Analysis of expression of chorionic gonadotrophin transcripts in prostate cancer by quantitative Taqman and a modified molecular beacon RT-PCR

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Abstract

Expression of human chorionic gonadotrophin (hCG) is associated with trophoblastic, testicular and other malignancies such as bladder, pancreatic, cervical, breast and prostate cancer. In the prostate, however, hCG expression, associated with neuroendocrine cells, is also found in normal tissue. Of the six highly homologous genes that all encode the β -subunit of hCG, the $\beta 7$ gene is reportedly the only gene expressed in several non-transformed tissues. The $\beta 3$, 5 and 8 genes would be variably expressed in malignant tissue and placenta, but not in normal tissue. To assess to what extent this expression difference can also be found in the prostate, we compared the levels of the different hCG β transcripts in concurrent normal and cancerous prostate tissues obtained from 17 patients. To

this end, we developed a Taqman real-time fluorescent RT-PCR assay for hCG β , and a quantitative assay specific for the $\beta 3$, 5 and 8 genes, modified from the molecular beacon principle. This latter assay proved highly specific and capable of reliably distinguishing between these hCG β transcripts that differ in only one nucleotide. Surprisingly, median expression levels of hCG β were lower in prostate cancer when compared with normal tissue from the same patient. In contrast, hCG $\beta 3$, 5 and 8 transcripts were found in normal tissue and did not differ in prostate cancer, arguing against a specific role of these transcripts in the development of prostate cancer.

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Introduction

Serum human chorionic gonadotrophin (hCG) is widely used as a tumour marker in the management and follow-up of patients with trophoblastic or testicular malignancies. Other malignancies such as bladder (Marcillac *et al.* 1992, Iles *et al.* 1996), pancreatic and cervical carcinomas (Marcillac *et al.* 1992), breast (Agnantis *et al.* 1992, Bièche *et al.* 1998) and prostate (Sheaff *et al.* 1996) cancer also express hCG. In the prostate, hCG is one of the paracrine hormones produced by neuroendocrine cells (Bostwick *et al.* 1994, Sheaff *et al.* 1996) which could stimulate proliferation of adjacent (tumour) cells (Bonkhoff *et al.* 1991, Bonkhoff 1998). Of note, hCG is also expressed in normal prostate tissue (Dirnhofer *et al.* 1998). Serum (Broder *et al.* 1977) or urinary (Fukutani *et al.* 1983) hCG levels have been reported to be elevated in prostate cancer.

The specific β -subunit of the heterodimeric glycoprotein hCG is encoded by six genes located on chromosome 19q13.3 in close proximity to the highly homologous gene for luteinising hormone (LH), originally designated CG β -4 (Policastro *et al.* 1986). Of these genes, $\beta 1$ and $\beta 2$ are considered pseudogenes. All other genes encode the same protein, with the exception that the $\beta 7$ gene encodes an alanine (GCC) at position 117 as opposed to an aspartic acid (GAC) in the other hCG β genes 3, 5 and 8. The $\beta 7$ gene is reportedly the only gene expressed in several non-transformed tissues. The $\beta 3$, 5 and 8 genes would be variably expressed in placental and malignant tissue such as thyroid, breast, bladder and prostate cancer (Bellet *et al.* 1997), but not in normal tissue. In prostate cancer, however, transcripts from the $\beta 3$, 5 or 8 gene could be detected in only two out of ten patients (Bellet *et al.* 1997). Considering the controversy concerning the relevance of hCG expression in prostate cancer (Sheaff *et al.* 1996,

Table 1 Taqman real-time PCR primers and probes

	Sequence (5'–3')	Position	Accession number	Amplicon length
ATP-synthase 6			NC001807	87
Forward primer	acg cct aac cgc taa cat tac tg	8999–9021		
Reverse primer	ggg aag gtt aat ggt tga tat tga	9086–9064		
Probe	tca tgc acc taa ttg gaa gcg cca	9034–9057		
hCGβ			J00117	171
Forward primer	cta ctg ccc cac cat gac c	193–211		
Reverse primer	gca gag tgc aca ttg aca gct	364–344		
Probe	tcc tgc cgg ccc tgc ctc	228–245		
hCGβ3, 5 and 8			J00117	89
Forward primer	cac ccc ttg acc tgt gat ga	401–420		
Reverse primer	cgg gag tcg gga tgg a	490–475		
Probe	cgc ttc cag gac tcc aag cg	425–439		

Dirnhofer *et al.* 1998), we set out to quantify the expression levels of these different hCGβ transcripts in a panel of normal and malignant prostate tissues.

The highly conserved sequences of these genes make it difficult to discriminate between transcripts expressed from the different hCGβ genes. Earlier studies used restriction fragment length analysis (Lazar *et al.* 1995) or nested PCR with primers that differ in one nucleotide at the 3'-end (Bellet *et al.* 1997) to discriminate between these genes. To reliably quantify the expression of hCGβ transcripts in prostate cancer, we developed a Taqman real-time fluorescent RT-PCR assay for hCGβ. To quantify the expression of the malignancy-associated β3, 5 and 8 genes, we set up a modified molecular beacon assay (Tyagi & Kramer 1996). We compared the expression levels of these transcripts in concurrent normal and cancerous prostate tissues from 17 patients to assess to what extent hCGβ gene expression changes upon development of prostate cancer.

Materials and Methods

Tissues

Upon radical prostatectomy, tissue specimens (*n*=17) were snap frozen in liquid nitrogen. Frozen tissue was step sectioned with haematoxylin and eosin staining evaluation at regular intervals. As such, samples containing either >75% normal cells or >50% tumour cells respectively were obtained from each patient.

RNA isolation

Total RNA was isolated by disruption of ten frozen 20 μm sections in 1 ml TRIzol (Life Technologies, Breda, The

Netherlands) using a sterile pestle. After completing the manufacturer's protocol for TRIzol, the amount of RNA was measured spectrophotometrically.

cDNA synthesis

Purified total RNA (1.0 μg) was DNase I treated, denatured for 5 min at 90 °C, and immediately cooled on ice. Reverse transcriptase mix was added, containing first-strand buffer (Invitrogen, Breda, The Netherlands), 200 U Moloney murine leukaemia virus (Invitrogen), 20 U RNasin (Promega, Leiden, The Netherlands), 10 mM dithiothreitol, 4.75 μM random hexamers, and 600 μM deoxynucleotides. After annealing of the hexanucleotides for 10 min at 20 °C, cDNA synthesis was performed for 45 min at 42 °C followed by an enzyme inactivation step for 5 min at 95 °C. Reverse transcription efficiency was quantified for every sample by a separate reaction that included ³²P-labelled ATP. After correction for reverse transcription efficiency, all cDNA samples were diluted to 5 ng/μl and stored at –20 °C until use.

Taqman probe design

Taqman real-time PCR primers and probes (Table 1) were designed using the Primer Express Software (PE Applied Biosystems, Foster City, CA, USA). Primers and probes for hCGβ were designed intron spanning to avoid coamplification of genomic DNA.

Molecular beacon design

PCR primers were designed using the Primer Express Software (PE Applied Biosystems). A β3-, 5- and 8-specific probe (Table 1) was designed essentially according to the guidelines for molecular beacons described by

Figure 1 Design of an hCG β 3-, 5- and 8-specific probe based on the principles of a molecular beacon. Nucleotides 1–15 are complementary to the target sequence (hCG β 3, 5 and 8). Nucleotide 11 differs in the hCG β 7 mRNA (GCC vs GAC).

Tyagi & Kramer (1996) (www.molecularbeacons.com). However, to obtain a smaller loop sequence, thereby increasing the specificity to discriminate between hCG β transcripts (www.molecularbeacons.com), only one arm was attached 3' (shown in *italics* in Table 1). This arm was complementary to five nucleotides at the 5'-end of the hCG β sequence, thereby resulting in a probe of 20 nucleotides, with a loop of 10 nucleotides and 15 nucleotides (the loop and one arm of the stem) complementary to the target sequence (Fig. 1). The intramolecular configuration of the β 3-, 5- and 8- specific probe was modelled using the DNA Mfold program (<http://bioinfo.math.rpi.edu/~mfold/dna/>). Thermal denaturation profiles of the probe and hybrids formed between the probe and oligonucleotide targets (the β 3, 5 and 8 gene, the β 7 gene, or LH) were made to determine the annealing temperature for optimal specificity. The LH oligonucleotide showed even less affinity for the probe than the β 7 gene (not shown).

All primers were from Biolegio (Malden, The Netherlands). Probes were from PE Applied Biosystems.

Generation of specific standards

Specific standards were generated by primer-mediated mutagenesis. PCR amplification of hCG β in the choriocarcinoma cell line JAR was performed using the following primers: 5'-cac ccc ttg acc tgt gat gac ccc cgc ttc cag ga/cc tcc t-3' and 5'-gcc taa ctc ttc gga aat aac a-3'. PCR products were cloned into pCR 2.1-TOPO in DH5 α

cells using the TOPO-TA cloning kit (Invitrogen). Sequences were confirmed by cycle sequencing (AbiPrism 3700; PE Applied Biosystems) using M13 forward and M13 reverse primers. Plasmids were linearised using BspHI, quantified spectrophotometrically, and diluted to 10^7 copies/ μ l.

PCR

All PCR reactions were carried out in Taqman Universal PCR master mix (PE Applied Biosystems) with 500 nM of each primer and 100 nM of probe in a final volume of 50 μ l. Amplifications, with denaturation at 95 °C for 10 min, and 40 cycles of 15 s at 95 °C (melting) and 60 s at 58 °C (annealing and elongation), were performed in a ABI Prism 7700 Sequence detection system (PE Applied Biosystems).

Quantification

For quantification of the reference gene ATP-synthase 6 (Gerard *et al.* 2000), hCG β 3, 5 and 8 and total hCG β transcripts the choriocarcinoma cell line JAR was used as calibrator.

Statistics

Statistical analyses were performed using the SPSS statistical software program version 10.0.5. All data were checked for normal distribution (Shapiro–Wilk). Subsequent non-parametric (Wilcoxon signed ranks) or parametric paired sample *t*-tests were performed to test for differences between normal and cancerous tissues. Data that were not distributed normally are shown as median (1st, 3rd quartile) levels. Differences with a $P < 0.05$ were considered significant. The effect of addition of β 7-specific sequences on the calibrator curve was analysed by linear regression. Samples outside the 95% prediction interval were considered affected.

Results

Characterisation of ATP-synthase 6 as endogenous control

The standard curve for ATP-synthase 6 of threshold cycle (Ct) against increasing amounts of cDNA of the choriocarcinoma cell line JAR, used as calibrator, was linear ($r^2 > 0.99$) over a wide range of at least 4 orders of magnitude (Fig. 2). This calibration curve was parallel to those of hCG β and hCG β 3, 5 and 8 (Fig. 2), indicating that these assays exhibit similar amplification efficiencies. The normalisation against ATP-synthase 6 was validated by comparing the delta Cts between normal and cancerous prostate tissue ($n = 17$). A median difference in Ct of 0.56

Figure 2 Calibration curve, in triplicate, for the real-time quantitative RT-PCRs for ATP-synthase 6 (Δ), total hCG β ($\square$) and the hCG β 3, 5 and 8 ($\circ$) transcripts in the choriocarcinoma cell line JAR.

(interquartile range 1.7) was found, which is in accord with data previously described for a smaller number of samples, and is at the lower end of values found for several different housekeeping genes (Gerard *et al.* 2000). In fact, paired *t*-testing revealed no significant differences in ATP-synthase 6 expression between normal and cancerous prostate tissue (data not shown).

Characterisation of Taqman hCG β

The JAR cell line was also used to examine the efficiency and linearity of the Taqman hCG β assay (Fig. 2). The assay was linear ($r^2=0.99$) over a wide range of at least 4 orders of magnitude of amounts of JAR cDNA input.

Characterisation of hCG β 3, 5 and 8 assay

The hCG β 3, 5 and 8 assay was linear ($r^2=0.97$) over a wide range of at least 4 orders of magnitude of amounts of JAR cDNA input (Fig. 2).

Specificity

The specificity of the probe was checked using standards generated by primer-mediated mutagenesis and cloned into pCR 2.1. A range of 10^2 – 10^6 copies of the β 3, 5 and 8 standard were mixed with a similar range of β 7 standard (Fig. 3). The assay for hCG β 3, 5 and 8 was only significantly influenced after addition of 10^6 copies of β 7-specific standard to 10^2 – 10^4 copies of β 3, 5 and 8. For example, addition of 10^5 copies of β 7 standard did not influence the quantification of as little as 10^2 copies of β 3, 5 and 8, indicating cross-reactivity of <0.1%.

Signal generation

As the probe we devised is complementary to its target at the 5'-end, it might be susceptible to 5'-nuclease activity

Figure 3 Calibration curve for the molecular beacon based assay for hCG β 3, 5 and 8 transcripts. The calibrator (10^2 – 10^6 copies) was mixed with 10^2 ($\circ$), 10^3 (Δ), 10^4 ($\square$), 10^5 (∇) or 10^6 ($\diamond$) copies of a calibrator containing a single nucleotide mismatch specific for the β 7 gene. Dotted lines represent the 95% prediction interval of the linear regression.

during the annealing/elongation step. However, when Eppendorf Taq DNA polymerase (Eppendorf AG, Hamburg, Germany), deficient in exo- and endonuclease activity, was used instead of Amplitaq Gold (PE Applied Biosystems), similar fluorescence values were obtained (data not shown). This indicated that this probe generates fluorescence in a manner similar to molecular beacons, i.e. by a conformational reorganisation causing the fluorophore and the quencher to move away from each other.

To characterise the assays reported here, hCG β and hCG β 3, 5 and 8 expression levels were measured in a number of tumours, cell lines, and in four different placentas (Fig. 4). Remarkably, both prostate tumour cell lines tested (PC3 and DU145) expressed only hCG β 3, 5 or 8. In four different placentas, relative levels of β 3, 5 and 8 of 40–100% were found.

Expression of hCG β transcripts in normal and cancerous prostate tissue

hCG β expression, normalised against ATP-synthase 6 expression, was significantly lower in cancerous tissue when compared with adjacent normal tissue from the same patient ($P=0.015$, Table 2, Fig. 5).

The amount of hCG β 3, 5 and 8 that is expressed did not differ significantly between normal and cancerous prostate tissue from the same patient ($P=0.301$, Table 2, Fig. 5).

In spite of the lower levels of total hCG β with no significant change in hCG β 3, 5 and 8 expression levels,

Figure 4 Relative expression (percentage of total hCG β expression) of the tumour-associated hCG β 3, 5 and 8 genes in testicular tumours, cell lines and placentas.

there was no significant change in the relative levels (percentage of total hCG β transcripts as measured by the Taqman based assay) of hCG β 3, 5 and 8 ($P=0.435$, Table 2).

Discussion

Here we show, by real-time fluorescence quantitative RT-PCR, that in the prostate median expression levels of hCG β are lower in cancer when compared with normal tissue from the same patient. In contrast, levels of the malignancy-associated hCG β 3, 5 and 8 transcripts (Bellet *et al.* 1997) did not differ in our study.

Recently, it has been reported that hCG is not detected in normal prostate tissue, but is specific for low-grade prostate cancer (Daja *et al.* 2000) and/or associated with poor prognosis (Sheaff *et al.* 1996). Expression of hCG has been associated with neuroendocrine cells in the prostate (Bostwick *et al.* 1994, Sheaff *et al.* 1996). The neuroendocrine cell is a minor cell population in normal human prostate glands, but the number is increased in advanced hormone-refractory prostate carcinomas. These androgen-independent cells secrete a number of growth-promoting hormones with paracrine effects on the proliferation of adjacent cells (Bonkhoff *et al.* 1991, Bonkhoff 1998). However, our quantitative RT-PCR results show that the expression of hCG β is frequently downregulated in malignant prostate tissue. This would be in accord with some studies, showing that (i) immunoreactive hCG β is lower in carcinomas than in prostatic intraepithelial neoplasia (Bostwick *et al.* 1994), (ii) hCG β expression is lower in high-grade compared with low-grade prostate cancer (Daja *et al.* 2000), and (iii) that

both normal and cancerous prostate tissue express hCG β (Dirnhofer *et al.* 1998). However, our results argue against a specific role of hCG β in the development of prostate cancer.

The hCG β -subunit is encoded by six highly homologous genes. Expression of the β 3, 5 and 8 genes is restricted to placenta and a number of malignancies (Bellet *et al.* 1997). Furthermore, the same authors reported that in only two out of ten patients with prostate cancer could transcripts from the β 3, 5 or 8 gene be found (Bellet *et al.* 1997). This prompted us to investigate whether prostate cancer is associated with an upregulation of the expression of hCG β 3, 5 or 8 transcripts. We show here that only approximately 0.5–1% of all hCG β transcripts are from the hCG β 3, 5 or 8 genes (Table 2), and that there is no significant change in expression levels upon malignant transformation (Table 2, Fig. 4).

In a small series of placentas we found that the hCG β 3, 5 and 8 genes account for only 40% of total hCG β expression. This is at variance with data reported earlier (Miller-Lindholm *et al.* 1997). These authors showed, by means of restriction fragment length analysis, that in placentas the hCG β 3, 5 or 8 genes account for practically all hCG β expression (98–99.5%). This would also be true for the choriocarcinoma cell line JAR, whereas we found only approximately 80% of hCG β 3, 5 or 8 expression in this cell line. In the breast cancer cell line MCF7 we found almost exclusively hCG β transcripts from the hCG β 3, 5 and 8 genes, which, unlike our results in the bladder cell line T24, concurs with the study of Bellet *et al.* (1997). These discrepancies are most likely attributable to the differences in the employed assays, and the intricacies involved in discriminating between transcripts differing only in one nucleotide.

Table 2 Median expression levels in normal and cancerous prostate tissue. Differences were tested by Wilcoxon rank sum testing and considered significant at $P<0.05$

	Normal	Tumour	P-value
hCGβ/ATP-synthase 6	4.7×10^{-5}	1.5×10^{-5}	0.015
hCGβ3, 5 and 8/ATP-synthase 6	1.8×10^{-7}	2.0×10^{-7}	0.301
hCGβ3, 5 and 8/hCGβ (%)	0.62	0.87	0.435

The probe we devised was based on the molecular beacon principle described by Tyagi & Kramer (1996). Molecular beacons show excellent specificity in discriminating between single nucleotide variations (Marras *et al.* 1999, Tapp *et al.* 2000). However, the generation of fluorescence during amplification by molecular beacons, in our hands, was much less than when Taqman probes were used. This affected the sensitivity and specificity of the assay. Molecular beacons and Taqman probes, however, generate fluorescence in a dissimilar manner. The fluorescence of a molecular beacon is quenched when the fluorescent moiety and the quencher are in close proximity due to the annealing of the stem sequences.

Figure 5 Differences between expression levels of hCGβ (A) or hCGβ3, 5 and 8 (B) in normal and cancerous prostate tissue of 17 patients.

Upon hybridisation of the loop with a complementary sequence, the fluorophore and quencher move away from each other as the stem opens. The fluorescence generated corresponds with the amount of amplicon and this process is repeated each amplification round. Taqman probes, on the other hand, are quenched when both fluorophore and quencher are on the probe. The probe is degraded by the inherent nuclease activity of the *Taq* polymerase during elongation. This releases the fluorophore and fluorescence increases each round. The latter formation of fluorescence is more efficient. Therefore, we devised a molecular beacon that might be susceptible to the 5'→3' nuclease activity of *Taq* polymerase as the 5' stem sequence was complementary to the sequence of interest. With this beacon, the amount of fluorescence generated during amplification did not change, however, when a nuclease-deficient polymerase was used. Possibly, our probe is not susceptible to nuclease activity as its melting temperature is only slightly higher than the annealing temperature used in the assay, whereas the melting temperature of Taqman probes is 8–10 °C higher (Livak *et al.* 1995). Indeed, when we used our probe at 50 °C, 8 °C below the annealing temperature, approximately 3- to 5-fold more fluorescence was found (not shown). At this temperature, however, the probe is not specific for hCGβ3, 5 and 8.

The probe we describe here shows excellent specificity at the single nucleotide level. Whether or not the specificity is enhanced compared with the classical molecular beacon configuration (Marras *et al.* 1999, Tapp *et al.* 2000) has as yet not been tested. However, the use of a smaller loop in the design of a molecular beacon is considered to enhance its specificity (www.molecularbeacons.com), as a relatively greater proportion of the loop sequence is specific for the target sequence. Therefore, the molecular beacon design we show here could be a favourable modification for its application in the quantitative discrimination of single nucleotide variations.

In conclusion, lower median expression levels of hCGβ are found in prostate cancer as compared with those of normal prostate tissue from the same patient. Levels of the malignancy-associated hCGβ3, 5 and 8 transcripts (Bellet *et al.* 1997) do not differ between normal tissue and prostate cancer tissue. Thus, in prostate cancer, there is no clear association of (specific) hCGβ expression with malignancy. Whether hCGβ3, 5 and 8 transcripts are

associated with malignant transformation in other tissues, such as bladder, breast, thyroid, etc (Bellet *et al.* 1997), remains to be confirmed with the specific, quantitative assays reported here.

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References

- Agnantis NJ, Patra F, Khaldi L & Filis S 1992 Immunohistochemical expression of subunit beta hCG in breast cancer. *European Journal of Gynaecological Oncology* **13** 461–466.
- Bellet D, Lazar V, Bièche I, Paradis V, Giovangrandi Y, Paterlini P, Lidereau R, Bedossa P, Bidart M & Vidaud M 1997 Malignant transformation of nontrophoblastic cells is associated with the expression of chorionic gonadotropin beta genes normally transcribed in trophoblastic cells. *Cancer Research* **57** 516–523.
- Bièche I, Lazar V, Nogues C, Poynard T, Giovangrandi Y, Bellet D, Lidereau R & Vidaud M 1998 Prognostic value of chorionic gonadotropin beta gene transcripts in human breast carcinoma. *Clinical Cancer Research* **4** 671–676.
- Bonkhoff H 1998 Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. *Prostate Supplement* **8** 18–22.
- Bonkhoff H, Wernert N, Dhom G & Remberger K 1991 Relation of endocrine–paracrine cells to cell proliferation in normal, hyperplastic, and neoplastic human prostate. *Prostate* **19** 91–98.
- Bostwick DG, Dousa MK, Crawford BG & Wollan PC 1994 Neuroendocrine differentiation in prostatic intraepithelial neoplasia and adenocarcinoma. *American Journal of Surgical Pathology* **18** 1240–1246.
- Broder LE, Weintraub BD, Rosen SW, Cohen MH & Tejada F 1977 Placental proteins and their subunits as tumor markers in prostatic carcinoma. *Cancer* **40** 211–216.
- Daja MM, Aghmesheh M, Ow KT, Rohde PR, Barrow KD & Russell PJ 2000 Beta-human chorionic gonadotropin in semen: a marker for early detection of prostate cancer? *Molecular Urology* **4** 421–427.
- Dirnhöfer S, Berger C, Hermann M, Steiner G, Madersbacher S & Berger P 1998 Coexpression of gonadotropic hormones and their corresponding FSH- and LH/CG-receptors in the human prostate. *Prostate* **35** 212–220.
- Fukutani K, Libby JM, Panko WB & Scardino PT 1983 Human chorionic gonadotropin detected in urinary concentrates from patients with malignant tumors of the testis, prostate, bladder, ureter and kidney. *Journal of Urology* **129** 74–77.
- Gerard CJ, Andrejka LM & Macina RA 2000 Mitochondrial ATP synthase 6 as an endogenous control in the quantitative RT-PCR analysis of clinical samples. *Molecular Diagnosis* **5** 39–46.
- Iles RK, Persad R, Trivedi M, Sharma KB, Dickinson A, Smith P & Chard T 1996 Urinary concentration of human chorionic gonadotrophin and its fragments as a prognostic marker in bladder cancer. *British Journal of Urology* **77** 61–69.
- Lazar V, Diez SG, Laurent A, Giovangrandi Y, Radvanyi F, Chopin D, Bidart JM, Bellet D & Vidaud M 1995 Expression of human chorionic gonadotropin beta subunit genes in superficial and invasive bladder carcinomas. *Cancer Research* **55** 3735–3738.
- Livak KJ, Flood SJA, Marmaro J, Giusti W & Deetz K 1995 Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* **4** 357–362.
- Marcillac I, Troalen F, Bidart JM, Ghillani P, Ribrag V, Escudier B, Malassagne B, Droz JP, Lhomme C, Rougier P, Duviard P, Prade M, Lugagne PM, Richard F, Poynard T, Bohuon C, Wands J & Bellet D 1992 Free human chorionic gonadotropin beta subunit in gonadal and nongonadal neoplasms. *Cancer Research* **52** 3901–3907.
- Marras SA, Kramer FR & Tyagi S 1999 Multiplex detection of single-nucleotide variations using molecular beacons. *Genetic Analysis* **14** 151–156.
- Miller-Lindholm AK, LaBenz CJ, Ramey J, Bedows E & Ruddon RW 1997 Human chorionic gonadotropin–beta gene expression in first trimester placenta. *Endocrinology* **138** 5459–5465.
- Policastro PF, Daniels-McQueen S, Carle G & Boime I 1986 A map of the hCG beta–LH beta gene cluster. *Journal of Biological Chemistry* **261** 5907–5916.
- Sheaff MT, Martin JE, Badenoch DF & Baithun SI 1996 Beta hCG as a prognostic marker in adenocarcinoma of the prostate. *Journal of Clinical Pathology* **49** 329–332.
- Tao YX, Bao S, Ackermann DM, Lei ZM & Rao CV 1997 Expression of luteinizing hormone/human chorionic gonadotropin receptor gene in benign prostatic hyperplasia and in prostate carcinoma in humans. *Biology of Reproduction* **56** 67–72.
- Täpp I, Malmberg L, Rennel E, Wik M & Syvanen AC 2000 Homogeneous scoring of single-nucleotide polymorphisms: comparison of the 5'-nuclease TaqMan assay and Molecular Beacon probes. *BioTechniques* **28** 732–738.
- Tyagi S & Kramer FR 1996 Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnology* **14** 303–308.

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