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2019-08-18

Lintula , S , Mirtti , T , Rannikko , A , Bützow , A , Lempiäinen , A , Stenman , J , Stenman , U-H & Hotakainen , K 2019 , ' Tumor expression of human chorionic gonadotropin beta mRNA and prognosis of prostate cancer treated by radical prostatectomy ' , Scandinavian Journal of Clinical & Laboratory Investigation , vol. 79 , no. 6 , pp. 424-430 . https://doi.org/10.1080/00365513.2019.1

http://hdl.handle.net/10138/311848
https://doi.org/10.1080/00365513.2019.1639214

unspecified
acceptedVersion

Tumor expression of human chorionic gonadotropin beta mRNA and prognosis of prostate cancer treated by radical prostatectomy

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Tumor expression of human chorionic gonadotropin beta mRNA and prognosis of prostate cancer treated by radical prostatectomy

Abstract

The beta subunit of human chorionic gonadotropin (hCGβ) is encoded by six genes (*CGB*) classified as type I and type II. *CGB* mRNA is produced in large amounts by trophoblastic tissues and in small amounts by several cancerous tissues including prostate cancer and by a few benign tissues, including the prostate. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was used to study the expression levels of all *CGB* mRNAs together (total *CGB* mRNA) and the two types of *CGB* mRNA separately in non-cancerous (n = 74) and cancerous prostatic tissue obtained by radical prostatectomy (n = 193). RNA was isolated from formalin-fixed paraffin-embedded (FFPE) samples and mRNA levels of *CGB* were correlated with disease-specific survival. Total *CGB* mRNA concentrations were significantly lower ($p < 0.0001$) in cancerous than non-cancerous prostatic tissue. Separate analysis of type I *CGB* and type II *CGB* mRNA showed that both type I *CGB* ($p < 0.0001$) and type II *CGB* mRNA ($p = 0.007$) are lower in cancerous tissue than in non-cancerous tissue. Low type II *CGB* mRNA level in cancerous tissue of was associated with shorter cancer-specific survival ($p = 0.001$) of prostate cancer patients treated by radical prostatectomy.

Key words: Human, Chorionic gonadotropin beta, , expression, prostate, cancer,

Introduction

Prostate cancer is the most common non-skin cancer in males. Its incidence has increased dramatically since the introduction of opportunistic screening based on determination of prostate-specific antigen (PSA) and biopsy of patients with elevated serum concentrations of PSA. While this has reduced mortality, it has also led to overdiagnosis and overtreatment [1]. These can be reduced by active surveillance of patients with favorable Gleason score, which is the most reliable prognostic factor. However, additional prognostic factors are needed.

Human chorionic gonadotropin (hCG) is a heterodimeric hormone consisting of an α (hCGα) and a β subunit (hCGβ). hCG is produced in large amounts by the placenta during pregnancy and also by trophoblastic tumors and many gonadal germ cell tumors. Isolated production of hCGβ is also observed in 30 – 50% of most non-trophoblastic tumors, and elevated serum concentration of hCGβ is associated with several cancers [2-3]. hCGβ is encoded by six *CGB* genes: *CGB1*, *CGB2*, *CGB* (originally called *CGB3*), *CGB5*, *CGB7*, and *CGB8* [4]. *CGB*, *CGB5*, and *CGB8* produce one protein-coding mRNA transcript each, while *CGB7* has three protein coding transcripts and two non-coding transcripts, the so-called processed transcripts. *CGB1* and *CGB2*, earlier considered pseudogenes, have two transcripts each, with two different translation initiation sites (AUG_1 and AUG_2). It has been speculated that the AUG_2 site is a common start site for all hCGβ proteins, while the AUG_1 site in *CGB1* and *CGB2* using a different open reading frame would encode a non-hCGβ protein, the structure and function of which are unknown [5].

Expression of the various *CGB* genes at the mRNA level has been found in several non-trophoblastic tumors, including those of the breast, thyroid, kidney, bladder, and prostate [6-10]. *CGB*7 and, according to recent information, *CGB2*, encode an alanine (GCC) at position 117, whereas *CGB*, *CGB5*, *CGB*8, and *CGB1* encode aspartic acid (GAC) at this position. Based on the difference at amino acid 117, *CGB*, *CGB5*, and *CGB*8 have been called type II genes, while *CGB7* is classified as a type I gene. The type I gene has been reported to be expressed in many non-

transformed tissues and type II genes in placental and malignant tissues [6]. However, other studies show that expression of the various genes is not specific for cancerous or non-cancerous tissues [5, 11]. Furthermore, increased expression of *CGB1* and *CGB2*, which were earlier considered pseudogenes, has been found in ectopic and molar pregnancies and in the male reproductive tract [12].

To further evaluate the role of hCGβ in prostate cancer, we studied the expression levels of *CGB* mRNAs in non-cancerous and cancerous prostate tissue and compared these using disease-specific and overall survival as endpoints.

Materials and Methods

Cell lines

As standards for quantitative RT-PCR (RT-qPCR) of *CGB*, we used mRNA isolated from T24 bladder cancer (type I genes) and JEG-3 trophoblast cell lines (type II genes) (American Type Culture Collection, Rockville, MD, USA). The cells were grown at 37°C in a humidified atmosphere with 5% CO_2. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 000 units/l penicillin, and 100 µg/l streptomycin.

Patients and Samples

Clinical characteristics of the prostate cancer patients are shown in Table 1. FFPE (formalin-fixed paraffin-embedded) samples were collected from 193 prostate cancer patients undergoing radical prostatectomy during 1984-1998. Median follow-up time was 16.1 years (range 0.57 – 26.10 years).

RNA was extracted from FFPE samples of all 193 patients and adjacent non-cancerous tissue were extracted from 74 patients out of 193. Total sample number and number of analyzed samples with different methods as well as number of samples with detectable amounts of different mRNAs are shown in Table 2.

SUPPLEMENTAL TABLE S1. MIQE checklist.

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	**E**	Experimental Group (A): FFPE samples from patients with Prostate cancer, Control groups (B): B1) non-ca tissue from same FFPE samples, B2)FFPE samples from BRH patients
Number within each group	**E**	A: n= 193, B1= 74, B2= 47
Assay carried out by core lab or investigator's lab?	D	
Acknowledgement of authors' contributions	D	
SAMPLE		
Description	**E**	FFPE Prostate tissue from Radical Prostatectomy
Volume/mass of sample processed	D	
Microdissection or macrodissection	**E**	macrodissection
Processing procedure	**E**	
If frozen - how and how quickly?	**E**	not frozen
If fixed - with what, how quickly?	**E**	fixed with 4% formalin (pH 7.4) overnight after 0-2h from prostatectomy
Sample storage conditions and duration (especially for FFPE samples)	**E**	room temperature appr. 1-26 years
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	**E**	Total RNA was extracted by the protocol of Chomczynski and Sacchi with minor modifivcations (described in text)
Name of kit and details of any modifications	**E**	No kit was used
Source of additional reagents used	D	
Details of DNase or RNAse treatment	**E**	The extracted total RNA was then DNase-treated by adding to 10 µg of RNA, 5 µl of 10x DNase buffer (100 mM Tris, pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$) and 10 units of DNase I and incubating for 30 min at 37°C, after which the DNase was inactivated for 5 min at 70°C. RNA was then precipitated with 5 µl of 2 M sodium acetate (pH 4.0) and 140 µl of 100% ethanol overnight at -20°C, after which the sample was centrifuged at 5200 g for 30 min at 4°C. The supernatant was carefully removed and the RNA pellet washed with 200 µl of 70% ethanol, after which the sample was centrifuged at 5200 g for 20 min at 4°C. The supernatant was removed, and the RNA was dissolved in 20 µl of RNase-free water.
Contamination assessment (DNA or RNA)	**E**	reverse transcription controls (W/O enzyme) were performed in order to assess the absence of DNA in the RNA sample
Nucleic acid quantification	**E**	RNA concentration was determined by measuring te absorbance at 260 nm UV light
Instrument and method	**E**	NanoDrop 1000 (Thermo Scientific)
Purity (A260/A280)	D	1.9-2.0
Yield	D	500-1000 ng/µl
RNA integrity method/instrument	**E**	RNA integrity was not determined (RNA from FFPE samples is degraded)
RIN/RQI or Cq of 3' and 5' transcripts	**E**	Not applicable
Electrophoresis traces	D	
Inhibition testing (Cq dilutions, spike or other)	**E**	We think that standard curve is sufficient to rule out the presence of inhibitors in RT or PCR
REVERSE TRANSCRIPTION		
Complete reaction conditions	**E**	Total RNA (450 ng) was denaturated for 5 min at 70°C and multiplex reverse-transcribed in 5 µl reaction volume with RevertAid Premium reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) at 55°C for 30 min using gene-specific antisense primers (Table 2a). After reverse transcription, the unbound reverse transcription primers were degraded by Exonuclease I (New England Biolabs, Ipswitch, MA, USA) at 42°C for 45 min.
Amount of RNA and reaction volume	**E**	Amount of RNA:450 ng, reaction volume 5 µl
Priming oligonucleotide (if using GSP) and concentration	**E**	Gene specific primers for CGB type 1 and type 2 as well as Total CGB and GAPDH. All in concentration 100 µM
Reverse transcriptase and concentration	**E**	RevertAid Premium reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) concentration 200U/µl
Temperature and time	**E**	Temperature:55°C,Time:30 min
Manufacturer of reagents and catalogue numbers	D	
Cqs with and without RT	D*	
Storage conditions of cDNA	D	
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	**E**	

Sequence accession number	**E**	ENSG00000111640 (GAPDH); ENSG00000267631 (CGB1); ENSG00000104818 (CGB2); ENSG00000104827 (CGB); ENSG00000189052 (CGB5); ENSG00000196337 (CGb7); ENSG00000213030 (CGB8)
Location of amplicon	D	
Amplicon length	**E**	(SYBR Green chemistry-PCR): GAPDH 127bp, CGB total 130 bp; TaqMan chemistry GAPDH 97 bp, type 1 and type 2 CGB 98 bp
In silico specificity screen (BLAST, etc)	**E**	BLAST
Pseudogenes, retropseudogenes or other homologs?	D	
Sequence alignment	D	
Secondary structure analysis of amplicon	D	
Location of each primer by exon or intron (if applicable)	**E**	
What splice variants are targeted?	**E**	All known spice variants
qPCR OLIGONUCLEOTIDES		
Primer sequences	**E**	Table 2a and 2b in paper
RTPrimerDB Identification Number	D	
Probe sequences	D**	
Location and identity of any modifications	**E**	No modifications were done
Manufacturer of oligonucleotides	D	
Purification method	D	
qPCR PROTOCOL		
Complete reaction conditions	**E**	Real-time qPCR for GCB type I and type II as well as for GAPDH separately were carried out with SensiFAST Probe Lo-ROX Kit (Bioline) with 4 pmol of each primer and 2 pmol of hydrolysis probes in a final volume of 10 µl using Applied Biosystem 7500 Fast Real-Time PCR System (Life Tecnologies, Carlstad, CA) and thermocycling conditions 95 °C for 5 min, 40 cycles at 95 °C for 10s, at 65 °C for 20s. Endpoint RT-qPCR for total CGB and GAPDH separately was carried out with Dynamo HS SYBR Green 2 x master Mix (Thermo Fisher Scientific) with 5 pmol of each primer in a final volume of 10.9 µl using Applied Biosystem 7500 Fast Real-Time PCR System (Life Tecnologies, Carlstad, CA) and thermocycling conditions 95 °C for 15 min, 10 cycles at 95 °C for 30s, at 63 °C for 30s and 30 cycles at 95 °C for 10s, at 63 °C for 30s. Following SYBR Green-based end-point RT-qPCR, the specificity of the amplification products was verified by melting curve analysis. All reactions were run in duplicate and for all samples no-RT -controls were run to exclude possible DNA contamination as well as NTC-controls to exclude PCR contamination and primer-dimers. In addition to calibration curves, positive control sample was included in each run.
Reaction volume and amount of cDNA/DNA	**E**	reaction volume: 10µl, amount of cDNA: 2µl
Primer, (probe), Mg++ and dNTP concentrations	**E**	Real_time qPCR: Probe conc. 200nM, primer conc.400nM, dNTP conc. Mg and dNTP was included to Sensifast Lo-Rox Kit. Endpoint RT-PCR: Primer conc: 500nM, Dynamo HS Sybr Green 2X master mix(contains a hot-start version of a modified Tbr DNA polymerase, SYBR Green I, optimized PCR buffer, 5 mM MgCl2, dNTP
Polymerase identity and concentration	**E**	Real-time qPCR: Sensifast (Bioline), endpoint RT-qPCR: hot-start version of a modified Tbr DNA polymerase (Thermo Fisher Scientific)
Buffer/kit identity and manufacturer	**E**	Real-Time qPCR: SensiFAST Probe Lo-ROX Kit (Bioline); Endpoint RT-qPCR: Dynamo HS SYBR Green 2 x master Mix (Thermo Fisher Scientific)
Exact chemical constitution of the buffer	D	
Additives (SYBR Green I, DMSO, etc.)	**E**	Endpoint RT-qPCR: 5 x ROX (Thermo); (SYBR Green was included to kit in both systems)
Manufacturer of plates/tubes and catalog number	D	
Complete thermocycling parameters	**E**	Real-time qPCR:thermocycling conditions 95 °C for 5 min, 40 cycles at 95 °C for 10s, at 65 °C for 20s; Endpoint RT-qPCR thermocycling conditions 95 °C for 15 min, 10 cycles at 95 °C for 30s, at 63 °C for 30s and 30 cycles at 95 °C for 10s, at 63 °C for 30s
Reaction setup (manual/robotic)	D	
Manufacturer of qPCR instrument	**E**	Applied Biosystem 7500 Fast Real-Time PCR System (Life Tecnologies)
qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	

Specificity (gel, sequence, melt, or digest)	**E**	Melting curve analysis, ramping from 63 ˚C to 95 ˚C. All reactions were run in duplicate and for all samples no-RT - controls were run to exclude possible DNA contamination as well as NTC-controls to exclude PCR contamination and primer dimers
For SYBR Green I, Cq of the NTC	**E**	Cq of the NTC was over 40 and in most cases not seen at all
Standard curves with slope and y-intercept	**E**	Endpoint RT-qPCR: Total CGB: y=-3.176 x 26.4, GAPDH y=3.381 x 25.7. Real-time qPCR: hCGbeta type Iy=-3.423 x 31.2, hCGbeta type II y=-3.379 x 22.2, GAPDH y= 3.482 x 21.8
PCR efficiency calculated from slope	**E**	Endpoint RT-qPCR: Total CGB: 106.8%, GAPDH 97.8%. Real-time qPCR: hCGbeta type I 92,4%, hCGbeta type II 97,6%, GAPDH r2 =93,7%.
Confidence interval for PCR efficiency or standard error	D	
r2 of standard curve	**E**	Endpoint RT-qPCR: Total CGB: r2 =0.984 GAPDH r2 =0.992. Real-time qPCR: hCGbeta type I r2 =0.944, hCGbeta type II r2 =0.986, GAPDH r2 =0.998.
Linear dynamic range	**E**	Standard curves for totalCGB mRNA and GAPDH for endpoint RT-qPCR were linear over 6 orders (CGB, $r^2 = 0.98$) or 5 (GAPDH, $r^2 = 0.99$) of magnitude. The standard curves for real-time RT-qPCR for CGB type I and II were linear over 6 orders (CGB type I, r2 = 0.94 and CGB type II, r2 = 0.98) of magnitude and for GAPDH over 7 orders (r2 = 0.99) of magnitude
Cq variation at lower limit	**E**	2 ˚C
Confidence intervals throughout range	D	
Evidence for limit of detection	**E**	Lowest standard in standard curve
If multiplex, efficiency and LOD of each assay.	**E**	Lowest calibrator of each gene was used as a limit of detection in each run: for total CGB mRNA and GAPDH mRNA in endpoint RT-qPCR, 0.01 ng of placental RNA, and in real-time RT-qPCR for CGB type I, 4 ng of T24 bladder carcinoma cell line RNA and for CGB type II and GAPDH, 0.005 ng of the JEG chorion carcinoma cell line RNA.
DATA ANALYSIS		
qPCR analysis program (source, version)	**E**	7500 Sofware v.2.0.6
Cq method determination	**E**	The threshold is used to specify Cq values of samples
Outlier identification and disposition	**E**	None of Cq was disposed
Results of NTCs	**E**	NTCs did not give any signal or there was very high Cq value (>40)
Justification of number and choice of reference genes	**E**	Based on earlier reports, GAPDH was chosen as a reference gene. The suitability of it has been explained in the text
Description of normalisation method	**E**	Normalisation were done by calculating the ratio of CGB/GAPDH mRNAs
Number and concordance of biological replicates	D	
Number and stage (RT or qPCR) of technical replicates	**E**	All RT-qPCR reactions were performed in duplicate
Repeatability (intra-assay variation)	E	
Reproducibility (inter-assay variation, %CV)	D	
Power analysis	D	
Statistical methods for result significance	**E**	Differences between various categories were analyzed using Mann-Whitney U-test. Correlation between hCGβ mRNA and clinical variables was analyzed by Spearman rank-order correlation test. Kaplan-Meier survival analysis and log-rank test were used to estimate the effect of CGB gene expression on prostate cancer-specific survival and overall survival.
Software (source, version)	E	IBM SPSS software (version 22.0)
Cq or raw data submission using RDML	**D**	

the RNA was precipitated by adding a double volume of absolute ethanol and incubating at -70°C overnight. The precipitate was washed twice with 500 µl of 70% ethanol and centrifuged at 5200 *g* for 10 min at 4°C to re-pellet the sample. After the final wash, the pellet was dried at room temperature and dissolved in 20 µl of RNase-free water.

The extracted total RNA was then DNase-treated by adding to 10 µg of RNA, 5 µl of 10x DNase buffer (100 mM Tris, pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$) and 10 units of DNase I and incubating for 30 min at 37 °C, after which the DNase was inactivated for 5 min at 70°C. RNA was then precipitated with 5 µl of 2 M sodium acetate (pH 4.0) and 140 µl of 100% ethanol overnight at -20 °C, after which the sample was centrifuged at 5200 g for 30 min at 4 °C. The supernatant was carefully removed and the RNA pellet washed with 200 µl of 70% ethanol, after which the sample was centrifuged at 5200 g for 20 min at 4 °C. The supernatant was removed, and the RNA was dissolved in 20 µl of RNase-free water. Total RNA from cell lines was isolated by Qiagen RNeasy as described by the manufacturer.

Reverse Transcription and Exonuclease I treatment

Total RNA (450 ng) was denaturated for 5 min at 70 °C and multiplex reverse-transcribed in 5 µl reaction volume with RevertAid Premium reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) at 55°C for 30 min using gene-specific antisense primers for *CGB* and *GAPDH* (Table 3a, 3b). After reverse transcription, the unbound reverse transcription primers were degraded by Exonuclease I (New England Biolabs, Ipswitch, MA, USA) at 42°C for 45 min.

Primers and probes

Primers for endpoint RT-qPCR (total *CGB* and *GAPDH*) as well as primers and hydrolysis probes for TaqMan Chemistry RT-qPCR (*CGB* type I, *CGB* type II and *GAPDH*) were designed using OLIGO Primer Analysis Software, version 7.0 (Molecular Biology Insights, Colorado Springs, CO, USA) and their specificity was verified with BLAST. All known splicing variants of different genes were included. The primers were purchased from TAG Copenhagen (Copenhagen, Denmark). Sequences of primers and probes as well as all their target sequences and length of amplicons are shown in Tables 3a and 3b.

PCR

Real-time qPCR for *GCB* type I and type II as well as for *GAPDH* separately were carried out with SensiFAST Probe Lo-ROX Kit (Bioline) with 4 pmol of each primer and 2 pmol of hydrolysis probes in a final volume of 10 µl using Applied Biosystem 7500 Fast Real-Time PCR System (Life Technologies, Carlstad, CA) and thermocycling conditions 95 ˚C for 5 min, 40 cycles at 95 ˚C for 10s, at 65 ˚C for 20s.

Endpoint RT-qPCR for total *CGB* and *GAPDH* separately were carried out with Dynamo HS SYBR Green 2 x master Mix (Thermo Fisher Scientific) with 5 pmol of each primer in a final volume of 10.9 µl using Applied Biosystem 7500 Fast Real-Time PCR System (Life Tecnologies, Carlstad, CA) and thermocycling conditions 95 ˚C for 15 min, 10 cycles at 95 ˚C for 30s, at 63 ˚C for 30s and 30 cycles at 95 ˚C for 10s, at 63 ˚C for 30s. Following SYBR Green-based end-point RT-qPCR, the specificity of the amplification products was verified by melting curve analysis.

All reactions were run in duplicate and for all samples no-RT - controls were run to exclude possible DNA contamination as well as NTC-controls to exclude PCR contamination and primer-dimers. Positive control sample was included in each run.

Quantification of RT-PCR results

Three different dilution of RNA isolated from cell lines or placental tissue were used as calibrators in every PCR run to confirm inter-assay reproducibility as follows: For total *CGB* mRNA and *GAPDH* mRNA in endpoint RT-qPCR, we used 100 ng, 1 ng, and 0.01 ng of placental RNA. In real-time RT-qPCR for *CGB* type I, 400 ng, 40 ng, and 4 ng of T24 bladder carcinoma cell line RNA and for *CGB* type II and *GAPDH*, 50 ng, 0.05 ng, and 0.005 ng of the JEG chorion carcinoma cell line RNA was used. Lowest calibrator of each gene was used as the limit of detection in each run. The relative expression of total *CGB* as well as *CGB* type I and II mRNAs were calculated by normalizing mRNA against *GAPDH* mRNA in each sample.

Suitability of *GAPDH* as a reference gene was tested between benign (n = 111) and malignant (n = 333) sample cohort. In this experiment 1000 molecules of genomic DNA was used as an internal control in DNase-treated RNA-samples and the ratio of mRNA to genomic DNA was measured. No difference of *GAPDH* mRNA expression levels between malignant and benign samples were seen (data not shown).

Statistical Analyses

Statistical analyses were performed using IBM SPSS software (version 22.0). Differences between various categories were analyzed using Mann-Whitney U-test. Correlation between *CGB* mRNAs and clinical variables was analyzed by Spearman rank-order correlation test. Kaplan-Meier survival analysis and log-rank test were used to estimate the effect of *CGB* gene expression on prostate cancer-specific survival and overall survival. Survival time was calculated from the date of radical prostatectomy to the date of last follow-up or death. In analysis of cancer-specific survival, death

from prostate cancer was considered as the event. In analysis of overall survival, the event was all causes of death. All tests were two-sided and P-values of less than 0.05 were considered significant.

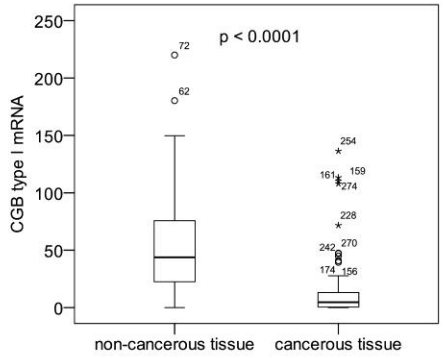
Results

Sensitivity and specificity of the assays

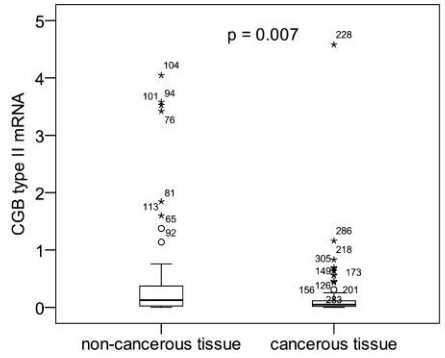
Dynamic range of each gene was measured by standard curves. Standard curves for total *CGB* mRNA and *GAPDH* for endpoint RT-qPCR were linear over 6 (*CGB*, $r^2 = 0.98$) or 5 orders (*GAPDH*, $r^2 = 0.99$) of magnitude (Suppl. Figure S1.).

Figure 1. CGB mRNA levels in non-cancerous and cancerous prostate tissue. The significance of the difference was calculated by the Mann–Whitney U-test. Type I (A), type II (B) and total CGB mRNA (C) expression levels in non-cancerous and cancerous prostatic tissue showing significantly higher expression in non-cancerous tissue.

A


p < 0.0001
CGB type I mRNA
250
200
150
100
50
0
72
62
254
161
159
274
228
242
270
174
156
non-cancerous tissue
cancerous tissue

B


p = 0.007
CGB type II mRNA
5
4
3
2
1
0
228
104
101
94
76
81
113
65
92
286
218
305
173
156
201
non-cancerous tissue
cancerous tissue

C

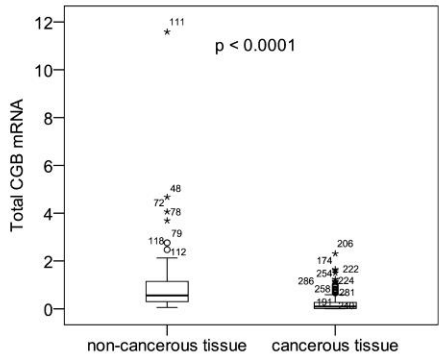
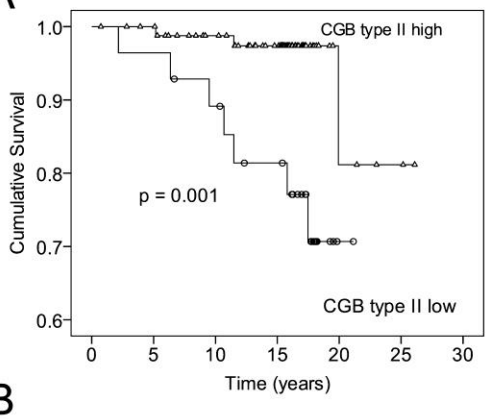
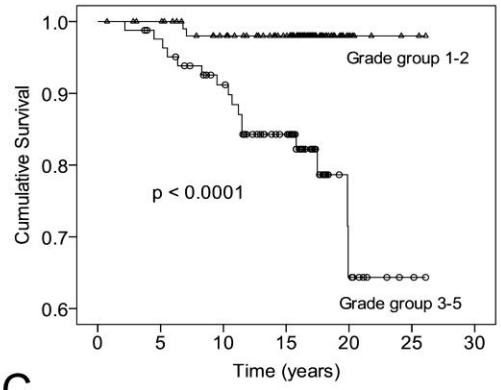

p < 0.0001
Total CGB mRNA
12
10
8
6
4
2
0
111
48
72
78
79
118
112
206
174
222
254
224
286
258
281
non-cancerous tissue
cancerous tissue

Figure 2. Kaplan–Meier analysis of cancer specific survival of prostate cancer patients. (A) Tissue mRNA concentration of CGB type II in the lower quartile predicts shorter disease specific survival of prostate cancer patients ($p = .001$). (B) Grade group 3–5 predicts shorter disease specific survival of prostate cancer patients ($p < .0001$). (C) Tumor stage 3–4 predicts shorter disease specific survival of prostate cancer patients ($p < .0001$).

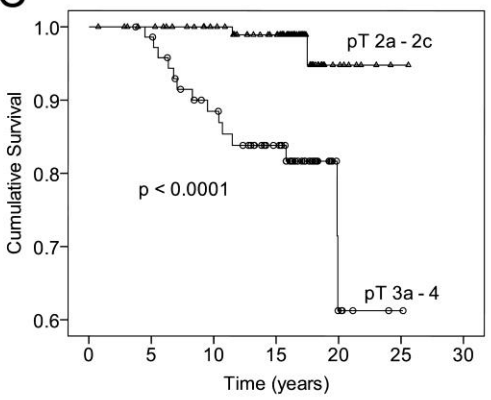
A


CGB type II high
p = 0.001
CGB type II low
Cumulative Survival
1.0
0.9
0.8
0.7
0.6
0
5
10
15
20
25
30
Time (years)

B


Grade group 1-2
p < 0.0001
Grade group 3-5
Cumulative Survival
1.0
0.9
0.8
0.7
0.6
0
5
10
15
20
25
30
Time (years)

C


pT 2a - 2c
p < 0.0001
pT 3a - 4
Cumulative Survival
1.0
0.9
0.8
0.7
0.6
0
5
10
15
20
25
30
Time (years)

The standard curves for real-time RT-qPCR for *CGB* type I and II were linear over 6 orders (*CGB* type I, $r^2 = 0.94$ and *CGB* type II, $r^2 = 0.98$) of magnitude and for GAPDH over 7 orders ($r^2 = 0.99$) of magnitude (Suppl. Figure S2.). Primers for type I and II are common to all genes (*CGB*1, 2, 3, 5, 7 and 8). Probe to type I *CGB* detects only *CGB*7 and probe to type II *CGB* detects *CGB*1, 2, 3, 5 and 8 (Table 3b). The specificity of *CGB* type I and II hydrolysis probes were studied using dilutions of T24 and JEG-3 cell lines, respectively. As shown before, type I mRNA was expressed in the T24 cell line and type II mRNA in JEG-3 cells [16]. The efficiency of different RT-qPCR methods were between 92-106 % (Suppl. Table S1).

Expression of type I and II CGB transcripts in non-cancerous and cancerous prostate tissue

CGB type I and II mRNA was determined in 114 malignant and 54 non-cancerous samples. RT-qPCR results for *CGB* type I mRNA (gene 7) were detected for 84% (96/114) of the tumor samples and for 91% (49/54) of the samples from adjacent non-cancerous tissue. *CGB* type II mRNA (genes 1, 2, 3, 5, 8) was detected in 96% (109/114) of the tumor samples and in 98% (53/54) of adjacent non-cancerous tissue. The difference between cancerous tissue and non-cancerous tissue was statistically significant for both type I *CGB* and type II *CGB* mRNA (Mann-Whitney U-test; $p < 0.0001$ and $p = 0.007$, respectively) (Figure 1a and b), showing strong downregulation of both mRNA types in cancer tissue.

Expression of total CGB mRNA in non-cancerous and cancerous prostate tissue

Total *CGB* mRNA expression was determined in 178 of 193 malignant prostatic tissues, in 62 of 74 non-cancerous prostatic tissues, and in all 47 BPH samples by RT-qPCR system, which detects all

ten hCGβ protein-coding transcripts from genes *CGB* 1, 2, 3, 5, 7, and 8. Total *CGB* expression was detected in 79% (141/178) of the tumor samples and in all non-cancerous (62/62) and BPH (47/47) samples. The median level of total *CGB* mRNA differed significantly between cancerous and non-cancerous tissue, showing strong downregulation in cancer (Mann-Whitney U-test; $p < 0.0001$) (Figure 1c).

Correlation between clinical characteristics and survival

There was a highly significant inverse correlation between total *CGB* mRNA and tumor stage (Spearman's rho correlation test; $p = 0.002$, correlation coefficient -0.240) and serum PSA ($p = 0.019$, correlation coefficient -0.187). The total *CGB* mRNA correlated positively with both type I ($p < 0.0001$, correlation coefficient 0.402) and type II mRNAs ($p < 0.0001$, correlation coefficient 0.299). No correlation between total *CGB* and Gleason score was found ($p = 0.102$, correlation coefficient -0.124).

Type I mRNA correlated negatively with tumor stage ($p = 0.003$, correlation coefficient -0.280), but showed no correlation with serum PSA or Gleason score. Type II mRNA correlated negatively with Gleason score ($p = 0.003$, correlation coefficient -0.271) and tumor stage ($p = 0.014$, correlation coefficient -0.232), but showed no correlation with serum PSA.

In Kaplan-Meier analysis, patients with low *CGB* type II mRNA (below the 25 percentile), Grade group 3 - 5, or pT 3-4 all correlated with shorter cancer-specific survival (Figure 2a, b, and c), but none of them with overall survival. In Kaplan-Meier analysis, the type I mRNA or total *CGB* mRNA expression did not correlate with cancer-specific survival or overall survival.

Discussion

We found that both type I and type II *CGB* genes are expressed in non-cancerous and cancerous prostate tissue and report for the first time that they both are significantly lower in cancerous than non-cancerous prostatic tissue. Furthermore, in Kaplan-Meier analysis, low type II *CGB* mRNA is associated with short cancer-specific survival. As expected, Gleason score and stage were also strongly associated with cancer-specific mortality. Dirnhofer et al. [11] found expression of *CGB* genes in both cancerous and BPH tissue and further detected hCG protein in culture fluid of BPH tissue explants. Span et al. [10] noted lower expression of total *CGB* mRNA in cancerous than non-cancerous prostatic tissue, but they detected no significant difference between these tissues in expression level of type II genes. They did not study the expression level of type I genes. We show here for the first time that type I genes are also downregulated in cancer. This might explain the lower expression of total *CGB* mRNA in cancer found by Span et al. [10]. Despite the stronger downregulation of type I, only low expression of type II mRNA was a significant prognostic factor in the present study.

In publicly available databases there are only few studies with *CGB* mRNA expression data. In cBioPortal [17] *CGB* mRNA can be found in six different cohorts, one with survival data. In that study, mRNA upregulation of all *CGB* genes was found in approximately 5% of the patients (n= 498). In Kaplan-Meier analysis no difference was found between cases with mRNA upregulation and cases without mRNA upregulation of any *CGB* gene. In ProgGeneV2 [18] two additional cohorts have information about *CGB* mRNA. No differences between high and low expression of *CGB* gene types I or type II was seen in Kaplan-Meier analysis. When individual *CGB* genes were analyzed, patients with high *CGB1* mRNA expression had statistically shorter relapse free survival. However, the sample size in the cohorts was small (n = 110, and n = 91) and follow-up time was shorter (5 to 8 years) than in the present study.

Earlier, Bostwick et al. [19] showed that hCG protein expression can be detected in prostate and prostate cancer tissue by immunohistochemistry (IHC). The expression localized to

neuroendocrine cells and was more common in non-cancerous prostatic tissue (100%) than in intraepithelial neoplasia (30%) or adenocarcinoma (22%) [19]. Their results are in agreement with the mRNA results of Span et al. [10] and our findings. The adverse prognosis associated with reduced *CGB* mRNA expression in prostate cancer is surprising as elevated serum concentrations of hCGβ protein have been found to indicate adverse prognosis in many other cancers [9, 20-23]. Interestingly, we have previously observed a discrepancy between hCGβ concentrations in serum and tissue expression of *CGB* mRNA also in kidney and bladder cancer [24-25]. Moreover, increased expression of type II *CGB* genes has been shown to correlate with early relapse in breast cancer [26]. The cause of the discrepancy in expression of *CGB* mRNA between prostate and breast cancer tissue is unclear. One potential explanation is different expression or function of transcription factor Ets-2, which is involved in protein kinase A-stimulated *CGB5* expression [27]. Ets-2 is upregulated in tumor stromal fibroblasts and has been shown to promote angiogenesis in breast cancer [28]. However, in the prostate cancer VCaP cell line, overexpression of wild-type Ets-2 results in decreased migration, invasion, and proliferation. A mutation (R437C) in the Ets-2 gene has opposite effects in these cells, indicating that Ets-2 has different roles in prostate and breast cancer [29].

In our earlier study on bladder cancer, we found that the ratio of type II to type I (primers detecting *CGB*2 and *CGB*7) mRNA increased with increasing stage and grade of the tumor. In the present study, we found a significant decrease of type I mRNA (primers detecting only *CGB*7), and the ratio of type II to type I mRNA was significantly higher ($p < 0.0001$) in cancerous tissues than in non-cancerous tissues, consistent with our earlier results for bladder cancer [8]. No correlation, however, was found between type II to type I *CGB* mRNA ratio and survival time of the patients in the present prostate cancer material.

Our present RT-PCR assay measures both *CGB1* and *2* together with the classical type II genes, *CGB3*, *CGB5* and 8 whereas the method of Span et al measured only *CGB*3, 5 and 8.

Thus, the difference between the results in our studies and that those of Span et al [10] may due to decreased expression of *CGB*1 and/or *CGB*2 in prostate cancer tissue. However, separate RT-PCR methods for *CGB*1 and 2 are needed to show their real expression levels and correlation with aggressive disease.

A strength of this study is the very long follow-up (median 16 years, maximum 26 years), allowing long-term prognosis to be reliably evaluated. However, we studied patients treated by radical prostatectomy, and thus, whether *CGB* gene expression correlates with relapse and survival also in patients under active surveillance remains unknown. Our patients cohort consisted of hormone-naïve (no neoadjuvant treatment) surgically treated patients. Thus, the biological differences between samples are not affected by any preoperative treatments, and the disease specific variation between patients is well detectable. However, as the study is retrospective, we cannot fully control the effects of any possible secondary treatments to the patients' survival. An important future goal is to determine whether survival can be predicted on the basis of *CGB* gene expression also in these patients.

We conclude that reduced *CGB* type II mRNA expression in prostate cancer tissue is associated with adverse outcome of prostate cancer patients treated by radical prostatectomy.

Acknowledgments

We thank Laura Mäkelä and Anne Ahmanheimo for excellent technical assistance. This work was supported by Finnish Funding Agency for Technology and Innovation (TEKES). No potential conflict of interest was reported by the authors.

Tables

Table 1. Clinical characteristics of PCa patients (n = 193)

Age at surgery, years; median (range)	64 (45 - 76)
Years of collection	1984 - 1998
Dead from PCa	18
Dead from other cause	72
Tumor Gleason score	
≤ 6	56
7	118
8 - 9	17
NA	2
ISUP Grade group	
1	56
2	52
3	66
4	14
5	3
NA	2
Pathological tumor stage	
2	110
3	72
4	1
NA	10
Patient follow-up, years; median (range)	16.10 (0.57 - 26.10)
Serum PSA, µg/l	
<4	13
4 - 10	66
> 10	95
NA	26
Patients receiving secondary therapy	53
Patients receiving tertiary therapy	24

Table 2. Number of samples analyzed with different RT-qPCRS.

Total no. of samples	Malignant 193		Benign 74	
	samples analysed	samples with detectable CGB mRNA (%)	samples analysed	samples with detectable CGB mRNA (%)
Total *CGB* mRNA	178/193	141/178 (79%)	62/74	62/62 (100%)
Type I *CGB* mRNA	114/193	96/114 (84%)	54/74	49/54 (91%)
Type II *CGB* mRNA	114/193	109/114 (96%)	54/74	53/54 (98%)

Table 3a. Primers used with SYBR Green Q-RT-PCR chemistry (endpoint RT-qPCR).

Primer	Sequence	Gene Accession Number

GAPDH (127 bp)		
forward	GGAAATTCCATGGCACCGTCA	ENSG00000111640
reverse	CGACGTACTCAGCGCCAGCATC	
CGB total (130 bp)		
forward	CTGCCCCGTGTGCATCACC	ENSG00000267631 (CGB1)
reverse	CTCGAAGCGCACATCGCGGTA	ENSG00000104818 (CGB2)
		ENSG00000104827 (CGB)
		ENSG00000189052 (CGB5)
		ENSG00000196337 (CGb7)
		ENSG00000213030 (CGB8)

Table 3b. Nucleotide sequences of primers and probes used with TaqMan probe RT-qPCR chemistry.

Primer/Probe	Sequence	Gene Accession Number
GAPDH (97 bp)		
forward primer	GCCCCAGCAAGAGCACAA	ENSG00000111640
reverse primer	TGTGAGGAGGGGAGATTCAGT	
probe	FAM-ACTCCCCAGCAGTGAGGGTCT-MGB	
CGB type I and II (98 bp)		
forward primer	CTGCTGTTGCTGCTGCT	ENSG00000267631 (CGB1)
reverse primer	TTCTCCACAGCCAGGGT	ENSG00000104818 (CGB2)
		ENSG00000104827 (CGB)
		ENSG00000189052 (CGB5)
		ENSG00000196337 (CGB7)
		ENSG00000213030 (CGB8)
CGB type I/probe	FAM- ACATGGGCATCCAGGGAGATGC –MGB	ENSG00000196337 (CGb7)
CGB type II/probe	VIC-ACATGGGCATCCAAGGAGCCG-MGB	ENSG00000267631 (CGB1)
		ENSG00000104818 (CGB2
		ENSG00000104827 (CGB)
		ENSG00000189052 (CGB5)
		ENSG00000213030 (CGB8)

Figure legends

Figure 1. *CGB* mRNA levels in non-cancerous and cancerous prostate tissue. The significance of the difference was calculated by the Mann-Whitney U-test. Type I (A), type II (B) and total *CGB* mRNA (C) expression levels in non-cancerous and cancerous prostatic tissue showing significantly higher expression in non-cancerous tissue.

Figure 2. Kaplan-Meier analysis of cancer specific survival of prostate cancer patients. A. Tissue mRNA concentration of CGB type II in the lower quartile predicts shorter disease specific survival of prostate cancer patients ($p = 0.001$). B. Grade group 3-5 predicts shorter disease specific survival of prostate cancer patients ($p < 0.0001$). C. Tumor stage 3-4 predicts shorter disease specific survival of prostate cancer patients ($p < 0.0001$).

Supplemental Figure S1. Standard curves for total *CGB* (A) and *GAPDH* (B) endpoint RT-qPCR using dilutions of placental RNA.

Supplemental Figure S2. Standard curves for *CGB* type I RT-qPCR using dilutions of cell line JEG-3 RNA (A), *CGB* type II using dilutions of cell line T4 RNA (B) and *GAPDH* using dilutions of cell line JEG-3 RNA(C).

References

1. Schroder F, Hugosson J, Carlsson S, et al. Prostate cancer screening in Europe - Authors'reply. Lancet 2015;385:1507-8.

2. Alfthan H, Haglund C, Roberts P, et al. Elevation of free beta subunit of human choriogonadotropin and core beta fragment of human choriogonadotropin in the serum and urine of patients with malignant pancreatic and biliary disease. Cancer Res. 1992;52:4628-33.

3. Stenman UH, Tiitinen A, Alfthan H, et al. The classification, functions and clinical use of different isoforms of HCG. Hum Reprod Update 2006;12:769-84.

4. Talmadge K, Boorstein WR, Vamvakopoulos NC, et al. Only three of the seven human chorionic gonadotropin beta subunit genes can be expressed in the placenta. Nucleic Acids Res. 1984;12:8415-36.

5. Burczynska BB, Kobrouly L, Butler SA, et al. Novel insights into the expression of CGB1 & 2 genes by epithelial cancer cell lines secreting ectopic free hCGbeta. Anticancer Res. 2014;34:2239-48.

6. Bellet D, Lazar V, Bieche I, et al. Malignant transformation of nontrophoblastic cells is associated with the expression of chorionic gonadotropin beta genes normally transcribed in trophoblastic cells. Cancer Res. 1997;57:516-23.

7. Lazar V, Diez SG, Laurent A, et al. Expression of human chorionic gonadotropin beta subunit genes in superficial and invasive bladder carcinomas. Cancer Res. 1995;55:3735-8.

8. Hotakainen K, Lintula S, Jarvinen R, et al. Overexpression of human chorionic gonadotropin beta genes 3, 5 and 8 in tumor tissue and urinary cells of bladder cancer patients. Tumour Biol. 2007;28:52-6.

9. Hotakainen K, Lintula S, Ljungberg B, et al. Expression of human chorionic gonadotropin beta-subunit type I genes predicts adverse outcome in renal cell carcinoma. J Mol Diagn. 2006;8:598-603.

10. Span PN, Thomas CM, Heuvel JJ, et al. Analysis of expression of chorionic gonadotrophin transcripts in prostate cancer by quantitative Taqman and a modified molecular beacon RT-PCR. J Endocrinol. 2002;172:489-95.

11. Dirnhofer S, Berger C, Hermann M, et al. Coexpression of gonadotropic hormones and their corresponding FSH- and LH/CG-receptors in the human prostate. Prostate 1998;35:212-20.

12. Rull K, Hallast P, Uuskula L, et al. Fine-scale quantification of HCG beta gene transcription in human trophoblastic and non-malignant non-trophoblastic tissues. Mol Hum Reprod. 2008;14:23-31.

13. Epstein JI, Egevad L, Amin MB, et al. The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. Am J Surg Pathol 2016;40:244-52.

14. TNM Classification of Malignant Tumours, 7th Edition Leslie H. Sobin (Editor), Mary K. Gospodarowicz (Editor), Christian Wittekind (Editor) ISBN: 978-1-444-35896-4 August 2011 Wiley-Blackwell.

15. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156-9.

15. Aldaz-Carroll L, Richon S, Dangles-Marie V, et al. Specific detection of type II human chorionic gonadotropin beta subunit produced by trophoblastic and neoplastic cells. Clin Chim Acta 2015;444:92-100.

17. http://www.cbioportal.org/index.do

18. http://watson.compbio.iupui.edu/chirayu/proggene/database/ index.php

19. Bostwick DG, Dousa MK, Crawford BG, et al. Neuroendocrine differentiation in prostatic intraepithelial neoplasia and adenocarcinoma. Am J Surg Pathol. 1994;18:1240-6.

20. Marcillac I, Troalen F, Bidart JM, et al. Free human chorionic gonadotropin beta subunit in gonadal and nongonadal neoplasms. Cancer Res. 1992;52:3901-7.

21. Vartiainen J, Lehtovirta P, Finne P, et al. Preoperative serum concentration of hCGbeta as a prognostic factor in ovarian cancer. Int J Cancer 2001;95:313-6.

22. Hedstrom J, Grenman R, Ramsay H, et al. Concentration of free hCGbeta subunit in serum as a prognostic marker for squamous-cell carcinoma of the oral cavity and oropharynx. Int J Cancer 1999;84:525-8.

23. Iles RK, Persad R, Trivedi M, et al. Urinary concentration of human chorionic gonadotrophin and its fragments as a prognostic marker in bladder cancer. Br J Urol. 1996;77:61-9.

24. Hotakainen K, Lintula S, Stenman J, et al. Detection of messenger RNA for the beta-subunit of chorionic gonadotropin in urinary cells from patients with transitional cell carcinoma of the bladder by reverse transcription-polymerase chain reaction. Int J Cancer 1999;84:304-8.

25. Hotakainen K, Ljungberg B, Haglund C, et al. Expression of the free beta-subunit of human chorionic gonadotropin in renal cell carcinoma: prognostic study on tissue and serum. Int J Cancer 2003;104:631-5.

26. Span PN, Manders P, Heuvel JJ, et al. Molecular beacon reverse transcription-PCR of human chorionic gonadotropin-beta-3, -5, and -8 mRNAs has prognostic value in breast cancer. Clin Chem. 2003;49:1074-80.

27. Johnson W, Jameson JL. Role of Ets2 in cyclic AMP regulation of the human chorionic gonadotropin beta promoter. Mol Cell Endocrinol. 2000;165:17-24.

28. Wallace JA, Li F, Balakrishnan S, et al. Ets2 in tumor fibroblasts promotes angiogenesis in breast cancer. PLoS One 2013;8:e71533.

29. Grasso CS, Wu YM, Robinson DR, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012;487:239-43.