. NM_033183). These duplex sets were distinct in target but *CGB2* siRNA duplexes cross-hybridized significantly (100%) with *CGB1*, and the *CGB8* siRNA duplexes significantly cross-hybridized (>95%) with all classical *CGB* genes (Table I). Bladder cancer cell line SCaBER cells were transfected with siRNA duplexes using CodeBreaker™ siRNA Transfection Reagent (Promega, UK) according to the manufacturer's protocol. A total of 5×10^3 cells per well were plated into 100 μl complete growth medium in 96-well plates (BD Falcon, Oxford, Oxfordshire, UK) and allowed to grow for 24 h (until they were 50% confluent). Cells were then transfected with siRNA at a final concentration of 15 nM, 20 nM and 25 nM of siRNA in sets of six replicates. Plates were incubated for a further 72 h at 37˚C and 5% CO_2. A non-specific siRNA targeting enhanced green fluorescent protein (EGFP) was included as a transfection response control. Negative controls were cells which had been treated with the transfection reagents (CodeBreaker™ siRNA; Promega), but without any siRNA.

Determination of secreted hCG and hCGβ protein by cancer cells. The media from confluent cell cultures grown in 75 cm^2 flasks from before and after specific *CGB* gene silencing were harvested for hCGβ protein quantification. hCGβ concentration was estimated using a two-site FBT-11 immunoenzymetric assay, described previously (27, 29-32). Cell numbers post-culture, were estimated using a haemocytometer and used to normalise protein concentrations (1×10^6 cells). The assay was calibrated against a

standard curve of recombinant hCGβ (Sigma) (range of 50 pg/l to 0.5 pg/l) which had been calibrated against the first international reference preparation of hCGβ (batch 75/551; NIBSC, Potters Bar, UK). Intact hCG concentrations were quantified using the USA hCG Reference Service in-house intact hCG ELISA, utilising the antibody combination of McAb2119 with 4001-POD (33). This was calibrated against the 3rd International Standard preparation of hCG (batch 75/589; NIBSC). The limit of detection (lowest standard) for the intact hCG assay was 0.75 pg/l.

Cell proliferation assay following siRNA transfection. For viability assays, cells were seeded in complete growth medium, as described above. Growth medium was replaced with 20 μl of CellTiter 96® AQueous® One Solution Cell Proliferation Assay reagent (Promega) and cells were incubated at 37˚C for 1-4 h until colour was well-developed in a humidified atmosphere with 95% air, 5% CO_2. 3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS) is a tetrazolium salt reduction-type assay method - absorbance at 490 nm was measured on a Fluostar OPTIMA (BMG Labtech, Aylesbury, Buckinghamshire, UK), and optical density was measured and expressed as percentage change relative to control cultures in 96-well plates. Data were normalised against the optical density achieved for the untreated controls, and expressed as a percentage change in cell number. Data were then pooled between plates and expressed as mean and standard deviation change in normalised cell populations from quintuplicate experiments. Statistical significance was conducted using Stats Direct™ (Altrincham, Cheshire, UK) software in ANOVA, or Friedman's two-way analysis (data were not normally distributed).

Results

Detection and sequencing of CGB gene transcripts. The *CGB* genes were detected by two separate sets of primers that differentiated the *CGB* genes 1/2 and the classical *CGB* genes *3, 5, 7* and *8*. In all cancer cell lines tested (and placenta control), transcripts for classical *CGB* genes were detected (Figure 2A – product 81 bp) and sequences matched those predicted in the GenBank database.

The transcripts of *CGB1* and *-2* genes were found only in cell lines SCaBER, RT112, C2235 and placenta (Figure 2B – product 231 bp and 396 bp). All *CGB1* and *-2* products were sequenced, and aligned with the known *CGB 1/2* sequences in the GenBank database. In regard to *CGB 1/2* transcripts, sequence analysis identified that the products arose largely from the expression of *CGB2* alone (Figure 3).

No product appeared to be derived from genomic DNA, as no high molecular mass sequences (containing introns) were observed.

Relative quantification of the level of CGB gene expression. *CGB* gene expression in the cancer cell lines was quantified and compared to the expression level of *CGB* genes in term placenta. Classical *CGB* genes were expressed at the highest level in the placental tissue and to a much lesser extent by cancer cell lines; in some cases less than 1% of that seen in term placenta. The exceptions were by the bladder cancer cell line T24 at 1.6%, SCaBER at 3.1%, and by the two breast cancer cell lines C2335 at 6.3% and C2238 at 25.0% of that seen in the placental control tissue (Table II).

CGB1 and *-2* mRNA was detected in two out of three bladder cancer cell lines (SCaBER and RT112), where the expression level was significantly higher in comparison to term placenta, both 128-fold higher. The breast cancer cell line C2335 demonstrated a 32-fold higher expression of *CGB1/2* mRNA compared to the placental control sample (Table II).

Detection of hCGβ protein. Immunostaining for hCGβ scored positively in most of the tested non-trophoblastic cancer cell lines, albeit at different levels. Significantly, bladder cancer cell line SCaBER and breast cancer cell line C2235 exhibited very weakly-positive cellular staining. The remaining cell lines stained more positively, as did the placental positive control (Table II). However, the presence and quantity of free hCGβ protein secreted into the culture media (as measured by specific hCGβ ELISA) was significant in only three of the cancer cell lines: The highest level of hCGβ protein in media was seen in the bladder cancer cell line SCaBER (4.4 ng/10^6 cells/24 h), breast cancer cell line C2235 (2.3 ng/10^6 cells/24 h) and bladder cancer cell line RT112 (0.78 ng/10^6 cells/24 h). In the remaining cancer cell lines (T24, PC-3, LN-CAP, C2238), no hCGβ was detected in the media (<0.5 ng/10^6 cells/24 h). No intact (αβ heterodimer) hCG of pregnancy was detected in any culture medium (Table II).

Effect of siRNA duplex silencing. Following siRNA control transfection, secreted hCGβ protein levels ranged from 3-4.6 pg/l for bladder cancer cells. siRNA *CGB2* oligo- duplex (25 nM) reduced secretion by 96%, while *CGB8* oligo-duplex reduced secretion by 42% of controls. At all concentrations, we observed that siRNA duplexes effectively inhibited *CGB* expression (Figure 4A). We further examined whether viability of cells in culture could be changed after silencing to determine the impact of hCGβ secretion on survival. At all concentrations of siRNA, *CGB2* oligo-duplex reduced cell numbers significantly: 25 nM by 44% ($p<0.0001$), 20 nM by 24% ($p=0.0011$), and 15 nM by 19% ($p=0.02$) compared to controls (Figure 4B). In contrast, the control siRNA directed against the sequence of an unrelated protein (EGFP) failed to produce any significant reduction in relative cell numbers, with reduction of only 13% ($p=0.22$). When *CGB8* gene was targeted by siRNA oligo-duplex, only the highest concentration (25 nM) resulted in a significant reduction of cell growth by 23% compared to control ($p=0.0004$) (Figure 4B). Non-specific silencing of control EGFP sequence had no effect on cell numbers (1.01%, $p=0.42$).

Discussion

The overexpression and secretion of ectopic hCGβ by epithelial cancer cells is well-reviewed (1, 34-37) such that vaccines

Table I. *Detail of siRNA sequences used for silencing CGB genes. siRNA sequences used for silencing CGB2 (and 1) and CGB8 (and CGB, CGB5, -7 and -8); their location on mRNA mapping to base-pairs on exon 1 (according to GenBank accession number NM_033378 for CGB2 and NM_033183 for CGB8, respectively) and the degree of homology between the siRNA duplexes and the different CGB gene sequences.*

Target gene							
Nucleotide position in exon 1	siRNA duplex Sequence	Between-gene homology (%)					
		CGB1	*CGB2*	*CGB3*	*CGB5*	*CGB7*	*CGB8*
CGB 2 position: 188	Sense 1: CAGUGCUUGCGGAAGAUAU dTdT Antisense 1: AUAUCUUCCGCAAGCACUG dTdT	100	100	37	37	37	42
CGB 2 position: 152	Sense 2: CACGGAGACUCAAUUUACU dTdT Antisense 2: AGUAAAUUGAGUCUCCGUG dTdT	100	100	42	37	37	37
CGB 2 position: 209	Sense 3: CGCUAAGAGAGAGACAUGU dTdT Antisense 3: ACAUGUCUCUCUCUUAGCG dTdT	100	100	42	42	42	42
CGB 8 position: 280	Sense 1: UCACUCCAGCAUCCUACAA dTdT Antisense 1: UUGUAGGAUGCUGGAGUGA dTdT	28	28	84	95	100	100
CGB 8 position: 322	Sense 2: AGGUUUAAAGCCAGGUACA dTdT Antisense 2: UGUACCUGGCUUUAAACCU dTdT	42	42	95	95	95	100
CGB 8 position: 135	Sense 3: AGUCUCUGAGGUCACUUCA dTdT Antisense 3: UGAAGUGACCUCAGAGACU dTdT	0	0	100	95	95	100
EGFP	Sense 1: GCAAGCUGACCCUGAAGUUCAU dTdT Antisense 1: GAACUUCAGGGUCAGCUUGCCG dTdT	0	0	0	0	0	0

against hCGβ have been developed and discussed as adjuvant treatments in cancer (1, 38-40). However, despite the wealth of evidence in the field, the molecular mechanisms are not well-defined and occasionally result in discordant data. Previous studies have failed to correlate *CGB* mRNA expression with protein expression as detected by immunohistochemistry and immunoassays (8).

Several molecular studies have been conducted on placental tissues, choriocarcinoma and epithelial cancer (particularly bladder) to try and establish a model for *CGB* gene expression. These are not necessarily comparable as the placenta expresses both α and β subunits to form and secrete the pregnancy hormone intact hCG; choriocarcinomas appear to secrete intact hCG and excess free subunits, and epithelial carcinomas express and variably secrete only the free β-subunit hCG.

Due to the high level of homology between the *CGB* gene cluster sequences, it is difficult to identify base pair sequences that are sufficiently specific enough to differentiate individual *CGB* genes. In all earlier studies, specific *CGB1* and *-2* gene expression was excluded from primer design in the belief that these were non-functioning genes (pseudogenes) simply because no functional hCG product has been identified at the time (10). In placental and choriocarcinoma tissue, all *CGB* gene expression was based solely upon mRNA detection (11) and cancer studies have shown that *CGB* mRNA is detectable in both normal and carcinoma tissue. Attempts to differentiate *CGB* expression revealed that only *CGB7* (then termed *CGB* type I) was transcriptionally active in both tumor and normal tissue and the remaining classical genes (termed type II) were indicative of cancer (22, 24, 41). In our study, it was not assumed that *CGB1* and *-2* genes were transcriptionally silent and we designed specific primers for *CGB1* and *-2* amplification which spanned intron 1 to allow detection and identification of theoretical spliced variants (11, 16-18).

Critically, our study clearly shows the expression of *CGB1* and *-2* genes by non-trophoblastic cancer cell lines and at mRNA levels vastly greater than those seen in the placental control tissue. Furthermore, sequencing reveals that this is largely due to the expression of *CGB2* rather than that of *CGB1*. *CGB1* and *CGB2* only vary by two nucleotides at positions 185 and 223 (accession numbers NM_033377.1 and NM_033378.1, respectively). The predominant nucleotides detected at these sequence positions were C and G, indicative of *CGB2* (Figure 3).

The molecular basis of earlier studies to distinguish between *CGB* gene expression was that type I gene (*CGB7*) encodes an alanine at position 117, whilst type II encode an aspartic acid (24), and it is this which is associated with malignant transformation of epithelial cells. At first, this appears to be inconsistent with our findings, but it is entirely plausible that the technology employed previously (22, 41) was incomplete. Indeed, the competitive oligonucleotide priming (COP) assay employed (which relies on differential binding on a single base pair mismatch) would have hybridised with *CGB1* and *-2* mRNA. Examining GenBank *CGB* gene sequences (Table III), it was found that mRNA arising from *CGB1* has the GAC codon (ASP) and *CGB2* gene has GCC codon (ALA) at amino acid position 117. Thus, of mRNA discarded, that arising from

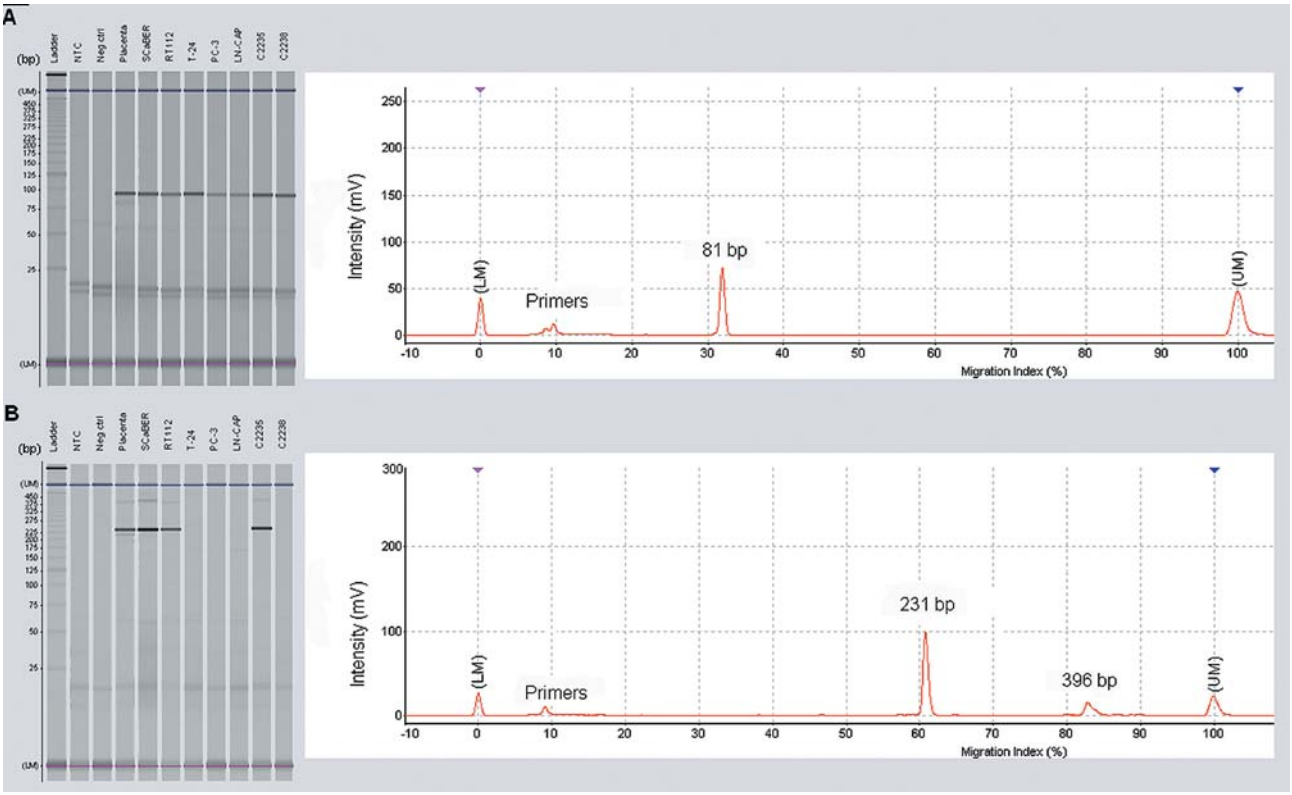

Figure 2. *Electrophoresis and electropherogram of qPCR products on Microchip Electrophoresis System MultiNA. Separation of real-time PCR products and analysis by electrophoresis system MultiNA. A: Amplification of classical CGB genes by quantitative PCR. The desired product is seen in appropriate lanes: placenta, SCaBER, RT112, T24, C2235 and C2238. B: PCR products obtained during amplification of genes CGB1 and -2. The product 231 bp corresponds to calculated specific product of CGB1 and -2, whilst the primers are the bands below 25 bp. In addition +166 bp splicing variant was amplified (with calculated by MultiNa software mass 396 bp). The position of upper (UM) and lower (LM) marker dyes of the MultiNA separation are indicated, as is the position of the base pair reference mass markers. The ladder (Invitrogen) shows the bands between 25 bp and 450 bp. Any slight difference in size of product is caused by variations in separation on different microchips. Non-template control (NTC) and negative control (Neg ctrl) are indicated. Representative electropherograms from MultiNA electrochip system of separated PCR products amplified (A) with CGB, CGB5,-7 and -8 primers with a mass of 81 bp (B) and with CGB1 and -2-specific primers with predominant product with a mass of 231 bp (calculated 229 bp), and less abundant 396 bp. LM and UM were used for calibration according to the manufacturer. The axes of the plot indicate migration index (x) and fluorescence intensity (y). Each peak in the electropherogram represents a DNA fragment of PCR product or primer which migrates according to molecular weight. Migration index is inversely proportional to the length of DNA fragments and is used by software to calculate exact mass.*

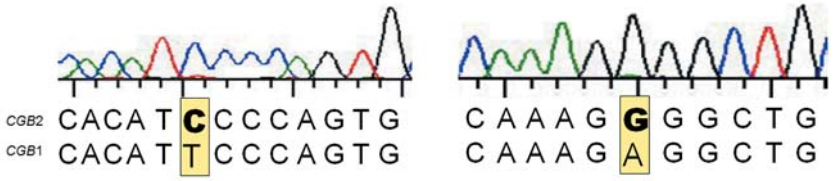

Figure 3. *Sequencing analysis of CGB1 and -2 qPCR products. Sections of specific sequencing of CGB1 and -2 PCR products (both 231 bp and 396 bp) were sequenced indicating the single marker nucleotide differences between CGB1 and CGB2 mRNA (at nucleotides 185 and 223 - accession numbers NM_033377.1 and NM_033378.1 respectively). This demonstrates that expression of CGB2 mRNA is the predominant source of CGB1/2 RT-PCR product detected in qPCR with CGB1/2-specific primers.*

CGB1 would have registered as type I and that arising from *CGB2* as type II. Here we found that abundant mRNA from *CGB2* correlated with expressed and secreted free hCGβ protein and confounds the COP assay.

A more recent study, using similar real-time PCR to our study, claimed they were unable to detect any increase in gene *CGB1* and *-2* expression (23); it should be noted that they exclusively studied normal breast and breast tumour samples.

Table II. *Relative quantification of CGB gene expression by cancer cell lines and placental tissue compared with detection of hCGβ by immunohistochemistry, and hCGβ and intact hCG secretion into culture media by specific ELISA. Average crossing points (Cp) were normalised to the GAPDH level in order to correct for variation in total cDNA concentration between samples. Normalised expression was compared as relative to that detected in the placental tissue extract which was adopted as an internal calibrator. ND: Not detected, gene expression level was, therefore, not quantified. The immunoreactivity score was graded using a semi-quantitative scoring system which scored both the percentage of positively stained cells and the intensity of immunopositivity as negative (–ve), uncertain (+/–), weakly positive (+), strongly positive (++) and intensely positive (+++). Scoring was based on positivity seen in control placental tissue. The level of hCGβ protein secreted into the culture media by the tumor cell lines (10^6 cells cultured for 24 h) was determined by specific immunoassay for free hCGβ and intact hCG.*

Cell line or tissue	Normalised Cp		Level relative to placenta		Immunostaining for hCGβ	Secretion of hCGβ (ng/1×10^6 cells/24 h)	Secretion of hCG (ng/1×10^6 cells/24 h)
	CGB(3),5,7,8	CGB1/CGB2	CGB(3),5,7,8	CGB1,2			
Placenta	22	34	1	1	+ + +	-	-
SCaBER	27	27	0.0313	128	+	4.4	<0.5
RT112	30	27	0.0039	128	+ +	0.78	<0.5
T24	28	ND	0.0156	ND	+ + +	<0.5	<0.5
PC-3	31	ND	0.002	ND	+ + +	<0.5	<0.5
LN-CAP	30	ND	0.0039	ND	+ +	<0.5	<0.5
C2235	26	29	0.0625	32	+	2.3	<0.5
C2238	24	ND	0.25	ND	+ ++	<0.5	<0.5

Table III. *Alignment of CGB gene mRNA corresponding to exon 3 coding for amino acid 117. Sequence alignment shows that the triplet codon variant GCC (alanine) previously considered unique to hCGβ protein arising from transcription of CGB7 is also found in CGB2. The location of the mutation corresponds to amino acid position 117 usually aspartic acid (GAC). The mRNA GenBank accession number and base pairs position of sequences are indicated in Bold.*

Accession number	Gene	Sequence
NM_033043 bp726-785	*CGB5*	TGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAG**GAC**TC
NM_033183 bp726-785	*CGB8*	TGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAG**GAC**TC
NM_000737 bp726-785	*CGB*	TGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAG**GAC**TC
NM_033142 bp726-785	*CGB7*	TGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAG**GCC**TC
NM_033378 bp-571-630	*CGB2*	TGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAG**GCC**TC
NM_033377 bp-571-630	*CGB1*	TGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAG**GAC**TC

In this study, we also found contradictory patterns of *CGB* gene expression in the breast cancer cell lines: C2238 did not secrete hCGβ into culture media and did not express *CGB1* and *-2* genes, but did stain strongly for hCGβ on immunohistochemistry and expressed relatively high levels (25% of that of third trimester pregnancy) of classical *CGB* genes. C2235 secreted high levels of hCGβ into culture media, expressed high levels of *CGB1* and -2 genes, but stained weakly for hCGβ on immunohistochemistry and expressed relatively low levels (6% of that of third trimester pregnancy) of classical *CGB* genes.

Most significantly, in this study, such *CGB2* gene expression was only found where hCGβ protein was detected as a secreted (culture media) product. However, the multiple splice variants arising from *CGB1* and *-2* genes had been presumed by others to translate into a non hCGβ-like protein. This proposed protein was purported to initiate translation from an alternative ATG start site (ATG_1) located 47 nucleotides upstream from the conserved classical *CGB* mRNA start site (Figure 1). Interestingly ATG_1 is not present in the other *CGB* genes due to splicing. Moreover, putative 155- and 163-amino-acid proteins (translated from an ATG_2 ORF within genes *CGB1* and *-2* respectively) are of a comparative size to 'classical' CGB gene transcripts and share up to 98% sequence homology. In the present study of *CGB1* and *-2* gene expression by epithelial cancer, we found that the predominant mRNA spliced variant arising from *CGB2* is a fully-spliced 231-bp product

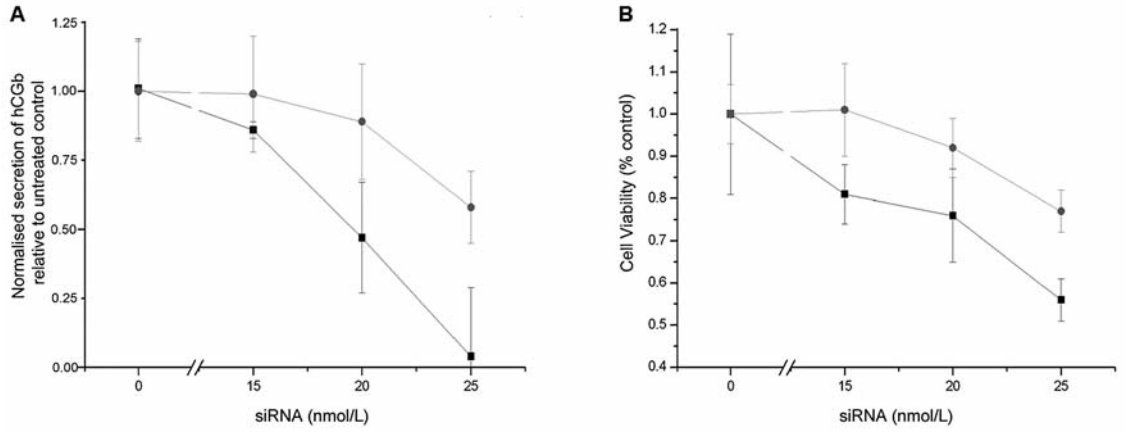

Figure 4. *Effect of silencing of CGB1 and -2 and classical CGB genes on bladder cancer cell line SCaBER. A: Secretion of hCGβ into culture media of SCaBER cancer cells treated with increasing concentrations of siRNA targeting CGB1 (—■—) and -2 or CGB, CGB5, -7 and -8 (—●—). Values are expressed as percentage of untreated control and each point is the mean of six replicates of the assay ran in duplicate; the bars represent standard deviation about the mean. EGFP silencing represents non-specific effects of introducing a non-functioning siRNA.B: Influence of RNA silencing on SCaBER cell viability – increasing concentration of siRNA (nmol/l) and relative level of viable cells are shown. MTS assay values are expressed as a percentage that of untreated control. Results are the mean from six replicates of the assay. The bars represent standard deviation about the means. EGFP silencing represents non-specific effects of introducing a non- functioning siRNA.*

(shown in the electropherogram, Figure 2B), which sequenced as predicted, and would therefore have been available for translation to a mature hCGβ-like protein.

It has been proposed that the expression of *CGB* type II genes significantly increases in many non-trophoblastic malignant tissues and may correlate with elevated levels of hCGβ in patients' serum. The results obtained by our study showed expression of all classical *CGB* genes by all non-trophoblastic cell lines. However, this does not correlate with secreted protein levels. Immunohistochemical detection indicates that protein expressed might be held intracellularly for the majority of cancer cell lines, and not secreted. A possible explanation may be that transcription and translation of the *CGB* gene cluster does not result in secretion *per se*, and that more specific gene or cellular processing may be required for active autocrine/paracrine expression. The levels of mRNA arising from classical *CGB* genes in our cell lines are generally less that 1% of that found in third trimester placental tissue. Interestingly, in one breast cancer cell line (C2238), mRNA levels were as high as 25% of that seen in placental tissue, yet no secreted hCGβ protein was detected.

As already stated, transcriptional messages were detected in all cell lines using primers designed to amplify classical *CGB* genes, including the non-hCGβ protein-secreting bladder cancer cell line T24. However, mRNA arising from *CGB1* and *-2* gene was only detected in placental tissue, breast cancer cell line C2235, and bladder cancer cell lines RT112 and SCaBER (Figure 2B). Furthermore, these are the only cell lines studied which secrete hCGβ protein into their culture media (Table II). The high level of *CGB1* and *-2* gene expression in these cancer cell lines may be evidence of their functionality, and this is supported by the interesting correlation with protein secretion. This complex condition of expression may explain ectopic hCGβ secretion by tumors when simple 'all *CGB*' mRNA detection in a tumor fails to correlate with circulating levels found in patients' blood and urine, as reviewed by Butler and Iles (1), and explain why molecular and immunological hCGβ studies often differ. In much the same way as antibody selection has become important in immunoassays detecting the right 'kind' of hCG for the management of trophoblastic diseases (42), we might find ourselves having to use the right kind of molecular markers to improve the diagnostic and prognostic power of hCGβ in epithelial cancer.

More recently it has been shown that *CGB1* and *-2* is expressed in the placenta (14), testes (13, 43) and in ovarian tissue (44). Moreover, in testis, the predominant *CGB1* and *-2* mRNA transcript is also a fully-spliced mRNA with translation initiation from AUG_2. This was also proposed to give rise to an hCGβ protein homologous to the protein coded by classical *CGB* genes. Similarly, we have now demonstrated that the major transcribed splice variant of *CGB2* mRNA is fully-spliced and would result in a recognisable hCGβ peptide in agreement with these findings (13, 43). A +166 splice variant was also detected arising from *CGB2* gene expression, but at lower levels in the hCGβ-secreting cancer cell lines. It has been suggested that these are CGB2a and CGB2b proteins (13, 43) and that the splice variant +166 bp encodes a novel peptide using a different open reading frame starting from ATG1. However, the function of this peptide remains unclear.

To study the possible effect of any hCGβ protein derived from expression of *CGB1* and *-2*, we inhibited its

translation by specifically targeting the mRNA with a mix of siRNA duplexes for different regions within exon 1 of *CGB1* and *-2*, and *CGB8* (with high homology to other classical *CGB* genes – Table I). Silencing of *CGB* genes with siRNA duplexes resulted in the reduction of the viable cancer cell number (Figure 3B). However, at all concentrations of siRNA, *CGB2* oligo-duplex cell numbers were reduced significantly more than if siRNA targeted classical *CGB* mRNA. This was also reflected in the amount of hCGβ protein secreted by the exposed cells, with *CGB2* oligo-duplex siRNA effectively abolishing secretion (Figure 3A).

Non-specific silencing of *CGB* gene expression was shown to induce increased apoptosis of HeLa cells (20) and inhibit growth of SCaBER cells *in vitro* (21). The significant reduction of gene expression was demonstrated using a modified U1 snRNA in which the first 10 nucleotides were substituted with a complementary *CGB* gene sequence present in exon 3 (20). However, in neither of these studies was any distinction made between which of the six *CGB* genes were silenced. Moreover, silencing of any gene coding beta subunit of human chorionic gonadotropin could lead to reduction not only of free hCGβ, but also of heterodimeric hCG known to be produced by HeLa cells (45-47). Here by distinguishing the target region more specifically' we were able to examine the respective influence of gene groups on cancer cell viability.

Specific targeting of *CGB1* and *-2* is much more effective in reducing cancer cell numbers than silencing classical *CGB* genes, or general silencing of 'all' *CGB*, and although it was once considered to be a pseudogene, *CGB2* may now be considered an active gene which is important for cancer growth. Furthermore, CGB2 may interact at the mRNA level with other gene transcripts such as the snRNA, *SNAR-G2* which is encoded adjacent to *CGB2* (13, 43, 48) (Figure 1). The profile of *CGB1* and *-2* gene expression during the first trimester correlates with successful pregnancies (14, 16) and particularly elevated levels of *CGB1/2* mRNA were reported in ectopic and molar pregnancies (12). It can be postulated that these pregnancy implantation associations, together with data present here, indicate a role for *CGB1* and *-2* (possibly only *CGB2*)-derived free hCGβ in the invasive process (elevated free hCGβ is pronounced in early pregnancy).

In conclusion, up-regulation of *CGB2* gene expression in common epithelial cancer cells suggests an important function for this presumed pseudogene in ectopic hCGβ secretion and oncogenesis. This finding could play a significant role in cancer diagnostics by differentiating aggressive from non-aggressive tumors.

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