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PRACE POGLĄDOWE

położnictwo

hCG – related molecules and their measurement

Gonadotropina kosmówkowa – czego nie mierzą komercyjne testy

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Abstract

Measurements of human chorionic gonadotropin (hCG) synthesized by trophoblast cells is a powerful tool of pregnancy monitoring. It was showed that similarly to pregnancy also trophoblastic and nontrophoblastic malignancies produce variety of hCG molecules. In urine and serum of both pregnant women and tumors patients a fifteen various forms of hCG, such as: regular hCG, hyperglycosylated hCG and predominant hyperglycosylated hCG free β, were identified. These forms might be useful in order to recognize between physiological and pathological pregnancies as well as cancers.

Even the presence of these different hormone variants is well documented the commercially available biochemical tests detecting hCG failed to identified and distinguish among these forms. Especially hard is to identify glycan chains linked to heterodimer. Thus, a detailed analysis of hCG-related molecules produced during physiological and pathological condition, together with a new tests development are needed.

Key words: **chorionic gonadotropin** / **hCG** / **hCGβ** / **H-hCG** / **hCG assays** /

Streszczenie

Wykrywanie ludzkiej gonadotropiny kosmówkowej (hCG) produkowanej przez komórki trofoblastu jest wykorzystywane do wykrywania i monitorowania rozwijającej się ciąży.

Oprócz ciąży szereg nowotworów pochodzenia zarówno trofoblastycznego jak i nietrofoblastycznego cechuje synteza i wydzielanie różnych form gonadotropiny kosmówkowej. Dotychczas w surowicy i moczu kobiet ciężarnych oraz u osób z chorobami nowotworowymi zidentyfikowano piętnaście różnych form hCG. Najczęściej występującymi cząsteczkami są: regularna hCG, hiperglikozylowana hCG i hiperglikozylowana wolna podjednostka beta. Cząsteczki te mogą być wykorzystane do rozróżnienia pomiędzy ciążą fizjologiczną a patologiczną, czy rozpoznania nowotworu rakiem

Niestety dostępne na rynku testy diagnostyczne nie są wstanie wykryć i rozróżnić poszczególnych form hCG. Szczególnie trudne w identyfikacji są reszty cukrowcowe związane z hormonem. Szczegółowa analiza cząsteczek hCG produkowanych w określonych warunkach fizjologicznych jak i patologicznych powinna pozwolić na opracowanie nowych testów pozwalających na ich identyfikację.

Słowa kluczowe: **gonadotropina kosmówkowa** / **hCG** / **hCGβ** / **H-hCG** / **testy hCG** /

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Introduction

The relationship between placental hormone – human chorionic gonadotropin (hCG) and progesterone produced by the *corpus luteum* has been showed by Hirose in 1920 [1]. Till today it is believed that the primary function of hormone is regulation of pregnancy development and its maintenance. Under physiological conditions, hCG is produced by trophoblast cells and is responsible for the processes such as: implantation, placentation, angiogenesis, embryo development, and immune tolerance [2]. Synthesis of hCG starts in blastocyst after the hatching, however beta subunit of the hormone presence was reported at the 2PN (2 pronuclear) stage in embryos [3]. Thus, the measurement of hCG concentration is a powerful tool of pregnancy monitoring.

The differences of the hormone's level mark some pathological conditions like: failing pregnancies, hydatidiform mole, gestational trophoblastic diseases, choriocarcinomas, germ cell malignancy as well as nontrophoblastic tumors of various origin [4, 5]. However in urine and serum of both pregnant women and tumor patients a various forms of hCG were identified [2, 6]. These forms might be used as a disease-specific markers. Unfortunately commercially available assays fail to detect and distinguished among these forms. Thus, a detailed analysis of hCG-related molecules produced during physiological and pathological condition together with a new tests development are needed.

CG structure

Human chorionic gonadotropin like other gonadotropins (LH, FSH, TSH) is a heterodimeric hormone composed of two non-covalently bonded subunits: alpha and beta [7, 8]. Highly conservative alpha subunit is common for all gonadotropins and this is specific beta subunit, which determines the biological properties of each hormone [7, 9].

The formation of a biologically active hCG molecule requires the expression of genes encoding both alpha and beta subunits. Consisting of a ninety-two amino acids alpha subunit (P01215, UniProtKB/Swiss-Prot) is encoded by a single gene located on chromosome 6 (6q14-q21, NC_000006.11). β-subunit (P01233, UniProtKB/Swiss-Prot) comprises a 145 amino acids and it is encoded by a six allelic genes (*CGB*, *CGB5*, *CGB6*, *CGB7*, *CGB8*, *CGB9*) located in LHB/CGB cluster on chromosome 19 (19q13.32, NC_000019.9). hCG subunits are separately synthesized in the endoplasmotic reticulum (ER). After assembling a functional heterodimer is modified in ER and Golgi apparat (GA) and then secreted out of the cell [10].

For full biological activity, human chorionic gonadotropin must undergo a series of post-translational modifications whose purpose is primarily the proper conformation of hormone.

The polypeptide chains shortening is particularly important for beta subunit, as its unveiling amino acids of C-terminal peptide (CTP). CTP determines half-life of hCG in a blood, and binding to LHCGR receptor [11].

Disulfide bridges, which are subunit-specific and essential for functional heterodimer formation are formed in ER. It was showed that hCG 3-D structure is stabilized by a cysteine knot motif [12]. Because of its presence, as well as the hormone's functional and structural homology to members of cystine-knot growth-factor superfamily (such as: TGFB, PDGFB, VEGF or NGF) it is postulated that hCG belongs to this family and may act as a growth factor [4].

The numerous and various function of hCG both in pregnancy and cancer are however related mainly to its glycosylation pattern. Sugar residues (including: acetylglucosamine, mannose, galactose, fucose and sialic acid) forming straight and branched chains are attached to both hormone subunits. hCG contains four N-linked (linked to asparagine residues via *N-glycosidic bonds)* and four O-linked oligosaccharides (linked to *serine and threonine residues via O*-glycosidic bonds) [1]. On both α- and β-subunit there are two N-linked oligosaccharides. 4 O-linked oligosaccharides are attached to the C-terminal peptide region of the β-subunit (Figure 1) [1].

The glycosylation pattern of hCG variants affects their molecular weight, which varies from 36 to 40 kDA [13]. In

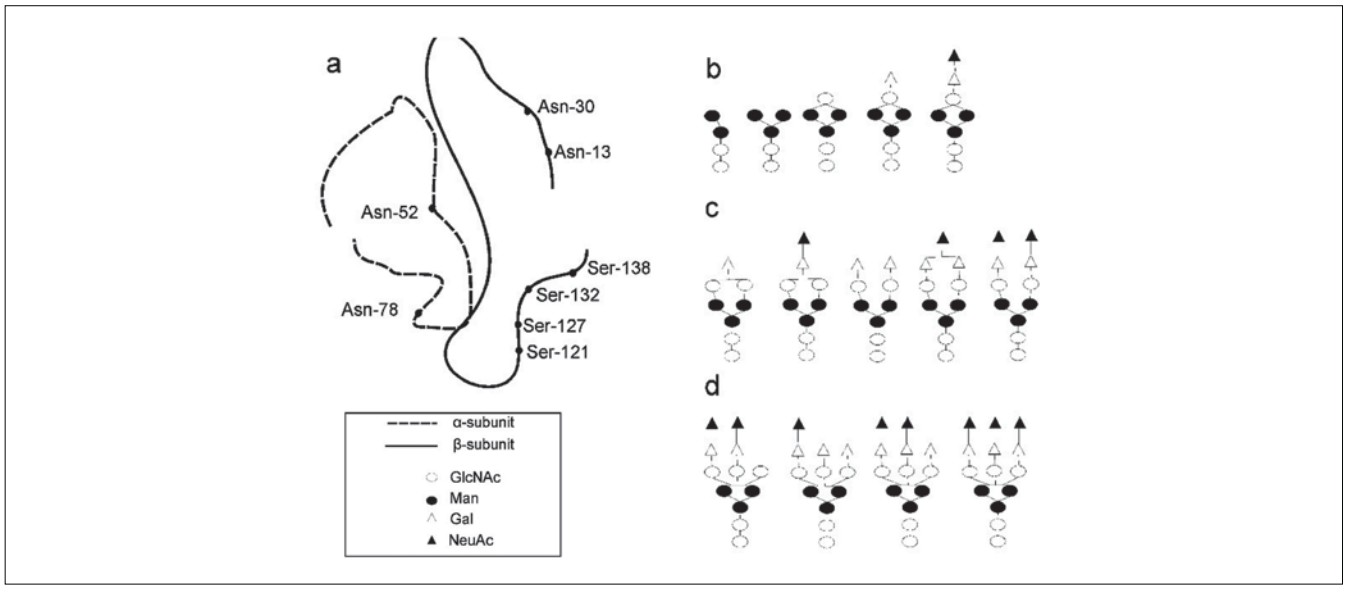

Figure 1. Glycosylation pattern of hCG molecule: A – structure of hCG molecule ; B – monoantenary oligosaccharides residues linked to hCG, C – biantenary structure of sugars linked to hCG, D – triantenary structure of sugars linked to hCG.

Table I. The most common forms of hCG-related molecules.

no	symbol	name	description	IFCC approval
1	hCG	intact hCG	biologically active; present in urine and serum of pregnant women and cancer patient [35]	yes
2	hCGn	nicked hCG	non active; occurs in plasma and serum of pregnant women and cancer patients [37]	yes
3	hCGα	free α-subunit	non active; present in plasma and serum, especially in cancer patients; according to some reports hCGα is produced by trophoblast cells only and is not incorporated into hCG; measurement of hCG and hCGα may be used as a screening for pregnancies at risk for fetal chromosome abnormalities [38]	yes
4	hCGβ	free β-subunit	unstable; occurs in plasma of pregnant women and cancer patients [4]	yes
5	hCGβn	nicked free β-subunit	unstable; commonly occurs in urine, detected in plasma both during pregnancy and cancer [39]	yes
6	hCGcf	β core fragment	major form in urine of pregnant women and molar and hyperemesis gravidarium patients [39]; in plasma on a very low concentration level [39]	yes
7	H-hCG	hyperglycosylated hCG	detectable in serum and urine of pregnant women and cancer patient [15]; responsible for promotion of invasion, growth and malignancy [1]; may be useful in screening for Down syndrome in the first trimester of pregnancy [40]	no
8	H-hCGβ	hyperglycosylated hCGβ	detectable in serum and urine of cancer patient; promote growth, invasion and metastasize [15]	no
9	hCG lacking the CTP	hCG lacking the CTP	identified in urine of some cancer patients [39]	no
10	O-hCGα	O-glycosylated alpha subunit	produced by placenta during pregnancy and by nontrophoblastic cancers [41]	no

case of so called regular hCG (synthetized by the trophoblast) oligosaccharides represent up to 40% of molecule total mass. Due to differences in the number and types of oligosaccharides, as well as the types of bonds several different variants of hCG may be distinguished [14]. In urine and serum of both pregnant women and tumors patients a fifteen various forms of hCG were identified [1, 15]. Five of them: regular chorionic gonadotropin (hCG), hyperglycosylated chorionic gonadotropin with additional sugar residues (H-hCG), hyperglycosylated chorionic gonadotropin beta subunit (H-hCGβ), free alpha subunit (hCGα) and O-glycosylated alpha subunit (O-hCGα) are produced by the placenta and nontrophoblastic cancer [6]. The additional ten variants represent degradation products of both regular and hyperglycosylated hormone. Enzymatic cleavage by macrophages' protease and leukocyte elastase leads to the production of nicked chorionic gonadotropin (hCGn). This form is unstable, and rapidly splits into: a nicked free ß-subunit and free alpha-subunit [16]. Then leukocyte elastase attacks the hCG molecules and cleave off the ß-subunit C-terminal peptide. When the remaining molecules are filtered through the kidney, further degradation occurs and ß-core fragment is released (Table I). These products of enzymatic destruction functions appear to be negligible biological value [15, 17].

The hyperglycosylated variants of hCG have not been admit to International Federation of Clinical Chemistry and Laboratory Medicine standards, yet. Six preparation has now been established as the first WHO International Reference reagents for hCG immunoassays (Table I).

Still the presence of hyperglycosylated forms of hCG has been well do documented [17]. These forms might be useful in order to recognize between physiological and pathological pregnancies as well as cancers and in near future might become a new markers of these conditions.

Different form of hCG in pregnancy and cancer

Regular hCG is a major hCG variant produced through the almost whole period of both normal and abnormal pregnancies. It is also the principal molecule produced by individuals with hydatidiform moles or pregnancies comprising solely trophoblast tissue [13]. While its reduced level marks spontaneous abortion and ectopic pregnancy [18], a double level of regular hCG is observed in Down syndrome pregnancy (Table II) [19].

Hyperglycosylated chorionic gonadotropin, when compared to regular hCG, is characterized by one and a half as much of carbohydrate chains linked to asparagine residues and a doubled number of oligosaccharides linked *via* O-glycosidic bonds [20]. H-hCG together with hyperglycosylated free beta subunit is produced by extravillous invasive cytotrophoblast cell in the first week of pregnancy, following implantation of the fetus. It is postulated that H-hCG is an autocrine factor promoting growth and invasion of cytotrophoblast during early pregnancy [21] as well as choriocarcinoma cells *in vivo* and *in vitro* [22, 23].

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Table II. Concentration of hCG in serum samples in pregnancy and pregnancy disorders.

condition		hCG level
early pregnancy (3-4 weeks)		22-239 mIU/ml [1]
general pregnancy (6 weeks - term)		16.850 mIU/ml [1]
ectopic pregnancy	patients with an ectopic mass or fluid in the pouch of Douglas	>1500 IU/l [42]
	patients without an ectopic mass or fluid in the pouch of Douglas	>2000 IU/l [42]
complete hydatidiform mole		>100.000 mIU/ml [1]
partial hydatidiform mole		median: 48.900 mIU/ml (range of 11.600 to 220.114 mIU/ml) [1]
choriocarcinoma		>1.000.000 mIU/ml [43]
placental site trophoblastic tumor		median: 30 mIU/ml (range of 1 to 231 mIU/ml) [1]

Table III. hCG-related molecules serum level [2].

condition	hCG		H-hCG		H-hCGβ	
	presence	level – comparing to 2nd trimester pregnancy	presence	level – comparing to 2nd trimester pregnancy	presence	level – comparing to 2nd trimester pregnancy
spontaneous abortion	+	=	+	↓	+	↓
ectopic pregnancy	+	=	+	↓	+	↓
invasive hydatidiform mole	+	↑	+	=	+	=
invasive trophoblastic diseases	+	↑	+	↓	+	↑
non-invasive trophoblastic disease	+	=	-	↓	-	↓
21 trisomy	+	↑	+	↑	+	↑
18 trisomy	+	↓	+	↓	no data	no data
13 trisomy	+	↓	+	↓	no data	no data
choriocarcinoma	+	↑	+	↑	+	=
testicular germ cell malignancy	+	↓	+	↑	+	=
non-gestational malignancies	+	↓	+	↓	+	↑

Legend:
"+" – presence, "- " – lack; "=" – no changes in hCG level, "↓" – decrease of hCG level, "↑" – increase of hCG level

The differences of H-hCG as well as H-hCGβ levels mark some pathological conditions like: failing pregnancies (early pregnancy loss, spontaneous abortion or ectopic pregnancy), hydatidiform mole, choriocarcinoma, invasive and non-invasive gestational trophoblastic diseases, as well as retrodifferentiation to cytotrophoblast cells and cancer advancement in testicular germ cell malignancy cases (Table III).

In Down syndrome pregnancies the limitation of villous cytotrophoblast cells differentiation leads to these cells accumulation and in consequence to the increase of hyperglycosylated hCG. Thus, H-hCG can be used as an improved marker for Down syndrome, in both the first and second trimesters of pregnancy [24].

Excessively large N- and O-linked oligosaccharides forms of hCG have also been demonstrated to be produced by non-gestational cancer cells [25, 26]. Their expression was detected in cancer of: brain, pancreas, lung, breast, kidney, colon, ovaries and endometrium [4, 5]. These tumor derived H-hCG has been shown to contain increased amounts of triantennary N-glycans [13], abnormal biantennary N-glycans [27], and biantennary core-2 type O-glycans [13, 27]. What is more the detection of free H-hCGβ in serum or β-subunit core fragment in urine samples correlates with poor grade and advanced stage cancer, or poor outcome malignancy [1]. Therefore, these particular forms might be used as markers allowing to detect and monitor: pregnancy, gestational trophoblastic diseases and cancer.

Table IV. Automated diagnostic analysers and kits used for determination of hCG level in blood samples.

diagnostic system/kit	detection limit	detecting molecules	
		hCG	hCGβ
Abbott Architect /Architect total βhCG	2.0 mIU/ml	yes	yes
Abbott AxSYM /AxSYM total βhCG	2.0 mIU/ml	yes	yes
Beckman Access /Acces total βhCG 5th IS	0.5 mIU/ml	yes	yes
Ortho Vitros /Vitros total βhCG II Reagent Pack)	0.70 mIU/ml detection 2.39 mIU/ml quantification	yes	yes
Perkin-Elmer /AutoDelfia hCG KIT	0.5 mIU/ml	yes	no
Roche Elecsys /free βhCG	0.1 mIU/ml	no	yes
Roche Elecsys /HCG+β Intact human chorionic gonadotropin + the β-subunit	0.1 mIU/ml	yes	yes
Roche Elecsys/HCG STAT Human chorionic gonadotropin, STAT (Short Turn Around Time	0.5 mIU/ml	yes	no
Siemens Centaur /Access Total PhCG (5 th IS)	0.6 mIU/ml	yes	yes
Siemens Immulite 2000/HCG	0.4 mIU/ml	yes	yes
Tosoh AIA/ST AIA-PACK HCG	2.5mIU/ml	yes	no
Tosoh AIA/ST AIA-PACK HCGII	2.0 mIU/ml	yes	no
Tosoh AIA/ST AIA-PACK βHCG	0.5 mIU/ml	yes	yes
Tosoh AIA/ST AIA-PACK βHCGII	0.5 mIU/ml	yes	yes

hCG assays

The first pregnancy test, the rabbit bioassay, was developed in the 1920s [28]. For four decades this test was the only way to measure hCG, and thus to detect pregnancy. The development of polyclonal antibodies in 1960s originated with a new – the agglutination inhibition test [29]. In 1967 the discovery of the competitive immunoassay allows hCG radioimmunoassay development, which became the first fast and sensitive method and gave rise to a variety of commercial hCG tests, both over the counter or fully automatic tests used in clinic till now [1].

The first hCG assays identified the whole heterodimeric molecule. Since alpha subunit of hCG and LH is identical, both hormones were picked up by the tests. It is in 1973 only, thanks to antibodies recognizing hCG beta subunit, the hCG-specific pregnancy test was develop [30]. Two years later monoclonal antibodies were utilized and their commercial availability and low price leaded to hCG detection and quantification further improvement [31].

Nowadays two-antibodies immunometric assays, based on sensitive antibody enzyme-labeling and high sensitivity fluorimetric and chemiluminescent tracers are used. These assays contain at least two antibodies that recognize different epitopes on hCG molecule; the first antibody immobilise the hormone on a carrier, the second one allows the precise determination of the hormone amount [1].

Currently there are more than 100 types of antibodies recognizing hCG-related molecules which are commercially available. Most of them bind hCG thanks to the interaction with one of its 26 known epitopes [1, 32]. This fact creates some problems – since hCG-assays do not utilized the same sets of antibodies, various tests can detected different forms of hCG. On the other hand they can detected the same forms of hormone but with different sensitivity (Table IV). Thus, the results obtained with different hCG assays may vary greatly.

The most tests detecting hCG utilised two antibodies: first directed against beta subunit core (aa 1-91) of hCG, and the second one recognising the CTP of beta subunit (aa 92–145). It was showed that this second antibody, which is commonly utilised by commercially available tests, does not bind glycosylated hCG efficiently. Thus, it does not allow to detect all hCG molecules secreted during pregnancy, by choriocarcinomas and other cancers [1].

Only particular antibodies directed toward regular hCG was showed to cross-react with the hyperglycosylated form of hormone [33]. So far, the most reliable method of H-hCG measurement is based on the usage of two monoclonal antibodies, including B152 antiobody. The antibody has more than 99% specificity for H-hCG [33]. As it was reported, B152 interacts with carbohydrate and peptide backbone structure of hCG, however detailed characterization of its recognition patterns was not completed [20, 27].

Meanwhile, the differences in hCG assays specificity are critical especially in case of cancer patients. Both hCG and hCGβ measurement is crucial for diagnosis and monitoring of placental trophoblastic and testicular cancers as well as nontrophoblastic tumors (30%–60% of which produce hCGβ, but not hCG) [34]. Therefore the recognition of particular hCG forms, including H-hCG and free H-hCGβ, correlating with poor grade and advanced stage cancer or poor outcome malignancy, might improve cancer diagnostic. Unfortunately because of H-hCG highly heterogeneous carbohydrate structure, a reference material for this form of hormone has not been established yet. Thus, it is impossible to use H-hCG-related molecules in cancer diagnostic. However, they might be recognized and distinguished, thus used as a markers,using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI) [35].

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Conclusions

Human chorionic gonadotropin plays a key role in pregnancy development and maintaining. Thus, the measurement of hCG concentration in serum and urine of pregnant women is a powerful tool for detection and monitoring of pregnancy and pregnancy related disorders. Recently it was showed that similarly to pregnancy also non-trophoblastic malignancies produce variety of hCG-related molecules, namely: regular hCG, hyperglycosylated hCG and predominate in case of cancer – hyperglycosylated hCG free beta subunit of hormone. Even the presence of these different variants is well documented, still the commercially available tests failed to detected and distinguish among them.

Therefore the development of a new test identifying all hCG-related molecules in one assay is needed. Such a "total hCG" test would be an ideal in order to monitor pregnancy, pregnancy disorders and cancer cases.

On the other hand the recognition of particular, disease-specific variants of hCG might be a crucial for precise diagnosis. This, however requires a detailed characteristic of hCG-related molecules, evaluation of their specific epitopes and a new antibodies development.

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