Significance of TGFBR3 allelic loss in the deregulation of TGFβ signaling in primary human endometrial carcinomas

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Abstract. Downregulation of betaglycan (β-glycan) [transforming growth factor β receptor type III (TGFβR3)], which belongs to co-receptors of the TGFβ pathway, occurs in a broad spectrum of primary human malignancies. However, in the case of endometrial cancer (EC), the mechanisms responsible for genetic alterations are still unknown. Therefore, we investigated allelic imbalance at the TGFBR3 locus (1p33-p32) in the context of β-glycan mRNA and protein expression, as a possible genetic event determining β-glycan deregulation in EC patients. Study of β-glycan allelic imbalance in 48 primary human ECs was performed with the use of three different microsatellite markers, spanned within or in direct proximity to the TGFBR3 locus. Real-time PCR and western blotting were used for β-glycan mRNA and protein quantification methods, respectively. Altogether, 25 of 39 (64%) informative cases and 25 of 48 (52%) of all specimens showed allelic imbalance in at least one microsatellite marker, concomitantly with decrease at both the β-glycan transcript and protein levels. Interestingly, 54% (15/28), 36% (8/22) and 35% (7/20) of informative ECs displayed allelic loss in DIS188, DIS435 and DIS1588 microsatellite markers, respectively. It is worth pointing out that 5 out of 39 (13%) informative cases showed loss of heterozygosity (LOH) at two microsatellite markers. Microsatellite instability (MSI) was found in two markers, but to a very strictly limited extent. None of the clinicoprognostic features was found to be of significance. Our results suggest that LOH in the TGFBR3 locus may be one of the mechanisms responsible for loss of β-glycan expression. No correlation of LOH at the TGFBR3 locus with clinicopathological parameters suggests that allelic imbalance may be an early genetic event during neoplastic transformation of human endometrium.

Introduction

Betaglycan (β-glycan) [transforming growth factor β receptor type III (TGFβR3)] belongs to the membrane-bound accessory receptors involved in signal initiation and propagation in cellular pathway, activated by transforming growth factor-β types (TGFβs) (1). Biochemically, this receptor is a proteoglycan encoded by 225,660 bp TGFBR3, which is located on chromosome 1p33-p32 (NCBI reference sequence: NG_027757.1). It has two promoters but in the majority of tissues, the proximal type dominates over a distal one. TGFBR3 is divided into 18 exons and encodes a protein of 851 amino acids (2). The molecular weight of β-glycan protein is approximately 300 kDa, due to heparin and chondroitin sulphate modifications (3-5). It is ubiquitously expressed in almost all cell types, in approximately 200,000 particles/cell, and forms the non-covalently linked homodimers (6).

Although, structural analysis of β-glycan revealed lack of any well-known signaling motif in its amino-acid sequence, it plays a vital role in signal mediation in the TGFβ pathway. Studies on TGFβ binding caused by β-glycan demonstrated its affinity to TGFβ isoforms and inhibit A, BMP-4 and -7, as well as to GDF-5. β-glycan shows the highest effect on TGFβ2-induced signal initiation, which itself binds poorly to the TGFβ type II receptor (TGFβRII). This suggests the particular role of β-glycan in TGFβ2 signaling (7-10). Signal mediation via β-glycan is based on TGFβ factor binding by its extracellular domain, with simultaneous ligand concentration on the cell surface and complex formation with TGFβRII receptor. As a consequence, the β-glycan cytoplasmic region promotes interaction of intracellular domains of heterodimeric TGFβRII and I receptors, as well as trans-phosphorylation of TGFβRI by TGFβRII. After activation of TGFβRII and TGFβRI receptors, β-glycan dissociates from this complex and phosphorylated TGFβRII receptor propagate signal downstream to the cellular TGFβ effector, Smad proteins (11). In addition to the above-mentioned mechanism of action, in physiological conditions, the β-glycan extracellular domain undergoes proteolytic cleavage (ectodomain shedding) by metalloproteases (MT1-MMP and MT3-MMP) and plasmin, resulting in the sequestration of a soluble form of β-glycan (sol-β-glycan) in the extracellular matrix (ECM). Sol-β-glycan is thought to modulate the signal induced by TGFβ factors, competing with...
TGFβ receptors for their ligands. Sol-β-glycan functions as an antagonist of TGFβ signaling, suggesting a potential role as an antitumor agent in future therapies (12-17). Another proposed mechanism of signaling orchestration in the TGFβ pathway is the steric effect, which is a result of β-glycan modification by residues of glycosaminoglycans (GAG). Oncogenic Ki-ras seems to be involved in post-translational GAG attachment, resulting in increased responsiveness to TGFβ proliferative stimuli and downregulation of p21 in colon cancer (18). Mythreye and Blobe (19) reported that GAG modifications are necessary for inhibition of cell migration. Compared to controls, TGFBR3ΔGAG mutants displayed increased migratory properties, as shown either in ovarian cancer (Ovca429) or in normal ovarian surface epithelial (NOSE007) cell lines. The GAG chains inhibit TGFβ induced signaling by preventing formation of TGFβRII-TGFβRI complexes, as confirmed in an LLC-PK1 model (20).

Some studies suggest an un questioned role of alterations in the TGFβ signaling pathway in many human diseases, in particular cancer (10,21-23). A large number of factors activating the TGFβ cascade result in the regulation of opposed processes, what is known as the pleiotropic effect on cells. TGFβs inhibit cancer development and progression early in neoplastic transformation, whereas they contribute to acquisition of a metastatic phenotype in more advanced clinical stages (11). Our recent data clearly demonstrated downregulation of β-glycan mRNA and its relationship with clinical and pathological parameters (24,25). It suggests that in the case of ECs, TGFβ deregulation may be a result of impaired TGFβ2 signaling, which is caused by β-glycan. However, a literature search (Medline® database) has revealed that there are no studies evaluating the role of allelic loss of β-glycan (TGFβR3) in primary human ECs.

The results obtained are the basis for the search of potential molecular mechanisms responsible for the β-glycan decline in primary human ECs. The aim of our present study was to evaluate loss of heterozygosity (LOH) as a potential mechanism responsible for downregulation of β-glycan in primary human ECs. We also correlated the prevalence of allelic loss with clinical and pathological variables of uterine malignancies.

### Materials and methods

**Patient material.** Tissue samples from women having undergone surgery for primary ECs were collected in the Second Department of Gynecology, Lublin Medical University, Lublin, Poland, between 2010 and 2014. The study group consisted of 48 EC specimens and matched 48 normal tissue samples. None of the patients had received hormonal therapy, radiation therapy or chemotherapy before surgery. The mean age of the patients was 62 years, ranging from 46 to 81 years. At surgery, tissue specimens were immediately subdivided into two portions; one was fixed in buffered formalin (pH 7.4) for routine pathological examination, and the other was stored at -70°C until further analysis. The clinical stage was assigned according to a recently established FIGO classification system (26). World Health Organization classification was applied to determine the pathological grading. Myometrial and lymph node invasion and VSI (vascular space invasion) were evaluated as well. Clinicopathological variables of the EC samples are depicted in Table I. The study cohort was subdivided into two age groups: the first group consisted of women <60 years of age (n=21; 44%), the second group of women ≥60 years (n=27; 56%). The Independent Ethics Committee of the Lublin Medical University, Lublin, Poland approved the tissue collection and subsequent experiments, and all the women enrolled provided their informed consent.

<table>
<thead>
<tr>
<th>Patients’ age (years)</th>
<th>No. of patients (%)</th>
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<tbody>
<tr>
<td>&lt;60</td>
<td>21 (44)</td>
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<tr>
<td>≥60</td>
<td>27 (56)</td>
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<tr>
<th>Clinical stage*</th>
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<td>I</td>
<td>22 (46)</td>
</tr>
<tr>
<td>II</td>
<td>15 (31)</td>
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<tr>
<td>III</td>
<td>7 (15)</td>
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<td>IV</td>
<td>4 (8)</td>
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<th>Histological gradeb</th>
<th>No. of patients (%)</th>
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<tr>
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<td>9 (19)</td>
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<tr>
<td>G2</td>
<td>34 (71)</td>
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<tr>
<td>G3</td>
<td>5 (10)</td>
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<th>Depth of myometrial invasion</th>
<th>No. of patients (%)</th>
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<tbody>
<tr>
<td>&lt;1/2</td>
<td>21 (44)</td>
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<tr>
<td>&gt;1/2</td>
<td>27 (56)</td>
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<tr>
<th>Vascular space invasion</th>
<th>No. of patients (%)</th>
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<tr>
<td>Not present</td>
<td>37 (77)</td>
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<tr>
<td>Present</td>
<td>11 (23)</td>
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<tr>
<th>Lymph node invasion</th>
<th>No. of patients (%)</th>
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<tr>
<td>Not present</td>
<td>36 (75)</td>
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<tr>
<td>Present</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Not assessed</td>
<td>11 (23)</td>
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### RNA isolation and real-time polymerase chain reaction (PCR).
Total RNA was extracted according to a modified Chomczynski and Sacchi protocol. Afterwards, RNA (1 µg) was retro-transcribed using RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, Canada) according to manufacturer's recommendation. Real-time PCR was performed using TaqMan® probes (Life Technologies, Carlsbad, CA, USA), and in line with the protocol on Mastercycler® Eppendorf. The catalogue numbers of probes were: Hs00234259_m1 for TGFBR3 (β-glycan) and Hs99999905_m1 for GAPDH. The relative expression level was normalized to GAPDH, and calculated using the following equation: 2<sup>ΔCt</sup> x 1,000.

### Western blotting.
Tissue samples were homogenized in lysis buffer containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.4),
5 mM MgCl₂, 0.5% Triton X-100 and 1 mM PMSF. For each sample, protein concentration was evaluated by Lowry protocol (27). The proteins (30 µg/well) were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (7.5%) and transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA) using the semi-dry system. The membranes were incubated overnight at 4°C with primary antibodies after prior blocking with 5% dry non-fat milk. Following extensive washing with Tris-buffered saline with 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies for 1 h and visualized with Novex HRP Chromogenic Substrate-TMB (Invitrogen Inc., Carlsbad, CA, USA). β-actin served as loading control. After TMB visualization, blots were incubated at 50°C for 45 min in stripping buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 0.8% β-mercaptoethanol, rinsed with running water, and rehydrated with methanol. Immune-identification of β-actin was carried out. Quantitative analysis was performed by measuring IOD by GelProAnalyzer v. 3.0 for Windows™ software (Media Cybernetics, Baltimore, MD, USA). The following commercially available antibodies were applied: primary: rabbit polyclonal anti-β-actin antibodies (sc-1616, Santa Cruz Biotechnology, MD, USA). The following commercially available antibodies were applied: primary: rabbit polyclonal anti-β-actin antibodies (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) against carboxy-terminus of actin of human origin (dilution: 1:1,000); secondary: goat anti-rabbit polyclonal antibodies (A9169, Sigma-Aldrich, Schnelldorf, Germany) against whole molecule (dilution: 1:20,000), rabbit anti-goat polyclonal antibodies (A8919, Sigma-Aldrich) against whole molecule (dilution: 1:5,000).

**DNA isolation and PCR.** Total genomic DNA was isolated according to the phenol/chloroform protocol. Briefly, tissue was minced and homogenized with denaturing solution consisting of 10 mM EDTA, 10 mM Tris/HCl (pH 8.0), 0.5% SDS. Following overnight incubation at 55°C with 20 µl of proteinase K (10 mg/ml), an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) mixture was added to the samples. After centrifugation, the aqueous phase was precipitated with an equal volume of isopropanol. DNA pellet was washed twice with 70% ethanol, re-suspended in TE buffer and stored at -70°C for further analysis. The quality and quantity of DNA was estimated spectrophotometrically with a BioPhotometer Plus (Eppendorf, Hamburg, Germany).

PCR was performed in Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). Primers used in the reaction were fluorescently labeled (Sigma-Aldrich). Table II presents the TGFBR3 microsatellite markers and PCR conditions applied. Briefly, the PCR reaction was carried out in a total volume of 12.5 µl and the mixture consisted of 1X PCR buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin] dedicated for the JumpStart™ Taq DNA polymerase (Sigma-Aldrich), 5 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM of each dNTP and 0.625 U of JumpStart™ Taq DNA polymerase. Sequences of primers were adopted from the National Center for Biotechnology Information database-NCBI (www.ncbi.nlm.nih.gov).

**LOH analysis.** After PCR, the samples were mixed with solution containing deionized formamide, GeneScan-350 TAMRA (Life Technologies) dye size standard, and loading buffer (blue dextran, EDTA). They were denatured, chilled on ice, and separated in 5% Long Ranger (BioWhittaker Molecular Applications, Rockland, ME, USA) containing 6 M urea. PCR products were analyzed using DNA Sequencer ABI PRISM 377 (Applied Biosystems). Allele lengths were determined with GeneScan v. 3.1.2 and Genotyper v. 2.5 software (Applied Biosystems). Amplification of microsatellite markers yielded one or two allele peaks, depending upon whether the individual is homozygous (non-informative cases) or heterozygous (informative cases) for that marker. Loss of heterozygosity (LOH) was defined when one allelic band from tumor DNA disappeared completely or when the signal intensity (allelic ratio ≤0.5) was reduced <50% in the tumor DNA compared with the paired normal DNA pattern. Allele ratios were calculated only for informative cases according to the following formula: \(T_1/T_2/N_1/N_2\), where \(T_1\) and \(N_1\) are the values for shorter length allele product peak of tumor and normal sample, and \(T_2\) and \(N_2\) are the values for longer length allele product peak of tumor and normal sample, respectively (28). All samples were analyzed in replicates.

**Statistical analysis.** Statistical tests were performed applying GraphPad Prism v. 5.00 software for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as mean ± standard error of mean (SEM). P-values were
calculated using two-tailed paired Student's t-test. The statistical analysis of allelic loss included the assessment of the association between the prevalence of LOH and clinical and pathological variables using Fisher's two-tailed exact test. \(P<0.05\) was considered to indicate a statistically significant difference.

**Results**

**β-glycan expression pattern.** Real-time PCR and western blotting were used for β-glycan mRNA and protein quantification methods, respectively. \(TGFBR3\) expression was found to be significantly downregulated in the EC samples compared to that noted in the normal tissues (\(P<0.001\)) (Fig. 1A). Moreover, β-glycan mRNA decline corresponded to its protein decrease, as demonstrated in all EC samples studied. The decrease of β-glycan protein expression in ECs was highly significant (\(P<0.001\)) (Fig. 1B). Examples of immunoblots of β-glycan in normal and cancer samples, β-actin served as a control for the amount of protein loading. Antibodies are described in Materials and methods. Data are presented as mean ± SEM. P-values were estimated by the paired Student's t-test. ***\(P<0.001\).

<table>
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<th>Marker</th>
<th>Informative cases/total cases</th>
<th>LOH-positive cases (%)</th>
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<tr>
<td>D1S188</td>
<td>28/48</td>
<td>15 (54)</td>
</tr>
<tr>
<td>D1S435</td>
<td>22/48</td>
<td>8 (36)</td>
</tr>
<tr>
<td>D1S1588</td>
<td>20/48</td>
<td>7 (35)</td>
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**Correlation between β-glycan LOH and clinicopathological variables of ECs.** The correlation between LOH in the \(TGFBR3\) locus and clinical and pathological variables of EC samples is presented in Table IV. None of the clinicoprognostic features, including clinical staging, histological grading, myometrial invasion, VSI and age at diagnosis, was found to be of significance. Moreover, additional analysis of LOH occurrence in ECs in relation to combined clinicopathological variables, i.e., FIGO stage/myometrial invasion, histological grade/myometrial invasion, FIGO stage/age at diagnosis, histological grade/age at diagnosis did not reveal any significant differences (data not shown). Therefore, the presence of LOH in the \(TGFBR3\) locus did not appear to determine the malignancy of the sporadic uterine neoplasms, and probably is an early event during endometrial transformation in humans.
Discussion

EC is one of the leading causes of cancer-related mortality among females with approximately 320,000 new cases and 76,000 deaths worldwide in 2012. In Poland, 5426 new cases were diagnosed in 2011 (29). A significantly higher incidence rate is observed in developed countries in contrast to less developed ones. An increase in the EC incidence rate should...
be expected in the near future due to the intensified aging of human societies (30).

Other studies suggest that cancer development and progression appears to be associated with alterations in the TGFβ signaling cascade (31). Disturbed signal mediation in the TGFβ pathway triggers its development from a tumor suppressor, early in neoplastic transformation, to a cancer-promoting and -metastatic agent in advanced clinical stages of the disease (32). A particular role in neoplastic transformation has been reported in the case of β-glycan, which acts as a tumor inhibitory agent suppressing cancer cell migration, invasion, proliferation and angiogenesis (6,11). β-glycan downregulation is responsible for impaired ligand presentation to TGFβ canonical receptors - TGFβRII and TGFβRI (33). Loss of β-glycan expression results in impaired signaling driven by TGFβ2 isoform, as TGFβ2 possesses the highest and exclusive affinity to β-glycan (33-35). Besides TGFβ2 isoform signal mediation, β-glycan reduction may favor development of an immunotolerant tumor microenvironment as an immune suppressor and stimulator of Treg cells (36). Furthermore, involvement of β-glycan in cancer development through non-canonical TGFβ signaling pathways cannot be excluded. Significant alterations in β-glycan expression have been reported in several human neoplasms originating from different tissues, such as breast, endometrial, ovarian, pancreatic, prostate, bladder, liver, lung and renal carcinomas (24,37-46). According to previous studies, chromosome 1p exhibits a meaningful allelic imbalance in a number of primary human malignancies (47).

Our current results are in line with those previously published by Florio et al (48), who reported a significant downregulation of β-glycan expression with concomitant decrease in the inhibit α-subunit in the case of EC. Current results concerning β-glycan protein expression are not in line with those published previously by our group (24), where ELISA assay was performed. Presently, protein expression was analyzed by western blotting as a more specific and acknowledged method. Moreover, we applied different primary antibodies which corresponded to a region within amino acids 88-74 of the fully processed and mature human β-glycan C-terminus may lead to immunodetection of non-functionally synthesized β-glycan particles with abrogated trafficking to the cell membrane and might also cross-react with endoglin due to the sequence similarities.

Indirect epigenetic regulation was reported in bladder urothelial tumor microenvironment as an immune suppressor and stimulator of Treg cells (36). Besides TGFβ2 isoform signal mediation, β-glycan reduction may favor development of an immunotolerant tumor microenvironment as an immune suppressor and stimulator of Treg cells (36). Furthermore, involvement of β-glycan in cancer development through non-canonical TGFβ signaling pathways cannot be excluded. Significant alterations in β-glycan expression have been reported in several human neoplasms originating from different tissues, such as breast, endometrial, ovarian, pancreatic, prostate, bladder, liver, lung and renal carcinomas (24,37-46). According to previous studies, chromosome 1p exhibits a meaningful allelic imbalance in a number of primary human malignancies (47).

The data of our study indicate LOH in the TGFBR3 region located at chromosome 1p, and its association with a decrease in β-glycan in sporadic human ECs. These results suggest that allelic loss at the TGFBR3 region may be the mechanism through which EC cells escape from TGFβ-mediated suppression. Similar results have been achieved in studies on allelic imbalance in different human neoplasms, in particular in those derived from hormone-dependent tissues (38,39,41,42). In breast carcinomas, the mechanisms responsible for down-regulation of β-glycan included LOH in microsatellite markers (DIS1588 and DIS188), in half of the samples analyzed (38). The study of the DIS1588, DIS2804 and DIS435 microsatellite markers in prostate carcinomas showed LOH in 37.5% of samples studied (41). Comparable results of allelic imbalance in the β-glycan locus have been achieved in investigations of non-small cell lung carcinomas, where LOH was reported in 38.5% of cases, at least in one microsatellite marker (DIS1588, DIS188 or DIS2804) (42). Despite the high percentage of LOH-positive cases in the TGFBR3 locus in our study (52% of the samples investigated; 64% of informative cases), other mechanisms causing mRNA and protein downregulation in ECs cannot be excluded.

Studies on cancer cell lines revealed that loss of β-glycan expression could be due to altered epigenetic regulation, as observed in prostate and ovarian tumor cell lines, where β-glycan expression was restored after treatment with methylenetransferase and histone deacetylase inhibitors (39,41). Indirect epigenetic regulation was reported in bladder urothelial and renal cell carcinoma (23,49). B-glycan expression in these tumor types appeared to be positively controlled by the GATA3 transcription factor (23,49). Increased methylation of GATA3 was found to result in a decline in β-glycan at the transcriptomic level (49). The molecular mechanisms determining β-glycan expression alterations appear to involve other mechanisms as well, since neither genetic aberrations nor allelic imbalance were found to be associated with β-glycan expression in hepatocellular carcinoma (44). Transcriptional regulation is suggested to play an exclusive role. Moreover, the lack of a statistical significance of LOH occurrence and single or combined clinicopathological parameters of EC samples strongly supports the hypothesis that β-glycan allelic imbalance may be an early genomic event during endometrial neoplastic transformation that does not determine cancer aggressiveness.

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References


