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Advances in Brief

Expression of Human Chorionic Gonadotropin β Subunit Genes in Superficial and Invasive Bladder Carcinomas[1]

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Abstract

Increased serum levels of human chorionic gonadotropin β subunit (hCGβ) were described previously in patients with bladder cancer. To obtain insight into such production of hCGβ, the expression of hCGβ 7, 8, 5, and 3 genes in bladder carcinomas and normal urothelia was investigated by reverse transcription PCR. Surprisingly, hCGβ mRNAs were detected in both normal urothelial and carcinomatous cells. However, tumor progression was characterized by different patterns of transcription of the hCGβ genes; the β7 gene was the only gene transcribed in normal urothelia and T_a tumors included in this study, whereas in addition to β 7, genes β 5, 8, and 3 were transcribed in T_1 to T_4 tumors. Moreover, transcription levels of the latter three genes increased with the stage of the disease. These observations show that dramatic modifications in the expression of hCGβ genes accompany progression of bladder carcinomas.

Introduction

hCG[3] is a dimeric hormone produced by the trophoblast during pregnancy (1). It belongs to a glycoprotein hormone family, which includes LH, follitropin, and thyrotropin, and is composed of two noncovalently linked subunits, designated α and β. Whereas the α subunit common to the four hormones is encoded by a single gene located at position 6q14–q21, the hCGβ subunit is encoded by a cluster of six genes (β 7, 8, 5, 1, 2, and 3) located at position 19q13.3, adjacent to the β4 gene coding for the hLHβ subunit (2, 3). All six hCGβ genes were found to be transcribed in trophoblastic cells (4). However, different levels of mRNA have been detected, with the β 5, 8, and 3 genes being preferentially expressed. Only genes β 7, 8, 5, and 3 present the open reading frame of the hCGβ subunit protein. They can be distinguished by variations in the sequence of either the 5′ transcribed untranslated regions (4) or the promoters (5). Such variations account for differences observed in the levels of their expression in trophoblastic cells (5).

The dimeric hormone hCG, as well as its free β subunit, are also produced by gestational trophoblastic and gonadal cancers (6–8). Moreover, the presence of the free hCGβ subunit has been demonstrated clearly in sera of patients with nontrophoblastic and nongonadal neoplasms (8). In particular, preferential production of the free hCGβ subunit in the absence of dimeric hCG was found in the sera of patients with bladder cancers, and its presence correlated with the stage of the tumor (8, 9).

Bladder cancers are divided into two groups, superficial (T_{is}, T_a, and T_1) and invasive (T_2, T_3, and T_4). Characterization of the molecular genetic pathways involved in the progression of bladder carcinomas is important for the identification of prognostic factors as well as for the development of new therapeutic strategies (10–12). In this study, we investigated the expression of genes hCGβ 7, 8, 5, and 3 in both normal bladder and bladder carcinomas at various stages of the disease. Surprisingly, hCGβ mRNAs were detected by RT-PCR in both normal and neoplastic tissues, but tumor progression was characterized by dramatic changes in the expression of the different hCGβ genes.

Materials and Methods

Tissue RNA Extraction. Four normal urothelia and 34 bladder transitional cell carcinoma fragments were obtained from patients after surgery at the Hôpital Henri Mondor and at the Institut Gustave Roussy, in accordance with protocols approved previously by the human studies committee of each hospital. The proportion of tumor cells in surgical fragments of bladder carcinomas was estimated by visual examination of sections stained with hematoxylin and eosin. Adjacent pathological material sections with a high tumor cell content (>80%) were used for RNA extraction. Three first-trimester placentae were obtained from women undergoing legal abortions. RNA was purified in cesium chloride gradients (13).

Oligonucleotides. Two pairs of primers, β1-β2 and β3-β4 (DNAgency, Malvern, PA), were designed to commonly amplify the hCGβ 7, 8, 5, and 3 mRNA, without amplification of contaminating DNA, hCGβ 1, hCGβ 2, or hLHβ mRNA (Fig. 1*A*). For this purpose, one primer in each pair was placed at the junction between two exons and contained, therefore, in its 3′ end, a mismatch with any contaminating DNA; β1 was placed upstream of the hLHβ transcription start site; β3 presented, at its 3′ end, a 2-bp mismatch with the hLHβ sequence (CC *versus* TG), and oligonucleotide β1 did not match with the hCGβ 1 and 2 mRNA sequences (4). One primer in each pair was fluorescein-labeled at its 5′ extremity by adding Fluorprime during oligonucleotide synthesis (Pharmacia, Uppsala, Sweden).

hCGβ mRNA Pattern. The identification of each hCGβ 7, 8, 5, and 3 mRNA was performed by *Hha*I and *Dra*I restriction endonuclease analysis of the β1-β2 RT-PCR products (Fig. 1*A*). One μg of total RNA was reverse transcribed under 20 μl using the Gene Amp RNA PCR kit (Perkin-Elmer Cetus, Foster City, CA), according to the manufacturer's instructions. The equivalent of 250 ng cDNA (5 μl RT product) was amplified under 50 μl using 2.5 units of Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM $MgCl_2$, and 10 pmol of each of the β1 and β2 primers. PCR were processed through 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C on a 9600 Perkin thermocycler. Restriction endonuclease analysis was performed in a 20-μl volume reaction using 5 units of *Hha*I and 2.5 units of *Dra*I according to the manufacturer's instructions (Biolabs, Beverly, MA).

hCGβ mRNA Steady-State Level. The RT-PCR method was used to quantify hCGβ mRNA. To take into account the extent of possible degradation of the RNA preparations, TFIID transcripts were quantified and used as an

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[3] The abbreviations used are: hCG, human chorionic gonadotropin; LH, lutropin; RT, reverse transcription; IQS, internal quantitative standard.

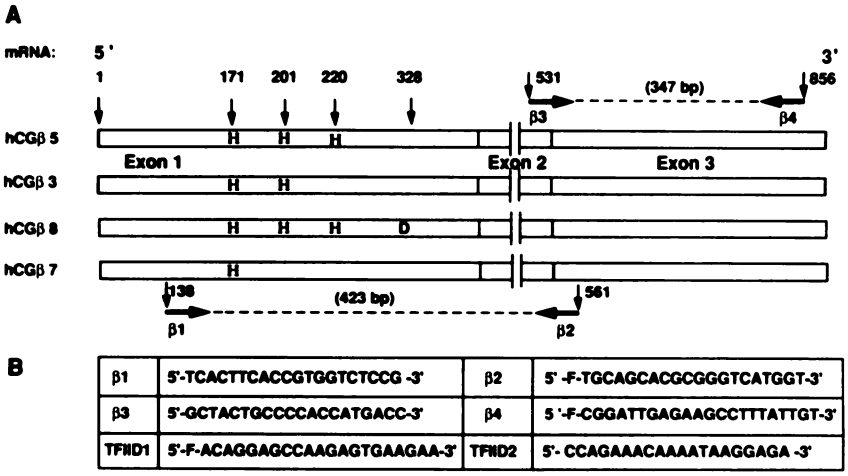

Fig. 1. *A*, schematic representation of hCGβ 5, 3, 8, and 7 mRNAs. The three exons are indicated by *boxes* (exon 2 is represented in part), and the numbering system is based on Hollenberg *et al.* (5). *Horizontal arrows*, sites of binding of the primers; *vertical arrows*, sites of restriction recognized by enzymes *Hha*I (*H*) and *Dra*I (*D*). *B*, the nucleotide sequence of the primers for PCR amplification of hCGβ and TFIID mRNAs. *F*, fluorescein dye.

endogenous mRNA control (TFIID primers are presented in Fig. 1*B*). Therefore, each sample was normalized on the basis of its TFIID content, and final results were expressed as hCGβ:TFIID ratios. To monitor variability in the PCR assay, we used an IQS matched to the target sequence of interest (hCGβ or TFIID) but differing from it by virtue of a 12-bp internal insertion, created by PCR. The insert enables the distinction between wild-type and IQS PCR products by electrophoretic separation. Quantitation was based on the determination of their relative amounts (14). The RT products, obtained as described previously, were first diluted 1:4 with a mix containing 1 mM of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 5 mM $MgCl_2$. The subsequent PCR protocol included 4 μl of the diluted RT (equivalent to 50 ng cDNA) and 16 μl of a common master mix containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM $MgCl_2$, 6 pmol of each primer β3 and β4, 1 unit Taq DNA polymerase, and known amounts of hCGβ IQS. The same protocol was used to quantify TFIID mRNA using appropriate primers and IQS. PCR were processed through 35 cycles as described.

Electrophoretic Separation and PCR Product Quantification. Labeled restriction fragments or quantitation PCR products were coelectrophoresed with size standards (Genescan 500 ROX) on a 373 ABI DNA sequencer and analyzed with Genescan 672 software (Applied Biosystems, Foster City, CA). The software identifies the length of the fragments and calculates the area under each curve expressed in relative fluorescence units, which is correlated with PCR product amounts. Statistical interpretation of the data was performed using the Mann and Whitney *U* test (two-tailed).

Results

hCGβ mRNA Pattern. The length of the restricted labeled fragments enabled the distinction between the different hCGβ 7, 8, 5, and 3 mRNAs: β7, 391 bp; β3, 360 bp; β5, 341 bp; and β8, 233 bp. The peak area of the fluorescent signal, corresponding to each restricted fragment, was used to calculate the percentage of each hCGβ expressed gene. Normal urothelia ($n = 4$) and T_a tumors ($n = 6$) displayed a unique β7 mRNA pattern, whereas 45% of T_1 bladder carcinomas ($n = 11$) and 95% of invasive (T_2 to T_4) bladder carcinomas ($n = 17$) displayed a β 5, 8, or 3 mRNA pattern in addition to β7. The $\beta_{5,8,3}$:$\beta_{7,5,8,3}$ ratio of the mRNA content was calculated in each tissue sample. Within tumors of the same stage, the ratio was variable from one sample to another (Fig. 2). The mean values of the $\beta_{5,8,3}$:$\beta_{7,5,8,3}$ ratios for normal bladders and superficial and invasive tumors were 0.0, 0.08, and 0.49, respectively. There was no statistically significant difference in the ratio values between normal bladders and superficial tumors (U = 24; $P = 0.23$). In contrast, a statistically significant difference was found in ratio values between normal bladders and invasive tumors (U = 2; $P = 0.004$) and between superficial tumors and invasive tumors (U = 34; $P = 0.0001$). Indeed, hCGβ 5, 8, or 3 mRNA was detected in 21 of 34 tumor samples (61%). Among those samples, 13 tumor tissues expressed the three hCGβ 5, 8, and 3 genes, 3 tissues expressed two genes, and 5 tissues expressed only one gene (data not shown).

hCGβ mRNA Steady-State Level. Thousand-fold higher amounts of hCGβ IQS were necessary to obtain equivalent amplification of target and IQS in first-trimester placental tissues in comparison to bladder tissues. In contrast, TFIID target and IQS were coamplified using identical amounts of TFIID IQS in both placentae

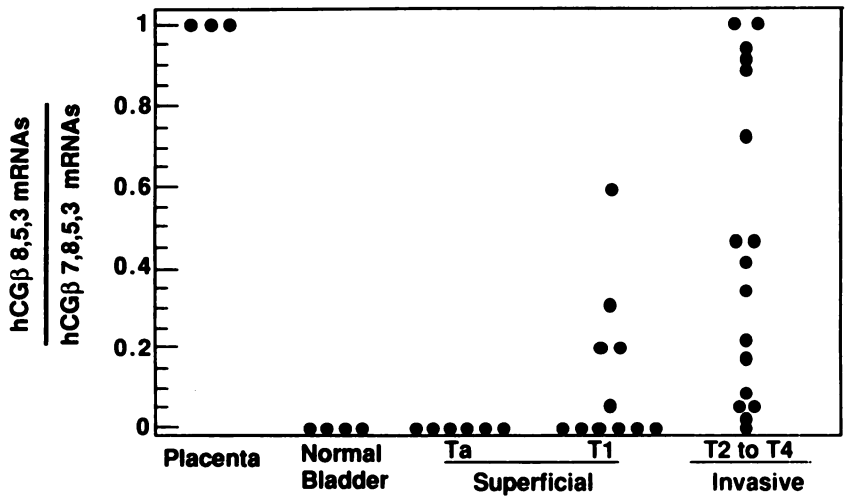

Fig. 2. hCGβ mRNA pattern in normal and tumoral bladder tissues as compared to first-trimester placenta specimens. The identification of each hCGβ mRNA was performed by restriction endonuclease analysis of the β1-β2 PCR products. Electrophoresis was performed on an ABI DNA sequencer, and the peak area corresponding to each restriction fragment was measured. The $\beta_{8,5,3/7,8,5,3}$ ratios of the hCGβ mRNA are represented for each sample.

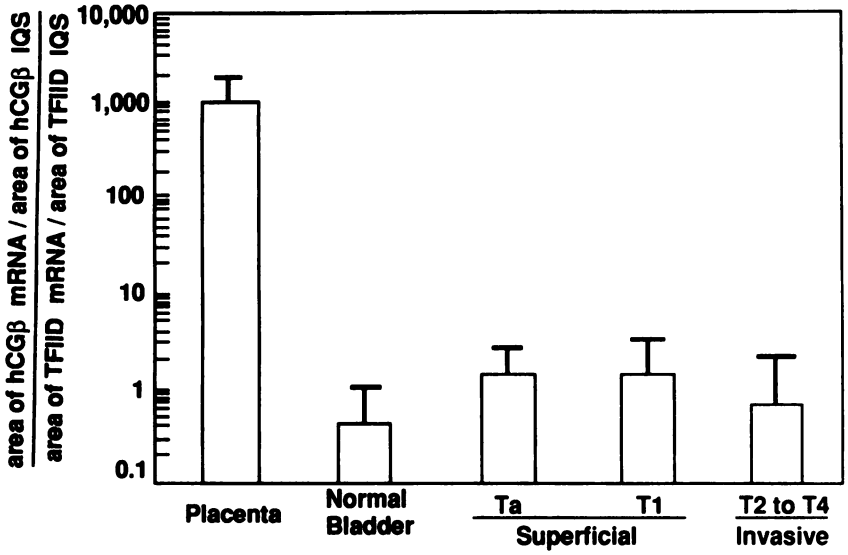

Fig. 3. hCGβ mRNA mean steady-state levels in normal and tumoral bladder tissues as compared to first-trimester placenta specimens. For each group, standard deviation is indicated. Quantification was achieved by RT-PCR using IQS DNA. Electrophoresis was performed on an ABI DNA sequencer, and the peak areas were measured. hCGβ mRNA levels were normalized, by measuring in a similar manner, the number of transcripts encoding the housekeeping gene TFIID. All experiments were done in duplicate; *bars*, SD.

and bladders. This result indicated that, whereas the steady-state level of TFIID transcripts was similar in both trophoblastic and bladder tissues, the steady-state level of hCGβ mRNAs was dramatically different in these tissues. The steady-state levels of hCGβ mRNAs in placental and bladder tissues, normalized with those of TFIID, are presented in Fig. 3. No significant difference was found in the steady-state levels of total hCGβ mRNAs between normal bladders and invasive tumors (U = 15; P = 0.79). The expression of hCGβ including β7, 5, 8, and 3 genes in superficial tumors was significantly higher than in invasive tumors (U = 199; P = 0.007).

Discussion

The aim of this study was to characterize the hCGβ mRNA pattern in a series of normal and tumoral bladder tissues at various stages of disease and to compare the pattern observed in urothelial cells to that displayed by normal trophoblastic cells. To investigate the steady-state level of each of the hCGβ 7, 5, 8, and 3 mRNAs, we developed an original strategy based on the RT-PCR method, combined with restriction endonuclease analysis and competitive PCR quantitation.

Similar levels of hCGβ mRNA were detected in both normal and tumoral bladder tissues, but the overall steady-state level of hCGβ mRNAs in bladder tissues was found to be a thousand-fold lower than that detected in first-trimester placental tissues. In contrast to trophoblastic cells from normal placenta, which preferentially express hCGβ 5, 8, and 3 genes (4), only β7 mRNA was detected in four normal urothelia. Indeed, the transcription of hCGβ genes had been observed already in normal tissues other than the trophoblast; hCGβ mRNAs were detected by RT-PCR in normal testis (15), and there is evidence for hCG production by human pituitary cells (16). Although the limited number of normal urothelia included in our study does not enable extrapolation to all bladder controls, it is striking that distinct β genes were transcribed by different normal tissues. The present observation in normal bladder, when compared with previous data observed in normal trophoblast, suggests that regulation of β7 gene transcription within the hCGβ cluster varies with the differentiation of the tissue. At least two mechanisms might account for such distinct regulation: differences in the sequence of the distal promoter of the β7 and β 5, 8, and 3 genes (5); and variability in the degree of methylation of the hCGβ cluster, depending on the origin of the cells (17). Finally, it is noteworthy that both the hCG and free hCGβ subunit are detected at very low levels in serum and urine of men and nonpregnant women (8, 18). Until now, such production was thought to originate from the pituitary (16), but other normal tissue, such as the bladder urothelium, might also be responsible for the presence of the free hCGβ subunit in biological fluids.

Whereas only hCGβ 7 mRNA was detected in normal urothelia and T_a tumors, simultaneous expression of the β7 and β 5, 8, or 3 genes was detected in 45% of T_1 and 95% of invasive (T_2 to T_4) tumors. In fine, hCGβ 5, 8, or 3 gene expression was detected in 61% of bladder carcinomas. Qualitative ($\beta_{5,8,3}$:$\beta_{7,5,8,3}$ hCGβ mRNAs ratio) as well as quantitative (steady-state mRNA levels) analyses of β 7, 5, 8, and 3 mRNA patterns indicated that the level of expression of the β 5, 8, and 3 genes is correlated with the tumor stage. Interestingly, the simultaneous presence of hCGβ 5, 8, or 3 and hCGα mRNAs was detected in two superficial tumors (11%) and seven invasive tumors (41%; data not shown). This molecular pattern is close to that observed in trophoblastic cells. This observation is in agreement with the hypothesis that the origin of hCGβ productive bladder cancer cells involves metaplasia of the carcinomatous tissue into a tissue similar to trophoblast tissue (7, 8, 19). Nevertheless, the secretion of hCG-related proteins is quantitatively and qualitatively different in productive bladder tumors and in placenta; while dimeric hCG is preferentially produced by normal trophoblastic cells in pregnant women, bladder carcinomas preferentially produce the free hCGβ subunit (8, 9, 20).

It is tempting to speculate that the acquired expression of the β 5, 8, and 3 mRNAs in invasive bladder carcinomas is responsible for the increase in hCGβ serum levels (≥100 pg/ml) detected in 47% of patients with bladder carcinomas (8). Indeed, we observed that both the expression of the hCGβ 5, 8, or 3 genes and the presence of the hCGβ subunit in the sera of patients with bladder carcinomas (8, 9) correlate with the stage of the disease. However, the use of highly sensitive fluoroimmunoassays (2 pg/ml) shows that low serum hCGβ levels (<100 pg/ml) are detected in normal individuals (8, 18). The interest of determining the pattern of hCGβ mRNAs might be to enable the distinction between expression of hCGβ genes by normal tissues and expression by invasive bladder carcinomas, as well as to determine whether combined ultrasensitive serological assays (15, 18) and analysis of the pattern of hCGβ gene expression in surgical fragments optimizes the management of patients with bladder carcinomas.

Taken together, the present results demonstrate that the pattern of expression of various hCGβ genes differs in normal urothelium and bladder carcinomas and that progression of the disease is characterized by increased levels of expression of β 5, 8 and 3 genes.

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