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Genetic polymorphism of Klotho gene and bladder cancer risk

Polimorfizm genu Klotho a ryzyko raka pęcherza moczowego

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Abstract

Introduction: Bladder cancer is the most frequent tumor of the urinary tract in Poland. Klotho gene can act as an suppressor gene. Therefore, variability of this gene might be implicated in the carcinogenesis of urinary bladder. The aim of the study was analysis of the association between the g.33590184 G>A (rs1207568), g.33634983 C>T (rs564481), g.33628193 G>C (rs9527025) polymorphisms of the Klotho gene and bladder cancer risk.

Materials and methods: The study included 96 patients diagnosed with transitional cell carcinoma of the bladder (TCC) and 114 healthy, cancer-free individuals. Three selected polymorphisms were typed by PCR with confronting two-pair primers (PCR-CTPP) and Real Time PCR with TaqMan probes.

Results: The GA and AA genotypes of the rs1207568 polymorphism increased the risk of bladder cancer (OR = 1.86, 95% CI [1.04-3.33], p = 0.03 and OR = 6.58, 95% CI [1.27-34.02], p = 0.01, respectively). Individuals who were heterozygous and homozygous for the A variant had 2.10-fold higher risk of bladder cancer (OR = 2.10, 95% CI [1.20-3.65], p = 0.009). On the other hand, heterozygous subjects and homozygous carriers of the wild-type allele (G) had a decreased bladder cancer risk (OR = 0.19, 95% CI [0.04-0.95], p = 0.043). Also, the occurrence of bladder cancer was positively correlated with the presence of the GC genotype of the rs9527025 polymorphism (OR = 2.84, 95% CI [1.57-5.15], p = 0.0001).

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Conclusions: Two polymorphisms of Klotho gene (rs1207568 and rs9527025) may play a role in susceptibility to bladder cancer.

Key words: genetic polymorphism, Klotho, bladder cancer, SNP

Streszczenie

Wstęp: Rak pęcherza moczowego jest najczęściej występującym nowotworem układu moczowego w Polsce. Sugeruje się, że gen Klotho może pełnić funkcje genu supresorowego. W związku z tym, polimorfizm genu Klotho może mieć wpływ na proces transformacji nowotworowej pęcherza moczowego. Celem przedstawionych badań był analiza związku pomiędzy występowaniem wybranych polimorfizmów pojedynczych nukleotydów g.33590184 G>A (rs1207568), g.33634983 C>T (rs564481), g.33628193 G>C (rs9527025) a ryzykiem zachorowania na raka pęcherza moczowego.

Materiały i metody: Do badań włączono 96 pacjentów ze zdiagnozowanym przejściowokomórkowym rakiem pęcherza moczowego (TCC; transitional cell carcinoma) oraz 114 osób zdrowych, u których nie stwierdzono choroby nowotworowej. Występowanie trzech wybranych polimorfizmów analizowano przy użyciu techniki PCR z dwiema parami przeciwstawnych starterów (PCR-CTPP; PCR with confronting two-pair primers) oraz metody Real Time PCR z sondami fluorescencyjnymi TaqMan.

Wyniki: Genotypy GA i AA polimorfizmu rs1207568 wpływają na wzrost ryzyka zachorowania na przejściowokomórkowego raka pęcherza moczowego (OR = 1,86, 95% PU [1,04-3,33], p = 0,03 oraz OR = 6,58, 95% CI [1,27-34,02], p = 0,01, odpowiednio). U osób będących heterozygotami lub homozygotami pod względem allela A wykazano ponad 2-krotnie wyższe ryzyko zachorowania na raka pęcherza moczowego (OR = 2,10, 95% PU [1,20-3,65], p = 0,009). Natomiast w przypadku nosicieli allela G, w układzie homozygotycznym lub heterozygotycznym, obserwowano spadek ryzyka zachorowania na badany nowotwór (OR = 0,19, 95% PU [0,04-0,95], p = 0,043). W przypadku polimorfizmu rs952705 wykazano, że genotyp GC zwiększa ryzyko zachorowania na przejściowokomórkowego raka pęcherza moczowego (OR = 2,84, 95% PU [1,57-5,15], p = 0,0001).

Wnioski: Dwa spośród badanych polimorfizmów genu Klotho (rs1207568 i rs9527025) mogą mieć wpływ na predyspozycję do zachorowania na przejściowokomórkowego raka pęcherza moczowego.

Słowa kluczowe: polimorfizm genów, Klotho, rak pęcherza, SNP

Introduction

In Europe, bladder cancer is the 5th most commonly diagnosed cancer type and the 9th leading cause of cancer mortality [1]. In addition, bladder cancer is the most frequent tumor of the urinary tract in Poland [2]. It affects men more frequently than women. Typical of solid tumors, bladder cancer incidence increases with age. Tumor of the bladder rarely occur before the age of 40 – 50, arising most commonly in the seventh decade of life [1]. The most important risk factors for the development of bladder cancer are smoking and occupational exposure to toxic chemicals, such as aromatic amines and polycyclic aromatic hydrocarbons [3]. Recent studies have facilitated even more rapid progress in the identification of the molecular mechanisms involved in bladder cancer initiation and progression. Many transforming and tumor suppressor genes, such as FGFRs (fibroblast growth factor receptors), Ras, p53 and RB (retinoblastoma) may be involved in bladder carcinogenesis [4, 5]. The fibroblast growth factor receptors (FGFRs) play a role in tumor associated angiogenesis, as some receptors (FGFR3) are mutated and others (FGFR 1, 2, and 4) are abnormally expressed in bladder cancers. Overexpression of wild-type FGFR4 in bladder tumors can be associated with progression and aggressive clinical behavior of this cancer [5]. Recent studies indicate that Klotho (KL) protein can modulate FGF/FGFR signaling and act as tumor suppressor [6]. The Klotho gene named after a Greek goddess who spins the thread of life, was originally identified as

a gene mutated in a mouse strain that inherits a premature aging syndrome in an autosomal recessive manner [7, 8]. The Klotho gene is composed of five exons and encodes a type I single-pass transmembrane protein of 1,012 amino acid long (~130 kDa). The intracellular part of Klotho protein is very short (10 amino acid) and has no known functional domains. The extracellular domain is composed of two internal repeats with homology to family 1 glycosidases that hydrolyze β-glucosidic linkage in saccharides, glycoproteins, and glycolipids [9, 10]. The extracellular domain is also subject to ectodomain shedding. As a result, the entire extracellular domain is released into the extracellular space and is detectable in blood, urine, and cerebrospinal fluid [11, 12]. Thus, the Klotho protein exist in two forms: membrane Klotho and secreted Klotho. Membrane Klotho functions as a coreceptor for endocrine FGF23 that regulates excretion of phosphate and synthesis of active vitamin D [13-16]. Secreted Klotho functions as a humoral factor with pleiotropic activities, including suppression of growth factor signaling, suppression of oxidative stress, and regulation of ion channels and transporters [17, 18]. Furthermore, secreted Klotho protein may modify glycans of receptor tyrosine kinases, such as insulin- and IGF-1 receptors and FGFRs, which inhibits their activity and/or alters cell surface abundance [19]. Goetz et al. [20] suggested that the association of Klotho protein with FGFR not only enhances the binding affinity of the receptor for endocrine FGFs such as FGF23 but at the same time also suppresses the binding and activation of FGFR by FGF8 subfamily ligands and possibly other paracrine FGFs [20].

We hypothesized that genetic polymorphisms in Klotho gene may be associated with development of bladder cancer. To test our hypothesis we evaluated the association between the g.33590184 G>A (G-395A, rs1207568), g.33634983 C>T (C1818T, rs564481), g.33628193 G>C (C370S, rs9527025) polymorphisms of the Klotho gene and bladder cancer.

Table 1. Characteristics of the bladder cancer patients and healthy participants

Characteristic	No. of patients (%)	No. of healthy participants (%)	p[a]
Sample size	96	114	–
Age, years	66.3 ± 8.1	67.8 ± 12.2	–
Histologic cell type Transitional cell carcinoma	96 (100)	–	–
Sex Male Female	68(70.8) 28 (29.2)	46 (40.3) 68 (59.7)	<0.001
Grade 1 or 2 3	64 (66.6) 32 (33.4)	–	–
Stage Noninvasive pTa pTis pT1 Muscle invasive pT2a or pT2b pT3a or pT3b pT4	41 (42.7) 22 (22.9) 1 (1.0) 18 (18.7) 55 (57.3) 21 (21.8) 23 (23.9) 11 (11.7)	–	–
Disease recurrence Yes No	15 (15.6) 81 (84.4)	–	–

[a] χ^2 test

Material and methods

Patients

The study included 96 patients diagnosed with transitional cell carcinoma of the bladder at the 2nd Clinic of Urology, Medical University of Łódź, Poland between 2009 and 2012. The gender was 68 male and 28 female. The mean age of patients was 66.3 years. Clinicopathological characteristics of participants are shown in Table 1. The control group consisted of 114 healthy individuals (46 male and 68 female) recruited from the 2nd Clinic of Urology, Medical University of Łódź, Poland, from periodic health check-ups. The controls were genetically unrelated cancer-free individuals and frequency matched to the cases based on age (mean age 67.8 years). This study was approved by the Ethical Committee of the Medical University of Łódź. Each subject donated a venous blood samples of ~ 2 mL, 250 µL of which was used for genomic DNA extraction. Blood samples of all patients and controls were collected into ethylenediaminetetraacetic acid (EDTA) tubes and store at -20°C until further use.

DNA preparation and storage

Genomic DNA was obtained from a 250 µl aliquot of blood using a commercially available AxyPrepTM Blood genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA), according to the manufacturer's instructions. DNA purity and concentration were determined spectrophotometrically at 260 and 280 nm. The purified genomic DNA was stored in TE buffer at -20°C.

Table 2. Sequence of primers and probes used for the genotyping of Klotho gene

SNP	Method of genotyping	Sequence (5'-3')	Reference
rs1207568	PCR-CTPP	F1:GTTTCGTGGACGCTCAGGTTCATTCTC	(21)
		F2:GAGAAAAGGCGCCGACCAACTTTC	
		R1:GATCCCGCCCC CAAGTCGGGA	
		R2:GTCCCTCTAGGATTTCGGCCAG	
rs564481	PCR-CTPP	F1:CTCAGTTTACCGACCTGAATGTTTACCTG	(21)
		F2:CAGATCGCTTTACTCCAGGAAATGCAC	
		R1:GTCCAGGGAGAAGCGAAAATGTGTAACA	
		R2:5'- GAGCTCTTGAAAGCACAGTCGGGC-3'	
rs9527025	TaqMan® assay	F:AGTTCATCAAAGGAACTGCT GACTT	(22)
		R:CTTCATGTGAGGGTCCAAAA GTT	
		S1 (allele G) FAM-TTGCTCTTTGCTTTGGACCCACCT-BHQ1	
		S2 (allele C) VIC-TTGCTCTTTCCTTTGGACCCACCTT-BHQ1	

PCR-CTPP

The genotyping of g.33590184 G>A (G-395A, rs1207568) in the promoter region and g.33634983 C>T (C1818T, rs564481) in exon 4 was performed using PCR with confronting two-pair primers (PCR-CTPP) assay (21). In this assay, confronting pairs of primers (four primers in all) are used as shown in Table 2. The regions containing studied polymorphism were amplified by PCR with these primers with the initial denature at 95°C for 1 min, at 65°C (rs1207568) or 69°C (rs564481) for 1 min, at 72°C for 1 min and additionally at 72°C for 10 min. PCR products were visualized on a 2% agarose gel with ethidium bromide staining. Genotyping was performed as follows: rs1207568 – 252, 175 bp for GG genotype, 252, 175, 121 bp for GA genotype, 252, 121 bp for AA genotype (Fig. 1) and rs564481 – 416, 291 bp for CC genotype, 416, 291, 179 bp for CT genotype, and 416, 179 bp for TT genotype (Fig. 2).

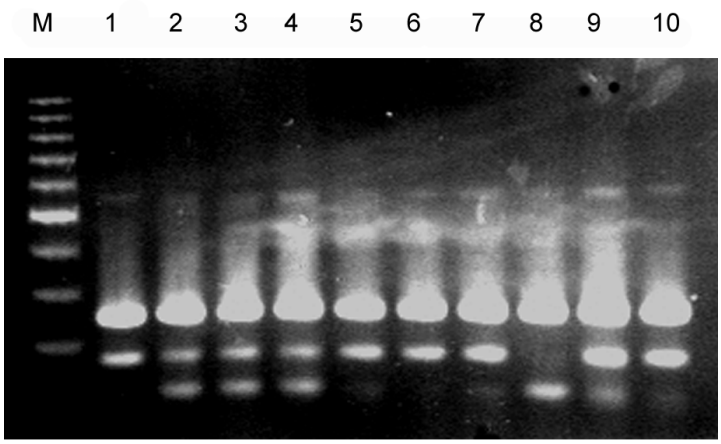

Fig. 1. Genotypes of the g.33590184 G>A (G-395A) polymorphism (rs1207568) determined by PCR-CTPP method and analyzed by 2% agarose gel electrophoresis stained with ethidium bromide and viewed under UV.

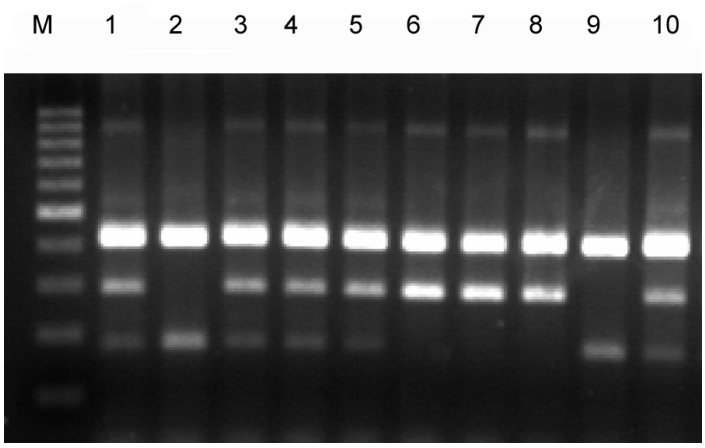

Fig. 2. Genotypes of the g.33634983 C>T (C1818T) polymorphism (rs564481) determined by PCR-CTPP method and analyzed by 2% agarose gel electrophoresis stained with ethidium bromide and viewed under UV.

TaqMan® Assay

Genotyping for g.33628193 G>C (C370S, rs9527025) allelic variant in exone two was performed with using TaqMan® genotyping assay in which a fluorogenic probe, consisting of an oligonucleotide labeled with both a fluorescent reporter dye (FAM or VIC) and a quencher dye, is included in a typical PCR. Amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence. Sequences of pair of primers and two probes are shown in Table 2 (22). The PCR thermal cycling was as follows: initial denaturing at 95°C for 10 min; 45 cycles of 95°C for 30 sec and 60°C for 60 sec were carried out and fluorescence was measured at the end of each cycle and at endpoint. Detection was carried out by qPCR in an Eppendprf realplex thermocycler (Eppendorf, Germany). All samples were determined in duplicate and genotypes were assigned by gene identification software (RealPlex 2.0, Eppendorf, Germany).

Statistical data analysis

Genotype distributions were evaluated for agreement with Hardy–Weinberg equilibrium by the Chi-square test. All genotype distributions of Klotho gene fit Hardy–Weinberg equilibrium. Unconditional multiple logistic regression models were used to calculate odds ratios (ORs) and 95 % confidence intervals (CIs) for the association of genotype with bladder cancer risk. Genotype data were analyzed with the homozygote of the common allele as the reference group. Variants of homozygotes and heterozygotes were combined to evaluate the dominant effect. Reported p values were two sided. Probabilities were considered significant whenever p value was lower than 0.05. All analyses were completed using STATA software (version 11.0 Stata-Corp., Texas, USA).

Results

The PCR-CTPP and Real Time TaqMan® PCR analyses were successful for all cases and controls. The characteristics of the patients and controls are presented in Table 1. There were significantly more male among the patients than controls (70.8% vs 40.3%, $p<0.001$). The genotype and allele distributions of the g.33590184 G>A (rs1207568), g.33634983 C>T (rs564481) and g.33628193 G>C (rs9527025) Klotho polymorphisms in bladder cancer patients and controls are summarized in Table 3. In our study, all the observed genotype frequencies were in agreement with Hardy-Weinberg equilibrium calculated for the cases and controls ($p>0.05$). As shown in Table 3 the differences in the frequency distributions of genotypes of the rs1207568 and rs9527025 polymorphisms between the cases and controls were statistically significant ($p<0.05$).

Table 3. Associations between Klotho SNPs and bladder cancer risk

SNP genotype	Cases (%)/Controls (%)	OR (95% CI)[a]	p
rs1207568			
GG	45 (46.9)/ 74 (64.9)	1.00	
GA	43 (44.8)/ 38 (33.3)	1.86 (1.04-3.33)	0.03
AA	8 (8.3)/ 2 (1.8)	6.58 (1.27-34.02)	0.01
p-trend[a]	0.002		
GA or AA vs. GG[b]	51 (53.1)/ 40 (35.1)	2.10 (1.20-3.65)	0.009
GA or GG vs AA[c]	88 (91.7)/ 112 (98.2)	0.19(0.04-0.95)	0.043
rs564481			
CC	43 (44.8)/ 48 (42.1)	1.00	
CT	39 (40.6)/ 54 (47.4)	0.81 (0.45-1.45)	0.47
TT	14 (14.6)/ 12 (10.5)	1.30 (0.54-3.14)	0.55
p-trend[a]	0.88		
CT or TT vs. CC[b]	53 (55.2)/ 66 (57.9)	1.15 (0.87-1.52)	0.32
CC or CT vs. TT[c]	82 (85.4)/ 102 (89.5)	1.41 (0.92-2.19)	0.11
rs9527025			
GG	67 (70.0)/ 79 (69.3)	1.00	
GC	27 (28.1)/ 33 (28.9)	2.84 (1.57-5.15)	0.0001
CC	2 (1.9)/ 2 (1.8)	1.84 (0.25-13.65)	0.55
p-trend[a]	0.98		
GC or CC vs. GG[b]	29 (30.2)/ 35 (36.4)	1.02 (.57-1.85)	0.94
GG or GC vs. CC[c]	94 (97.9)/ 112 (98.2)	1.19 (0.16-8.62)	0.86

[a] Testing additive genetic model (Cochran-Armitage test for trend); [b] Testing dominant genetic model; [c] Testing recessive genetic model

An association between bladder cancer and the GA and AA genotypes of the rs1207568 polymorphism was found. Both genotypes increased the risk of bladder cancer occurence (OR = 1.86, 95% CI [1.04-3.33], p = 0.03 and OR = 6.58, 95% CI [1.27-34.02], p = 0.01, respectively). Moreover, we found that individuals who were heterozygous and homozygous for the A variant had 2.10-fold higher risk of bladder cancer (OR = 2.10, 95% CI [1.20-3.65], p=0.009). On the other hand, heterozygous subjects and homozygous carriers of the wild-type allele (G) had a decreased bladder cancer risk (OR = 0.19, 95% CI [0.04-0.95], p=0.043). In addition, the occurrence of bladder cancer was positively correlated with the presence of the GC genotype of the rs9527025 polymorphism (OR=2.84, 95% CI [1.57-5.15], p = 0.0001).

Discussion

In recent years, the role of genetic factors in bladder cancer development has been extensively investigated. Numerous case-control studies have confirmed the association between single nucleotide polymorphisms (SNPs) and bladder cancer [23-25]. We analyzed three single nucleotide polymorphisms in Klotho gene. One SNP (rs1207568) is located in the promoter region of Klotho gene. We also analyzed a G to C transversion at the position g.33628192 (rs9527025) located in the second exon of this gene. This polymorphism causes change of cysteine to serine at the amino acid position 370 (C370S) of Klotho protein. The third SNP (rs564481) is located within exon 4 and is synonymous mutation (His589His) [26]. We found significant association between rs1207568 and rs9527025 polymorphisms and bladder cancer risk in Polish population.

To our knowledge, this is the first study to report an association of SNPs in Klotho with the risk of bladder cancer. Polymorphisms of Klotho gene have

been studied for their associations with risk for a variety of disease, such as osteoarthritis, ischemic stroke, coronary artery disease, metabolic syndrome and type 2 diabetes [22, 26-30]. There are a few studies analyzing the correlation between Klotho SNPs and breast and ovarian cancer risk [31, 32]. A functional variant of Klotho, termed KL-VS, contains six sequence variants in complete linkage disequilibrium, two of which result in amino acid substitutions F352V and C370S. The presence of phenylalanine at a position equivalent to position 352 in the human Klotho gene is highly conserved among species and its substitution to valine may alter the excretion and enzymatic activity of the protein [31]. Wolf et al. [31] analyzed the association between KL-VS and cancer risk among BRCA1/BRCA2 mutation carriers of Ashkenazi origin. Among BRCA1 carriers, heterozygosity for the KL-VS allele was associated with increased breast and ovarian cancer risk and younger age at breast cancer diagnosis [31]. The effect of KL-VS variant on breast and ovarian cancer risk in non-Jewish BRCA1/BRCA2 mutation carriers has not been reported [32].

Moreover, recent studies indicate that Klotho protein can act as tumor suppressor [6]. Analysis of KL protein expression in breast tissue revealed high Klotho expression in normal breast samples but very low expression in breast cancer [33, 34]. Klotho gene expression was also downregulated in cell lines and tissue samples of human gastric, colorectal, cervical and lung cancer [35-39]. In the case of cervical cancer samples, loss of Klotho mRNA was observed in invasive carcinoma but not during the early, preinvasive phase of primary cervical tumorigenesis. Abramovitz et al. [6] showed that Klotho expression is downregulated in pancreatic adenocarcinoma. Overexpression of KL, or treatment with soluble Klotho, reduced growth of pancreatic cancer cells *in vitro* and *in vivo* [6].

In conclusion, our work shows that genetic polymorphisms of the Klotho gene may be associated with development of bladder cancer. These findings may be helpful in increasing our understanding of carcinogenesis of urinary bladder.

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