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Microsatellite polymorphism in the P1 promoter region of the IGF-1 gene is associated with endometrial cancer

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Abstract. Endometrial carcinoma (EC) is the most common type of gynecological malignancy. Studies have demonstrated that the insulin growth factor (IGF) pathway is implicated in the development of endometrial tumors and that the serum levels of IGF-1 are affected by estrogen. Most EC cells with high microsatellite instability (MSI-H) accumulate mutations at a microsatellite sequence in the IGF-1 gene. The present study investigated the CA repeat polymorphism in the P1 promoter region of the IGF-1 gene among Caucasian females with endometrial hyperplasia, EC and healthy control subjects, whose blood serum and surgical tissue specimens were analyzed. Differences or correlations between the analyzed parameters [serum levels of IGF-1 and IGF binding protein (IGFBP)-1 and IGFBP-3 as well as estrogens among the polymorphisms] were verified using the χ^2, Mann-Whitney U, Kruskal-Wallis or Spearman's rank correlation tests. A PCR amplification and DNA sequencing analysis was used for identification of $(CA)_n$ repeats in the P1 region of IGF-1. ELISA was used to determine the blood serum levels of IGF-1, IGFBP-1, IGFBP-3 and estrogens. Furthermore, IGF-1 was assessed in endometrial tissues by immunohistochemical analysis. The present study indicated no statistically significant differences between serum levels of IGF-1, IGFBP-1, IGFBP-3 and estrone, estriol and estradiol in the control and study groups. A significant correlation was identified between the IGF-1 levels and estrone levels in the MSI-H polymorphism (r=-0.41, P=0.012) as well as a highly negative correlation between IGF-1 levels and the estradiol levels in the MSI-H polymorphism (r=-0.6, P=0.002). Genotypes without the 19 CA allele were predominantly found in EC. Furthermore, statistical analysis indicated that the number of IGF-1-expressing cells was significantly elevated in MSI-H type 18-20 (P=0.0072), MSI-L type 19-20 (P=0.025) and microsatellite-stable MSS type 19-19 (P=0.024) compared with those in the MSI-H 20-20 genotype. The present study suggested that it is rather likely that the polymorphisms in the IGF-1 promoter are associated with EC in Caucasian females with regard to its development. In the present study, polymorphisms of the IGF-1 promoter may have been introduced during the genesis of EC and contributed to it by leading to aberrant expression of IGF-1.

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Introduction

Endometrial carcinoma (EC) is the most common type of gynecological malignancy and are categorized as follows: Type I EC is estrogen-dependent and often occurs in postmenopausal women, accounting for >85% of cases, whereas type II EC is not estrogen-dependent (1,2). In estrogen-induced endometrial carcinogenesis, insulin-like growth factor-1 (IGF-1) has an important role. Estrogens increase the expression of IGF-1 in tissues, and IGF-1 is required to mediate their mitogenic effects on the endometrium (3,4). In addition, estrogens modulate IGF-1 signaling by regulating the expression in other members of the IGF family, including the ligands insulin receptor substrate-1 (IRS-1) and IGF binding proteins (IGFBPs) (5). However, the exact mechanisms of estrogen-induced EC have remained elusive.

Stimulation of uterine epithelial cell proliferation by estradiol was indicated to be mediated by the IGF1/IRS-1/phosphoinositide-3 kinase (PI3K)/AKT pathway, which targets the activity of mitotic kinase cyclin-dependent kinase (Cdk1)/cyclin B (6). Under specific conditions, deregulation of Cdks may be essential for DNA damage and cancer development (7). Recently, Tang *et al* (8) also demonstrated that estrogen and IGF-1 act synergistically to promote the development of lung adenocarcinoma in mice, which may be associated with the activation of mitogen-activated protein kinase (MAPK) signaling pathways, in which estrogen receptors beta 1 and beta 2 as well as IGF1 receptor (IGF1R) have important roles.

Genes encoding for the human protein IGF-1, located in the long arm of chromosome 12 (12q22-24.1), cover an area of

~90 kbp and contain six exons separated by long (1.9-50 kbp) introns. The sequence of the IGF-1 gene is highly conserved and its transcription is controlled by the two promoters P1 and P2, while it is estimated that ~90% of IGF-I transcripts are controlled by P1. The P1 promoter region of the human genome comprises 322 nucleotides located in the 5'-untranslated region (5'UTR) and exon 1 of the regulatory region at 1,630 bp. The most highly conserved region is a 322-nucleotide sequence in the 5'UTR. The P1 promoter region lacks typical sequences of other genes, such as TATA or CCAAT elements, lacking defined transcriptional start points and also GC-rich areas or CpG islands. The P1 promoter has five sections, HS3A, HS3B, HS3C, HS3D and HS3E, which are protected from DNase digestion. HS3D is thought to be responsible for the regulation of IGF-I expression by estrogens (9,10). 5' Cytosine-adenosine (CA)$_n$ repeats in the P1 promoter region of the IGF-I gene, 1 kb upstream of the transcription site, are highly polymorphic microsatellites comprising a variable length of repeat sequences. The number of CA repeats ranges between 10 and 24 with the most common allele containing 19 CA repeats (192 bp), characteristic for Caucasian genotypes (9,11). Numerous studies suggested that the number of CA repeats in the promoter region is inversely correlated with the transcriptional activity. The involvement of the polymorphism of CA promoter dinucleotide repeats in clinical conditions, including cancer, diabetes and cardiovascular diseases as well as parameters including birth weight, adult body height and IGF-1 serum levels, has remained controversial (12,13).

It is well known that IGF-1 is produced in most organs and tissues where it can function in an autocrine as well as a paracrine manner to stimulate cell growth. However, the liver is the major source of circulating IGFs. The activity of IGF-1 is mediated through IGF1R, a tyrosine kinase receptor that can bind to IGF-1 and IGF-2 to initiate activation of two principal downstream signaling pathways, including the Ras-Raf-extracellular signal-regulated kinase signaling pathway, the PI3K/Akt and the MAPK signaling pathway. The MAPK signaling pathway is primarily responsible for cell growth and proliferation (14).

The bioavailability of IGF-1 is regulated by the circulating concentration and cellular expression of six IGFBPs, which are expressed in human endometrium. Among them, IGFBP-1 has the highest abundance and competes with type I IGF receptor for binding of IGF in the endometrium. Due to its high affinity, the majority of IGF-1 circulates in a complex with IGFBP-3 and IGFBP-1 (15).

To the best of our knowledge, microsatellite polymorphisms in the P1 promoter region of the IGF-1 gene have not been previously studied in human EC. The present study investigated the correlation between the circulating levels of IGF-1, IGFBP-1, IGFBP-3 and estrogens in various types of microsatellite polymorphism in the P1 promoter region of the IGF-1 gene in patients with EC.

Materials and methods

Ethics statements. The study was approved by the Ethics Committee of the Medical University of Lublin (Lublin, Poland; Resolution of the Bioethics Comittee no. 0254/263/2011). Written informed consent was obtained from all subjects included, and the study was performed in accordance with the principles of the Helsinki Declaration.

Patient samples. Patient samples used for the assessment of IGF-1 levels as well as CA repeat analysis of the P1 promoter region of the IGF-1 gene comprised: i) Peripheral blood obtained from the antecubital vein prior to surgery from 82 patients enrolled in the present study and ii) tissue sections embedded in paraffin (Sigma-Aldrich, St. Louis, MO, USA) from patients who underwent surgery at the Department of Gynecological Oncology and Gynecology, Medical University of Lublin (Lublin, Poland) between November 2010 and December 2014.

A total of 33 tissue samples were taken from post-menopausal women with type I EC [endometroid type adenocarcinoma; G2 stage according to the International Federation of Gynecology and Obstetrics (FIGO) criteria from 2009 (16)]. The number of samples classified as FIGO stages Ia, Ib, II and IIIb were 12 (37.5%), 12 (37.5%), 4 (12.5%) and 4 (12.5%), respectively. Diagnosis was performed histologically by two independent pathologists. Furthermore, tissue samples from 32 post-menopausal women with hyperplasia simplex (HS; non-atypical) were used. The control group consisted of endometrial tissue samples from 27 patients referred to the department for diagnostic procedures of uterine bleeding in which histopathological examination found endometrium proliferativum. Patients with hormone replacement therapy, other types of cancer, systemic diseases, ischemic heart disease, peripheral vascular diseases, thyroid diseases and/or other endocrine diseases as well as liver and bile duct diseases were excluded from the study. The average age of the patients with EC was 64 years. The mean age in the groups of patients with EC and HS was higher than that in the control group (64.2 years [range, 56-78 years] and 62.8 years [range, 50-71 years] vs. 60.1 years [range, 47-68 years], respectively; P=0.01.

Enzyme-linked immunosorbent assay (ELISA). ELISA was used to assess the plasma levels of IGF-1, IGFBP-1, IGFBP-3, estrone, estriol and estradiol using the following kits: Human IGF-I Quantikine ELISA kit (cat. no. DG100; R&D Systems, Minneapolis, MN, USA), IGFBP-1 ELISA kit (cat. no. DEE001; Demeditec Diagnostics GmbH; Kiel-Wellsee, Germany), human Insulin-like Growth Factor Binding Protein-3 ELISA kit (cat. no. E03A; Mediagnost, Reutlingen, Germany), Estrone ELISA kit (cat. no. EIA-4174), Estriol ELISA kit (cat. no. EIA-3717) and Estradiol ELISA kit (cat. no. EIA-2693) (all from DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions.

DNA isolation from peripheral blood cells. DNA was isolated from peripheral blood cells using the QIAamp DNA mini kit (cat. no. 51306; Qiagen, Hilden, Germany).

DNA isolation from paraffin-embedded tissue sections. Paraffin-embedded tissue blocks fixed in 10% buffered formalin (Sigma-Aldrich) were cut into two or three 4-μm sections using a microtome (model SM 2000R; Leica Biosystems GmbH, Nussloch, Germany) with a razor blade (Feather Microtome Blade Type R35; Feather Safety Razor Co., Ltd., Osaka, Japan), which was cleaned with ethanol between samples. A fresh cutting blade was used for the cutting of each of the paraffin blocks. The sections obtained were placed in a 1.5-ml test tube containing polypropylene (Sigma-Aldrich) and stored at 4°C for future analysis.

The isolation of DNA from archived paraffin tissues was performed using a Maxwell® 16 Instrument for Nucleic Acid and Protein Purification device (cat. no. AS1250; Promega Corp., Madison, WI, USA) equipped with its designated software for automated DNA isolation with use of the Maxwell 16 FFPE Plus LEV DNA Purification kit (cat. no. AS1135; Promega Corp.). Quantitative analysis of the DNA obtained was performed using a Novaspec II automatic spectrophotometer (GE Healthcare, Little Chalfont, UK). The resulting DNA was used as template for polymerase chain reaction (PCR) amplification followed by analysis of CA repeats in the P1 promoter region of the IGF-1 gene.

Analysis of CA repeats in the P1 region of IGF-1. Analysis of $(CA)_n$ repeats of the IGF-1 gene located 1 kb upstream of the transcription start site was performed using PCR and fragment analysis. PCR was performed in 15-μl volumes consisting of 100 ng genomic DNA, 3.75 pmol forward primer (5'-AAG AAAACACACTCTGGCAC-3') fluorescently labeled with FAM (Polish Academy of Science, Warsaw, Poland), 3.75 pmol reverse primer (5'-ACCACTCTGGGAGAAGGGTA-3'; Roche Diagnostics, Mannheim, Germany), 0.01 mM deoxynucleotide triphosphate (Polish Academy of Science), 1.5 mM $MgCl_2$ (Fermentas, Poznan Poland), 1X PCR buffer (Fermentas) and 0.6U HiFi DNA polymerase (cat. no. N1003 05; Novazym, Poznan, Poland). The analysis was performed using a thermal cycler (Tgradient Thermocycler, Biometra, Goettingen, Germany). Amplification cycles included one cycle of 4 min at 94˚C; 28 PCR cycles consisting of 5 sec at 94˚C (denaturation), 30 sec at 60˚C (annealing) and 1 min at 72˚C (elongation), and a final 30-min elongation step at 65˚C. PCR product size analysis was performed on an automated ABI 3130 sequencer camera XL (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and determined by comparison with the GS600LIZ internal size markers (Applied Biosystems). The estimation of CA repeat numbers in each of the analyzed specimens was based on an extrapolation to the previously developed specific allelic ladder (17). The ladder marker consisted of 14 sequenced amplifications representing alleles with 7, 9, 11, 13 and 23 CA repeats.

Tissues were classified as microsatellite instability-high (MSI-H) when at least two of the five loci showed MSI [non19/non 19] and as MSI low (MSI-L) when only one locus showed MSI [19/non 19 and/or non 19/ 19]. If none of the microsatellite sequences was mutated, the tumor was classified as microsatellite stable [MSS; 19-19] (9).

Immunohistochemical analysis of IGF-1 expression. Immunohistochemical staining for IGF-1 was performed using the DAKO LSAB+System-HRP set (Rabbit, Mouse, Goat, DAB+; cat. no. K0679; Dako North America, Carpinteria, CA, USA). Dako Antibody Diluent with Background Reducing Components (cat. no. S3022; Dako) was used to prepare dilutions. Tissue sections (4 μm) were prepared from paraffin-embedded tissue. Following de-paraffinization and re-hydration, sections were incubated with Dako Target Retrieval Solution (pH 9, 10X, cat. no. S2367, Dako) at 95-99˚C for 20 min. The sections were rinsed three times for 5 min each in Tris-buffered saline (TBS; pH-7.6; Dako) and endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 5 min. Following rinsing with distilled water, samples were immersed in TBS for 5 min and incubated with 5 μg/ml primary goat polyclonal antibody directed against human IGF-1 (cat. no. 18773; Sigma-Aldrich) at room temperature for 15 min. The sections were then incubated with secondary biotinylated anti-goat polyclonal immunoglobulin (1:1,000 dilution; included in the kit mentioned above). at room temperature for 10 min. Following rinsing as above, samples were incubated with streptavidin conjugated to horseradish peroxidase for 10 min. Following rinsing, antibodies were visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Dako) for 5 min. After rinsing with distilled water, cell nuclei were stained with Meyer's hematoxylin (Dako). Following dehydration with an ethanol series, samples were rinsed in xylene, mounted in mounting medium (Consul Mount™, Thermo Shandon™, Thermo Fisher Scientific, Inc.) and studied using an optical microscope (Axioskop 40; Carl Zeiss, Oberkochen, Germany).

Quantitative scoring of slides. Evaluation of immunohistochemical staining was performed by two independent pathologists using Cell-2 software, version 4.1 (Poznan University of Medical Sciences, Poznan, Poland). The scoring method was based on analysis of the distribution of colors and their optical density. The software identifies cells with an optical density greater than the background and classifies them as immunoreactive on the basis of the color ratio. To determine the percentage of positive cells in the sections, the number of immunopositive cells was divided by the total cell count. A minimum of 5,000 cells was counted and the number of sections was 99, 96 and 71 for the EC, HS and CG group, respectively. An investigator who was blinded with regard to the identity of the samples performed all analyses.

Statistical analysis. Differences or correlations between the analyzed parameters were verified using multi-way tables and homogeneity or independence were tested using the χ^2 test. Due to the skewed distribution of measurable parameters evaluated on the basis of the Shapiro-Wilk test, the analysis of differences between the studied sub-groups was performed by non-parametric tests. Comparison of two independent groups was performed using the Mann-Whitney U test. To compare more than two groups, the Kruskal-Wallis test and multiple comparisons/post-hoc tests were performed. Bivariate correlations between study variables were determined by calculating Spearman's rank correlation coefficients. Analysis assumed a 5% error of inference and the associated significance level of $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using Statistica software version 8.0 (StatSoft, Krakow, Poland).

Results

Allelic distribution of CA repeats in the IGF-1 gene P1 promoter in DNA isolated from serum and tissue samples from patients with EC. DNA from the blood and tissue of patients with EC, HS and normal controls was isolated and the occurrence of CA repeats situated in the P1 promoter region of the IGF-1 gene as well as the serum and tissue levels of IGF-1 were compared between the groups. The IGF1 genotype distribution in the total cohort and sub-categories is shown in Table I. The length range of CA repeats in the DNA of the study subjects was 17-21.

Depending on the single nucleotide CA polymorphism in the study group, subjects were assigned to three genotypes: MSS, presence of 19-19 (CA)19 repeat alleles; MSI-L, presence of only one (CA)19 allele [19/non and/or non 19/ 19]; and MSI-H-lack of (CA)19 repeat alleles [non19/non 19]. The most common genotype of blood cells and tissue samples from the control group was MSS [homozygote (CA)19 repeat], which was identified in 20 out of 27 subjects (74%). In the HS group, the MSS genotype was identified in 62.5% of blood cell specimens and 68.7% of tissue samples, and in the EC group, 21.2% of blood cell specimens and 9% of tissue specimens were of the MSS genotype. Statistical analysis revealed no significant association between serum and tissue genotype frequency in any of the study groups (P>0.05) (Table I). However, the most frequent genotype in the control group was MSS (P<0.01), while MSI-H was most frequent in the EC group (P<0.01). This suggests that mutations in the IGF-1 promoter are common in EC and may be associated with its genesis (Table I).

Analysis of blood serum IGF-1, IGFBP-1 and IGFBP-3 levels in study groups of women. The blood serum levels of IGF-1, IGFBP-1 and IGFBP-3 in the experimental groups are shown in Table II. No statistically significant differences in IGF-1, IGFBP-1 and IGFBP-3 serum concentrations between the control, HS and EC groups were detected.

Correlation of blood serum IGF-1, IGFBP-1 and IGFBP-3 levels with the IGF-1 genotype among patients with EC. To further evaluate whether blood serum levels of IGF-1, IGFBP-1 and IGFBP-3 were linked to the genotype of IGF-1 and the occurrence of EC, the IGF-1 levels were correlated with IGFBP-1 and IGFBP-3 levels within the MSS and MSI-H genotypes. Due to the low number of patients, the MSI-L group was excluded from this analysis. IGF-1 levels were positively correlated with IGFBP-3 in the MSS genotype of IGF-1 (r=0.38, P=0.019), while a high and negative correlation with IGFBP-1 (r=-0.67, P=0.001) was identified for the MSI-H genotype (Table III).

Blood serum estrone, estriol and estradiol levels. The serum levels of estrone, estriol and estradiol in the control, HS and EC groups are shown in Table IV. No significant differences in the serum concentrations of these estrogens were identified between any of the groups.

Correlation of blood serum IGF-1 with estrone, estriol and estradiol levels in MSS and MSI-H genotypes among patients with EC. The present study further assessed whether blood serum levels of IGF-1 were correlated with estrogen levels for the individual genotypes MSS and MSI-H (Table V). Due to the low number of patients, group MSI-L was excluded from this analysis. A significant correlation was identified between the IGF-1 levels and estrone levels in the MSI-H genotype group (r=-0.41, P=0.012) as well as a highly negative correlation between IGF-1 levels and the estradiol concentration in the MSI-H genotype group (r=-0.6, P=0.002).

Quantitative scoring of immunohistochemical samples. The present study investigated the association of IGF-1 expression in tissues with IGF-1 genotypes. Expression levels of IGF-1 in tissue samples assigned to control, HS or EC groups confirmed by histopathological diagnosis were determined by immunohistochemical scoring (Fig. 1) and are expressed graphically as the median and range in Figs. 2 and 3. No statistically significant differences in the number of IGF-1-expressing cells were identified between the control, HS and EC groups (P>0.05) (Fig. 2). Of note, the number of IGF-1-expressing cells was significantly higher in the MSI-H-type 18-20 (P=0.007), MSI-L-type 19-20 (P=0.025) and MSS 19-19 (P=0.024; characteristic for healthy tissues) genotypes compared with that in the MSI-H type 20-20, which is characteristic for EC (Fig. 3).

Discussion

Several studies have demonstrated that the IGF pathway has an important role in gynecological cancer types in general and endometrial tumors in particular (18-20). The results of an epidemiological study shoed that elevated levels of IGF-1 are correlated with an increased risk of the development of numerous types of cancer (21). IGF-1 expression and signaling regulate the transition of the pre-menopausal endometrium through the proliferative, secretory and menstrual cycles and have a significant role in the development of EC. A case-cohort study by Gunter *et al* (22) that included 250 EC patients and 465 controls suggested an association between the risk for EC and the serum levels of IGF-1, IGFBP-3, insulin and estradiol. Furthermore, Ayabe *et al* (23) reported elevated IGF-1 and decreased IGFBP-1 levels in post-menopausal EC patients. However, Petridou *et al* (24) indicated that EC was positively correlated with IGF-2 serum levels and inversely associated with IGF-1. Cao *et al* (25) indicated that low serum levels of IGFBP-1 and high levels of IGF-1 are sufficient to elevate the risk of prostate cancer. Low levels of circulating IGFBP-1 were also shown to be able to predict the risk of pancreatic cancer (26). The present study found no statistically significant differences between serum levels of IGF-1, IGFBP-1 and IGFBP-3 in the control, HS and EC groups. However, a correlation between serum levels of IGF-1 and a polymorphism in the CA repeat in the 5' untranslated region of the IGF-1 promoter was identified. Serum levels of IGF-1 were positively correlated with IGFBP-3 in the MSS and MSI-L groups and negatively correlated with IGFBP-1 in the MSI-H group. Analysis of the polymorphic repeat in the P1 promoter of the IGF-1 gene in the serum and tissue showed no statistically significant differences. Polymorphism changes were identical in the serum and tissues of the control groups (EC and HS). Moreover, IGF-1, IGFBP-1, IGFBP-3, estrone, estriol and estradiol levels in the blood serum of the control group and patient study group showed no statistically significant differences. By contrast, a significant negative correlation between the plasma levels of IGF-1 and IGFBP-1 as well as between estrone and estradiol levels was observed in EC patients with IGF-1 polymorphisms of the MSI-H type. Therefore, the expression of IGF-1 was assessed in tissues. No statistically significant differences in the number of IGF-1-expressing cells were identified between the control and study groups; however, the number of IGF-1-expressing cells was significantly higher in tissues from patients of the MSI-H type 18-20, the MSI-L type 19-20 and the MSS type 19-19 (characteristic for healthy tissues) compared with that in the MSI-H type 20-20, which is characteristic for EC. These results confirmed the notion that

Table I. Comparison of microsatellite instability evaluation (CA repeat) in DNA isolated from peripheral blood cells and paraffin tissues of patients from the study and the control group.

	Control group (n=27)		Non-atypical hyperplasia simplex (n=32)		Endometrial cancer (n=33)	
Groups	N (serum)	N (tissue)	N (serum)	N (tissue)	N (serum)	N (tissue)
IGF-1 $(CA)_n$ genotype						
CA17/18	0	0	0	2	5	5
CA17/19	2	1	0	1	3	1
CA17/21	0	0	3	1	5	4
CA18/19	2	1	0	2	0	2
CA18/20	0	1	2	2	6	5
CA18/21	0	0	2	0	4	6
CA19/19	20	20	20	22	7	3
CA19/20	0	1	3	2	0	1
CA19/21	2	2	2	0	0	1
CA20/20	1	1	0	0	3	5
IGF-1 $(CA)_n$ genotype						
Group 1						
MSS	20	20	20	22	7	3
MSI-L	6	5	5	5	3	5
MSI-H	1	2	7	5	23	25
P-value[a]	0.067[a]		0.098[a]		0.087[a]	
Group 2						
19 allele present	26	26	25	27	20	17
19 allele absent	1	1	7	5	13	16
P-value[b]	0.098[b]		0.881[b]		0.922[b]	

[a]Comparison of CA repeats in DNA isolated from serum and tissue between control, non-atypical hyperplasia simplex and endometrial cancer groups; [b]comparison of CA repeats in DNA within control, non-atypical hyperplasia simplex and endometrial cancer patient groups between serum and tissue. IGF, insulin-like growth factor; MSS, microsatellite stable; MSI-L, microsatellite instability low; MSI-H, microsatellite instability high; N, number of subjects with the respective genotype.

Table II. Analysis of IGF-1 (ng/ml), IGFBP-1 (ng/ml), IGFBP-3 (ng/ml) levels in blood serum of patients from the HS and EC groups and the control group.

Group	N	Mean	SD	Me	Q1	Q3	Min-Max	P-value
IGF-1								
Control	27	178.9	85.1	152.6	125.4	206.1	100-314	
HS	32	180.1	114.9	171.4	142.8	221.2	101-232	0.21
EC	33	209.9	60.6	193.4	121.8	230.1	102-340	0.50
IGFBP-1								
Control	27	5.4	4.4	4.0	2.6	5.8	2.6-5.8	
HS	32	5.0	5.0	3.3	2.4	5.5	1.2-7.7	0.57
EC	33	6.8	5.9	4.9	3.0	9.1	1.7-13.2	0.43
IGFBP-3								
Control	27	1689.8	542.7	1551.3	1370.0	1893.3	942-2387	
HS	32	1712.9	589.9	1705.3	1407.1	1749.7	1104-2654	0.46
EC	33	1725.6	412.6	1706.0	1482.9	1956.7	1104-2417	0.46

P-values refer to comparison with control group. N, number; Me, median; SD, standard deviation; Q1, lower quartile; Q3, upper quartile; Min-Max, minimum-maximum range; EC, endometrial cancer; HS, non-atypical hyperplasia simplex; IGFBP, insulin-like growth factor binding protein.

Table III. Spearman rank correlation coefficients for the correlation of blood serum levels of IGF-1 with IGFBP-1 and IGFBP-3 levels for the MSS and MSI-H genotypes among patients with EC.

	MSS		MSI-H	
Parameter	IGFBP-1	IGFBP-3	IGFBP-1	IGFBP-3
IGF-1	0.12	0.38	-0.67	-0.21
P-value	0.2311	0.0191[a]	0.0007[a]	0.3830

[a]P<0.05. IGF, insulin-like growth factor; MSS, microsatellite stable; MSI-L, microsatellite instability low; MSI-H, microsatellite instability high; EC, endometrial cancer.

Table IV. Analysis of estrone (pg/ml), estriol (ng/ml) and estradiol (ng/ml) levels in the blood serum of patients with EC.

Hormone/group	N	Mean	SD	Me	Q1	Q3	Min-Max	P-value
Estrone								
Control	27	45.6	133.4	33.8	23.7	167.5	9-132	
HS	32	54.0	59.6	39.0	16.7	66.3	7-103	0.65
EC	33	71.0	47.3	35.5	23.7	80.9	10-228	0.72
Estriol								
Control	27	2.7	1.6	2.5	1.8	5.2	1-8.8	
HS	32	2.4	1.1	2.4	2.0	3.3	1.3-1.6	0.87
EC	33	2.6	0.6	1.9	1.5	2.6	1.4-5.5	0.74
Estradiol								
Control	27	39.7	83.7	24.5	12.6	67.5	7.7-108	
HS	32	68.2	31.4	32.1	20.9	46.7	1.6-214	0.22
EC	33	51.4	76.7	25.9	7.8	94.0	3.3-214.8	0.60

P-values refer to comparison with control group. N, number; Me, median; SD, standard deviation; Q1, lower quartile; Q3, upper quartile; Min-Max, minimum-maximum range; EC, endometrial cancer; HS, non-atypical hyperplasia simplex.

Table V. Spearman rank correlation coefficients for the correlation of blood serum levels of IGF-1 with levels of estrone, estriol and estradiol in patients with EC of the MSS and MSI-H genotypes.

	MSS			MSI-H		
	Estrone	Estriol	Estradiol	Estrone	Estriol	Estradiol
IGF-1	0.21	0.11	0.27	-0.41	-0.11	-0.6
P-value	0.401	0.453	0.290	0.012[a]	0.700	0.002

[a]P<0.05. IGF, insulin-like growth factor; MSS, microsatellite stable; MSI-L, microsatellite instability low; MSI-H, microsatellite instability high; EC, endometrial cancer.

(CA)$_n$ microsatellite repeat polymorphisms in the P1 promoter of IGF-1 themselves are not the primary regulatory elements of IGF-1 expression.

Cleveland *et al* (27) reported that IGF-1 genotypes which include alleles with less than 19 CA repeats appeared to be associated with an increased risk of breast cancer. In line with this, several further studies have also found an association between the number of IGF-1 CA repeats and the risk for breast cancer (28-30), while others have not (31). Zecevic *et al* (32) suggested that IGF-1 variant genotypes modify the risk of hereditary forms of cancer. The serum levels of IGF-1 are highly influenced by estrogen. Liang *et al* (33) indicated that IGF signaling has an important role in estrogen-induced endometrial carcinogenesis. While the molecular mechanisms of estrogen-induced expression of IGF-1 have largely remained elusive, it has been reported that estrogen treatment increased the mRNA expression of IGF-1, possibly through regulation and modulation of the IGF-1 promoter and

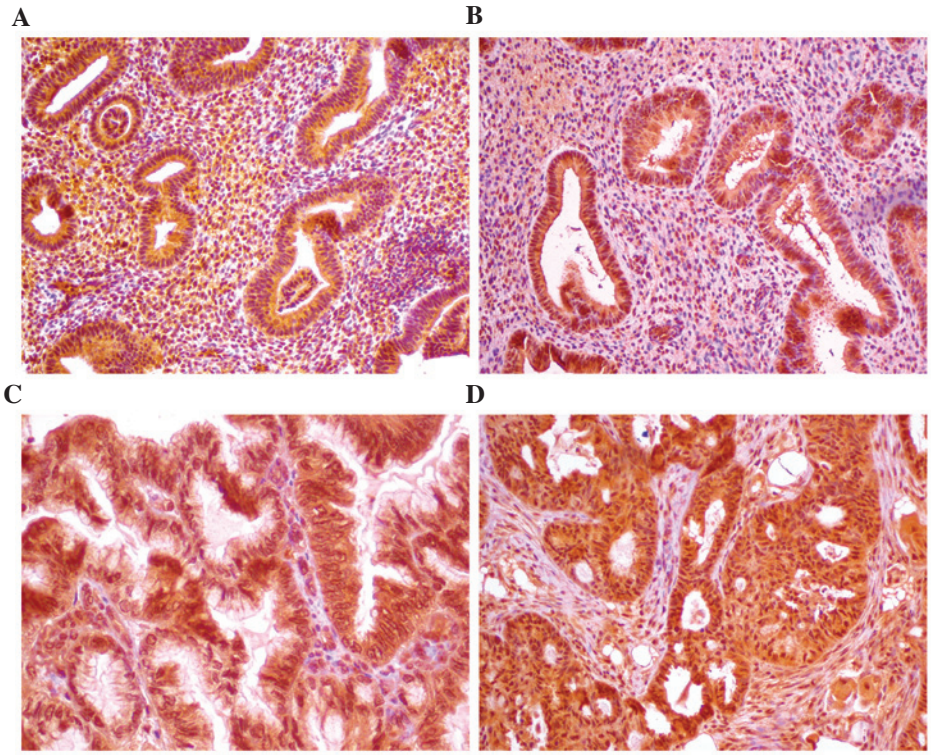

Figure 1. Immunohistochemical analysis of insulin-like growth factor-1 expression in the control and the study group. (A) Control group: Proliferative endometrium; (B) HS-group: Simple hyperplasia; (C) EC-group: Endometrioid adenocarcinoma G2; (D) EC group: Endrometroid adenocarcinoma, G2 (magnification, x100 for A, B and D; x200 for C). Samples shown in C and D are from the same patient.

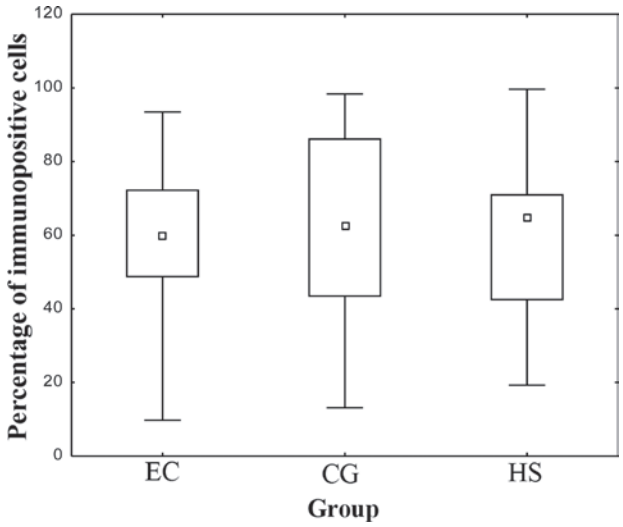

Figure 2. IGF-1 expression in the endometrial tissues in the study group. Small squares indicated the median value, boxes indicate the 25-75% range and bars indicate the minimum-maximum range. IGF, insulin-like growth factor; EC, endometrial cancer; HS, non-atypical hyperplasia simplex; CG, control group.

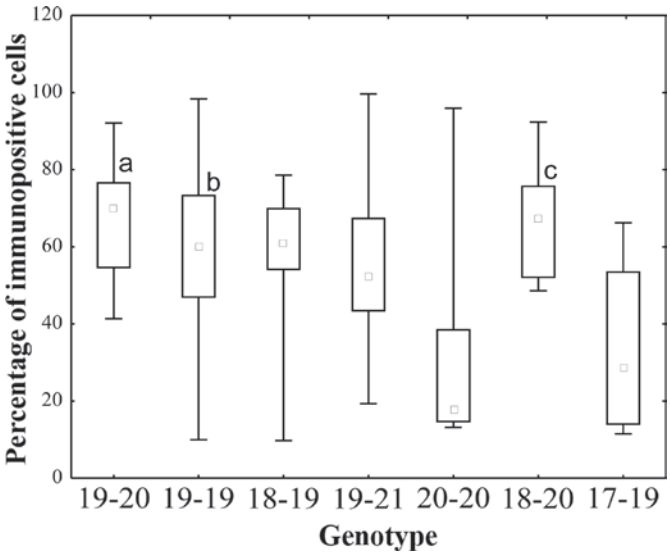

Figure 3. IGF-1 expression in the endometrial tissues classified as microsatellite instability high (20-20, 18-20), low (19-20, 19-21, 17-19, 18-19) or microsatellite stable (19-19). The number of IGF-1-expressing cells was significantly higher in the MSI-L-type 19-20 ([a]P=0.025), MSS type 19-19 ([b]P=0.024) and MSI-H-type 18-20 ([c]P=0.0072) vs. the MSI-H type 20-20. Small squares indicated the median value, boxes indicate the 25-75% range and bars indicate the minimum-maximum range. IGF, insulin-like growth factor; MSS, microsatellite stable; MSI-L, microsatellite instability low; MSI-H, microsatellite instability high.

CCAAT-enhancer-binding protein transcription factors (34). Estrogens are known to increase IGFBPs, which regulate the bioavailability and activities of IGFs, and which either enhance or inhibit the action of IGFs, while also being able to act independently of IGFs.

When the endometrium is exposed to unopposed estrogen, the risk of hyperplasia and EC increases, which, however, may be reduced by sex hormone binding globulin, progesterone and further steroid hormones and factors (35). Estrone is a precursor of estradiol, the principal estrogen, whose metabolic waste product is estriol. All of these estrogens act upon the endometrium through estrogen receptors, resulting in the induction of growth factors, including the epidermal growth factor, IGF-1 and growth-enhancing proto-oncogenes, such as c-fos and

c-myc (36). All hormones can also act via non-genomic pathways to control cell function and proliferation (35). The present study revealed a negative correlation between the serum levels of IGF-1 and estrone and estradiol concentrations in patients with EC (r=-0.41, P=0.012).

Little is known regarding autocrine activation of the IGF-1 system and estrogens in endometrial tissues. The difference in IGF-1 levels among oral contraceptive users with and without the 19 repeat allele suggests that this allele may be associated with a conformational change in the IGF-1 promoter region, possibly involving the estrogen response element (27).

The immunohistochemical analysis performed in the present study indicated that in tissues with a genotype other than (CA)19, the number of IGF-1 expressing cells was significantly higher. This finding confirmed the finding that IGF-1 genotypes other than (CA)19 show elevated levels of IGF-1 in tissues, which may be responsible for autocrine stimulation of cancer development (37). However, as all cancer types, EC is a multifactorial disease and carcinogenesis is a result of multiple gene mutations leading to aberrant expression of proteins, which regulate cell functions and the polymorphism assessed in the present study leading to aberrant IGF-1 expression may be one of them.

In the present study, the association between CA sequence polymorphisms in the IGF-1BP1 promoter region, IGF-1 levels and endometrial cancer development in comparison to healthy individuals was examined. The study revealed that the length of the CA repeat sequence in women with non-atypical hyperplasia simplex and endometrial cancer ranged from 17-21 bp. A similar distribution of CA polymorphisms was observed among the control group. However, among healthy individuals, ~74% were homozygote carriers of 19 CA repeats (MMS) according to serum and tissue analysis, while 62.5 and 68.7% of patients with non-atypical hyperplasia simplex were of the MMS type according to serum and tissue analysis, respectively. However, the MMS type was only detected in the serum of 22.2% and in the tissue of 9.0% of patients with EC. A previous bioinformatics study by our group showed that the CA repeat region of the P1 promoter of IGF-1 is able to form DNA loop structures, which may serve as a recognition site for transcriptional modulators of the IGF-1 gene (38). Thus, changes in the number of CA repeats may have an influence on IGF-1 promoter activity and be associated with EC; however, further studies are required to confirm this.

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